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In the coming year, it is my vision to have JJBS publish a combination of manuscripts documenting rigorous studies in the area of biological sciences, and one or more manuscripts from distinguished scholar on recent advances in molecular biology. As you read throughout this volume of JJBS, I would like to remind you that the success of our journal depends directly on the number of quality articles submitted for review. Accordingly, I would like to request your participation and colleagues by submitting quality manuscripts for review. One of the great benefits we can provide to our prospective authors, regardless of acceptance of their manuscripts or not, is the feedback of our review process. JJBS provides authors with high quality, helpful reviews to improve their manuscripts.

Moreover, and as always, my thanks are also extended to the Hashemite University and Jordanian Scientific Research Support Fund for their continuous financial and administrative support to JJBS. I would like to highlight and proudly thank the group of authoritative reviewers, both local and international, who have done an outstanding work. We are honored to have you on our review list and many thanks for your valuable mentorship and contributions you provided to authors. Indeed, we count on your excellent reviews to include only high quality articles worthy of publication in JJBS. Together, we strive to make JJBS reach a remarkable rank among other international journals. I very much appreciate your support to make JJBS one of the most authoritative journals in biological sciences.

March 2018

Prof. Khaled H. Abu-Elteen
Editor-in-Chief
The Hashemite University
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First Record of *Anilocra physodes* (Isopoda, Cymothoidae) on the *Phycis blennoides* (Pisces; Phycidae) with Morphological Characters and Hosts Preferences

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Abstract

Anilocra physodes (Linnaeus, 1758) (Isopoda, Cymothoidae) is reported for the first time on *Phycis blennoides* (Brünnich, 1768) (Pisces; Phycidae) from the North Aegean Sea Coasts of Turkey. The present paper aims to present the morphological characters of *Anilocra physodes* from Turkey. Some morphological characters of this parasitic isopod are illustrated. A new host species for *Anilocra physodes* and the host's preferences with it, according to family characteristics, habitat selections, feeding habits, are presented.

Keywords: *Anilocra*, Cymothoidae, Isopoda, morphology, *Phycis*, Turkey.

1. Introduction

Cymothoids are ectoparasitic isopods on the body, fins, or inside the buccal or the branchial cavities of numerous freshwater and marine fishes. They are the protandrous hermaphrodite (Bariche and Trilles, 2005). Cymothoids are serious parasites currently affecting a number of fish farms in the World (Sarusic, 1999; Papapanagiotou *et al*., 1999; Papapanagiotou and Trilles, 2001).

The family Cymothoidae includes 43 genera according to Hadfield *et al*. (2017). Although that Cymothoidae family is well-known, there are some deficiencies from the taxonomic point of view. Studies concerned with molecular and morphological are needed on this family according to some researchers (Poore and Bruce, 2012; Martin *et al*., 2013; Hadfield *et al*., 2016).

Fifty-one species in the genus *Anilocra* were listed by The World Register of Marine Species (Bruce and Schotte, 2008). Two species (*Anilocra physodes* and *Anilocra frontalis*) were reported from Turkish waters, but these studies include limited information about the morphology of mouth-parts (Öktener and Trilles, 2004; Kırkım, 1998).

The present study aims to report a new host species for *Anilocra physodes* and its host preference according to family characteristics, habitat selections, feeding habits.

2. Material and Methods

Seventy greater forkbeard, *Phycis blennoides* (Brünnich, 1768) (Pisces; Phycidae) were collected from the North Aegean Sea in 2014. Collected parasites were fixed in 70% ethanol. Mouthparts and pleopods were dissected using a Wild M5 stereo microscope. The dissected parts were mounted on slides in a glycerin-gelatine mounting medium. The pleopods were stained with methylene blue. The appendages were drawn with the aid of a camera lucida (Olympus BH-DA). The photos were taken with the aid of Canon camera (EOS 1100D) attached to the microscope. Measurements were taken in millimeter (mm) with a micrometric program (Pro-way). Scientific names, synonyms were checked with the WoRMS Editorial Board (2018). The information of feeding habits, habitat characteristics of the host were prepared according to Froese and Pauly (2017). Specimens of *Anilocra physodes* were deposited in the collections of the Muséum National d'Histoire Naturelle (MNHN), Paris, France (MNHN-IU-2013-18754).

3. Results

Anilocra physodes (Linnaeus, 1758) (Figures 1-5)

Synonyms

Oniscus physodes Linne, 1758: 636. —Linne, 1767: 1060. —Fabricius, 1787: 241

Asellus physodes Olivier, 1789: 255

Cymothoa physodes Fabricius, 1793: 507

Idotea physodes Fabricius, 1798: 320

Anilocra cuvieri Leach, 1818: 350. —Desmaret, 1825: 306. —White, 1847: 109. —Lucas, 1850: 250. —Ellis, 1981: 123. —Bruce, 1987: 91

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Anilocra mediterranea Leach, 1818: 350.—Desmaret, 1825: 306.—Audouin, 1826: 94, pl.11, fig.10-11.—Edwards, 1833: 321-334, pl.14.—Edwards, 1839: 66, fig.1.—Edwards, 1840: 257.—White, 1847: 108.—Lucas, 1850: 250.—Hope, 1851: 32.—Heller, 1866: 741.—Barcelo Combis, 1875: 67.—Bullar, 1877: 254-256.—Stalio, 1877: 234.—Mayer, 1879: 165-179.—Stossich, 1880: 46.—Saint-Loup, 1885: 175-176.—Gourret, 1891: 13-14, pl.I, fig.8.— Ide, 1892: 106, pl.VII, fig.84-92.—Bolivar, 1892: 132.—Gerstaecker, 1901: 255,257, taf.XXVI, fig.2.—Gourret, 1907: 50,89.—Coulon, 1908: 92.—Gibert i Olive, 1919-1920: 87.—Zimmer, 1926-1927: 746.—Gunther, 1931: 1-79.—Demir, 1952-1954: 363-364, fig.150,tab.VI(fig.3).—Balcells, 1953: 550.—Fain-Maurel, 1966: 7-10, fig.1-3.—Ellis, 1981: 123

Canolira albicornis Guérin, 1832-1835: 48.—Gerstaecker, 1901: 257

Anilocra physodes Edwards, 1840: 257.—Lucas, 1849: 77.—Hope, 1851: 32.—Heller, 1866: 741.—Stalio, 1877: 234-235.—Stossich, 1880: 46.—Schioedte and Meinert, 1881: 131-139, tab.IX, fig.4-7.—Carus, 1885: 441.—Saint-Loup, 1885: 175-176.—Buen, 1887: 14.—Bolivar, 1892: 132.—Gerstaecker, 1901: 255-257, taf.XXVI, fig.2.—Tattersall, 1905: 85.—Gourret, 1907: 89.—Nierstrasz, 1915: 80.—Buen, 1916: 363.—Nierstrasz, 1918: 115.—Gibert i Olive, 1919-1920: 87.—Monod, 1923a: 16-18.—Dudich, 1931: 18.—Monod, 1931: 496.—Nierstrasz,1931: 130.—Montalenti, 1941: 357-362, fig.9-11.—Montalenti, 1948: 63-67, tab.VII, 1-6, fig.24-25.—Holthuis, 1950: 7.—Amar, 1951: 530.—Balcells, 1953: 550.—Remy and Veillet, 1961: 54.—Lee, 1961: 470.—Trilles, 1962: 114-118, fig.8-9.—Trilles, 1964a: 110-116.—Trilles, 1964b: 365-369.—Trilles, 1964c: 127-134.—Trilles, 1965: 575-594.—Cicero, 1965: 119, 122-123,125-128, fig.5.—Quintard-Dorques, 1966: 10-11.—Fain-Maurel, 1966: 7-10, fig.1-3.—Trilles, 1968: 85-101, phot.18-21, pl.XXV-XXIX.—Macquart-Moulin, 1969: 266.—Berner, 1969: 93.—Trilles, 1969: 433-445.—Lagarrigue and Trilles, 1969: 117-136, phot.2.—Roman, 1970: 501-514.—Trilles and Raibaut, 1971: 80-81, pl.II.—Ktari-Chakroun and Azouz, 1971: 21.—Romestand, Trilles and Lagarrigue, 1971: 447-450.—Geldiay and Kocataş, 1972: 19, 23-24, fig.I.—Trilles and Raibaut, 1973: 275-276,280.—Romestand, 1974: 571-591, fig.1-13.—Thampy and John, 1974: 580-582.—Trilles, 1975: 347-354, fig.1-74, pl.I.—Lombardo, 1975: 301-316, fig.1-4, fig.5A-C.—Capape and Pantoustier, 1976: 203.—Romestand, Voss-Foucart, Jeuniaux and Trilles, 1976: 981-988.—Trilles, 1977: 10-12.—Romestand, Janicot and Trilles, 1977: 171-180, p.I-IV.—Romestand and Trilles, 1977: 91-95.—Rokicki, 1977: 178.—Holthuis, 1978: 29.—Brusca, 1978: 10.—Romestand and Trilles, 1979: 195-202.—Trilles, 1979: 514.—Romestand, 1979: 423-448, pl.I-IV.—Quignard and Zaouali, 1980: 357.—Williams and Williams, 1980: 578.—Renaud, Romestand, Trilles, 1980: 467-476, pl.I.—Brusca, 1981: 127.—Ellis, 1981: 123.—Korner, 1982: 248-250.—Radujkovic, 1982: 155-161.—Radujkovic, Romestand, Trilles, 1984: 161-181.—Rokicki, 1985: 95-122.—Rokicki, 1984: 1-220, figs.1-68.—Sartor, 1987: 49.—Segal, 1987: 351-360.—Bruce, 1987: 91.— Wägele, 1987: 1-398.—Trilles, Radujkovic and Romestand, 1989: 279-306, fig.1.—Avdeev, 1990: 32-42, fig.1-6.

Anilocra edwardsii Saint-Loup, 1885: 175-176.—Carus, 1885: 441.—Buen, 1916: 363

Anilocra frontalis Monod, 1923b: 84-85

Anilocra mediterranea Sanada, 1941: 209

Livoneca motasi Vasiliu and Carausu, 1948: 176-180, pl.1, fig.1-21

Nec Anilocra physodes (Linnaeus, 1758): Holthuis, 1950: 7.—Fryer, 1968: 40.—Lincoln, 1971: 185, fig.1.—Holthuis, 1972: 22-23, pl.I.—Lanzing and Connor, 1975: 360.—Holthuis, 1975: 65.—Huwae, 1977: 23

Host: Phycis blennoides

Locality: Babakale Port

Infection site: Caudal peduncul

Prevalence: 7.14%

Mean intensity: 1

Total parasite number: 5

Dissected parasite number: 4

Female morphological characteristics: Body (Figure 1) length varies from 25 to 35 mm. Body expands from anterior to posterior, later narrower at 7. pereonite. Body about 2-2.5 times as long as wide. The width of the head is about 2 times the head length. The eyes are small, 0.33 times at head width. Coxal plates visible in dorsal view, posterior margins with sharpened. Pereon longest at pereonite 1, shortest at pereonite 7. Pereon widest at pereonite 6, most narrow at pereonite 1. All pleonites visible in dorsal, the first pleonite distinctly narrow, 2-5. pleonites slightly wider. Pleon 1 largely and pleon 2 partially concealed by pereonite 7. Pleotelson 0.75 times as length as width, posterior margin broadly rounded. Pleotelson not wider than seven pleonite.

Antennula (Figures 2b, 3b) composed of 8 articles, antenna longer than antennula. Antenna (Figures 2 a, 3a) composed of 9 articles, extending to the middle of 1. pereon. Maxillula (Figures 2i, j, 3f) with four terminal spines, one long and three short. Maxilla (Figure 2e, f, 3d) medial and lateral lobe with 2 spines. Mandible (Figures 2 c, d, 3c) palp third article distinctly shorter than others. First and second article without seta, the third article with 16-20 seta. Maxilliped (Figures 2g, h, 3e) article 3 with three hooked spines.

Pereopods (Figures 4a-g) 1-5 nearly in size, 6-7 pereopods longer than others. The behind edge of carpus at all of pereopods include with one setae while only the front edge of the propodus, carpus, merus at seventh pereopod with various seta. Pleopods (Figures 5a-e) 1-3 nearly in size, pleopods 4-5 smaller than others. Pleopods 1 to 5 having peduncle medial margin with 4 hooks. The proximomedial lobe of pleopod 3-5 developed. Fifth pleopod with three curved structures. Uropods (Figure 4h). beyond margin of pleotelson. Exopod slightly larger than endopod. Endopod beyond slightly margin of pleotelson. Uropod peduncle without spines.

Figure 1. *Anilocra physodes* ♀

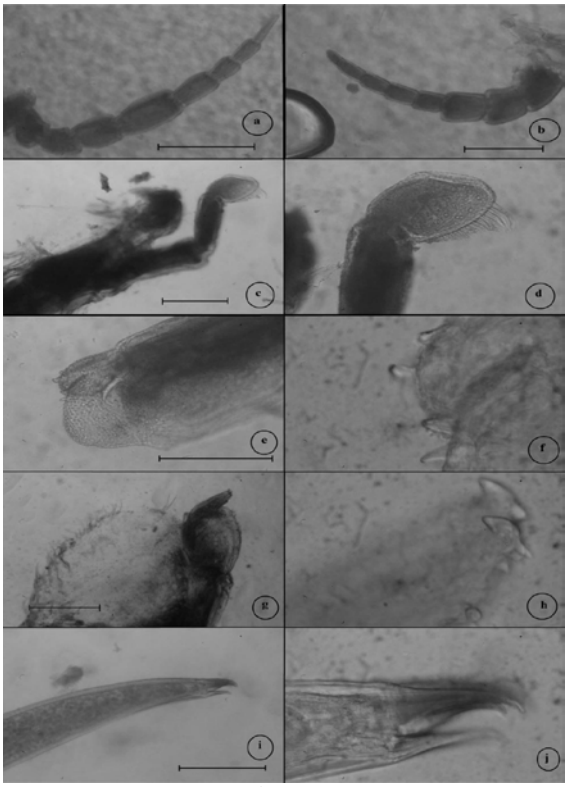

Figure 2. *Anilocra physodes* ♀, a) antenna (1.16mm), b) antennula (1.73mm), c) mandible (0.32mm), d) distal of mandible, e) maxilla (0.43mm), f) distal of maxilla, g) maxilliped (0.35mm), h) distal of maxilliped, i) maxillula (0.67mm), j) distal of maxillula.

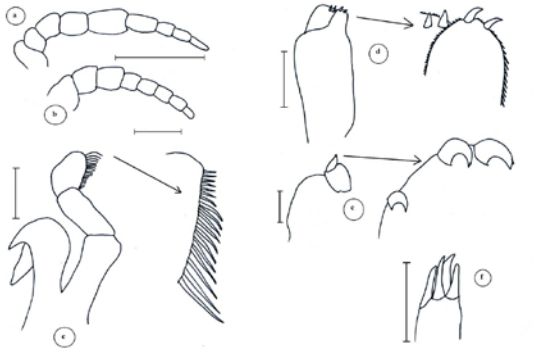

Figure 3. *Anilocra physodes* ♀, a) antenna (1.16mm), b) antennula (1.73mm), c) mandible (0.46mm), d) maxilla (0.43mm), e) maxilliped (0.35mm), f) maxillula (0.18mm).

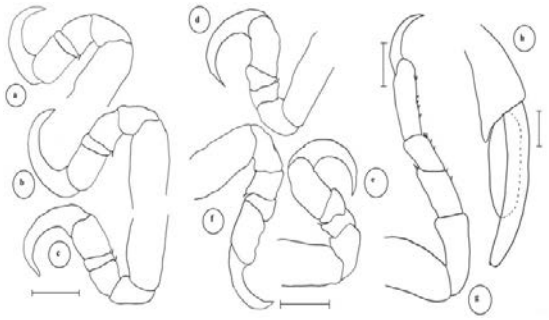

Figure 4. *Anilocra physodes* ♀, a) Pereopod I, b) Pereopod II, c) Pereopod III, d) Pereopod IV, e) Pereopod V, f) Pereopod VI, g) Pereopod VII (1.81mm), h) Uropod (0.72mm).

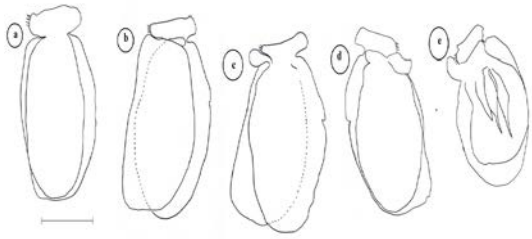

Figure 5. *Anilocra physodes* ♀, a) Pleopod I, b) Pleopod II, c) Pleopod III, d) Pleopod IV, e) Pleopod V (2.51mm).

4. Discussion

Anilocra physodes has been reported from North Atlantic Ocean, Mediterranean Sea, Adriatic Sea (Trilles, 1994). It is associated with Actinopterygii and Elasmobranchii (Table 1). The hosts' parasitism with *Anilocra physodes* was examined according to family characteristics, 28% of 57 hosts belong to Sparidae, %30 to Carangidae, Mugilidae, Centracanthidae, Sciaenidae, Mullidae, Scorpaenidae, 44% to 25 different families. The host's parasitism with *Anilocra physodes* was examined according to habitat selections; 40% of 57 species host fish species are demersal, 26% to benthopelagic, 16% to pelagic-neritic, 11% reef-associated, 5% pelagic-oceanic, 2% bathydemersal. The host parasitism with *Anilocra physodes* according to feeding habits; 68% of 57 species host fish species are carnivorous, 30% omnivorous, %2 herbivorous.

It may be said that this parasite selects the fishes with carnivorous and demersal character. In the present study, the examined *Phycis blennoides* is carnivorous and demersal character fish. It is fit as a preferred host for *Anilocra physodes*.

Table 1. *Anilocra physodes* and hosts

Host species	References
Boops boops	Balcells (1953); Berner (1969); Trilles and Raibaut (1973); Romestand *et al.* (1976); Trilles *et al.* (1989); Akmirza (1998); Charfi-Cheikhrouha *et al.* (2000); Bariche and Trilles (2005); Perez-del-Olmo (2008).
Diplodus annularis	Berner (1969); Trilles and Raibaut (1973); Papoutsoglou (1976); Trilles (1977); Trilles *et al.* (1989); Akmirza (2000); Charfi-Cheikhrouha *et al.* (2000); İnnal *et al.* (2007).
Spondyliosoma cantharus	Holthuis (1972); Trilles and Raibaut (1973); Dollfus and Trilles (1976); Akmirza (2000); Charfi-Cheikhrouha *et al.* (2000); Ramdane *et al.* (2007).
Pagellus erythrinus	Balcells (1953); Berner (1969); Trilles *et al.* (1989); Akmirza (2000); Bariche and Trilles (2005); İnnal *et al.* (2007); Kırkım *et al.* (2008).
Lithognathus mormyrus	Charfi-Cheikhrouha *et al.* (2000); Bariche and Trilles (2005); İnnal *et al.* (2007).
Merluccius merluccius	Balcells (1953); Trilles and Raibaut (1973); Trilles *et al.* (1989).
Spicara smaris	Demir (1952-1954); Berner (1969); Geldiay and Kocataş (1972); Trilles (1977); Trilles *et al*. (1989); Kırkım *et al.* (2008).
Diplodus vulgaris	Papoutsoglou (1976); Akmirza (2000); Öktener *et al.* (2010).
Mullus surmuletus	Papoutsoglou (1976).
Scorpaena porcus	Papoutsoglou (1976).
Umbrina cirrosa	Papoutsoglou (1976).
Solea solea	Papoutsoglou (1976).
Serranus scriba	Papoutsoglou (1976); Kırkım *et al.* (2008); Öktener *et al*. (2009).
Torpedo sp	Gibert i Olive (1919-1920).
Trigla sp	Gibert i Olive (1919-1920).
Lichia sp	Gibert i Olive (1919-1920).
Scorpaena sp	Gibert i Olive (1919-1920).
Naucrates ductor	Gibert i Olive (1919-1920).
Sardina pilchardus	Gibert i Olive (1919-1920), Lee (1961).
Liza ramada	Trilles (1977).
Sciaena sp	Trilles (1977).
Lophius piscatorius	Stalio (1877).
Oblada melanura	Berner (1969); Papoutsoglou (1976); Akmirza (2000);, Öktener *et al.* (2010).
Pagellus sp	Montalenti (1948); Geldiay and Kocataş (1972).
Dentex dentex	Trilles and Raibaut (1973); Trilles and Öktener (2009).
Pagellus acarne	Bariche and Trilles (2005).
Pagrus auriga	Trilles and Raibaut (1973).
Pomatomus saltatrix	Trilles and Raibaut (1973).
Pagrus caeruleostictus	Trilles *et al.* (1989); Bariche and Trilles (2005).
Sarpa salpa	Berner (1969); Papoutsoglou (1976).
Sciaena umbra	Charfi-Cheikhrouha *et al.* (2000); Kırkım *et al.* (2008).
Uranoscopus scaber	Charfi-Cheikhrouha *et al.* (2000).
Serranus hepatus	Trilles *et al.* (1989).
Trachinus draco	Trilles *et al.* (1989).
Atherina boyeri	Trilles *et al.* (1989).
Sparisoma cretense	Thorsen *et al.* (2000).
Siganus luridus	Shakman *et al.* (2009).
Trisopterus capelanus	Berner (1969).
Sparus aurata	Oğuz and Öktener (2007); Kırkım *et al.* (2008).
Spicara maena	Berner (1969); Dollfus and Trilles (1976); Akmirza (2001); Öktener *et al.* (2010);
Spicara sp	Montalenti (1948); Trilles and Raibaut (1973).
Squatina squatina	Nierstrasz (1918).
Zeus faber	Rokicki (1985).
Sphyraena chrysotaenia	İnnal *et al*. (2007).
Liza aurata	İnnal *et al*. (2007).
Raja clavata	Capape and Pantoustier (1976).
Trachurus trachurus	Oğuz and Öktener (2007).
Dentex macrophthalmus	Kırkım *et al.* (2008).
Dicentrarchus labrax	Kırkım *et al.* (2008).
Labrus merula	Kırkım *et al.* (2008).
Chromis chromis	Öktener *et al*. (2009).
Conger conger	Öktener *et al*. (2009).
Belone belone	Öktener *et al*. (2009).
Diplodus sargus	Akmirza (2000).
Mullus barbatus	Roman (1970).
Mugil cephalus	Roman (1970).
Scomber japonicus	Akmirza (1997).

Anilocra physodes was also reported in the cephalopod *Loligo vulgaris* from the northern Tyrrhenian Sea (western Mediterranean) by Gestal *et al.* (1999). There are the symbiotic associations of *Anilocra physodes*, such as that betwen *Obelia geniculata* and *Anilocra physodes* (Stechow, 1921), between epiphytes and *Anilocra physodes* (Öktener *et al.*, 2010). There are some reports as feeding source among diets of some fish (Pais, 2002; Narvaez *et al*., 2015; Châari *et al*., 2016).

The number of articles on antennula and antenna found in the present study agree with findings of Schioedte and Meinert (1881), Montalenti (1948), Trilles (1975), Kussakin (1979), Kırkım (1998). The maxillula with four terminal spines found in the present study is compatible with Trilles (1975), while two spines found by Kussakin (1979). The medial lobe and lateral lobe with two spines of maxilla found in this study are compatible with the findings indicated by Kussakin (1979), while medial lobe with 2 spines and lateral lobe 4 spines found by Trilles (1975), medial lobe 1 spine and lateral lobe with 2 spines found by Montalenti (1948). The third article with setae on the lateral margin of the mandible palp found in this study are compatible with the descriptions of Trilles (1975), Kussakin (1979), while without setae found by Montalenti (1948). Three spines on article 3 of the maxilliped of

ovigerous female observed in this study are compatible with the descriptions of Trilles (1975), while five spines found by Kussakin (1979).

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Karyomorphology of Five *Allium* species from Nagaland, North-Eastern Region of India

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Abstract

The *Allium* species {*A. chinense* (2n=4x=32), *A. tuberosum* (2n=4x=32), *A. hookeri* (2n=22), *A. ascalonicum* (2n=2x=16) and *A. sativum* (2n=2x=16)} with basic chromosome number (x= 8) were compared karyomorphologically using different quantitative and asymmetry parameters. The total sum of long arm (Σq) was observed high (61.70 µm) in *A. hookeri* followed by *A. ascalonicum* (58.87µm), *A. chinense* (58.44µm), *A. tuberosum* (57.87µm) and *A. sativum* (56.78µm), and an exact reverse trend was observed for total sum of short arm (Σp). The maximum mean value of arm ratio was observed in *A. hookeri* (1.75±0.144), covariance total chromosome length (A_2=CV_{CL}) in *A. tuberosum* (31.66), mean centromere asymmetry (M_{CA}) in *A. ascalonicum* (1.10), and covariance centromere index (CV_{CI}) in *A. hookeri* (21.10). The value of relative chromatin (VRC or ACL) was observed similar in tetraploids and diploids. Pearson correlation ($p \leq 0.05$ and $p \leq 0.01$), PcoA and cluster analysis showed the strong interrelationship of studied parameters among the *Allium* species. The karyotic formula (KF) and chromosome categorization (on the basis of chromosome length) was drawn for the *Allium* species (*A. chinense, A. tuberosum, A. hookeri, A. ascalonicum* and *A. sativum*) as follows 26m+5sm+1st (B_7+C_{19}+D_6), 25m+5sm+2st (B_{13}+C_{19}), 12m+9sm+1st (C_{19}+D_3), 12m+3sm+1st (C_{13}+D_3) and 14m+2sm (A_1+B_9+C_6), respectively. Stebbin's classification showed 2A and 1B type of chromosomal asymmetry among *Allium* species.

Key words: Karyotypic Formula, Principle Coordinate Analysis, Cluster Analysis, Inter or Intra Chromosomal Analysis, Stebbins Classification, *A. chinense, A. tuberosum, A. hookeri, A. ascalonicum, A. sativum.*

1. Introduction

The importance of chromosome investigation for basic dissimilarity has been transformed in modern periods. The qualitative or quantitative explanation of chromosome structure has been merged with molecular techniques for a better understanding of the structure, number and behaviour of chromosomes in an organism (genus or species. The interdisciplinary research approach of chromosome has revealed the possible types of karyotypic variation (within and between), systematic relationships, phylogeny and evolution of the related taxa.

The chromosomal symmetry or asymmetry leads to the symmetric or asymmetric differences in the genomic content of an individual and vice-versa. Therefore, the chromosome morphology (or chromosome karyotypes/ idiograms) is an important tool to establish uniqueness among the plant or animal species. The unique quality of a plant or animal species may be improved for various needs through a hybridization program. There is a need to know the chromosome number and structure of every possible organism {especially crops and Rare, Endangered and Threatened (RET) species} for genetic improvement by development of hybridization program (conventional as well as molecular) where both chromosome number and structure can be manipulated.

Allium chinense and *A. bakeri* Regel are known as synonyms to each other and both belong to the Alliaceae family (Bah *et al*., 2012; Allardice, 1997). It has been reported that *A. chinense* supports sub-genus *cepa* in the section of saccuniferum (Dutta and Bandyopadhyaya, 2014). It has been reported that *A. chinense* is a tetraploid (2n=4x=32) plant but some other plants with deviation in chromosome numbers (2n=3x=24; 2n=24 and 2n=33) were also reported (Dubouzet *et al*., 1993; Gohil and Kaul, 1980). Mukherjee and Roy (2012) reported that *A. tuberosum* is a tetraploid (2n=4x=32) plant. *A. hookeri* (subgenus *Amerallium*) is an important member of family Alliaceae. *A. hookeri* recorded chromosome number 2n=22, which is the most common, except for a few (33 and 44 chromosome number) as reported from Yunnan (Sen, 1974; Jha and Jha, 1989; Yi-Xiang *et al*., 1990; Rui-Fu *et al*., 1996). Both *A. ascalonicum* and *A. sativum* were reported as diploid (2n=2x=16) species.

Although very few studies are found on the karyomorphology (not from the Nagaland) of the *A. hookeri* (Ved Brat, 1965; Sharma *et al*., 2011; Toijam *et al*., 2013), *A. tuberosum* (Mukherjee and Roy, 2012; Ramesh, 2015) and *A. sativum* (Konvicka and Levan, 1972), we did not come across reports on *A. chinense* and *A. ascalonicum* from Nagaland as well as adjoining North-Eastern region of India and at National level. The present paper aims to conduct a karyomorphological study of five

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Allium species (*A. chinense, A. tuberosum, A. hookeri, A. ascalonicum* and *A. sativum*), collected from the different parts of the Nagaland, India which may provide additional information to the published data on the *Allium* karyomorphology at world, national or regional levels.

2. Materials and Method

Bulbs of *Allium* species (*A. chinense, A. tuberosum, A. hookeri, A. ascalonicum* and *A. sativum*) were collected from the different parts of the Nagaland and maintained in the Department for root tips. The chromosomal analysis was done according to the conventional root tip squash method (Sharma and Sharma, 1980). The root tips were pre-treated with saturated PDB for 3 h then fixed in carnoy's 1 (3:1 ethanol: glacial acetic acid) solution for 24 h and stored in preservative (70% v/v ethanol) at 4^0C for further use.

2.1. Preparation of slides

Each root tip was washed with distilled water (5 min) and then treated with 1N HCl (15 min). The hydrolysed root tips washed repeatedly with distilled water and stained (5 min) with acetocarmin (2% w/v) and then squashed. Approximately, 10 slides were analysed for each species and the best three slides were observed for number, size and morphology of the chromosomes. The metaphase stages were photographed by Leica digital microscope. The SPSS ver. 16 and ImageJ was used to analyse and measure the long and short arms (µm) of chromosomes and idiograms were prepared.The chromosome classification was done according to the Levan *et al.* (1964).

2.2. Karyotype variation study

The following are the different parameters used to study the karyotypic variations: chromosome number (2n), total chromosome length (TCL), basic chromosome number (x), total haploid chromosome length (THL), mean centromere asymmetry (MCA), covariance of centromere index (CV_{CI}), covariance of total chromosome length (CV_{CL}), mean (q_{Mean}) and summation (Σq) of long arm (q), mean (p_{Mean}) and summation (Σp) of short arm (p), mean arm ratio (AR_{Mean}), mean (RCL_{Mean}) and summation (ΣRCL) of relative chromosome length (RCL), average chromosome length (ACL), mean ($p+q_{Mean}$), summation (Σp+q), difference summation (Σp-q), standard deviation ($p+q_{S.D.}$), variance (V_{p+q}) and covariance (CV_{p+q}) of total chromosome length (p+q), mean (CI_{Mean}) and standard deviation ($CI_{S.D.}$) of centromeric index (CI), karyotypic formula (KF), chromosome categorization and Stebbins classification.

The other indices were also used to analyse the karyotype asymmetry, such as A, A_1, A_2, AI, AsK%, SYi, Rec, TF%, Value of Relative Chromatin (VRC), Centromeric Gradient (CG), Dispersion Index (DI) and Disparity Index (Dis. I).

The detailed formulas for calculations of the different parameters are presented in the form of a table (Table 1)

Table 1. Detailed Formulae used for calculation of different parameters of *Allium* species

Formulae	References
$\mathbf{TF\% = \frac{Total\ sum\ of\ short\ arm\ lengths}{Total\ sum\ of\ chromosome\ lengths} \times 100}$	Huziwara, 1962
$\mathbf{AsK\% = \frac{Length\ of\ long\ arm\ in\ chromosome\ complements}{Total\ sum\ of\ chromosome\ length\ in\ a\ set} \times 100}$	Arano, 1963
$\mathbf{SYi = \frac{Mean\ length\ of\ short\ arms}{Mean\ length\ of\ long\ arms} \times 100}$	Greilhuber and Speta, 1976
$\mathbf{Rec = \frac{Total\ sum\ length\ of\ each\ chromosome/\ Longest\ chromosome}{Total\ number\ of\ chromosomes} \times 100}$	Greilhuber and Speta, 1976
$\mathbf{A1 = 1 - \frac{\Sigma Mean\ length\ of\ short\ arms/\Sigma Mean\ long\ arms\ of\ each\ chromosome\ pair}{Number\ of\ homologous\ chromosome\ pairs} \times 100}$	Romero-Zarco, 1986
$\mathbf{A2 = \frac{Standard\ deviation\ of\ chromosome\ length}{Mean\ chromosome\ length} \times 100}$	Romero-Zarco, 1986
$\mathbf{A = \frac{\Sigma\ Difference\ of\ long\ and\ short\ arms}{\Sigma Sum\ of\ long\ and\ short\ arms} / Number\ of\ homologous\ chromosome\ pairs}$	Watanabe *et al*., 1999
$\mathbf{AI = \frac{Covariance\ of\ chromosome\ length \times Covariance\ of\ centromeric\ index}{100}}$	Arano and Saito, 1980
$\mathbf{CVCL = A2 \times 100 = \frac{Standard\ deviation\ of\ chromosome\ length}{Mean\ chromosome\ length} \times 100}$	Arano and Saito, 1980
$\mathbf{CVCI = \frac{Standard\ deviation\ of\ centromeric\ index}{Mean\ centromeric\ index} \times 100}$	Arano and Saito, 1980
$\mathbf{CG = \frac{Median\ length\ of\ short\ arm}{Median\ length\ of\ chromosome} \times 100}$	Lavania and Srivastava, 1999
$\mathbf{CV = \frac{Standard\ deviation\ of\ chromosome\ length}{Mean\ chromosome\ length} \times 100}$	Lavania and Srivastava, 1999
$\mathbf{Dispersion\ Index\ (DI) = \frac{CG \times CV}{100}}$	Lavania and Srivastava, 1999
$\mathbf{MCA = A \times 100}$	Peruzzi and Eroglu, 2013; Peruzzi and Altinordu, 2014
$\mathbf{Disparity\ index\ (Dis.I) = \frac{Longest\ chromosome - Shortest\ chromosome}{Longest\ chromosome + Shortest\ chromosome} \times 100}$	Mohanty *et al*., 1991
$\mathbf{VRC = \Sigma Total\ Length\ of\ chromosomes/n}$	Dutta and Bandyopadhyaya, 2014

2.3. Chromosome categorization

Chromosomes were categorized on the basis of their length as follows: Type A=5.00µm and above, Type B=4.00µm-4.99µm, Type C=3.00µm-3.99µm, Type D=2.00µm-2.99µm, Type E=1.00µm-1.99µm, and Type F=0.99µm and below.

3. Results

The *Allium* species were collected locally from the different regions of the Nagaland (North Eastern region of India) and the chromosome number from mitotic metaphase images and karyomorphology (karyotype and idiogram) were studied (Figure 1).

The quantitative parameters, such as Chromosome Number, CN (2n=2x), mean length and summation (Σ) of short arm (p), mean length and summation (Σ) of long arm (q), mean Arm Ratio (AR), Average Chromosome Length (ACL), mean and summation (Σ) Relative Chromosome Length (RCL) of *Allium* species (*A. chinense*, *A. tuberosum*, *A. hookeri*, *A. ascalonicum* and *A. sativum*), were analysed and reported in Table 2. The quantitative parameters, such as mean, Standard Deviation (SD), Variance (V), Covariance (CV) and summation (Σ) of total chromosome length (p+q), summation (Σ) of difference between short and long arm (p-q), mean and Standard Deviation (SD) of Centromeric Index (CI), Karyotypic Formula (KF), THL and chromosome categorization, were recorded and presented in Table 3. The inter- and intra-chromosomal quantitative asymmetric indices were calculated and presented for all *Allium* species in Table 4. The Pearson correlation between the inter and intra chromosomal asymmetry indices was performed and the indices, such as A_2, AI, SYi, TF%, CG, Dispersion index and Disparity index showed negative correlation and the indices AsK%, Rec, VRC, CV_{CI} showed positive correlation but not significant for all the indices (Table 5). The Stebbins classification, based on the ratio of longest and shortest chromosome and the proportion of their arm ratio, was provided (Table 6), and, based on that, 2A type of karyotype asymmetry was observed in all the species except *A. ascalonicum* (Table 7).

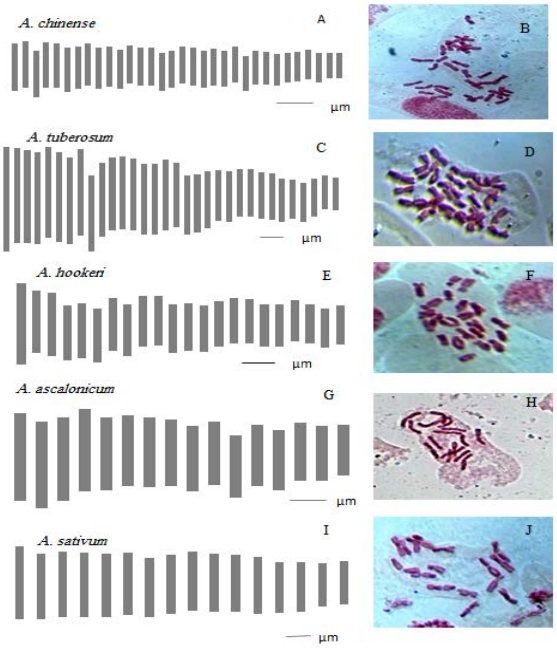

Figure 1. Idiograms (a,c,e,g,i) and Mitotic metaphase (b,d,f,h,j). A) *A. chinense*, B) *A. tuberosum*, C) *A. hookeri*, D) *A. ascalonicum*, E) *A. sativum*

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Table 2. Quantitative karyomorpho-parameters of *Allium* species.

Allium species	CN	BCN (x)	$p_{(mean±S.E.)}$	$q_{(mean±S.E.)}$	Σp	Σq	AR $_{(Mean±S.E.)}$	$RCL_{(mean±S.E.)}$	ΣRCL	ACL
chinense	32	8	1.29±0.053	1.82±0.059	41.58	58.44	1.47±0.082	0.72±0.031	23.14	3.12
tuberosum	32	8	1.31±0.093	1.80±0.097	42.11	57.87	1.50±0.105	0.72±0.036	22.50	3.12
hookeri	22	11	1.74±0.119	2.80±0.129	38.33	61.70	1.75±0.144	0.63±0.044	14.01	4.54
ascalonicum	16	8	2.57±0.150	3.67±0.194	41.12	58.87	1.522±0.150	0.68±0.065	10.89	6.24
sativum	16	8	2.70±0.127	3.54±0.114	43.23	56.78	1.33±0.052	0.75±0.028	12.10	6.25

Table 3. Quantitative karyomorpho-parameters of *Allium* species (Continued).

Allium species	$p+q_{(mean±S.E.)}$	p+q (S.D.)	Σ(p+q)	THL	Σ(p-q)	$V_{(p+q)}$	$CV_{(p+q)}$	CI $_{(Mean±S.E.)}$	CI(S.D.)	KF	Chromosomes category
chinense	3.12±0.087	0.495	99.97	49.985	16.72	0.246	15.87	41.53±1.119	6.334	26m+5sm+1st	$B_7+C_{19}+D_6$
tuberosum	3.12±0.174	0.988	100.02	50.01	15.60	0.976	31.61	41.48±1.324	7.491	25m+5sm+2st	$B_{13}+C_{19}$
hookeri	4.54±0.189	0.891	100	50.00	22.47	0.794	19.62	38.16±1.717	8.053	12m+9sm+1st	$C_{19}+D_3$
ascalonicum	6.25±0.263	1.054	99.99	49.995	17.75	1.112	16.87	41.17±1.807	7.230	12m+3sm+1st	$C_{13}+D_3$
sativum	6.25±0.219	0.877	100	50.00	13.07	0.770	14.03	43.03±0.922	3.689	14m+2sm	$A_1+B_9+C_6$

Table 4. Quantitative inter and intra karyomorphological indices of *Allium* species.

Allium species	Inter and Intrachromosomal quantitative asymmetric indices													
	A	A_1	$A_2=CV_{CL}$	AI	AsK% $_{(Mean±S.E.)}$	SYi	Rec	TF%	VRC	CV_{CI}	CG	DisI	DispI	M_{CA}
chinense	0.005	95.60	15.86	2.418	58.50±1.125	70.87	77.90	41.51	3.12	15.25	41.44	6.57	30.40	0.50
tuberosum	0.004	95.71	31.66	5.714	58.59±1.359	72.77	62.13	42.10	3.12	18.05	41.25	13.05	51.73	0.40
hookeri	0.010	92.70	19.62	4.139	61.85±1.710	62.14	61.59	38.33	4.54	21.10	28.92	5.67	35.41	1.00
ascalonicum	0.011	91.10	16.86	2.960	58.80±1.808	70.02	78.21	41.12	6.24	17.56	43.31	7.30	26.82	1.10
sativum	0.008	91.81	14.03	1.202	56.96±0.926	76.27	79.82	43.23	6.25	08.57	43.75	6.13	25.78	0.80

Table 5. Pearson correlation among the different quantitative chromosomal asymmetry indices.

	A	A_1	A_2=CV_{CL}	AI	AsK%	SYi	Rec	TF%	VRC	CV_{CI}	CG	Dispersion Index	Disparity Index	M_{CA}
A	1	-0.922*	-0.527	-0.304	0.373	-0.449	0.173	-0.468	0.800	0.159	-0.295	-0.628	-0.610	1.000**
A_1		1	0.575	0.485	-0.022	0.086	-0.369	0.111	-0.969**	0.192	0.004	0.565	0.677	-0.922*
A_2			1	0.931*	0.177	-0.034	-0.778	-0.028	-0.591	0.489	-0.118	0.922*	0.983**	-0.527
AI				1	0.504	-0.385	-0.887*	-0.378	-0.589	0.772	-0.392	0.751	0.918*	-0.304
AsK%					1	-0.985**	-0.696	-0.987**	-0.224	0.863	-0.940*	-0.191	0.222	0.373
SYi						1	0.566	0.999**	0.164	-0.835	0.905*	0.315	-0.076	-0.449
Rec							1	0.569	0.513	-0.727	0.711	-0.475	-0.819	0.173
TF%								1	0.139	-0.826	0.912*	0.323	-0.069	-0.468
VRC									1	-0.407	0.218	-0.496	-0.700	0.800
CV_{CI}										1	-0.672	0.219	0.483	0.159
CG											1	0.274	-0.204	-0.295
Dispersion Index												1	0.873	-0.628
Disparity Index													1	-0.610
M_{CA}														1

Table 6. Stebbins classification based on ratio of longest and shortest chromosome and arm ratio of longest and shortest chromosome.

Ratio longest/shortest chromosome		Proportion of arm ratio of longest chromosome and shortest chromosome <2:1			
		1.00 (1)	0.99-0.51 (2)	0.50-0.01 (3)	0.00 (4)
<2:1	(A)	1A	2A	3A	4A
2:1-4:1	(B)	1B	2B	3B	4B
>4:1	(C)	1C	2C	3C	4C

Table 7. Karyotype asymmetry in *Allium* species based on Stebbins classification.

Allium species	Ratio longest/shortest chromosome	Proportion of arm ratio of longest chromosome and shortest chromosome	Stebbins karyotype asymmetry
chinense	1.72	0.74	2A
tuberosum	1.57	0.87	2A
hookeri	1.40	0.88	2A
ascalonicum	1.39	1.94	1B
sativum	1.39	0.80	2A

Recently, statistically correct six parameters (2n, x, THL, M_{CA}, CV_{CL} and CV_{CI}) have been suggested to analyse principle coordinates (PcoA) and chromosome asymmetry. In the present study, we used seven parameters including Total Chromosome Length (TCL) in the earlier parameters to analyse PcoA and phylogram (UPGMA) (Figures 2-3). The inter- (CV_{CL}) and intra- (M_{CA}) chromosomal asymmetry were performed and reported (Figure 4).

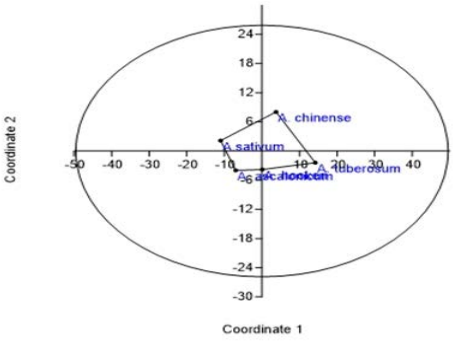

Figure 2. Principle coordinates analysis (PcoA) using six parameters among *Allium* species.

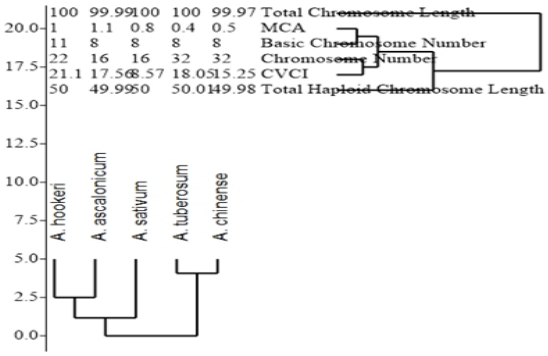

Figure 3. Two Way Euclidean Paired Group Cluster Analysis using six parameters among Allium species.

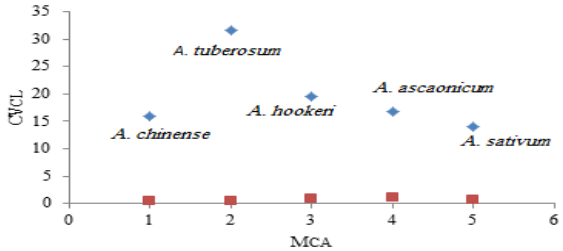

Figure 4. Quantitative inter and intra chromosomal asymmetry among the *Allium* species.

4. Discussion

The chromosome count of *Allium* species was similar as reported by the other studies (Dutta and Bandyopadhyaya, 2014; Mukherjee and Roy, 2012; Sharma *et al*., 2011). Some other studies suggested different chromosome count for presented species (Dubouzet *et al*., 1993; Gohil and Kaul, 1980; Sen, 1974; Jha and Jha, 1989; Yi-Xiang *et al*., 1990; Rui-Fu *et al*., 1996; Sharma and Gohil, 2013; Gohil and Koul, 1973; Talukdar and Sen, 2000). Therefore, doubt continues to remain for the chromosome count in the species analyzed as well as many other *Allium* species (Figure 1).

The mean short arm (p_{mean}) and long arm (q_{mean}) observed maximum in *A. ascalonicum* and *A. sativum*, on the other hand, total sum of long arm (Σq) and Arm Ratio (AR) was maximum in *A. hookeri* which suggests that chromosomes of *A. hookeri* are longer than others. The Average Chromosome Length (ACL) and Value of Relative Chromatin (VRC) are exactly similar and increases from polyploidy to *A. hookeri* to diploids. It may suggest the origin and speciation of the species from diploids to *A. hookeri* to ploids which was supported by the phylogram of the species (Table 2).

The total mean chromosome length ($p+q_{mean\pm S.E.}$) of *Allium* species (*A. chinense* and *A. tuberosum*) and (*A. ascalonicum* and *A. sativum*) was recorded similar (3.12±0.087 and ±0.174) and (6.25±0.263 and ±0.219), respectively. The earlier species were tetraploid and, later, diploid. The mean chromosome length was high for *A. ascalonicum* (2n=16) and *A. sativum* (2n=16) than *A. hookeri* (2n=22), A. *chinense* (2n=32) and *A. tuberosum* (2n=32). It suggests that the diploid species (*A. ascalonicum* and *A. sativum*) have more compact and larger chromatin and may be involved in the formation of the tetraploids (*A. chinense* and *A. tuberosum*). The diploid and polyploidy *Allium* species might have taken the same evolutionary process during the evolution in time and space. The maximum variance (V_{p+q}) and covariance (CV_{p+q}) in chromatin length were observed in *A. ascalonicum* and *A. hookeri*, respectively. *A. ascalonicum* showed more variations between the chromosomes while *A. hookeri* varied within the chromosomes. The mean centromeric index (CI) was recorded 43.03±0.922 (*A. sativum*), 41.53±1.119 (*A. chinense*), 41.48±1.324 (*A. tuberosum*), 41.17±1.807 (*A. ascalonicum*) and 38.16±1.717 (*A. hookeri*), respectively. The high centromeric index suggests that most of the chromosomes are in median region as the chromosomal arms are not exactly equal to make strict metacentric chromosomes (M). The position of centromere is variable in chromosomal arm which depends on the centromeric index of chromosome and suggest the symmetry or asymmetry among the chromosomes. The *Allium* species were recorded with sub-telocentric chromosomal region (st) (centromere near to the terminal region of the chromosome arm) except *A. sativum*. The Karyotypic Formula (KF) and chromosome categorization (on the basis of chromosome length) were drawn for the *Allium* species (*A. chinense, A. tuberosum, A. hookeri, A. ascalonicum* and *A. sativum*) as follows 26m+5sm+1st ($B_7+C_{19}+D_6$), 25m+5sm+2st ($B_{13}+C_{19}$), 12m+9sm+1st ($C_{19}+D_3$), 12m+3sm+1st ($C_{13}+D_3$) and 14m+2sm ($A_1+B_9+C_6$), respectively. The method of measurement of chromosome arms may affect the karyotype asymmetry or symmetry. The chromosomal categorization suggested that *A. sativum* (2n=2x=16) shared its maximum genome with the tetraploids, *A. chinense* (2n=4x=32) and *A. tuberosum* (2n=4x=32) while *A. ascalonicum* and *A. hookeri* shared their maximum genome with *A. chinense* (2n=4x=32) (Table 3).

The inter- or intra-chromosomal asymmetry may be measured from the shifting of centromeric position from median to subterminal or it may be the difference in relative size between the individual chromosome. Stebbins (1971) classified the chromosomal asymmetry on the basis of variation in chromosome length and centromeric position. The higher value of the indices suggested the more asymmetric chromosome complement, while the lower value indicates towards less asymmetric or more symmetric chromosome complement.

The intra-chromosomal asymmetry (A_1) was recorded maximum in *A. tuberosum* and *A. chinense* followed by *A. hookeri*, *A. sativum* and *A. ascalonicum*. The approximate similar chromosomal asymmetry between (*A. tuberosum* and *A. chinense*) and (*A. sativum* and *A. ascalonicum*) indicates the similar genome size or chromosome numbers. The inter-chromosomal asymmetry (A_2) was recorded with maximum chromosome variation in *A. tuberosum* followed by *A. hookeri*, *A. ascalonicum*, *A. chinense* and *A. sativum*. The covariance of the total chromosome length which is a variation within the chromosome of a complement recorded maximum for the *A. tuberosum* with maximum variable chromosomes than others. The measurement of the chromosomal variation with other species (A_2) did not follow the pattern of chromosomal variation within the same species (A_1). The asymmetry index (AI) of chromosomes of a species exactly followed the chromosomal variation with other species (A_2). It also suggests that the total asymmetry of chromosomes of a species is the measure of the covariance of the total chromosome length of a species. Also, the asymmetry indices (SYi, Rec and TF%) provides an average degree of symmetry over whole karyotype of a species. The Value of Relative Chromatin (VRC) ranged from 3.12-6.25µm in all the species, which is very little as compared to the earlier reports in *A. chinense* (27.38 and 26.89) and *A. tuberosum* (26.31 and 26.03) (Dutta and Bandyopadhyaya, 2014) (Table 4).

In the present study, the index A_1 and M_{CA} showed highly negative correlation (-0.922*) and perfect positive correlation (1.000**) with the index A at $p\leq0.05$ and $p\leq0.01$, respectively. The intra-chromosomal asymmetry index may not be dependent on the centromeric asymmetric position variation; the inter-chromosomal asymmetry index (A), however, may be fully or partially dependent on the centromeric position variation in a chromosome. The index A_1 showed highly negative correlation with VRC (-0.969**) and M_{CA} (-0.922*) at $p\leq0.01$ and $p\leq0.05$, respectively. It suggests that A_1 does not dependent on the VRC and M_{CA} for the chromosomal asymmetry. The index A_2 showed highly positive correlation with AI (0.931*), dispersion index (0.922*) and disparity index (0.983**) at $p\leq0.05$ and $p\leq0.01$, respectively. AI showed highly negative (-0.887*) and positive (0.918*) correlation with Rec and disparity index

at $p \leq 0.05$, respectively. AsK% showed highly negative correlation with SYi (-0.985**), TF% (-0.987**) and CG (-0.940*) at $p \leq 0.01$ and $p \leq 0.05$, respectively. SYi showed highly positive correlation with TF% (0.999**) and CG (0.905*) at $p \leq 0.01$ and $p \leq 0.05$, respectively. TF% showed highly positive correlation with CG (0.912*) at $p \leq 0.05$. It was reported that the indices TF% and AsK% perfectly positive or negative correlated with the index SYi. The present results suggested that indices TF% and AsK% highly positive (0.999**) and negative (-0.985**) correlated with SYi which agrees with other authors (Paszko, 2006; Peruzzi *et al*., 2009) (Table 5).

The Stebbins classification, based on the ratio of longest and shortest chromosome and the proportion of their arm ratio, was provided (Table 6) and, based on that, ratio (1.72, 1.52, 1.40, 1.39 and 1.39) and proportion of their arm ratio (0.74, 0.87, 0.88, 1.94 and 0.80), among the Allium species (*A. chinense*, *A. tuberosum*, *A. hookeri*, *A. ascalonicum* and *A. sativum*), were recorded respectively, and 2A type of karyotype asymmetry was observed in all the species except *A. ascalonicum* (Table 7). The Stebbins chromosomal asymmetry (2A) for *A. chinense* in present study supported the earlier reports of *A. chinense* collected from the other parts of North Eastern region (Shillong, Meghalaya) as well as the rest of India (Dutta and Bandyopadhyaya, 2014). The resemblance of karyotype asymmetry may be due to similar type of geographical and climatic conditions in Meghalaya and Nagaland as both are hilly states and near to each other. The earlier reports on *A. tuberosum* (collected from Kolkata, India) and *A. hookeri* (Darjeeling, West Bengal and NBPGR, Uttarakhand) suggested 2B and 3B type of Stebbins karyotype asymmetry, but in present result it showed 2A type of karyotype asymmetry in both the cases (Dutta and Bandyopadhyaya, 2014; Sharma *et al*., 2011). The difference in the karyotype asymmetry may be because of the distance factor in collection site, climate conditions and growth factor of States Kolkata, West Bengal and Uttarakhand which are very far from each other. *A. ascalonicum* and *A.sativum* showed 1B and 2A type of karyotype asymmetry, respectively.

The karyotypic formula, Stebbins classification and value of relative chromatin may be different in the species because of the different methods and application used to measure long and short arm of the chromosomes.

Recently, it has been reported that the karyological characters should be described by quantitative parameters which are statistically correct and without redundancy (Peruzzi *et al*., 2009). The quantitative parameters, such as chromosome number (2n), basic chromosome number (x), Total Haploid Length (THL) of the chromosome (rough estimation of genome size), mean centromeric asymmetry (M_{CA}), covariance of total chromosome length (CV_{CL}) and covariance of centromeric index (CV_{CI}), were suggested for karyomorphological calculation and its study. The suggested parameters were statistically correct and measures three different features of a karyotype without redundancy. The quantitative parameters measure the intra-chromosomal variation (M_{CA}), heterogeneity in the centromere position (CV_{CI}) and inter-chromosomal variation or asymmetry (CV_{CL}).

In present study, we used seven parameters including Total Chromosome Length (TCL) in the earlier parameters to analyse PcoA, phylogram (UPGMA), and inter- and intra-chromosomal asymmetry. The seven parameters as suggested (including TCL) were used to locate the ordinates on the x and y axis of principle coordinates (PcoA) of the five *Allium* species. All the *Allium* species were well distributed in all the quadrates of x and y axis. The distribution indicated that the taken species are not redundant and belong to different species; they also differ karyomorphologically (Figure 2). The same parameters were also used to draw the phylogram (UPGMA) and *A. ascalonicum* and *A. sativum* grouped or placed together in the phylogram. It seems that other species evolved, diverged and speculated from them in time and space (Fig. 3). The covariance of the chromosome length (CV_{CL}) was compared with mean centromeric asymmetry (M_{CA}) and suggested a variation in the species from each other. Intra-chromosomal variation was observed but the centromere variation seems to be near to the axis (Figure 4).

5. Conclusion

The other *Allium* species, such as *A. wallichii* Kunth. (2n=2x=16), *A. roylei* Stearn (2n=2x=16), *A. ampeloprasum* L. (2n=2x=16), *A. schoenoprasum* L. (2n=2x=16), *A. cepa* var. *cepa* Helm. (2n=2x=16), *A. cepa* var. *aggregatum* G. Don (2n=2x=16), *A. fistulosum* L. (2n=2x=16), *A. prattii* Wight (2n=2x=16), *A. stracheyi* Baker (2n=2x=14), *A. macranthum* Baker (2n=4x=28), *A. cepa* var. *viviparum* (Metzger) Alefeld (2n=3x=24; $8^{II}+8^{I}$), *A. porrum* L. (2n=4x=32) and *A. griffithianum* Boiss. Syn. *A. rubellum* M. Bieb. (2n=4x=32), has been observed around the North-Eastern region as well as Eastern Himalaya of the Indian sub-continent; therefore, it may be suggested that *Allium* species may be collected, maintained and preserved in these regions to be scientifically identified at molecular level to reduce the chance of misidentification and redundancy of the species.

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Utilization of Extracted Protein from Fish Fin and Chicken Feather Waste for Alkaline Protease Production by Indigenous Bacteria

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Abstract

Microbial Alkaline Proteases (APs) are of considerable interest in view of their activity and stability at alkaline pH. The present study aims to utilize keratin and collagen extracted from Fish Fin (FF) and Chicken Feather (CF) waste, respectively, for the production of AP enzyme by indigenous bacteria. Both wastes can be sources of solid waste contamination; hence, they were investigated for AP production through microbial degradation. The proteins extracted were added into the production medium containing the bacterial suspension, and assayed for AP production. The process parameters were optimized by One Factor At a Time (OFAT) and the optimum conditions for CF and FF were pH 9.0, temperature of 28°C for CF and 40°C for FF, incubation period was 6 and 10 days for CF and FF, respectively. The optimum carbon source was galactose and glucose for CF and FF, respectively, and the optimum nitrogen source was ammonium chloride and beef-extract, respectively. The inoculum size of 1.5 mL and a protein volume of 0.5 and 2.0 mL for CF and FF, respectively, was recorded. The present study indicates that the protein was successfully extracted from the waste used and degraded by AP enzyme that produced and optimized using OFAT by indigenous bacterial isolates.

Keywords: Animal waste; Environmental pollution; Indigenous bacteria; Protein Extraction.

1. Introduction

Proteases are a group of enzymes that hydrolyze the peptide bond of proteins, breaking them into polypeptides or free amino acids. They constitute 59% of the global market for industrial enzymes (Deng *et al.*, 2010). They have a wide range of application in detergents, leather, food and pharmaceutical industries (Bhaskar *et al.*, 2007 and Jellouli *et al.*, 2009). The sources of proteases include all forms of life, including plants, animals, and microorganisms. Based on their acid-base behavior, proteases are classified into three groups which are acid, neutral and alkaline proteases. The acid proteases perform best at a pH range of 2.0 - 5.0 and are mostly produced by fungi. Proteases with pH optima of 7.0 are called neutral proteases, mainly of plant origin. Proteases that have optimum activity at a pH range of 8 and above are classified as APs, mostly produced by microorganisms. Proteases produced from microorganisms play an important role in several industries, such as detergent, tanning, photographic and pharmaceutical industries (Gupta *et al*., 2002). However, the high cost and lack of long-term stability under storage and process conditions often hampered their applications (Binod *et al.*, 2013; Cavaco-Paulo and Gubitz, 2003). Pollution can be described as the introduction of contaminants which may harm or discomfort living beings into the environment. Pollutants can be in the form of naturally occurring substances or energies; however, they are considered contaminants when in excess of the natural levels (Santos, 1990). The decomposition of nutrients is facilitated by gastrointestinal bacteria through the secretion of physiologically active enzymes, amino acids, and vitamins (Sugita *et al.*, 1997). Besides, few proteolytic bacteria have been previously reported to be related to fresh water and marine fish processing wastes (Sudeepa *et al.*, 2007; Triki-Ellouz *et al.*, 2003). The catalytic properties of AP proved that it is a suitable candidate for industrial applications as in tannery and detergent formulations (Fouzia *et al*., 2017). The present study aims to extract, utilize and optimize the keratin and collagen from CF and FF, respectively, for the production of AP enzyme by indigenous bacteria. The process was optimized to achieve zero solid waste from animal sources.

2. Materials and Methods

2.1. Samples Collection and Preparation

Two types of solid waste, namely Chicken Feather (CF) and Fish Fin (FF) were collected from the wet market, Kuantan area, Pahang, Malaysia, from Sept. 2015

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to April 2016. The wastes moved to the lab immediately, washed, dried at room temperature, and blended into small pieces to a size range of about 2-3 mm (Raid *et al*., 2017).

2.2. Extraction of Keratin from Chicken Feathers

The extraction of keratin from the CF was done as described by Gupta *et al*. (2012). About 25 g of ground CF was added into 1 L of 0.5M sodium metabisulfite (pH 5.0) and incubated at 30 °C with continuous stirring for 6 h. After the incubation period, the solution was filtered and centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected and filtered again using filter paper (Whatman 125 mm Ø). Then, 100 mL of the CF filtrate was added into a beaker and placed on a magnetic stirrer before adding 100 mL of ammonium sulfate drop wise. The mixture was later kept in the chiller at 4 °C for further experiments.

2.3. Extraction of Collagen from Fish Fin Wastes

The extraction of collagen from the FF was done as described by Hashemi-jokar (2014). About 5 g of the ground FF was added in 100 mL of 1M NaOH and stirred continuously using magnetic stirrer for 6 h. To get rid of the stirred non-collagen proteins, the suspension was centrifuged at 7000 rpm for 5 minutes. The pellets obtained were washed with distilled water and mixed by vortex before centrifuging again. Then, 0.5M of acetic acid was added to the pellets and kept for 3 days before centrifuging at 2000 rpm for 1 h. The supernatant was removed and the pellets washed with distilled water. The insoluble materials were soaked in 0.5M EDTA at pH 8.0 for 5 days to remove calcium. After 5 days, the pellets were washed with distilled water by centrifuging at 10,000 rpm for 1 hour. The distilled water was discarded and 0.5M of acetic acid was added until it covered the insoluble pieces. Then, ammonium sulfate was added at a ratio of 1:1 and the mixture centrifuged again at 8000 rpm for 5 minutes. The protein content of the pellets was determined using Lowry *et al*. (1951) method.

2.4. Ammonium Sulfate Precipitation

The crude protein filtrate (100 mL) was poured into a beaker and stirred before slowly adding particles of 80% ammonium sulfate into the filtrate. The calculation of the solid ammonium sulphate to be added at any concentration was obtained by the chart of (Gomori, 1955) as mentioned by (Dixon and Webb, 1964). The solution was centrifuged at 10,000 rpm for 5 minutes; the formed solids were carefully gathered by rinsing with distilled water. More precipitates were formed by the addition of more ammonium sulfate into the supernatant. The protein content was determined by Lowry method of protein assay.

2.5. Inoculum and Production Media Preparation

The bacterial suspension was prepared by mixing 1 g of the waste with autoclaved distilled water. The solution was shaken for three to five minutes; 1 mL of the clear supernatant from the waste suspension was inoculated into 50 mL nutrient broth and incubated for 48 h at 37ºC. M9 minimal salts stock solution (5X) (Sigma-Aldrich) was used (g/L): $Na_2HPO_4.7H_2O$, 64; KH_2PO_4, 15; NaCl, 2.5; NH_4Cl, 5 (Stukus, 1997), after autoclaving, the media was mixed uniformly and cooled down. Then, 2 mL of 1M $MgSO_4$, 0.1mL of 1M $CaCl_2$, and 20 mL of 20% glucose was added to the autoclaved stock solutions. 200 mL of the M9 medium was mixed with 0.5 mL of the extracted protein from CF and FF and inoculated with 1 mL of the bacterial suspension in a conical flask before incubating at 37 °C for 48 h. After the incubation period, the solution was centrifuged to get the Cell-Free Filtrate (CFF) used for AP assay (Raid *et al*., 2017).

2.6. Alkaline Protease Assay

Alkaline protease was determined using the Folin-Lowry method as described by Nisha and Divakaran (2014). A 1.25 mL of Tris buffer (100 mM, pH 9) and 0.5 mL of 1% aqueous casein solution was added into 0.25 mL of CFF and incubated for 30 minutes at 30 °C. Next, 3 mL of 5% Trichloroacetic Acid (TCA) was added and incubated for 10 minutes at 4 °C before centrifuging at 5000 rpm for 15 minutes. The supernatant (0.5 mL) was added to 2.5 mL of 0.5M of sodium carbonate, mixed and incubated for 20 minutes. Thereafter, 0.5 mL of Folin reagent was added and analyzed under UV-Vis at 660 nm. The concentration of protease was measured using a tyrosine standard graph (Takami *et al*., 1989). One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per milliliter per minute under the stated experimental conditions.

2.7. Optimization of the Parameters

To optimize the culture conditions for maximum AP production, different process parameters, such as initial pH-values (5.0, 6.0, 7.0, 8.0 and 9.0), temperature (20, 30, 40, room temperature (RT) and 50°C), carbon sources (glucose, starch, maltose, galactose, xylose, lactose and fructose), nitrogen sources (yeast extract, beef extract, peptone, urea, ammonium chloride, sodium nitrate and ammonium sulfate), incubation periods (2, 4, 6, 8 and 10 days), extracted protein volume (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mL), and bacterial inoculum sizes (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mL), were studied using One Factor At a Time method (OFAT). The effect of these parameters on the production of AP was investigated. All statistics were performed are shown as means ± standard deviations (SD) with sample size (n) indicating the number of independent experiments, and analysis of variance (ANOVA) was used to analyze the observed differences ($p<0.05$).

3. Results and Discussions

3.1. Total Protein Determination

Different quantities of FF and CF samples were used for the extraction of protein content. Table 1 shows the protein contents of the extracted samples determined by the Lowry method of protein estimation. A high protein content was observed in the supernatants of all the samples. The waste pellets were completely dissolved during the extraction steps, leaving no waste from the FF and CF. However, the protein content of FF was lower (0.852 ± 0.050 mg/mL) compared to that of CF (1.183 ± 0.035 mg/mL). The protein content of the samples was higher compared to the control. This may be due to the higher surface area of waste pellets which made more room for the interaction of the chemicals with the small pieces of FF waste. The indigenous bacteria grew well in

the minimal salt medium containing CF as the only carbon source, and degraded 91% of the CF in a period of 7 days (Avinash *et al*., 2011). Similar results were reported for other bacterial isolates (Williams *et al*., 1991) and fungal strains (Kaul and Sumbali, 1999). The pH of the medium was continuously monitored during the course of CF degradation; a gradual increase was observed from an initial value of 7.0 to 8.5; which suggests the possible deamination of peptides and amino acids resulting in the production of ammonia. Such alkalization of media was also reported in the case of keratolytic fungi (Avinash *et al*., 2011).

3.2. Alkaline Protease Assay

The AP activity of the extracts was determined in the presence of the extracted protein by the indigenous bacteria. The concentration of AP was slightly higher in CF (0.254 ± 0.001 U/mL) compared to FF (0.246 ± 0.014 U/mL). This might be due to the amount and/or the type of protein present in each sample (Table 1). The higher the protein content t in the sample, the higher the production of AP enzyme. Casein served as the substrate; tyrosine was liberated during the enzymatic degradation of the Casein either as amino acids or peptide fragments. Folin's reagent was used to develop the color from the reaction with free tyrosine. Hence, the higher the amount of tyrosine from casein, the higher the produced chromophores, and the stronger the protease activity.

Table 1. Screening of AP production and extracted protein content by indigenous bacterial isolates in both FF and CF waste samples

Samples	AP activity (U/mL)	Protein content (mg/mL)
Control	0.0273 ± 0.002	0.058 ± 0.004
CF	0.254 ± 0.001	1.183 ± 0.035
FF	0.246 ± 0.014	0.852 ± 0.050
F-value	3099.706	33.619
P-value	0.00[a]	0.03[a]

Each value represents the AP activity and protein content extracted from CF and FF waste by indigenous bacterial isolates in column of means compared to control. P-value= [a]Significant at $p < 0.05$.

3.3. Optimization of Enzyme Production

The effect of temperature on AP enzyme activity was determined at different temperatures, as presented in Figure 1. The AP enzyme was active at the temperature range of 20–50°C, with an optimum at RT (27 ± 2°C) and 40°C for both CF (0.362 ± 0.016 U/mL) and FF (0.342 ± 0.030 U/mL), respectively. The activity decreased rapidly above this temperature range.

Earlier reports have shown that protease production is maximum at 30°C, and there is a reduction in the enzyme production above this range as the enzyme undergoes thermal inactivation. The enzyme production can be affected by temperature through changes in the physical properties of the cell membrane (Goma, 2013). Nisha and Divakaran (2014) also reported that protease production was highest at 40°C using *Bacillus subtilis*. The incubation temperature to be used generally depends on the microorganisms (Gomaa, 2013). However, in the present study, a mixed culture was used, which explained why the highest temperature for alkaline protease production from the extracted proteins was different. At high temperatures, the enzyme was inactivated, resulting in the low enzyme activity at 50°C for both CF (0.195 ± 0.000 U/mL) and FF (0.201 ± 0.002 U/mL). The effect of temperature on the enzyme production was studied by varying the temperature from 24 to 39°C, with an increment of 3°C while keeping the other parameters constant. It was found that protease production was maximum (397.19 U/g) at 33°C. Also, the enzyme production was favored at a temperature range of 30 to 39°C, showing the ability of the organism to reduce protease enzyme over a wide range of temperature (Renganath *et al*., 2017).

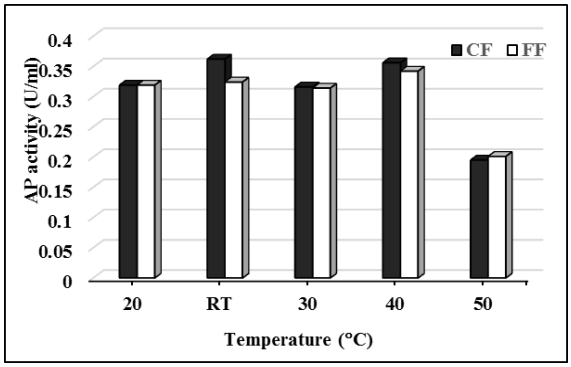

Figure 1: Effect of temperature on AP activity against extracted proteins of CF and FF samples.

Media pH strongly affects many enzymatic processes and transport of compounds across cell membranes. The maximum AP production was achieved at pH 9.0 in both CF (0.217 ± 0.013 U/mL) and FF (0.277 ± 0.034 U/mL) as substrates (Figure 2). The enzymes were inactivated in the acidic medium, resulting in low enzymatic activity. In addition, as the pH deviates from the optimal level, the enzymatic process can be altered. This indicates the low level of enzyme saturation due to pH effect on their stability (Dixon and Webb, 1979). The organism was efficient in protease production at alkaline pH conditions compared to neutral pH (Renganath *et al*., 2017). The maximum protease production was achieved at medium pH 9, while the least was recorded at medium pH 5. The production of protease increased as the pH of the medium increased towards pH 9. After pH 9, there was a decrease in the enzyme production, suggesting a stimulation of enzyme production at alkaline pH. This could be indicative of the alkalophilic nature of the microorganism (Sunita *et al*., 2016).

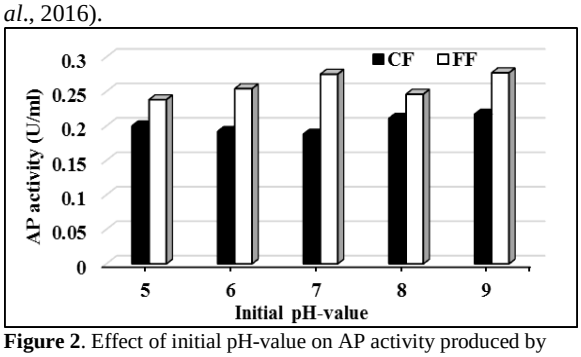

Figure 2. Effect of initial pH-value on AP activity produced by indigenous bacterial isolates from protein extracted based waste

To investigate the effect of different carbon sources on AP production by indigenous bacteria, the glucose of M9

medium was substituted with equal amounts of complex or simple carbon sources. It was observed that galactose showed the highest AP production (0.230 ± 0.011 U/mL), followed by glucose and starch (0.206 ± 0.008 U/mL) in the presence of CF as substrate. Meanwhile, glucose showed the highest AP production (0.233 ± 0.004 U/mL), followed by lactose (0.169 ± 0.010 U/mL) in the presence of FF as substrate (Figure 3). A similar effect of galactose on AP production was observed by Pant *et al*. (2015) who found that galactose gave the maximum amount of alkaline protease, while sucrose gave the lowest amount. The present study shows that the lowest enzyme activity was in the presence of maltose for CF (0.201 ± 0.004 U/mL) and starch for FF (0.148 ± 0.007 U/mL). Generally, the results obtained indicate that monosaccharide sources produced a higher amount of alkaline protease compared to disaccharide and polysaccharides. The difference in enzymatic activities on the carbon sources between CF and FF may be due to the different types of protein present in each sample. Since a mixed culture of microorganisms was used for both samples, different types of microbes utilized different carbon source to produce alkaline protease. The production of alkaline protease was dependent on the available carbon and nitrogen sources in the medium. The addition of carbon sources in the form of either monosaccharides or polysaccharides could influence the production of an enzyme (Sudharshan *et al*., 2007).

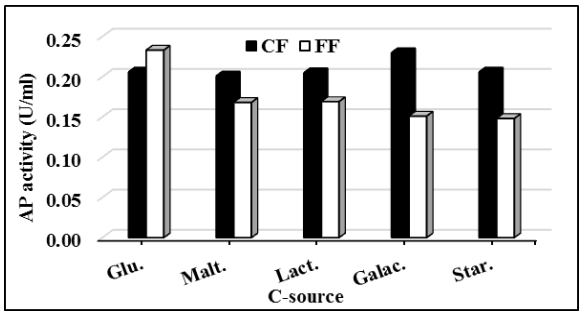

Figure 3. Effect of carbon source on AP production from extracted proteins of CF and FF waste by indigenous bacteria

Various types of nitrogen sources (including organic and inorganic) were evaluated in the M9 medium containing the extracted proteins (Figure 4). The nitrogen source in the M9 medium was substituted with a nitrogen equivalent. It was found that beef extract (0.350 ± 0.000 U/mL) and ammonium chloride (0.414 ± 0.050 U/mL) served as the best organic and inorganic sources for enhancing AP activity in presence of both CF and FF extracted proteins, respectively. Nisha and Divakaran (2014) reported that beef extracts gave the highest alkaline protease production compared to yeast extract, ammonium sulfate, ammonium chloride, urea, and peptone. The requirement for a specific nitrogen source for protease production differs from organism to organism, and also, the alkaline protease biosynthesis depends on the presence of both nitrogen and carbon sources in the production medium (Kole *et al*., 1988). The outcome of the present study is in the line with the findings of Shafee *et al*. (2005) who reported that beef extract, among the different organic nitrogen sources and ammonium chloride among the inorganic nitrogen sources, leads to a high proteolytic activity by *Bacillus sp.* after 48 h of incubation.

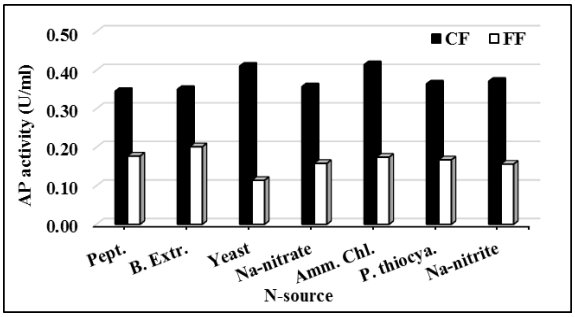

Figure 4: Effect of nitrogen source on AP production from extracted proteins of CF and FF wastes by indigenous bacteria

The optimum volume of the extracted protein required for maximum activity of AP was 0.5 mL (0.364 ± 0.016 U/mL) in CF and 2.0 mL (0.251 ± 0.060 U/mL) in FF (Figure 5). The production of alkaline protease was higher when 0.5 mL of the protein was inoculated. This may be due to the presence of more active sites for more substrate binding. Hence, more alkaline protease enzyme can be produced from lesser enzyme volumes compared to higher protein volume. Conclusively, the lowest enzymatic activity can be observed in the absence of proteins due to the reduced rate of substrate binding. Chandran *et al*. (2016) reported an increase in the protein content at 0.5% (v/v) protein volume. The proteins isolated from 0.1-0.5% (w/v) of the substrates were used as the substrate for the production of protease. The maximum protein content of 0.5% contrasted with our results at 5% protein content (the equivalent of 2.5 mL); though, 2 mL was found as the highest level for AP enzyme production.

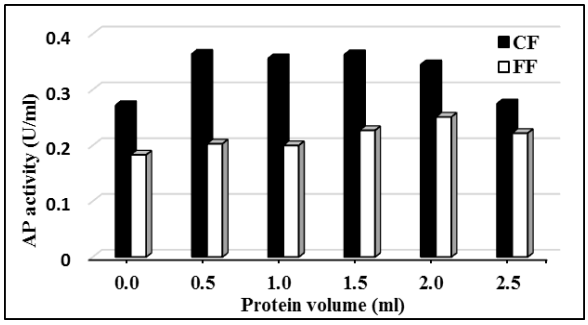

Figure 5. The effect of extracted protein volumes on AP activity by indigenous bacteria

To investigate the effect of incubation period on the production of AP enzyme, the M9 medium was inoculated and incubated at different periods ranging from 2-10 days. The maximum AP production was found after 6 and 10 days of incubation, with enzymatic activities of 0.290 ± 0.001 U/mL and 0.336 ± 0.019 U/mL in the presence of CF and FF, respectively (Figure 6). As for the CF, the production of protease declined at 10 days of incubation because, the enzyme production could have ended with auto proteolysis (Nisha and Divakaran, 2014). However, the production of AP was high after 10 days of incubation with FF likely due to the different proteins in FF compared to CF. The proteins in the FF can yield more alkaline protease when incubated for 10 days. In addition, Kaur *et al*. (1998) reported that the synthesis of enzymes can be associated with the growth of the cell and the incubation period.

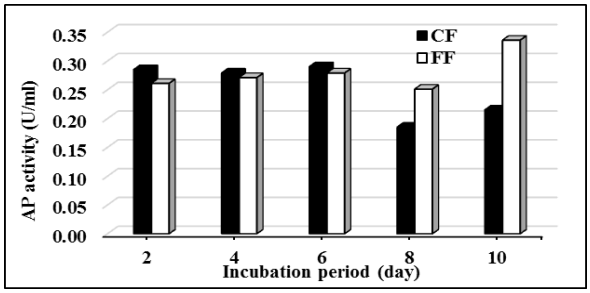

Figure 6. The effect of incubation period on AP activity by indigenous bacteria

The effect of various inoculum sizes (0.25-1.5 mL) was tested and the results are presented in Figure 7. The maximum AP activities of 0.308 ± 0.001 U/mL and 0.450 ± 0.013 U/mL were found with 1.5 mL of the indigenous bacterial inoculum in the presence of CF and FF extracted protein, respectively. Generally, it can be concluded in the present study that large sizes of bacterial inoculum produced the maximum amounts of protease in the presence of both CF and FF. This is because the increased bacterial concentration can increase AP production as more bacteria will be available to degrade the protein in the production medium. On the other hand, smaller inoculum sizes gave the lowest AP production because of the lack of enough bacteria to degrade the protein. Moreover, an upgraded distribution of dissolved oxygen and high nutrient uptake can increase AP production. However, 0.5 mL of the inoculum gave considerable results for FF as AP synthesis with small inoculum size had larger surface areas which contributed to more protease production (Shafee et al., 2005). Renganath *et al*. (2017) reported the highest the protease activity with an inoculum size of 15% after studying a concentration range of 5 to 25%. Furthermore, Divakar *et al*. (2006) reported a higher protease activity with inoculum a concentration of 20% using Wheat bran as the substrate.

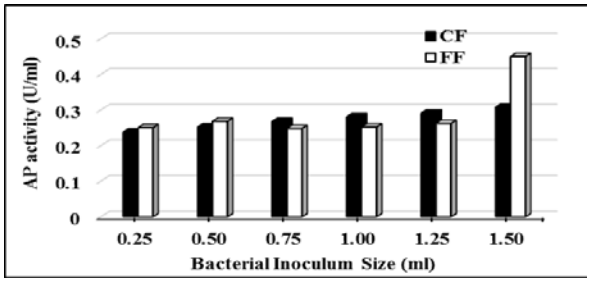

Figure 7. The effect of inoculum size on AP activity by indigenous bacteria

4. Conclusion

The ability to produce AP from waste chicken feathers and fish fins using indigenous bacteria was investigated. The M9 minimal media efficiently supported the production of AP from the extracted CF and FF proteins. The process parameters were optimized for optimum AP production and to reduce the cost of the AP production process industrially. Furthermore, the protein extraction process completely utilized the waste materials, leaving no solid waste afterward. Moreover, CF and FF are inexpensive protein sources for keratin and collagen needed for the cost-effective production of AP. It is revealed that CF and FF can be a potential source of alkaline proteases for use as bacterial additives in many industrial applications. These proteases have good activities at high alkaline pH levels and wide temperature ranges; thereby, permitting their wide biotechnological application potentials in many industries.

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Authors' Contributions

Study Design-Raid D. Thanoon and Essam A. Makky; Data Collection-Raid D. Thanoon and Rubaaini Subramaniam; Statistical Analysis-Essam A. Makky; Data Interpretation- Raid D. Thanoon, Rubaaini Subramaniam and Essam A. Makky; Manuscript Preparation and Literature Search-Raid D. Thanoon and Rubaaini Subramaniam; Funds Collection-Essam A. Makky; Manuscript Revision and Supervision-Essam A. Makky, Mashitah M. Yusoff.

Conflict of Interest Disclosure

The above-mentioned manuscript has not been published before and is not under consideration for publication anywhere else. The publication of this article was approved by all authors, as well as by the responsible authorities.

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Antimicrobial and Antioxidant Activities of Crude Methanol Extract and Fractions of *Andrographis paniculata leaf* (Family: Acanthaceae) *(Burm. f.) Wall. Ex Nees*

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Abstract

Assessment of the antimicrobial and antioxidant activities of different solvent fractions of crude methanol extract of *Andrographis paniculata* leaf was carried out to investigate their medicinal properties. The test samples, crude methanol eextractt and its n-hexane, ethyl acetate, chloroform and water soluble fractions were tested against five clinical isolates: *Enterobacter cloacae, Escherichia coli, Salmonella typhi, Staphylococcus aureus* and *Candida albicans.* All the test samples showed antimicrobial activity against the test organisms, except for *Escherichia coli* which showed resistance to all the samples at the studied concentrations and *Candida albicans* which showed resistance to all the samples except for ethyl acetate with diameter zones of inhibition ranging from 11.5-17.5 mm and water soluble fractions with diameter zones of inhibition ranging from 11.5-13.0 mm; both in concentration dependent manner. The highest flavonoid content (41.79±0.44 µg QE/mg) and phenolic content (26.79±0.66 µg GAE/mg) were shown by the crude methanol extract and the n-hexane fraction, respectively while the chloroform fraction showed the least concentrations for both flavonoid (11.66±0.12 µg QE/mg) and phenolic (16.17±0.61 µg GAE/mg). *In vitro* antioxidant study using 2, 2-diphenyl-1-picrylhydrazyl scavenging assay showed that the crude methanol extract had the highest free radical scavenging activity with average percentage inhibition of 54.50±0.10 while the hexane fraction showed the least with average percentage inhibition of 11.36±0.10. Conclusively, the various solvent fractions of crude methanol extract of *Andrographis paniculata*; especially the ethyl acetate fraction could be considered a remedy for various infections and diseases which are associated with both the test organisms and free radicals.

Key words: *Andrographis paniculata,* Fractions, Antimicrobial, Antioxidant, Phenolic, Flavonoid.

1. Introduction

Andrographis paniculata Nees, commonly called "King of Bitters or Creat or Green Chirayta,'' is an important medicinal plant which belongs to the family Acanthaceae. It is a renowned annual traditional herbaceous crop with immense therapeutic properties (Datta *et al*., 2012) and it is widely cultivated and used in South Asia, India and China. In Ayurvedic formulations, it is one of the most extensively used plants (Okeke *et al*., 2001). It is a hardy and erect herb which grows mainly as an under-shrub in tropical, moist deciduous forest. It has glabrous leaves, about 8.0cm long and 2.5cm broad and white flowers with rose- purple spots on the petals (Nirlep, 2016). The stem is dark green, about 0.3- 1.0m in height and 2-6mm in diameter (Zhang, 2004; Niranjan *et al*., 2010). Some of its vernacular names include; Chuan xin Lian (Chinese), Kalmegh (Urdu), Kirayat (Hindu), Aluy (Philippines), Andrograhis (Spanish/Russian), Senshinren (Japenese) and India echinacea (Indian) (Jarukamjorn and Nemoto, 2008; Mishra *et al*., 2007; Sharma and Sharma, 2013). It is known as *Bhui-neem*, meaning "neem of the ground," since the plant, though being a small annual herb, has a similar strong bitter taste as that of the large Neem tree (Neha, 2016) and in Malaysia, it is known as *Hempedu Bumi*, which literally means 'bile of earth' since it is one of the most bitter plants which are used in traditional medicine. It is sometimes locally referred to as 'Ewe Jogbo' (Jogbo leaf) because of its bitterness but popularly called 'Mejemeje' (seven-seven) among 'Yoruba' speaking natives in Nigeria because an average dosage comprises of seven leaves eaten raw once or twice daily for about five days in the treatment of febrile illness or chronic debility and in the treatment of hypertension (Dada-Adegbola *et al*., 2014). The whole part of *A. paniculata* as well as its roots and aerial parts have been found useful for medicines over the years (Agbolahor *et*

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al., 2014) although (Aniel *et al*., 2010) have stated that the parts of the plant mostly used for medicinal purpose are the leaves and roots. Some of the chemical constituents that have been found in *A. paniculata* are: Diterpenes, flavonoids, iterpenoid, lactones, alkanes, alkaloids, glycoside, tannins, saponins ketones, aldehydes, paniculides, farnesols, polyphenols, arabinogalactan, and several sub-units of andrographolides (Niranjan *et al*., 2010; Akbar, 2011; Sharma and Joshi, 2011).

A. paniculata has been reportedly used for many years to successfully combat various diseases, such as skin infections, herpes, dysentery, fever, sore throat, lower urinary tract infections, gastrointestinal tract and upper respiratory tract infections, inflammation, diarrhea, pneumonia, tonsillitis, gastroenteritis, pyelonephritis and laryngitis (Shalini and Narayanan, 2015; Wangboonskule *et al*., 2006; Mishra *et al*., 2007; Sharma M and Joshi, 2011; Dhiman *et al*., 2012). It has been used as an immune system booster and for the treatment of many chronic infections (Nirlep, 2016; Chandrasekaran *et al*., 2009). Its hepatoprotective effect has also been reported (Abdulaziz *et al*., 2014). It has been reported as antidote for snakebite and poisonous insects (Dhiman *et al*., 2012; Samy *et al*., 2008), anti-diabetes and anti-malarial agent (Agarwal *et al*., 2009; Mishra *et al*., 2009). However, some of its adverse effects may include gastric instability, loss of appetite, diarrhea, metallic taste and allergic reactions; it is not recommended for pregnant women (Sachin and Kailasam, 2017). Various solvent extracts of *A. paniculata* have also been reported for a wide spectrum of pharmacological activities which include antiviral, choleretic, hypoglycemic, hypocholesterolemic, anti-microbial, antioxidant, anti-inflammatory, immunological, antivenomous, antithrombotic, anticancer and antimalaria properties (Hosamani *et al*., 2011; Anurag *et al*., 2017; Sheeja *et al*., 2006; Kumar *et al*., 2001; Mishra *et al*., 2004). In recent times, the main alkaloid in *A. paniculata*; andrographolide has been reportedly confirmed for its anti-HIV activity (Nirlep, 2016).

Although many pharmacological activities of various solvent extracts of *Andrographis paniculata* have been reported, no literature has reported the antioxidant and antimicrobial activities of the various solvent fractions of the crude methanol extract of its leaves; hence the need for the present study.

2. Materials and Methods

2.1. Collection, Preparation and Extraction of Sample

Fresh and healthy leaves of *Andrographis paniculata* were obtained from its plant in a local farm in Ibadan, Oyo state, Nigeria and identified by a specialist in the Botany department of the University of Ibadan, Oyo state, Nigeria. The leaves were oven dried at 50 ^{o}C for 24 hours. 88 grams of the oven dried leaves was pulverized with the use of a laboratory blender (LEXUS MG-2053 OPTIMA) and extracted by maceration in 450 mL methanol, shaken and left for 48 hours. The mixture was filtered and the residue was re-macerated in another 350 mL methanol for 24 hours (three more times) in order to obtain adequate quantity of extract. The filtrates were combined and concentrated under reduced pressure at about 40 ^{o}C with the use of a vacuum rotary evaporator (Eyela N-1001) and this yielded a dark green semi-solid extract. The total average weight of the methanol extract obtained was 18.568 g.

2.2. Fractionation of Crude Methanol Extract

A portion of the crude methanol extract of *A. paniculata* leaves was reconstituted in distilled water and then fractionated successively (by liquid-liquid extraction method) into n-hexane, chloroform and ethyl acetate. Each of the resulting solvent fractions; n-hexane, chloroform, ethyl acetate as well as the water soluble fraction was collected and concentrated under reduced pressure at about 40 ^{o}C with the use of a vacuum rotary evaporator (Eyela N-1001). The methanol extract of *A. paniculata* and its fractions were immediately assayed for their antimicrobial and antioxidant activities using various standard methods.

2.3. Media Preparation

Nutrient agar and Potato Dextrose agar (Rapid Labs) were prepared following manufactures instruction. The media were sterilized in the autoclave at 121 ^{o}C for 15 minutes.

2.4. Antimicrobial Activity

Clinical isolates from stock cultures from Babcock University Teaching Hospital, Ilishan-Remo, Ogun State, Nigeria were used. These included *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter cloacae*, *Salmonella typhi* and *Candida albicans*. The already prepared agar plates were inoculated with 24 hour- old culture by uniformly streaking the surface of the agar in order to achieve uniform distribution of the test organism. A heat sterilized 10 mm cork borer was then used to make wells in the already inoculated medium with the number of wells bored and labelled corresponding to the number of concentrations of plant samples to be tested against each test organism. 100 µL of each concentration of the plant samples was then dispensed into corresponding wells of each set of organisms and allowed to stand for 30 minutes before being incubated at 37 ^{o}C for 24 hours. The inhibitory effect of the crude methanol extract of *A. paniculata* and its fractions on organism growth was assessed after 24 hours of incubation by visual analysis of the growth in each well and values were recorded. Dimethyl sulfoxide (DMSO) was used as the blank while 0.05 % ciprofloxacin was used as the positive control for bacteria while 0.05 % fluconazole for the fungus. All analyses were performed in triplicate. Minimum Inhibitory Concentration (MIC) of the extracts on the test organisms was done at varying concentrations and the results were recorded. The work benches were disinfected while the pathogenic organisms and the materials were autoclaved after use to avoid any form of contamination. Gloves and laboratory coats were also won as personal protective measures against the pathogenic organisms.

2.5. Antioxidant Activity

2.5.1. Free Radical Scavenging Activity

The ability of the crude methanol extract of *A. paniculata* and its fractions to scavenge free radicals was determined according to the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) spectrophotometric method of Mensor *et al*. (2001). One mL of a 0.3 mM DPPH methanol

solution was added to a 2.5 mL solution of the plant sample of various concentrations (200, 400, 600, 800 and 1000 µg/mL) or standard (2, 4, 6, 8 and 10 µg/mL), shaken thoroughly for one minute and allowed to react in the dark at room temperature for 30 minutes. The absorbance of the resulting mixture was measured at 518 nm on a UV-Visible spectrophotometer (JENWAY 6305) and converted to percentage antioxidant activity (AA %), using the formula:

$$AA\ \% = \frac{[(Abs_{control} - Abs_{sample})}{Abs_{control}} \times 100$$

Methanol (1.0 mL) plus extract solution (2.5 mL) was used as blank. 1ml of 0.3 mM DPPH plus methanol (2.5 mL) was used as a negative control. Standard solutions of Gallic acid served as positive controls. This assay was carried out in triplicates for each sample and concentration. The IC_{50} value represented the concentration of the plant sample (extract/ fraction) which scavenged 50 % of the DPPH free radical and this was obtained from the linear regression analysis (Stoilova *et al*., 2007).

2.5.2. Total Phenolic Content

The concentration of phenolics in plant sample (extract/fraction) of *A. paniculata* was determined using the method of Singleton *et al*. (1999). The reaction mixture was prepared by mixing 0.5 mL of methanol solution of extract/fraction (containing 100 µg/mL), 2.5 mL of 10 % Folin-Ciocalteu's reagent dissolved in water and 2.5 mL 7.5 % $NaHCO_3$. Blank containing 0.5 mL methanol, 2.5 mL 10 % Folin-Ciocalteu's reagent dissolved in water and 2.5 mL of 7.5 % of $NaHCO_3$ was concomitantly prepared. The samples were thereafter incubated in a thermostat at 45 ºC for 45 minutes. The absorbance was determined using spectrophotometer (JENWAY 6305) at a wavelength of 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solutions of Gallic acid to obtain a calibration curve (R^2 = 0.8752). Based on the measured absorbance, the concentration of phenolics was read from the calibration curve and expressed in terms of Gallic acid equivalent (mg of GA/g of extract/fraction).

2.5.3. Total Flavonoid Content

Analysis of total flavonoid content of the plant sample (extract/fraction) was done by using aluminum trichloride spectrophotometric method of (Dewanto *et al*., 2002). Quercetin was used as the reference substance. One milliliter of each sample in methanol (containing 100 µg/mL) was diluted with distilled water (4 mL) in a 10 mL volumetric flask. 5 % $NaNO_2$ solution (0.3 mL) was then added to each flask. At 5 minutes, 10 % $AlCl_3$ (0.3 mL) was added and at 6 minutes, 1.0 M NaOH (2 mL) was added. Distilled water (2.4 mL) was then added to the reaction flask and shaken thoroughly. Absorbance of the resulting reaction mixture was then read on a UV-Visible spectrophotometer (JENWAY 6305) at 510 nm. Reagent blank; containing 1 mL methanol in place of the extract was simultaneously prepared and treated in the same manner as the samples. A calibration curve was also prepared by repeating the same procedure for standard solutions of Quercetin (2 to 10 µg/mL, R^2 = 0.986). Based on the measured absorbance of the sample, the total Flavonoid Content was determined from Quercetin calibration curve and results expressed as mg Quercetin Equivalent per gram (mg QE g^{-1}) of the sample on a dry weight basis. The analysis was carried out in triplicates for each sample.

3. Results

The results of the percentage yield of methanol extract of *A. paniculata* as well as its various solvent fractions are shown in Table 1. The results showed that the yield of the methanol extract was 21.10 % of the extracted leaves while its solvent fractions; hexane, ethyl acetate, chloroform and water soluble were 13.10 %, 37.72 %, 11.87 % and 34.16 %, respectively.

Table 1. Percentage yield of methanol extract of *Andrographis paniculata* leaf and its fractions

Test sample	Percentage yield (%)
MEE	21.10±1.03
NHF	13.10±0.99
CHF	11.87±0.07
EAF	37.72±0.76
WSF	34.16±1.21

Data are expressed as mean ± standard error of three replicates. MEE= Crude methanol extract, NHF= n-Hexane fraction, CHF= Chloroform fraction, EAF= Ethyl acetate fraction, WSF= Water soluble fraction

Table 2 shows the results of the zones of inhibition of methanol extract of *A. paniculata* leaf and its fractions. *E. coli* showed resistance to all the test samples at the respective concentrations used. *Enterobacter cloacae* also showed resistance to the n-hexane fraction but showed susceptibility to methanol extract and the remaining fractions.

Table 3 shows the antimicrobial effect of 0.05 % Ciprofloxacin and 0.05% fluconazole on the test organisms. The standard antibiotic, Ciprofloxacin, was found to inhibit the growth of all the test bacteria except *Salmonella typhi* which showed resistance to the antibiotic while *Candida albicans* showed significant susceptibility to 0.05 % fluconazole.

The results presented in Table 4 shows the zones of inhibition of *Candida albicans* by methanol extract of *A. paniculata* leaf and its fractions. *Candida albicans* was resistant to methanol extract, n-hexane and chloroform fractions but was susceptible to ethyl acetate and water soluble fractions. The best inhibition of *Candida albicans* was, however, shown by the ethyl acetate fraction.

The results presented in Table 5 shows the MIC of the methanol extract and its fractions against the bacteria and *Candida albicans*. All the extracts showed MIC of 1 mg/mL for *Staphylococcus aureus* except chloroform which was 5 mg/mL. For *Salmonella typhi*, the MICs of methanol extract and chloroform fraction were 2 mg/mL and 4 mg/mL, respectively, while the MICs of other fractions was 1 mg/mL. All the extracts showed MIC of 1 mg/mL for *Enterobacter cloacae* except n-hexane and water soluble fractions with MIC values of > 20 and 5 mg/mL, respectively. On *Escherichia coli* all the samples

showed MIC of > 20 mg/mL. For *Candida albicans*, MIC values of ethyl acetate and water soluble fractions were 5 and 10 mg/ml, respectively. Meanwhile, methanol extract, n-hexane fraction and chloroform fraction did not show any inhibitions at concentrations lower than 20 mg/ml against *Candida albicans*.

The results of the total flavonoid and phenolic contents of the crude methanol extract of *A. paniculata* leaves and its fractions are represented in Table 6. The crude methanol extract showed the highest flavonoid content (41.79 µg QE/mg) while the least was shown by the chloroform fraction (11.66 µg QE/mg). The flavonoid contents of the others are: 28.77 µg QE/mg, 19.42 µg QE/mg and 17.11 µg QE/mg for water soluble fraction, ethylacetate fraction and n-hexane fraction respectively. However, n-hexane fraction showed the highest phenolic content (26.79 µg QE/mg) while chloroform fraction showed the least (16.17 µg GAE/mg). The crude methanol extract, ethylacetate fraction and water soluble fraction showed phenolic content of 24.96 µg GAE/mg, 20.54 µg GAE/mg, and 20.50 µg GAE/mg, respectively.

Meanwhile, the DPPH scavenging activity of the extract and its fraction are presented in Table 7. From the results, the crude methanol extract gave the best activity with an average percentage inhibition of 54.50 of the DPPH free radical. The ethyl acetate and water soluble fraction showed about the same DPPH scavenging activity while the least was shown by the n-hexane fraction with an average percentage inhibition of 11.36 of the DPPH free radical.

Table 2. Bacterial susceptibility pattern to methanol extract of *Andrographis paniculata* leaf and its fractions

Organism	Fraction	Diameter zones of inhibition (mm) at various concentrations of extract/ fractions			
		5 mg/mL	10 mg/mL	15 mg/mL	20 mg/mL
S. aureus	MEE	16±0.02	14±0.03	15±0.02	16±0.05
	NHF	13±0.12	14±0.07	14±0.01	14±0.11
	CHF	12±0.11	15±0.10	16±0.22	17±0.03
	EAF	14±0.02	15±0.02	15±0.13	15±0.11
	WSF	13±0.04	13±0.13	14±0.01	16±0.03
S. typhi	MEE	13±0.14	15±0.11	17±0.02	19±0.13
	NHF	11±0.02	15±0.02	15±0.04	18±0.04
	CHF	14±0.01	17±0.10	20±0.14	22±0.06
	EAF	17±0.22	19±0.04	22±0.21	23±0.05
	WSF	14±0.04	15±0.03	15±0.07	15±0.01
E. cloacae	MEE	15±0.01	16±0.01	17±0.01	17±0.05
	NHF	R	R	R	R
	CHF	15±0.12	16±0.04	16±0.05	17±0.01
	EAF	15±0.02	16±0.01	17±0.03	17±0.03
	WSF	12±0.02	13±0.01	13±0.04	13±0.01
E. coli	MEE	R	R	R	R
	NHF	R	R	R	R
	CHF	R	R	R	R
	EAF	R	R	R	R
	WSF	R	R	R	R

Data are expressed as mean ± standard error of three replicates. MEE= Crude methanol extract, NHF= n-Hexane fraction, CHF= Chloroform fraction, EAF= Ethyl acetate fraction, WSF= Water soluble fraction, R= Resistant

Table 3. Antimicrobial activities of 0.05 % Ciprofloxacin and 0.05 % fluconazole against the test organisms

Test organisms	Diameter zone of inhibition (mm)
Escherichia coli	22±1.43
Salmonella typhi	R
Staphylococcus aureus	19±1.22
Enterobacter cloacae	20±1.12
Candida albicans	21±0.55

Data are expressed as mean ± standard error of three replicates. MEE= Crude methanol extract, NHF= n-Hexane fraction, CHF= Chloroform fraction, EAF= Ethyl acetate fraction, WSF= Water soluble fraction, R= Resistant

Table 4. Susceptibility pattern of *Candida albicans* to the methanol extract of *Andrographis paniculata* leaf and its fractions

	Diameter zones of inhibition (mm) at various concentrations of extract/fractions (mg/ml)				
Test sample	20 mg/mL	40 mg/mL	60 mg/mL	80 mg/mL	100 mg/mL
MEE	R	R	R	R	R
NHF	R	R	R	R	R
CHF	R	R	R	R	R
EAF	11.5±0.51	12.5±0.51	13±0.13	14±0.66	17.5±0.81
WSF	11.5±0.81	12±0.11	12.1±0.21	12.2±0.022	13.0±0.02

Data are expressed as mean ± standard error of three replicates. MEE= Crude methanol extract, NHF= n-Hexane fraction, CHF= Chloroform fraction, EAF= Ethyl acetate fraction, WSF= Water soluble fraction, R= Resistant

Table 5. Minimum Inhibitory Concentration (mg/ml) of the methanol extract of *Andrographis paniculata* leaf and its fractions

Extracts	*Staphylococcus aureus*	*Salmonella typhi*	*Enterobacter cloacae*	*Escherichia coli*	*Candida albicans*
MEE	1	2	1	> 20	> 100
NHF	1	1	>20	> 20	> 100
CHF	5	1	1	> 20	> 100
EAF	1	1	1	> 20	5
WSF	1	4	5	> 20	10

MEE= Crude methanol extract, NHF= n-Hexane fraction, CHF= Chloroform fraction, EAF= Ethyl acetate fraction, WSF= Water soluble fraction

Table 6. Phenolic and Flavonoid contents of methanol extract of *Andrographis paniculata* leaf and its fractions

Extracts (1000 µg/ml)	Flavonoid (µg QE/mg)	Phenolic (µg GAE/mg)
Methanol extract	41.79±0.44	24.96±1.00
Hexane fraction	17.11±0.51	26.79±0.66
Ethyl acetate fraction	19.42±0.21	20.54±0.51
Chloroform fraction	11.66±0.12	16.17±0.61
Residual fraction	28.77±0.35	20.50±0.43

Data are expressed as mean ± standard error of three replicates. MEE= Crude methanol extract, NHF= n-Hexane fraction, CHF= Chloroform fraction, EAF= Ethyl acetate fraction, WSF= Water soluble fraction

Table 7. DPPH Scavenging activity of methanol extract of *Andrographis paniculata* leaf and its fractions

Extract	Average % inhibition of DPPH	IC50 (µg/mg)
Methanol	54.50±0.10	536.04±1.11
N-Hexane	11.36±0.10	4422.38±1.24
Ethyl acetate	42.61±0.21	703.34±1.44
Chloroform	26.97±0.20	1166.17±1.34
Residual	42.60±0.71	1203.24±1.11

Data are expressed as mean ± standard error of three replicates. MEE= Crude methanol extract, NHF= n-Hexane fraction, CHF= Chloroform fraction, EAF= Ethyl acetate fraction, WSF= Water soluble fraction

4. Discussion

The emergence of multi-drug resistance in human pathogens which threatens the efficacy of commonly used antibiotics (Bandow *et al*., 2003) and the increasing cost of synthetic drugs have necessitated a search for new antimicrobial substances from other sources, especially plants, by pharmaceutical industries. Plants are known to possess a variety of compounds to protect themselves against a variety of their own pathogens and can therefore be considered as potential sources of different classes of pharmaceutical substances. Many synthetic drugs are usually accompanied with a number of side effects when compared with medicinal plants which on the other hand are natural and are perceived to have little or no side effects; they are considered safer, easily accessible and of lower cost (Ghosh *et al*., 2008; Kumar and Pandey, 2012). However, others have argued that determining the precise pharmacological activity and side effects or toxicity of a singular active chemical compound usually present in synthetic drugs is considerably easier as against numerous chemicals normally contained in medicinal plants (Philomena, 2011). This may be attributed to the complexity of interactions and synergies that might occur amongst the numerous chemicals found in crude plant extracts (Philomena, 2011). Several studies have reported that medicinal plants have one or more of their parts which

contain substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (Sofowora, 2008). However, the selective extraction or isolation of each individual pharmaceutical compound from medicinal plants could be time consuming, stressful and capital intensive.

In the present study, leaves of *A. paniculata* were extracted with methanol. The crude methanol extract of the leaves was then reconstituted in water and successively partitioned with n-hexane, chloroform and ethyl acetate to give n-hexane, chloroform, ethyl acetate and water soluble fractions. The crude methanol extract and its fractions were analyzed for their antimicrobial and antioxidant activities. The results of the present study revealed that the extract of *A. paniculata* leaf and its resulting fractions had a significant antimicrobial activity on the test organisms as well as considerably promising antioxidant potential. This could be due to the presence of significant amount of polyphenolic compounds determined in the extract and fractions of *A. paniculata*. Polyphenolic compounds have been reported to serve many functions in plants; some of which are cell wall strengthening, antibacterial and antifungal activities (Furiga *et al*., 2008). Due to the complexity of compound mixture in the plant extract, antioxidant and antimicrobial potential of its fractions did not follow a particular order. This may also be due to the difference in the polarity of the partitioning solvent which may result in extraction of compounds of varied properties in the different fractions.

Andrographis paniculata has been reported to have antibacterial effect on both Gram positive and Gram negative bacteria (Aniel *et al*., 2010; Neha, 2016). In the present study, all the test organisms were susceptible to the test samples except *E. coli* which showed complete resistance to the crude methanol extract and its entire fractions at the studied concentrations. This is in consonant with the findings of (Suparna *et al*., 2014) who also reported strong resistance of *E. coli* to leaf extracts of *A. paniculata*. *Salmonella typhi* on the other hand showed the highest susceptibility to the crude extract as well as its fractions especially at 20 mg/mL concentration. Other studies, carried out on the leaf of *A. paniculata*, used higher concentrations: 100, 200 and 500 mg/mL (Suparna *et al*., 2014) and 750 mg/mL (Aniel *et al*., 2010). It is generally known that the activities of antimicrobials increase with concentration. The results of the present study, however, showed that the inhibitory activity of the crude extract and its fractions against the test organisms was slightly concentration dependent. It is important to note also that *C. albicans* showed strong resistance to all the test samples except the ethyl acetate and water soluble fractions. Ethyl acetate fraction, however, showed the best anti-*Candida* activity. This may imply that ethyl acetate was the most suitable solvent for the extraction of compounds with good anti- *Candida* activities from the crude methanol extract of *A. paniculata*. *Salmonella typhi* showed resistance to the standard substance; 0.5 % Ciprofloxacin, but was susceptible to all the test samples. This might be due to the large array of compounds in the complex mixture of the fractions which may work synergistically to enhance the antimicrobial potency of the fractions. Meanwhile, the importance of efficient liquid-liquid separations has been pointed out to be critical in achieving optimum plant performance (Cusack *et al*., 2009) especially for pharmacological purposes.

The relatively high yield obtained for crude methanol extract in the present study may be due to the ability of methanol to extract both polar and non-polar compounds from plants. Previous authors (Siddhuraju and Becker, 2003) reported the efficiency of methanol for extracting high amount of pharmaceutically important phtyochemicals, such as the polyphenolic compounds from plants. However, the highest and lowest fraction yields were obtained for ethyl acetate and chloroform fractions of the crude methanol extract, respectively. This variation in yield may also be due to variations in polarity of the partitioning solvents used as well as differences in extractability of bioactive compounds. The polarity of extraction solvents has been suggested to play an important role in the ability of plant extracts to exhibit potential antimicrobial activities (Siddhuraju and Becker, 2003; Jigna *et al*., 2006; Sultana *et al*., 2007).

Antioxidants protect the body from the damaging effect of free radicals either by suppressing the formation of the free radicals, scavenge them before they do damage to body cells or repair damage that has been done by them. Medicinal plants are known to contain loads of phytochemicals with outstanding antioxidant properties. One of the most important groups of these phytochemicals is the polyphenolics which are renowned for their free radical scavenging ability (Ravipati *et al*., 2012; Ogasawara *et al*., 2007). In the present study, the free radical scavenging ability of the crude methanol extract and fractions was determined through the degree of discoloration of the methanol solution of DPPH. In the presence of an active free radical scavenger, the absorption vanishes and the resulting discoloration is stoichiometric at a selected range with respect to the degree of reduction (Janaina *et al*., 2009). The solution loses color with increase in concentration of antioxidant as electrons are taken up by DPPH radical from the antioxidant (Calliste *et al*., 2001). The present study reveals that the best antioxidant activity in terms of DPPH scavenging strength was displayed by methanol extract. This could be attributed to its possession of the highest flavonoid content. Flavonoids are a group of polyphenols with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory actions (Wang *et al*., 2006; Frautchy *et al*., 2001; Clavin *et al*., 2007). However, even though the n-hexane fraction contained the highest phenolic content, it showed the least free radical scavenging strength. It is therefore important to note that flavonoid and phenolic are not the only phytochemicals that confer antioxidant properties on plants. Other classes of phytochemicals, such as carotenoids, tannins, volatile oils, α-tocopherols, and ascorbic acid have also been reported to enhance the antioxidant ability of plants (Javanmardi *et al*., 2003; Amarowicz, 2007). Recent findings suggest that diets rich in polyphenolic substances play an important role in combating oxidative stress related disorders due of their antioxidant activities. Hence, polyphenolic constituents of *A. paniculata* could possess the capability to counteract oxidative stress related disorders.

5. Conclusion

The crude methanol extract of *A. paniculata* leaf and its fractions showed considerable antimicrobial and antioxidants activities. The Ethyl acetate fraction in comparison with other fractions showed the best antimicrobial activities. It was therefore concluded that the ethyl acetate fraction of the leaves' crude methanol extract contained most of the bioactive components with both antibacterial and anti-Candida activities. However, the best antioxidant activity was exhibited by the crude methanol extract and this was attributed to its possession of highest flavonoid content. The present study further supports the traditional use of this plant for the treatment of various infections and diseases, such as food poisoning, typhoid, diarrhea, urinary tract infection, boil, skin rashes, inflammation, aging, heart disease, cataracts etc. for which the test organisms (bacteria and fungus) and free radicals may be implicated or associated with. The present study of antioxidant and antimicrobial evaluation of the different fractions of methanol extract of *A. paniculata* leaves forms a primary platform for further phytochemical and pharmacological studies. In addition to carrying out researches on the phytotoxicity of the plant as well as establishing a safe dosage regime, further works on the characterization, isolation and purification of the active compounds from the extract is imperative. This would pave way for further evidence based investigations to ascertain whether whole plant extracts are better for pharmacological purposes than pure compounds extracted from them or vice versa.

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Optimization of Factors Influencing Cellulase Production by Some Indigenous Isolated Fungal Species

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Abstract

The aim of the present study is to isolate and characterize efficient cellulose degrading fungi from their common natural habitats and optimize the factors influencing the production of cellulase enzyme. Fungi were isolated from soil, tomatoes and oranges samples which were collected from different sites in Khartoum and Khartoum North. Cellulase production was studied after cultivation of fungi in cellulose containing media. The conditions were optimized by using production media containing Carboxymethyl Cellulose (CMC) and inoculated by fungi with different biotechnological parameters, such as temperatures (28 ºC, 37 ºC, and 50 ºC), pH (3.0, 5.0 and 9.0) and substrate specificity of carbon (lactose, maltose and sucrose) & nitrogen sources (urea, yeast extract and sodium nitrate). Among eight initial isolates, three isolates (A, B, and C) were finally screened as the most efficient cellulase producer fungal isolates. These isolates were confirmed as (A) *Aspergillus niger* (B) *Fusarium solani* and (C) *Trichoderma viride*. Out of these isolates, the maximum zone of hydrolysis (54.33±1 mm) was obtained for 'isolate A, whereas the minimum zone of clearance (19.67±1mm) was recorded for *Trichoderma viride.* Cellulase activity and amount of cellulase produced by the three test microorganisms were determined and compared. The results obtained from the fermentations showed that *Aspergillus niger* produced the highest amount of cellulase among the test microorganisms (2.9 IU/ml) at pH 5 and temperature of 50 ºC on Day 5 of fermentation.

Key words*:* Cellulase, Cellulose, *Trichoderma, Fusarium, Aspergillus.*

1. Introduction

Cellulose is the most abundant component of plant biomass, exclusively in plant cell walls (Lee *et al*., 2002). Cellulose is totally insoluble in water (Lederberg, 1992). It is a linear, unbranched homopolysaccharide consisting of glucose subunit joined together via 1-4 glycosidic linkages. Individual cellulose molecules (polymer) vary widely in length and are usually arranged in bundles or fibrils (Walsh, 2015). Within the bundles, cellulose molecules can occur in crystalline or paracrystalline (amorphous) structures (Walter, 1998).

The hydrolysis of cellulose can be done by using enzymes known as cellulases to produce glucose, which can be used for the production of ethanol, organic acids and other chemicals (Koomnok, 2005). Cellulase refers to a class of enzyme that catalyze the hydrolysis of 1, 4 β-D glycosidic linkages in cellulose. These enzymes are mainly produced by fungi, bacteria and protozoans (Jagdish and Pawandeep, 2012).

Cellulases can convert world's most abundant biopolymer, 'cellulose' into reducing sugars and used in many biotechnological applications (Bhat, 2000). A hefty portion of these applications are accounted for instance cotton handling, paper reusing and as creature encourage added substances. (Yano *et al*., 2012). It is used for bioremediation, wastewater treatment and also for single cell protein (Alam *et al*., 2005). Cellulases are likewise utilized for deinking of fiber surfaces in paper ventures and to improve mash seepage in material businesses. (Penttila *et al*., 2004).

This compound is significant in nourishment sciences, like sustenance handling in espresso, drying of beans by for effective cleansing of juices when utilized blended with pectinases. It is also useful in plant protoplast isolation, plant viruses' investigations, metabolic and genetic modification studies (Chandara *et al*., 2005; Shah, 2007). This enzyme has also pharmaceutical importance, treatment of phytobezons - a type of bezoar cellulose existing in human's stomach - (Ali and Saad, 2008).

Fungi are the main natural agents of cellulose degradation; they are widely distributed in nature and used for commercial production of cellulases. Most of the fungi elaborate one or more cellulolytic enzymes including endoglucanase, exoglucanase and β- glucosidase (Bhat and Hazlewood, 2003). Amongst fungi, species of *Trichoderma* and *Aspergillus* are well known for cellulolytic potential (Lee *et al*., 2002).

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Generation of cellulases by fungal isolates requires ideal conditions for their development which prompts the obtaining of extracellular chemical molecules. The development conditions and additionally extracellular protein generation conditions are probably going to shift among test microorganisms. The real parts of generation medium, like carbon and nitrogen sources and physical parameters like temperature, pH and brooding time, were observed to be basically influencing the cellulase creation consequently should be enhanced for each isolate (Polyanna *et al*., 2011).

Therefore, the present study aims to investigate high level production of extracellular Cellulases by *Trichoderma*, *Aspergillus* and *Fusarium* and optimizing cultural parameters to enhance cellulase enzyme production.

2. Materials and Methods

2.1. Area of Study

The present study was conducted at the Department of Microbiology and Molecular Biology, Faculty of Science and Technology, Al-Neelain University, Khartoum – Sudan. All experiments were done aseptically in the laboratory of microbiology.

2.2. Collection of Sample

Five random soil, tomatoes and oranges samples were collected from different areas in Khartoum and Khartoum north. The samples were then brought to the laboratory of Microbiology.

2.3. Isolation of Test Microorganisms

Potato Dextrose Agar (PDA) (Hi-media, India) was prepared according to the manufacturers' recommendations with pH 5.6, sterilized at 121 ºC and 15 Ibs for 15 minutes and poured into the pre-sterilized Petri dishes, the plates were let to solidify at room temperature.

One gram of soil was transferred to aliquots of 10 mL sterile distilled water in test tube. It was shaken vigorously at constant speed for 10 min. The soil suspension was then subjected to serial tenfold dilutions. An amount of 0.1 ml soil suspension from the appropriate dilution (10^{-2}) was transferred to Petri dish containing PDA medium. While an infected spot in tomato and orange samples were swabbed and placed on the middle of the plate of PDA media. The plates were incubated for 5 days at 28ºC. The well-growing spread single colonies were picked up and further sub-cultured on potato dextrose agar (Aneja, 2005).

To confirm their purity and their viability, the isolates were examined macroscopically by determination their culture characteristics. Also they examined microscopically using Lactophenol cotton blue stain technique and compared with those listed in standard reference books (Domsch *et al*., 1980).

2.4. Screening for Cellulase Enzyme Production

Associated fungi were tested for their ability to produce cellulase enzyme by the plate assay method using 1% carboxymethyl cellulose in a modified basal salt media. According to Hankin and Anagnostakis, (1977) at the incubation period, 0.1% Congo red solution was added and counterstained with 1 M NaCl for 15 minutes. The zone of cellulose hydrolysis appears as a clear area around the colony.

2.5. Production of Cellulase Enzyme

Cellulase production was carried out by using cellulose as the sole carbon source in 250ml an Erlenmeyer flask containing broth media. The composition of the medium was in (g/l in distilled water peptone (0.1%), urea (0.03%), $MnSO_4.7H_2O$ (0.0016%), $ZnSO_4.7H_2O$ (0.0014%), $(NH_4)_2SO_4$ (0.14%), $MgSO_4.7H_2O$ (0.03%), $FeSO_4.7H_2O$ (0.05%), $CaCl_2$ (0.01%), $CON_2O.6H_2O$ (0.0029%), KH_2PO_4 (0.2%) and cellulose (1%), the pH value of the media was 5. For the cellulolytic fungi test, 1% CMC, 1.5% agar and 1 ml of Triton-X-100 were also added to the media.

An Erlenmeyer flask (250ml) containing this media was autoclaved at 121ºC and 15 Ibs for 15 minutes. The cellulose medium was inoculated with two plugs (5mm diameter) of fungi isolates from 5 days old culture and incubated on a shaker (Orbital shaker, Gerhardt, Bonn) at 121rpm. After 5 days of cultivation the culture filtrates were filtered off (Whatman filter paper No.1) and transferred into falcon tube to centrifuge (China), at 10,000 rpm for 15 minutes to remove cell debris. The supernatants were used to determine the cellulolytic activity by the standard assay method (Jadhav *et al*., 2013).

2.6. Cellulase Assay for Enzyme Production

Filter paper activity (FPase) for total cellulase activity in the culture filtrate was determined according to the standard method (Eveleigh *et al*., 2009). Aliquots of appropriately diluted cultured filtrate as enzyme source were added to Whatman no. 1 filter paper strip (1 × 6 cm; 50 mg) immersed in one milliliter of 0.05 M sodium citrate buffer of pH 5.0. After incubation at 50ºC for 1 hour, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method (Ghose, 1987). One unit of filter paper (FPU) activity was defined as the amount of enzyme required to release 1 μmole reducing sugars from filter paper per ml per min under standard assay condition (Gilna and Khaleel, 2011).

2.7. Optimization of Culture Conditions for Cellulose Enzyme Production under Submerged State Fermentation (SmF)

Certain factors were examined and optimized to obtain the highest enzymatic yields, those include:

2.7.1. Effect of pH on Cellulase Enzyme Production:

To determine optimal pH, fungus cultures were cultivated in a 250 mL flask containing 50 mL optimized medium with different pH values 3.0, 5.0 and 9.0. The pH of the medium was adjusted by using 1 N HCl and 1 N NaOH. The flasks were kept in stationary stage at 28 ºC for 5 days of cultivation. After 5 days of incubation the culture broths were filtered off (Whatman No.1 filter paper) and transferred into falcon tube to centrifuge at 10,000 rpm for 15 minutes to remove cell debris. The supernatants were used to assay cellulase enzyme activity by using DNS method (Ghose, 1987). The absorbance was measured using UV-Spectrophotometer at 540nm.

2.7.2. Effect of Temperature on Cellulase Enzyme Production:

In order to determine the optimum temperature for cellulase production by the fungal species, fermentation was carried out at 28 ºC, 37 ºC, and 50 ºC. The flasks were incubated for 5 days of cultivation. After 5 days of incubation the culture broths were filtered off (Whatman No.1 filter paper) and transferred into falcon tube to centrifuge at 10,000 rpm for 15 minutes to remove cell debris. The supernatants were used to assay cellulase enzyme activity by using DNS method (Ghose, 1987). The absorbance was measured using UV-Spectrophotometer at 540nm.

2.7.3. Effect of Carbon Sources on Cellulase Enzyme Production:

Various carbon compounds namely, sucrose, lactose and maltose were used. The broth was distributed into different 250 ml flasks and 1% of each carbon sources were then added before inoculation of the strain and after culture inoculation, the flasks were incubated for 5 days at 28 ºC. After 5 days of incubation the culture broths were filtered off (Whatman No.1 filter paper) and transferred into falcon tube to centrifuge at 10,000 rpm for 15 minutes to remove cell debris. The supernatants were used to assay cellulase enzyme activity by using DNS method (Ghose, 1987). The absorbance was measured using UV-Spectrophotometer at 540 nm.

2.7.4. Effect of Nitrogen Sources on Cellulase Enzyme Production:

The fermentation medium was supplemented with organic and inorganic compounds (Sodium nitrate, urea and yeast extract) replacing the prescribed nitrogen source of the fermentation medium. After 5 days of incubation the culture broths were filtered off (Whatman No.1 filter paper) and transferred into falcon tube to centrifuge at 10,000 rpm for 15 minutes to remove cell debris. The supernatants were used to assay cellulase enzyme activity by using DNS method (Ghose, 1987). The absorbance was measured using UV-Spectrophotometer at 540 nm.

3. Results and Discussion

3.1. Test Microorganisms

Eight initial fungal isolates including molds (*Aspergillus*, *Trichoderma*, *Fusarium*, *Penicillium*, *Alternaria*, and *Curvularia*) and yeasts (Two *Candida* species) were isolated from soil samples, tomatoes and oranges. Three isolates were selected as active cellulase producers, namely *Aspergillus niger*, *Trichoderma viride* and *Fusarium solani*. Other excluded either as saprophytes or non-cellulase producers.

Figure 1. Morphological and cultural characteristics of *Apergillus niger* in PDA using 10 X objective lens

Figure 2. Morphological and cultural characteristics of *Trichoderma viride* in PDA using 10 X objective lens

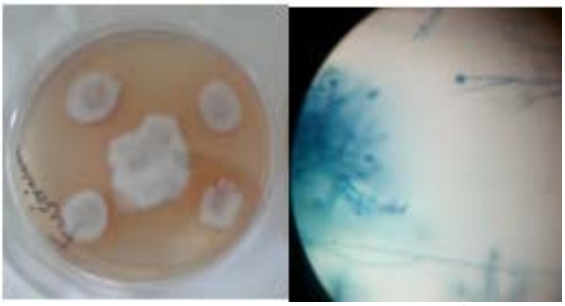

Figure 3. Morphological and cultural characteristics of *Fusarium solani* in PDA using 10 X objective lens

3.2. Screening of Fungi for Cellulase Enzyme Activity

Screening of fungi for their cellulase activity was carried out by the hydrolysis of substrate incorporated in the basal salt medium. After an incubation for 15 minutes, enzyme activities were detected by the appearance of zones either by substrate clearances or coloration and discoloration around the fungal colonies. Three fungal isolates, *Apergillus niger*, *Trichoderma viride* and *Fusarium solani*, showed the highest zone around the colony (Figure 4 (a-d)), used for further study. All the fungal isolates exhibited cellulase activity.

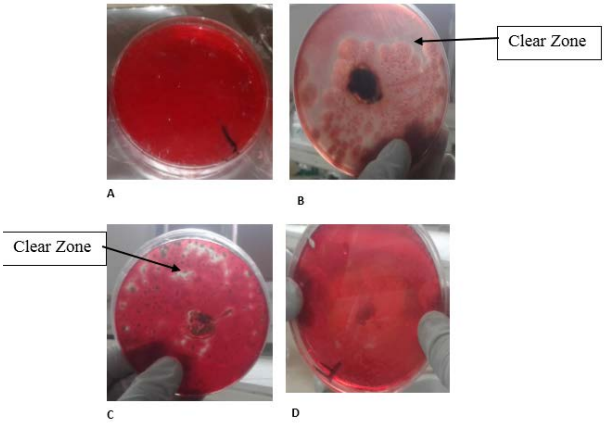

Figure 4. Screening for cellulase enzyme production in BSM at 28 ºC for 15 minute using 1% Congo red (a) Negative control (un-inoculated Petri plate) (b) *A. niger* (substrate clearance) (c) *T. viride* (discoloration around the fungal colonies) (d) *F. solani* (change of indicator colour entire the Petri plate from deep red to pink which indicates the cellulase production)

3.3. Cellulase Enzyme Assay

The cellulase enzyme was detected as yellow coloration after the addition of 3 ml DNS reagent to mixture of sodium citrate and metabolite filtrate of pre-inoculated basal medium (Figure 5).

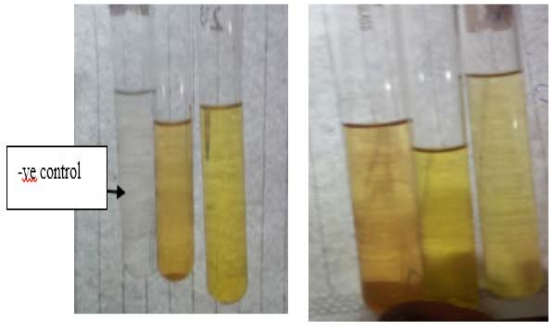

Figure 5. Production of cellulase indicated by the yellow color (Right), the clear solution indicates the negative control which was mixture of sodium citrate and metabolite filtrate of pre-inoculated basal medium without the addition of DNS reagent (Left)

3.4. Optimization of Culture Conditions for Cellulase Enzyme Production

3.4.1. Effect of pH on Cellulase Enzyme Production

Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes and affects product stability in the medium. The optimal pH varies with different microorganisms and enzymes. All the three isolates were allowed to grow in media of different pH values 3, 5, and 9. The maximum enzyme activity was observed in medium of pH 5.0 in case of *Aspergillus niger* (1.3U/ml) showed (Figure 6, Table 1). The findings of the present study are in agreement with many scientists, such as Beldman *et al*. (1985) who reported that *Aspergillus* species grow and metabolize well in acidic pH medium between pH 3.0 –5.0. Their study investigated that maximum cellulase production from *A. oryzae* was reported when the pH of the medium was adjusted to 5. Ali *et al*. (1991) also stated similar results and he noted that there are certain fungal genera have an ability to produce cellulases include *Aspergilli*. Also the present findings were in line with Pothiraj *et al*. (2006) who determined that the *Aspergillus niger* and *Aspergillus terreus*, *Rhizopus stolonifer* and *Trichoderma* species have an ability to produce cellulases. Shafique and Bajwa, (2009) revealed that pH was the key factor that affects the production of cellulases enzymes from *T. harzianum*. The findings of the present study are more than the findings of Lee *et al*. (2002) who noted that CMCase, Avicelase and FPase activities exhibit a pH optimum of approximately 4.

Table 1. Yield of cellulase enzyme U/ml under different pH value

pH value	Test microorganisms		
	A. niger	*F. solani*	*T. viride*
3	0.91	1.14	0.35
5	1.3	1.25	0.46
9	0.53	0.16	0.18

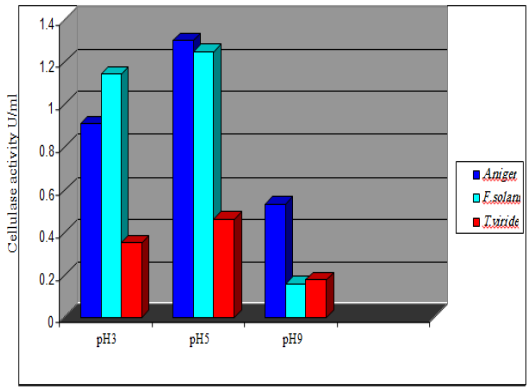

Figure 6. Effect of pH on enzyme production, the most effective pH value was 5. *A. niger* and *F. solani* produce pronounced cellulase at acidic pH and less at alkaline pH

3.4.2. Effect of Temperature on Cellulase Enzyme Production

The results of the test made at different temperatures value (28 ºC, 37 ºC, and 50 ºC) showed that the optimal temperature for cellulase activity (2.9 U/mL) produced by *A. niger* at 50 °C (Table 2, Figure 7). Many researchers have reported different temperatures for maximum cellulase production either in flask or in fermenter studies using *Aspergillus* sp. and *Trichoderma* sp. suggesting that the optimal temperature for cellulase production also depends on the strain variation of the microorganism (Lu *et al*., 2003). The present results disagreed with Immaneul *et al*. (2007) who estimated the optimum temperature for cellulase enzyme production by *A. niger* and *A. fumigatus* at 40 ºC.

Table 2. Yield of cellulase enzyme U/ml under different temperature

Temperature (ºC)	Test microorganisms		
	A. niger	*F. solani*	*T. viride*
28	2.3	2.6	0.96
37	2.19	1.35	1.81
50	2.9	2.44	0.35

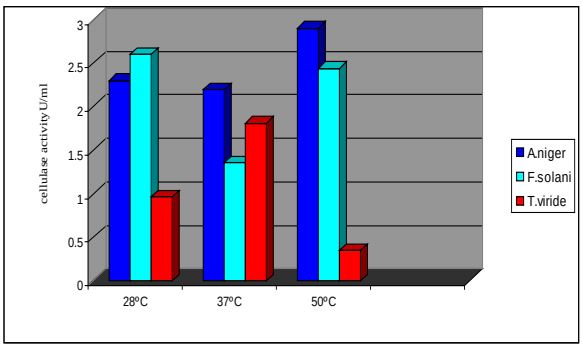

Figure 7. Effect of temperature on enzyme production, *A. niger* and *F. solani* have a wide range of temperature to produce cellulases while *T. viride* produces cellulases with very good amount at ambient temperature 37 ºC. The production of cellulase was reduced when *T. viride* grown at high temperature (50 ºC)

3.4.3. Effect of Carbon Sources on Cellulase Enzyme Production

Various sources of carbon, such as lactose, maltose and sucrose, were used to replace glucose which was the original carbon source in growth media. Results obtained showed that *Fusarium solani* in presence of sucrose brought about the maximum cellulase production compared to other carbon sources (Table 3, Figure 8). This result is in line with Asma *et al*. (2012) who reported that sucrose was the most effective as a sole carbon source for the cellulolytic enzymatic activity. Also, the findings of the present study are in line with Kilikian *et al*. (2014) who estimated that the production of cellulases by certain species of *Trichoderma* was enhanced by the presence of sucrose as a carbon source. Findings of the present study are contrary to those of Vinod *et al*. (2014) who observed that glucose was a sole carbon source.

Table 3. Yield of cellulase enzyme U/ml under different carbon sources

Carbon source	Test microorganisms		
	A. niger	*F. solani*	*T. viride*
Sucrose	1.44	2.14	2.12
Lactose	2.09	1.9	0.53
Maltose	1.18	1.49	0.15

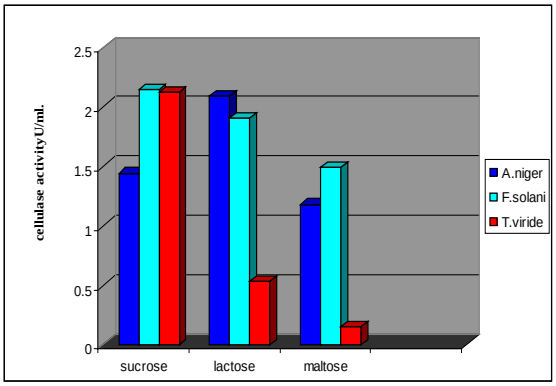

Figure 8. Effect of Carbon sources on enzyme production, the best Carbon source for cellulase production by all test fungi was sucrose; *A. niger* and *F. solani* have an ability to utilize other Carbon sources (lactose and maltose) and produce cellulases but *T. viride* affected negatively when subjected to medium containing lactose and maltose

3.4.4. Effect of Nitrogen Sources on Cellulase Enzyme Production

Results indicate that the sources of nitrogen greatly affected the production of cellulase enzyme. Sodium nitrate was the best nitrogen source for *Aspergillus niger* (Table 4, Figure 9). The current results are in accordance with Swati *et al*. (2014) who reported that inorganic nitrogen source sodium nitrate was found to enhance mean activities. Also similar results were reported by different workers with *Alternaria* spp. including *Alternaria helianthi*, *Alternaria triticina* and *Alternaria sesame* Jha and Gupta, (1988). But the finding is in disagreement with the work of Pothiraj and Eyini (2007) who reported that good cellulase production can be obtained with the

organic nitrogen sources, such as yeast extract and peptone.

Table 4. Yield of cellulase enzyme U/ml under different nitrogen source

Nitrogen source	Test Microorganisms		
	A. niger	*F. solani*	*T. viride*
Yeast extract	0.43	1.8	1.7
Urea	0.99	0.55	2
$NaNO_3$	2.64	2.4	1.74

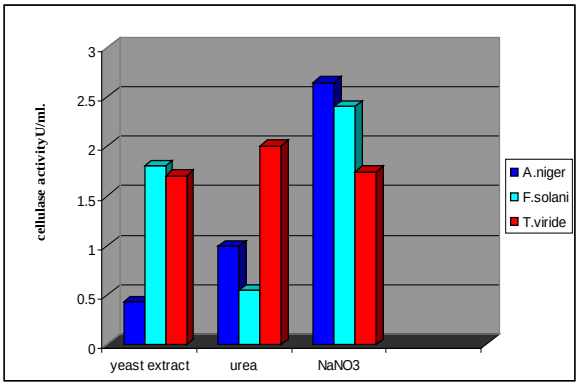

Figure 9. Effect of Nitrogen sources on enzyme production, production of cellulase by *T. viride* was not affected by the different sources of nitrogen and the amount almost similar. *A. niger* produces cellulase significantly when grown in $NaNO_3$ and less significant when use urea and yeast extract as nitrogen sources. *F. solani*, significantly produces cellulases when grown in $NaNo_3$ and yeast extract while produces less amount when grown in medium use urea as nitrogen source

4. Conclusion

The results of the present study showed that all isolated fungi which were confirmed as (A) *Apergillus niger* (B) *Fusarium solani* and (C) *Trichoderma viride* have cellulolytic enzymatic activity. The best result obtained from the fermentations showed that *Aspergillus niger* produced the highest amount of cellulase among the test microorganisms (2.9 IU/ml) at pH 5 and temperature of 50ºC on Day 5 of fermentation.

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Growth Promotion and Phytopathogen Inhibition Potentials of Lemon Grass (*Cymbopogon citratus*) Endophytic Bacteria

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Abstract

Fresh and apparently healthy leaves and roots of lemon grass were collected and surface - sterilized using 70% (v/v) Ethanol, 3% (v/v) sodium hypochlorite solution and sterile distilled water. Isolation of endophytic bacteria was achieved using culture technique while, characterization was done based on morphological, biochemical and microscopic characteristics. Growth promotion potentials of some selected isolates were tested using tomato and millet seeds. Similarly, antagonistic potentials against *Fusarium oxysporum* were evaluated. A total of 16 endophytic bacteria were isolated and identified as *Bacillus* spp (3 isolates), *Escherichia coli* (1 isolate), *Klebsiella pneumoniae* (3 isolates), *Micrococcus* spp (3 isolates), *Pseudomonas* spp (1 isolate), *Rhizobium* spp (2 isolates) and *Staphylococcus aureus* (3 isolates). Growth promotion test showed that, only *K. pneumoniae* significantly improved ($P < 0.05$) the germination time, germination percentage, shoot length and fresh weight of tomato seeds. None of the bacteria showed evidence of improving any of the parameters of germination of millet seeds. All the endophytic bacteria significantly inhibited ($P < 0.05$) the growth of *F. oxysporum. S. aureus* yielded the largest (21.30 mm) while, *Bacillus cereus* yielded the smallest (17.2 mm) zone of inhibition. Moreover, all the isolates especially *S. aureus* significantly inhibited ($P < 0.05$) the growth of *F. oxysporum*. In conclusion, Lemon grass harbours a variety of endophytic bacteria some of which showed potentials of enhancing the emergence and development of tomato seedling, and also have antagonistic activity against *F. oxysporum.*

Keywords: Endophytic bacteria, Lemon grass, *Fusarium oxysporum*, Growth promotion, Biocontrol.

1. Introduction

The recent surge in the need to exploit the health benefits that microbial inoculants may give to plants as well as, the desire to reduce the use of chemicals due to health and ecological concerns, has fuelled interests in studying an array of bacteria and fungi called "Endophytes". Hallmann *et al.* (1997) defined endophytic bacteria as all bacteria that can be detected inside surface-sterilized plant tissues or extracted from inside plants and having no visibly harmful effect on the host plants. This definition includes internal colonists with apparently neutral behaviour as well as symbionts. It also includes bacteria, which migrate back and forth between the surface and inside of the plant during their endophytic phase.

Bacterial endophytes are found in a variety of plants, ranging from herbaceous plants, such as maize and beet, to woody plants (Ryan *et al*., 2007). Bacteria belonging to the genera *Bacillus* and *Pseudomonas* are easy to culture, and the cultivation-dependent study has identified them as frequently occurring endophytes (Seghers *et al*., 2004). *Bacillus* sp. and *Enterobacter* sp. were found in maize (Surette *et al*., 2003; McInroy and Kloepper, 1995), *Klebsiella pneumoniae* in soybean (Kuklinsky-Sobral *et al*., 2004), *Rhizobium leguminosarum* in Rice (Yanni *et al*., 1997), *Rhizobium* in carrot and rice (Surette *et al*., 2003), *Escherichia coli* in Lettuce (Ingham *et al*., 2005). Indeed, numerous reports have shown that endophytic microorganisms can have the capacity to control plants (Sturz *et al*., 1997; Duijff *et al*., 1997; Krishnamurthy and Gnanamanickam, 1997), insects (Azevedo *et al*., 2000) and nematodes (Hallmann *et al.* 1997, 1998). In some cases, they can also accelerate seedling emergence, promote plant establishment under adverse conditions (Chanway, 1997) and enhance plant growth (Bent and Chanway, 1998).

Cymbopogon citratus, commonly known as the Lemon grass, is a tropical herb that is popular in south East Asia and Africa. The plant has a plenty of medicinal uses, prominent among which is its application as antihelmintic, aphrodisiac, appetizer and laxative. It is used in Ayurvedic medicine in the treatment of epilepsy, leprosy and bronchitis (Parrotta, 2001).

Strobel *et al*. (2004) reported that, close to 300,000 different plant species exist on the earth each of which hosts one or more endophytes. Only a fraction of these plants have been fully explored relative to their endophytic biology. In view of the medicinal and other uses of *C. citratus*, a study on its endophytic microorganisms would be of great impact. In an earlier study, Deshmukh *et al.* (2010) reported 24 different fungal species belonging to 21 genera isolated from the leaves and rhizomes of *C.*

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citratus. To the best of our knowledge, no previous studies have been done regarding the endophytic bacteria of the same plant, hence the need for this study. The current study, therefore, aims at evaluating the plant growth promotion and biocontrol potentials of endophytic bacteria isolated from *C. citratus*.

2. Materials and Methods

2.1. Sample Collection

For the isolation of endophytic bacteria, fresh and apparently healthy leaves and roots of *C. citratus* were collected using a sterile scissors, during the rainy season from the Botanical Garden of the Department of Biological Sciences Bayero University Kano Nigeria. All samples were immediately transported in sterile bags to the Microbiology laboratory of Bayero University Kano for analysis.

2.2. Sample Pre-Treatment and Surface Sterilization

Upon the arrival of the samples at the laboratory, they were processed immediately without any delay as follows: The leaves and roots of the plant were washed separately under running tap water to remove adhering soil particles, and the majority of microbial surface epiphytes. The samples were then subjected to surface sterilization procedure as follows: An initial wash in sterile distilled water to remove adhering soil particles, 1 minute immersion in 70% (v/v) ethanol, followed by a 2 minute immersion in 3% (v/v) sodium hypochlorite and finally, a three times rinse in sterile distilled water (Hallman *et al*., 1997).

2.3. Isolation of Endophytic Bacterial Isolates

To target a wide range of endophytes, five different isolation media were used, i.e., Yeast extract sucrose agar (Yeast extract 4.0 g; Sucrose 20.0 g; KH_2PO_4 1.0 g; $MgSO_4$ 0.5 g; Agar 15.0 g in 1.0 L distilled water, pH adjusted to 6.2 ± 0.2 and autoclaved at 121 ^oC for 15 minutes) which is selective for the isolation of *Rhizobium* species, Nutrient agar (Oxoid), MacConkey agar (Oxoid), Nutrient broth (Oxoid), yeast extract agar (Sigma-Aldrich) and Brain heart infusion agar (Oxoid).

The isolation followed the protocol of Sheng *et al*. (2008) with some modifications. Each of the collected *C. citratus* samples was aseptically homogenized in a sterile blender (Panasonic MS-337N) and a three-fold serial dilution was carried out after which, 1 mL aliquot from each dilution was inoculated in triplicates on the various growth media using pour plating method. The cultures were then placed in an incubator (Gallenkamp series) at room temperature for 48 hours. Individual colonies were picked and streaked on fresh culture media for purification to generate pure cultures. Control cultures of the surface-sterilized but unhomogenized leaves of the plant were also prepared and incubated at similar conditions with the test culture plates.

2.4. Morphological and Biochemical Characterization of the Bacterial Isolates

Cell morphology of the pure cultures obtained was determined by the Gram staining method (Bartholomew, 1962). Biochemical tests, such as catalase, coagulase, oxidase, indole, methyl red, Voges-Proskauer urease activity, citrate utilization, cellulose hydrolysis, starch hydrolysis and triple sugar iron tests were done according to the procedures described by Cappuccino and Sherman (2000). Endospore staining and capsule staining were also carried out.

2.5. Evaluation of Plant Growth Promoting Effects of the Endophytic Bacteria on Tomato and Millet Seeds

A total of nine isolates were randomly selected and tested using Petri plate trials in order to evaluate their growth promotion effects on tomato and millet seedlings. A loopful growth of each bacterial isolate was inoculated in 10 mL of Luria-Bertani (LB) broth **(**HIMEDIA**)** in a test tube, and incubated for 24hrs. Tomato and millet seeds were obtained from the Department of Crop Protection, Bayero University Kano. The seeds were surface-sterilized by immersing in 70% ethanol (1 minute) and 2% sodium hypochlorite (2 minutes) and then rinsed thoroughly in sterile distilled water. The surface-sterilized seeds were added to the inoculated LB medium (ten per test tube), and incubated for 24 hrs to allow bacterial penetration. Another set of ten surface sterilized seeds of tomato and millet each, were inoculated in sterile LB broth for 24 hrs in order to serve as negative control. The culture fluid was then aseptically decanted and the treated seeds from the test tubes were then planted in Petri dishes layered with moistened cotton wool. Seedlings were grown at room temperature with regular watering. After 10 days of nursing, growth parameters, such as height, fresh weight, number of leaves of the seedlings, and time of germination of the seeds, were both measured. The test was conducted in triplicates as adopted by Ji *et al*. (2014).

2.6. Evaluation of Antagonistic Effect of the Endophytic Bacteria against F. Oxysporum

Fusarium oxysporum, a soil-borne fungal pathogen of plants was collected from the culture collections of the Plant Biology Department of Bayero University Kano. The identity of the fungus was authenticated by sub-culturing on potato dextrose agar (BIOMARK Laboratories). The culture was incubated at room temperature for five days. Morphological characteristics and reverse pigmentation of the fungus on PDA were noted and recorded. A sterile needle was used to pick a small portion of the mycelium of the test fungus, and this was transferred on to a drop of lacto phenol cotton blue on a clean glass slide. The preparation was then carefully emulsified so as to disperse the inoculum. A cover slip was placed carefully and finally; the preparation was viewed under the microscope using × 100 oil immersion objectives. Features, such as the nature of hyphae, spore types and spore attachment, were observed and recorded. Final authentication was done by making reference to Benson (1998). A needle-full mycelial mat of freshly cultured *F. oxysporum* was picked using a straight wire loop, and placed on one side of a Petri dish containing PDA and the fresh culture of the endophytic bacterial isolate was streaked on the other side of the plate. A minimum of 35 mm separation was maintained between the organisms. The PDA plates were incubated at 28^o C for 7 days. The antagonistic effects of the bacterial endophytes against the fungus were confirmed by inhibition zones

formed between the bacterial endophytes and the fungus. A PDA plate inoculated with *F. oxysporum* only, served as the control. The test was carried out in triplicates (Ji *et al*., 2014).

2.7. Statistical Analysis

All data obtained (in triplicates) were tested for statistical significance using the Statistical Package for Social Science (SPSS) version 21.0. General linear model multivariate analysis was used to test the data obtained from the germination tests of tomato and millet seeds and means were separated using Least Significant Difference (LSD). Data from the antagonistic tests of the endophytic bacteria on *F. oxysporum* were tested using one-way ANOVA. Means were separated using LSD. All analyses were carried out at 5% level of significance.

3. Results

3.1. Occurrence and Morphological Characteristics of Endophytic Bacteria of Lemon Grass

The various endophytic bacteria and their frequency of occurrence are represented in Table1. A total of 16 endophytic bacteria were isolated. Among these, 10 (62.5%) were isolated from the roots, while the remaining 6 (37.5%) were isolated from the leaves of the plant. The bacteria belong to the genera *Bacillus*, *Escherichia*, *Klebsiella*, *Micrococcus*, *Pseudomonas*, *Rhizobium* and *Staphylococcus*.

Table 1. Distribution of Endophytic Bacterial Genera in the Roots and Leaves of Lemon Grass

Bacterial isolates	Root	Leaves
Bacillus	2	1
Escherichia	1	0
Klebsiella	2	1
Micrococcus	2	1
Pseudomonas	0	1
Rhizobium	2	0
Staphylococcus	1	2
Total	10 (62.5%)	6 (37.5%)

3.2. Growth Promotion Potentials of the Endophytic Bacteria

This was carried out to evaluate the potentials of the isolates in enhancing tomato and millet seeds germination. The effects of the bacteria on the germination of tomato seeds are presented in Table 2. Statistical analysis of the result showed significant difference between the mean values of all the germination parameters when tested jointly ($P < 0.05$). A separate ANOVA conducted between subjects showed significant difference ($P < 0.05$) between the mean values of germination time, germination percentage, length of shoot, fresh weight. No significant difference ($P > 0.05$) was observed between the mean values of the number of leaves. Multiple comparison tests showed that, only the mean germination time of *K. pneumoniae* (2.0 days), and *E. coli* (3.3 days) were shorter than the corresponding value yielded by the control (3.7 days). However, it is only the mean germination time of *K. pneumoniae*-treated seeds that was statistically different ($P < 0.05$) from all others including the control. Similarly, the germination percentage of 100 and 96.7 were recorded for *K. pneumoniae*, and *E. coli*-treated seeds, respectively. As with germination time, only the germination percentage of *K. pneumoniae*-treated seeds was statistically greater ($P < 0.05$) than that of all others, including the control. For shoot length, only *K. pneumoniae*-treated seeds (4.80 cm) yielded better than the control (4.20 cm). The values were also found to be statistically different ($P < 0.05$). The mean fresh weight yielded by *K. pneumoniae*-treated seeds (0.050 g) and *S. aureus* (0.040 g) were greater than the value yielded by the control (0.033 g). However, only the mean fresh weight of *K. pneumoniae*-treated seeds was statistically different ($P < 0.05$) from that of the control.

The result of the germination test of millet seeds, as presented in Table (3), show the control yielding the mean germination time, mean germination percentage, number of leaves and shoot length of 2.6 days, 45.3%, 1 leaf, and 4.0 cm, respectively. None among the endophytic bacteria-treated seeds yielded better results in all the parameters tested. However, the mean fresh weight results showed yields of 0.040, 0.033 and 0.033 g from *E. coli*, *K. pneumoniae*, *and Micrococcus* spp treated seeds, respectively, and these were higher than the fresh weight of 0.030 g yielded by the control. However, the values were not significantly different ($P < 0.05$) from one another and the control.

Table 2. Effects of Endophytic Bacteria on Tomato Seeds Germination

Endophytic Bacterium	Germination Time (Days)	Germination Percentage	Number of Leaves	Length of Shoot(cm)	Average Fresh Weight(g)
Bacillus subtilis	5.5 ± 0.29	90 ± 0.00	2 ± 0.00	3.4 ± 0.31	0.030 ± 0.00
Bacillus cereus	4.0 ± 0.00	46.7 ± 3.33	2 ± 0.33	4.2 ± 0.10	0.030 ± 0.00
Escherichia coli	3.3 ± 0.33	96.7 ± 3.33	2 ± 0.00	3.1 ± 0.03	0.030 ± 0.00
Klebsiella pneumoniae	2.0 ± 0.00	100 ± 0.00	2 ± 0.33	4.8 ± 0.42	0.050 ± 0.00
Micrococcus spp	6.3 ± 0.33	53.3 ± 3.33	2 ± 0.00	3.1 ± 0.35	0.020 ± 0.00
Micrococcus luteus	7.0 ± 0.33	53.3 ± 3.33	2 ± 0.00	4.2 ± 0.09	0.031 ± 0 .00
Rhizobium spp	4.3 ± 0.33	26.7 ± 3.33	1 ± 0.00	2.7 ± 0.15	0.022 ± 0.00
Staphylococcus aureus	6.3 ± 0.33	63.3 ± 3.33	2 ± 0.33	4.2 ± 0.20	0.040 ± 0.00
Control	3.7 ± 0.33	93.3 ± 6.67	2 ± 0.33	4.2 ± 0.17	0.033 ± 0.00

Results are values of three replicates ± the S.E (Standard error)

Table 3. Effects of the Endophytic Bacteria on Millet Seeds Germination

Endophytic Bacterium	Germination Time (Days)	Germination Percentage	Number of Leaves	Length of Shoot(cm)	Fresh Weight(g)
Bacillus subtilis	8.4 ± 0.18	23.6 ± 0.89	1.0 ± 0.00	3.6 ± 0.03	0.020 ± 0.02
Bacillus cereus	8.3 ± 0.10	24.0 ± 0.58	1.0 ± 0.00	2.9 ± 0.03	0.030 ± 0.00
Escherichia coli	5.4 ± 0.10	34.3 ± 1.20	1.0 ± 0.00	3.7 ± 0.05	0.040 ± 0.00
Klebsiella pneumoniae	6.3 ± 0.10	34.0 ± 1.00	1.0 ± 0.00	3.7 ± 0.03	0.033 ± 0.00
Micrococcus spp	7.03 ± 0.03	34.0 ± 2.10	1.0 ± 0.00	3.1 ± 0.01	0.033 ± 0.00
Micrococcus luteus	3.5 ± 0.00	21.3 ± 1.33	1.0 ± 0.00	1.2 ± 0.00	0.010 ± 0.02
Rhizobium spp	5.4 ± 0.09	40.0 ± 0.00	1.0 ± 0.00	2.8 ± 0.06	0.030 ± 0.00
Staphylococcus aureus	6.3 ± 0.08	40.0 ± 0.00	1.0 ± 0.00	1.2 ± 0.06	0.030 ± 0.00
Control	2.6 ± 0.07	45.3 ± 0.88	1.0 ± 0.00	4.0 ± 0.03	0.030 ± 0.00

Results are values of three replicates ± the S.E (Standard error)

3.3. Antagonistic Effects of the Endophytic Bacteria against F. oxysporum

The selected endophytic bacteria showed varying degree of inhibitory activity against the phytopathogen *F. oxysporum*. The result, as presented in Table 4, shows that all the means were statistically greater ($P < 0.05$) than the control, indicating the ability of the test endophytic bacteria in the inhibition of *F. oxysporum.* There was a significant difference ($P < 0.05$) between all the mean values of zone of inhibition. *S. aureus* and *Bacillus subtilis* yielded the highest zone of inhibition of 21.3 and 20.2 mm, respectively. However, there was a significant difference ($P < 0.05$) between the sizes of zone of inhibition yielded by the two bacteria. On the other hand, *Bacillus cereus* which produced a zone of 17.2 mm has the lowest inhibitory activity.

Table 4. Antagonistic Effects of Some Endophytic Bacteria against *F. oxysporum*

Endophytic Bacterium	Mean Zone of Inhibition (mm)
Bacillus subtilis	20.2 ± 0.17
Bacillus cereus	17.2 ± 0.12
Escherichia coli	18.5 ± 0.20
Klebsiella pneumoniae	19.2 ± 0.15
Micrococcus spp 1	18.1 ± 0.10
Micrococcus luteus	18.2 ± 0.12
Staphylococcus aureus	21.3 ± 0.21
Control	12.7 ± 0.15

Results are values of three replicates ± the S.E (Standard error)

Qualitative detection of enzymes, such as cellulase, catalase, amylase, urease and oxidase, was carried out and the distribution of some of the enzymes among the test bacteria is represented in Table 5.

Table 5. Distribution of some enzymes among the test bacteria

Isolate	Catalase	Cellulase	Urease	Amylase
Bacillus subtilis	+	+	-	+
Bacillus cereus	+	+	-	+
Escherichia coli	+	-	-	+
Klebsiella pneumoniae	+	-	+	-
Staphylococcus aureus	+	-	+	+
Micrococcus luteus	+	+	+	+
Micrococcus spp	+	+	-	+
Rhizobium spp	+	+	-	+

+: Positive, **-:** Negative

4. Discussion

The result showed that the roots of *C. citratus* contain higher population of endophytic bacteria more than the leaves. This is most probably due to the fact that, the roots are the primary sites of infection as opined by Kobayashi and Palumbo (2000) and Hallmann *et al*. (1997). Similarly, Rosenblueth and Martinez-Romero (2004) found that, in most plants, the number of bacterial endophytes is higher in the roots than the above-ground tissues. Moreover, most endophytic bacteria are soil-borne and, therefore, colonize the roots region first and subsequently spread to other parts of the plants. Interestingly, opposite pattern of distribution was observed among the endophytic fungi that colonize same plant as reported by Deshmukh *et al*. (2010) who, in a study of fungal endophytes of *C. citratus* in two sites in India, reported 53% and 50% compared with 25% and 23% of fungi isolated from the leaves and rhizomes of the two sites, respectively. Furthermore, the isolates obtained in the present study are similar to the common endophytic bacteria isolated from different plants by different workers

at different times as reported by Ryan *et al*. (2007) as well as Rosenblueth and Martinez-Romero (2006).

The result shows that *K. pneumoniae* has potentials of promoting the growth of tomato seeds by ways of either shortening the length of germination period, improving the chances of seed germination, raising the length of shoot, improving weight gain or both. The mechanisms through which endophytes promote plant growth are many. These include: improved cycling of nutrients and minerals, phytoremediation (Ryan *et al*., 2007), phosphate solubilisation activity (Verma *et al*., 2001; Wakelin *et al*., 2004), Indole acetic acid production (Lee *et al*., 2004), production of a siderophore (Costa and Loper, 1994), and supply of essential vitamins to host plants among others (Pirttila *et al*., 2004).

All the tested bacteria showed antagonistic activity against the plant pathogen, *F. oxysporum* and, the activity was highest in *S. aureus* followed by *B. subtilis*. The result shows some agreement with the work of Ji *et al*. (2014) who reported the antagonistic activity of 12 endophytic diazotrophic bacteria isolated from Korean rice cultivars on mycelial growth of all the isolates of *F. oxysporum* tested. They further reported 4 species of both *Bacillus* and related genus *Paenibacillus* among the seven species with the highest antagonistic activity. The result also agrees with the work of Kim *et al*. (2008) who reported the antagonistic effects of 7 out of 20 *Bacillus* spp isolated from manure and cotton waste composts against soil borne fungi, *F. oxysporum*, *Rhizoctonia solani*, *Phytophthora casici* and *Sclerotinia sclerotium*. This in-vitro antagonistic effect of the endophytic bacteria against *F. oxysporum* is best explained by the mechanism of antibiosis. Several studies have indicated the ability of endophytic bacteria to exude compounds with antibiotic properties and biocontrol potentials. Notable among these include compounds, such as oligomycin A, kanosamine, zwittermicin A, and xanthobaccin produced by *Bacillus* spp (Compant *et al*., 2005). This further proves the potential application of these bacteria more especially *S. aureus* and *B. subtilis* as biocontrol agents of plant diseases and also potential sources of natural bioactive compounds.

The growth promotion and pathogen inhibition of the test bacteria might also be associated with the enzymes produced by the test bacteria. The bacteria were found to possess a variety of enzymes, such as catalase, cellulase and urease. Kuhad *et al*. (2011) reported the application of cellulase in plant pathogen and disease control, as well as plant growth and flower production. Catalase was reported to reduce the toxicity of hydrogen peroxide in plants (Felton *et al*., 1991), while urease when combined with nitrification inhibitors prevents loss of Nitrogen and improves yield (Freney, 1997).

5. Conclusion

The present study has shown that the internal tissues of *C. citratus* harbour a diverse range of endophytic bacteria that offer benefits to other plants in terms of growth promotion and pathogen inhibition. However, qualitative assay procedures that screen the useful bacteria for the production of useful enzymes, bioactive compounds and metabolites may reveal the answers for the potentials of these endophytic bacteria not only in growth promotion and biocontrol but possibly other areas.

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Comparing the Total Coliform and Fecal Coliform for Recreational Waters in Public Swimming Areas in the Kingdom of Bahrain

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Abstract

The Kingdom of Bahrain has an annual rainfall of about 78 mm with limited surface water resources and poor underground recharge. The rapid increase in urbanization, during the last 20 years, has increased the demands for additional water supplies. Recycling of such limited resources would also necessitate monitoring effluents coming off wastewater treatment plants. The monitoring process requires continuous and laborious work by dedicated water laboratories to verify water supply safety. Herein, the total number of coliforms in two public swimming areas/beaches (Zallaq and Hidd) were evaluated, and the results were compared to those from effluents of the main sewage treatment plant in Bahrain (Tubli Water Pollution Control Centre, WPCC). The results indicated a high Most Probable Number (MPN) for Tubli (MPN annual average value of 504 CFU/100 mL) compared to the other two sites Hidd (102 CFU/100 mL) and Zallaq (47 CFU/100 mL). The number of fecal coliforms was estimated using both biochemical and molecular approaches. According to estimates by EMB cultures and PCR among the total coliforms, *E. coli* (fecal indicator) constituted 37.3% for Tubli WPCC effluents, and less than 30% for the two public swimming areas in Hidd and Zallaq.

Keywords: Coliform; Fecal coliform; Recreational waters; Bathing waters; Kingdom Bahrain.

1. Introduction

Insufficient water resources in arid areas with scarce and erratic rainfalls are adding to the costs spent by developing countries to maintain the infrastructure of water facilities. In the Kingdom of Bahrain, the annual rainfall is about 78 mm with limited surface water resources and poor underground recharge. The rapid increase in urbanization during the last 20 years has increased the demands for additional water supplies. Recycling of such limited resources would also necessitate monitoring effluents coming off wastewater treatment plants. The monitoring process requires continuous and laborious work by dedicated water laboratories to test water supply safety. Many worldwide water safety programs test water samples and check for indicator microorganisms. Upon obtaining data, regulations and access to recreational waters are coordinated with local organizations and governmental agencies (Efstratiou *et al.*, 2009).

In 1914, the US Public Health Service Drinking Water Standards set the criteria for testing the quality of water for drinking and bathing (Efstratiou *et al.*, 2009). The test uses conventional techniques, e.g., the multiple tube fermentation/Most Probable Number (MPN) to test for presence of coliforms. Coliforms are Gram-negative, rod shape *Enterobacteriaceae*. A subset of this group is the fecal coliforms (e.g., *Escherichia coli*), which indicates contamination of test samples with human waste/sewage outlets (Rompre *et al.*, 2002). Fecal coliforms are used as indicator for the presence of pathogenic microorganisms (Efstratiou *et al.*, 2009).

The objectives of the present study are to monitor the total number of coliforms (TC) in two public swimming areas/beaches (Zallaq and Hidd) and to compare the results obtained with effluents of the main sewage treatment plant in Bahrain (Tubli Water Pollution Control Centre-WPCC). The present study also estimates the percentage of fecal coliforms at the three aforementioned seashores. The study used both conventional and molecular approaches to estimate and enumerate the microorganisms.

To the best of our knowledge, the two selected beaches have not been previously investigated for the presence of coliforms even though that these were public swimming areas. The numbers obtained would be useful as references for future studies and further analysis.

2. Materials and Methods

2.1. Isolates

Collection of samples was performed between October and June for three successive years (2014 - 2016). The samples were collected at 1.0-meter depth offshore. Collection of samples was performed during the daytime at

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high tides (two liters for each location/site in sterile containers). The samples were processed within two hours after sampling by transfer of seawater into lauryl sulfate broth (for overnight incubation) and subsequent culture on Eosin Methylene Blue (EMB) for isolation (Figure 1). Fifty-one isolates were stored in 30 % glycerol at -20°C for further analysis and polymerase chain reaction.

2.2. Conventional Microbiology

Standard procedures for isolation and enumeration of coliforms using the presumptive (MPN) and confirmative tests (Grabow *et al*., 1981; SABS, 1984; Grabow, 1990; ISO, 1990; ISO, 1994; Standard Methods, 1995; Grabow, 1996) were followed by biochemical assessments to complete the identification of isolates.

2.3. Molecular Typing and Restriction Digests

Amplification of the 16S rDNA was carried in 100 µL total volumes. The reaction components were 2.5 units of HotstarTaq DNA polymerase (QIAGEN), 200 µM dNTPs and 0.5 µM of both primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and U1492R (5'-GGTTACCTTGTTACGACTT-3') (Jiang *et al*., 2006). The following run conditions were used (BIO-RAD iCycler): initial denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 56°C for 1 minute, and extension at 72°C for one minute. The final extension was performed at 72°C for ten minutes (Noble *et al*., 2003; Kampfer *et al*., 2011; Aghababaee *et al*., 2012).

The produced amplicons (1.5 kb) were digested with *Eco*RI at 37°C for 2 hours (Figure 1). The PCR fragments and restriction digests were run on 0.8-1.3 % agarose gels for visualization.

2.4. Statistical Analysis

Logistic regression analysis, with the presence of coliforms as dependent variable and the investigated years as categorical independent variable, was used to assess whether there is a significant positive relationship between locations during consecutive years and the log values for the MPN numbers obtained for each site.

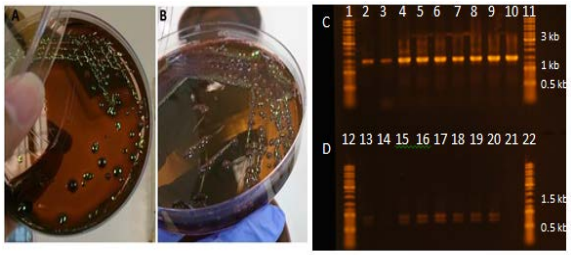

Figure 1. Obtained isolates from confirmatory tests on EMB plates and PCR. **A.** Metallic green sheen and fish-eye purple colony. **B.** Green sheen, purple un-nucleated and flat nucleated purple colony. **C.** Agarose gel electrophoresis of PCR products. The PCR amplicons indicates the detection of the 16S rDNA gene in the isolates. In numeric order from left to right, Lanes 1 and 11 is GeneRuler DNA ladder (ThermoFisher Scientific). Lanes 2 and 3 references strain *E. coli* MG1655. Negative control was run in (D) lane 21. Lanes 4-10 runs for selected isolates. Band size was 1.5 kb. **D.** Restriction digests of the PCR products in (C) by *Eco*RI. In numeric order from left to right, Lanes 12 and 22 is GeneRuler DNA ladder (ThermoFisher Scientific). Lane 13 reference strain *E. coli* MG1655. Lanes 14-20 runs for selected isolates. Band sizes 0.7 and 0.8 kb.

3. Results and Discussion

The present study was carried out during the months of winter and early summer as we expected high tides during these seasons. High tides might change the number of counted coliforms (St Laurent *et al*., 2014). The locations studied had the following coordinates: Dry-dock beach at Hidd (26.19595986, 50.662142), Tubli effluent (26.196866, 50.565727), Zallaq/Al Jazair beach (25.989600, 50.461081) (Figure 2).

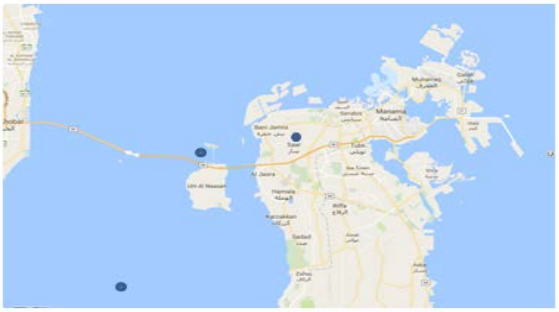

Figure 2. Map of the Kingdom of Bahrain showing study sampling sites. The blue marks, from left to right: Zallaq, Tubli, and Hidd.

Two of the studied areas are known public swimming beaches Zallaq and Hidd. The third location was selected as a control (Tubli-WPCC). Physical parameters for studied areas showed alkaline pH for both Hidd and Tubli (pH 8), while close to neutral for Zallaq (pH 7.2). Salinity was higher for Zallaq (54 PSU), than for Hidd (44 PSU) and Tubli (44 PSU). Figure 3 shows the total coliform values obtained for each site for three successive years (2014-2016). The numbers indicate high MPN for Tubli (MPN nine months' average value of 504 CFU/100 mL) compared to the other two sites; Hidd (102 CFU/100 mL) and Zallaq (47 CFU/100 mL). The number of fecal coliforms was estimated using both biochemical and molecular tests. According to estimates by EMB cultures and PCR among the total coliforms, *E. coli* (a fecal indicator) constituted 37.3 % for Tubli WPCC effluents, and less than 30 % for the two public swimming areas in Hidd and Zallaq.

Compared to previous data, Tubli WPCC continues to contribute to the total coliforms and fecal coliforms found in Tubli bay (Qureshi and Qureshi, 1990; Qureshi *et al*., 1993; Mahasneh *et al*., 1997). The effluents of Tubli bay potentially pose health issues due to the highly pathogenic species detected previously (Amin, 1988; Qureshi and Qureshi, 1992). They found that most of the species were resistant to a panel of routinely used antibiotics in the public health sector (Amin, 1988; Qureshi and Qureshi, 1992). Thus, there is a necessity for a quick intervention by local environmental agencies to control the spread of antibiotic resistant strains. However, in the current study, the obtained numbers for Tubli WPCC effluents (504 CFU/100 ml) were not significant in terms of introduced pathogenic species as set by Efstratiou, *et al.* (2009) (Figure 3). Efstratiou *et al.* (2009) indicated that a value of 1000 CFU/100 mL of total coliforms is needed to indicate the presence of pathogenic species, such as *Salmonella* spp. in seawater. Moreover, our values indicate a reduction in total coliforms compared to previous values obtained during a study in 1993-1994 (Al-Sayed *et al*., 2005). The

log mean values of CFU/mL were around 5-6 (Al-Sayed *et al*., 2005), while the mean log value in our study was around 0.7 CFU/mL. This indicates that in the last 20 years, Tubli WPCC has increased their standards and quality of released effluents, hence achieving a reduction in the TC by almost 86%. In regards to both Hidd and Zallaq, most numbers of total coliforms are still within the limits set for public use and are considered safe < 100 CFU/100 mL (Figure 2) (Efstratiou *et al*., 2009).

The 16S rDNA PCR and the restriction digest showed similar sizes and patterns as depicted by http://insilico.ehu.eus/PCR/ using the sequences of both primers 27F and U1492R as inputs and selecting - apply to all *Escherichia* as the target microorganism with allow a mismatch of 2 (San Millán *et al*., 2013). No new species were identified by PCR, as both typical PCR amplicon sizes (Figure 1) and the restriction fragments produced were identical to that obtained *in silico* (two DNA fragments of 0.7 and 0.8 kb) (Suardana, 2014).

The statistical analysis presented in (Table 1) and (Figure 3) shows a positive trend among different swimming areas and their corresponding log mean values for the MPN numbers upon successive years of study, $r(18)= 0.599$, $p < 0.005$. Indicating a significant relationship between the numbers of coliforms detected and the sites studied. The standard deviations obtained for readings of Zallaq and Tubli are smaller than that for Hidd (Table 1 and Figure 3). This variability in the reported readings of Hidd area could be attributed to the fact that Hidd is more open to the high tides and open sea as illustrated in (Figure 2).

Table 1. The log mean value CFU/100 mL at different sites

Year	Location	Nine months log mean values for the MPN TC coliforms (Standard deviation)
	Zallaq	1.66 (±0.024)
2014	Hidd	1.86 (±0.179)
	Tubli	2.69 (±0.017)
	Zallaq	1.70 (±0.024)
2015	Hidd	1.90 (±0.179)
	Tubli	2.70 (±0.017)
	Zallaq	1.65 (±0.024)
2016	Hidd	2.19 (±0.179)
	Tubli	2.72 (±0.017)

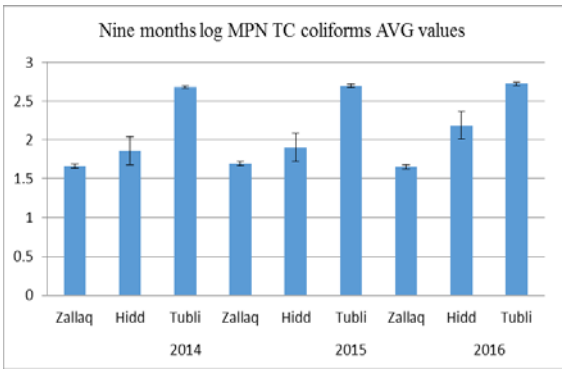

Figure 3. Total coliform estimates using the MPN method. Nine months' log average values (CFU/100 mL) of seawater for the three studied areas (years 2014-2016). Error bars refer to the standard deviation obtained for each site.

4. Conclusions

According to the estimates done by both EMB and PCR, *E. coli* (as fecal indicator) constituted 37.3% of the total coliforms isolated in Tubli WPCC effluents, and less than 30% for the two public swimming areas in Hidd and Zallaq. Compared to previous data, Tubli WPCC continues to contribute to the total coliforms and fecal coliforms found in Tubli bay. However, the obtained numbers for Tubli WPCC effluents are not significant in terms of introduced pathogenic species. Moreover, the data indicate a reduction in total coliforms compared to previous values obtained during a study in 1993-1994.

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Evaluation of Six Imported Accessions of *Lupinus albus* for Nutritional and Molecular Characterizations under Egyptian Conditions

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Abstract

White lupin (*Lupinus albus* L.) is an annual grain-legume widely harvest and cultivated in Egypt and also worldwide. Lupin seeds are utilized as food for the human and livestock nutrition. The aim of the present study is to estimate the genetic diversity among the seven genotypes of *L. albus*. In addition, the field performance of these genotypes was evaluated to drought tolerant under Egyptian conditions. Also, the macro- and microelements in the seeds of the white lupin were determined. In the present investigation, high significant differences among seven white lupin genotypes were observed for field performance traits under water deficit. Two accessions *L. albus* CGN 10106 and CGN 10108 were tolerant of drought compared with other genotypes. Thus, these genotypes recorded the highest seed yield when exposed to water stress. The macro- and microelement contents of seven white lupin genotypes were found to be different based on the genotype. In the present study, slight differences were observed in the total protein bands among the seven genotypes, thus polymorphism is low (28.57% polymorphism). These genotypes varied in expression from strong to low both Polyphenol Oxidase (PPO) and Peroxidase (POX) isozymes. So, the highest expression of antioxidant enzymes was recorded in genotypes tolerant of drought CGN 10106 and CGN 10108. On the other hand, Random Amplified Polymorphism DNAs (RAPD); Inter Simple Sequence Repeats (ISSR) assays recorded the percentage of polymorphism 47.96% and 29.82%, respectively. The Nei genetic similarity index ranged from 0.74 to 0.88 using UPGMA. These results are important in the breeding programs for the selection process of parental strains that feasibility the prediction of crosses to generate hybrids with the best performance.

Keywords: White lupin, yield, isozyme, RAPD, ISSR.

1. Introduction

White lupin (*Lupinus albus* L.; 2n = 50) is a member of the family *Fabaceae* (El-Enany *et al*., 2013; EL-Harty *et al*., 2016; Prusinski, 2017). It is sown as a crucial rotational yield. In addition, white lupin is beneficial in the diseases and weeds controlling in a crop rotation in a mixed agriculture program; lupin fixes nitrogen ($N_{2)}$ gas of the *atmosphere* and improving of the soil fertility. *L. albus* is an efficient scavenger of phosphorus (P) ascribable the existence of proteoid roots which release organic acids and make P more available (Neumann and Martinoia, 2002). White lupin seeds are utilized for the human and livestock nutrition (Barnevelda, 1999).

The genetic variability of *Lupinus* species has been characterized by agronomical and morphological characters (Andres *et al*., 2007), biochemical (Vaz *et al*., 2004) and molecular markers, such as Random Amplified Polymorphism DNAs (RAPD), Inter Simple Sequence Repeats (ISSR) and Amplified Fragment Length Polymorphism (AFLP) (Talhinhas *et al*., 2003). Estimation of genetic variability depending on the morphological properties is not very authoritative, as it may be affected by the environment and the number of traits with recognized inheritance is few. Molecular markers have the distinguished advantages of being independent of climatic changes.

White lupin seeds are beneficial source of macro- and microelement contents. Essential elements are divided to macronutrients [Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), Manganese (Mg) and Sulfur (S)] and micronutrients [(Iron (Fe), Copper (Cu), Manganese (Mn), Zinc (Zn), Boron (B), Molybdenum (Mo), Nickel (Ni) and Chlorine (Cl)] and the classification depends on the relative abundance in the plants. A significance of mineral constitution is due to their nutritional characterizations and good health effects, also needed for a healthy diet (Kırbaşlar *et al*., 2012). Iron is required for haemoglobin (Hb) and myoglobin (Mb) synthesis (Saleh-e-in *et al*.,

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2008). Sodium (Na), K and Ca also impact heart functions (Rajurkar and Damame, 1997). Numerous metabolic processes need Mg, Fe and K. Zn plays main role in several immunological and biochemical functions (Özcan *et al*., 2013).

The aim of this study is to estimate the genetic diversity among the seven genotypes of *L. albus*. In addition, the field performance under water stress and the essential and non-essential elements of seven white lupine genotypes were determined.

2. Materials and Methods

2.1. Plant Materials

The six white lupin accessions imported from (Centre for Genetic Resources, The Netherlands) and one local cultivar Balady were used in this investigation. Names, pedigree and origin of lupin genotypes are presented in Table 1. These materials were evaluated in 2015/16 growing season in field experiment under water stress conditions in a Randomized complete block design with three replications. Plants received two only irrigations through the whole season. The plot size of one row was 0.60 m x 4 m. Lupin seeds were planted on 13th of November in hills with 0.25 m apart on one side of ridge in Delta region at Shebin El-Kom, Menofiya Governorate, Egypt. Yield components and the other related traits, plant height (cm), No. of pods/plant, No. of seed/pod, No. seeds/plant, 100 seed weight (g) and seed yield/plant (g) were measured at harvesting.

Table 1. Pedigree of genotypes used in this study.

No.	Accessions	Type	Name	Country
1	*Lupinus albus* CGN 10105	Research material	N106/50	Italy
2	*L. albus* CGN 10106	Research material	N107/50	Italy
3	*L. albus* CGN 10108	Research material	N121/50	Italy
4	*L. albus* CGN 10109	Research material	N122/50	Italy
5	*L. albus* CGN 10112	Land variety	Przehendowski Wezesnv	Poland
6	*L. albus* CGN 10113	Land variety	Kisordai Feheroiragu	Hungary
7	*L. albus* cv. Balady	Land variety	Balady	Egypt

2.2. Macro- and Micro-Nutrient Analyses

The seeds of seven white lupin genotypes were milled after being oven-dried at 40°C, and then kept in sealed vials for further analyses. A portion of the dried samples was dissolute in acids mixtures to be digested as described by Cottenie *et al*., (1982).

Macro- and micro-nutrients were determined in the digested aliquots. Magnesium, iron, manganese, zinc and copper were measured by atomic absorption spectroscopy (AAS, Unicam 939 AA Spectrometer); sodium, potassium and calcium by flame emission (Cottenie *et al*., 1982). Total nitrogen was determined by kjeldahl method and phosphorus was determined by ammonium-vanadate and molybdate method according to Motsara and Roy (2008).

2.3. Electrophoretic Analysis of Protein by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was done according to (Laemmli, 1970) as modified by (Studier, 1973).

2.4. Polyphenol Oxidase (PPO) and Peroxidase (POX) Isoforms.

For the assay of antioxidant enzymes Peroxidase (POX) and Polyphenol Oxidase (PPO) were extracted based on the method described in (Stagemann *et al*., 1985). PPO and POX isozymes were separated by Native-Polyacrylamide Gel Electrophoresis (Native-PAGE). The activities of POX and PPO were determined according to (Baaziz *et al*., 1994).

2.5. Extraction of Genomic DNA

Young plant leaves of seven white lupin genotypes were soaked in liquid nitrogen for DNA extraction using 2% (CTAB) cetyl trimethyl ammonium bromide (Borsch *et al*., 2003).

2.6. RAPD Analysis

A total of five primers were used to amplify DNA (Mahfouze *et al*., 2012) (manufactured by Bioneer, New technology certification from ATS Korea). The total reaction mixture was 25 µl contained 10X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs mixed, 10 *p*mol primer, 1.25 U *Taq* polymerase and about 150 ng genomic DNA. RAPD-PCR amplification was performed in thermal cycler (Biometra Inc., Germany). The temperature profile was as follows: An initial denaturation at 94°C for 3 min; followed by 35 cycles of denaturation temperature 94°C for 5 min; annealing temperature 37°C for 1 min and extension temperature 72°C for 1 min, final extension at 72ºC for 5 min.

2.7. ISSR Profiles

A total of four anchored ISSR primers were used to amplify DNA (Life Technologies, Gaithersburg, Md.). Each 25-µl amplification reaction consisted of 2.5 µl 10X PCR buffer, 2.5 µl 25 mM $MgCl_2$; 0.5 µl 40 mM dNTPs; 1 µl *Taq* DNA polymerase (1 unit/µl); 2 µl 0.4 µM primer. Amplification was carried out in DNA thermocycler (Biometra, Germany) under the following conditions: one cycle of 94°C at 3 min, followed by 28 cycles of denaturation temperature 94°C/45 sec, annealing temperature 52°C/30 sec and extension temperature 72°C/2 min at; a final extension 72°C/6 min.

Amplification products were separated on a 1.5% agarose gel containing 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) and 0.5 µg/ml ethidium bromide at 90 V. Gels were analyzed by UVI Geltec version 12.4, 1999-2005 (USA).

2.8. Data Analysis

A matrix for SDS-PAGE, POX, PPO, RAPD and ISSR combined was generated by scoring reproducible bands as 1 for their presence and as 0 for their absence across the genotype. Genetic similarity coefficients were computed according to (Nei and Li, 1979). The data were subsequently used to construct a dendrogram using the un-

weighted pair group method of arithmetic averages (UPGMA) (Sneath and Sokal, 1973) employing sequential, agglomerative hierarchic and non-overlapping clustering (SAHN). All the computations were carried out using the software NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 2.02 (Rohlf, 2000). Correlation coefficients were calculated using similarity coefficients obtained from combined SDS-PAGE, POX, PPO, RAPD and ISSR analysis.

2.9. Statistical Analysis

The data were analyzed by ANOVA procedure of program SPSS (1995) statistical procedures version 21 (Chicago, USA) (www.spss.com).

3. Results

3.1. Field Performance

The analysis of variance of all traits studied is presented in Table 2. Highly significant differences among seven white lupin genotypes were recorded for all the traits studied, indicating the presence of a considerable genetic diversity among the tested lupin genotypes. Also, these variations among genotypes might partially reflect their different genetic backgrounds.

Mean performance of seed yield and its componenets for the seven lupin genotypes are presented in Table 3. All the six forign genotypes exhibited exceeded the plant height of the local landraces Balady. With regard to the number of pods/plant means ranged from 3.67 pods for the genotype CGN 10105 to 12 pods per plant for the genotype CGN 10108. Significant differences were found among genotypes for number of seeds/pod and the highest number found in genotype CGN 10106 (5.67 seeds/pod). Concerning the number of seeds/plant, genotype CGN 10106 had a larger number of seeds/plant (63.67), followed by CGN 10108 (58.67 seeds/plant). Also, results showed that the genotypes CGN 10106 and CGN 10108 had the highest values of seed yield. Moreover, these lines had the highest values for 100 seed weight.

Table 2. Mean square values for all studied characters among seven lupin genotypes evaluated in the growing season 2015/16.

S.O.V	D.F	Plant height.(cm)	No. of Pods/ plant	No. of seeds/pod	No. of seeds/plant	100 seed weight (g)	Seed yield /plant (g)
Reps	2	1.286	0.190	0.190	22.333	0.136	2.086
Genotypes	6	200.603**	28.857**	1.937**	974.429**	38.69**	146.17**
Error	12	26.175	1.857	0.746	22.667	1.069	2.451

** Significant at $p = 0.01$; * significant at $p = 0.05$.

Table 3. Mean performance and standard error for all studied characters in seven white lupin genotypes evaluated in the growing season 2015/16.

Genotypes	Plant height (cm)	No. of Pods/ plant	No. of seeds / Pod	No. of seeds / plant	100 seed weight (g)	Seed yield /plant(g)
L.albus CGN 10105	58.67±1.85	3.67±0.33	5.67±0.32	21.00±3.00	33.85±0.25	7.09±0.98
L. albus CGN 10106	70.00±2.88	11.33±0.88	5.67±0.33	63.67±1.8	34.78±0.57	22.13±0.56
L. albus CGN 10108	69.00±2.08	12.00±0.88	5.0±0.57	58.67±1.33	36.58±0.26	21.43±0.36
L. albus CGN 10109	64.33±2.60	9.0±0.57	5.30±0.33	48.0±4.16	34.37±0.51	16.50±1.43
L. albus CGN 10112	62.33±0.65	6.0±0.58	5.33±0.67	31.33±2.40	33.20±0.09	10.39±0.76
L. albus CGN 10113	52.67±1.45	6.66±0.60	3.66±0.33	24.0±0.01	25.71±1.17	6.17±0.81
L. albus cv. Balady	48.00±1.53	5.67±0.66	4.0±0.58	22.66±3.92	30.53±0.24	6.92±1.21

3.2. Macro- and Micro-Elements of White Lupin Seeds

Studying the nutrients' content in the seeds of *L. albus* are important from a human nutritional point of view as well as from the preferences of which genotype is best for agriculture. Generally, content of macro- and micro-nutrients in white lupin seeds based on the genotype (Tables 4 and 5). The highest content of N was recorded for genotype CGN 10106; however, the differences in this element's content, among the studied genotypes, cannot be statistically considered. CGN 10113 seeds showed the best genotype in K, P and Na contents reached 1.78, 0.334 and 0.289 g 100 g^{-1} dried material, respectively; followed by the seeds of Balady cultivar. The influence of the genotypes on the contents of Ca and Mg were not statistically significant. Nevertheless, the data showed that the CGN 10105 and CGN 10112 genotypes were the highest content of Mg and Ca, respectively, compared with the other genotypes (Table 5). On the other hand, the results revealed that the reduction of the K contents was depicted in CGN 10105 and CGN 10108 genotypes. In addition, the lowest contents of Na were found in CGN 10112 compared with the other genotypes (Table 5).

Among all the examined genotypes, CGN 10112 and CGN 10113 accessions and Balady cultivar had the superior content of the micronutrients (Tables 6 and 7). Wherein the nutrients increased as follow: Fe by (0.14 – 0.35), Mn by (0.7 – 1.5), Zn by (0.3 – 0.7), and Cu by (0.11 – 0.34) fold, compared with the other studied genotypes. Furthermore, the minimum contents of Fe and Cu nutrients were exhibited in the CGN 10106 genotype. According to, Mn and Zn were recorded in CGN 10108 and CGN 10105 genotypes, respectively. The results indicated the significant influence of the genotypes on the content of some nutritional elements in the white lupin seeds (Table 7).

Table 4. Analysis of variance of macronutrients element contents (%) in white lupin genotypes

S.O.V	D.F	N	P	K	Ca	Mg	Na
Reps	2	0.886	0.043	0.056	0.001	0.003	0.001
Genotypes	6	0.450**	0.005**	0.122**	0.027ns	0.005ns	0.003**
Error	12	0.131	0.001	0.009	0.030	0.004	0.001

** Significant at $p = 0.01$; * significant at $p = 0.05$., ns = not significant.

Table 5. Mean values ± SE for macronutrients element (%) in seven white lupin genotypes studied (in milligrams per 100 gram)

Genotypes	N	K	P	Mg	Ca	Na
L. albus CGN 10105	4.13±0.35	1.28±0.068^{b}	0.26±0.004^{c}	0.22	1.09	0.24±0.012^{b}
L. albus CGN 10106	4.30±0.32	1.32±0.083^{b}	0.23±0.014^{c}	0.11	1.16	0.23±0.003^{b}
L. albus CGN 10108	3.65±0.19	1.28±0.021^{b}	0.24±0.015^{c}	0.11	1.28	0.24±0.009^{b}
L. albus CGN 10109	4.03±0.362	1.29±0.014^{b}	0.27±0.002^{c}	0.14	1.09	0.218 ± 0.006^{b}
L. albus CGN 10112	3.67±0.098	1.38±0.067^{b}	0.297±0.007^{b}	0.12	1.34	0.211±0.012^{b}
L. albus CGN 10113	3.27±0.372	1.78±0.140^{a}	0.334±0.013^{a}	0.111	1.26	0.289±0.003^{a}
L. albus cv. Balady	3.38±0.142	1.64±0.027^{a}	0.319±0.008^{b}	0.114	1.23	0.286±0.011^{a}

Values followed by the same letter are not significantly different according to Duncan's multiple range test ($p < 0.05$).

Table 6. Analysis of variance of micronutrients element contents (%) in white lupin genotypes.

S.O.V	D.F	Fe	Mn	Zn	Cu
Reps	2	0.001	78.619	7.620	0.147
Genotypes	6	260.475**	17414.52**	9.982**	1.989**
error	12	18.329	201.76	3.237	0.116

** Significant at $p = 0.01$; * significant at $p = 0.05$.

Table 7. Mean values ± SE for micronutrients element (%) in seven white lupin genotypes studied (in milligrams per 100 gram).

Genotypes	Fe	Mn	Zn	Cu
L. albus CGN 10105	78.34±1.17	194.33±2.96^{c}	7.77±0.296	6.15±0.406^{b}
L. albus CGN 10106	66.53±2.84	194.67±2.60^{c}	8.65±1.16	6.00±0.037^{b}
L. albus CGN 10108	70.40±0.58	153.33±10.98^{d}	9.01±2.39	6.22±0.243^{b}
L. albus CGN 10109	76.53±0.267	222.67±4.05^{c}	9.54±0.427	7.25±0.017^{a}
L. albus CGN 10112	87.86±1.07	268.67±10.08^{b}	12.87±0.712	7.63±0.070^{a}
L. albus CGN 10113	89.7±0.91	384.33±10.34^{a}	11.29±0.442	7.41±0.151^{a}
L. albus cv. Balady	88.83±1.93	275.33±8.25^{b}	11.36±0.981	8.02±0.163^{a}

Values followed by the same letter are not significantly different according to *Duncan's* multiple range test ($p < 0.05$).

3.3. SDS-PAGE

The electrophoresis of the total protein extracted from the leaves of seven white lupin genotypes determined by SDS-PAGE as shown in Figure 1. SDS-PAGE revealed that, seven *L. albus* genotypes were rich with protein content depending on number of bands on the gel. The electrophoregrams were estimated depending on their molecular masses. A total of 14 polypeptide chains were recorded ranging from 4.5 to 250 kDa; ten of these were monomorphic (71.43%), while four were polymorphic (28.57% polymorphism). The highest number of polypeptides scored in accessions CGN 10106 and CGN 10113 (14 polypeptides), followed Balady cultivar (13 bands) and CGN 10109 (12 subunits). However, the lowest number of subunits detected in accessions CGN 10105, CGN 10108 and CGN 10112 (ten subunits). On the other hand, one unique band with molecular weight 170 kDa scored in two accessions CGN 10106 and CGN 10113 (Figure 1).

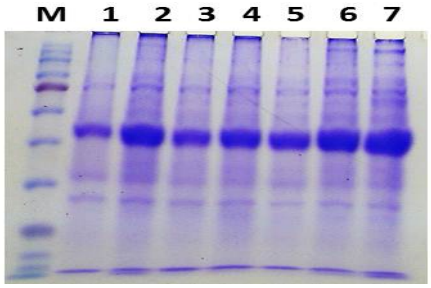

Figure 1. SDS-PAGE banding patterns of leaf protein extracted from seven genotypes *Lupinus albus*. Lane M: Marker protein. Lane 1: CGN 10105; lane 2: CGN 10106, lane 3: CGN 10108; lane 4: CGN 10109; lane 5: CGN 10112; lane 6: CGN 10113 and lane 7: Balady cultivar.

3.4. POX and PPO Isozymes

Isozyme spectra of two tested isoforms (PPO and POX) were determined by native-PAGE in leaves of seven white lupin genotypes as shown in Figure 2. POX recorded three isoforms with *Rf* value ranging of 0.293 to 0.693. The highest expression was scored in CGN 10105, CGN 10106, CGN 10108 and CGN 10109 (three alleles), followed by CGN 10113 (two isoforms). However, the lowest expression was found in two genotypes CGN 10112 and Balady cultivar (one isoform) (Figure 2).

The Isozyme spectra of PPO of all studied *L. albus* genotypes, composed of four detectable bands with *Rf* value, ranged from 0.320 to 0.620 (Figure 2). Accessions CGN 10108 and CGN 10109 had a strong expression (four alleles). On the contrary, accession CGN 10106 gave a weak expression (two isoenzymes). However, the other genotypes recorded a moderate expression (three isoforms) (Figure 2).

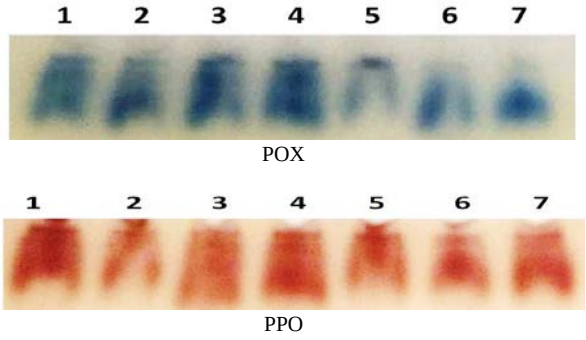

Figure 2. POX and PPO isozyme profiles of seven genotypes Lupinus albus.. Lane 1: CGN 10105; lane 2: CGN 10106, lane 3: CGN 10108; lane 4: CGN 10109; lane 5: CGN 10112; lane 6: CGN 10113 and lane 7: Balady cultivar.

3.5. RAPD Analysis

Five decamer RAPD primers (10 nucleotide length) from arbitrary nucleotide sequences were used to amplify seven *L. albus* genotypes (Figure 3 and Table 8). A total number of 98 scorable bands were amplified by five RAPD primers (19.6 bands per primer), ranging from 90 to 1730 bp (RAPD-2) (Figure 3 and Table 8). Fifty-one out of 98 fragments were common bands (52.04%), 47 loci were polymorphic (47.96%). The number of fragments per primer varied from 16 (RAPD-1) to 26 (RAPD-2). Primer RAPD-1 scored the highest number of polymorphism (75%), followed by primer RAPD-5 (58.82%). However, Primer RAPD-3 recorded the lowest number of polymorphism (29.41%). On the other hand, 14 out of the 98 were unique markers (14.29%) (Table 8). CGN 10105 appeared the maximum number of positive and negative markers (six) with molecular sizes (+180; +191 and +500 bp) and (-700; -703 and -1510 bp), respectively. However, CGN 10108, CGN 10109 and Balady cultivar scored two bands of (+1730 and -185 bp); (+160 and +382); and (+730 and +781) bp, respectively. In contrast, CGN 10106 revealed the minimum number of specific bands (one) of +133 bp (Table 8).

Table 8. RAPD-PCR analysis, a total number of loci, monomorphic, polymorphic, unique loci of seven *L. albus* genotypes

Primer Code No.	Primer sequences	Size range of the scorable loci (bp)	Total loci	No. of monomorphic loci	No. of polymorphic loci	% Polymorphism	Unique loci	Molecular size of markers (bp)
RAPD-1	GTTTCGCTCC	160 -1600	16	4	12	75	3	+160; +730; -703
RAPD-2	AACGCGCAAC	90-1730	26	16	10	38.46	2	+1730; -482
RAPD-3	CCCGTCAGCA	133-805	17	12	5	29.41	3	+133;+382;+500
RAPD-4	GGACGGCGTT	100-1510	22	12	10	45.45	2	-185; -1510
RAPD-5	AAGCCCGAGG	180-1500	17	7	10	58.82	4	+180; +191;+ 781; -700
Total		**90-1730**	**98**	**51(52.04%)**	**47**	**47.96%**	**14**	**14.29%**

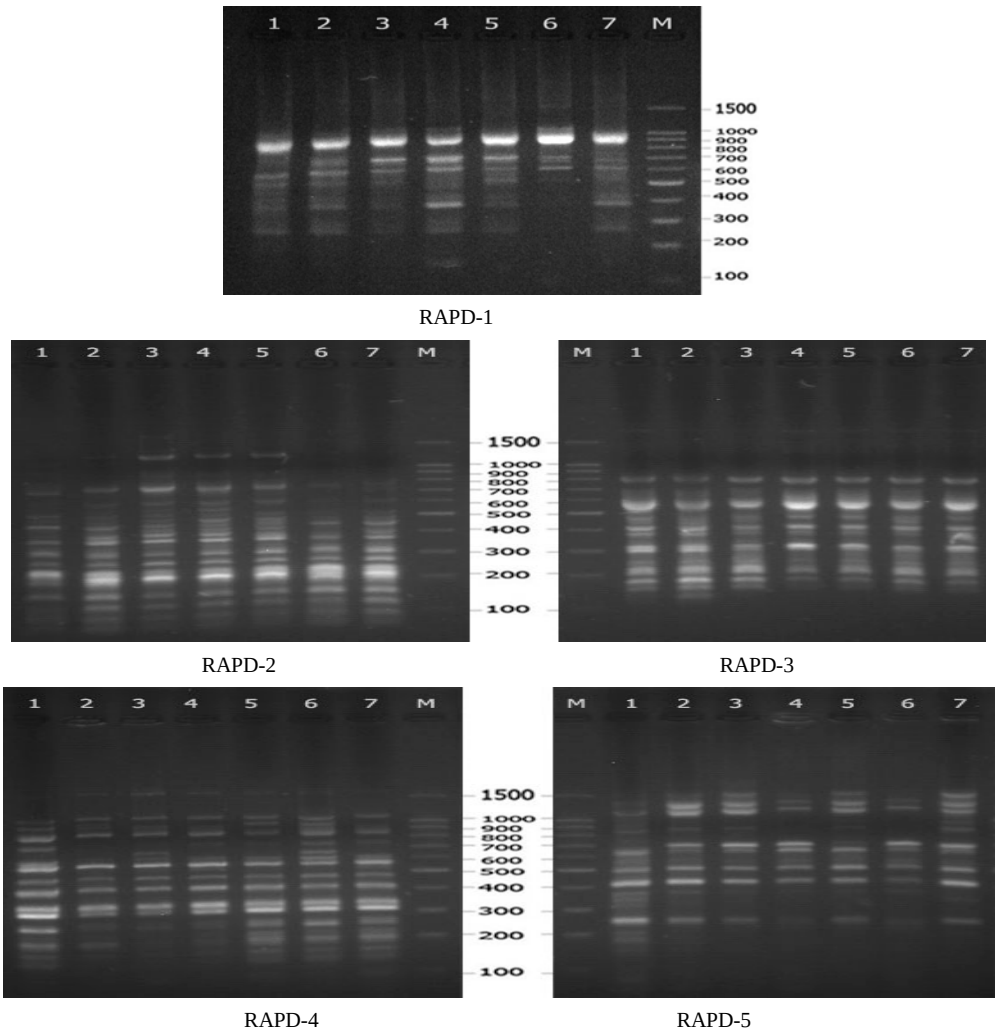

Figure 3. Amplified products of RAPD-PCR using of five primers for analyzed seven genotypes *Lupinus albus*. Lane M= DNA ladder 100 bp. Lane 1: CGN 10105; lane 2: CGN 10106, lane 3: CGN 10108; lane 4: CGN 10109; lane 5: CGN 10112; lane 6: CGN 10113 and lane 7: Balady cultivar.

3.6. ISSR Profiles

The four primers were used in ISSR loci, produced a total of 57 amplified fragments, ranging from 185 (ISSR-4) to 1500 (ISSR-1) bp. Forty amplicons were monomorphic (70.18%), 17 were polymorphic (29.82%) of the total number of bands (Figure 4 and Table 9). The number of amplicons using single primers ranged from 11 (ISSR-2) to 21 (ISSR-1) with a mean of 14.25 bands per primer. The highest degree of polymorphic among accessions for each primer was 33.33% for primers (ISSR-1 and ISSR-4), followed by ISSR-3 (30.77%). However, the lowest degree of polymorphism was scored in primer ISSR-2 (18.18%). The polymorphism of all amplification fragments was 29.82% for the genotypes investigated. Furthermore, seven out of the 57 bands were unique markers (12.28%). On other hands, CGN 10105 record the maximum number of unique loci (four) with molecular sizes (+433; +491; +619 and +1200 bp). Followed, CGN 10109 scored three specific bands of (+530; +595 and +715 bp) (Table 9). On the contrary, the other accessions have not showed any markers.

Table 9. ISSR-PCR analysis, a total number of loci, monomorphic, polymorphic, unique loci of seven *L. albus* genotypes.

Primer Code No.	Primer sequences	Size range of the scorable loci (bp)	Total loci	No. of monomorphic loci	No. of polymorphic loci	% Polymorphism	Unique loci	Molecular size of markers (bp)
ISSR-1	$(CA)_6AC$	191-1500	21	14	7	33.33	2	+433, +1200
ISSR-2	$(CT)_8GC$	216-740	11	9	2	18.18	1	+619
ISSR-3	$(GA)_6CC$	202-1441	13	9	4	30.77	1	+491
ISSR-4	$(CAC)_3GC$	185-850	12	8	4	33.33	3	+530, +595, +715
Total		**185-1500**	**57**	**40(70.18%)**	**17**	**29.82%**	**7**	**12.28%**

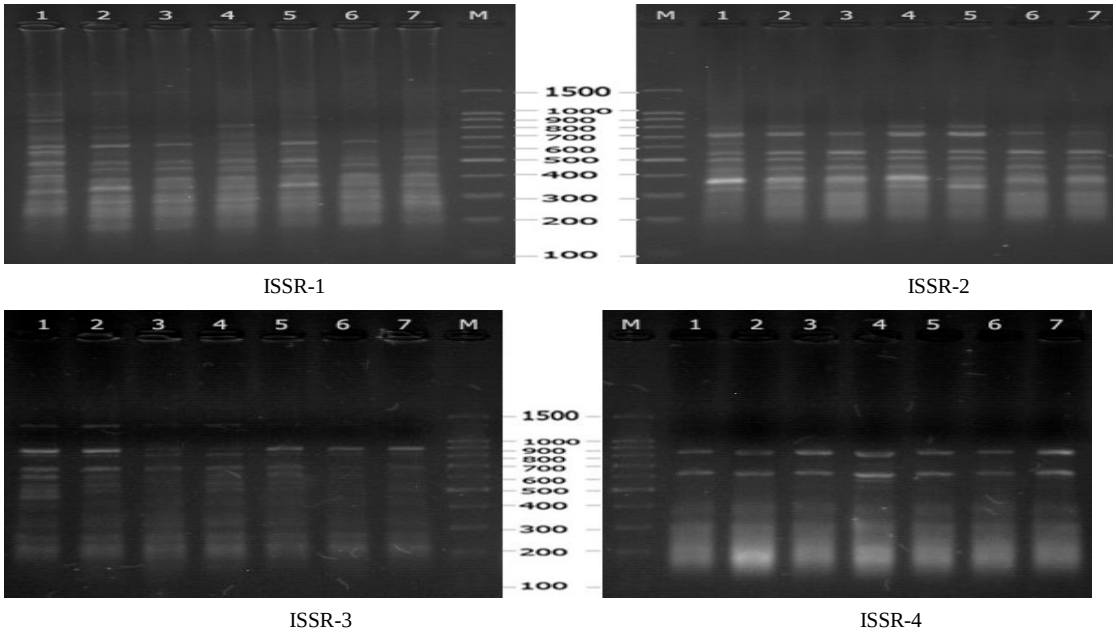

Figure 4. Amplified products of ISSR-PCR using of four primers for analyzed seven genotypes *Lupinus albus*. Lane M= 100 bp DNA ladder. Lane 1: CGN 10105; lane 2: CGN 10106, lane 3: CGN 10108; lane 4: CGN 10109; lane 5: CGN 10112; lane 6: CGN 10113 and lane 7: Balady cultivar.

3.7. Cluster Analysis

The Nei genetic similarity index ranged from 0.74 between (CGN 10105 and CGN 10108); (CGN 10105 and CGN 10109); (CGN 10105 and CGN 10113) and (CGN 10109 and CGN 10113) to 0.88 between (CGN 10108 and CGN 10109) (Table 10). The genetic identity between *L. albus* genotypes felled into the range of 0.74 to 0.88 as shown in the UPGMA tree (Figure 5)

Three major groups were observed. The lupin genotypes CGN 10106; CGN 10113 and Balady cultivar were put in the first group (I): (similarity range 0.74 to 0.83), whereas the accessions CGN 10108, CGN 10109 and CGN 10112 were placed within the second group (II): (similarity range 0.74 to 0.88). However, CGN 10105, which were put in the third group: (similarity range 0.74 to 0.81), referred to be the most distinct but joined with groups I and II (Figure 5).

Table 10. Genetic similarity and genetic distance statistics for seven genotypes of *L. albus.*

Accessions	*L. albus* CGN 10105	*L. albus* CGN 10106	*L. albus* CGN 10108	*L. albus* CGN 10109	*L. albus* CGN 10112	*L. albus* CGN 10113	*L. albus* cv. Balady
L. albus CGN 10105	1.00						
L. albus CGN 10106	0.81	1.00					
L. albus CGN 10108	0.74	0.81	1.00				
L. albus CGN 10109	0.74	0.80	0.88	1.00			
L. albus CGN 10112	0.77	0.81	0.86	0.86	1.00		
L. albus CGN 10113	0.74	0.83	0.79	0.74	0.78	1.00	
L. albus cv. Balady	0.79	0.83	0.80	0.78	0.82	0.83	1.00

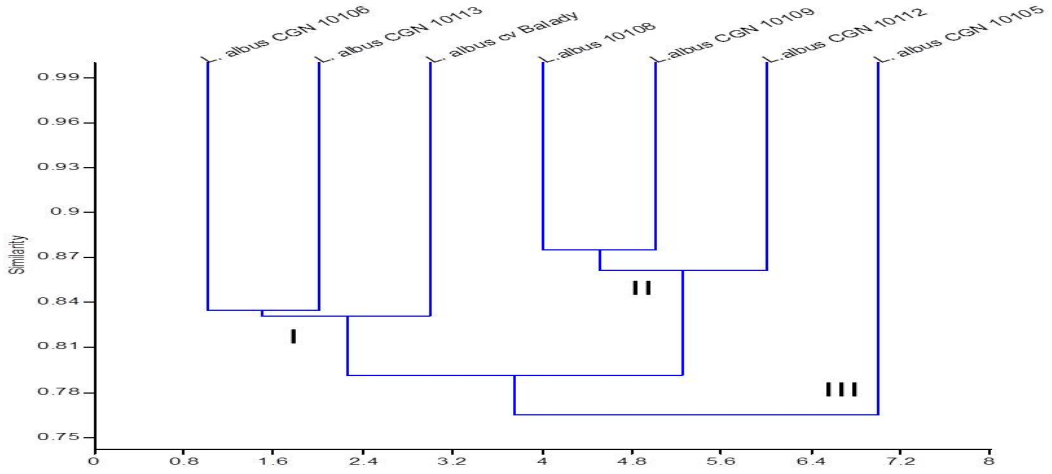

Figure 5. UPGMA dendrogram of seven genotypes *Lupinus albus* depend on Jaccard"s similarity coefficient

4. Discussion

White lupin (*L. albus* L.), is a member of the family *Fabaceae*. Lupin seeds are used as source of a protein for the human and animal nutrition due to their nutritional value (high in lipids, protein and dietary fiber). The present study is conducted to characterize the genetic relationships among six white lupin imported from Centre for Genetic Resources, and one local Egyptian cultivar Balady. Seven genotypes of white lupin seeds were exposed to water deficit. Our results showed highly significant differences in the field performance and drought tolerant among tested seven genotypes. Two accessions CGN 10106 and CGN 10108 were tolerant of drought compared with other genotypes. Thus, these genotypes recorded the highest seed yield when exposed to water stress. These results are in agreement with Annicchiarico *et al*., (2010), Mut *et al.,* (2012) and EL-Harty *et al*., (2016) who found significant differences among the Egyptian landraces of white lupin in crop components in various environments but seasonal variance was non-significant for the number of branches, plant height, seeds pod^{-1} and pods $plant^{-1}$.

In the present investigation, the content of the macronutrients in the studied genotypes was compared with those of other studies on different legume seeds, including *L. albus*, carried out by Özcan *et al*., (2013). From the previous results, it is observed that the highest concentration of N recorded in (CGN 10106); (K, P and Na (CGN 10113 and Balady); Mg and Ca (CGN 10105 and CGN 10112) genotypes. However, the maximum contents of micro-nutrients were scored in genotypes CGN 10112, CGN 10113 and Balady. Concentration of micronutrients in the studied white lupin seeds was of less values than those in the *L. albus* reported by Özcan *et al*., (2013) and of similar or higher values than those reported in other varieties (Bartkiene *et al*., 2016). In general, essential elements are necessary for physiological and metabolic processes in the human body (Alsafwah *et al*., 2007; Bartkiene *et al*., 2016).

Several biochemical (protein and isozyme) and molecular markers (RAPD and ISSR) have been used to assess the genetic diversity of the seven *L. albus* genotypes. In the present study, slight differences were observed in the total protein bands among seven *L. albus* genotypes, thus polymorphism was low (28.57%). These results are in agreement with Muzquiz *et al*., (2011) who mentioned that lupins contained the major storage proteins, such as albumins and three globulin kinds: α, β, γ-conglutin (Melo *et al*., 1994). β- conglutin is the main constituent; it acts the largest heterogeneity among white lupin species, showing several polypeptide chains with molecular weights ranging from (15 to 72 kDa). The α-conglutin fraction consists of heavy polypeptide subunits with molecular masses from (31 to 63 kDa) and a lighter polypeptide subunit of 20 kDa, and γ-conglutin, usually the minor constituent, including two polypeptide chains (the first chain 17 kDa and the second chain 27-30 kDa) (Melo *et al*., 1994).

Isozyme spectra of PPO and POX isoforms were determined by native-PAGE in leaves of seven *L. albus* genotypes. Our results revealed that seven white lupin genotypes varied in expression from strong to low both PPO and POX isozymes. The highest expression of antioxidant enzymes was recorded in two genotypes tolerant of drought CGN 10106 (POX) and CGN 10108 (POX and PPO). Therefore, antioxidant isozymes play a main role in the tolerance of the plant to water stress. These findings agree with those obtained by Horáček *et al*., (2009) who found that the variations in the isozyme have a great importance in the plants' breeding programs, especially in defense reactions to biotic and abiotic stresses.

All the assays used in the present study were able to uniquely fingerprint each of the seven white lupin genotypes. RAPD recorded the highest percentage of polymorphism 47.96%. On the contrary, for ISSR gave 29.82% polymorphism. Comparing between RAPD and ISSR loci, the ISSR has the capability of scoring more polymorphism to the primers barely amplify the non-coding regions of the white lupin genome, which are highly polymorphic. The RAPD loci amplifies both coding and non-coding DNA sequence of the white lupin genome, but when it amplifies in one region it does not amplify in another, decreasing the possibility of amplifying the most polymorphic sequences. According to reproducibility, the ISSR profile was indicated to be more specific in that it employs greater primers and needs higher annealing temperatures lessens the non-reproducibility that is so highly linked with RAPD (McGregor *et al*., 2000). Yorgancilar *et al*., (2009) used ISSR and RAPD loci to estimate the genetic variability among 20 old world lupin accessions and obtained that there are relationships between Egyptian and some American accessions and found that American genotype was screened from Egyptian origin materials. Talhinhas *et al*., (2003) as well as Al-Rawashdeh and Al-Rawashdeh (2015) reported that the low genetic similarity among *Lupinus* spp is most unlikely to be due to the differences in coding. You *et al*., (2005) and Yuan *et al*., (2005) mentioned that both RAPD and ISSR loci are dominant markers and their combination showed that *L. albus*, *L. luteus* and *L. angustifolius* were put in three different groups with minor genetically distances between the individuals of each group. Also, ISSR profiles are recognized to be more sensitive than RAPD technique which is again confirmed here.

Our results showed that biochemical (protein and isozymes) molecular (RAPD and ISSR) markers are beneficial to characterize the genetic diversity and evaluation of genetic distances among seven *L. albus* genotypes. Also, a combination among these assays could detect polymorphism in the tested seven *L. albus* genotypes to distinguish each genotype from the others by the unique band. Moreover, these results are important in the breeding programs for the selection process of parental strains that feasibility the prediction of crosses to generate hybrids with the best performance and drought tolerant.

5. Conclusion

The seven white lupine genotypes showed different responses under water deficit stress conditions. Analysis of variance (ANOVA) revealed that there are significant differences among the seven tested genotypes under water deficit stress. Two accessions, *L. albus* CGN 10106 and CGN 10108, were tolerant against drought compared with

other genotypes. The macro- and micro-element contents of seven *L. albus* genotypes were found to be different based on the genotype. The highest content of N was recorded for genotype CGN 10106. However, CGN 10113 seeds showed the best genotype in K, P and Na contents; this is followed the seeds of Balady cultivar. Nevertheless, CGN 10105 and CGN 10112 genotypes scored the highest content of Mg and Ca, respectively. On the other hand, the results revealed that the reduction of the K contents was depicted in CGN 10105 and CGN 10108 genotypes. In addition, the lowest contents of Na were found in CGN 10112. RAPD and ISSR analyses recorded the percentage of polymorphism 47.96% and 29.82%, respectively. The Nei genetic similarity index among genotypes ranged from 0.74 to 0.88, based on biochemical and molecular markers combined using UPGMA. So, these genotypes could be used in the future white lupin breeding programs in Egypt.

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Direct Bioconversion of Sorghum Straw to Ethanol in a Single-step Process by *Candida* species

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Abstract

The present study explores the potential of *Candida* species to convert sorghum straw biomass to ethanol. Two strains of *Candida* species (*C. tropicalis* and *C. shehatae*) were used to produce ethanol by distillation of fermented sorghum straw medium. These yeasts exhibited high amylolytic, cellulolytic and fermentative ability and were used for bioconversion of sorghum straw [2.5 - 15 % (w/v)] at pH (4.0 – 7.0). The yeasts were capable of producing ethanol from solutions containing 7.5 % sorghum straw. Ethanol production during optimization of growth parameters showed that *C. tropicalis* produced more ethanol (38.12 g/L) than *C. shehatae* (30.32 g/L), except optimization of incubation temperature where *C. shehatae* produced more ethanol (43.96 g/L) than *C. tropicalis* (35.10 g/L). The present study suggests cellulolytic yeasts, such as *C. tropicalis* and *C. shehatae*, for direct ethanol production from lignocellulosic material.

Keywords: Ethanol; Direct bioconversion; cellulolytic yeast; *Candida* species; Lignocellulosic.

1. Introduction

Bioethanol production is being considered an alternative source of energy due to the prediction that there will be exhaustion of fuel energy supply (Ariyajaroenwong *et al*., 2012). Bioethanol is mainly produced from sugar or starchy biomass (Agbogbo and Coward-Kelly, 2008) which poses a competition for the raw materials with food industry. In the last decade, attention started to shift to lignocellulosic feed stocks for ethanol production through multistage process including pretreatment, enzymatic hydrolysis, sugar fermentation and process design. Most of the processes developed toward industrial scale involve the addition of enzymes for cellulose and hemicellulose hydrolysis and use of specific yeast strains engineered to utilize pentose and hexose sugars during fermentation process (Bettiga *et al*., 2009). Both achieving effective biomass hydrolysis and complete sugar conversion are essential for an economical process (Kurian *et al*., 2010).

A process that aims at circumventing this multistage and cost prohibitive, such as critical cost-increasing item, is the direct microbial conversion or Consolidated Bioprocessing (CBP) is considered necessary (Lynd *et al*., 2002). In CBP, an organism or a mixed culture of organisms simultaneously produce hydrolytic enzyme and ferment the pentose and hexose sugars into ethanol or other valuable products without the addition of cellulolytic enzymes. This alternative process is envisaged to reduce energy consumption of the overall process of ethanol production (Lynd *et al*., 2002). *Pichia stipitis*, *Candida shehatae*, and *Pachysolan tannophilus* are known to use both pentose and hexose sugars (Agbogbo and Coward-Kelly, 2008). The advantage of the single-step bioconversion is that the process is carried out in one bioreactor where hydrolysis and fermentation take place at the same time. Microbial conversion of lignocellulosic materials to ethanol is performed by the action of xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) (Khan and Dwivedi, 2013). These metabolic capacity has been reported in several yeast species, such as, *Debaryomyces hansenii*, *Meyerzyma guillermondii* and *Candida parapsilosis* (Lourenco *et al*., 2014; Latif and Rajoka, 2002). *Candida* species are asporogenous diploid yeast, which can utilize a very large variety of carbon sources including many sugars, disaccharides, phenols, alkanes, alkane derivatives, and fatty acids (Sanchez *et al*., 2009).

Huge volumes of cellulosic materials, such as sorghum straw, are renewable resources being generated as waste from various agro allied industries (Das and Singh, 2004). These potential can be exploited as sustainable resource for production of many organic fuels and bioenergy. They can reduce greenhouse gas emissions, enhance energy security, improve the economy, dispose problematic solid wastes, and improve air quality (Das and Singh, 2004).

Bioconversion of corn straw into ethanol seems to be one of the solutions to the increasing demands of energy. Although Oyeleke and Jibrin (2009) had produced bioethanol from guinea corn and millet husk through acid

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hydrolysis and fermentation with *Aspergillus niger* and *Zymomonas mobilis*. Wakil *et al*. (2013) also produced bioethanol from palm oil mill effluent using moulds and yeast, but literature has been silent on single-step production of bioethanol through consolidated bioprocesses. The present study presents here reports on the production of ethanol from sorghum straw by *Candida* species in a single step process.

2. Materials and Methods

2.1. Source of Microorganisms

Four *Candida* species (*C. tropicalis*, *C. shehatae*, *C. utilis* and *C. krusei*) were obtained from the Culture Collection Centre of the University of Agriculture, Abeokuta, Nigeria. The cultures were maintained on Yeast Extract Peptone Dextrose (YEPD) agar slant at 4 ^{o}C and sub-cultured twice a month.

2.2. Screening of Yeasts

2.2.1. Screening for Amylolytic Yeasts

Yeast isolates were qualitatively screened for using Gram iodine solution. Purified yeast isolates were grown on agar plates containing 1% starch agar which were inoculated with pure yeast isolates and were incubated at 30 ˚C for 3 days. The plates were flooded with grams iodine solution, colonies forming clear zones were selected for quantitative screening (Kareem *et al*., 2009). Quantitative screening was carried out using YEPD broth containing $MgSO_4.7H_2O$, 0.03 g; $FeSO_4.7H_2O$, 0.5 g; $MnSO_4.H_2O$, 0.16 g; $ZnSO_4.7H_2O$, 0.14 g. Culture media were inoculated with pure yeast isolates and incubated under shaking condition (150 rpm) at 30 ˚C for 3 days, amylase production was quantified using the method of Kareem *et al*. (2009).

2.2.2. Screening for Cellulolytic Yeast

Yeast isolates were screened for cellulose qualitatively using congo red test. Purified yeast isolates were grown on agar plates containing 1% carboxyl methyl cellulose (CMC). Plates were inoculated with pure yeast isolates and were incubated at 30 ˚C for 3 days and flooded with 1% Congo red solution for 30 min and de-stained with 1 M NaCl solution for 20 min (Saliu, 2012). Quantitative screening was carried out using modified YEPD which consist of 1% CMC, NH_4NO_3, 0.2 g; KH_2PO_4, 0.5 g; $CaCl_2.2H_2O$, 0.03 g; $MgSO_4.7H_2O$, 0.03 g; $FeSO_4.7H_2O$, 0.5 g; $MnSO_4.H_2O$, 0.16 g; $ZnSO_4.7H_2O$, 0.14 g; Tween-80, 0.1 g. Culture media were inoculated with pure yeast isolates and were incubated under shaking condition (150 rpm) at 30 ˚C for 3 days and cellulase production was quantified according to the method of Saliu (2012).

2.2.3. Screening for Ethanol Producing Yeast

Purified yeast isolate were screened for fermentative ability using YEPD broth prepared in test tubes containing inverted Durham tube (Wakil *et al*., 2013). Test tubes were inoculated and incubated at 30 ˚C for 3 days, isolates were selected based on the volume of gas in Durham tube during the incubation period (Brooks, 2008). Quantitative screening was carried out by distillation using 5% starch according to the method of Wakil *et al*. (2013).

2.3. Selection of Starters

Two *Candida* spp (*C. tropicalis* and *C. shehatae*) with best amylolytic, cellulolytic and ethanol producing abilities were selected from the four *Candida* species obtained.

2.4. Determination of Fermentative Parameters of Selected Yeasts

Enzymes released from selected yeast were used for hydrolysis of corn and sorghum straw (10 % w/v). Each product of hydrolysis was fermented by the yeasts. Using the method of Lazarova *et al*. (1987), fermentative parameters of selected yeasts were determined using 10 mL needle and syringe inverted into injection bottles. Carbon dioxide productivity, volumetric ethanol productivity, theoretical alcohol recovery, actual alcohol recovery and fermentation efficiency were determined.

2.5. Processing of Substrate

Sorghum straws were collected from a farm at Kishi in Oyo State, Nigeria. The straws were oven dried at 70 ^{o}C for 2 hours and grounded into powdered using an electric blender (Philips INO23) and was sieved using 40 mm mesh. 10 % of the straw was used for fermentation.

2.6. Ethanol Production

2.6.1. Fermentation of Sorghum Straw

Yeast strains were grown in a 1 L Erlenmeyer flask that contained 700 mL of basal medium containing: NH_4NO_3 1.2 g; KH_2PO_4 0.8 g; $CaCl_2.2H_2O$ 0.3 g; $MgSO_4.7H_2O$ 0.3 g; $FeSO_4.7H_2O$ 0.4 g; $MnSO_4.H_2O$ 1.5 g; $ZnSO_4.7H_2O$ 1.3 g; Tween-80 0.15 g; peptone 0.75 g, yeast extract 0.3 g; glucose 5 g and 10 % sorghum straw. The pH of the medium was adjusted to 5.5 prior to sterilization. The flask was inoculated with 5 % yeast suspension and incubated at 30 ºC for 96 hours (Hashem *et al*., 2013). Fermented corn straw was analyzed for ethanol production at 24, 48, 72 and 96 hour.

2.6.2. Fractional Distillation

Distillation of the fermented medium was carried out using 100 mL of each fermented medium which was dispensed into round-bottom flasks fixed to a distillation column enclosed in running tap water. A conical flask was fixed to the other end of the distillation column to collect the distillate. A heating mantle with temperature adjusted to 78 ˚C was used to heat the round bottom flask containing the fermented sample (Wakil *et al*., 2013).

2.6.3. Determination of Quantity of Ethanol Produced

The distillate collected over a slow heat at 78°C was measured using a measuring cylinder, and expressed as the quantity of ethanol produced in g/L by multiplying the volume of distillate collected at 78°C by the density of ethanol (0.8033 g/mL). Gram/L is equivalent to the yield of 100 g of dried substrate (Wakil *et al*., 2013).

2.7. Optimization the Fermentation Conditions of Ethanol Production

2.7.1. Effect of Substrate Concentration

Ethanol production was carried out at constant pH, incubation temperature and inoculum concentration using

various substrate concentrations (5%, 7.5%, 10% 12.5%) of sorghum straw. Samples were taken at 72 hours of incubation. Ethanol productions by yeast stains were determined as previously described.

2.7.2. Effect of Temperature

Ethanol was produced from the substrates in flasks inoculated with yeast cells. The flasks were incubated at different temperature (30 , 35 , 40 , 45 , 50 and 60 ^{o}C). Other growth conditions were constant. Ethanol productions by yeast stains were determined as previously described.

2.7.3. Effect of pH

Effect of pH on ethanol production, using the selected yeast strains, was studied by conducting experiments at different pH (4.0, 4.5, 5.0, 5.5 and 6.0) while all other parameters were kept constant. Ethanol productions by yeast stains were determined as previously described.

2.7.4. Effect of Inoculum Concentration

Effect of inoculum concentration on ethanol production by the selected yeast strains was carried out using YEPDA medium incorporated with sorghum straw. The medium was sterilized and inoculated with varying yeast suspension of 5, 7.5, 10, 12.5 and 15 %. Other growth conditions were constant. Ethanol productions by yeast stains were determined as previously described.

2.7.5. Statistical Analysis

All the experiments were performed in triplicates and the results were presented as mean ± standard deviation and were also analyzed by ANOVA using statistical software SPSS version 17. 0.

3. Results

3.1. Screening of Yeasts

All the yeast strains tested positive for amylase and cellulase production by showing clear zones on starch and carboxyl methyl cellulose (CMC) agar. *Candida tropicalis* produced the highest halo zone (39.0 mm) followed by *C. shehatae* (36.0 mm), while the least was observed in *C. utilis* (10.0 mm) (data not shown). Result of the quantitative screening showed that the highest amylase activity was produced by *C. tropicalis* (319.50 U/mL) while *C. utilis* had the least amylase activity (136.46 U/mL). Highest cellulase activity was produced by *C. tropicalis* (174.67 U/mL) followed by *C. shehatae* (161.38 U/mL) while the least cellulase activity was observed in *C. utilis* (100.18 U/mL) (Table 1). Screening for ethanol production showed that *C. tropicalis* had the best ethanol producing ability (31.96 g/L), followed by *C. shehatae* (26.13 g/L) while *C. krusei* produced the least (13.63 g/L) (Table 1).

Two yeasts (*C. tropicalis* and *C. shehatae*), which displayed the best amylolytic, cellulolytic and ethanol producing abilities, were selected for bioethanol production in submerged fermentation.

Table 1. Screening for amylase, cellulase and ethanol production in yeast isolates.

Yeast Isolates	Enzyme activity (U/mL)		Ethanol (g/L)
	Amylase	Cellulase	
C. shehatae	246.63±11.76	161.38±23.21	26.13±6.27
C. krusei	171.84±80.62	112.31±14.98	13.63±0.90
C. utilis	136.46±35.64	100.18±9.44	16.32±2.17
C. tropicalis	319.50±34.63	174.67±24.54	31.96±10.58

Each value is a mean of 3 readings ± standard deviation

3.2. Measurement of Fermentative Parameters of Yeasts on Hydrolyzed Sorghum Straw Medium

Fermentative parameters (carbon dioxide productivity and volumetric ethanol productivity) of the yeasts on hydrolyzed sorghum straw are presented in Figure 1. *Candida tropicalis* had the highest carbon dioxide production (3.93 L/L.h) while *C. shehatae* had (3.81 L/L.h) (Figure 1). Maximum volumetric ethanol production was achieved by *C. tropicalis* (9.43 g/L.h) while *C. shehatae* had (9.14 g/L.h) (Figure 1). Total alcohol recovery, actual alcohol recovery and fermentation efficiency of the yeasts were presented in Table 2. The yeasts had total alcohol recovery of 4.0 %. *Candida tropicalis* had maximum actual alcohol recovery and fermentation efficiency of 1.68 % and 42 %, respectively, while *C. shehatae* had actual alcohol recovery and fermentation efficiency of 1.55 % and 39 %, respectively (Table 2).

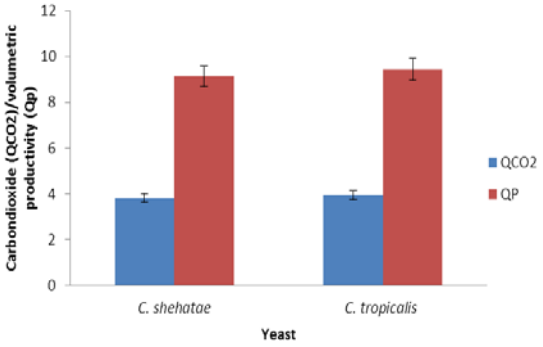

Figure 1. Carbon dioxide productivity and volumetric ethanol productivity of yeasts on hydrolysed sorghum straw medium

Note: QCO_2; Carbon dioxide productivity (L/L.h) ; Qp; Volumetric ethanol productivity (g/L.h)

Table 2. Fermentation parameters of yeasts on hydrolyzed sorghum straw medium.

Yeast	Total Alcohol Recovery (%)	Actual Alcohol Recovery (%)	Fermentation Efficiency (%)
C. shehatae	4.0±0.014	1.55±0.025	39±1.52
C. tropicalis	4.0±0.014	1.68±0.015	42±2.08

Each value is a mean of 3 readings ± standard deviation

3.3. Ethanol Production from Sorghum Straw

The result, presented in Figure 2, shows production of bioethanol from sorghum straw. Volume of ethanol increased with increased fermentation time with the two yeasts. The two yeasts produced ethanol throughout the fermentation period. Ethanol production by *C. tropicalis* was higher than that of *C. shehatae* (Figure 2). *Candida*

tropicalis produced maximum quantity of ethanol (16.25 g/L) at 72 hour of fermentation while *C. shehatae* produced 12.50 g/L at 72 hour. Further increase in fermentation time decreased ethanol production (Figure 2). Although the two yeasts had almost the same volume of ethanol at 24 hour of fermentation, rapid bioethanol production was observed in *C. tropicalis* after 48 hour (8.10 – 14.80).

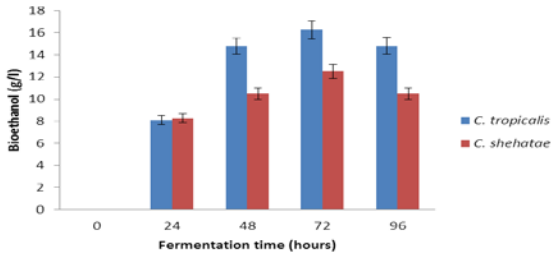

Figure 2. Ethanol production from sorghum straw

3.4. Optimization of Fermentation Conditions of Ethanol Production

3.4.1. Effect of Substrate Concentration on Ethanol Production

Ethanol productions at different substrate concentration of sorghum straw by the two yeasts are summarized in Figure 3. Ethanol production increased gradually with the use of 5 % to 7.5 % and thereafter declined. *Candida tropicalis* produced the highest volume of ethanol (28.65 g/L) awhile *C. shehatae* produced (22.08 g/L) (Figure 3). Bioethanol production with 15 % sorghum straw concentration with the two yeasts produced the least volume of ethanol.

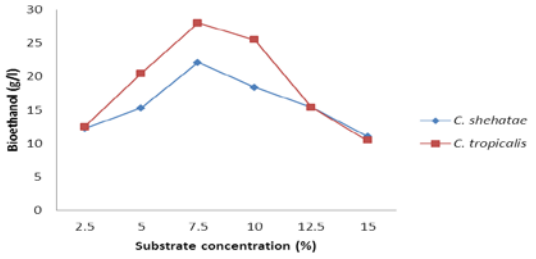

Figure 3. Effect of substrate concentration on ethanol production

3.4.2. Effect of pH on Bioethanol Production from Sorghum Straw

Ethanol production at different pH from sorghum straw by yeast strains are shown in Figure 4, with pH 5.5 having highest yield of ethanol. Ethanol production by direct conversion with *C. tropicalis* (35.81 g/L) was the highest among the yeast strains. On the other hand *C. shehatae* produced lowest volume of ethanol (17.0 g/L) during fermentation period. Fermentation at pH 7.0 produced the least volume of bioethanol (Figure 4).

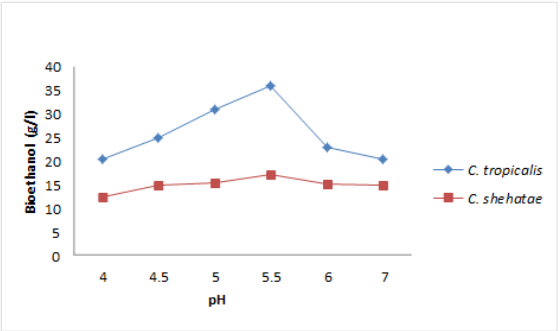

Figure 4. Effect of pH on bioethanol production from sorghum straw

3.4.3. Effect of Inoculum Concentration on Bioethanol Production

Result, presented in Figure 5, shows the effect of inoculum concentration on ethanol production from sorghum straw. Inoculum concentration of 7.5 % was observed as the optimum for ethanol production. Highest ethanol production was observed with *C. tropicalis* fermented sorghum straw (38.12 g/L) while *C. shehatae* produced least ethanol (30.32 g/L) during fermentation (Figure 5). Bioethanol production with 7.5 % inoculum concentration produced highest volume of ethanol, followed by 5 % inoculum concentration while 15 % produced the least bioethanol (Figure 5).

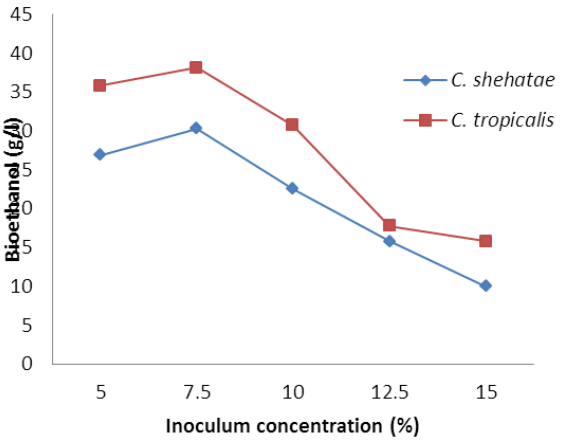

Figure 5. Effect of inoculum concentration on bioethanol production

3.4.4. Effect of Incubation Temperature on Bioethanol Production

Experimental data, presented in Figure 6, shows the effect of different incubation temperature ranging from 30 °C to 60 °C on bioethanol production by yeast strains grown in medium containing sorghum straw. The Figure indicates that the selected yeast strains were able to produce bioethanol from sorghum straw with all temperature. *Candida shehatae* produced the highest volume of ethanol during the fermentation (43.98 g/L) while *C. tropicalis* produced 35.1 g/L (Figure 6). Fermentation at 40 °C produced the highest volume of ethanol, followed by 35 °C while at 60 °C; *C. shehatae* produced 15.62 g/L and *C. tropicalis* produced 12.52 g/L which is the least volume of ethanol produced.

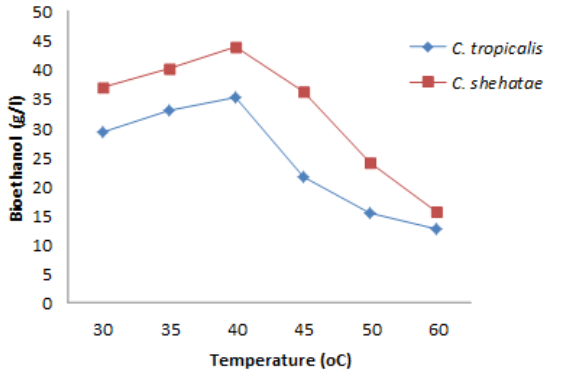

Figure 6. Effect of incubation temperature on bioethanol production

4. Discussion

Yeasts are industrially important unicellular microorganism due to their ability to hydrolyze polysaccharide to monomers and their fermentative role in biosynthesis of ethanol. They are found everywhere and thus can be easily isolated from the environment. *Candida* species are used in the present study for the production of ethanol. One factor that could have been responsible for the presence of *Candida* spp in the environment is the nature of the yeast. Yeast, especially *Candida* spp are known to adapt best in high temperature and low pH (4-5). These observations are in agreement with earlier studies (Bey *et al*., 2011; Boonmak *et al*., 2011). *Candida tropicalis* and *C. shehatae* used in the present study were identical to those earlier reported by Rai *et al*. (2012) who identified *Candida* spp as yeast used for saccharification of sugar cane baggase which shows that *Candida* spp are cellulolytic yeast. Idowu and Edema (2003) also identified *Candida* spp as cellulolytic yeasts that can digest food materials.

In this present study, all the yeast strains (*C. tropicalis*, *C. shehatae*, *C. utilis* and *C. krusei*) produced amylase, cellulase and ethanol with *C. tropicalis and C. shehatae* having the highest production, thus they were selected as very high ethanol producing yeast strains. These observations are in agreement with earlier studies by Limtong *et al*. (2012) who state that yeast species, such as *Candida shehatae*, *Pachysolen tannophilus*, *Scheffersomyces (Pichia) stipites*, had been reported to assimilate cellulose and ferment it to ethanol. *Candida shehatae* and *C. tropicalis* were introduced as new stains and are used to study the effect of fermentation conditions on their efficiency in ethanol production.

Yeasts growth is usually accompanied with fermentation. They have been referred to as being respiro-fermentative organisms (Aransiola, 2006). Actively growing yeasts are likely to be actively fermenting. Carbon dioxide production, volumetric ethanol productivity, theoretical alcohol recovery and fermentative efficiency are important parameters to be studied in ethanol producing yeasts (Nwachukwu *et al*., 2006). Analysis of fermentation parameters in the fermentation of hydrolysed corn and sorghum straw showed the difference in the fermentative parameters of the yeasts. *Candida tropicalis* fermented sorghum straw had the highest carbon dioxide productivity and fermentation efficiency; this may be due to the yeast to easily use up sugars present in the hydrolysed sorghum straw (Tahmina and Capareda, 2011).

Yeast may be confronted with different environmental factors that can cause the loss of yeast cell viability and decreased fermentation rates (Hashem *et al*., 2013). Fermentation period is an important factor in ethanol production. Results from the present work show that ethanol increased gradually with increasing in incubation time with each of the yeast used: *C. tropicalis* and *C. shehatae* and reached their maximum at 72 hours of fermentation and dramatically decreased with further extension of time with each of the yeast. These findings are in agreement with those of Kurian *et al*. (2010) who reported that highest ethanol production by yeasts at 72 hours. *Candida tropicalis* was found to be better than *C. shehatae*. This may be due to the fact this yeast has more cellulolytic enzyme (xylose isomerase) which is responsible for the breakdown of lignocellulolytic materials to glucose (Aristidou and Penttila, 2000). Latif and Rajoka (2002) confirmed *C. tropicalis* as the major yeast that has enzyme xylose reductase which is responsible for the bioconversion of lignocellulolysic materials. *Candida shehatae* had been also recorded as naturally occurring yeast that is xylose-fermenters (Khan and Dwivedi, 2013).

Production of ethanol was affected by sorghum straw concentration between 5 and 12.5 %. *Candida tropicalis* gave the highest yield. Production of ethanol decreased by increasing substrate concentration above 7.5 %, this could be due to decrease in sugar utilization which results in reduction of total ethanol production (Reddy and Reddy 2006). Increase in sorghum straw concentration could have also led to high concentration of complex sugars in the fermentation medium and this could have had a high inhibitory effect on yeast growth and their capability to produce ethanol (Wakil *et al*., 2013). This has been reported by Pratt-Marshall *et al*. (2003) who observed that the fermentation of high gravity worts has a negative effect on the yeast performance due to the elevated osmotic pressure. High substrate concentration leads to decrease ethanol production. This reduction could be due to increase in ethanol production at high sugar concentration which exerts high toxicity on yeast and the nutrients may be deficient at the final stage of the fermentation (Hashem *et al*., 2013). This is in agreement with the work of Kumar and Murthy (2011) who reported 6% xylose concentration for maximum ethanol productivity of *Pichia stipitis*, which is comparable with the present study.

Ethanol production varies with changes in physical parameters, such as temperature and pH of the production medium. The effect of initial pH of the fermentation media on ethanol production showed that the highest ethanol concentration was obtained by *C. tropicalis* in medium with initial pH 5.5. Any change in this parameter induces morphological changes in microbes (Bodade *et al*., 2010). Russell (2003) also recorded that yeast prefers an acidic pH and its optimum pH is 5.0-5.2 but brewing yeast can grow at the pH range of 3.5 to 6.0.

Inoculum concentration of 7.5 % produced the highest volume of ethanol. Although inoculum concentration is known to play a vital role in the production of microbial metabolites; however, higher concentration of cell did not

lead to improved ethanol yield. This may be attributed to substrate limitations or product inhibition and also supported by the finding of Mahoney (2003). The results of Kourkoutas *et al*. (2004) confirmed our results, where they observed maximum ethanol from *S*. *cerevisiae* at 10% inoculum size, whereas Anxo *et al*. (2008) observed the highest ethanol production by *S. cerevisiae* at 5.0% v/v inoculum size. Lower ethanol biosynthesis at lower inoculum size is probably due to the less cells which are insufficient to use the fermentation medium for enzyme maximal activity, while the decreased yield at higher inoculum size might probably due to nutritional imbalance caused by tremendous growth resulting in autolysis of cells (Shafei and Allam, 2010).

Fermentation temperature has a significant effect on ethanol production. *Candida shehatae* was observed to adapt and produced ethanol at high temperature than *C. tropicalis.* This may be due to the fact this yeast strain code for genes that help to tolerate high temperature. In industry, it is commonly believed that 20 -35 ^{o}C is the ideal range for fermentation and at higher temperatures, almost all fermentation would be problematic (Phisalaphong *et al*., 2006; Aldiguer *et al*., 2004). However, in the present study, when the temperature was increased to 40 ^{o}C, the yeast still produced high volume of ethanol. Using a higher fermentation temperature, similar to the optimal temperature for cellulolytic activity, it may be possible for direct microbial conversion process to improve the final efficiency (Yan *et al*., 2012). In addition, volume of ethanol was found to decline at temperature above 40 ^{o}C, the reason might be that fermentation at higher temperature might disrupt enzyme activity and membrane function (Aldiguer *et al*., 2004).

A recent finding shows that approximately 35 g/L of ethanol had been produced form agricultural waste (Cutzu and Bardi, 2017), while 38.12 g/L of ethanol was produced from the present study. Conversion of lignocellulosic material into ethanol still has economic, technical and environmental obstacles, thus different feedstocks and methods should be studied to make it more feasible. Bioethanol production method has to be efficient (high energy yields), cost effective (energy return on investment) and environmentally beneficial, in order to be feasible. Also single-step production of bioethanol is economically feasible; therefore, more research and technological development are needed. As a recommendation, governmental policies are important to promote bioethanol research and make its price competitive with other sources of energy. Moreover, there should be participation of all stake holders to enhance energy security.

5. Conclusion

From the present study, it is concluded that cellulolytic yeasts (*C. tropicalis* and *C. shehatae*) can produce ethanol directly from sorghum straw using a single-step approach. These yeasts produced appropriate hydrolytic enzymes thus no external enzymes were required. The direct conversion of sorghum straw to ethanol by *C. tropicalis* and *C. shehatae* is significant in single-step production of bioethanol

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Effect of *Solanum nigrum* Methanol Leaf Extract on Phenylhydrazine Induced Anemia in Rats

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Abstract

Anemia is a global health problem affecting both developed and developing countries, characterized by low level of haemoglobin in the blood. The effect of *Solanum nigrum* methanol leaf extract on phenyl hydrazine induced anemia in rats was investigated using an automatic counter. Forty-two (42) Albino rats were induced anemia through intraperitoneal injection of phenylhydrazine at 10mg/kg for 8 days. Packed cell volume was taken after some hours to ensure that the rats were anemic; those with packed cell volume less than 29% were grouped into seven groups of six rats each. Methanol extract of *Solanum nigrum* was administered at 100, 200, 300 and 400 mg/kg/body weight to groups 4, 5, 6 and 7 for three weeks orally by gastric intubation. Result obtained revealed that oral administration of *S. nigrum* methanol leaf extract to rats previously treated with phenylhydrazine significantly (p<0.05) increased the packed cell volume, haemoglobin, red blood cells, mean corpuscular volume, mean capsulated haemoglobin, and platelets in a dose dependent manner but decreased the white blood cells, lymphocytes and neutrophils within three weeks. Phytochemical analysis of the plant revealed the presence of alkaloids, saponins, flavonoids, phenols and tannins. The extract also contains substantial amount of vitamins A, K, B_6, C, E, and folic acid. Mineral elements, such as iron, magnesium, calcium, zinc and copper, were also observed in the plant extract. Results obtained also revealed that the methanol leaf extract of *S. nigrum* exhibited strong antioxidant activity measured using 2, 2-Diphenyl-l-Picryl Hydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP) assay at different concentrations of the methanol extract (20, 40, 60, 80 and 100 mg/mL). The findings of the present study suggest that *S. nigrum* methanol leaf extract contains hematinic properties thus, justifying the use of the plant in the management of anemia in north eastern Nigeria.

Keywords: Anaemia, *Solanum nigrum*, Vitamins, Mineral elements, Phytochemicals, Phenyl hydrazine, Haematological Parameters.

1. Introduction

Anemia is a medical condition in which the red blood cells count is less than normal. It is evidenced by a reduced quality or quantity of red blood cells. It is devastating effects on health, physical and mental productivity affect the quality of life and translate in to significant economic losses for individuals and for countries with high anemia prevalence (Diallo *et al*., 2008). Anemia is one of the world's most widespread health problems. It affects more than one third of the world's population. In almost all the developing countries, between one third and one-half of the females and children are anemic. The prevalence among pregnant women and children under two years of age is typically more than fifty percent (WHO, 2002).

Anemia has multiple causes categorized as poor, insufficient or abnormal red blood cells production, excessive red blood cells destruction and excessive red blood cell loss (Dacia and Lewis, 2004). According to WHO 2005, several factors are associated with anemia; these are iron deficiency, micronutrient deficiency, malaria, parasitic infestation and HIV infection.

A good number of medicinal plants are traditionally employed to alleviate anemia. Some of these plants include *Spinacia oleracea*, *Telfeira occidetallis*, *Jatropha curcas*, *Waltheria indica and Spondias mombin* (Luka *et al*., 2014; Dina *et al*., 2006). *Solanum nigrum* is a species in the Solanum genus, native to Eurasia and introduced in the America, Australia, and South Africa. The plant has a long history of medicinal usage, dating back to ancient Greece. Plant parts are used in traditional medicine. The juice of the plant is used on ulcers and other skin diseases. The fruits are used as a tonic, laxative, appetite stimulant, and for treating asthma and "excessive thirst." The plant *Solanum nigrum* (black night-shade) commonly known as *kumbi* in *Hausa* is a widely used plant in oriental medicine where it is considered to be antitumor, antioxidant, anti-inflammatory, hepatoprotective, diuretic, and antipyretic (Jain *et al*., 2011). *Solanum nigrum* is also used in the

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north eastern Nigeria to treat anemia. The present study, therefore, seeks to scientifically look at the antianemic potential of *Solanum nigrum* on phenylhydrazine induced anemia.

2. Materials and Methods

2.1. Plant Material

Solanum nigrum leaf was collected in March from farm in vunoklang, Girei Local Government Area of Adamawa State and was authenticated by a Botanist in the Department of Plant Science, Modibbo Adama University of Technology, Yola. The fresh leaf sample was shade-dried for 7 days and milled into coarse powder using a manual blender. The coarse material was sieved using 0.3mm endicott test sieve to obtain a fine powder.

2.2. Preparation of Plant Extract

Powdered sample (1 kg) was extracted with 1.5 L of methanol by cold maceration for 48 h (Trease and Evans, 1989). The solvent extract was then concentrated by evaporating the solvent at 50° C using rotary evaporator and vacuum oven to obtain a dry powder.

2.3. Quantitative Analysis of Phytochemicals

The presence of alkaloids, saponins, flavonoids, total phenols and tannins were determined using the methods of Trease and Evans (1989), Harbone (1973) and Sofowora (1993); Alkaloid was determined using the method of Trease and Evans (1989); Saponin was determined using the method of Harbone (1973); Flavonoid was determined using the method of Harbone (1973); Total phenol was determined using the method of Sofowora (1993); Tannin was determined using the method of Harbone (1973).

2.4. Vitamin Analysis

Vitamin analysis was carried out for vitamin A, K, B_6, C, E and Folate using the method of AOAC (2000).

2.5. Elemental Analysis

The method of AOAC (1990) was used to determine iron, magnesium, calcium, zinc and copper.

2.6. Determination of Antioxidant Activity

2.6.1. Determination of DPPH (2, 2-diphenyl-l-picryl hydrazyl)

DPPH (2,2-diphenyl-l-picryl hydrazyl) radical scavenging assay was determined using the method described by Sasidharan *et al*. (2007). The free radical scavenging activity of the extract was measured by the decrease in absorbance of methanol solution of DPPH. Different concentration of the plant extracts (20, 40, 60, 80 and 100 mg/mL in methanol) was added at an equal volume (10 mL) to methanol solutions of DPPH (400 mg/mL) and incubated for 30 minutes. The absorbance was measured at 517 nm using spectrophotometer (VIS 721, PEC MEDICAL USA). A different concentration of L-ascorbic acid ((20, 40, 60, 80 and 100 mg/mL) was used as standard antioxidant. The antioxidant activity of the leaf extract was compared with L-ascorbic acid. Values obtained were converted in to percentage antioxidant activity using the equation below:

$$\% \text{ DPPH antiradical scavenging capacity} = \frac{\text{absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of blank}} \times 100$$

2.6.2. Determination of Ferric Reducing Antioxidant Power (FRAP Assay)

Ferric Reducing Antioxidant Power (FRAP Assay) was determined using the method described by Banerjee *et al*. (2008). Various concentration (20, 40, 60, 80 and 100 mg/100 mL of the methanol leaf extract was mixed with 1mL of 0.2 M sodium phosphate buffer (pH 6.6) and 1 mL of 1% potassium ferricyanide in separate test tubes. The reaction mixtures were incubated in a temperature-controlled water bath at 50 ^{0}C for 20 min, followed by the addition of 1 mL of 10 % trichloroacetic acid. The mixtures were then centrifuged for 10 min at room temperature. The supernatant obtained (1 mL) was added with 1 mL of de-ionized water and 200 µL of 0.1 % $FeCl_3$. The blank was prepared in the same manner as the samples except that 1% potassium ferricyanide was replaced by distilled water. The absorbance of the reaction mixture was measured at 700 nm. L-ascorbic acid was used as standard. The reducing power was expressed as an increase in $A_{700\ nm}$ after blank subtraction (Banerjee *et al*., 2008). Percentage inhibitory activity was calculated as follows:

$$\% \text{ inhibitory activity} = \frac{\text{absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

2.7. Experimental Animals

Forty-two male albino rats (90 ± 10 g) were obtained from National Veterinary Research Institute (NVRI) Vom, Plateau State. The animals were maintained under standard laboratory conditions and had a free access to standard finisher feeds and water for two weeks for acclimatization before the commencement of the experiments. All animal experiments were conducted in compliance with NIH guidelines for Care and Use of Laboratory Animals.

2.8. Induction of Anemia

Anemia was induced in rats by daily injection (intraperitoneally) of Phenylhydrazine (PHZ) at 10 mg/kg for 8 days (Yeshoda *et al*., 1942). No death was recorded. Rats that developed anemia with PCV less than 29% were recruited for the study (Agbor *et al*., 2005).

2.9. Experimental Design

Forty-two (42) Albino rats weighing 90 ± 10g (eight weeks old) were assigned into 7 groups (n=6) animals per groups. The rats were administered different doses of the methanol extract of *S. nigrum* orally by gastric intubation daily for 3 weeks following Demo *et al*. (2007).

2.10. Collection of Blood Samples

GROUP	TREATMENT
I	Normal Control
II	Experimental Control (Anemia + No treatment)
III	Standard Control (feroton 10 mg/kg body weight)
IV	Treatment group (100mg\kg body weight)
V	Treatment group (200mg\kg body weight)
VI	Treatment group (300mg\kg body weight)
VII	Treatment group (400mg\kg body weight)

At the end of the three weeks' experimental period, the albino rats were sacrificed under chloroform. A blood sample was collected by cardiac puncture. About 3mL of blood was collected into an EDTA sample bottle for haematological assay and sample bottles labeled accordingly for all the 7 groups.

2.11. Determination of Hematological Parameters

The Red Blood Cell count (RBC), White Blood Cell count (WBC), Haemoglobin concentration (HGB), Mean Capsulated Haemoglobin (MCH), Mean Corpuscular Volume (MCV) and Platelets (PLT) were assayed using an automatic counter (Sysmex K21, Tokyo, Japan), as described by Dacie *et al*. (2001).

2.12. Statistical Analysis

Experimental data were analyzed using one-way analysis of variance (ANOVA) and LSD multiple range test to determine significant differences between means. The difference between the means was regarded as significant at $p<0.05$ and the differences of the mean was expressed using SPSS software version 23.

3. Results

3.1. Phytochemical Screening

Quantitative phytochemical screening of *S. nigrum* methanol leaf extract revealed the presence of alkaloids, saponins, flavonoids, total phenols and tannins. Total phenols and flavonoids were found to be highest while tannins, saponins and alkaloids were found to be lowest.

Table 1. Quantitative Phytochemical Content of *Solanum nigrum* Methanol Leaf Extract (mg GAE/g)

Parameters	Sample
Alkaloids	3.70 ± 0.03
Saponins	3.62 ± 0.06
Flavonoids	43.67 ± 1.08
Total Phenols	70.60 ±2.15
Tannins	1.89 ± 0.22

Values are Mean ± SD for 3 determinations

3.2. Vitamins Composition of Solanum nigrum Methanol Leaf Extract

The Vitamins composition of *S. nigrum* methanol leaf extract revealed the presence of high amounts of Vitamin C. The plant extract was also found to contain vitamin A, vitamin K, vitamin B_6, vitamin E and Folic acid in substantial quantities.

Table 2. Vitamins Composition of *Solanum nigrum* Methanol Leaf Extract

Vitamins	Concentration
Vitamin A (µg/100g)	400.66 ± 06.02
Vitamin K (µg/100g)	42.14 ± 0.10
Vitamin B_6 (Mg/100g)	14.23 ± 0.01
Vitamin C (Mg/100g)	45.18 ± 0.02
Vitamin E (IU/100g)	10.72 ± 0.02
Folic acid (µg/100g)	1100.61 ± 10.01

Values are mean ± SD for 3 determinations

3.3. Mineral Composition of Solanum nigrum Methanol Leaf Extract

Table 3 shows the level of some mineral elements in *S. nigrum* methanol leaf extract. Magnesium was found to be highest in the plant extract followed by calcium and iron while copper and zinc were found to be lowest.

Table 3. Mineral Composition of *Solanum nigrum* Methanol Leaf Extract in mg/100g

Mineral Element	Concentrations
Iron	13.01±0.01
Magnesium	247.59±4.12
Calcium	17.33±0.03
Zinc	0.07±0.01
Copper	2.12±0.12

All values are mean ± SD for 3 determinations

3.4. DPPH Radical Scavenging Activity Solanum nigrum Methanol Extract

The result of DPPH radical scavenging activity of the *S. nigrum* methanol extract is as presented in Table 4. The plant extract significantly ($p<0.05$) exhibited a high radical scavenging activity at the different concentrations of the plant extract (50, 100, 150, 200 and 250 mg/mL) compared to L-ascorbic acid at the same concentrations. The radical scavenging activity was also found to be dose-dependent. Significantly higher ($p<0.05$) radical scavenging activity was observed at the highest concentration of the plant extract (250 mg/mL) while the lowest activity was observed at 50mg/mL of plant extract.

Table 4. DPPH Radical Scavenging Activity of *S. nigrum* Methanol Leaf Extract in %

Concentration (mg/mL)	Methanol Extract	Ascorbic Acid
50	56.00 ±0.65 [a]	42±1.01
100	67.53±1.42 [a]	58±0.26
150	78.24±1.32 [a]	65±1.13
200	86.92±1.68 [a]	72±2.15
250	97.08±1.20 [a]	81±1.92

All values are mean ± SD for 3 determinations. [a]=Significantly ($p< 0.05$) higher compared to ascorbic acid

3.5. Ferric Reducing Antioxidant Power (FRAP) of Solanum nigrum Methanol Leaf Extract

The ferric reducing antioxidant power of *S. nigrum* methanol leaf extract revealed the antioxidant power of the plant extract in percentage. The antioxidant power of the plant was found to be dose-dependent. Significantly higher (p<0.05) antioxidant power was observed at 250 mg/mL while the least antioxidant power was observed at the lowest concentration (50 mg/mL) of both the plant extract and ascorbic acid. The ferric reducing antioxidant power of the methanol extract of *S. nigrum* was significantly (p<0.05) higher at 200 mg and 250 mg compared to the antioxidant power of ascorbic acid at the same concentration. No significant (p<0.05) difference was observed in the antioxidant power of the plant extract and ascorbic acid at the concentrations of 50, 100 and 150 mg/mL.

Table 5. Ferric Reducing Antioxidant Power (FRAP) of *Solanum nigrum* Methanol Leaf Extract in %

Concentration (mg/mL)	Methanol Extract	Ascorbic acid
50	39.22 ± 0.64	37.42±0.61
100	48.26±0.22	46.64±0.72
150	57.48±0.46	55.55±0.45
200	75.55±0.48 [a]	63.25±0.65
250	89.64±0.62 [a]	74.24±3.12

All values are mean ± SD for 3 determinations. [a]=significantly higher compared to Ascorbic acid

3.6. Effect of Solanum nigrum Methanol Leaf Extract on PCV

Table 6 shows the progressive effect of *S. nigrum* methanol leaf extract on PCV levels of rats in percentage. Administration of Phenylhydrazine to rat significantly reduced the PCV levels of rats. Administration of plant extract to rats revealed a dose dependent increase in PCV levels of rats compared to control. The PCV level of rats increased significantly (p<0.05) at 300 and 400 mg extract concentration compared to control. Results also indicate significantly (p<0.05) higher PCV levels in rats treated with 400 mg/kg *S. nigrum* (65.60±1.03 %) compared to rats treated with standard drug (59.50 ± 1.32 %). A progressive increase in PCV levels was observed with days of treatment. A significantly higher (p<0.05) increase in PCV was observed at day 21 of treatment in all the groups compared to day 0. However, rats in group 6, treated with 400 mg of the plant extract, had significantly higher (p<0.05) PCV at day 21 compared to other groups treated with different concentrations of the plant extract.

3.7. Effect of Solanum nigrum Methanol Leaf Extract on Different Hematological Indices

Table 7 shows the effect of *S. nigrum* methanol leaf extract on different hematological indices. Significantly (p>0.05) lower values of PCV, HGB, MCV, MCH, PLT and RBC were observed in the experimental control group compared to normal rats. The result of the study showed that rats treated with 400mg/kg had significantly (p>0.05) lower levels of WBC count, lymphocytes and neutrophils compared to Negative control. However, an increase in HGB, MCV, MCH, platelets and RBC values was observed in a dose dependent manner compared to negative control when the extract was administered to the different groups. The PCV was significantly (p>0.05) higher at 400 mg/kg body weight compared to groups administered 100, 200 and 300 mg/kg body weight. Significant (p>0.05) increase was also observed in PCV of rats in groups 3, 4, 5, 6 and 7 compared to control group. Values for MCV, PLT and RBC were found to be significantly (p>0.05) higher at 400 mg of *S. nigrum* extract compared to the different extract concentrations administered. The levels of PCV and RBC were observed to be significantly (p>0.05) higher at 400 mg of the extract compared to normal rats.

Table 6. Effect of *Solanum nigrum* Methanol Leaf Extract on PCV Levels of Rats (%)

GROUP	DAY 0	DAY 7	DAY 14	DAY 21
Normal	44.83 ± 1.38	45.67 ± 1.20	46.23 ± 1.15	47.33 ± 1.45 [b]
PHZ Negative control	28.33 ± 0.88[a]	30.67 ± 0.67[a]	31.00± 1.15[a]	36.33 ± 1.45[a]
Standard Control (feroton 10mg/kg/ body wt)	27.75 ± 0.25[a]	40.00 ± 3.19[b]	48.00± 1.73[b]	59.50 ± 1.32[b]
S.*nigrum* 100 mg/kg/ body wt	28.26 ± 0.62[a]	34.59 ± 0.46[a]	40.38± 1.6 [a b]	48.20± 2.15 [b]
S.*nigrum* 200 mg/kg/ body wt	27.67 ± 0.33[a]	36.67 ± 0.88[a]	44.67± 1.86[b]	55.30± 1.20 [b]
S.*nigrum* 300 mg/ kg/ body wt	28.25± 0 .48[a]	39.70 ± 0.85 [a b]	50.25±0.85[b]	59.00 ± 1.29[b]
S.*nigrum* 400 mg/ kg/ body wt	28.40 ± 0.51[a]	43.40 ± 1.63[b]	53.80± 0.58[b]	65.60 ± 1.03[bc]

Values are Mean ± SEM, (n = 6). [a=]Significantly (p< 0.05) lower compared to normal, [b=] Significantly (p< 0.05) higher compared to negative control, [c=] Significantly (p< 0.05) higher compared to different extract concentrations. Wt= body weight.

Table 7. Effect of *Solanum nigrum* Methanol Leaf Extract on Hematological Indices

GROUP	PCV (%)	WBC (*10)	HGB (g/dl)	MCV (fl)	MCH (g/dl)	PLT (*10)	LYM (%)	NEU (%)	RBC (*10)
Normal	47.33 ± 1.45 [b]	11.87 ± 2.02	14.15 ± 0.35 [b]	78.73 ± 0.32 [b]	30.20 ± 1.64 [b]	479.67 ± 3.84 [b]	28.33± 1.20	39.00 ± 3.61	5.77±0.02 [b]
PHZ Negative control	36.33 ± 1.45[a]	18.07 ± 0.27[c]	9.23 ± 0.32 [a]	43.27 ±0.85[a]	23.37 ± 0.83[a]	411.67 ±1.45[a]	8.30±0.88[c]	60.35 ±0.88[c]	3.27 ± 0.17 [a]
Standard control	59.50 ± 1.32[b]	11.40 ± 0.78	12.15 ± 0.26 [b]	74.33 ± 0.27[b]	31.60 ± 1.27[b]	479.30 ± 3.52[b]	41.00±0.91	47.25 ± 0.75	7.40 ± 0.08[b]
S.*nigrum* 100 mg /kg / body wt	42.30 ± 2.10	14.00 ± 0.80	10.20 ± 0.16 [a]	62.74 ± 0.44 [b]	26.43 ± 0.20	424.13 ± 2.12[a]	45.04±1.28	57.10 ± 3.42	4.50 ± 0.30 [b]
S.*nigrum* 200 mg /kg / body wt	55.30 ± 1.20 [b]	8.00 ± 0.10 [a]	12.80 ± 0.15 [b]	72.87 ± 1.94 [b]	29.43 ± 0.19 [b]	454.33 ± 3.21[a b]	40.40±2.22	52.20 ± 3.93[c]	6.50 ± 0.10 [b]
S.*nigrum* 300 mg / kg / body wt	59.00 ± 1.29[b]	7.93 ± 1.63[a]	13.87 ± 0.51[b]	74.42 ± 2.83 [b]	30.10 ± 0.88 [b]	474.67 ± 4.25 [b]	34.25±1.03	47.67± 1.83	7.13 ± 1.63 [b]
S.*nigrum* 400 mg / kg per body weight	65.60 ± 1.03[bc]	6.30 ± 1.47[a]	14.26 ± 0.27 [b]	79.40 ± 0.66[bc]	31.24 ± 0.71 [b]	481.60 ± 3.11[bc]	34.67± 2.53	36.25 ± 1.11	7.51 ± 0.07[bc]

Values are Mean ± SEM, (n = 4). [a=]Significantly (p< 0.05) lower compared to normal; [b=]Significantly (p< 0.05) higher compared to negative control; [c=]Significantly (p< 0.05) higher compared to different extracts concentration

4. Discussion

The phytochemical analysis carried out on the methanol leaf extract of *S. nigrum* revealed the presence of high content phenols and flavonoids as well as alkaloids saponins and tannins. Results of phytochemical analysis seems to be in agreement with the findings of Temitope and Omotayo (2012). It has been reported that Phenylhydrazine causes oxidative damage to red cells by increasing the formation of reactive oxygen species (Clemens *et al*., 1984). These phytochemicals protect cells as powerful antioxidants which prevent or repair damage done to red cells by free radicals or highly reactive oxygen species. Adewoye *et al*. (2012) stated that some of the biological functions of flavonoids include protection against allergies, free radicals, platelet aggregation microorganisms, ulcers, hepatotoxins and tumors. The presence of these phytochemicals might have contributed to the antihaematinic activity of *Solanum nigrum* observed in the present study.

The vitamins content of the plant (Table 2) revealed the presence of an appreciable amount of some haematinic vitamins, such as folic acid, vitamin A, vitamin C, vitamin K, vitamin $B_{6,}$ and vitamin E. Deficiency of folic acid and other vitamins constituents in erythropoiesis has been reported to cause macrocytic, megaloblastic and pernicious anemia (Chanarin *et al.*, 2004). These haematinic agents have been found to be effective in relieving the symptoms of anemia in pregnancy and infancy. Vitamins A and C contribute to the uptake of iron while vitamin C enhances the intestinal absorption of non-haem iron by reducing ferric ion to a ferrous form or by forming a soluble complex in the alkaline PH of the small intestine thereby increasing /enhancing iron absorption (Demodara, 2013). This probably was the reason for the observed increase in haemoglobin observed in the present study. The methanol extract of *S. nigrum* has contributed in the faster reversal of the phenylhydrazine induced anemia in rats treated with the extract for three weeks. A similar outcome was observed when anemic rats were treated with *Tectona grandis* (Diallo *et al*., 2008).

Table (3) shows the mineral composition of *S. nigrum.* Calcium and magnesium are useful in the formation of blood and intracellular and extracellular fluids of body cells. They also function as constituents of bones, teeth and in regulation of nerve and muscle function (Brody *et al*., 2004; Ogbe *et al*., 2010). The value of iron obtained in the present study (15.01±0.03 mg/100g) is higher than the values reported for some selected leafy vegetables in Nigeria. Iron is a part of the haemoglobin, myoglobin and

cytochrome. Findings by Nasima *et al*. (2004) reported that zinc plays a major role in the synthesis of haemoglobin. Zinc deficiency has been associated with anemia and erythrocyte fragility. Zinc is also a cofactor for RBC-SOD thereby protecting the integrity of the cell and oxidative stress (El-Nawawy *et al*., 2002). Copper is also an active agent in haemoglobin synthesis. Copper containing enzymes catalyze the oxidation of ferrous iron to ferric iron. It is necessary for the absorption and use of iron in the formation of haemoglobin (Whitney and Rolfes, 2001). Potassium is necessary in the management of sickle cell anemia. It plays a major role in heart beat and assist in nerve impulse transmission. Abnormal activation of potassium chloride co transport system was found to be involved in cell potassium loss and dehydration seen in sickle cell anemia (Agoreyo and Nwaeze, 2009). While magnesium is part of the protein making machinery, together with calcium, magnesium is involved in muscle contraction and blood clotting (Demo *et al*., 2007)

The radical scavenging activity of the plants revealed that both DPPH and FRAP activity of the methanol extract of *S. nigrum* exhibited significantly higher ($p< 0.05$) antioxidant activity compared to L-ascorbic acid. A study by Turaaskar (2013) revealed that most anti-anemic compounds are known for their free radical scavenging activity that reverses anemic conditions. The scavenging activity of free radicals and reactive oxygen species is in a dose dependent manner with reference to DPPH and FRAP antioxidant determination. This probably is due to the presence of phytochemicals in the leaf extract. According to Lv *et al*. (2013), good antioxidant activities exhibited by plants extracts are due to the presence of poly-phenolic compound. Administration of the methanol extract of *S. nigrum* significantly ($p<0.05$) increased the haematological parameters in the experimental groups in a dose dependent manner. A significant increase was observed in the levels of PCV, HB, platelets, MCV, MCHC, neutrophils and RBC. A similar result was obtained by Asuquo (2013) when ethanol leaf extract of yellow mombin was administered to rats. However, a significant decrease ($p<0.05$) was observed in the levels of WBC, lymphocytes and neutrophils. The white blood cells, lymphocytes and neutrophils are indices of immunology of the body against infection; thus, a significant decrease was seen in these parameters when methanol leaf extract of *S. nigrum* was administered. Treatment with 400 mg/kg/body weight was found to be more effective in ameliorating the effect of phenylhydrazine than other doses. A similar result was obtained by Vamsee *et al*. (2004) when a curry leaf was administered to anemic rats at 400 mg/kg body weight.

5. Conclusion

In conclusion, results obtained from the present study indicate that the methanol leaf extract of *S. nigrum* possesses anti-anemic potentials and this may be attributed to the phytochemicals, antioxidant vitamins, such as folic acid, vitamin C, and minerals, such as iron, zinc and calcium content of *S. nigrum* leaf. The present study, therefore, supports the therapeutic use of the plant in the traditional medicine for the treatment of anemia.

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Antimicrobial Activity of Endophytic Fungi from Leaves and Barks of *Litsea cubeba* Pers., a Traditionally Important Medicinal Plant of North East India

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Abstract

The present research work was carried out to study the endophytic fungal flora associated with the leaves and barks of *Litsea cubeba* and antibacterial activity of the crude metabolites produced by the endophytes. *L. cubeba* is an endemic plant to Southeast Asia and is commonly known as Mezankari in Assam. A total of 12 morphologically different endophytic fungi were isolated from *L. cubeba*. *Acremonium falciforme* was the most dominant fungi that inhabited both the leaves and barks of *L. cubeba* and, respectively, had 42.41% and 31.42% relative frequency of dominance. The ethyl acetate extracts of the crude metabolites of all the isolates, showed antagonistic activity against at least one of the tested bacteria. *Acremonium falciforme* showed the highest zone of inhibition (12.3±0.50 mm) against *Staphylococcus epidermidis* (MTCC 435). The results of the present study indicated that the isolated endophytes produced bioactive compounds which might have potential application in pharmaceutical industry.

Keywords: *Acremonium falcifome*, Antimicrobial activity, Endophytic fungi, Inhibition zone, *Litsea cubeba*.

1. Introduction

Endophytes are microorganisms colonizing healthy plant tissues without causing overt symptoms or apparent injuries to the host (Bills, 1996). Since the discovery of endophytes in Darnel, Germany, in 1904, various investigators have defined endophytes differently depending on the perspective from which the endophytes were being isolated and subsequently examined (Strobel and Daisy, 2003). The most common endophytes in plants were fungi (Tayung, 2008). According to Petrini (1991) endophytes, include all those fungi that during quite a prolonged period of their life remain present in the living internal tissues of their host without producing any symptoms. Mostly Ascomycetes, Deuteromycetes and Basidiomycetes class of fungi are reported as endophytic fungi (Petrini, 1986; Dayle *et al.*, 2001). Many genera and species of fungi belonging to first two classes could live endophytically in plants (Khan, 2007; Dissanayake *et al.*, 2016). Fungi are a rich source of many therapeutic substances. Metabolites of endophytic *Fusarium* sp. isolated from *Selaginella pallescens*, collected from Guanacaste Conservation Area of Costa Rica, showed antifungal activity (Brady and Clardy, 2000). The secondary metabolites produced by *Guignardia sp.* was active against *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Geotrichum* sp. and *Penicillium hennebertii*. Phomopsilactone, an antifungal compound, was isolated from *Phomopsis cassia*, an endophyte of *Cassia spectabilis* (Silva *et al.*, 2005). Nineteen out of 73 endophytic fungi produced antimicrobial compounds that inhibited several plant and human pathogens (Tuppad and Shishupala, 2014). Katoch *et al.* (2014) observed that twenty-six endophytic fungi isolated from *Bacopa monnieri* possessed antimicrobial activity against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Candida albicans.*

Litsea cubeba is an important medicinal plant. The fruit and leaf of *L. cubeba* produce an essential oil that primarily contains Citral and 1,8- Cineol, respectively (Ho *et al.*, 2010). This oil exhibited cytotoxic activity against human lung, liver and oral cancer cells besides antimicrobial activity (Ho *et al.*, 2010). It is also used as a raw material for the synthesis of Vitamin-A. In Assam, it is economically important and is widely used as a secondary food plant for the Muga silkworms (*Antheraea assamensis*), which yields valuable golden yellow muga silk fiber ("the golden fiber"). The medicinal as well as the economic importance of *Litsea cubeba* enthused us to carry out the present investigation on endophytic fungi, which has been properly explored so far as a source of noble compounds. Till now, meagre work has been done related to isolation and bioactivities of endophytic fungi

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associated with *L. cubeba*. The objectives of the present work, therefore, were to isolate the endophytic fungi associated with *L. cubeba* and to investigate the antibacterial properties of their secondary crude metabolites against some important bacterial pathogens.

2. Materials and Methods

2.1. Collection of the Plant Materials

The present study was conducted in the Botanical Garden, Department of Botany, Gauhati University, Guwahati, Assam, which is located between 25°45' N to 26°25' N latitude and 91°10' E to 92°E longitudes, at an altitude of 62.0 masl. The present study was conducted between March 2012 and February 2013. Healthy leaves and barks of *L. cubeba* were collected aseptically. Three samples each from the bark and leaf totaling to six samples were collected for isolation of endophytic fungi. The samples were immediately brought to the laboratory in sterilized bags and were kept in a refrigerator at 4°C until they were processed. The materials were used for the analysis within 24 hours.

2.2. Isolation of Endophytes

Samples were washed thoroughly with distilled water, air-dried and were cut aseptically into about 2 cm long and 0.5 cm broad segments with a sterile knife and were surface-sterilized. A total of 144 segments were made from the plant, 72 each from barks and leaves for isolation of the endophytic fungi. For surface-sterilization, segments were immersed in 70% ethanol for 3 minutes and 4% aqueous solution of sodium hypochlorite for 5 minutes there after again with 70% ethanol 1 minute and 0.1% mercury chloride ($HgCl_2$) for 3 minutes (Bills and Polishook, 1993; Strobel, 2002). Finally, the segments were rinsed with sterile distilled water until the traces of $HgCl_2$ were washed off. The efficiency of surface sterilization was ascertained for every segment following the imprint method of Schulz *et al*. (1993). After surface drying under sterile conditions (Arnold *et al.*, 2000) in laminar air flow chamber to remove the excess water, segments were inoculated in plates containing Czapeck-Dox-Agar (CDA), Potato-Dextrose-Agar (PDA) media (Hi-Media, India) and media amended with bark and leaf extracts separately. Bark and leaf extracts were prepared by boiling 500 g of the plant's bark and leaf in 250 ml of distilled water separately for 10-15 minutes (Tayung, 2008). The preparation was cooled and filtered through sterile Whatman No.1 filter paper to get the bark and leaf extracts. The medium was supplemented with streptomycin (50 µg/ml) to prevent bacterial contamination. The plates were sealed with parafilm and then incubated at 25±1°C until the mycelium appeared surrounding the segments. The plates were checked every other day continuously for 30 days. The individual fungal colonies were transferred onto other plates with PDA for pure culture and pure culture was maintained on PDA slants.

2.3. Identification of Isolates

The fungal endophytes were identified based on their morphological and reproductive characters using identification manuals of Nagamani *et al.* (2006) and Gilman (1950). Sporulation was induced in non-sporulating isolates by inoculating them in different media and incubating them at different temperatures for different period of time. Those without distinct morphological and reproductive characters were recorded as mycelia sterilia.

2.4. Production of Crude Metabolites

All the isolates were cultivated to produce crude metabolites according to the protocols of Phongpaichit *et al.* (2007). Endophytic fungal isolates were grown in 1000 ml Erlenmeyer flask containing 500 ml potato dextrose broth media and incubated at 25±1ºC for 3-4 weeks under a stationary condition. The crude fermentation broth was filtered using Whatman filter paper No. 1 and the supernatant was blended thoroughly and centrifuged at 3600 rpm for 10 minutes. Finally, the crude metabolite was extracted three times with ethyl acetate and then it was concentrated to dryness by using rotary vacuum evaporator (Model: EYELA/NVC-2100) at 40°C. The resulting extracts from each isolate was diluted with Dimethyl Sulfoxide (DMSO) at a concentration of 10 mg/ml. The solution was sterilized by filtration through 0.4 µm Cellulose Acetate (hydrophilic) filter and was examined for antimicrobial activity against some bacteria.

2.5. Antibacterial Activity Assay

It was assayed by Kirby-Bauer disc diffusion method (Bauer *et al.*, 1996). The antimicrobial activity of the crude extract was determined against two-gram negative, viz. *Escherichia coli* (MTCC 443) and *Klebsiella pneumoniae* (MTCC 619), and two gram-positive, viz. *Bacillus subtilis* (MTCC 441) and *Staphylococcus epidermidis* (MTCC 435) bacteria. The test organisms, except for *S. epidermidis*, were collected from the Institute of Microbial Technology (IMTECH), Chandigarh, India. *S. epidermidis* was collected from Regional Institute of Medical Sciences (RIMS), Imphal, India. Prior to testing, the pathogens were cultured in Nutrient broth at 28±1°C until their growth was observed. Then, with sterile cotton-buds swabbing was done on the Nutrient Agar (NA) medium in Petri dishes using the four test bacteria, after solidification. The sterile paper disc (0.6 cm in diameter) soaked in crude extract was placed on the NA media to evaluate of antimicrobial activity. Tetracycline antibiotic discs (10 µg/disc) was used as positive control and discs immersed with DMSO were used as negative control in the experiment. The plates were incubated at 28±1°C for 4-7 days and diameter of the inhibition zone was measured. Three replicates were maintained in each case.

2.6. Data Analysis

The Colonization Frequency (CF %) of endophytic fungi was calculated using the following formula, given by Fisher and Petrini (1987):

$$CF = (N_{COL} / N_{t)} \times 100$$

where, N_{COL} = Number of bark/leaf segments colonized by specific fungus; N_t = Total number of bark/leaf segments plated.

Frequency of dominant endophytes was calculated as percentage colony frequency divided by sum of percentage of colony frequency of all endophytes x 100 (Kumaresan and Suryanarayanan, 2002).

Similarity co-efficient ($SC = 2w/a+b+c$) was calculated to compare the endophytic colonization in different organs of the plants, by using Carroll and Carroll (1978) formula

and was expressed as a percentage, where: a = the sum of colonization frequency for all fungal species in a tissue; b,c = the similar sum for another tissue; w = the sum of lower colonization frequencies for fungal endophytes in common between the tissues.

2.7. Statistical Analysis

Standard error was calculated for the antimicrobial activity assay using Microsoft office excel 2016. One-way analysis of variance (ANOVA) was used to analyze the differences between the number of isolates of the endophytic fungi in the media amended with plant extract and un-amended medium followed by Least Significant Difference (LSD) test. P value of less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. Endophytic Fungi Isolated from L. cubeba:

A total of sixty-nine isolates were obtained from healthy barks and leaves of *L. cubeba*. Thirty-six isolates were obtained from bark and thirty-three from leaf samples. Out of sixty-nine isolates, sixty-three isolates belonged to eight different genera. Four isolates did not show any reproductive structures, i.e., they did not sporulate due to which they could not be identified. These four isolates were termed as mycelia sterilia (Table 1). The occurrence of Ascomycota was the highest both in leaves and bark as compared to Chytridiomycota and mycelia sterilia (Figure 1). Species of *Acremonium* and *Nigrospora* were the most frequently isolated endophytes during the present investigation. Along with *Acremonium* and *Nigrospora*, genera, like *Allomyces*, *Penicillium*, *Aureobasidium*, *Periconia*, *Chaetomium* and *Acrophialophora*, were also isolated in the present work (Table 1). The colony morphology and microphotograph of *Nigrospora sphaerica* and *Acremonium falciforme* have been shown, respectively, in Figure 2 (a & b) and Figure 3 (a & b). Colonizing Frequency (%) and the frequency of dominance (%) were highest for *Acremonium falciforme* (Table 1).

Table 1. Occurrence, Colonizing Frequency (%) and Frequency of dominance (%) of endophytic fungi isolated from different parts of *L. cubeba*

Plant part	Endophytic fungi	Total no. of isolates	Colonizing Frequency (%)	Frequency of dominant Endophytes (%)
Bark	1.*Nigrospora sphaerica*	10	13.89	28.56
	2.*Acremonium falciforme*	11	15.28	31.42
	3. *Periconia hispidula*	2	2.78	5.72
	4.*Allomyces arbuscula*	4	4.17	8.57
	5. *Aureobasidium sp.*	1	1.39	2.86
	6. *Chaetomium sp.*	1	2.78	5.72
	7.*Penicillium chrysogenum*	2	1.39	2.86
	8. Mycelia sterilia (1)	1	1.39	2.86
	9. Mycelia sterilia (2)	1	1.39	2.86
	10. Mycelia sterilia (3)	1	1.39	2.86
	11. Mycelia sterilia (4)	2	2.78	5.72
Leaf	1.*Nigrospora sphaerica*	12	16.67	36.37
	2.*Acremonium falciforme*	14	19.44	42.41
	3.*Allomyces arbuscula*	3	1.39	3.03
	4.*Penicillium chrysogenum*	2	4.17	9.10
	5. *Acrophialophora* sp.	1	2.78	6.06
	6. Mycelia sterilia (3)	1	1.39	3.03

The Colony frequency was calculated based on 72 segments of plant parts plated

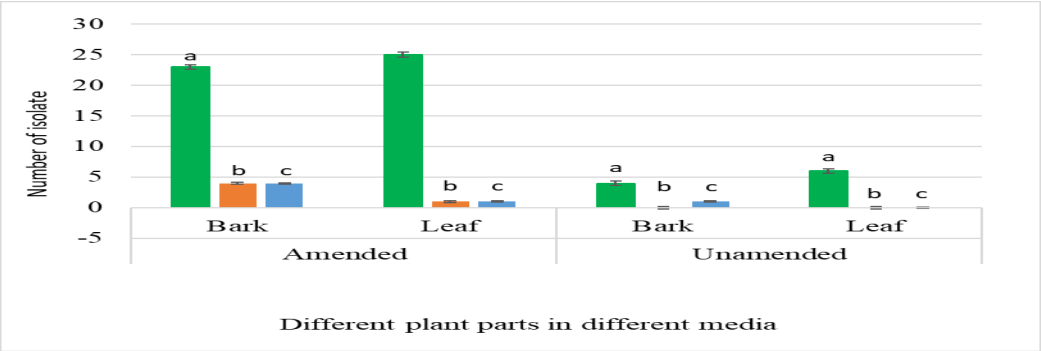

Figure 1. Occurance of (a) Ascomycota, (b) Chytridiomycota and (c) unidentified (sterile) fungal endophytes isolated from bark and leaf of *L. cubeba* using amended and unamended media. Results are expressed as Mean±SE. Isolation of the fungal endophytes (a, b and c) from bark and leaf are significantly different in media amended with plant extract from unamended media (p<0.05).

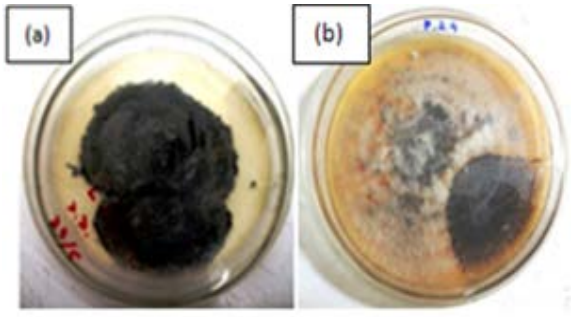

Figure 2. Colony morphology of (a) *Nigrospora sphaerica* and (b) *Acremonium falciforme*

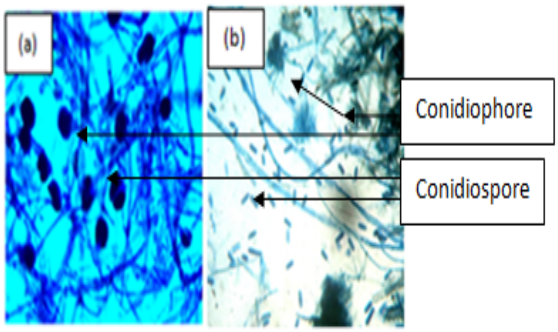

Figure 3. Photo micrograph of (a) *Nigrospora sphaerica* and (b) *Acremonium falciforme*

3.2. Effect of Different Media on the Growth of Endophytic Fungi

The endophytic fungi grew optimally and produced spores on Potato Dextrose Agar media than other media used. A significant difference in the number of isolates of the endophytic fungi was observed when the medium was amended with bark and leaf extracts than un-amended medium (Figure 1) (P<0.05). Out of sixty-nine fungal isolates of *L. cubeba*, 58 isolates were obtained from sample segments placed in media amended either with bark or leaf extracts.

3.3. Organ Specificity of Endophytic Fungi in the Host

The recovery of endophytes from the bark of *L. cubeba* (52.17%) was more than that of leaf (47.83%). The colonization frequency, in case of bark, was 48.63%, while the same for leaf was 45.84%. The fungi, viz. *Periconia hispidula*, *Aureobasidium sp.*, *Chaetomium sp.*, mycelia sterilia (1), mycelia sterilia (2) and mycelia sterilia (4) were isolated only from bark showing their organ specificity. Moreover, the fungus *Acrophialophora sp.* colonized only leaf segments showing its organ specificity for leaves (Table 1). The similarity coefficient between leaf and bark was 35.29%.

3.4. Antimicrobial Activity of Ethyl Acetate Extracts of Crude Metabolites against Some Bacteria

Ethyl acetate extracts of crude metabolites of all the isolates were tested for antimicrobial activity against four test bacteria. Amongst all, the crude extract of *Nigrospora sphaerica* showed activity against all the four test microbes and it inhibited *B. subtilis* mostly (Table 2, Figure 4a). *Acremonium falciforme* showed the highest zone of inhibition of 12.3±0.50 mm diameter, against *S. epidermidis* (Table 2, Figure 4b).

Table 2. Zone of inhibition of crude metabolites obtained from different endophytic fungi isolated from *L. cubeba* against different gram-positive and gram-negative bacteria

	Zone of inhibition (mm)			
	Gram- positive bacteria		Gram- negative bacteria	
Endophytic fungi	Se	Bs	Kp	Ec
Nigrospora sphaerica	4 ±0.75	8±0.25	6±0.29	3±0.36
Acremonium falciforme	12.3±0.50	5±0.9	2±0.25	-
Periconia hispidula	-	5± 0.55	5±0.29	-
Allomyces arbuscula	-	7±0.32	9±0.19	-
Aureobasidium sp.	-	3±0.50	2.15±0.75	-
Chaetomium sp.	-	7 ±0.45	-	3±0.35
Penicillium chrysogenum	-	3±0.61	2±0.16	-
Acrophialophora sp	-	2 ±0.19	-	-
Mycelia sterilia (1)	-	2±0.23	-	-
Mycelia sterilia (2)	-	3±0.32	-	-
Mycelia sterilia (3)	-	-	3±0.70	-
Mycelia sterilia (4)	2±0.12	3±0.29	-	-
Tetracycline	18.8±0.21	15.05±0.19	4±0.6	13±0.15
Negative control	0	0	0	0

Positive control: Co-assayed antibiotics (Tetracycline-30mcg/disc). Negative control: Sterile disc (5 mm diameter) immersed in Dimethyl sulphoxide (DMSO)

Se=*Staphylococcus epidermidis*, *Bs=Bacillus subtilis*, *Kp= Klebsiella pneumoniae*, *Ec=Escherichia coli*. Data mean of three replicates ± SE.

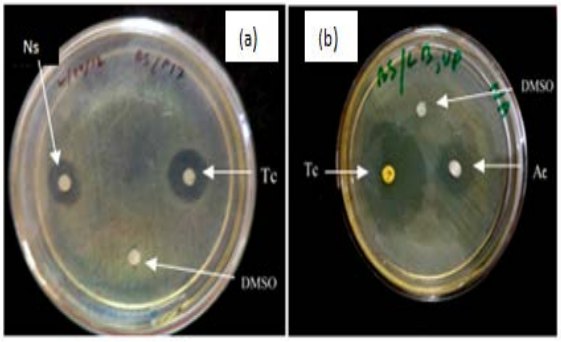

Figure 4. Antibacterial activity of the metabolites produced by (a) *Nigrospora sphaerica* (Ns) against *Bacillus subtilis* and (b) Antibacterial activity of the metabolites produced by *Acremonium falciforme* against *Staphylococcus epidermidis.* Co-assayed antibiotic-tetracycline (Tc-30mcg/disc) and Negative control-Dimethyl Sulfoxide (DMSO).

4. Discussion

Litsea cubeba is of great economic importance due to its high medicinal properties. Meager work has been done on endophytes associated with *L. cubeba.* Over exploitation of these plants for medicinal and commercial purposes has threatened the existence of this plant. Therefore, the present work was carried out with an aim to study the endophytic fungi associated with *L. cubeba* so

that edophytes may be used for antimicrobial metabolites instead of plants, thus conserving the plants. The major objectives of the present work include studying the occurrence of endophytic fungi and to screen and evaluate these microorganisms for the presence of antimicrobial bioactive metabolites. The endophytic fungi were isolated from bark and leaf samples of the plant to screen organ specific endophytes regarding the host plant. Endophytes were generally not considered as organ-specific microbes and it is likely that many of the species isolated from bark may also occur in leaves (Dix *et al.*, 1995). A similar type of study was carried out to evaluate the organ specificity of the endophytes in the host plant. The endophytic fungi viz., *Nigrospora sphaerica*, *Acremonium falciforme*, *P. chrysogenum*, *Allomyces arbuscula* were recovered both from bark and leaf samples of *Litsea cubeba* while some were restricted to a particular organ of the plant. The fungus *Aureobasidium* sp., *Chaetomium* sp., *Periconia hispidula*, and the three mycelia sterilia were isolated only from the bark throughout the study period. The fungus *Acrophialophora* sp. was isolated only from the leaf samples. Tejesvi *et al.* (2005) while working on the endophytic fungi of *Terminalia arjuna* found that the distribution of some taxa and their density was more in inner bark segments compared to the twigs. Chareprasert *et al*. (2006) also recovered more endophytic fungi from leaves of *Tectona grandis L.* and *Samanea saman* Merr. Tejesvi *et al.* (2005) isolated *Chaetomium* and *Penicillium* from *T. arjuna*, a medicinal plant. Some species of endophytes, like *Nigrospora* sp., *Penicillium* sp., *Chaetomium* sp., *Aspergillus* sp. Etc., were isolated from *Rauvolfia serpentina*, a medicinal plant (Daleyi, 2002). *Nigrospora* sp., *Penicillium* sp., *Chaetomium* sp. were also isolated during the present work. Endophytic fungi, *Nigrospora sphaerica*, was also isolated from medicinal plants, viz. *Adhatoda vasica*, *Costus igneus*, *Coleus aromaticus* and *Lawsonia inermis* by Amirita *et al*. (2012). The genera *Penicillium* was amongst the most commonly isolated genera (Santos *et al.*, 2003). *Aureobasidium pullulans*, endophytic fungi isolated from grapevine **(***Vitis vinifera*), play a potential role as biological control agents against grapevine pathogens (Martini *et al.*, 2009). *Aureobasidium pullulans* were also isolated from *L. cubeba* during the present work. *Acremonium sp.*, the dominant endophytic fungi of *L. cubeba* of the present work, was also isolated from grass and found to be antagonistic towards several grass pathogens (White and Cole, 1985).

The culture media can affect the endophytic fungi that produce secondary metabolites. In the present experiment recovery of endophytic fungi differed in different media. The PDA media appeared as the suitable media for isolation of a large number of isolates. It might be due to the nature of carbon and nitrogen constituents of the media (Tayung, 2008). A large number of species were isolated from *L. cubeba* on media amended with bark and leaf extracts due to the addition of some extra nutrients through the host plant part extracts which had a positive effect on the growth of endophytes. This indicates the presence of some substances in the host plant which encourage the growth of the endophytes.

Endophytic fungi are by now recognized as a potential source of anti-microbial secondary metabolites (Strobel and Daisy, 2003; Li *et al.*, 2005; Huang *et al.*, 2008) that could be used for various medicinal purposes. The crude extracts of some endophytic fungi, namely *Acremonium* sp., *Aspergillus terreus*, *A. flavus*, *Alternaria* sp., showed an antimicrobial activity against pathogenic *E. coli*, *Proteus mirabilis*, *S. typhi*, *K. pneumoniae* (Kalyanasundaram *et al.*, 2015). Nwakanma *et al.* (2016) studied antagonistic activity of the crude secondary metabolites of 16 different endophytic fungi isolated from leaves of Bush mango against *E. coli*, *S. aureus*, *P. aeruginosa*, *B. subtilis*, *P. chrysogenum* and *A. fumigatus.* Pinheiro *et al.* (2017) also found that, among seventeen endophytic fungi isolated from *Bauhinia guianensis*, the fungus *Exserohilum rostratum* showed the highest activity against *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 25923), *B. subtilis* (ATCC6633) and *S. typhimurium* (ATCC14028). During the present investigation, some of the isolates of *L. cubeba* also showed a very good antimicrobial activity against some microbes, which are of pharmaceutical importance. Endophytic fungi isolated from *L. cubeba*, produced antimicrobial secondary metabolites as most of the crude extracts showed an inhibitory activity against all the test organisms. Gram-positive test bacteria (*B. subtilis*) was more sensitive to the crude extracts of the isolated endophytes than that of gram-negative bacteria which supported the findings of Rakshith and Sreedharamurthy (2011) and Dzoyem *et al.* (2017).

The results of the present work, thus, suggest that *L. cubeba* harbor some endophytic fungi producing antimicrobial secondary metabolite which may have noble compounds. These endophytes may be used as source of therapeutic agents in pharmaceutical industries. However, further investigation is needed for the characterization of these endophytes within the host plant, proper establishment of their role and chemical characterization of secondary metabolites produced by them for their future applications as bio-control and pharmaceutical agents (Dissanayake *et al.*, 2016).

5. Conclusion

The present study reveals that a total of sixty-nine isolates, thirty-six isolates from bark and thirty-three from leaf samples, sheltered *L. cubeba*. These sixty-nine isolates, excluding four mycelia sterilia isolates, belonged to 8 different genera. *Acremonium falciforme* was the most dominant and potent endophyte showing highest antimicrobial activity against *Staphylococcus epidermidis* (MTCC 435). All the isolates showed antimicrobial activity against the test organisms. Thus, it can be concluded from the present investigation that endophytic fungi, isolated from *L. cubeba*, can be used for pharmaceutical purposes. More aggressive investigation is required to better understanding of the metabolomics and endophyte biology of *L. cubeba*.

Conflict of Interest

No conflicts of interest have been declared by the authors.

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Morphometric Relationships of the Tank goby *Glossogobius giuris* (Hamilton, 1822) in the Gorai River using Multi-linear Dimensions

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Abstract

The present study illustrates the first complete and inclusive information of morphometric relationships, including Length-Weight Relationships (LWRs) and Length-Length Relationships (LLRs), using a total of 13 linear dimensions of *Glossogobius giuris* (Hamilton, 1822) in the Gorai River, southwestern (SW) Bangladesh. Also meristic characters, including various fin-rays of the tank goby, have been studied. In total, 229 specimens of *G. giuris* were collected occasionally from the Gorai River during March 2016 to February 2017 by a variety of local fishing gears (e.g., cast, gill, and square lift net). Fin rays and scales (including lateral line scale) were counted by a magnifying glass. Different morphometric lengths were measured to 0.01 cm, and whole Body Weight (BW) was estimated ± 0.01 g for each individual. The fin formula of *G. giuris* is: dorsal, D_1. VI; D_2. 8–11 (II–III/8–11); pectoral, P_1. 17–22 (II–VI/14–19); pelvic, P_2. 10–13 (II–III/8–10); anal, A. 7–12 (II–IV/5–8); and caudal, C. 16–21 (IV–VIII/12–13), correspondingly. In the present study, Total Length (TL) varied from 4.3 to 26.9 cm and BW ranged from 0.67 to 146.55g. All LWRs were highly significant ($p < 0.0001$) with r^2 values ≥ 0.975. Based on r^2 value, LWR by BW *vs.* TL, BW *vs.* SL and BW *vs.* PoAnL were good fitted models among 13 equations. The present study would be very valuable for species recognition and stock assessment of tank goby in the Gorai River, SW Bangladesh and in adjoining ecosystems.

Keywords: Tank goby; Fin rays; *Glossogobius giuris;* Meristic; Morphometric.

1. Introduction

The tank goby *Glossogobius giuris* (Hamilton, 1822), belonging to the family Gobiidae, is a benthopelagic; amphidromous species occurring in sea-, brackish- and fresh-waters. It is the only species of diverse genus *Glossogobius*, found in Bangladesh, locally known as Bele (Rahman, 2005), Bhaila in India, Tank goby in Malaysia, Goby in Philippine (Freose and Pauly, 2016). *G. giuris* inhabits streams, canals, ditches and ponds. This goby fish is broadly distributed in coastal and estuarine as well as fresh waters alongside the coasts of East Africa, the Red Sea and the Indian subcontinent to China (Freose and Pauly, 2016). It is very rich in protein and micronutrients and has high market value (Islam and Joadder, 2005; Islam *et al.*, 2014). This fish is one of the dominant species in the Gorai River (SW Bangladesh); hence, it is an important capture species for small- and large- scale fishermen (Costa *et al.*, 1999; Hossain *et al.*, 2009).

Studies on morphometric and meristic features can be constructive tools for exact identification of any species and its classification (Begenal and Tesch, 1978; Jayaram, 1999; Hossen *et al.*, 2016). Moreover, in fisheries research, appraising the well-being of individuals as well as evaluating the life history and the morphological traits of populations of different locality greatly rely on morphometric characters (King, 2007; Hossain, 2010; Hossain *et al.*, 2013).

To the best of our knowledge, a few studies, including morphometric and meristic characters, Length-Weight Relationships (LWRs), food and feeding habits, reproduction and breeding performance, have been conducted on this species from other habitats (Hossain *et al.*, 2009; Mollah *et al.*, 2012; Islam and Mollah, 2013; Hossain, 2014; Islam *et al.*, 2014; Kaur and Rawal, 2015; Qambrani *et al.*, 2015; Hossain *et al.*, 2016; Saha *et al.*, 2016); however, no sound studies on this issue, covering a large number of linear dimensions, have been conducted yet from the Gorai River. Therefore, the present study is designed to describe the morphometric and meristic characteristics of *G. giuris* systematically using large number of specimens from small to larger sizes over a study period of one year from the Gorai River (SW Bangladesh).

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2. Materials and Methods

In the present study, a total of 229 individuals of *G. giuris* (Figure 1) were collected occasionally from the Gorai (distributary of Ganges River) River (Latitude: 23° 32' N; Longitude: 89° 31' E), SW Bangladesh during March 2016 to February 2017 from artisanal fishers. The samples were caught using various types of traditional fishing gears, i.e., cast net (mesh size ranges: 1.5 - 2.5 cm), gill net (mesh size ranges: 1.5–2.0 cm), and square lift net (mesh size: ~2.0 cm). The fresh samples (dead fish) were instantly chilled in ice on site and preserved with 10% buffered formalin after arrival in the laboratory.

Total Body Weight (BW) of each individual was taken using a digital electric balance with 0.01 g accuracy and different linear dimensions, i.e., lengths (Table 1 and Fig. 2) were estimated to the nearest 0.01 cm using digital slide calipers. The LWR was estimated using the equation: $W = a \times L^b$, where W is the body weight (BW, g) and L is the 13 different lengths in cm. The regression parameters a and b were calculated by linear regression analyses based on natural logarithms: ln (W) = ln(a) + b ln(L). Moreover, 95% Confidence Limit (CL) of a and b and the co-efficient of determination (r^2) were estimated. Extremes outliers were removed from the regression analyses according to Froese (2006). A t-test was used to confirm whether b values obtained in the linear regressions were significantly different from the isometric (b = 3) value (Sokal and Rohlf 1987). A total of 12 LLRs were estimated by linear regression analysis (Hossain *et al*., 2006). Best/ good model for both LWRs and LLRs was selected based on the highest value of determination r^2. Total number of fin rays and scales from different body parts (including the lateral line) were counted by using magnifying glass. Statistical analyses were performed using Graph Pad Prism 6.5 software. All statistical analyses were considered significant at 5% ($p < 0.05$).

Figure 1. A photo of *Glossogobius giuris* which was collected from the Gorai River, southwestern Bangladesh

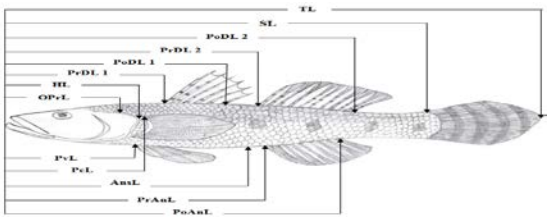

Figure 2. Showing the morphometric measurements of *Glossogobius giuris* in the Gorai River, southwestern Bangladesh

Table 1. Morphometric measurements of the *Glossogobius giuris* (Hamilton, 1822) (n = 229) captured from the Gorai River, southwestern Bangladesh

Min, minimum; Max, maximum; SD, standard deviation; CL, confidence limit for mean values; TL, total length; SL, standard length; BW, body weight; PrDL 1,1st Pre-dorsal length; PoDL 1,1st Post-dorsal length; PrDL 2, 2nd Pre-dorsal length; PoDL 2, 2nd Post-dorsal length; HL, Head length; OprL, Opercular length; PcL, Pectoral length; PvL, pelvic length; AnsL, anus length; PrAnL, pre-anal length; PoAnL, post-anal length; *, weight in g).

Measurements	Min (cm)	Max (cm)	Mean ± SD	95% CL	%TL
TL (Total length)	4.3	26.9	12.803±5.893	12.036-13.571	
SL (Standard length)	3.3	19.5	9.867±4.535	9.276-10.457	76.728
PrDL 1(1st Pre-dorsal length)	1.2	7.8	3.679±1.705	3.457-3.901	37.291
PoDL 1(1st Post-dorsal length)	1.5	10.1	4.810±2.226	4.520-5.099	37.401
PrDL 2(2nd Pre-dorsal length)	1.9	11.6	5.658±2.594	5.320-5.995	43.996
PoDL 2(2nd Post-dorsal length)	2.5	15.6	7.564±3.470	7.112-8.016	58.822
HL (Head length)	0.9	6.3	2.914±1.362	2.737-3.092	22.664
OprL (Opercular length)	0.8	6.2	2.781±1.372	2.602-2.959	21.625
PcL (Pectoral length)	0.9	6.3	2.964±1.410	2.781-3.148	23.051
PvL (Pelvic length)	1.1	6.4	3.110±1.449	2.922-3.299	24.188
AnsL(Anus length)	1.4	11.8	5.535±2.710	5.182-5.888	43.045
PrAnL(Pre-anal length)	1.9	12.3	6.000±2.766	5.640-6.360	46.658
BW (Body weight)	0.67*	146.55*	27.731±33.217	23.406-32.056	

3. Results

The body of *G. giuris* is elongated and moderately compressed, mouth oblique with prominent lower jaw and flattened head. The body color is brownish yellow with 5 to 6 dark and rounded spots on its sides. Dorsal, pectoral and caudal fins mottled with small spots where darkest spots are found along the spine of second dorsal fin. Pelvic fins united but attached to the body only from their anterior part. The morphometric measurements of *G. giuris* are shown in Figure2.

The fin formula of *G. giuris* is: dorsal, D_1. VI; D_2. 8–11 (II–III/8–11); pectoral, P_1. 17–22 (II–VI/14–19); pelvic, P_2. 10–13 (II–III/8–10); anal, A. 7–12 (II–IV/5–8); and caudal, C. 16–21 (IV–VIII/12–13), respectively. A completed lateral line is present. There are about 32-33 scales in lateral line and 5.5 scales above lateral line and 6.5 scales below the lateral line. All morphometric and meristic measurements are presented in Table 1 and 2, respectively. In the present study, TL was ranged from 4.3 to 26.9 cm (mean ± SD = 12.80±5.89) and the BW was varied from 0.67 to 146.55g (mean ± SD = 27.731±33.217). The standard length (76.73%) contains the high percentage of TL (Table 1).

The regression parameters (*a* and *b*), with their 95% confidence intervals for LWRs, coefficients of determination (r^2) of *G. giuris*, are given in Table 2. All LWRs were highly significant ($p < 0.0001$) with r^2 values ≥ 0.975. Based on r^2 value, LWR by BW *vs.* TL, BW *vs.* SL and BW *vs.* PoAnL were good fitted models among the 13 equations.

Also, the LLRs are presented in Table 3 and all LLRs were also highly correlated with r^2 values ≥ 0.990. According to r^2 value, LLR by TL *vs.* SL; TL *vs.* PoDL 2; TL *vs.* PrAnL and TL *vs.* PoAnL were good fitted models among 12 equations.

Table 2. Descriptive statistics and estimated parameters of the length-weight relationships of *Glossogobius giuris* (Hamilton, 1822) (*n* = 229) from the Gorai River, southwestern Bangladesh

Equation	Regression parameter		95% CL of *a*	95% CL of *b*	r^2
	a	*b*			
BW= $a \times TL^b$	0.0102	2.910	0.0096-0.0102	2.882-2.937	0.995
BW= $a \times SL^b$	0.0222	2.902	0.0209-0.0236	2.875-2.929	0.995
BW= $a \times$ PrDL 1^b	0.4168	2.849	0.3974-0.4370	2.812-2.886	0.990
BW= $a \times$ PoDL 1^b	0.1862	2.876	0.1773-0.1957	2.844-2.908	0.993
BW= $a \times$ PrDL 2^b	0.1104	2.907	0.1046-0.1165	2.875-2.939	0.993
BW= $a \times$ PoDL 2^b	0.0487	2.895	0.0460-0.0515	2.866-2.924	0.994
BW= $a \times HL^b$	0.8289	2.827	0.7797-0.8812	2.770-2.885	0.976
BW= $a \times OprL^b$	1.1287	2.672	1.0669-1.1941	2.617-2.726	0.975
BW= $a \times PcL^b$	0.8422	2.775	0.7977-0.8892	2.725-2.825	0.981
BW= $a \times PvL^b$	0.6699	2.853	0.6336-0.7082	2.804-2.903	0.983
BW= $a \times AnsL^b$	0.1747	2.687	0.1654-0.1846	2.654-2.720	0.991
BW= $a \times PrAnL^b$	0.0998	2.869	0.0950-0.1049	2.841-2.897	0.994
BW= $a \times PoAnL^b$	0.0529	2.877	0.0500-0.0558	2.849-2.905	0.995

n, sample size; *a* and *b* are regression parameters; CL, confidence intervals for mean values; r^2, co-efficient of determination

Table 3. The estimated parameters of the length-length relationships ($Y = a + b \times X$) of *Glossogobius giuris* (*n*= 229) from the Gorai River, southwestern Bangladesh

Equation	Regression parameters		95% CL of *a*	95% CL of *b*	r^2
	a	*b*			
TL = $a + b\times$ SL	0.0003	1.298	-0.0976 to 0.0982	1.289-1.307	0.997
TL = $a + b\times$PrDL 1	0.1166	3.448	-0.0095 to 0.243	3.417-3.479	0.995
TL = $a + b\times$PoDL 1	0.0947	2.642	-0.0231 to 0.2126	2.620-2.665	0.996
TL = $a + b\times$PrDL 2	-0.0232	2.267	-0.1392 to 0.0927	2.249-2.86	0.996
TL = $a + b\times$PoDL 2	-0.0217	1.696	-0.1249 to 0.0814	1.683-1.708	0.997
TL = $a + b\times$ HL	0.2509	4.307	0.0714-0.4305	4.251-4.363	0.990
TL = $a + b\times$OprL	0.9208	4.273	0.7457-1.0959	4.217-4.330	0.990
TL = $a + b\times$PcL	0.4735	4.160	0.2988-0.6483	4.106-4.213	0.991
TL = $a + b\times$PvL	0.2190	4.046	0.0365 - 0.4015	3.993-4.0990	0.991
TL = $a + b\times$AnsL	0.7912	2.170	0.6831-0.8994	2.153-2.188	0.996
TL = $a + b\times$PrAnL	0.0415	2.127	-0.0612 to 0.1442	2.111-2.143	0.997
TL = $a + b\times$PoAnL	0.0618	1.711	-0.0381 to 0.1616	1.698-1.723	0.997

SL, standard length; ; PrDL 1,1st Pre-dorsal length; PoDL 1,1st Post-dorsal length; PrDL 2, 2nd Pre-dorsal length; PoDL 2, 2nd Post-dorsal length; HL, Head length; OprL, Opercular length; PcL, Pectoral length; PvL, pelvic length; AnsL, anus length; PrAnL, pre-anal length; PoAnL, post-anal length; *a*, intercept; *b*, slope; CL, confidence limit for mean values; r^2, co-efficient of determination.

4. Discussion

The present study illustrates the first complete information on morphometric (LWRs and LLRs) and meristic characteristics of *G. giuris* from the Gorai River, southwestern Bangladesh. In this study, a total of 229 individuals from small to larger body sizes were used; however, it was not possible to collect *G. giuris* smaller than 4.3 cm TL, which can be attributed that the fishermen failed to catch the smaller size or selectivity of fishing gears (Hossain *et al.*, 2012; Hossain *et al.*, 2016a, b). In the present study, the maximum length was found 26.9 cm TL, which is quite close to the study of Talwar and Jhingran, 1991 (30 cm) but lower than the maximum recorded value of 50.0 cm SL (Eccles, 1992). The absence of maximum sizes of *G. giuris* in the Gorai River might be due to either the absence of larger-sized individuals in the populations in the fishing grounds (Hossain *et al.*, 2016c, d; 2017) or fishermen did not go where the larger size exist. Indeed, maximum length is a helpful fool to estimate the growth parameters (i.e., asymptotic length, growth coefficient), thereby important for fisheries resource planning and management (Ahmed *et al.*, 2012; Hossain, 2016b, 2017).

The allometric co-efficient (*b*) values of LWRs may vary between 2.0 and 4.0 (Carlander, 1969); however, values ranging from 2.5 to 3.5 are more common (Froese, 2006). In the present study, most of the *b* values were within the limit (2.67–2.91) indicating negative allometric growth pattern for *G. giuris* in the Gorai River, SW Bangladesh which was dissimilar with Hossain *et al.* (2009) (*b*= 3.07-3.09). However, the *b* values may vary in the same species due to the amalgamation of one or more factors including variations of growth in different body parts, sex, physiology, preservation methods and differences in the observed length ranges of the specimens collected (Tesch, 1971; Hossen *et al*., 2016; Hossain *et al*., 2015; 2017, Nawer *et al.*, 2017), which were excluded during the present study. In addition, all LLRs were highly correlated, which is not in accordance with Hossain *et al.* (2009). However, the present study found the best/good model among equations using a number of different lengths based on coefficient of determination, which would be very effective for comparison with any future studies.

In the present study, 6 fin rays in 1st dorsal fin and 8–11 in 2nd dorsal fin, 17–22 pectoral fin rays, 10–12 rays in attached pelvic fin, 7–12 anal fin rays and 16–21 caudal fin rays were observed, which was more or less similar with the studies done by Talwar and Jhingran (1991) and Rahman (2005). Besides, we found a total of 32-33 scales in lateral line, which is in agreement with Rahman (2005).

In conclusion, these findings would be a helpful tool for taxonomists to recognize *G. giuris* and for fishery managers/ biologists to instigate the stock assessment of the remaining stocks of this species in the Gorai River, SW Bangladesh and other subtropical countries. Also, these results will impart an important baseline for future studies within the Gorai River and surrounding ecosystems.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of the present paper.

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Lactobacillus rhamnosus Ability of Aflatoxin Detoxification

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Abstract

The present study was conducted to isolate and identify *Lactobacillus rhamnosus* from locally fermented dairy products collected from different markets in Irbid city, Jordan. Thereafter, the ability of *Lb. rhamnosus* to detoxify aflatoxins (AFs) was investigated *in vitro* after incubation on 37°C in MRS medium and in artificial intestine fluid (AIF). Three *Lb. rhamnosus* out of nine different species of Lactic Acid Bacteria (LAB) isolated from 15 fermented dairy products samples were identified. The isolates were characterized based on their morphological, microscopic, cultural and biochemical properties. The selection of isolates as probiotics depended on their abilities to grow in pH levels between 2 to 6 and their tolerance to grow at 1.0 % bile salts concentrations, Furthermore, *Lb. rhamnosus* was able to adhere to mucus onto the intestine surface at 54.7%. The ability of *Lb. rhamnosus* of AFs detoxification has significantly (p<0.05) increased with the increase in incubation periods, and the detoxification percentage after 72h incubation in each MRS medium and AIF, was 76% and 81.6%, respectively.

Keywords*: Lb. rhamnosus*, Aflatoxins, Probiotics, Detoxifications.

1. Introduction

Mycotoxins are secondary metabolites of molds that contaminate over than 25% of the human food (Moss, 2002). They have been found in homes, agricultural settings and food; they could be able to cause different human health problems, because they have wide toxic effects, ranging from short-term mucous membrane irritation to damaging the internal organ, depression of the immune system and cancer (Williams, 2004; Mohamad *et al*., 2015). Almost the diseases related to causes by mycotoxins were related to consuming contaminated food (Hussein *et al*., 2015). The most important kind of mycotoxin is the aflatoxins group which include aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) and the metabolites. These aflatoxins were well-characterized biologically and toxicologically (Wagacha and Muthomi, 2008). Aflatoxins are among the most potent mutagenic and carcinogenic substances. They were classified by the International Agency for Research on Cancer (IARC) as a Class 1 human carcinogen (IARC, 2002). They are associated with many chronic health risks, including the induction of cancer, immune suppression, digestive, blood and nerve defects (Bryden, 2007).

Aspergillus flavus and *A. parasiticus* are capable of colonizing a wide variety of food commodities including maize, oilseeds, spices, groundnuts, tree nuts, milk, and dried fruit (Thalij *et al*., 2015). Ability of these fungi to produce aflatoxins depends on multiple climate factors, such as drought stress, rainfall, suitability of crop genotype, insect damage, and agricultural process (Mohammed *et al*., 2005; Wu and Khlangwiset, 2010). These foods were the main sources of human exposure to aflatoxin because they are so highly consumed worldwide and unfortunately they are also the most susceptible crops to aflatoxins contamination (Thalij *et al*., 2015).

Various physical and chemical methods have been developed to decrease the aflatoxins toxicity, but these methods have many limitations, such as loss of nutrition products, organoleptic qualities, undesirable health effects and high cost of equipment (Hussein *et al*., 2014). These disadvantages have stimulated recent prominence on biological methods of degradation of aflatoxins (Basappa and Shantha, 1996).

Lactobacillus is a broad genus from Lactic Acid Bacteria (LAB) characterized by formation of lactic acid as a major metabolites product of carbohydrate utilization. It is a genus of gram-positive, non-spore-forming, microaerophilic and some other characteristics (Satokari *et al*., 2003). LAB are common and usually being inhabitants of the GI and the vagina in the bodies of humans and animals (Hammes and Vogel, 1995).

Several publications have reported *in vitro* ability of binding by LAB and some species of yeast with mycotoxins, such as aflatoxin B1 (Hernandez-Mendoza *et al*., 2009; Hernandez-Mendoza *et al*., 2010). Some species of LAB were reported to be the strongest binder of aflatoxin (Fazeli *et al*., 2009). The interaction was influenced by the peptidoglycan structure and, more

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accurately, by its amino acid composition (Niderkorn *et al*., 2009). The LAB, which have been used as probiotics, were considered potential mycotoxin decontaminating microorganisms because of their ability to bind these toxic metabolites (Hernandez-Mendoza *et al*., 2010).

The objectives of the present study are to investigate the ability of *Lb. rhamnosus* isolated from local fermented dairy products to degrade AFs produced by *A. parasiticus* after incubation in MRS medium and AIF.

2. Materials and Methods

2.1. Lactobacillus rhamnosus Isolation and Identification

Lactobacillus rhamnosus isolation was conducted from Locally Dairy Fermented (LDF) samples that were collected from different markets in Irbid city in Jordan. Fifty mL of each LDF sample were mixed with 10 mL of MRS broth medium (Oxoid, UK) and incubated statically under aerobic conditions at 37 ˚C for 48 h. The last two series of dilution mixture of each sample were spread on MRS agar plates supplemented with 1.1 mM bromocresol purple, and incubated anaerobically at 37˚C for up to 48 h, (Sujaya *et al*., 2001). Single yellow colonies were selected randomly from the MRS agar plates, then transferred into test tubes containing 10 ml of MRS broth, and incubated at 37˚˚C for 24 h under aerobic conditions. The pure colony isolates were streaked onto MRS agar plates. The isolated bacterium was examined by comparing its bacterial colony and cell morphology, gram staining properties, acid and gas production from different carbohydrates as carbon sources. Fermentation was observed after incubation for 24 and 48 h anaerobically at 30˚˚C and 37˚˚C. In addition, identification was completed using other biochemical profiles and combined with the descriptions contained in Bergey's Manual of Systematic Bacteriology (Kandler *et al*., 1986). The bacteria were maintained by routine subculture at 4 °C in slant tubes with MRS agar for further investigation (Kozaki *et al*., 1992).

2.2. Tolerance Ability of Lb. rhamnosus to Low pH Values

Five mL from MRS medium tubes were adjusted to pH levels at 2, 3, 4, 5 and 6 using optimal amount from artificial gastric juice. *Lb. rhamnosus* cells were pre-cultured in 5mL of MRS broth at 37˚C for 24 h under aerobic conditions, then a 1ml of aliquot of the culture broth was harvested by centrifugation at 15000 g for 5 min and washed twice with PBS. The bacterial cells were suspended in 100 µL of PBS and incubated with 5mL of MRS broth medium at various pHs levels at 37 ˚C for 3 h under aerobic conditions. After incubation, 50 µL of the culture broth were appropriately diluted with PBS then streaked on MRS agar plates. Viable cells were counted after anaerobic incubation at 37 ˚C for 48 h (Sultana *et al*., 2000).

2.3. Tolerance Ability of Lb. rhamnosus to Bile Salts

This test was performed by inoculating 100 µl of bacterial cells pre-cultured at 37 ˚C for 24 h in 5mL of MRS broth containing bile salts (BDH, UK) at 0.3, 0.5 and1% and then incubated at 37 ˚C for 4 h under aerobic conditions. Viable cells were counted as described by Deshpande *et al*. (2014).

2.4. Adhesion Properties of Lb. rhamnosus

The adhesion assay, to screen the ability of *Lb. rhamnosus* bacteria to adhere to cells, was performed using the Adhesion Index (AI) (Gratz *et al*., 2004). The assay procedure was completed according to Lee *et al*. (2003).

2.5. Aflatoxin Production

The aflatoxin was produced from *Aspergillus parasiticus* NRRL 2999 which was obtained from College of Agricultural Tikrit University, Laboratory of Food Science Department. Then, an assurance of mold strains was done on the basis of morphological characteristics using Scotch tape preparation and cultural characteristics after cultivation on malt extract agar and potato dextrose agar, according to (Sammson *et al*., 1992). Thereafter, a fermentation of rice was done by the method of Boller and Schroeder (1973). Successfully fermented rice was then steamed to kill the fungus, dried and ground to a fine powder. The aflatoxin content in rice powder was measured by ELISA as follows:

2.6. Aflatoxin Assay

Aflatoxin extraction was performed according to Kawamura *et al*. (1988). An aliquot of each rice fermented at 2 g was shaken for 10 min at 150 rpm with 10 mL methanol: water (70:30, v/v). The crude extract was then filtered through Whatman No. 1 and diluted in PBST (PBS + 0.05% Tween 20) for intracellular (ic-ELISA) determination.

Aflatoxins were determined by a monoclonal antibody-based ic-ELISA using Aflatoxin ELISA Test Kits (Shenzhen Lvshiyuan Biotechnology Co., Ltd. Guangdong, China) sensitivity: 0.1ppb and as the product protocol procedure. This test kit was based on the competitive enzyme immunoassay for the qualitative-quantitative detection of Aflatoxins in the rice. The coupling antigen was pre-coated on the micro well stripes. The AFs in the sample and the coupling antigens pre-coated on the micro-well stripes compete for anti-Aflatoxin antibodies. After the addition of the enzyme conjugate, the TMB substrate was added for coloration. The Optical Density (OD) value of the testing sample has a negative correlation with the AF concentration in the sample. This value was compared to the standard curve and the AF concentration was subsequently obtained. The average absorbance was calculated from the individual absorbance obtained from triplicate wells and the results were expressed as percentage of binding. This ELISA procedure was completed according the description guide from the manufacturing company (Shenzhen Lvshiyuan Biotechnology Co., Ltd. Guangdong, China).

2.7. Assessing the Ability of Lb. rhamnosus to Detoxify AFs

Lb. rhamnosus was activated in MRS broth at 37°C for 24 h., and viable counts (approximately 1.5×10^8cfu/ml) were calculated by McFarland procedure (Winn *et al*., 2006). One ml of activated culture was inoculated into 100 mL of fresh MRS broth. Aflatoxin was added to make the mixtures containing 2.5µg/ml of aflatoxin. MRS broth containing aflatoxins was used as a control and was not inoculated with activated culture. Each mixture was incubated at 37°C for 72 h., with shaking at 150 r/min. At 0, 24, 48, and 72 h of fermentation, 5 ml of fermented

broth was taken out and centrifuged (14,000×g, 10 min, at 4°C) with ultra-centrifuge (Sigma-Aldrich), supernatant fluid was filtered through 0.22-µm filter twice and kept at 4°C before it was analyzed for aflatoxins content (Niderkorn *et al*., 2006).

2.8. Assessment of Lb. rhamnosus Ability to Detoxify AFs in Artificial Intestinal Fluids

The ability of *Lb. rhamnosus* to detoxify AFs against simulated intestinal fluids was tested as described by Fernandez *et al*. (2003), with some modifications. One ml of 24 h., culture-broth was harvested by centrifugation at 14000 g for 5 min at 4 °C, washed with sterilized PBS, and suspended in 100 mL of PBS. The cell suspension was added to 900 ml of AIF (RICCA CHEMICAL COMPANY, USA). The bacterial suspensions were incubated at 37 °C for up to 72 h. with agitation at 160 rpm. Fifty mL of the Aliquots of the mixture were taken at each 0, 12, 24, 36, 48, 60 and 72 h., of incubation, and were used for an appropriate dilution then they were streaked on MRS agar plates (in triplicates) and were incubated at 37 °C for 48 h., under anaerobic conditions, followed by counting of viable cells.

Another amount of 50 ml from suspension, at different incubation periods, was used to detect the AFs contents after extraction according to Kawamura *et al*. (1988). The suspension was shaken for 10 min at 150 rpm with 10 ml methanol: Water (70:30,v/v). The crude extract was then filtered through Whatman No.1 and was diluted in PBST (PBS+0.05% Tween 20) for ic-ELISA determination. Aflatoxins were determined by a monoclonal antibody-based ic-ELISA using Aflatoxin ELISA Test Kits (Shenzhen Lvshiyuan Biotechnology Co.,Ltd. Guangdong, China) and the procedures were completed according to the same steps mentioned above.

2.9. Statistical Analysis

Data were analyzed by the ANOVA analysis, using the general linear model of the Statistical Analysis System (SAS Institute, 2001). Significant treatment differences were evaluated using Duncan's multiple-range test (Duncan, 1955). All statements of significance are based on the 0.05 level of probability.

3. Results and Discussion

3.1. Isolation and Identification of Lb. rhamnosus

Isolation of the *Lb. rhamnosus* from the fermented dairy products was carried out using the morphological characteristics, after cultivation on MRS media. The growth on these media has been observed because it contained all nutrients needed to grow well. The colony appeared as restricted and in a pale yellow color in central of pellucid zone for each species.

Nine pure isolates were primarily assigned as different lactobacilli species. Since they appeared as Gram-positive, rods shapes were straight and they were cultivated on MRS- $CaCO_3$ in an aerobic environment and showed ability to utilize the $CaCO_3$. Moreover, they were catalase negative and unable to produce NH_3 from arginine (Gilliland, 1990).

The assurance diagnosis process for species level was completed with biochemical test after obtaining subcultures of pure colonies from each isolate on MRS media. Three isolates out of nine appeared as hetero-fermentative and gas producing; they were tentatively identified as *Lb. rhamnosus* (Tables 1). Also, *Lb. rhamnosus* differed from some other *lactobacillus* spp. in its capability to grow in pH range from 2 to 6, and at 25 to 45 °C while it was not able to grow at 10 °C. In addition, the isolates were capable of fermenting all carbohydrates when used as carbon sources, excepted D-arabinose and D-xylose. On the other hand, the other *Lactobacillus* spp. had a different fermentation action.

These results of biochemical tests of *Lb. rhamnosus* were in agreement with accurate data found in Berge's Manual Guide at Holt *et al*. (1994).

Table 1. Characteristics tests of *Lb. rhamnosus*

Phenotypic, cultures and biochemical tests characteristics		*Lb. rhamnosus*
shape of colony		appearance on MRS agar is pale yellow
Shape under microscope		Rods, usually straight
Gram stain reaction		+
Catalase activity		-
CO_2 from glucose		-
NH_3 from arginine		-
Growth at pH	2.0	+
	3.0	+
	4.0	+
	5.0	+
	6.0	+
Growth at Temp. °C	10	-
	35	+
	40	+
	45	+
Sugar fermentation	D-arabinose	-
	D-ribose	+
	D-xylose	±
	D-galactose	+
	D-mannose	+
	D-maltose	+
	D-lactose	+
	D-glucose	+
	D-sorbose	+
	L-rhamnose	+
	D-turanose	+

+, positive; —, negative; ±, undetermined

3.2. Parameters for Probiotics Characteristics

Optimal bacterial species, which were selected as probiotics, should have many characteristics, such as the ability to grow in stomach acidity, the resistance to bile salts and the capability to adhere to intestine epithelial cells.

The tolerance of *Lb. rhamnosus* to different pH levels after cultivation on MRS medium at 37 °C for 48 h is illustrated in Table 2. The *Lb. rhamnosus* showed an ability to grow in pH levels between 2 to 6. These results were in agreement with another study by Ali (2011) who found the same results for some lactic acid bacteria. The LAB tolerance to the acidic environment may indicate that they contain lipoteichoic acid and hydrophobic amino acids in S-layer proteins of cell wall of these bacteria (Frece *et al*., 2005). The tolerance of *Lb. rhamnosus* to different bile salts concentrations appeared important to

evaluate the bacterial species to be used as probiotics especially in cases of oral intake by the organisms.

Lb. rhamnosus showed an ability to survive at certain bile salts concentrations (Morelli, 2000). The results in Table 2 indicate that the *Lb. rhamnosus* were able to grow at 0.3 to 1.0% bile salts concentrations.

This result was in harmony with that of Shi *et al*. (2012) who found that *Lb. rhamnosus* was able to grow in 0.3 to 1.0% of bile salts. Generally, the LAB, which were capable of growing with bile acids, were found to contain the bile salts hydrolase which function by stimulating the fraction of bile salts conjugated with glycine or taurine amino acids for carrying out the non-conjugated bile salts, which in turn is described as less dissolving and exerting with feces, and replacing with other new bile salts through manufacturing in liver from cholesterol. These results were in agreement with Aries and Hill (1970).

The ability of *Lb. rhamnosus* cells to adhere to rats' intestine mucus surface was illustrated in Figure 1. The results indicated that the *Lb. rhamnosus* was able to adhere at 54.7%. The adhesion ability was the essential characteristic for using *Lb. rhamnosus* as probiotics. The capability of adhesion refers to the S-layer protein in the cell wall, the protein percentage in this layer was 10 to 15% from total proteins the cell contains (Bezkorovaing, 2001).

Table 2. Tolerances Ability of *Lb. rhamnosus* to different levels of pH and bile salts

LAB Species	pH levels				
	2.0	3.0	4.0	5.0	6.0
Lb. rhamnosus	-	+	+	+	+
	Bile salts concentrations (%)				
	0.3	0.5	0.75	1.0	
	+	+	+	+	

+: *positive reactions* - : *negative reaction*

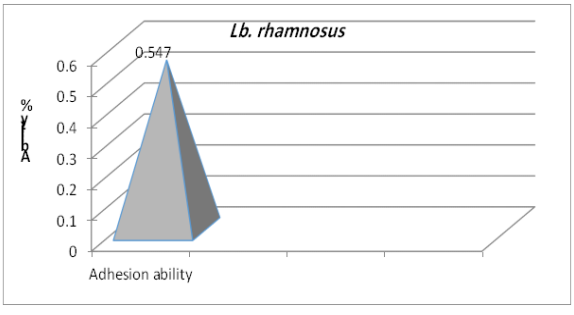

Figure 1. Adhesion Ability of Lb. rhamnosus with intestine cells

3.3 Lb. rhamnosus Ability of AFs Detoxification

The *Lb. rhamnosus* Ability of AFs Detoxification after 72 h. of incubation in MRS media was summarized in Table 3. The results showed that adding AFs to MRS media without inoculation of *Lb. rhamnosus* caused an insignificant change (p<0.05) in AFs concentrations through the incubation periods from 0 to 72 h. On the other hand, the incubation of MRS medium that contains AFs with *Lb. rhamnosus* caused a significant decrease in the AFs concentration, this happened with 0, 24, 48 and 72 h., at 2.5, 1.9, 1.2 and 0.6µg/mL, respectively, and the detoxification percentage was at 0.0, 25, 52 and 76%, respectively.

The results showed that the *Lb. rhamnosus* have the ability to degrade AFs contents in medium, and this degradable activity increased with the increase of incubation periods of *Lb. rhamnosus* with AFs.

Table 3. *Lb. rhamnosus* Ability of AFs Detoxification

Incubation periods (hours)	AF Concentration (µg/ml) in		Detoxification %
	AFs in MRS	AFs + *Lb.rhamnosus*	
0	2.5^{a} ±0.04	2.5^{a} ±0.04	0^{d} ±0.00
24	2.3^{a} ±0.06	1.9^{b}±0.06	25^{c}±2.14
48	2.5^{a} ±0.06	1.2^{c}±0.06	52^{b} ±2.51
72	2.4^{a}±0.02	0.6^{d} ±0.02	76^{a} ±4.37

a-d: Values within columns followed by different letters for different significance levels at 0.05.

The ability of degradation of AFs concentrations at 2.5 µg/mL in artificial intestine fluid during incubation with *Lb. rhamnosus* at 37°C with different period times from zero to 72 h are shown in Table 4. The *Lb. rhamnosus* counts have significantly (p<0.05) increased with the increase in the incubation periods to 72 h and the maximum count was at 48 h of incubation. The AFs concentration in the artificial intestine fluid was significantly removed by the *Lb. rhamnosus* fermentation in artificial intestine fluid and with the 0, 12, 24, 36, 48, 60 and 72 h of incubation and at 2.46, 2.24, 1.86, 1.27, 1.02, 0.74 and 0.46 ηg/mL, respectively. The AFs detoxification percentage at 72 h of incubation was 81.6%. The Mechanism of the AFs removal is shown in Tables 3 and 4. The AFs were bond by *Lb. rhamnosus* in each liquid medium and artificial intestine fluid was assayed at toxin concentration. The mechanism of aflatoxins detoxification by *Lb. rhamnosus* occurs by the interaction between ingredients of its cell wall with aflatoxins. The nature of binding is poorly understood till this moment; it also differs according to the types of ingredients. The binding between aflatoxins and bacterial cell wall ingredients modifies aflatoxins structures and gets a new structure form. The binding of *Lb. rhamnosus* and some other LAB species with all types of aflatoxins were conducted by same mechanism, because the activity of all types of aflatoxins depends on the same active groups, such as double bonds, OH, CH_3, etc. (Huang *et al*., 2017).

The results showed that *Lb. rhamnosus* was able to bind the AFs and the level of binding varied between the incubation times, the AFs concentration decreased in the MRS medium and in fluid with the increase of incubation time. These results were in agreement with Hernandez-Mendoza *et al*. (2009) who found LAB species' ability to bind the AFB1.

These results confirm the role of a cell wall-related physical phenomenon as opposed to a metabolic degradation reaction, and are consistent with the results reported by Haskard *et al*. (2000). The cell wall peptidoglycans of LAB was found by Teniola *et al*. (2005) as responsible for AFs removal. On the other hand, Niderkorn *et al*. (2009) reported that treatments affecting bacterial wall polysaccharides, lipids and proteins caused an increase in the binding with AFs, while those degrading peptidoglycan partially decreased it.

Table 4. *Lb. rhamnosus* Ability of AFs Detoxification in artificial intestine fluid.

Test types	Incubation periods (h.)						
	0	12	24	36	48	60	72
Lb. rhamnosus accounts (CFU/mL)	8.17^{e}±0.72	8.96^{d}±0.49	9.32^{c}±0.85	9.83^{b}±0.76	10.51^{a}±0.74	9.41^{c}±0.81	8.29^{e}±0.57
AFs concentration (ηg/mL)	2.46^{a}±0.05	2.24^{a}±0.06	1.86^{b}±0.03	1.27^{c}±0.01	1.02^{c}±0.02	0.74^{d}±0.07	0.46^{d}±0.02
AFs detoxification (%)	1.6^{g}±0.08	10.4^{f}±0.93	25.6^{e}±2.22	50.8^{d}±3.64	60.2^{c}±3.26	71.4^{b}±4.26	81.6^{a}±4.44

a-d: Values within rows followed by different letters for different significance levels at 0.05.

4. Conclusion

The present study investigated the ability of *Lactobacillus rhamnosus* to detoxify aflatoxins. It was isolated from locally fermented dairy products and identified by morphological, microscopic, cultural and biochemical characteristics. The results indicated the high ability of *Lactobacillus rhamnosus* in aflatoxins detoxification. This ability increased with the increase of incubation periods. These findings are important to food industry and public health; thus, aflatoxin is believed to possess high toxicity among various types of secondary metabolites produced by a larger number of *Aspergillus* spp. Many foods, such as grains (corn, sorghum, and millet), peanuts, beans, and nuts (almonds, pistachios, etc.), may support the growth of *Aspergillus*, and may be contaminated with aflatoxins.

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Expression of Biotransformation and Antioxidant Genes in the Liver of Albino Mice after Exposure to Aflatoxin B1 and an Antioxidant Sourced from Turmeric (*Curcuma longa*)

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Abstract

The present investigation aims to determine the effects of aflatoxin B1 (AFB1) on biotransformation and antioxidant genes and the protective effects of curcumin, present in turmeric (*Curcuma longa*) powder (TMP). Specifically, the study included four groups of albino mice were fed for 30 days on diet Group I: Control, Group II: animals fed on the conventional basal diet supplemented with 0.5% food grade TMP that supplied 74 mg/kg total curcuminoids. Group III contained animals reared on conventional basal diet supplemented with 1.0 ppm AFB1 supplied by ground aflatoxin culture material (760 ppm AFB1). Finally, Group IV comprised of albino mice fed with basal diet supplemented with 1.0 ppm AFB1 and 0.5% TMP that supplied 74 mg/kg of the body weight. After treatment, a number of physical parameters were assessed (gain in body weight, average quantity of feed intake and relative liver weight) and it was found that the subjects fed on diet containing curcumin and aflatoxin B1experienced ameliorative effect on the impact of aflatoxin B1 and performed better on growth and liver weight parameters. RNA extracted from the mice liver successfully was subjected to quantitative real time PCR analysis (Q PCR) and the results revealed no high significant difference in the expression of *CAT* gene between studied groups with probability value ≥0.005. However, at the other hand a decreased in expression statically of *SOD, GPx, GST, EH* genes was observed while there was an increased Synchronous and consistent expression of *CYP1A1* and *CYP2H1* genes display in the studied groups in the current study.

Keywords: Aflatoxin B1, *Curcuma longa*, Gene expression, Antioxidant.

1. Introduction

Fungi contaminate the food in a variety of ways including food spoilage and toxicity depending on their biological make-up and the eco-physiological conditions (Ahmadib *et al*., 2011). Some fungi are toxigenic, synthesising one or more mycotoxins (Ahmed *et al*., 2013), having impact on humans and certain animals when ingested in large quantities. Amongst these, Aflatoxins (AF), especially Aflatoxin B1 (AFB1), are wide spread in distribution, potent contaminants of foodof humans and animals, and result in food security issues and mortality. (Kumar *et al*., 2017). These toxins travel in the food chain and causing wide spread impact in a variety of organisms apparently not under the direct influence of the fungus (Moosavy *et al*., 2013). Recognizing the significance of food security and these toxins rendering the food unsuitable for human consumption has led to a large number of studies (Moosavy *et al*., 2013) on various aspects of the toxicity and remedies thereof. When consumed by Albino mice, AFB1 causes invariably cell damage, production of free radical, and lipid peroxidation in different organs (Dheeb,2013; Nogueira *et al*., 2015). Protective effects against damage due to oxidative are normally redressed by antioxidants that obstruct the free radicals like reactive oxygen species (ROS) reactions. Various studies have been reported the healing effect of aflatoxins through use of medicinal plants (Abdulmajeed, 2011) and the curative role of curcuminoid pigments extracted from roots and rhizomes of turmeric (*Curcuma longa*) (WH, 2009; Bayram *et al*., 2008) is one such example. The present study was therefore initiated to investigate and evaluate this curative role of curcumin and its influence on the expression of biotransformation and anti-oxidant function genes, using Quantitative real time PCR.

2. Materials and Methods

2.1. Animals and Experimental Design

A total 40 male Albino mice (age 7weeks) obtained from the National Centre of Research and Drugs

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Monitoring in Baghdad were employed in the study. The mice were allowed to adapt for two weeks in the Biotechnology Research Centre at Al Nahrain University before the commencement of the study. The animals were reared separately in clean, disinfected and pathogen-free facility fed on commercially available assorted pellets and tap water *ad libitum* (Jun *et al*., 2006). Experiment design "A completely randomized design" was adopted with ten replicates of eight Albino mice assigned to each of four dietary treatments: Group I: Control, Group II: animals fed on the conventional basal diet provided with TMP 0.5% food grade that supplied total curcuminoids74 mg/kg. Group III contained animals reared on conventional basal diet supplemented with 1.0 ppm AFB1 supplied by ground aflatoxin culture material (760 ppm AFB1). Finally, Group IV comprised of albino rats fed with basal diet supplemented with 1.0 ppm AFB1 and 0.5% TMP that supplied 74 mg/kg of the body weight. During the 30 days' dietary treatment, mortality of the animals was recorded as and when noticed and mice were inspected daily for any health related anomalies. At the end of the treatment, mice were sacrificed, the liver was weighed and the tissue collected, immediately frozen in liquid nitrogen, and stored in a freezer at-80 °C until RNA extraction in order to use it in molecular analysis and determination expression of biotransformation and antioxidant genes. Dietary AFB1 concentrations and the content of all the diets were confirmed by the ELISA technique performed using an ELISA kit (Wacoo *et al*., 2017). All treatments on present experiments were approved based on The Committee Ethics of Baghdad University ID: 2398.

2.2. Extraction of Curcuminoid and Determination of the Concentration

The potent curcuminoid was extracted from naturally grown, commercially available Turmeric. A sample of (10) g powdered root and rhizomes was extracted using hexane (50 mL) as a solvent. Re-extracted done to Hexane extracted powder (I gm) with methanol (20 ml) for 2 hours, aliquot centrifuged at 13,000 rpm for 5 min. The supernatant (1 mL) was removed, diluted methanol (4 mL) and total curcuminoid content (curcumin, bis-dimethoxy curcumin and dimethoxy curcumin) was ascertained by High Performance Liquid Chromotography following Gowda *et al*. (2009). To achieve highest possible degree of precision, Sampler and Column (Hitachi Model L–7200 autosampler, 250 x 4.6 mm HyperSil reverse phase C18 column) were employed. Following Dheeb (2015), Hitachi D-7000 data acquisition interface and Concert Chrome software were used to gather data at a detection wavelength of 425 nm. The mobile phase was a mixture of methanol, acetonitrile and acetic acid (1:11:10) with a flow rate of 1mL/min. The curcumin standards were established following Gowda *et al*. (2009). The total curcuminoid content of TMP was determined by adding up the concentrations of the three curcuminoids: curcumin, bis-dimethoxy curcumin and dimethoxy curcumin (Buchau *et al*., 2007) .

2.3. Gene Expression Study of Biotransformation and Antioxidants

2.3.1. RNA Isolation and cDNA Synthesis

An RNA extraction kit (Promega) was used for extraction of Total RNS following the manufacturer's instructions. Although not considered mandatory step, more complete DNA removal was carried out by treating isolated RNA with RNase-free DNase I (Biobasic, Canada) for 20 min at 37°C. Quantus Florometer was used to detect the concentration of extracted RNA in order to detect the goodness of samples for downstream applications. For 1 µL of RNA, 199 µL of diluted Quanty Flour Dye was mixed. After 5min incubation at room temperature, RNA concentration values were detected.

The DNase I was inactivated at 65°C for 10 min. The integrity of the RNA was ascertained by gel electrophoresis (1.5% agarose gel containing 0.5% (v/v) ethidium bromide) following Rassin *et al* (2015). First-strand cDNA was synthesised from 500 ng of total RNA using a Reverse Transcription system (Bioneer, Korea) with an oligo-dT_{15} primer. The reaction solution was used as a template for reverse transcriptase polymerase chain reaction (RT-PCR) .

2.3.2. Amplification of Biotransformation and Antioxidant Genes

Target gene and housekeeping B-actin (reference gene) cDNA were amplified using biotransformation and antioxidant primers in order to measure the expression patterns of genes involved in antioxidant function. Primers were as follows:

Catalase (CAT) Forward 5′GGGGAGCTGTTTACT GCAAG-3′ and a reverse primer5′TTCCATTGGCTATG GCATT-3′, product size 139bp; super oxide dismutase (SOD, GPx, GST biotransformation genes EH, CYP 2H1, CYP 1A1,), Forward5′- AGGGGGTCATCCACTTCC-3′ and a reverse primer 5′ CCCATTTGTGTTGTCTCCAA-3′, product size 122 bp; glutathione peroxidase (GPx), Forward 5′- TTGTAAACATCAGGGGCAAA-3′and a reverse primer 5′ TGGGCCAAGATCTTTCTGTAA-3′, product size 140 bp; glutathione S-transferase (GST) Forward GCCTGACTTCAGTCCTTGGT-3′ and a reverse primer 5′ 5′- CCACCGaATTGACTCCATCT -3′, product size 131 bp.

The following primers were used for biotransformation genes: epoxide hydrolase (EH) Forward5′-AAAGGGACAGAAGCCTGACA -3′ and a reverse primer 5′ CCTCCAGTGGCTCAGTGAAT-3′,product size 128 bp; cytochrome P450'sCYP2H1 Forward 5′-ATCCCCATCATTGGAAATGT-3′ and a reverse primer 5′ TCGTAGCCATACAGCACCAC -3′,product size 137 bp; cytochrome CYP 1A1Forward 5′CACTTTCTGCCTGCTCCTG-3′ and a reverse primer 5′ GGTCCTTCCTCAGCTCCAG -3′, product size 125 bp polymerase chain reaction (PCR) was initiated by employing cDNA template on a Lab net Thermocycler (USA) at the following conditions : (95°C for 5 min and 40 cycles at 95°C for 1 min, 60°Cfor 45 s and 72°C for 1 min). Primers were designed according to Livak *et al*., (2017)using the Primer3 program with an annealing temperature of 60 °C (Al-Tekreeti *et al*., 2017).

2.4. Gene Expression Analysis Study through SYBR Green Real-Time RT-PCR

The gene expression of biotransformation and antioxidant was evaluated through use of SYBR real-time RT-PCR using Exicycler real time PCR (Bioneer, Korea) following Al-Mashhadani (2014). Quantitation of relative expression was determined by the following equation (Yarru, 2008):

Gene expression (Quantity) $=10^{[(CT-b)/slop]}$

Gene for Glyceraldehyde Phosphate Dehydrogenase (GAPDH) was used as the endogenous control gene in the qRT-PCR experiments.

2.5. Quantitative Real Time PCR (qRT–PCR)

The expression levels of antioxidant genes CAT, SOD, GPx, GST, biotransformation genes EH, CYP 2H1, and CYP 1A1, were estimated by One Step qRT-PCR. To confirm the expression of target gene, quantitative real time one step qRT-PCR sybr Green assay was used. Primers sequences for each gene were prepared. The mRNA levels of endogenous control gene GAPD H were amplified and used to normalize the mRNA levels of the up genes , reaction volume and Thermal Cycler Programming summarized in Table 1 and 2.

Table 1. Reaction volume and components of RT qPCR

Components	Conc.	Volume(µL)\Reaction
GoTaqqPCR master mix	2X	10
RT mix	10µM	0.4
Forward Primer	10µM	2
Revers Primer	1-2ng	2
RNA	-	4
RNase-free water		1.6
Total per reaction		20

Table 2. Thermal Cycler Programming

Steps	˚C	min:sec	Cycles
cDNA Synthesis	37	15min	1
Initial Denaturation	95	5 min	1
Denaturation	95	30 sec	40
Annealing	60	30 sec	
Extension	72	30 sec	
Melt	65-90		1

2.6. Statistical Analysis

Data were analysed using the model procedures of SAS. The differences of values of the investigated parameters among different groups of the subjects (mean ± standard error) were assessed by analysis of variance using SAS version 7.5, (difference $p<=0.05$ and 0.001) (Choi *et al*.,2010).

3. Results

3.1. Influence of Dosage of AFB1on Total Body Weight and Some Properties of Mice Organs

Data for the animals belonging to control and experimental groups regarding average food intake, gain in weight, and liver weight (% BW) after 30 days of experimentation are presented in Table 3. The data reflects that the mice treated with TMP alone (Group II) and the control (Group I) had similar pattern of weight gain and feed intake while the mice in Group III(receiving dose of AFB1) had significantly lower feed intake and corresponding weight gain. For animals receiving 0.5% TMP (74 mg/kg curcuminoids) together with AFB1 (Group IV), feed intake and body weight both increased, suggesting a protective action of the curcuminoids present in TMP. The results of the present investigation are in conformity with some previous studies investigating the effects of AFB1 (Choi *et al.*, 2008, Livak *et al.*, 2008),and other chemicals (Yarru, 2008, Livak *et al.*, 2008).The condition of the mice exposed to AFB1 together with TMP (Group IV) and the ones receiving dose of TMP alone (Group II) are a reflection of curcumin present in turmeric works as an antioxidant through inhibition of the biotransformation of AFB1 to aflatoxicol in the liver (Yarru, 2008, Livak *et al.*, 2008), and may also have antimutagenic and anticarcinogenic effects (Yarru, 2008, Cleveland *et al.*,2009). In another study (Hismiogullari, 2014; Livak *et al.*, 2008), where mice were fed on different concentrations of turmeric powder over a period of49 days, the animals experienced a positive effect on liver enzymes that directly or indirectly reflect a healthier liver status.

Table 3. Average feed intake, weight gain, feed efficiency and relative liver weight in mice during the study.

Treatment	Av. Feed intake (gm/mice)	Av. Body weight gain (gm/ mice)	Wt of liver (% BW)
I(Control)	1047.3±0.22 a	33.5± 0.12a	1.74 ±0.44b
II(animals fed on the conventional basal diet supplemented with 0.5% food grade TMP that supplied 74 mg/kg total curcuminoids)	857.8±0.75b	26.0 ±0.34c	1.37±0.19a
III(animals reared on conventional basal diet supplemented with 1.0 ppm AFB1 supplied by ground aflatoxin culture material (760 ppm AFB1)	905.5±0.73b	27.6 ±0.32b	1.09± 0.09ab
IV (animals feed basal diet supplemented with 1.0 ppm AFB1 and 0.5% TMP that supplied 74 mg/kg of the body weight)	1004.9±0.20a	29.9 ±0.18ab	1.016±0.007ab

*$p \leq 0.05$. *different letter means significant difference between the treatment

3.2. Quantitative Real Time PCR Results

The expression of genes responsible for antioxidant and biotransformation functions was ascertained using the quantitative real time PCR techniques show in Figure 1. The results of the present investigation showed similar pattern of gene expression among the four groups receiving different dietary treatments for the catalase (CAT).

However, super oxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase(GST) and epoxide hydrolase (EH) genes exhibited a decreased activity in animals treated with 1 ppm AFB1 (Group III). These results may be interpreted as the effects of AFB1are neutralized by inclusion of curcumin in the diet (Group IV). Increased expression of CYP1A1 and CYP2H1 genes may also be attributed to curcumin's alleviation of the toxic effects of AFB1. The present results may be due to the presence of TMP stimulating the antioxidant system in the livers of albino mice in order to counteract the oxidative damage caused by AFB1 (Dwivedi, 2013, Cleveland *et al.*, 2009). The animals fed on diet containing AFB1exhibited an increased liver peroxide level and a decrease in SOD, GPx and CAT activities, and some studies have shown the supplementation of diets with the phenolic compounds of plants origin have reduced the free radical production and apoptosis in human hepatoma cells induced by AFB1(Helal *et al.*, 2012).

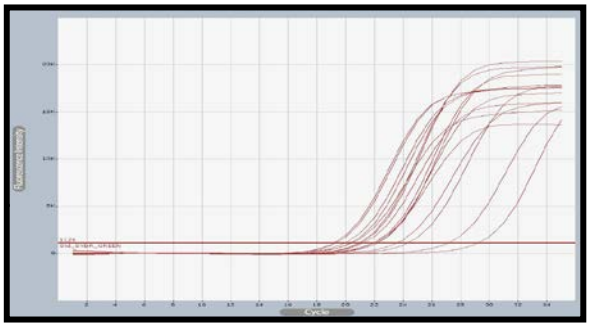

Figure 1, Quantitative Real Time PCR Curves of antioxidant genes CAT, SOD, GPx, GST, biotransformation genes, and GAPD Hwith threshold (177.8)

Some other studies have demonstrated the carbonyl functional group of curcuminoids to be responsible for its antimutagenic and anticarcinogenic effects (Buchau and Gallo, 2007), and curcumin also has inhibitory effects on superoxide anion generation and the biotransformation of Aflatoxin B1 to aflatoxicol in liver (Buchau and Gallo, 2007). Supplementation of turmeric is known to reduce the formation of adducts through modulation of cytochromeP450 function (Buchau and Gallo, 2007). Aflatoxin B1 is known to cause lipid peroxidation in liver (Buchau and Gallo, 2007), induces oxidative damage in the cell, and is also a potent carcinogen that forms DNA adducts (Buchau and Gallo, 2007). Feeding AFB1 to rats increased liver peroxide levels and was associated with a decrease in activities of superoxide dismutase, catalase, and glutathione peroxidase. Supplementation of root extracts of *Picrorhiza kurroa* and seeds of *Silybum marianum* ameliorated the effects of Aflatoxin B1, reduced peroxide levels, and returned antioxidant enzymes to control levels (Buchau and Gallo, 2007). Rosamarinic acid, a phenolic compound present in *Boraginaceae* species of plants reduced free radical production and apoptosis in human hepatoma cells induced by Aflatoxin B1(Buchau and Gallo,2007). The carbonyl functional group of curcuminoids was responsible for its antimutagenic and anticarcinogenic actions (Ahmeda *et al.*, 2014). Further, earlier reports suggest that curcumin has a strong inhibitory action on superoxide anion generation (Choi *et al.*, 2010) and biotransformation of Aflatoxin B1 to aflatoxicol in liver (Buchau and Gallo, 2007). Supplementation of turmeric is known to reduce the formation of adducts through modulation of cytochrome P450 function (Shivanoor *et al.*, 2016). The above findings demonstrated the possible mode of action of curcumin as an antioxidant and the results we obtained in the present study suggest that curcumin in TMP gave partial protection against aflatoxicosis. Current findings also suggest that curcumin may need to be supplemented at levels higher than 74mg/kg to achieve optimum protection against 1.0 ppm AFB1.

4. Discussion

In the present study, expression of gene for super oxide dismutase (SOD) was down-regulated in albino mice fed a diet containing AFB1 and this reduced expression of gene has been attributed to the superoxide anions accumulated within the mitochondria, thus leading to an oxidative stress and thereby hindering the cellular processes (Ahmeda *et al.*, 2014). Curcumin is known to boost the antioxidant activity especially through SOD (Thinarayanana *et al.*, 2017). The animals receiving dose of AFB1 together with curcumin had greater expression of SOD gene compared to those receiving AFB1 dose alone, an indication of ameliorative effects of curcumin in improving the antioxidant status in aflatoxin fed mice. Similarly, the expression of the GPx gene was decreased in mice fed on diet containing AFB1 (Group III) compared to the Control. Such a decrease in GPx expression may result in impaired conversion of hydrogen peroxide to water and thus maintaining hydroxyl radicals within hepatocytes (Buchau and Gallo, 2007). Decreased expression of GPx due to AFB1 was normalized in animals containing in group IV, receiving curcumin in the diet (Group III). The addition of curcumin seems to support the role of GPx in protecting against high cellular concentrations of hydrogen peroxide (Nouri *et al.*, 2015). Supplementation of curcumin, however, was not completely protective against the toxic effects of AFB1 since catalase levels remained low in these mice compared to controls. Like other genes, the expression of Glutathione S-transferase (GST) was decreased in mice exposed to AFB1 in their diet and potentially it was due to the ability of hepatic tissue to conjugate reactive metabolites1 . As a result, curcumin in the diet alleviated the negative effect of AFB1 on GST expression. Studies have established that the curcumin reduced iron-induced hepatic damage, AFB1 induced mutagenicity and hepatocarcinogenecity by inhibiting cytochrome P450 in the liver and also induction of antioxidant enzymes (Ahmeda *et al.*, 2014). The expression of Epoxide hydrolase (EH), GST and Cytochrome P450genes show that there was an increase in the expression of hepaticCYP1A1 and CYP2H1 genes in the albino mice fed on diet containing AFB1. The gene

CYP450 isoforms have previously been shown to be overexpressed, leading to hepatocellular injury and inducing death through chronic oxidative stress, excess ROS and transformation of aflatoxin B1 to toxic metabolite aflatoxin (Jesuthasan *et al.*, 2005). This toxic metabolite is produced from the oxidation of AFB1 by the CYP450 isoforms reactive intermediates, AFB1 -8,9-epoxide (AFBO), and aflatoxin M1 (aflatoxin M1) The fact that, in the present study, CYP450 isoform genes were up-regulated and epoxide hydrolase and GST genes were down-regulated in AFB1receiving group, compared to the control suggests that there is a greater chance of the formation of more toxic intermediate metabolites such as aflatoxin 8, 9 epoxide. Furthermore, down-regulation of these detoxification genes could reduce the ability of the mice to detoxify AFB1 which could lead to various toxicological effects. The present study highlights the antioxidant role of curcumin in different parameters investigated and it is linked to decrease CYP1A gene expression compared to mice fed on AFB1. It further suggests chemo protective action against the negative effects of aflatoxin B1 (Haas *et al.*, 2006) .

There are important interactions among the activities of several antioxidant enzymes and various ROS and cellular reactions, all of which could be responsible for some of the observations in the present study. The decrease in the expression and activity of SOD, GST and GPx observed in the present study are additive with respect to oxidative damage. On-enzymatic decomposition of hydrogen peroxide involving transition metals, such as iron, in a Fenton-type reaction can be more damaging to the cell than the production of the hydroxyl radical species (Giray *et al.*, 2008). Furthermore, increased levels of hydrogen peroxide within the cells reduce SOD activity (Mathuria *et al.*,2007), thereby increasing superoxide levels within the cell and reducing catalase activity. It is evident from the results of the present study that transcriptional activation ofCYP1A1 and CYP2H1 isoforms, in response to aflatoxin has the potential to increase oxidative stress. Also, these CYP 450 isoforms are involved in biotransformation of aflatoxin B1 to the highly toxic metabolite aflatoxin 8,9 epoxide (Buchau and Gallo,2007). CYP 450 isoforms oxidize Aflatoxin B1into two metabolites: the reactive intermediate, 71Aflatoxin B1 -8,9-epoxide (AFBO), and aflatoxin M1 (AFM1). Because of the importance of AFBO and AFM1 in the toxicity of AFB1, CYP450 isoforms play an important role in the well-known hypersensitivity of mice to Aflatoxin B1 (Magnoli *et al.*, 2011). Since genes coding for CYP 450 isoforms were up regulated and epoxide hydrolase and GST genes were downregulated in aflatoxin fed mice compared to controls in the present study, there is a greater chance for formation of more toxic intermediate metabolites such as aflatoxin 8, 9 epoxide. Furthermore, down-regulation of these detoxification genes could reduce the mice ability to detoxify Aflatoxin B1 which could lead to various toxicological effects. The inclusion of butylated hydroxyl toluene, an antioxidant in the diet has any chemo protective effects in Aflatoxin B1 fed mice (Osawa, 2007). "They observed decreased activity of hepatic microsomal CYP1A as well as conversion of Aflatoxin B1 to the putative toxic metabolite, AFB1-8,9-epoxide (AFBO), compared to controls (Matur *et al.*,2007). Similar to the above findings, the antioxidant, curcumin in the current study decreased CYP1A gene expression compared to mice fed Aflatoxin B1 suggesting chemo protective effects .

5. Conclusion

The above findings demonstrate the possible mode of action of curcumin as an antioxidant, and the results obtained in the present study suggests that curcumin present in TMP gave partial protection against aflatoxicosis. Results also suggest that curcumin may need to be supplemented at levels higher than 74 mg/kg in order to achieve optimum protection against 1.0 ppm AFB1. These results suggest that mice fed AFB1 had impaired antioxidant activities along with decreased growth, development, and detoxification mechanisms, making them susceptible to various other stressors. Furthermore, addition of TMP containing curcumin to the AFB1-contaminated diet partially protected mice against AFB1.

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Molecular Phylogeny of *Trametes* and Related Genera from Northern Namibia

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Abstract

Trametes Fr. is widely characterized as a polyporoid cosmopolitan genus which is presented in almost any type of forest environments. It is characterized by a combination of pileate basidiocarp, porous hymenophore, trimitic hyphal system and thin-walled basidiospores which do not react in Melzer's reagent. Dry polypores were collected from Northern Namibia and identified as *Trametes* species based on morphology. Molecular analysis of Internal Transcribed Spacer region 1 (ITS 1) and Internal Transcribed Spacer region 2 (ITS 2) of the collected material revealed inconsistency with morphological identification. The phylogenetic tree was reconstructed using the Neighbour Joining method and reliability for internal branches Assessment was done using the ML bootstrapping method with 500 ML bootstrap replicates applied to 44 unpublished sequences and sequences from GenBank database. Only specimens such as D1-D9, D11 and D13 and specimens F1, I2-I4 and K3-K6 were grouped in the trametoid clade together with *Trametes* species. Furthermore, the position of *Trametes trogii* (also known as *Coriolopsis trogii*) was confirmed to be outside the trametoid clade and more closely related to *Coriolopsis gallica*. The close relationships of *Pycnoporus* and *Trametes* were confirmed by grouping of *Pycnoporus sanguineus* in to trametoid clade. Alignment with GenBank sequences revealed identity to *Trametes* species with up to 99%. These results suggest that it is better to keep a single generic name of *Trametes* for the trametoid clade.

Key words: *Trametes* species, Phylogeny, Polyporoid, Namibia.

1. Introduction

Traditionally, fungal taxonomy was based mainly on morphological description of the fruit body, host specificity, and geographical distribution (Seo and Kirk, 2000; Olusegun, 2014). However, polyporoid fungi in the *Trametes* genus have a similar morphology and have proved to be challenging to identify based on this traditional technique (Ofodile *et al*., 2007). Ever since, mycologists have turned to molecular techniques to explain the taxonomic challenges in *Trametes* and related genera (Zakaria *et al*., 2009; Olusegun, 2014).

The *Trametes* classification has undergone extensive study and deliberation (Zhao *et al*., 1983). Although the Friesian (1835) description is widely accepted, there are ongoing studies to find a clear species delimitation for this Polyporoid group (Justo and Hibbett, 2011). *Trametes* Fr. is widely characterized as a polyporoid cosmopolitan genus which is presented in almost any type of forest environment. It is characterized by a combination of pileate basidiocarp, porous hymenophore, trimitic hyphal system and thin walled spores which do not react in Melzer's reagent (Tomšovsky *et al*., 2006; Carlson *et al*., 2014). *Trametes* Fr. was first named by Fries in 1835 because the hymenophore was considered a distinctive feature of the *Polyporus* genus and Fries wanted to accommodate *Coriaceous* species with a poroid hymenophore characterized by context continuously descending into the hymenophoral trama. At this stage, genera were created according to the hymenophore structure and were either grouped as lamellate, daedaleoid or regular (Trametoid) pores (Welti *et al*., 2012).

In 1886, Quèlet initially separated species by the shape of their pores but later considered other morphological features relevant to define new genera from the classical *Trametes*. The abhymenial surface of the tomentum was considered as a distinctive feature of the *Coriolus* group. Another description was suggested for a *Trametes* group consisting of all genera with di- or trimitic hyphal system with colorless, smooth and not amyloid basidiospores. This group included *Cerrena*, *Daedalea*, *Hexagonia*, *Pycnoporus*, *Coriolopsis*, *Datronia*, *Lenzites*, *Megasporoporus*, *Microporus*, *Trichaptum* and *Trametes* (Ko and Jung, 1999). Kavina and Pilát in 1936 also supported the view that hymenophoral morphology suggested by the Fries is devoid of generic systematic value. Therefore, species with lamellate, daedaleoid and poroid hymenophore were grouped into one, combining *Lenzites* and *Daedalea*. *Lenzites betulina* was combined with *Trametes sensu* Pilát (Welti *et al*., 2012).

Nobles (1958) further considered the significant role of wood rot type caused by the fungi as a distinguishing feature between the polypores. The white rot *Trametes* group was delineated from *Daedalea*, a brown rot fungus. In 1967, David argued that the heterocytic nuclear

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behavior with bipolar mating system separates *Funalia* and *Cerrena* from *Trametes* and *Coriolopsis* (Welti *et al*., 2012). In 1989, Corner observed many tropical species whose intermediate characters could change subject to basidiocarp conditions. The white flesh of *Trametes pubescens* would become brown due to age and mistakenly identified as *Coriolopsis polyzona* which has a brown context. As a result, Corner reported that pigment and rot type alone are not sufficient characters for species delimitation (Justo and Hibbett, 2011; Ko and Jung, 1999). Ryvarden's classification in 1991, corresponded with the widely accepted Friesian description of pileate basidiocarps, poroid hymenophore, trimitic hyphal system, smooth thin-walled basidiospores and white rot (Ko and Jung, 1999). Ryvarden also included all the genera synonymized by Corner in 1989, but excluded brown rot causing genera, such as *Daedalea* and *Fomitopsis* (Justo and Hibbett, 2011; Welti *et al*., 2012).

In 1995, Hibbet and Donoghue used the mitochondrial SSU rDNA to study the phylogeny of *Trametes* and related genera. It was concluded that trimitism in white rots was a common feature for all genera in the *Trametes* clade (Welti *et al*., 2012).

Ko (2000) used SSU mtDNA and ITS sequences to divide the Polyporaceae into two subgroups, where subgroup A contained: *Cryptoporus*, *Daedaleopsis*, *Datronia*, *Funalia*, '*Coriolopsis*' *gallica*, '*Tramella*' *trogii*, *Ganoderma*, *Lentinus*, *Microporus* and *Polyporus*. Subgroup B only had *Coriolopsis polyzona*, *Lenzites*, *Pycnoporus* and *Trametes*. Context pigmentation was also considered to be a distinctive feature for the identification of *Coriolopsis* Murril, which is now *Coriolopsis polyzona*. In 1881, *Pycnoporus* P. Karsten was created to distinguish trametoid specimens which had a brown or red (cinnabarin) colour (Ko and Jung, 1999).

These different concepts on the generic limits of *Trametes* have led to confusion and unresolved species delimitation in the genus (Carlson *et al*., 2014; Olusegun, 2014). It is not clear whether closely related genera, such as *Coriolopsis*, *Coriolus*, *Lenzites* and *Pycnoporus* in subgroup A, should be recognized as independent monophyletic genera or if they should be included in an enlarged *Trametes* genus (Welti *et al*., 2012).

Furthermore, during the past decade, researchers in Namibia developed an interest in studying the indigenous mushrooms of Namibia. Studies by Kadhila-Muandingi and Chimwamurombe (2012) and Ekandjo and Chimwamurombe (2012) focused mostly on medicinal mushrooms, specifically *Ganoderma* species. The problem remains that most Basidiomycetes in Namibia still need to be explored in order to document and preserve the Namibian mushrooms biota and biodiversity (Chang and Mshigeni, 2004).

Lastly, the incorrect taxonomy of many medicinal mushrooms jeopardizes the validity of current and future investigations of these mushrooms and their derivatives (Wasser, 2011; Zmitrovich *et al*., 2012). The use of general names like Turkey tail (*Trametes versicolor*) makes room for mistaken identity of specific species and type material (Wasser, 2011; Wasser, 2014). Therefore, there is a need for consistency in the identification of medicinal mushrooms, like *Trametes* species, to ensure that future investigations of their medicinal properties, composition and effectiveness are done on the right species. This study characterizes indigenous *Trametes* species from Northern Namibia in order to generate information on its genetic diversity. This study endeavors to confirm the identity of *Trametes* representatives from Northern Namibia using ITS region. The present work also attempts to reconstruct the phylogeny of indigenous Namibian *Trametes* using sequences from GenBank Database.

2. Materials and Methods

2.1. Material Studied

Dry mushrooms were collected from dead wood in three regions, namely Ohangwena, Omusati, and Oshana regions in Northern Namibia during late March and early April 2014. The samples were identified with pictures from Van der Westhuizen and Eicker (1994). The samples were recorded and kept in khaki paper bags labeled with name of village, region and host substrate type.

The collected mushrooms were dried in a cool shade for 6 hours and kept in a cool dry place. All visible sand and wood particles were removed before grinding the mushrooms to powder using sterilized mortar and pestles. For this study, samples C1-C4, C21, D1-D7, D9, D11, D13, I2-I4, J1-J4, J6-J9 and K3-K6 were collected from Ohalushu in Ohangwena region, while E1-E5 where collected from Okalumbi in Omusati region and M6 and M7 were collected from Omakango in Ohangwena region.

2.2. DNA Extraction

A Qiagen DNeasy® Plant Mini Kit (Hilden, Germany) and protocol was used to extract fungal genomic DNA from indigenous Namibian *Trametes* species and according to manufacturer's instruction. An adjustment was made to the protocol by reducing Buffer AE from 100 µL to 50 µL.

2.3. PCR and Sequencing

PCR amplification was performed in a 25 µl reaction consisting of 12.5 µL DreamTaq Green PCR Master Mix (2X), 10.5 µL nuclease free water, 1 µL ITS1-F primer (CTTGGTCATTTAGAGGAAGTA), 1 µL ITS2 primer (GCTGCGTTCTTCATCGATGC) and 1 µL DNA. PCR conditions were as follows: Pre-denaturation at 95°C for 4 mins, denaturation at 95°C for 30 s, annealing at 55°C for 1min and elongation at 72°C for 2 mins for 35 cycles. This was followed by a final extension of 72°C for 7 mins. PCR products were viewed using gel electrophoresis. Gel electrophoresis was performed in 0.5 % Tris-Borate EDTA (TBE) buffer. The DNA gel was prepared by dissolving 1 g agarose gel in 100 mL TBE buffer (1 %) and completely dissolving it by heating in the microwave. After slight cooling, 2.5 µL ethidium bromide was added to the gel before casting in a tray. Gel electrophoresis was run at 110 V for 60 minutes after which the gel was visualized under Ultra-Violet (UV) light. Sequencing of PCR product was performed in both directions using ITS1 and ITS2 primers at Inqaba Biotechnical Industries (Pty) Ltd in South Africa. 88 pairs of sequences of 200-250 base pairs were produced.

2.4. Alignment of Sequences

Sequences were analyzed and predicted by utilizing Chromas Lite201 version 2.1.1 (Queensland, Australia). A Local Alignment of the ITS1 and ITS2 sequences was performed in Bioedit to create contig sequences. A BLAST search was performed on the resulting 44 contig sequences using the NCBI GenBank database. The unpublished sequences as well as the sequences obtained from GenBank were aligned with Bioedit and Clustal W.

2.5. Phylogenetic Analysis

The Maximum Likelihood (ML) analysis was performed in MEGA version 6 (Tempe, USA). The phylogenetic tree was reconstructed using the Neighbour Joining method and reliability for internal branch assessment. It was done using the ML bootstrapping method with 500 ML bootstrap replicates.

3. Results

The mushrooms collected presented a morphology characteristic of *Trametes* species, although a high variation was observed in size and color of fruit bodies, size of pores, concentric zones and rigidity of mushroom upon breaking or tearing. Based on these morphological differences, at least four *Trametes* species were identified (Figure 1). Specimen A has a grey basidiocarp with 73 mm diameter with 1 to 2 mm wide pores underneath. Specimen B has a thin and dark brown basidiocarp 70 mm in diameter with black prickly 'hairs' on the top surface and 1mm sized pores on the hymenium. Specimen C was the smallest of the four types observed with 25 mm diameter basidiocarp and 1 mm sized pores. The basidiocarp was covered with distinct zones with different shades of grey, white and black. Specimen D basidiocarp was 45 to 75 mm in diameter with zones that have shades of tan, grey, black and white colour. The hymenium was covered with many small white pores ranging from 2-3 pores/millimeter. The mushrooms lacked a distinct pileus as they were attached directly to their host.

The ITS region of nuclear rDNA from 40 *Trametes* species from Northern Namibia was used for molecular identification. The sequences obtained were aligned with sequences from GenBank. Alignment with GenBank sequences revealed a variety of identities, with most sample species scoring 95-99 % similarity to the *Trametes* and *Coriolopsis* genera. The rest of samples showed similarity scores of 92-94 % to genera, such as *Hexagonia*, *Truncospora* and *Fomes*. For example, samples C1- C4 and C21 showed 99 % similarity to *Coriolopsis caperata* and D1-D7, D9, D11 and D13 are 99 % identical with *Trametes polyzona*. Samples E1, E3 and E4 showed 93 % similarity with *Truncospora macrospora*. Specimens F1, I2-I4, K3-K6 and M6 are 93-99 % identical with *Trametes* species, while E2, G6, J2, J6 and J7 resemble *Hexagonia* species with 92-94 % identity (Table 1).

The phylogenetic tree generated from the unpublished sequences and sequences from GenBank showed some variations. The phylogenetic tree has 8 major clades in total. Clade 1 contains specimens D1-D9, D11 and D13, and specimens F1, I2-I4 and K3-K6 as well as GenBank sequences *Trametes polyzona* (JN164979.1), *Coriolopsis polyzona* (FJ627248.1), *Trametes gibbosa* (FJ481048.1), *T. villosa* (KF573031.1), *T. hirsuta* (GU062274.1), *T. maxima* (JN164918.1), T. *cinnabarina* (AB735965.1), *Pycnoporus sanguineus* (AJ537499.1), *Trametes cubensis* (KJ654513.1), *T. orientalis* (AB735966.1), *T. elegans* (EU661879.1), *T. ljubarskii* (GU731579.1) and *T. marianna* (KC848334.1). Clade 2 contains specimens E1, E3 and E4 alongside *Truncospora macrospora* (JX941573.1). Clade 3 contains *Hexagonia tenuis* (KC414233.1), *Daedaleopsis* sp. (KF541330.1) and *Fomes fomentarius* (EF155494.1). Clade 4 contains specimens G6 and G9, while clade 5 contains specimens C1-C4, C21, M6, M7 and GenBank sequences *Coriolopsis caperata* (AB158316.1), *C. trogii* (KJ093492.1), *Funalia trogii* (EU273516.1), *Coriolopsis gallica* (JN165013.1), *Trametes suaveolens* (FJ478094.1) and *T. trogii* (HM989941.1). Clade 6 has *Hexagonia hirta* (KC867359.1), *C. aspera* (KP013018.1) and *H. apiaria* (KC867362.1). Clade 7 has specimens E2, E5, J1 and J3-J9, while Clade 8 only contains specimen J2. The specimens collected from Northern Namibia were distributed in 6 clades mainly alongside *Trametes* species *Truncospora* and more distantly *Coriolopsis* species.

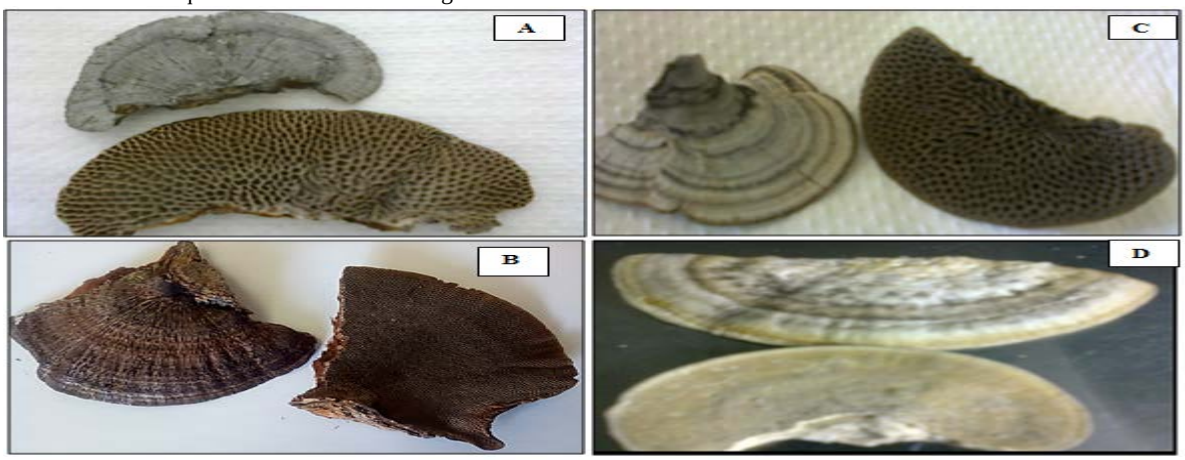

Figure 1. *Trametes* samples collected from Northern Namibia. The mushrooms collected had four distinct morphologies. Mushroom A had a grey basidiocarp with daedalean like pores. Mushroom B had black hair like structures with fine regular pores. Mushrooms C and D had distinct zones on the basidiocarp with different shades of tan, grey, white and black.

Table 1. Molecular identification of *Trametes* species from Northern Namibia based on ITS region

Sample	Organism name	Accession Number	Identity scores (%)
C1	*Coriolopsis caperata*	KF564288.1	99
C2	*Coriolopsis caperata*	KF564288.1	99
C3	*Coriolopsis caperata*	HQ323692.1	99
C21	*Coriolopsis caperata*	HQ323692.1	99
D1	*Trametes polyzona*	JN164979.1	99
D2	*Trametes polyzona*	JN164979.1	99
D3	*Trametes polyzona*	JN164979.1	99
D4	*Trametes polyzona*	KJ654516.1	99
D5	*Trametes polyzona*	JN164977.1	98
D6	*Trametes polyzona*	JN164980.1	99
D7	*Trametes polyzona*	JN164980.1	99
D9	*Trametes polyzona*	JN164978.1	99
D11	*Trametes polyzona*	KP013053.1	99
D13	*Trametes polyzona*	JX941573.1	99
I3	*Trametes marianna*	JQ806418.1	99
K3	*Trametes* sp	KP013021.1	99
M7	*Coriolopsis caperata*	AB158316.1	99
C4	*Coriolopsis caperata*	GQ372861.1	98
D5	*Trametes polyzona*	JN164977.1	98
M6	*Trametes trogii*	HM989941.1	98
I4	*Trametes ljubarski*	HM136871.1	97
K4	*Trametes ljubarski*	JQ806418.1	96
F1	*Trametes villosa*	KC414233.1	95
I2	*Trametes villosa*	KC848334.1	95
E2	*Hexagonia apiaria*	JX941573.1	94
J1	*Fomes* sp.	KC867359.1	94
J3	*Fomes* sp.	KF541332.1	94
J4	*Fomes* sp.	KC867362.1	94
J6	*Hexagonia apiaria*	KC867359.1	94
J8	*Fomes* sp.	KF541332.1	94
J9	*Fomes* sp.	KF541332.1	94
K6	*Trametes villosa*	JN164970.1	94
E1	*Truncospora macrospora*	KC867362.1	93
E3	*Truncospora macrospora*	JX941573.1	93
E4	*Truncospora macrospora*	HM136871.1	93
E5	*Fomes* sp.	KF573031.1	93
J2	*Hexagonia hirta*	HM136871.1	93
J7	*Hexagonia hirta*	KF541332.1	93
K5	*Trametes hirsuta*	JF439511.1	93
G6	*Hexagonia tenuis*	JN164995.1	92
G9	*Coriolopsis trogii*	JN164970.1	92

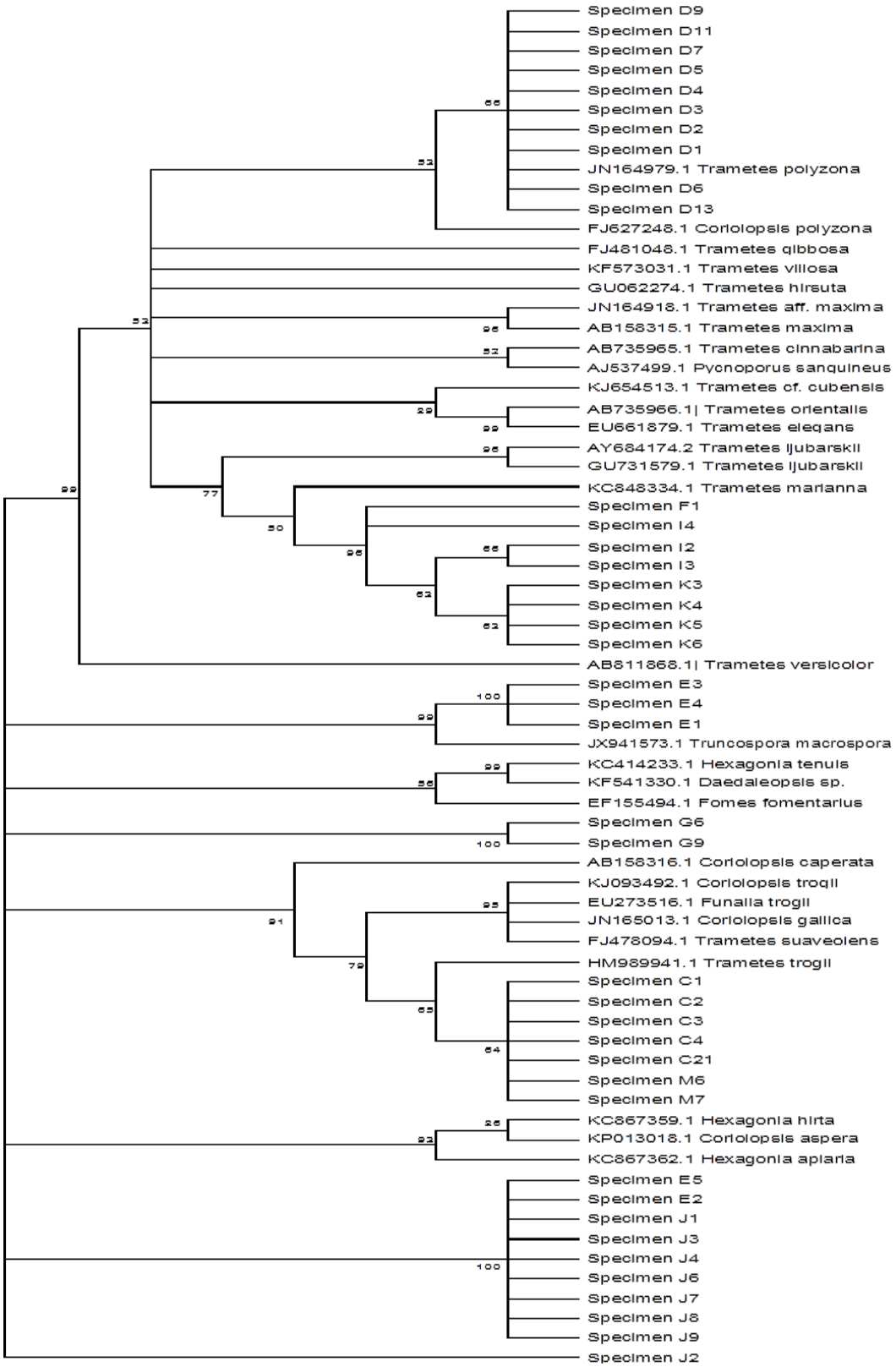

Figure 2. Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbour-Joining method [1]. The optimal tree with the sum of branch length = 1.40518603 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches [2]. The evolutionary distances were computed using the p-distance method [3] and are in the units of the number of base differences per site. The analysis involved 69 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 162 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [4].

4. Discussion

Dry *Trametes* specimens were collected from dead wood in three regions of Northern Namibia. The samples were identified positively with pictures from Van der Westhuizen and Eicker (1994). The fungi collected presented a morphology characteristic of *Trametes* species, although a high variation was observed in size and color of fruit bodies, size of pores, concentric zones and rigidity of the context upon breaking or tearing. Based on these morphological differences, at least four *Trametes* species were identified (Figure 1). Although these polypores were positively identified as *Trametes* species using morphologic features, molecular results show that not all the samples collected were indeed *Trametes* species. This proves that, although the traditional taxonomy of *Trametes* species was based on morphological features, it is not always reliable because these features are affected by nutrient status and growth conditions. Some species are so similar in their morphology that it is difficult to delineate them based on morphology alone (Gilbertson and Ryvarden 1987; Yang *et al*., 2010).

Maximum Likelihood and Neighbour Joining analysis was used to reconstruct a phylogenetic tree of *Trametes* species from Northern Namibia. The resulting phylogenetic tree showed 8 major clades in total. The placement of unpublished sequences into these different clades indicates that these sequences are not from the same ancestral origin as the sequences in GenBank (Olusegun, 2014). Sequences placed in the same clade share a common ancestor from whom they have inherited a set of unique characters (Baldauf, 2003).

It is interesting to observe that specimen D1-D9, D11 and D13 and specimens F1, I2-I4 and K3-K6 were all collected from Ohalushu village in Ohangwena region. However, specimens E2, E5, J1 and J3-J9 were also collected from Ohalushu village in Ohangwena region but they formed a separate clade. According to Olusegun (2014), these differences might occur because of geographical and environmental factors. Geographic factors are responsible for fungal diversity at a regional level in a radius of 1000-4000 km, while environmental factors may cause diversity at a local level within a radius of less than 1000 km.

In the phylogenetic tree obtained in the present study, specimens D1-D9, D11 and D13 and specimens F1, I2-I4 and K3-K6 were grouped in the trametoid clade together with *Trametes* species, such as *T. polyzona*, *T. gibbosa*, *T. villosa*, *T. hirsuta*, *T. maxima*, *T. cinnabarina*, *T. cubensis*, *T. orientalis*, *T. elegans* and *T. marianna*. These results are similar to those reported by Welti *et al*. (2012) and Carlson *et al*. (2014). Welti *et al*. (2012) reconstructed the phylogeny of the *Trametes* group using Bayesian analysis of ITS1-5.8S-ITS2 region and RPB2 protein coding gene to confirm the close relationship between the genera *Trametes*, *Coriolopsis* (*polyzona*) and *Pycnoporus*. One of the most recent and comprehensive works on *Trametes* is phylogeny using molecular data from the ribosomal Large Subunit (LSU) rRNA and ITS region as well as the RPB1, RPB2 and TEF1-alpha protein coding genes. Similar to the results obtained, the five-marker molecular analysis strongly supported a Trametoid clade which includes most *Trametes* species (*T. suaveolens*, *T. versicolor*, *T. maxima*, *T. cubensis*) and *Lenzites*, *Pycnoporus* and *Coriolopsis polyzona* species. Furthermore, the position of *T. trogii* (*Trametes trogii*) was confirmed to be outside the Trametoid clade and more closely related to *C. gallica* (Tomšovsky *et al*., 2006; Justo and Hibbett, 2011). The genus *Coriolopsis* is currently defined as polyphyletic with type species in the trametoid clade and two additional lineages in the core Polyporoid clade (Carlson *et al*., 2014). This explains why *Trametes trogii* was placed in a clade much further from other *Trametes* species but closer to *Coriolopsis gallica* and *C. trogii* (Figure 2). Except for the red color of *Pycnoporus* basidiocarp, it is morphologically similar to *Trametes*. Other biochemical characters between the two genera do not differ and molecular analysis of the ribosomal DNA groups the two genera in one clade (Tomšovsky *et al*., 2006), just as confirmed in the present study by grouping *Pycnoporus sanguineus* in the trametoid clade with *Trametes* species (Figure 2).

The authors support the decision to keep a single generic name of *Trametes* for the trametoid clade because, according to Justo and Hibbett (2011), which allows the preservation of the morphological concept of *Trametes*, the classification of additional species which may not yet be sampled or analysed and the classification of *Trametes* species using morphological features alone.

Any other decision to divide the trametoid clade is deemed extremely difficult or even impossible (Justo and Hibbett, 2011). Therefore, the question whether closely related genera, such as *Coriolopsis*, *Coriolus*, *Lenzites* and *Pycnoporus*, should be recognized as independent monophyletic genera, or whether they should be included in an enlarged *Trametes* genus (Welti *et al*., 2012) has been answered, albeit temporarily.

5. Conclusion

In conclusion, the present study is able to confirm for the first time, the identity of *Trametes* mushrooms from Northern Namibia using ITS region and to reconstruct the phylogeny of these indigenous *Trametes* using sequences from GenBank Database. From the results above, the authors identify specimens C1-C4, C21, D1-D7, D9, D11, D13, F1, I2-I4, K3-K6 as well as M6 and M7 to be *Trametes* species. Specimens E1, E3 and E4 are identified as *Pycnoporus* species.

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Determination of Water Quality and Detection of Extended Spectrum Beta-Lactamase Producing Gram-Negative Bacteria in Selected Rivers Located in Ibadan, Nigeria

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Abstract

The present study is designed to determine the occurrence of Extended Spectrum β-Lactamase (ESBL)-producing Gram-negative bacteria in water samples from selected rivers in Ibadan, Nigeria. Water samples were collected from three rivers and physicochemical analysis carried out. Isolated Gram-negative bacteria were identified using conventional biochemical method. Antimicrobial susceptibility test of the isolates was by disc diffusion technique while ESBL detection was by double disc synergy method. Physicochemical analysis showed that turbidity ranged between 17.7- 164.7NTU; total suspended solids between 0.45 -1.3mg/L; total dissolved solids between 246 - 735mg/L. The conductivity, Biological Oxygen Demand and Chemical Oxygen Demand were between 367-1061mg/L, 267.8-385.2mg/L, and 395.8-563.3mg/L, respectively; oil and grease was between 272.8 - 2067.9mg/L. A total of 207 β-lactam resistant Gram-negative bacteria were isolated, out of which 37 (17.9%) produced ESBL; 9(24.3%) were from Yemetu, 14 (37.8%) from Kudeti and 14 (37.8%) from Alaro rivers. Among the ESBL-producers, 35.1% were *Klebsiella pneumonia*e, while 91.9%, 73.0% and 64.9% of ESBL isolates showed resistance to Cefotaxime, Cefepime and Aztreonam, respectively; while resistance to Ciprofloxacin and Gentamicin was 8.1% and 18.9% respectively. The present study reveals the need for continuous pollution monitoring and proper management program of the rivers to prevent indiscriminate discharge of wastes harboring ESBL-producing bacteria into water bodies.

Keywords: Rivers, Extended spectrum β-lactamase, Gram-negative bacteria, Antibiotics, Resistance.

1. Introduction

One of the factors responsible for the global emergence of antibiotic resistance among enteric bacteria during the recent decades is the misuse of antibiotics (Chitanand *et al*., 2010). More so, the occurrence of Extended-Spectrum β-Lactamase (ESBL) production has been due to the use of cephalosporin in both clinical practices and animal husbandry (Canton *et al*., 2008; Castanheira *et al*., 2008). These strains of bacteria produce beta-lactamase enzymes that cleave to the beta-lactam ring thereby disrupting the action of antimicrobials leading to the development of resistance to most beta-lactam antibiotics including the first, second, third and fourth generation cephalosporins. The increase in the number of ESBL producing Gram-negative bacteria is a threat to healthcare because infections caused by these strains of organisms are difficult to treat, leads to increased medical costs and limited therapeutic options (Harris *et al*., 2015; Upadhyay and Joshi, 2015). Production of ESBL is common with many species of Gram-negative bacteria but is mainly detected in the family *Enterobacteriaceae* (Falagas and Karageorgopoulos, 2009).

One of the main sources of transmission of pathogenic organisms including antibiotic resistant bacteria is water. Moreover, multiple antibiotic resistant bacteria have been isolated from different water sources, such as rivers, groundwater, drinking water and recreation water (Marti *et al*., 2013; Ramirez-Castillo *et al*., 2013). The possibilities of human exposure to water bodies contaminated with ESBL–producing bacteria when used for recreation, irrigation, drinking and other domestic purposes is very high (Zhang *et al*., 2015). In Nigeria, most studies on ESBL producing bacteria focused on isolates from clinical origin particularly *E. coli*. Meanwhile, there is dearth of information on isolates from environmental samples. The present study is, therefore, aimed at evaluating the water quality of selected rivers in Ibadan as well as determining the occurrence of ESBL production in Gram negative bacteria isolated from selected rivers within Ibadan metropolis. These rivers are used for domestic purposes, such as washing and bathing.

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2. Materials and Methods

2.1. Description of the Study Area

The sites of the present study include: Alaro, Yemetu and Kudeti Rivers. Alaro River (with geographical position of 0812901N, 00593332E) is located in an industrialized environment, in Ibadan South West Local Government Area of Oyo State. There is discharge of effluents from industries into the river. Yemetu River (with geographical position of 0723318N, 00354303E) is along Oje Street behind Adeoyo hospital, in Ibadan North Local Government Area and Kudeti River (with geographical position of 0721909N, 00353845E) is along Idi-arere Kudeti road, in Ibadan South East Local Government Area of Oyo state. Yemetu and Kudeti rivers are located within residential areas, where open defecation is highly practised and domestic wastes are dumped in the rivers and along the river banks.

2.2. Sample Collection

Water samples were randomly collected between the months of May and July, 2015 into sterile containers and transported in an ice pack to the Laboratory, Department of Microbiology, University of Ibadan, for microbiological analysis. The geographical position of the sampling sites was determined and recorded.

2.3. Physico-Chemical Analysis of the Water Samples

The physico-chemical analysis of the water samples was carried out using standard analytical methods. Parameters, such as temperature and pH, were determined on the field using thermometer and pH meters respectively while turbidity was determined using turbidity meter. Total dissolved solid, electrical conductivity and dissolved oxygen were determined using an Extech digital meter (Extech Instruments, USA). Chemical Oxygen Demand (COD), suspended solids, acidity, alkalinity, chloride, Biochemical Oxygen Demand (BOD); and oil and grease were determined using standard methods (APHA, 2005).

2.4. Isolation of Beta-Lactams Resistant Gram-Negative Bacteria

Isolation of beta-lactams resistant Gram-negative bacteria was carried out as described by Lu *et al*. (2010) using MacConkey agar supplemented with filter-sterilised solution of ampicillin. Pour plate method was used for the inoculation as the supplemented media were dispensed into Petri dishes containing 1ml of the appropriate dilution of the samples. The plates were incubated at 37^{o}C for 18-24 hours. Distinct colonies presumptive of the target organisms were picked and further sub-cultured to obtain pure cultures. The isolates were characterized and identified using conventional biochemical method (Cheesbrough, 2008)

2.5. Antibiotic Susceptibility Tests

Antibiotic susceptibility test was carried out using the disc diffusion technique as described by Bauer *et al*. (1966). The antibiotics discs used were Cefotaxime (CXM, 30mg), Ceftazidime (CAZ, 30mg), Cefepime (FEP, 30mg), Aztreonam (AZ, 30mg), Imipenem (IMP, 10mg), Amoxicillin-Clavulanate (AMX, 30mg), Ciprofloxacin (CIP, 5mg), Gentamicin (CN, 10mg) and Florfenicol (FFL, 30mg) (Oxoid, UK). The susceptibility test was carried out using an overnight culture suspension of the test isolates adjusted to 0.5 McFarland Standard. The culture suspensions were inoculated unto the surface of Mueller Hinton agar plates with sterile swab sticks. The antibiotic discs were carefully placed on the inoculated plates with the aid of sterile forceps and incubated at 35±2˚C for 18-24 hours. The zones of inhibition were measured and interpreted based on Clinical and Laboratory Standards Institute (2017).

2.6. Detection of ESBL-Producing Bacteria Using Double Disc Synergy Test (DDST)

All the Ceftazidime (30mg) and Cefotaxime (30mg) resistant isolates were selected for ESBL detection using double disc synergy test previously described by Lu *et al*. (2010). The test was carried out using discs of amoxicillin-clavullanate and discs of ceftazidime (30mg) and cefotaxime (30mg) which were placed around amoxicillin-clavullanate disc at a distance of 15 to 20 mm from each other (center to center). The plates were incubated at 37^{o}C and after 18-24 hours of incubation, the plates were observed. Isolates producing ESBL were those with zones of inhibition around any of the cephalosporin discs with a clear-cut increase towards the amoxicillin-clavullanate disc. ESBL-positive *Klebsiella pneumoniae* ATCC 700603 and ESBL negative *Escherichia coli* ATCC 25922 strains were used as control.

3. Results

The physico-chemical analysis of the water samples from the rivers showed that Kudeti River had the highest temperature values (29^{o}C) while the pH ranged between 9.3 and 9.4. Except for the pH and DO, Yemetu River had the highest values for all the physico-chemical tested parameters (Table 1).

Out of the 207 beta-lactam resistant Gram-negative bacteria isolated from the rivers, 79 (38.2%) were from Alaro river, 62 (30.0%) from Kudeti river and 66 (31.9%) from Yemetu river while *Klebsiella* spp. had the highest occurrence rate (Table 2). The results of the susceptibility profile of the Gram-negative bacteria showed that resistance of bacteria from Yemetu, Kudeti and Alaro Rivers to cefotaxime were 68%, 58%, and 48%, respectively, while to cefepime, it was 55% (Yemetu), 40% (Kudeti) and 41% (Alaro). However, resistance of the bacteria was 3% (Yemetu and Kudeti) and 1% (Alaro) to imipenem (Table 3).

Table 1. Results of the Physico-chemical analysis of the water samples

Physicochemical parameters	Sampling points		
	Alaro	Kudeti	Yemetu
Turbidity (NTU)	17.7	17.7	164.7
Temperature (°C)	26	29	28
pH	9.4	9.4	9.3
Alkalinity (mg/L)	18.7	16.1	24.9
Acidity (mg/L)	2.0	1.6	1.9
Total Solids (mg/L)	246.5	652.6	736.3
Total Suspended Solids (mg/L)	0.5	0.7	1.3
Total Dissolved Solids (mg/L)	246	652	735
Electrical Conductivity (µS/cm)	367	945	1061
Dissolved Oxygen (mg/L)	8.4	8.4	8.4
Biological Oxygen Demand (mg/L)	267.8	296.4	385.2
Chemical Oxygen Demand (mg/L)	395.8	424.5	563.3
Nitrate (mg/L)	33.7	38.2	45.7
Chloride (mg/L)	38.6	27.5	78.2
Oil and grease (mg/L)	272.9	1400.1	2067.9

Key: NTU – Nephelometric Turbidity Unit, µS/cm - micro-Siemens per centimeter, mg/L – Milligram per Litre

Table 2. Occurrence of Beta-Lactam resistant Gram-negative bacteria isolates obtained from the rivers n(%)

	Sampling sites			
Genus	Alaro	Kudeti	Yemetu	Total
Escherichia spp.	8(10%)	19(31%)	5(8%)	32 (15%)
Klebsiella spp.	31(39%)	14(23%)	27(41%)	72 (35%)
Enterobacter spp.	13(17%)	15(24%)	7(10%)	35 (17%)
Pseudomonas spp.	8(10%)	3(4%)	7(10%)	18 (9%)
Salmonella spp.	4(5%)	5(8%)	11(17%)	20 (10%)
Proteus spp.	15(19%)	6(10%)	9(14%)	30 (14%)
Total	79(38.2%)	62(30.0%)	66(31.9%)	207(100%)

Table 3. Susceptibility profile of Gram-negative bacteria isolated from the rivers to selected beta-lactam antibiotics

	Yemetu River, n=66(31.9%)		Kudeti River, n=62(30%)		Alaro River, n=79(38.2)	
Antibiotics	I+R	S	I+R	S	I+R	S
IMP	14(21%)	53(80%)	10(16%)	52(84%)	8(10%)	71(90%)
CXM	55(83%)	11(17%)	48(77%)	14(23%)	59(75%)	20(25%)
CAZ	32(48%)	34(52%)	27(44%)	35(57%)	29(37%)	50(63%)
FEP	38(58%)	29(44%)	26(42%)	36(58%)	33(42%)	46(58%)
AZ	38(58%)	28(42%)	27(44%)	35(56%)	36(46%)	43(54%)
AMX	47(71%)	19(29%)	39(63%)	23(37%)	51(65%)	28(35%)

Out of the beta-lactam resistant Gram-negative bacteria, 37 (17.9%) were positive for ESBL production. These isolates belonged to the following genera: *Pseudomonas*, *Enterobacter*, *Klebsiella*, *Escherichia and Proteus* (Table 4). Exactly 37.8%, 24.3% and 37.8% ESBL producers from the rivers were from Alaro, Yemetu and Kudeti, respectively.

Furthermore, the resistance patterns of the ESBL-producing isolates to combinations of antibiotics showed that there were 19 different antibiotypes. The highest was 5(13.5%) in which three *K. pneumonia* and two *P. Mirabilis* showed resistant to a combination of both CXM and FEP. This was followed by 4(10.8%) antibiotypes that included a combination of CXM, FEP, AZ and FFC with one each of *K. pneumonia, P. mirabilis, E. aerogenes* and *P. putida;* one *P. mirabilis* also showed resistant to a combination of seven antibiotics that included CXM, FEP, AMX, AZ, CIP, FFC and CN (Table 5).

Table 4. Detection of ESBL-producing Isolates

		ESBL detected n (%)			
Isolates	Numbers screened	Alaro	Kudeti	Yemetu	Total ESBL detected n(%)
Escherichia spp.	32	_	2 (6.3)	_	2(6.3)
Klebsiella sp.	72	4(5.6)	5(6.9)	4(5.6)	13(18.1)
Enterobacter sp.	35	4(11.4)	3(8.6%)	1(2.9)	8(22.9)
Pseudomonas sp.	18	3(16.7)	3(16.7)	1(5.6)	7(38.9)
Salmonella sp.	20	_	_	_	-
Proteus sp.	30	3(10.0)	1(3.3)	3(10.0)	7(23.3)
Total	207	14(6.7)	14(6.7)	9(4.3)	37(17.9)

Table 5. Antibiotypes of ESBL-producing Gram–negative bacteria

Antibiotypes	*K. pneumoniae*	*P. fluorescens*	*P. mirabilis*	*E. coli*	*E. aerogenes*	*P. aeruginosa*	*P. putida*	Total n(%)
CXM AZ	2	0	0	0	0	0	0	2(5.4%)
CAZ AMX	1	0	0	0	0	0	0	1(2.7%)
CXM FEP	3	0	2	0	0	0	0	5(13.5%)
FEP AZ	1	0	0	0	0	0	0	1(2.7%)
CXM CAZ	0	0	0	0	1	0	0	1(2.7%)
CXM FFC	0	0	0	0	2	0	0	2(5.4%)
CXM FEP FFC	0	0	1	0	0	0	0	1(2.7%)
CXM FEP AZ	0	0	0	0	1	0	0	1(2.7%)
CXM CAZ AZ	1	0	0	0	1	0	0	2(5.4%)
CXM FEP AZ FFC	1	0	1	0	1	0	1	4(10.8%)
CXM CAZ FEP AZ	1	0	1	0	0	0	0	2(5.4%)
CXM FEP AMX AZ	0	0	0	1	0	0	0	1(2.7%)
CXM FEP AZ CIP FFC	1	0	0	0	0	0	0	1(2.7%)
CXM FEP AMX FFC CN	0	0	0	0	0	1	0	1(2.7%)
CXM CAZ FEP AZ FFC	0	1	0	0	1	0	0	2(5.4%)
CXM FEP AZ FFC CN	0	0	0	0	0	1	0	1(2.7%)
CXM FEP AMX AZ FFC CN	0	2	0	0	0	0	0	2(5.4%)
CXM CAZ FEP AZ FFC CN	0	1	0	0	0	0	0	1(2.7%)
CXM FEP AMX AZ CIP FFC CN	0	0	1	0	0	0	0	1(2.7%)

4. Discussion

The physico-chemical analysis of the water samples that showed pH values within the range 9.3 and 9.4 for the three rivers were within the international permissible limit of 6.5-9.5 (WHO, 2008). Likewise, the temperature of the rivers (26^{o}C-29^{o}C) was within the temperature permissible limit of less than 40^{o}C as recommended by the Federal Ministry of Environment of Nigeria (FMENV, 2001). While the turbidity (17.7 NTU) of the water samples collected from Alaro and Kudeti rivers were above the Standard Organisation of Nigeria (SON) permissible limits of 5.0 NTU (SON, 2007) the turbidity (164.7 NTU) obtained from Yemetu river was geometrically above the limit. The reason for this difference could be as a result of the location of the rivers. Yemetu River, for instance, receives inputs from a tertiary hospital, residents and the domestic wastes which could have led to the high turbidity value. The turbidity of both Alaro and Kudeti rivers were lower but comparably similar to the range of 24 and 28 NTU previously reported from another study conducted on a river in the same city of Ibadan (Adekambi and Falodun, 2015).

The pH range (9.3-9.4) of the rivers in the present study were higher compared to the pH range of 7.04 - 7.11 reported by Adekanmbi and Falodun (2015). The disparity in the pH values may be as a result of less anthropogenic activities impacting on the latter river compared to the rivers in the present study. These high pH values imply that the presence of basic salts (such as sodium and potassium salts) is likely to be prevalent in the river waters (John De Zuane, 1990). However, the pH range obtained in the present study is similar to a previous report from Turkey (Atici *et al*., 2008). The range of chloride quantity in the three rivers (38.6-78.2mg/l) is below the WHO permissible limit of 250mg/l chloride in water samples (WHO, 2008). Although, the range of Nitrate (33.7 - 45.7 mg/l) observed in the present study fell within the acceptable limit allowed (50 mg/l) in river water (WHO, 2008), the health implication associated with elevated concentrations of nitrate greater than 11mg/l in water is blue-baby syndrome (Methemoglobinemia) in children and Insulin-Dependent Diabetes Mellitus (IDDM) in adult when concentration exceeds 25 mg/l (Kostraba *et al*., 1992; Ward *et al*., 2005).

The total suspended solids (0.5-1.3 mg/l) and total dissolved solids (246-735mg/l) obtained from the present study were within the permissible limit of 30mg/l and 2000 mg/l, respectively (FMENV, 2001). However, the total suspended solids in the present study were lower while the total dissolved solids were higher compared to the 200mg/l and 320mg/l, respectively reported from the study carried out on another river (Ona River) in Ibadan, Nigeria (Osibanjo *et al*., 2011). The reason for the disparity could be as a result of an increasing measure of dissolved inorganic salts in Ona River. Moreover, the results of the biological oxygen demand (BOD) of the three rivers (267.8 mg/l-385.2 mg/l) were far above the permissible limit of 50 mg/l set by Federal Ministry of Environment (FMENV, 2001). Furthermore, the values of the Chemical Oxygen Demand (COD) of 563.3mg/l, 395.8 mg/l and 424.5mg/l obtained from Yemetu, Alaro and Kudeti Rivers, respectively, were all above the Federal Ministry of Environment permissible limit of 150 mg/l (FMENV, 2001) for surface waters. In addition, the value of the Dissolved Oxygen (DO) of 8.4 mg/l obtained from each river was above the permissible limit (5.0 mg/l) of the Federal Ministry of Environment (FMENV, 2001). Control

of indiscriminate discharge of wastes into these rivers is, therefore, imperative to forestall further deterioration of the river water.

The results of the oil and grease of the rivers (272.9-2067.86 mg/l) were far above the Federal Ministry of Environment permissible limit of 10mg/l (FMENV, 2001). The reason for this may be due to the urban runoff which conveys great amount of oil and grease from various auto-repair workshops within the vicinity of the sampling areas. For instance, close to Yemetu River bank is a large auto-repair workshop from where oil and grease discharges into the river. The results of the present study also showed that the most polluted of the three rivers was Yemetu River as revealed by the results obtained from the physicochemical analysis. The reason for the high level of pollution of the rivers could hence be largely attributed to anthropogenic activities that impacts on the river such as high practice of open defecation, improper disposal of wastes into the rivers as well as the release of industrial discharge into the Alaro River which is located in an industrialized locale.

Singal *et al*. (2005) and Reich *et al*. (2013) reported an increased prevalence of ESBL-producing *Enterobacteriaceae*. In the present study, 17.9% ESBL-producing Gram-negative bacteria predominantly of the family *Enterobacteriaceae* were detected and are similar to the recently reported 15.2% ESBL-producing bacteria of which, all the isolates belonged to the *Enterobacteriaceae* family from a study conducted on untreated hospital wastewater in the southern part of Nigeria (Egbule, 2016). In a study conducted in China, a higher prevalence of ESBL-producing isolates (69.6%), of the *Enterobacteriaceae*, from water samples collected from urban river, was reported (Lu *et al*., 2010). *Klebsiella pneumonia* having the highest occurrence rate among the ESBL-producing organisms in the present study is of great public health concern because it has been reported that the most common causative agent of nosocomial and community acquired infections are the members of the *Enterobacteriaceae* (Coque *et al*., 2008). The persistent and contagious nature of *Klebsiella* spp. may be as a result of resistance to harsh conditions due to the presence of capsules that gives protection to the cells (Paterson and Bonomo, 2003).

The observation from the present study that none of the ESBL-producing bacteria were resistant to imipenem except one of the *Enterobacter* species is similar and comparable to a previous study conducted in Malaysia in which all the 19 ESBL-producing bacteria from four different rivers were reported to be susceptible to imipenem (Tissera and Lee, 2013). It has been found that ESBL isolates are usually resistant to most β-lactam antibiotics and the implication of this is that few options are left for the treatment of ESBL-associated infections. Antibiotics susceptibility result that showed high resistance of the ESBL-producing isolates to cefepime, a fourth generation cephalosporin, is in agreement with previous reports of increasing emergence of resistance to fourth generation cephalosporins (Naumiuk *et al*., 2001; Grover *et al*., 2006). It was observed in the present study that all ESBL-producing *E. coli*, *P. mirabilis* and *Pseudomonas* spp. were resistant to cefepime, a fourth generation cephalosporin. Meanwhile, resistance to this antibiotic has been previously reported to be linked to the hydrolysis by blaCTX-M gene coded β-lactam enzyme (Paterson and Bonomo, 2003). However, ESBL-producing bacteria that exhibited high resistance to Cefotaxime (91.9%), Cefepime (73.0%), Aztreonam (64.9%) and Ceftazidime (37.8%), in the present study, is not in agreement with the total (100%) resistant to Cefotaxime and Ceftazidime and no resistant to Aztreonam reported from another study on surface water that included samples from various ponds, lakes and river in Dhaka city, Bangladesh (Nasreen *et al*., 2015). The reason for the differences may be as a result of the studied samples.

Gundogan and Yakar (2007) had previously reported a low resistance of the ESBL-producing isolates to ciprofloxacin and gentamicin which is also similar to the results of the present study. This, therefore, corroborates the assertion that ciprofloxacin and gentamicin can be effective in the treatment of infections caused by ESBL-producing bacteria. The resistance patterns of the ESBL producing isolates against the antibiotics tested in the present study showed that the majority were multidrug resistant (resistant to three or more classes of antibiotics); such multiple antibiotic resistance has been reported to be the outcome of the acquisition of resistance genes through genetic exchange and mutation as well as physiological mechanisms, such as the possession of specific proteins and efflux pump.

5. Conclusion

In conclusion, the present study shows that the studied rivers were not only contaminated with chemical impurities, they also contain ESBL-producing bacteria some of which harbor multidrug resistance features. These organisms could serve as potential risks of infection outbreaks on exposure; hence the need to put in place appropriate measures to prevent contamination of local surface waters. Furthermore, imipenem, ciprofloxacin and gentamicin showed good effect on the ESBL-producing isolates in the present study.

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Jordan Journal of Biological Sciences **Short Communication**

Arsenic Exposure from Bean Seeds Consumed in Owerri Municipal, Imo State, Nigeria: Can Insect Pest Detoxify the Metalloid during Infestation?

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Abstract

The common bean, *Phaseolus vulgaris* (L.) is one of the most important sources of protein worldwide. Bean consumption has increased in Nigeria particularly due to exorbitant cost of rice in the country. The present study determines the concentrations of total arsenic in samples of bean seeds obtained from the streets of Owerri city, Imo State, Nigeria using standard protocols. Bean seeds, sold in enclosed and open containers, are considered. The estimated dietary intake of total arsenic is compared with the arsenic benchmark dose lower confidence limit. Concentrations of total arsenic differed significantly across sampling locations. The pooled mean total arsenic was 0.47 µg/g while the estimated daily mean dietary intake of total arsenic was 32.9 µg/g for a typical adult in the place. This value is below the permissible limit of 18-480 µg/day for an adult at the average weight of 60 kg established by the European Food Safety Authority. This suggests that bean seeds, sold and consumed in Owerri Municipal, do not have inherent and acquired arsenic-associated health risks. Bean seeds, exposed to the bean bruchid, *Acanthoscelides obtectus* Say (Col., Bruchidae) for 60 days in the laboratory at a temperature of 29.7^{o}C and RH of 71.7%, revealed that the insect pest showed a potential for detoxification using feeding as a mechanism. Evaluation of the potentials of *A. obtectus* in the detoxification of bean seeds contaminated with arsenic under long storage duration is suggested.

Keywords: *Phaseolus vulgaris*, Safety evaluation, Arsenic, Health risks, *Acanthoscelides obtectus*, Detoxification.

1. Introduction

The common bean, *Phaseolus vulgaris* (L.), is commonly consumed by humans worldwide and is one of the main sources of protein in developing countries (Lopes *et al*., 2016). The common bean is the most important pulse in human diet (Larochelle *et al.*, 2016), and pulses are critical in achieving sustainable goals on food security, health and poverty alleviation. The exorbitant price of rice in Nigerian markets (167 % increases in cost over a period of two years) has led to an increase in bean consumption. Moreover, the global public advocacy on the health benefits of plant-based proteins favor bean consumption (Larochelle *et al*., 2016). It is simple to predict that the increased bean consumption will cause an upsurge in its production. Increases in bean consumption and production justify the need for safety assessment and risk management, ensuring that levels of environmental contaminants are below the maximum limits established by regulations or concerned authorities.

One of the contaminants of potential concern is arsenic, which can be released from anthropogenic sources, such as manufacturing of pesticides, smelting of metals, the use of arsenic-containing wood preservatives and production of pharmaceuticals (Adeyemi *et al*., 2017). Arsenic can also be released during glass production. Mining, metal smelting and burning of fossil fuels are the major industrial processes that contribute to arsenic contamination of the environment (soil, air and water). Apart from the man-made sources of environmental arsenic, there are natural sources. The earth's crust is an abundant natural source of arsenic and it occurs in at least 200 minerals, arsenopyrite, being the most common (FHE, 2017). According to Adeyemi *et al*. (2017), arsenic is the 20th most abundant element in the earth crust, its mean concentration in igneous and sedimentary rocks 2 mg/kg. Natural sources of arsenic may be found in inorganic and organic forms (FHE, 2017). The inorganic type is of geological origin and is found in ground water. Bangladesh, India and Taiwan have high concentrations. Organic arsenic is mainly found in sea-dwelling organisms; terrestrial species are not totally excluded. The source notwithstanding, arsenic is very toxic to human life (Adeyemi *et al*., 2017). It can be genotoxic and cytotoxic since it can induce micronuclei deoxyribonucleic acid strand breaks, chromosomes aberration and cell membrane distortion (Faita *et al.*, 2013). Indeed, arsenic contamination is of great concern to human and environmental health. If ingested, symptoms of arsenic poisoning include diarrhea, vomiting, vomiting blood, blood in the urine, cramping muscles, stomach pain and

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convulsions. Most times, the human skin, kidneys and liver are affected (Mazumder and Dasgupta, 2011). Considering the increase in human activities that potentially release arsenic to the environment, there is a need to carryout safety assessment for this toxic metalloid element. Most previous studies on dietary intake of arsenic used total arsenic in making inference (Roychowdhury *et al*., 2003; Marti-Cid *et al*., 2008). This has been confirmed in a recent study by Adeyemi *et al*. (2017). Therefore, the first two objectives of the present study are to determine the total arsenic levels in bean from different locations in Owerri city, Imo State, Nigeria and to estimate its daily dietary intake through bean consumption.

In literature, it has been recorded that postharvest insects cause nutrient loss during feeding (Lale, 2002; Nwosu, 2016). Unfortunately, the potential uses of insects in the detoxification of metalloids, such as arsenic, are still largely untested. Given that insect-association with stored beans is inevitable, it is worth investigating if insects can help to detoxify the toxic elements contaminating bean seeds. Thus, as a third objective, the present study sought to examine the potential of insects using the bean bruchid, *Acanthoscelides obtectus* Say (Col., Bruchidae) in the detoxification of arsenic. *A. obtectus* is one of the most important storage pests in Africa (Giga *et al*., 1992). Dry weight losses during storage of bean seeds are between 10-40% but where management is poor losses can be above 50% (Paul, 2007). This scientific communication on the detoxification of arsenic by *A. obtectus* could provide new insights in the use of insects in detoxification of toxic elements.

2. Materials and Methods

2.1. Sample Collection

Two bean samples were taken from markets/stores in each of the five prominent streets (n = 10) in Owerri Municipal, Imo State (5^o 28' 35.6" (5.4766^o) N; 7^o 1' 0.6" (7.0168^o) E. The streets sampled were Douglas, Whetheral, Rotibi, Okigwe Road and World Bank area. These streets (Douglas and Whetheral in particular) are exclusive points where commercial activities hold due to concentration of business outfits, such as markets, banks, mini shops and offices. During sample collection, bean sold in enclosed (sample coded a) and open containers (sample coded b) were considered. Samples were transferred immediately into clean plastic cork-containers and labeled also immediately according to point of collection. Sample digestion and determination of total arsenic were achieved the next day.

2.2. Determination of Total Arsenic

Total arsenic in each bean sample was determined using the method of Nardi *et al*. (2009). We added 1 mL 20 % (v/v) HNO_3 and 2 mL 30 % (v/v) H_2O_2 to 50 mg of the milled (electric mill) and sieved (0.4 mm screen) bean samples. The mixtures were digested for 40 min in a microwave oven. The digests were allowed to cool and their volumes brought up to 15 mL with distilled water. Arsenic was determined using an atomic absorption spectrometer (Varian Spectr AA 220FS). The accuracy and precision of the results were verified using four replications from each of the ten samples. Quality control using certified reference material (containing 1000 µg/ mL arsenic) was achieved through arsenic re-run as guided by Adeyemi *et al*. (2017).

2.3. Estimation of Daily Intake of Arsenic through Bean Consumption

The daily intake of arsenic was estimated using the formula explained in Adeyemi *et al*. (2017). The Estimated Daily Intake (EDI) in mg/day/individual or µg/day/individual = Cm X Mg, where Cm is the average concentration of element in a cereal sample (ng/g or µg/g) and Mg is the mass of bean consumed daily.

The quantity of bean consumed daily by a typical Nigerian adult (average weight 60 kg) was assumed to be 70 g which is the quantity of rice consumed daily by a typical Nigerian adult (Africa Rice Center, 2005).

2.4. Safety Evaluation

The estimated daily intake of arsenic was compared to the benchmark dose lower confidence limit value for arsenic. The standard is 0.3-8.0 µg arsenic per kg body weight/day. This is equivalent to 18-480 µg/day for an adult at the average weight of 60 kg (EFSA, 2009; Adeyemi *et al*., 2017). If the observed mean value falls within the range, it implied there is no arsenic health risks associated with bean consumption. On the contrary, if the observed mean value exceeds the range, it implied there is arsenic health risks associated with bean consumption.

2.5. Insect Culture

The bean bruchid, *A. obtectus* adults were isolated from naturally infested bean in Port Harcourt, Rivers State, Nigeria and authenticated using morphological characteristics (Lale, 2002). A total of 400 unsexed adults were identified and used to start the culture. Two plastic containers (height 28 cm; diameter 18 cm) with 300 g of landrace bean seeds were used and each received 200 beetles. Each container was covered immediately with muslin net (held in place with cut container-lid) for protection and ventilation. The parent beetles were allowed to feed on the culture seeds and lay eggs for a period of 8 days and thereafter, they were sieved out of the containers. The two set-ups were kept for 4 weeks for new adults to emerge under ambient temperature and RH of 29.7^oC and 71.7%, respectively. Two-week old emerged adults were used for the detoxification assay.

2.6. Insect Detoxification Assay

The experiments were conducted in the Crop Protection Laboratory of the Department of Crop and Soil Science, University of Port Harcourt, Rivers State, Nigeria. The mean ambient temperature and relative humidity of the laboratory were 29.7^oC and 71.7%, respectively. Fifty grammes of each of the 10 bean samples were weighed into ten different containers. Ten unsexed *A. obtectus* adults were introduced into each container and covered immediately with muslin net (held in place with cut container-lid) for protection and ventilation. These were arranged in a completely randomized design on laboratory bench. A control (without insects) of four replicates was designated. The bruchids were allowed to feed on the samples for 60 days. Post-infestation arsenic was determined using the above methods. Pre- and post-infestation arsenic levels were compared statistically to

ascertain the occurrence and extent of detoxification caused by the insect. Comparable weights of infested and un-infested bean samples were used to accommodate the weight loss in infested samples. The experiments were repeated four times.

2.7. Statistical Analysis

The assumption for homogeneity of group variance was tested prior to analysis of variance using Levene's test (Somta *et al*., 2008) for equality of variances. The outcome of Levene's test eliminated the need for data transformation. Data on total arsenic levels in the bean samples were statistically analyzed using one-way analysis of variance. Upon significance of the F-test, means were separated (at posthoc test) using Honestly Studentized range (HSD). The student-t test was used to compare the difference between pre- and post-infestation arsenic levels. Statistical inference was based on α level of 0.05 and the p-values were flagged by the analytical software, SPSS (Statistical Software for the Social Sciences) version 21.0 (Nwosu *et al*., 2017).

3. Results and Discussion

Table 1 presents the results for total arsenic concentrations in the bean samples. Total arsenic differed significantly ($P < 0.05$) among the samples collected at different locations in major commercial streets in Owerri Municipal, Imo State, Nigeria. The location-specific mean values of total arsenic ranged from < 0.001 to 1.08 µg/g. These values are much lower than those reported for rice samples from South West Nigeria (50.37-78 ng/g) (Adeyemi *et al*., 2017), India (70-80 µg/kg) (Williams *et al*., 2005), Brazil and China (223-360 µg/kg) (Meharg *et al*., 2008; Batista *et al*., 2011). The pooled mean total arsenic resulting from the study (n = 10) is 0.47 µg/g. This is also lower than the total arsenic (2.88 µg/g) in pooled samples (n = 10) in oysters collected from local market in Port Harcourt, Nigeria (Amayo *et al*., 2016). Geographical variation in total arsenic is consistent with the literature (Adeyemi *et al*., 2017) and summarily related to differences in environmental quality (soil, air, water and intensity of the anthropogenic sources). Spatial variation in the level of total arsenic recorded in the present study may be attributed to differences in the soils on which the beans were cultivated. This is because the grains of cereals planted in arsenic-rich soils appear to have higher concentration of arsenic (Sahoo and Mukherjee, 2014). Anthropogenic sources of arsenic are location-specific and probably, bean samples exposed and sold in locations with more sources of arsenic are potentially more vulnerable to environmental contamination. Therefore, it is not surprising that bean samples collected from the streets of Douglas and Whetheral had higher concentrations of total arsenic. Briefly, there are sources of arsenic pollution near the two streets that could lead to higher arsenic concentrations. As the centre of commercial activities with high human population and activities, the environmental quality of soil, air and water (which is obviously lower than the other streets) probably account for the higher arsenic concentrations observed. This analysis is supported by literature (Amayo *et al*., 2016; Adeyemi *et al*., 2017).

Reports of human exposure from the ingestion of food contaminated with arsenic are considerably documented (Roychowdhury *et al*., 2003). Exposure to toxic arsenic is inevitably associated with health problems for humans. At chronic exposure, human lungs, buccal cavity, pharynx and other important organs face increased chances of cancer (Adeyemi *et al*., 2017). These malicious effects of arsenic can be preventable through routine testing for contamination. The results of the present study show that the estimated daily dietary intake of total arsenic through bean consumption by typical adults in the area is 32.9 µg. This reveals that the total arsenic intake linked to the consumption of beans in major commercial streets in Owerri Municipal, Imo State, Nigeria does not constitute threat to the health of the residents.

Table 1. Levels of total arsenic in bean samples (n = 10) sold in major commercial streets in Owerri Municipal, Imo State, Nigeria during March 2017

Sample codes	Streets sampled	Total arsenic (µg/g)
1a and 1b	Douglas	1.08 ± 0.01^{a}
2a and 2b	Whetheral	0.76 ± 0.05^{a}
3a and 3b	Rotibi	0.02 ± 0.03^{b}
4a and 4b	Okigwe road	$<0.001\pm0.00^{c}$
5a and 5b	World Bank	$<0.001\pm0.00^{c}$

Data are means ± standard error of the means of four replications.
Means in a column followed by the same letter are not significantly different (α = 0.05) by HSD.
Pooled mean = 0.47 µg/g (used subsequently to compute the estimated daily intake of arsenic).
a = bean samples sold in enclosed container.
b = bean samples sold in open container.

After a two-month storage duration, some of the bean samples infested with *A. obtectus* lost some concentration of total arsenic (Table 2). However, loss of total arsenic due to insect feeding was not significant ($P > 0.05$). The highest percent detoxification that occurred in the study is 14.18 and on the contrary, some samples did not experience change in total arsenic level even when the insect fed for 60 days. Generally, it appears the beetles had an effect but only when arsenic values were higher (at Douglas and Whetheral) (Table 2). The other three sites had low arsenic values (≤ 0.02), that the beetles could not make much difference. From the observations above, there may be a threshold level of toxin that must be present for there to be any discernible effect of detoxification by the beetles. However, the result shows that the test insect can be potentially employed in achieving detoxification of bean contaminated with arsenic. At short exposure period, insect feeding was beneficial in reducing arsenic contamination especially added the fact that the test variety of bean was not broken down. Thus, there is a need to extend the storage duration beyond 60 days to observe what happens at prolonged storage conditions. Having the beetles feed longer may not make a difference at sites with low arsenic values but might make a difference on beans containing high arsenic concentrations (the present study provided the hint). Under the influence of insecticides and storage containers commonly used to protect stored bean seeds against beetle attacks in Nigeria, seeds can be successfully preserved for one year without being broken down. So, extension of the storage duration to examine the extent of detoxification may not be such a wasteful idea, the present study concludes.

Table 2. Pre- and post-infestation total arsenic and extent of detoxification caused by the bruchid insect, *Acanthoscelides obtectus* Say

Sample Codes	Streets Sampled	Pre-infestation total arsenic (µg/g)	Post-infestation total arsenic (µg/g)	Total arsenic lost (µg/g)	Percent Detoxification
1a and 1b	Douglas	1.08±0.01^{a}	0.92±0.01^{a}	0.16±0.01^{a}	14.81±0.01^{a}
2a and 2b	Whetheral	0.76±0.05^{a}	0.70±0.05^{a}	0.06±0.05^{b}	7.89±0.05^{b}
3a and 3b	Rotibi	0.02±0.03^{b}	0.02±0.03^{b}	0.00±0.03^{b}	0.00±0.03^{c}
4a and 4b	Okigwe road	<0.001±0.00^{c}	<0.001±0.00^{c}	<0.001±0.00^{b}	0.00±0.00^{c}
5a and 5b	World Bank	<0.001±0.00^{c}	<0.001±0.00^{c}	<0.001±0.00^{b}	0.00±0.00^{c}

t-values were not significant at $P > 0.05$.
Data are means ± standard error of the means of four replications. Means in a column followed by the same letter are not significantly different ($\alpha = 0.05$) by HSD.

4. Conclusion

In conclusion, beans consumed in the main commercial streets of Owerri Municipal at the time of the present study are safe for human health in regard to arsenic level. Total arsenic concentrations varied significantly among the locations sampled. Intra-spatial variation was linked to differences in the number and intensity of anthropogenic sources of the toxic element in these commercial streets. Variation in the concentration of arsenic (arsenic in soils where the bean was grown) may not be excluded. The present study found that the total arsenic contamination varies from place to place and this provides the rationale for location-specific analysis. The geographical variation is strongly related to environmental quality of soil, air, water and human activities. The present study has provided an important hint in the use of insects in the detoxification of toxic elements contaminating stored products. Further studies are suggested at long time storage duration.

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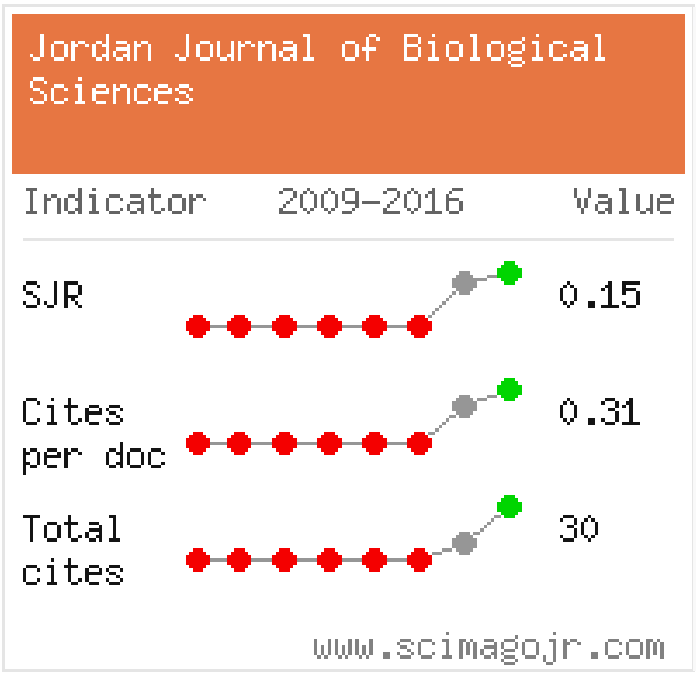
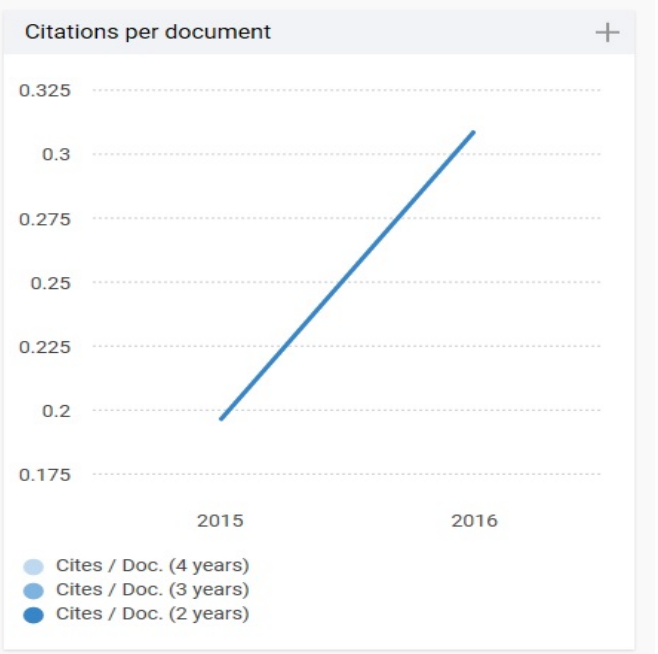
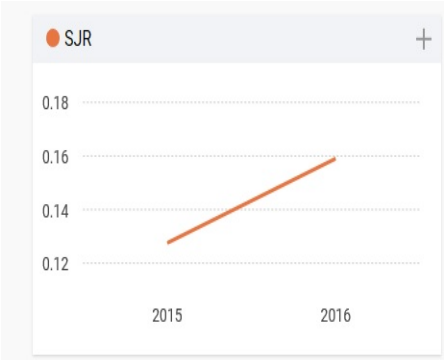
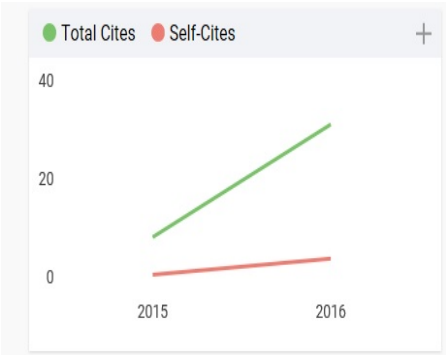
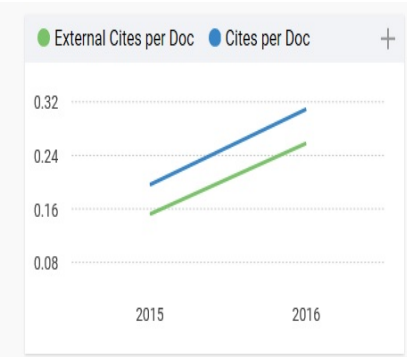
Appendix B

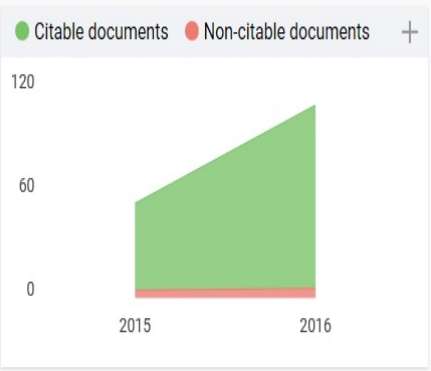


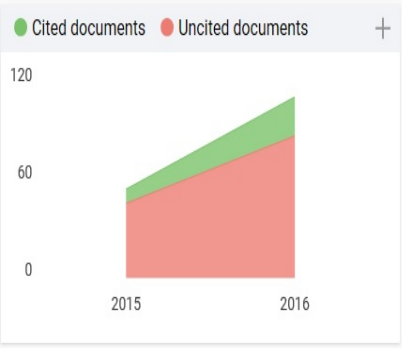












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