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EDITORIAL PREFACE

It is my pleasure to present the ninth volume of the *Jordan Journal of Biological Sciences* (JJBS) to the audience. JJBS is a refereed, peer reviewed quarterly international journal issued by the Jordanian Ministry of Higher Education and Scientific Research Support Fund in cooperation with The Hashemite University, Zarqa, Jordan. This journal publishes papers in Biological Sciences encompassing all the branches at molecular, cellular and organismal levels.

A group of distinguished scholars have agreed to serve on the Editorial Board. Without the service and dedication of these eminent scholars, JJBS would have never existed. Now, the Editorial Board is encouraged by the continuous growth of the journal and its formation into a true multidisciplinary publication. I am also honored to have the privilege of working with all members of the international advisory board served by a team of highly reputable researchers from different countries across the globe. I am also delighted with our team of national and international reviewers who are actively involved in research in different biological sciences and who provide authors with high quality reviews and helpful comments to improve their manuscripts.

JJBS has been indexed by SCOPUS, CABI's Full-Text Repository, EBSCO, Zoological Records and National Library of Medicine's MEDLINE\ Pub Med system and others. I would like to reaffirm that the success of the journal depends on the quality of reviewing and, equally, the quality of the research papers published.

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At the end of this preface, I would like to thank our readers and authors for their continuing interest in JJBS, and each member of our editorial and review boards for their continued hard work, support and dedication, which made it possible to bring another new issue of JJBS to the multidisciplinary international audience. My thanks are also extended to the Hashemite University and Jordanian Scientific Research Support Fund for their continuous support to Jordan Journal of Biological Sciences. I very much appreciate your support as we strive to make JJBS one of the most leading and authoritative journals in the field of Biological Sciences.

December, 2016

Prof. Ali Z. Elkarmi
Editor-in-Chief
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Antimalarial Activity of Natural Products Against Plasmodium Lactate Dehydrogenase Screened by Molecular Docking

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Abstract

Malaria is a parasitic disease which causes high mortality and morbidity rates all over the world. This disease is caused by four species of *plasmodium*: *P. vivax*, *P. malariae*, *P. ovale* and *P. falciparum*. The enzyme lactate dehydrogenase (LDH) catalyses the interconversion of L-lactate and pyruvate with the interconversion of NAD^+ as a cofactor. This enzyme is a possible new target to be exploited in the development of new antimalarial agents. Three X-ray structures of lactate dehydrogenase of *plasmodium* spp. were downloaded from protein data bank. A total of 120 natural products belongs to flavonoids, alkaloids, anthraquinones, coumarins, lignans, chalcones and iridoids were screened *in silico* against LDH. Molecular docking was performed by Hex 8.0.0 using NAD^+ as a positive control. Magnololide A had the highest binding affinities in terms of the total interaction energy in Kcal/mol. Eight analogs of magnololide A were also sketched by ChemBioDraw Ultra 11.0. Analog-7 (four-substituted) and analog-8 (Bromo and chloro substituted) had higher binding affinities than that of NAD^+ . Therefore, magnololide A and its halogenated analogs, rutin, amentoflavone, and hinokiflavone might be useful in the experimental design of anti-malarial agents.

Keywords: Nigeria, limestone, vegetation, families, Jaccard similarity index.

1. Introduction

Malaria infection extends from 60° north to 40° south of the globe where anopheline mosquitoes can live and breed. The disease affects more than 35% of the world population where ten millions are infected each year and two millions die. *P. falciparum* is prevalent in Africa, Middle East and South America while *P. vivax* in India and Far East. *P. ovale* and *P. malariae* in tropical regions of Africa (Goering et al., 2008).

The first antimalarial agent, quinine, is an alkaloid isolated from bark of Cinchona tree. In 1970, Chinese scientists extracted artemisinin from *Artemisia annua*, and its semisynthetic analogs were also used against quinine resistant *P. falciparum* (Bray et al., 2005; Enserink, 2007; Achan et al., 2011). The quinoline derivatives, such as chloroquine, may interact with the binding pocket of NADH as competitive inhibitor (Read et al., 1999).

P. vivax and *P. ovale* are characterized by dormant liver stages, referred to as hypnozoites, that are responsible for relapses of malaria in human (Mazier et al., 2009). *P. vivax* chloroquine resistance was initially discovered in New Guinea in 1989 (Rieckmann et al., 1989). In addition, there is evidence that *P. vivax* has developed resistance to

primaquine (Krudsood et al., 2008) and chloroquine (Oliveira-Ferreira et al., 2010).

Researchers try to identify new drugs pathways since the parasite had developed a resistance to most of the currently available antimalarial drugs (Krettli, 2009; Krettli et al., 2009), e.g., cyclic alkyl polyols, prenylated xanthenes and polyprenylated acylphloroglucinols (Marti et al., 2009; Roumy et al., 2009). LDH is considered a significant target for developing antimalarials since the parasite uses the enzyme on glycolysis to produce its own energy (Penna-Coutinho et al., 2011). LDH (EC number 1.1.1.27) catalyses the reversible conversion of L-lactate to pyruvate using NAD^+ as a cofactor. In the forward direction, a proton is taken from lactate and a hydride donated to NAD^+ . In the reverse direction, a proton is donated to pyruvate, and a hydride ion abstracted from NADH (Madern et al., 2004):



Protein-ligand docking is a computational tool to predict the most favourable structure of the complex formed between a given enzyme and a small-molecule, ligand (Sousa et al., 2006; Grosdidier and Fernandez-Recio, 2009). Sharma and Chetia (2013) used fourteen analogs of quinine and were docked against Plasmeprin II receptor using HEX docking software. The energy values ranged from -178.25 to

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-206.28 Kcal/mol. In the present study, docking of experimental structures of LDH in *Plasmodium* spp. with natural compounds is carried out in order to find an inhibitor to abolish the cofactor NAD⁺ binding to the enzyme as an attempt to find new antimalarials drugs acting on a novel.

2. Materials and Method

X-ray 3D structures of *Plasmodium* LDH were downloaded from Protein Data Bank (PDB) (Berman et al., 2000) at <http://www.rcsb.org/pdb/>. LDH of *Plasmodium vivax* has PDB ID: 2a92 and the others for *P. falciparum* having PDB IDs 1t2c and 1cet. The structures were visualized by Python Molecular Viewer, PMV (Sanner, 1999).

2.1. Multiple Sequence Alignment

Alignments of the three experimental structures were performed by Deep view/ Swiss-Pdb viewer (spdbv) a software maintained by SWISS Institute of Bioinformatics, Switzerland (Guex and Peitsch, 1997) and edited by BioEdit version 7.2.5, developed by Ibis Therapeutics, a division of Isis Pharmaceuticals Inc., California, USA (Hall, 1999).

2.2. Ligand Selection and Preparation

A total of 120 natural products were screened for LDH inhibition. The compounds were from different chemical scaffolds including groups of flavonoids, alkaloids, anthraquinones, coumarins, lignans, chalcones and iridoids. These compounds were either downloaded from ZINC database (<http://zinc.docking.org/>) (Irwin et al., 2012) or sketched by ChemBioDraw Ultra 11.0 developed by CambridgeSoft Corporation, Cambridge, USA (Strack, 2001). The .sdf format was converted to .pdb format using Open Babel software, a chemical tool box from University of Pittsburgh, Department of Chemistry, Pittsburgh, USA (O'Boyle et al., 2011). All ligands were energy minimized by ChemBioOffice Ultra 11.0 (Strack, 2001) to a minimum RMS gradient of 0.100. Molecular properties were predicted by ChemAxon, an online service by cheminformatics company at: www.chemicalize.org.

2.3. Molecular Docking

This method involves the search through different ligand orientations, called poses, within a given target protein, and the prediction of the binding modes and affinities (Sousa et al., 2013). Rigid protein-ligand docking of LDH/chain A was performed on the three crystal structures by Hex 8.0.0. A spherical polar Fourier protein docking algorithm developed by Dave Ritchie, Institut National de Recherche en Informatique et en Automatique (INRIA) at Loria, France (Ritchie and Venkatraman, 2010). The settings were: Grid dimension = 0.6, docking solutions = 100, an initial Steric Scan at N = 18, followed by a Final Search at

N = 25, receptor and ligand range 180 degrees. NAD⁺ was used as control.

2.4. Construction of Analogs

Eight analogs were sketched by ChemBioDraw Ultra 11.0 via adding hydroxyl, amine, chloride, methyl or carbonyl groups (analogs 1-5, respectively) to a phenolic ring of the ligand having the highest docking score. A second set of analogs were constructed via substitution of one or two hydroxyl groups by amine, fluoride or chloride and bromide (analogs 6-8, respectively) to the same ring of ligand according to the method Ashokan (2010) and Modi et al. (2013). All ligands were energy minimized by ChemBioOffice Ultra 11.0 (Strack, 2001) to a minimum RMS gradient of 0.100.

3. Results and Discussion

Rossmann et al. (1975) studied the crystal structure of human LDH. This Nicotinamide Adenine Dinucleotide (NAD) binding protein contains a pair of β - α - β - α - β units which is called Rossmann fold. Adjacent to the nicotinamide group of the cofactor is the substrate binding pocket. It is formed at the interface with the adjoining mixed α/β substrate binding domain (Read et al., 2010).

Figure 1 shows the three dimensional structure of LDH/chain A of *P. vivax* where the first pair of Rossmann appears to be composed of: β A (Pro21→Gly27), α A (Met30→Lys43), β B (Asp47→Asp53), α B (Met58→Ala73) and β C (Lys77→Ser81). The second pair is composed of the following; β D (Asp92→Thr97), α C (Leu113→Asn129), β E (Phe134→Val138), α D (Val142→Ser153) and β F (Lys159→Leu163).

The alignment of the three amino acid sequences of LDH, used in the present study, is shown in (Fig. 2), which points out the conserved glycines Gly27, Gly29 and Gly32. Lys22 is present at β 1 and Asp47 is present in β 2. The in silico study by Penna-Coutinho et al. (2011) suggested that NADH forms 22 hydrogen bonds with the plasmodial LDH in the residues Gly29, Met30, Ile31, Gly32, Asp53, Ile54, Tyr85, Thr97, Gly99, Phe100, Val138, Asn140 and His195. Brown et al. (2004) stated LDH of *P. vivax*, *P. malariae* and *P. ovale* exhibit 90-92% identity to *P. falciparum* in respect to LDH amino acid sequence. The amino acid residues of the catalytic and the cofactor sites in LDH are similar in *P. falciparum* and *P. malariae* while *P. vivax* and *P. ovale* have one substitution.

The region of the first 30-35 amino acids is called the "fingerprint" region. There are four features present in fingerprint region: (1) A phosphate binding sequence, GXGXXG; (2) a hydrophobic core of six amino acids; (3) a conserved positively charged residue (Arg or Lys); and (4) a conserved negatively charged residue (Glu or Asp) (Wierenga et al., 1985; Bellamacina, 1996).

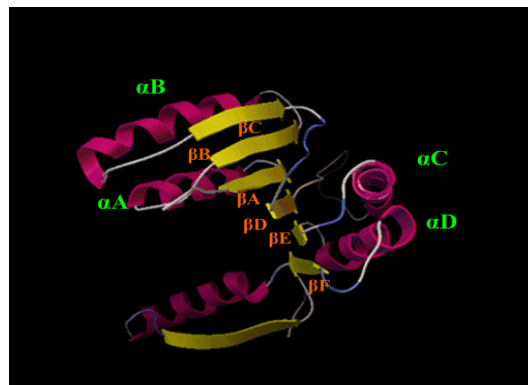


Figure 1. 3D structure of lactate dehydrogenase, *P. vivax* (PDB ID: 2a92A) showing secondary structures contributing to Rossmann folds; α -helices are pink in colour, α A- α D where as β -sheets are yellow in colour, β A- β F. The structure was visualized

	20	30	40	50	60	70	80	90	
1cetA	APKAKIVLVGSGMIGGV	MATLIVQKNLGD-VVLF	DIIVKNM	PHGKALDTSHTN	VMAYSNCKVSGSNTY	DDL	LAGSDVVIVTA		
1t2cA	APKAKIVLVGSGMIGGV	MATLIVQKNLGD-VVLF	DIIVKNM	PHGKALDTSHTN	VMAYSNCKVSGSNTY	DDL	LAGSDVVIVTA		
2a92A	TPKPKIVLVGSGMIGGV	MATLIVQKNLGD-VVMF	DVVKMPQ	GKALDTSHTN	VMAYSNCKVSGSNTY	DDL	LKADVVIVTA		
	100	110	120	130	140	150	160	170	
1cetA	GFT-----	NRLDLLPLNNKIMIE	IGGHIKKNCPNAFI	IIVVTNPVDVMVQLLH	QHS	GV	PKNII	IGLGGVLDTSRLK	
1t2cA	GFTKAPGKSDKEWNRD	LLPLNNKIMIEIGGHI	KKNCPNAFI	IIVVTNPVDVMVQLLH	QHS	GV	PKNII	IGLGGVLDTSRLK	
2a92A	GFTKAPGKSDKEWNRD	LLPLNNKIMIEIGGHI	KKNCPNAFI	IIVVTNPVDVMVQLLH	QHS	GV	PKNII	IGLGGVLDTSRLK	
	180	190	200	210	220	230	240	250	
1cetA	YYISQKLNVCPRDVNA	HIVGAHGNKMLKRYIT	VGGIPLQEFINNKLIS	DAELE--AIFDRTVNTA	LEIVNLHASP	YVA			
1t2cA	YYISQKLNVCPRDVNA	HIVGAHGNKMLKRYIT	VGGIPLQEFINNKLIS	DAELE--AIFDRTVNTA	LEIVNLHASP	YVA			
2a92A	YYISQKLNVCPRDVNA	LIVGAHGNKMLKRYIT	VGGIPLQEFINNKKIT	DEEVE--GIFDRTVNTA	LEIVNLLASP	YVA			
	260	270	280	290	300	310	320	330	
1cetA	PAAAIIEMAESYLKDL	LKKVLICSTLLEGQYG-HSDIF	GGTPVVLGANGVEQVIELQLN	SEEEKAKFDEAIAETKRM	KALA-				
1t2cA	PAAAIIEMAESYLKDL	LKKVLICSTLLEGQYG-HSDIF	GGTPVVLGANGVEQVIELQLN	SEEEKAKFDEAIAETKRM	KALA-				
2a92A	PAAAIIEMAESYLKDL	LKKVLICSTLLEGQYG-HSNIF	GGTPLVIGGTGVEQVIELQLN	AEEKTKFDEAVAETKRM	KALIH				

by Python Molecular Viewer (Sanner, 1999)

Figure 2. Multiple sequence alignment of *Plasmodium* LDH sequences by Deep view/Swiss-Pdb viewer software. 2a92A: LDH, chain A of *P. vivax*, 1t2cA and 1cetA: LDH, chain A of *P. falciparum*. Alignment was viewed by BioEdit version 7.2.5

Deep view/ Swiss-Pdb viewer (spdbv) software identified the largest cavity as shown in (Fig. 3) which has an area of 1940 Å² and a volume of 2658 Å³. The largest cavity is most frequently represents the ligand binding site (Singh *et al.*,

2011). However, LDH of *P. falciparum* displays structural and kinetic differences compared with human LDH suggesting that the enzyme can be a potential antimalarial target (Brown *et al.*, 2004).

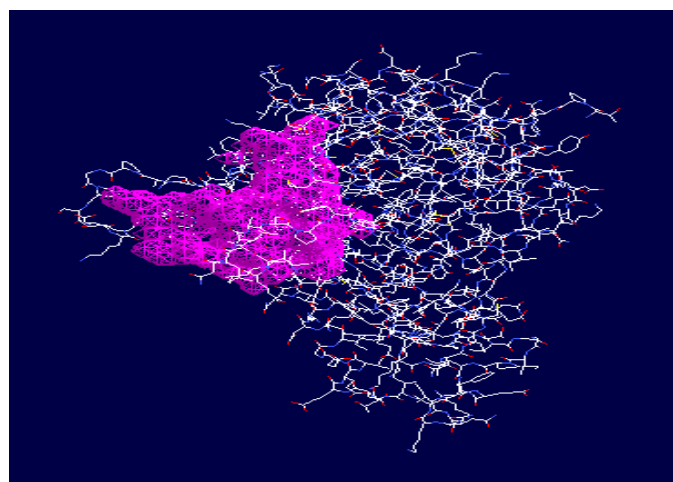


Figure 3. Binding cavity, pink in color, of 2a92A (LDH, chain A of *P. vivax*) where NAD⁺ cofactor associates to perform function, visualized by Deep view/Swiss-Pdb viewer (Guex and Peitsch, 1997).

Using NAD^+ as a control, a total of 120 natural compounds were docked against LDH to identify inhibitors which may interfere with cofactor binding. Different scoring systems are employed in docking software, Table (1) shows the total calculated interaction energy in Kcal/mol. Figure 4A shows the binding of NAD^+ with LDH/ chain A (PDB: 2a92), while Figure 4B shows the binding of magnolioside A ($\text{C}_{29}\text{H}_{36}\text{O}_{15}$) which is a phenolic compound having nine hydroxyl groups capable to form hydrogen bonds at the binding site of the receptor molecule. The polar surface area of the compound is higher than 90 \AA^2 , therefore, would not pass across the blood brain barrier and will not exert an activity in central nervous system or produce adverse effects there (Pajouhesh and Lenz, 2005). In contrast to magnolioside A which has only one violation, its mass, the other three compounds rutin, amentoflavone and hinokiflavone have two or more violations of Lipinski rule of five. Lipinski rule of five states that a candidate drug will be less orally absorbed when its mass higher than 500, logP value is greater than 5, H-bond donors are more than 5 and H-bond acceptors are more than 10 (Lipinski *et al.*, 2011). Rutin, amentoflavone and hinokiflavone have lower masses (610.52, 538.4 and 538.4 g/mol, respectively). Rutin, a glycosylated flavonoid, has the lowest logP (-1.06), a measure of lipophilic properties of a drug, but its H-bond donors are 10 and the H-bond acceptors are 16. The values of logP are 5.16 and 5.18 for amentoflavone and hinokiflavone, respectively. These two biflavonoids have their hydrogen bond donors 10 but their H-bond acceptors are 6 and 5, respectively.

Table 1: Docking results of the highest best compounds expressed in total interaction energy (Kcal/mol)*

Compound	2a92	1t2c	1cet
NAD^+	-	-	-
	361.15	329.12	349.36
Magnolioside A	392.28	334.39	314.62
Rutin	369.96	313.81	297.59
Amentoflavone	364.80	305.35	307.82
Hinokiflavone	361.99	352.13	339.50
Vicenin	354.14	301.90	323.25
Henryoside	350.66	319.29	335.19
6-O-Benzoylphlorigidioside B	338.04	294.88	287.67
Ducheside A	336.78	293.46	315.89
Silybin	334.44	307.75	315.81
Icariside	325.49	325.62	302.04

*Bold refers to the preferred docking values that are below that of NAD^+

To optimize Magnolioside, eight analogs were constructed either by adding functional groups (analogs 1-5, respectively) to magnolioside A or via substitution of one or two hydroxyl groups in the same ring (analogs 6-8, respectively). These analogs were also docked against LDH experimental structures. Table (2) shows the binding affinities in terms of total calculated interaction energy (Kcal/mol). The 7th (fluorinated analog) and 8th analogs (chlorinated and brominated analogs) had higher docking values (lesser in negative) than that of NAD^+ in all the experimental structures studied (Figures 4C and 4D). Table 3 represents the chemical names and molecular properties of magnolioside A and its analogs according to Chemaxon. Figures 5 and 6 show the chemical structure of analog-7 and analog-8 compared with magnolioside A, the parent compound and other ligands that had higher affinities than NAD^+

Table 2: Docking results of magnolioside A analogs in total interaction energy (Kcal/mol)*

Compound	2a92	1t2c	1cet
Analog-1	-372.00	-330.38	-328.61
Analog-2	-381.01	-346.03	-330.56
Analog-3	-408.28	-395.63	-341.56
Analog-4	-398.97	-350.31	-319.57
Analog-5	-389.64	-333.43	-321.63
Analog-6	-393.91	-325.70	-324.01
Analog-7	-427.58	-393.03	-355.13
Analog-8	-455.14	-438.85	-383.07

*Bold refers to the best docking values (binding affinities) that are higher than that of NAD^+

Table 3: Molecular descriptors of Magnolioside A and its analogs

Compound	IUPAC Name	Mass (g/mol)	logP ¹	R B ²	PSA ³
MagnoliosideA	(2R, 3R, 4R, 5R, 6R)-2-[2-(3, 4-dihydroxy-methyl phenyl) ethoxy]-5-hydroxy-6-(hydroxy methyl)-3-[[[(2S, 3R, 4R, 5R, 6S)- 3, 4, 5-trihydroxy-6-methyloxan-2-yl]oxy]-oxan-4-yl (2E)-3-(3, 4-dihydroxyphenyl) prop-2-enoate	624.5871	0.82	11	245.29
Analog-1	(2R, 3R, 4R, 5R, 6R)-3-hydroxy-2-(hydroxy methyl)-5-[[[(2S, 3R, 4R, 5R, 6S)- 3, 4, 5-trihydroxy-6-methyloxan-2-yl]oxy]-6-[2-(2, 3, 4-trihydroxyphenyl) ethoxy] oxan-4-yl (2E)-3-(3, 4-dihydroxyphenyl) prop-2-enoate	640.5865	0.52	11	265.52
Analog-2	(2R, 3R, 4R, 5R, 6R)-2-[2-(2-amino-3, 4-dihydroxyphenyl) ethyl]-5-hydroxy-6-(hydroxy methyl)-3-[[[(2S, 3R, 4R, 5R, 6S)- 3, 4, 5-trihydroxy-6-methyloxan-2-yl]oxy] oxan-4-yl (2E)-3-(3, 4-dihydroxyphenyl) prop-2-enoate	639.6018	-0.01	11	271.31
Analog-3	(2R, 3R, 4R, 5R, 6R)-2-[2-(2-chloro-3, 4-dihydroxyphenyl) ethoxy]-5-hydroxy-6-(hydroxy methyl)-3-[[[(2S, 3R, 4R, 5R, 6S)- 3, 4, 5-trihydroxy-6-methyloxan-2-yl]oxy] oxan-4-yl (2E)-3-(3, 4-dihydroxyphenyl) prop-2-enoate	659.032	1.42	11	245.29
Analog-4	(2R, 3R, 4R, 5R, 6R)-2-[2-(3, 4-dihydroxy-2-methyl phenyl) ethoxy]-5-hydroxy-6-(hydroxy methyl)-3-[[[(2S, 3R, 4R, 5R, 6S)- 3, 4, 5-trihydroxy-6-methyloxan-2-yl]oxy]-oxan-4-yl (2E)-3-(3, 4-dihydroxyphenyl) prop-2-enoate	638.6137	1.33	11	245.29
Analog-5	(2R, 3R, 4R, 5R, 6R)-2-[2-formyl-3, 4-dihydroxyphenyl) ethoxy]-5-hydroxy-6-(hydroxy methyl)-3-[[[(2S, 3R, 4R, 5R, 6S)- 3, 4, 5-trihydroxy-6-methyloxan-2-yl]oxy]-oxan-4-yl (2E)-3-(3, 4-dihydroxyphenyl) prop-2-enoate	652.5972	1.18	12	262.36
Analog-6	(2R, 3R, 4R, 5R, 6R)-2-[2-(3-amino-4-hydroxyphenyl) ethoxy]-5-hydroxy-6-(hydroxy methyl)-3-[[[(2S, 3R, 4R, 5R, 6S)- 3, 4, 5-trihydroxy-6-methyloxan-2-yl]oxy] oxan-4-yl (2E)-3-(3, 4-dihydroxyphenyl) prop-2-enoate	623.6024	0.29	11	251.08
Analog-7	(2R, 3R, 4R, 5R, 6R)-2-[2-(3-fluoro-4-hydroxyphenyl) ethoxy]-5-hydroxy-yl]-oxan-4-yl (2E)-3-(3, 4-dihydroxyphenyl) prop-2-enoate	626.5782	1.27	11	225.06
Analog-8	(2R, 3R, 4R, 5R, 6R)-2-[2-(4-bromo-3-chlorophenyl) ethoxy]-5-hydroxy-6-(hydroxy methyl)-3-[[[(2S, 3R, 4R, 5R, 6S)- 3, 4, 5-trihydroxy-6-methyloxan-2-yl]oxy] oxan-4-yl (2E)-3-(3, 4-dihydroxyphenyl) prop-2-enoate	705.929	2.80	11	204.83

¹logP: is the octanol-water partition coefficient, a measure of lipophilicity; ²RB: Rotatable bond count; ³PSA: Polar surface area

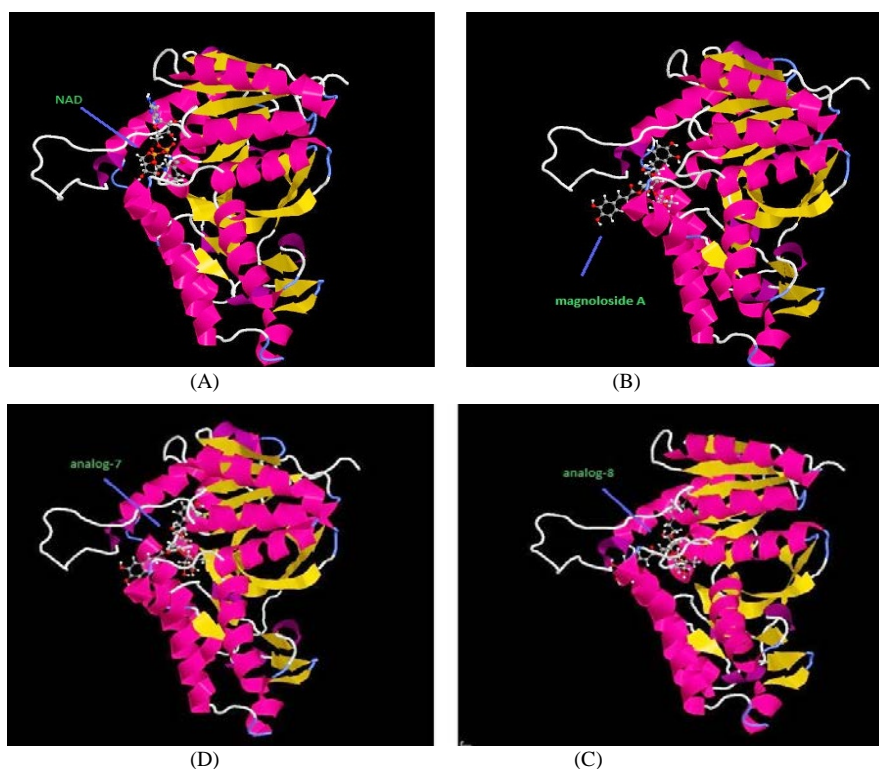


Figure 4. Docking results of and magnolioside A analogs against 2a92A using Hex 8.0.0 (A) NAD⁺ (B) magnolioside A (C) Analog-7 (D) Analog-8. α -helices are pink in color while β -strands are yellowish, visualized by Python Molecular Viewer (Sanner, 1999)

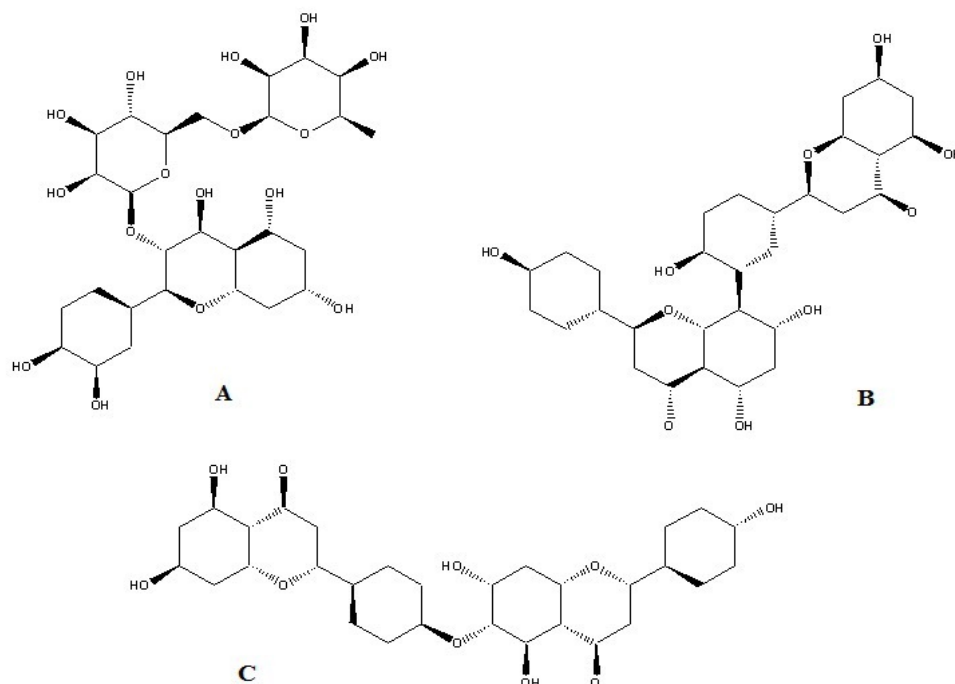


Figure 5. Chemical structure of (A) rutin (B) amentoflavone (C) hinokiflavone

There are many natural products screened for antimalarial activity. In a docking study of natural products against LDH of plasmodium species using ArgusLab and Swiss-dock tools, Panchal *et al.* (2013) found that apigenin, luteolin, ajmalicine, rosmarinic acid and swertiamarin might be lead compounds. However, docking experiments by Hex 8.0.0 showed that those compounds had their total interaction energy values of -234.66, -248.98, -258.03, -238.39 and -233.13 Kcal/mol, respectively. The compound magnolioside A and its analogs could be more effective *in vitro* than those obtained by Panchal *et al.* (2013) in binding LDH. Fifty compounds were screened *in silico* against lactate dehydrogenase of *P. falciparum* using Molegro Virtual Docker software (Penna-Coutinho *et al.*, 2011). Those having the best docking scores were itraconazole, atorvastatin and posaconazole with a MolDock score -218.5, -209.3 and -201.6 Kcal/mol, respectively, while NADH in the present study had -249.6 Kcal/mol.

The quassinoid isobruceine B is extracted from the roots and stems of *Picrolemma sprucei* while orinocinolide is extracted from *Simaba orinocensis* (Muhammad *et al.*, 2004; Pohlit *et al.*, 2009). The quassinoid, simalikalactone D was discovered in 1993 and extracted from *Simaba guianensis* (Cabral

et al., 1993). Simalikalactone D has an effective dose (ED_{50}) of 3.7 mg/kg/day against *P. yoelii* which infect rodents, suggesting a pharmacological activity *in vivo* (Bertani *et al.*, 2006). In Hex 8.0.0, docking of isobruceine B, orinocinolide and Simalikalactone D against 2a92A resulted in a total interaction energy of -290.19, -299.19 and -310.23 Kcal/mol, respectively, compared to magnolioside A, -392.28 Kcal/mol.

4. Conclusion

Molecular docking may be used in the drug design reducing time, cost and effort for *in vitro* screening of screening libraries of experimental compounds. Natural products and synthetic agents might be screened against new alternative targets in malaria parasites. This process requires pharmacokinetics and dynamics of these compounds to be investigated. Since possess the best results in terms of docking values, magnolioside A and its halogenated analogs might be used *in vitro* studies for screening inhibitors against the NAD^+ binding domain of LDH. A candidate drug acting on LDH of the parasite should not affect the metabolic pathways inside the human body.

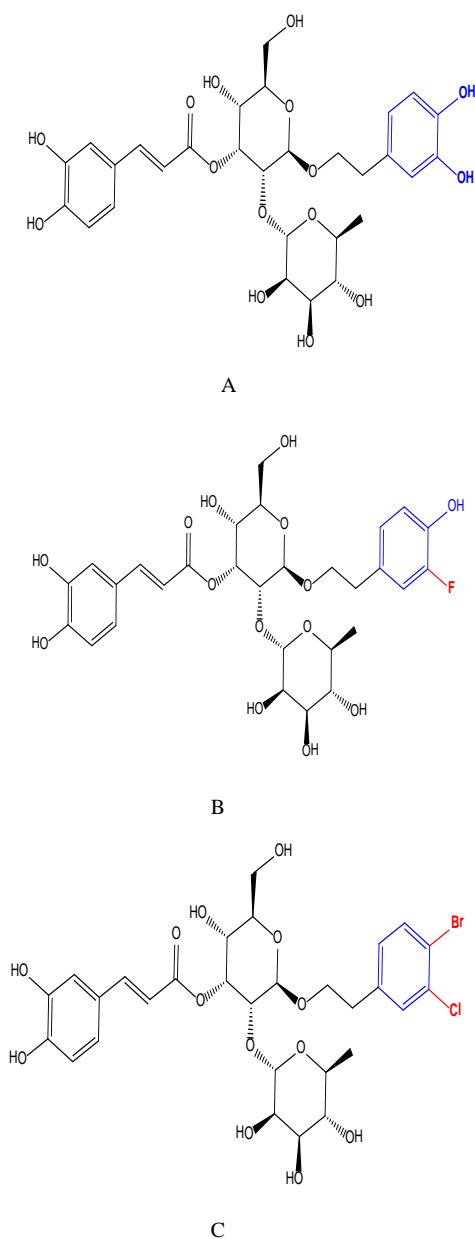


Figure 6. Chemical structure of (A) magnolide A, the ring where substitutions occurred is in blue color (B) analog-7, fluoride (colored red) replaces hydroxyl group (colored blue) (C) analog-8, chloride and bromide (appears red) replaces hydroxyl groups. Sketched by ChemBioDraw Ultra 11.0.

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Oral Toxicity of Thymol, α -Pinene, Diallyl Disulfide and Trans-Anethole, and Their Binary Mixtures against *Tribolium castaneum* Herbst Larvae (Coleoptera: Tenebrionidae)

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Abstract

Oral toxicity of thymol, α -pinene, diallyl disulfide and trans-anethole, as well as their binary combinations, was studied on the fourth larval instars of *Tribolium castaneum* Herbst. Serial dilutions of chemicals were prepared from the stock solution to obtain the concentrations between 2.5-200 μ l/ml prior mixing with 500 mg of the diet. LC₅₀ values were determined as 6.79, 12.85, 8.52 and 1.03 μ l/ml, for thymol, α -pinene, trans-anethole and diallyl disulfide after 24 hours and 3.74, 8.39, 6.48 and 0.68 μ l/ml after 48 hours of exposure, respectively. LC₅₀ values confirmed that diallyl disulfide and α -pinene were the highest and lowest toxic chemicals. The results show that combined treatment of thymol synergized α -pinene activity at 24 and 48 hours post treatments. In addition, "diallyl disulfide and thymol", "thymol and trans-anethole" had additive effects on *T. castaneum* larvae. Our results suggest that the combination of thymol and α -pinene compounds were significantly more effective than each compounds alone on *T. castaneum* larvae.

Keywords: Acute toxicity, Essential oil components, Botanical insecticides, Synergy, *Tribolium castaneum*

1. Introduction

Essential oils, as botanical insecticides, are the complex mixture of volatile compounds, whose bioactivities depend on chemical composition, synergistic or antagonistic effects (Isman, 2006). Essential oils are lipophilic and dissolvable in the lipid medium classified into monoterpenes, sesquiterpenes (both including hydrocarbons and oxygenated derivatives) and aliphatic compounds (acids, alcohols, aldehydes, alkanes, alkenes and ketones) (Tripathi et al., 2009; Ebadollahi, 2013). These compounds are considered as main bioactive chemicals, which are distributed in plant families, such as Asteraceae, Apiaceae, Zingiberaceae, Myrtaceae, Lamiaceae, Piperaceae, Poaceae, Rutaceae, Lauraceae, Cupressaceae, Graminaceae, Pedaliaceae etc. (Ebadollahi, 2013). Recently, researchers have shown an increased interest in bioactive effects of essential oils (including larvicidal, insecticidal, repellency, antifeedant, deterency, delay development, adult emergence and fertility) and their derivatives on insects (reviewed in Bakkali et al., 2008; Tripathi et al., 2009; Gonzalez-Coloma et al., 2010; Qin et al., 2010; Abbasipour et al., 2011; De Almeida et al.,

2011). They are insect neurotoxins that inhibit either GABA receptor or acetylcholinesterase (AChE) (Bakkali et al., 2008). Regarding their biological properties, some studies showed that complex essential oil compositions were more effective than pure compounds, which can be due to their synergistic effects. However, their modes of action are still obscure (Gonzalez-Coloma et al., 2010). Fumigant toxicity of essential oils have focused on stored beetles, such as *Sitophilus oryzae* L. (Col.: Curculionidae), *Sitophilus zeamais* M. (Col.: Curculionidae), *Tribolium castaneum* H. (Col.: Tenebrionidae) and *Rhyzopertha dominica* F. (Col.: Bostrichidae) (Rajendran and Sriranjini, 2008).

Despite that the red flour beetle *T. castaneum* is known as a key coleopteran insect model for genomic studies (Richards et al., 2008); it is a destructive pest of stored products feeding on different grain and products in stores (Garcia et al., 2005).

Thymol is a phenolic monoterpene with strong insecticidal and antimicrobial activities (Palaniappan and Holley, 2010; Kumrungsee et al., 2014). It disrupts GABA synapses function by binding to GABA receptors on membrane of postsynaptic neurons (Priestley et al., 2003).

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α -Pinene is an alkene terpene found in Apiaceae family, that possess insecticidal activity on pests, such as *Sitophilus granaries* L. (Col.: Curculionidae), *T. castaneum*, (Ebadollahi and Mahboubi, 2011), *Spodoptera litura* W. (Lep.: Noctuidae) and *Achaea janata* L. (Lep.: Noctuidae) (Rani et al., 2014). Despite playing a role as insecticide, antifeedant, repellency and development inhibition (Huang et al., 1998; Kim et al., 2010; Yang et al., 2014), α -Pinene is associated with attracting the ladybeetles *Chilocorus kuwanae* S. (Col.: Coccinellidae) (Zhang et al., 2009).

Diallyl disulfide is a major constituent of the essential oil of Alliaceae family (Block, 2010) preventing oviposition and exhibiting behavioral deterrence against insect adults like *S. zeamais*, *T. castaneum* (Huang et al., 2000), *Culex pipiens* L. (Dip.: Culicidae) (Ramakrishnan et al., 1989), *Sitotroga cerealella* O. (Lep.: Gelechiidae) (Yang et al., 2012) and *T. confusum* (Saglam and Ozder, 2013). Trans-anethole is an active terpene derived of phenylpropanoid which is the main compound in essential oils of anise and fennel plants (Ozcan and Chalchat, 2006; Heydarzade and Moravvej, 2012; Kim et al., 2013). It induces lethal effects during the larval development (Sousa et al., 2015) and inhibits acetylcholinesterase and butyrylcholinesterase activity in insects (Menichini et al., 2009).

It is hypothesized that the mixtures of essential oils from the different plant could improve the efficiency against insect pests (Hummelbrunner and Isman, 2001). In addition, plant essential oils are highly volatile components which extrinsic parameters, such as temperature, light and oxygen, show a crucial impact on their stability. Since essential oils process significant fumigant toxicity against insect pests, the application of alternative methods seems to be efficient at least to minimize degradation and improve stability of essential oils. The objectives of the present study are to determine the oral toxicity of thymol, α -pinene, diallyl disulfide and trans-anethole, individually and in binary mixtures to find the most effective compound on fourth larval instars of *T. castaneum*.

2. Materials and Method

2.1. Compounds

The chemicals including thymol (99%), α -pinene (98%), diallyl disulfide (80%) and trans-anethole (99.5%) were purchased from Sigma-Aldrich (Spain). Acetone (AR grade, Merck Germany) was used as the solvent in all experiments.

2.2. Insect Rearing

The method described by Mikhael (2011) was used for insect rearing. Adults of *T. castaneum* were collected from infested stores in Zabol, Iran then stock colonies were established. Insects were fed on wheat flour and yeast (10:1 w:w) under controlled conditions (30 \pm 2 °C, 70 \pm 2% R.H, 16:8 (L:D)) at Department of Plant Protection, University of Zabol, Zabol, Iran.

2.3. Bioassay

The experiments were carried out on the fourth larval instars of *T. castaneum* under laboratory conditions. Concentrations of 2, 4, 8, 16 and 20 μ l/ml of thymol, α -pinene, trans-anethole and 0.25, 0.5, 1, 2 and 4 μ l/ml of diallyl disulfide, were determined based on preliminary experiments. Then, 500 μ l of each concentration was added to 500 mg of larval diet. Diets were well mixed with chemicals, and then were allowed to evaporate their solvent for 15 min at room temperature. Controls were treated with acetone alone. The experiments were carried out in three replications with 10 larvae in each replication on plastic Petri dishes (diameter 6 cm). Larval mortality (lack of mobility was a criterion of larval mortality) was recorded 24 and 48 hours post treatments and LC₅₀ values were calculated. Probit analysis was used to calculate LC₅₀ and the corresponding 95 % CI values were obtained using Polo-Plus Software.

2.4. Insecticidal Activity of Binary Mixture of Chemicals

Acute effects of binary mixtures of different sublethal concentrations of compounds used in the survey were according to the described method above. Then the actual and expected mortalities of the treated larvae were compared using the following formula as were described by Trisyono and Whalon (1999): $Em = Oa + Ob (1 - Oa)$, where O and E are the observed and expected mortalities using first (Oa) and second chemicals (Ob) in the binary mixtures, respectively. Following Em formula, using X2' formula ($X2' = (Om - Em)^2 / Em$), where Om and Em are the observed and expected mortalities in the binary mixture. Additive, antagonistic or synergistic effects were determined. The values of X2' were compared to the values of X2 value in chi-square distribution table ($X2_{df=1, \alpha=0.05} = 3.84$). As described by Kumrungsee et al. (2014), when X2' values > 3.84 and < 3.84, synergistic and additive effects were represented, respectively. If the observed mortality were less than the expected one, it would be interpreted as antagonistic effect of the mixtures.

3. Results

3.1. Toxicity of Pure Compounds

Thymol, α -Pinene, trans-anethole and diallyl disulfide showed the oral toxicities against larvae of *T. castaneum* at different concentrations and all exposure times. LC₅₀ values were found to be 6.79, 12.85, 8.52 and 1.03 μ l/ml, for thymol, α -pinene, trans-anethole and diallyl disulfide 24 hours post-treatment, respectively (Table 1). Although after 48 hours post-treatment, LC₅₀ values of thymol, α -pinene, trans-anethole and diallyl disulfide were 3.74, 8.39, 6.48 and 0.68 μ l/ml, respectively (Table 1). LC₅₀ values confirmed that diallyl disulfide was the most toxic compounds. All chemicals showed a significant positive correlation between increasing

chemical concentrations, exposure times and increased larval mortality (Figure 1).

Table 1. Toxicity of different compounds ($\mu\text{l/ml}$) to early 4th instar larvae of *T. castaneum* at 24 and 48 hours post-treatment

Compound	N	Time (h)	LC ₅₀ (95% CL)	X ² (df)	Slope \pm SE	P-Value
Thymol	150	24	6.79 (4.86-9.25)	1.22 (3)	1.66 \pm 0.31	0.747
	150	48	3.74 (2.51-4.96)	1.82 (3)	1.93 \pm 0.33	0.610
α -pinene	150	24	12.85 (8.86-23.58)	1.13 (3)	1.32 \pm 0.30	0.769
	150	48	8.39 (5.60-13.29)	0.67 (3)	1.26 \pm 0.29	0.881
Trans-anethol	150	24	8.52 (6.07-12.42)	1.02 (3)	1.5 \pm 0.30	0.795
	150	48	6.48 (4.34-9.23)	1.79 (3)	1.43 \pm 0.30	0.616
Diallyl disulfide	150	24	1.03 (0.72-1.49)	0.41 (3)	1.45 \pm 0.27	0.939
	150	48	0.68 (0.47-0.92)	0.84 (3)	1.68 \pm 0.29	0.839

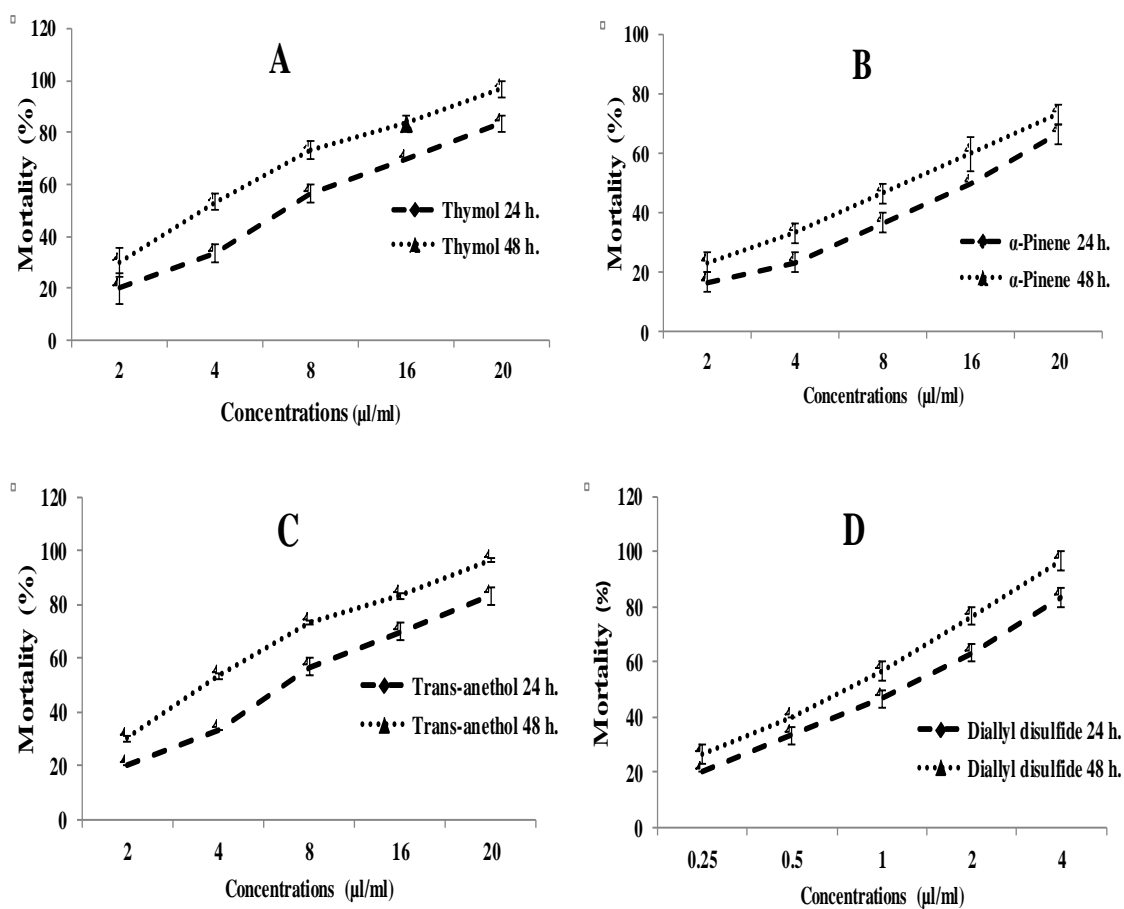


Figure 1. Relative toxicity of compounds to 4th instar larvae of *T. castaneum* at 24 and 48 hours post treatment. A) Thymol, B) α -pinene, C) Trans anethol, D) Diallyl disulfide

3.2. Combination of Pure Compounds

In all concentrations, combination of thymol with diallyl disulfide or trans-anethole showed additive effects (Table 2). Binary mixtures of thymol and α -pinene synergized oral toxicity

against *T. castaneum* (Table 2). In all concentrations, combinations of diallyl disulfide with trans-anethole or α -pinene and trans-anethole with α -pinene showed antagonistic effects 24 hours after exposure (Table 2).

Table 2. Relative toxicity of binominal mixtures of essential oil compounds to early 4th instar larvae of *T. castaneum* and measures of interactions at 24 hours post treatment

Compound A	Compound B	Concentrations (μ l/ml)	N	Larval mortality (%)				X ²	Effect
				Pure compound		Binary mixtures			
				O _a	O _b	E _m	O _m		
Diallyl disulfide	Thymol	0.25+2	30	20.00	20.00	36.00	46.67	3.16	Additive
Diallyl disulfide	Thymol	0.25+4	30	20.00	33.33	46.64	56.67	2.16	Additive
Diallyl disulfide	Thymol	0.5+2	30	33.33	20.00	46.64	50.00	0.24	Additive
Diallyl disulfide	Thymol	0.5+4	30	33.33	33.33	55.55	60.00	0.36	Additive
Diallyl disulfide	Trans-anethol	0.25+2	30	20.00	20.00	36.00	30.00	-	Antagonist
Diallyl disulfide	Trans-anethol	0.25+4	30	20.00	30.00	44.00	33.33	-	Antagonist
Diallyl disulfide	Trans-anethol	0.5+2	30	33.33	20.00	46.66	33.33	-	Antagonist
Diallyl disulfide	Trans-anethol	0.5+4	30	33.33	30.00	53.33	40.00	-	Antagonist
Diallyl disulfide	α -pinene	0.25+2	30	20.00	16.67	33.34	20.00	-	Antagonist
Diallyl disulfide	α -pinene	0.25+4	30	20.00	23.33	38.66	23.33	-	Antagonist
Diallyl disulfide	α -pinene	0.5+2	30	33.33	16.67	44.44	30.00	-	Antagonist
Diallyl disulfide	α -pinene	0.5+4	30	33.33	23.33	48.88	30.00	-	Antagonist
Thymol	Trans-anethol	2+2	30	20.00	20.00	36.00	43.33	1.49	Additive
Thymol	Trans-anethol	2+4	30	20.00	30.00	44.00	50.00	0.82	Additive
Thymol	Trans-anethol	4+2	30	33.33	20.00	46.66	53.33	0.95	Additive
Thymol	Trans-anethol	4+4	30	33.33	30.00	53.33	56.67	0.21	Additive
Thymol	α -pinene	2+2	30	20.00	16.67	33.34	56.67	16.32	Synergy
Thymol	α -pinene	2+4	30	20.00	23.33	38.66	60.00	11.78	Synergy
Thymol	α -pinene	4+2	30	33.33	16.67	44.44	66.67	11.12	Synergy
Thymol	α -pinene	4+4	30	33.33	23.33	48.88	73.33	12.23	Synergy
Trans-anethol	α -pinene	2+2	30	20.00	16.67	33.34	23.33	-	Antagonist
Trans-anethol	α -pinene	2+4	30	20.00	23.33	38.66	23.33	-	Antagonist
Trans-anethol	α -pinene	4+2	30	30.00	16.67	41.67	30.00	-	Antagonist
Trans-anethol	α -pinene	4+4	30	30.00	23.33	46.10	33.33	-	Antagonist

4. Discussion

In the present study, we demonstrated that the mixing of some secondary metabolites of plant essential oils can result in significant synergism to increase their toxicity. In general, fumigant and contact toxicity of essential oils have been successfully studied. Findings demonstrated that essential oils penetrate into insect respiratory system and cuticle (Kim *et al.*, 2010). Physical properties of essential oils, such as low vapor pressure, limit their application in stores. Although the application of traps baited with a mixture of diet and essential oil to prevent essential oil residues and food tainting in the products seems to be a useful technique to control the stored pests, there is a lack of data on the impact of essential oils on insect gustatory reaction and their antifeedent effects (Dubey, 2011; Hernández-Lambrano *et al.*, 2014). Koul *et al.* (2013) suggested that the relation between insect gustatory reaction and toxic effect of plant secondary metabolites is species specific, which may vary among insects.

Some monoterpenoids, such as thymol, 1,8-cineole, pulegone, Fenchone, S-carvone, γ -terpinene, geraniol and etc., were recommended as alternatives to synthetic insecticides against pests (Tripathi *et al.*, 2009; Lopez *et al.*, 2008, 2010; Kumrungsee *et al.*, 2014). In the present study, diallyl disulfide was the most toxic compound on *T. castaneum* fourth larval instar, followed by thymol, trans-anethole and α -pinene.

Many researchers have studied bactericidal and insecticidal activities of garlic, *Allium sativum* (Liliaceae) essential oil against pests. The authors found that diallyl disulfide, as a major compound of garlic essential oil was highly toxic against *T. confusum* and *S. oryzae* (Huang *et al.*, 2000; Yang *et al.*, 2010; Saglam and Ozder, 2013).

Trans-anethole exhibited fumigant toxicity (with LC_{50} = 5.02 mg/L air) on *S. oryzae* at 24 h. (Kim *et al.*, 2013). Kumrungsee *et al.* (2014) reported that thymol was highly toxic to *P. xylostella* (LD_{50} = 0.22 μ g/larva). Similarly, Kim *et al.* (2010) showed that thymol, α -pinene, camphene, p-cymene, and γ -terpinene were highly toxic to *T. castaneum* adults. Thymol binds GABA-gated chloride channels on the membrane of postsynaptic neurons and disrupts the function of GABA synapse (Priestley *et al.*, 2003). It was proposed that biological activities of monoterpenoids are associated with molecular configuration, position and nature of functional groups (Tripathi *et al.*, 2009). The minor structural diversity of monoterpenoids may be accounted for the significant differences in their mode of action of insecticidal activity.

Synergistic and additive effects of secondary metabolites are important in plant defenses against herbivores insects. The effects of minor constituents in a complex of plant secondary metabolites may be synergized and enhanced via different mechanisms. Some of the binary mixtures of essential oils and

volatile compounds potentially exhibit strong feeding deterrence in pests (Hummelbrunner and Isman, 2001; Faraone *et al.*, 2015). In the present study, combination of different concentrations of pure compounds including thymol and α -pinene caused synergistic effects on mortality of *T. castaneum*. In addition, combination of thymol with diallyl disulfide or trans-anethole resulted in additive mortality effect. Some studies have shown that combination of thymol and other compounds increased toxicity on insects, such as Hummelbrunner and Isman (2001) who demonstrated that thyme oil, containing thymol and carvacrol, showed greater toxicity effects than either of the pure compounds. In a similar study, the binary mixture of compounds "thymol and trans-anethole" were synergistic against *S. littura* larva (Hummelbrunner and Isman, 2001; Koul *et al.*, 2013). Singh *et al.* (2009) found that a combined treatment of thymol with 1,8-cineole or linalool significantly caused higher mortality than either treatment alone against the third instar of *Chilo partellus* S. (Lep.: Crambidae). However, combinations of "diallyl disulfide and trans-anethole", "diallyl disulfide and α -pinene" as well as "trans-anethole and α -pinene" were antagonistic in a mixture. Diallyl disulfide was the most toxic of all compounds. "Trans-anethole and α -pinene" caused reduction in the toxicity of diallyl disulfide, which suggests that both of these compounds may be competing for the same receptor site (Kumrungsee *et al.*, 2014). The combination of thymol with 1,8-cineole or linalool as well as 1,8-cineole with linalool mixtures showed an antagonistic effect against *P. xylostella* larvae (Kumrungsee *et al.*, 2014).

Synergistic power of pure compounds when they are mixed, may depend on compatibility of two constitutes as well as the used concentrations (or doses). Probably, the compounds that cause low mortality, their toxicity may increase in a suitable combination. In addition, Faraone *et al.* (2015) demonstrated that the use of essential oils, such as linalool and thymol, as synergistic agents with conventional pesticides (imidacloprid and spirotetramat) may lead to greater uptake of essential oils for crop protection. Our findings confirmed that α -pinene has low toxicity but combinations of "thymol and α -pinene" demonstrated the highest synergic effect on *T. castaneum*.

Unlike the synergism observed in some mixtures, in several cases the insecticidal activity of essential oil components was antagonized when mixed. We found that diallyl disulfide was most toxic alone but in binary mixture showed low effectiveness.

5. Conclusion

In conclusion, the individual monoterpenoids, and their mixtures showed a potential to be developed as possible natural insecticides for the

control of *T. castaneum* but needs further evaluation to enhance their insecticidal and antifeedant activity and effects on insect digestive physiology when fed on contaminated diets in the stores.

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In Vitro Antibacterial Activity of Selected Medicinal Plants Traditionally Used in Iran Against Plant and Human Pathogenic Bacteria

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Abstract

Medicinal plants are widely used for the treatment of different plant diseases and hospital infections caused by bacteria. The present study aims at determining the in vitro antibacterial activity of the medicinal plants traditionally used in Iran against the bacterial species associated with plant diseases and hospital infections. The antibacterial usefulness of methanol extracts of five medicinal plants is tested against seven Gram-negative bacteria and three Gram-positive bacteria using the agar disc diffusion method. Some of the plant extracts showed antibacterial activity against Gram-positive bacteria and Gram-negative bacteria. The high antibacterial activity against the Gram-positive species and Gram-negative was in the extract of *Geum urbanum* against *Bacillus subtilis* (+++) and *Pseudomonas aeruginosa* (+++). Consequently, plant *Geum urbanum* was subjected to gas chromatography-mass spectrometry (GC-MS) analysis. Results show that its antibacterial activity may be due to the presence of eugenol, phenolic acid and tannin. The present study finds clear evidence supporting the traditional use of the plants in treating plant diseases and hospital infections related to bacteria. These plant species showed a moderate to a high antibacterial activity against the bacteria tested.

Keywords: Medicinal plants; Antibacterial activity; Agar disc diffusion method; Inhibition zone; GC-MS analysis.

1. Introduction

Recently, antibiotics have been generated with a target of eliminating the microorganisms which were liable for many diseases as well as the emergence of antibiotic resistance and the failure of chemotherapy are increasing (Fankam et al., 2014). Furthermore, effects the extensive use of the synthetic medicines may lead to serious damages to many of human organs (Gupta et al., 2016). One strategy to avoid this is by using alternative therapeutic agents from plants that are effective against antibiotic resistant bacteria and have low cost (Wikaningtyas et al., 2016). Medicinal plants have great importance in the control of human diseases. Plant materials are calling secondary metabolites used as a source of numerous natural products as a crude extract or as purified products are employing in control and treatment of various

types of diseases globally (Cowan, 1999). The World Health Organization (WHO) has stated that medicinal plants are the richest and the best source for obtaining a variety of therapeutic agents (Gupta et al., 2016; Alo et al., 2012). Medicinal plants constitute credible sources for a huge number of modern antibiotics, several of which are usually based on their traditional folk medicine.

Khattab et al. (2016) reported that methanolic/chloroform extract of olive showed activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Iqbal et al. (2014) reported that the methanol extract of *Taraxacum officinale* was found to be effective against the tested bacterial pathogens *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus Subtilis*. Trabelsi et al. (2014) reported that *Citrus aurantium* (blossoms) extract was active only against *Staphylococcus aureus*. Soni et al. (2012) reported that the methanol extracts of *Datura*

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stramonium showed activity against Gram-positive bacteria and little or no antimicrobial activity has against *Escherichia coli* and *Pseudomonas aeruginosa*. Sudjana et al. (2009) have investigated the activity of a commercial extract derived from the olive leaves of *Olea europaea* and indicated that *Olea europaea* extract did not show broad-spectrum activity and has appreciable activity only against *Campylobacter jejuni*, *Helicobacter pylori* and *Staphylococcus* spp.

In the present study the antibacterial activity of seven medicinal plants against ten pathogenic bacteria was evaluated.

2. Materials and Method

Bacteria Isolates

All pathogenic bacteria used in the present study were included *Bacillus subtilis* (PTCC 1023), *Staphylococcus aureus* (PTCC 1431), *Rathayibacter toxicus* (ICMP 9525), *Escherichia coli* (PTCC 1330), *Pseudomonas aeruginosa* (PTCC 1074), *Pseudomonas syringae*, *syringae* (ICMP 5089), *Pseudomonas viridiflava* (ICMP 2848), *Xanthomonas campestris*, *campestris* (ICMP13), *Acidovorax avenae* and *Erwinia amylovora*. They

were obtained from Sari Agricultural Sciences and Natural Resources University (SANRU), the laboratory of microbiology, Sari, Iran. The isolates were subcultured on Nutrient Agar (NA) plates. The incubation condition was 37°C for quality control species and 27°C for plant bacteria for 24h. For the antibacterial activity test, a loopful of the organisms were inoculated individually into 5.0ml of nutrient broth and incubated at 37°C for quality control strains and 27°C for plant bacteria for 24h. 0.2ml from the 24 hours culture organism was dispensed into 10ml sterile nutrient broth and incubated for 3-5 hours to standardize the culture to 10⁶ CFU/ml (Abalaka et al., 2012).

Plants Materials

Different plants, such as *Geum urbanum* (leaves and roots), *Citrus aurantium* (blossoms), *Datura stramonium* (leaves and stems), *Olea europaea* (leaves), *Taraxacum officinale* (leaves and roots), were collected from North of Iran, Mazandaran province (Sari and Chalous cities). Table 1 shows the traditional uses and the plants parts used. The fresh samples were collected and washed with distilled water to remove impurities. The plants were shade-dried and pulverized to powder in a mechanical grinder.

Table 1. Traditional uses, parts used traditionally and bioactive or potentially bioactive components

Species (family)	Traditional uses	Parts used traditionally	Bioactive or potentially bioactive components
<i>Citrus aurantium</i>	Antidepressant, treating all types of negative emotional conditions, states of anxiety, menopause, insomnia, improves elasticity and antiseptic effects	Flowers, Peel, Leaves, Fruit	Adenosine, Asparagine, Tyrosine, Valine, Isoleucine, Alanine, limonexic acid, Geraniol, Eugenol, Menthol, Cinnamic aldehyde, Catechin tannins, Gallic, Tannins, Flavonoids, Polyphenols, Sterols, Polyterpenes and Alkaloids
<i>Geum urbanum</i>	Treatment acute diarrhea, astringent agent for inflammations of the mucosa, gums and treatment of hemorrhoids	Roots, Rhizomes, Leaves	Vicianose, Catechin, Gallic acid, Caffeic acid, Chlorogenic acid, Ellagic acid and Phenylpropanoid
<i>Datura stramonium</i>	Treatment of asthma, sinus infections, burns, ulcers, rheumatism, falling hair, hand ruff, madness, epilepsy, depression and relief of headache, asthma and bronchitis	Stem, Leaves, Flowers, Bark	Alkaloids, Atropine, Hyoscyamine, Scopolamine, Ascorbic-acid and Allantoin
<i>Olea europaea</i>	Analgesic and antiasthmatic	Fruit, Oil, Leaves	Alkaloids, Tannins, Carbohydrates and Proteins
<i>Taraxacum officinale</i>	Treat digestive disorders, infections, bile and liver problems	Flowers, Root, Leaves	Flavonoids, Caffeic, Chlorogenic acid, Terpenoids, Triterpenes and Sesquiterpenes

Preparations of Extracts

Methanol was used to extract the plant samples because it is known to be powerful extraction solvent for antibacterial compounds from plants (Vu *et al.*, 2016; Parekh *et al.*, 2005). The various parts of the plants were dried under shade at room temperature and then cut into small pieces. About 100g of powdered plant material was macerated in methanol (Kamali *et al.*, 2015). The extracted suspensions were filtered through Whatman No. 1 filter paper, and the filtrates were concentrated to dryness using a rotary evaporator (Vu *et al.*, 2016; Kamali *et al.*, 2015; Khaleel *et al.*, 2016). Extracts were then collected into a sterile goblet and were stored in the refrigerator at 4°C for future studies (Kamali *et al.*, 2015; Nagat *et al.*, 2016; Khaleel *et al.*, 2016). Subsequently, all the plant extracts were screened for their antimicrobial activity.

Antibacterial Activity of Extracts

The effects of extracts on bacterial growth were measured *in vitro* by agar disc diffusion method (Essawi and Srouf, 2000). 150µl of standardized bacterial suspension was spread over 20mm thick appropriate media with a swab. 20µl of the extracts (with different concentrations of 500, 250, 120, 60, 30 and 10 mg/ml DMSO) and 20µl of the DMSO were put on sterile paper disc and placed on the nutrient agar plates. Plates were incubated at 37°C for quality control strains and 27°C for plant bacteria for 24h. The assays were performed in four repeats (Kamali *et al.*, 2015).

Disc containing DMSO was used as control, which did not effect on the bacterial growth. The

measures were done with a ruler in millimeter (mm) and the zones around the discs recorded as inhibition zone for extracts (Kamali *et al.*, 2015; Khaleel *et al.*, 2016), according to the previously described method of Kamali *et al.* (2015) with some modifications.

Chromatographic Analysis by GC-MS

The essential oil of *Geum urbanum* was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) for identify and quantify the products composing it (Figure 1). The GC-MS analysis performed on an Agilent 7890A GC apparatus with a 5975 C mass spectrometer detector.

AHP5ms capillary column (30m* 4.6mm (id)* film thickness 0.25 µm) was used. The operating conditions were as follows: Carrier gas: helium, at a flow rate of 1ml/min; split ratio 1:10; injector temperatures: 250°C; detector temperatures: 290°C; sample size: 1µL of *Geum urbanum* essential oil, manual injection; oven temperature program: 50°C as an initial temperature, 5min isothermal raised to 280°C at a rate of 5°C/min, then isothermal at 280°C for 10min; ion source temperature: 230°C; energy ionization: 70eV; electron ionization spectra with a mass scan range of 50e550. The compounds of *Geum urbanum* essential oil were identified by comparing their Retention Indices (RI) mass spectra with National Institute of Standards and Technology (NIST 08) library data compounds were expressed as percentages of the peak area to the total oil peak area.

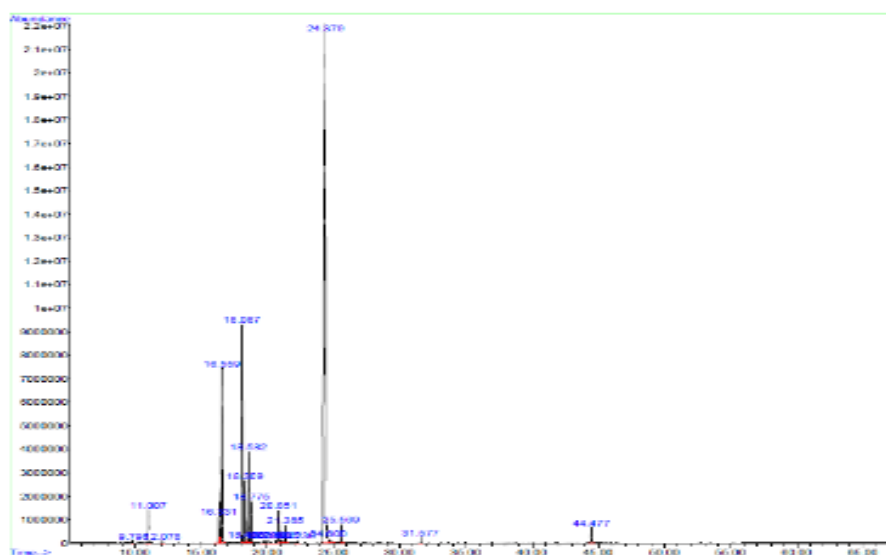


Figure 1: GC – MS chromatograph *Geum urbanum* roots

3. Results

Successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure.

The methanol extract of 5 plants belonging to 5 families were tested against 3 Gram-positive and 7 Gram-negative bacteria using agar disc diffusion method.

Antibacterial Activity

In the present study, zones of growth inhibition around the discs measured with agar disc diffusion method. The antibacterial activity against each bacterium was observed to be varied.

The results revealed that *Geum urbanum* leaves methanolic extracts showed the maximum activity against all bacteria, including *Pseudomonas aeruginosa* (+++), followed by *Pseudomonas viridiflava*, *Bacillus subtilis*, *Rathayibacter toxicus*, *Xanthomonas campestris*, *Acidovorax avenae*, *Staphylococcus aureus*, *Pseudomonas syringae*, *Erwinia amylovora* and *Escherichia coli*, respectively. The maximum antibacterial activity of *Geum urbanum* root extract was observed against: *Pseudomonas aeruginosa* (+++), followed by *Escherichia coli*, *Pseudomonas viridiflava*, *Rathayibacter toxicus*, *Pseudomonas syringae*, *syringae*, *Bacillus subtilis*, *Acidovorax avenae*, *Xanthomonas campestris*, *Staphylococcus aureus* and *Erwinia amylovora*. The antibacterial activity of *Datura stramonium* plant leave extract showed the activity against *Pseudomonas viridiflava*, followed by *Rathayibacter toxicus* (+++), *Bacillus subtilis* (+++) and did not show any inhibitory activity

against the other test pathogens. Stem extract of *Datura stramonium* showed activity against *Pseudomonas aeruginosa* (+++), *Rathayibacter toxicus* (+++). The *Citrus aurantium aurantium* (blossoms) extract showed the activity against *Pseudomonas viridiflava*, *Xanthomonas campestris* and *Staphylococcus aureus*. The *Olea europaea* (Leaf) showed moderate growth inhibition effects against *Staphylococcus aureus*. Leaves and roots extract of *Taraxacum officinale* did not show any antibacterial activity against the tested pathogens.

The highest antibacterial activity against the Gram-positive species was in the extract of *Geum urbanum* leave against *Bacillus subtilis* (+++). The most active extract against Gram-negative bacteria was extract of *Geum urbanum* for *Pseudomonas aeruginosa* (+++).

GC-MS analysis plays a key role in the analysis of components of plant origin. Among the five plants, *Geum urbanum* showed the significant antibacterial activity. Consequently, root (Figure 1) and leave (Figure 2) of *Geum urbanum* were subjected to GC-MS analysis.

The results of the analysis by GC-MS of the chemical composition of the *Geum urbanum* are presented in Table 2. The major constituents of the oil were Phytol (20.6%), Decane (11.1%), Limonene (10.5%), Beta-bisabolene (10%), Dodecane (9.9%), Bicyclo [3.1.1] hept-2-ene (4.9%) and Tetradecane (CAS) (4.6%). The root major phytoconstituents were Eugenol (57.5%), Benzeneacetonitrile (CAS) (13.1%), 6, 6-dimethyl (11.5%), Myrtenal (4.8%) and Benzeneacetonitrile (CAS) (2.6%).

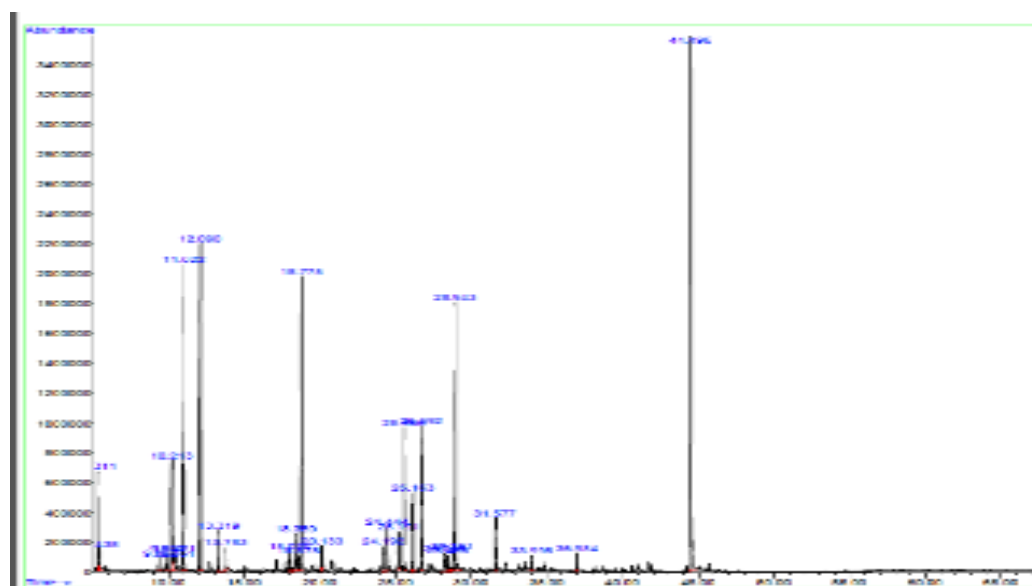


Figure 2. GC – MS chromatograph of *Geum urbanum* leaves

Table 2. The quantitation of antimicrobial activity for plant extracts measured by the agar disc diffusion method. The effectiveness of extracts is demonstrated by the size of the microorganism growth inhibition zone around the filter paper disc

Plant	Microorganism									
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>R. toxicus</i>	<i>E. coli</i>	<i>P. Syringae syringae</i>	<i>P. aeruginosa</i>	<i>P. viridiflava</i>	<i>E. amylovora</i>	<i>A. avenae</i>	<i>X. campestris</i>
<i>Geum urbanum</i> (leave)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>Geum urbanum</i> (root)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>Citrus aurantium</i> (blossoms)	-	+++	+	+	+	++	+++	-	+	+++
<i>Datura stramonium</i> (leave)	+++	-	+++	-	+	+	+++	+	++	++
<i>Datura stramonium</i> (stem)	-	-	+++	-	-	+++	-	+	-	-
<i>Olea europaea</i> (leave)	-	+	-	-	-	-	-	+	+	-
<i>Taraxacum officinale</i> (leave and root)	-	-	-	-	-	-	-	-	-	-

a) Diameter of the inhibition zone: no inhibition (-), 8-9.5 mm (+), 10-12 mm (++), > 12 mm (+++).

4. Discussion

Medicinal plants are widely used for the treatment of different plant diseases and hospital infections caused by bacteria. In this paper, the antibacterial activities of extracts from plants used by tribals in Iranian folklore medicine were reported. The results revealed that *Geum urbanum* methanolic extracts showed the maximum activity against the tested bacterial pathogens that the antibacterial activity of *Geum urbanum* may be due to presence of eugenol, phenolic acids and tannins (Kuczerenko *et al.*, 2011).

The *Datura stramonium* plant leave extract showed the antibacterial activity against gram positive bacteria *Bacillus subtilis*, *Rathayibacter toxicus* and Gram-negative bacteria *Pseudomonas viridiflava* and little or no antimicrobial activity has against *Escherichia coli* and *Pseudomonas aeruginosa*, our findings are in agreement with the study of Sony *et al.* (2012).

The *Citrus aurantium* (blossoms) plant extract showed the activity only against *Pseudomonas aeruginosa*. our findings are in according with Ammar *et al.* (2012). The *Olea europaea* (Leaf) showed moderate growth inhibition effects (9.8–10.4 mm) against *Staphylococcus aureus*. It is in accordance with Sudjana *et al.* (2009). *Taraxacum officinale* (leaves and roots) extracts did not show any antibacterial activity against the tested pathogens but Iqbal *et al.* (2014) reported that the

extract of *Taraxacum officinale* was found to be effective against the tested bacterial pathogens *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus Subtilis*. In the present study, we reported a high resolution GC-MS method for the evaluation of the chemical constituents *Geum urbanum* plant. The major bioactive compounds in *Geum urbanum* are eugenol, phenolic acids and tannins as mentioned by Kuczerenko *et al.* (2011).

The results obtained here in further encourage the use of *Geum urbanum* extract in antibacterial formulations due to the fact that *Geum urbanum* extract effectively kills pathogenic bacteria related to plant diseases and hospital infections (Vu *et al.*, 2016).

5. Conclusions

All the plant species evaluated in the present study are currently used traditionally for the treatment of various diseases (Table 1) and showed moderate to high antibacterial activity against the bacteria tested. The antibacterial activity of *Geum urbanum* was highly significant for *Pseudomonas aeruginosa* and *Bacillus subtilis*. Finally, the results of the present study clearly elucidate the antibacterial activities of these plants and provide an evidence to support their use in folk medicine.

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Authors' Contributions

Gh. K, T. T and S. Gh designed the experiment and revised the manuscript with co-author. A. G. conducted the experimental work, wrote the article and corrected it.

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Microbial Quality and Antibiotic Sensitivity Pattern of Isolated Microorganisms from Street Foods Sold in Akure Metropolis, Nigeria

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Abstract

The frequent incidence of food borne diseases had been attributed to the unhygienic status of street foods. Therefore, microbial quality and sensitivity pattern of isolated microorganisms from some street foods sold in Akure metropolis were examined. Some indicator parameters were also adopted to measure the sanitary qualities of the vendors' premises. The microbial load, occurrence of microorganisms and percentage of antibiotic resistance were determined using standard microbiological methods. The bacterial and fungal count obtained from examining street foods ranged from $(1.1 \times 10^2 - 1.08 \times 10^6)$ CFU g⁻¹ and $(1.0 \times 10^2 - 8.34 \times 10^4)$ SFU g⁻¹, respectively. *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Enterobacter aerogenes*, *Streptococcus lactis*, *Pseudomonas aeruginosa*, *Bacillus* spp, *Vibrio parahaemolyticus*, *Saccharomyces cerevisiae*, *Aspergillus* spp, *Penicillium* spp, *Rhizopus stolonifer*, *Mucor mucedo* and *Candida albicans* were isolated microorganisms from the street foods. The isolates show a varying degree of resistance to commonly used antibiotics. The percentage resistance shown by these isolates to the antibiotics ranged from 13.3% to 100 %. The microbiological status of the examined food samples suggested that there is the need to monitor the safety of these ready to eat foods sold on the streets. Hence, bodies saddled with the monitoring of such foods should establish effective measures to ascertain the safety of these foods for unsuspecting consumers.

Keywords: Food safety, foodborne, microbial load, food vendor, MDR.

1. Introduction

Street food vending and fast food enterprises are one of the major businesses that contribute to the socio-economic development in many countries (Rane, 2011). The street foods and fast foods had been the choice of many people especially the urban dwellers because the food is ready - to - eat, cheap, convenient, sweeten with a different flavor and accessible as immediate want (Rath and Patra, 2012). The busy activities and long-term schedule of individual per day have opened ways for the increased number of street- hawk foods and fast foods. These foods are commonly sold at bus stops, industrial sites, marketplaces, pupil school's gates, campuses, interstate highways and stalls at corner of the streets, where there are numerous consumers. This operation is carried out at locations that do not meet the sanitary qualities and specification of food

safety bodies (FAO/WHO, 2010). Although, street foods have partially alleviated the problem of food insecurity and hunger by its steady availability but major concerns still remain its hygienic status during production and marketing system.

Street foods are rich in high-level of white flour, sugar, polyunsaturated fat, salts, a combination of different spices, flavour and numerous additives to fascinate consumers. This content with other factors, such as moisture, exposure to air and temperature of storage, are supportive growth factors for different pathogenic microorganisms (FAO, 2009). Another important reason that necessitates the assessment of the microbial quality of street food is, several methods are adopted in the processing of these foods, which involves different people rendering assistance during the production to hasten readiness, in order to meet up with the choice of people within a short time.

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The incidence of foodborne diseases is increasing rapidly due to unhygienic methods of processing food (WHO, 2015a). Most microbial etiologic agents of foodborne diseases have developed resistance to commonly used antibiotics (CSPI, 2013). The transmission of antibiotic resistance genes to another pathogenic organism has contributed to food illnesses, higher morbidity and mortality rates (Economou and Gousia, 2015). It is therefore expedient, to carry out microbiological analysis to investigate the quality and safety of street foods in most of our cities and local communities. The present study is therefore undertaken to provide information on the microbial load, type of organisms and antibiotic sensitivity pattern of microorganisms isolated from street foods sold in Akure metropolis, Nigeria.

2. Materials and Method

Study Area

Akure is the capital of Ondo State in southwestern, Nigeria. The city has a population density of 387,100. It is occupied by students, farmers, civil servants, artisans. Its coordinates are 7°15'10" N 11°42'11" E. Tertiary institutions, shopping centers and hospitals are available in the city. The city has been classified as an oil-producing state, which has increased the human activities and state economy. During the present study, Akure metropolis was divided into five zones: Federal University of Technology, Akure (FUTA) and its environ as "zone A," Alagbaka and its environ as "zone B," King's Market and its environ as "zone C," Motor Parks in Akure as "zone D," and Highways as "zone E" (Figure 1). Some sanitary qualities were observed at the point of sample collection, such as closeness to drainage, presence of sanitary facility, the occurrence of flies, the presence of hand towel, closeness of the waste bin, hair cover on vendors, apron on the body and staff nail. The frequency of the hygienic status was calculated based on the number of samples collected

Collection of Food Samples

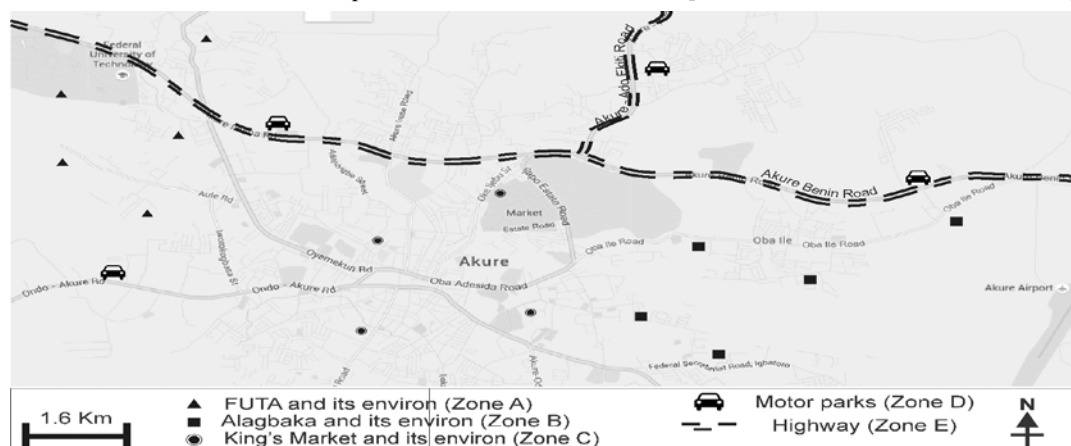
Food samples, such as popcorn, egg roll, meat pie, dough nut, sausage roll, hawk ready to eat foods (HRF), SNK (snacks from international food company) and foods from fast food joints (FSF), were collected from highly populated and busy areas in Akure metropolis. Food samples were aseptically collected from June 2013 to February 2014. The food samples in ice bag were transferred to the laboratory for immediate analysis.

Isolation of Microorganisms

Food samples were homogenized, and one gram of each homogenate was weighed out into 10 ml of sterile water as a stock solution. Serial dilution was carried out to obtain appropriate aliquot. Serially diluted sample (1.0 ml) was transferred into Petri dish containing Plate Count Agar (PCA, Lab M) for bacteria and Potato Dextrose Agar (PDA, Lab M) for fungi. The plates were incubated at 37 °C for 24 h and 48 h for bacteria and fungi, respectively. At the end of the incubation period, colonies were counted using the colony counter (TT-20, Techmel and Techmel, USA). The counts for each plate were expressed as colony forming unit per gram (CFU g⁻¹) for bacteria and spore forming unit per gram (SFU g⁻¹) for fungi. Microbial growth from the plate was subcultured to obtain pure cultures and these were kept at 4 °C for further study.

Identification of Microorganisms

Gram stain technique and biochemical tests were carried out according to the methods of (Cappuccino and Sherman 1999; Cheesbrough 2000) and the results obtained were interpreted according to Bergey's Manual of Systematic Bacteriology (Krieg et al., 2010). Some isolates were further confirmed by using the Analytical Profile Index (API 20 E and API 20 NE kits, API System, Biomerieux, Marcy-l'Etoile, France) and interpreted using API lab plus software. Identification of fungal isolates was carried out by staining with lactophenol blue and examined under a microscope. The feature characteristics of fungi



in each zone.

Figure 1. Map of Akure Metropolis showing zones where street foods were collected

were interpreted according to Samson et al. (2010).

Antibiotic Sensitivity Test

Antibiotic susceptibility test was carried out using the agar disc diffusion method following the recommendation of the Clinical and Laboratory Standards Institute (CLSI, 2012). The inoculum was prepared from 18 h old broth culture of each isolate and their absorbance was adjusted to 0.5 McFarland equivalent. Inoculum size (0.1 mL) was spread on Mueller-Hinton agar and the antibiotic discs were placed at the equidistance of the plate. The antibiotics used include gentamicin (GEN 10 µg), tetracycline (TET 25 µg), chloramphenicol, (CHL 30 µg), erythromycin (ERY 10 µg), amoxicillin (AMX 25 µg), cotrimoxazole (COT 25µg), nitrofurantoin (NIT 20 µg), nalidixic acid (NAL 30 µg), ofloxacin (OFL 5 µg), augmentin (AUG 30 g), streptomycin (STR 30 µg), ciprofloxacin (CFX 30 µg) obtained from Abtek Biological Ltd, Liverpool, L9 7AR, UK. The zones of inhibition were measured and interpreted according to CLSI (2012). The fungal isolates were tested against antifungal drugs, namely ketoconazole (15 µg), fluconazole (25 µg) and nystatin (1µg). This was done according to the standard methods described by CLSI (2009). The Multiple Antibiotic Resistance (MAR) index of the isolates was calculated as a/b , where 'a' represents the number of antibiotics to which the particular isolate was resistant to and 'b' represents the number of antibiotics to which the isolate was exposed.

Table 1: Hygienic status of the vendor's where ready to eat food samples were collected

Hygienic parameter	Frequency and Percent (%)				
	A	B	C	D	E
Closeness to drainage	10 (62.5)	7 (43.8)	14 (87.5)	NA	12 (75.0)
presence of sanitary facility	8 (50.0)	12 (75.0)	4 (25.0)	0 (0.0)	3 (18.8)
presence of hand towel	10 (62.5)	12 (75.0)	8 (50.0)	0 (0.0)	2 (12.5)
waste bin within	7 (43.8)	6 (37.5)	12 (75.0)	0 (0.0)	9 (56.3)
hair cover on Vendor	4 (25.0)	8 (50.0)	15 (93.8)	6 (50.0)	12 (75.0)
Apron on body	5 (31.3)	4 (25.0)	11 (68.8)	0 (0.0)	8 (50.0)
Vendor's nail well kept	13 (81.3)	10 (62.5)	8 (50.0)	5 (41.7)	4 (25.0)
Total number of area sampled	n=16	n=16	n=16	n=12	n=16

A =FUTA zone, B=Alagbaka zone, C= King's market zone, D= Highways, E= Motor parks, NA: not available, n= no of samples, value outside the bracket is the frequency while value inside the bracket is the percentage calculated for the hygienic parameter.

Table 2: Total Bacterial count (CFU g⁻¹) in sampled Street foods from different zones in Akure metropolis

Food samples	A	B	C	D	E
Pop corn	3.10 ^a x10 ²	5.60 ^c x10 ²	4.00 ^{ab} x10 ²	5.30 ^c x10 ²	5.10 ^{bc} x10 ²
Egg roll	3.60 ^a x10 ⁴	6.70 ^b x10 ⁴	6.40 ^b x10 ⁴	1.00 ^c x10 ⁵	5.70 ^b x10 ⁴
Meat pie	5.00 ^b x10 ⁵	2.60 ^a x10 ⁵	1.08 ^d x10 ⁶	6.70 ^c x10 ⁵	5.00 ^b x10 ⁵
Dough nut	2.40 ^a x10 ⁴	7.00 ^b x10 ⁴	1.10 ^c x10 ⁵	3.40 ^{ab} x10 ⁴	5.30 ^{ab} x10 ⁴
SNK	1.10 ^a x10 ²	1.10 ^a x10 ²	1.10 ^a x10 ²	1.10 ^a x10 ²	1.10 ^a x10 ²
Sausage roll	5.50 ^{ab} x10 ⁴	1.70 ^a x10 ⁴	1.20 ^c x10 ⁴	6.70 ^{bc} x10 ⁵	9.80 ^c x10 ⁴
HRF	4.60 ^{ab} x10 ⁵	7.40 ^{bc} x10 ⁵	7.70 ^c x10 ⁵	ND	3.70 ^a x10 ⁵
FSF	9.90 ^b x10 ⁴	9.90 ^b x10 ⁴	1.26 ^c x10 ⁵	ND	5.30 ^a x10 ⁴

Values are mean of replicates (n=3), means with different letters within a row, for each count obtained from food sample are significantly different by Duncan test (P < 0.05). A =FUTA zone, B=Alagbaka zone, C= King's market zone, D= Highways, E= Motor parks, ND= Samples are not available at zone, SNK: snack produced by an international food company, FSF: food samples from fast food joints, HRF: hawk ready to eat foods.

Statistical Analysis

Experiments were carried out in triplicates. Data obtained from the present study were analyzed by one-way analysis of variance (ANOVA) and means were compared with New Duncan's Multiple Range Test (SPSS 17.0 version). Differences were considered significant at $P \leq 0.05$

3. Results

The hygienic status of each vendor at different zones was recorded in Table 1. The frequency and percentage of the hygienic condition were reported based on the number of the area examined.

Table 2 shows the total bacterial count (CFU g⁻¹) obtained from examined street foods. The total bacteria count (CFU g⁻¹) for popcorn in all the zones ranged from 3.10×10^2 - 5.60×10^2 , egg roll (3.60×10^4 - 1.00×10^5), meat pie (2.60×10^5 - 1.08×10^6), dough nut (2.40×10^4 - 1.10×10^5), sausage roll (1.70×10^4 - 9.80×10^4), hawked ready to eat foods [HRF] (3.7×10^5 - 7.70×10^5), food samples from fast food [FSF] (5.30×10^4 - 9.90×10^4). Meat pie possessed highest bacteria count of 1.08×10^6 CFU g⁻¹ ($p < 0.05$), while snack produced by a food company (SNK) has the lowest and consistent bacteria count of 1.10×10^2 CFU g⁻¹.

Fungal count in street foods collected from different zones in Akure metropolis is reported in Table 3. The fungal count (SFU g⁻¹) obtained for popcorn in all zones ranged from 6.00×10^2 to 1.90×10^3 , egg roll (4.00×10^3 - 1.90×10^4), meat pie (9.60×10^3 - 2.20×10^4), doughnut (7.60×10^2 - 1.90×10^3), snack produced by a food company [SNK] (1.00×10^2 - 1.10×10^2), sausage roll (6.00×10^3 - 1.80×10^4), hawk ready to eat foods [HRF] (5.50×10^3 - 8.30×10^4) and food samples from fast food [FSF] (7.60×10^3 - 1.30×10^4). Meat pie examined

in zone E has 2.20×10^4 SFU g⁻¹, while snack produced by a food company (SNK) in zone A has a lowest fungal count of 1.00×10^2 SFU g⁻¹, which was not significantly different ($P < 0.05$) from what obtained in other zones.

Tables 4 and 5 show the occurrence of microorganisms isolated from the street foods with the highest occurrence of *S. aureus* (23.3 %) and *Saccharomyces cerevisiae* (34.3 %), respectively. Twelve bacteria and eight fungi were isolated from the sampled street foods from Akure in Nigeria.

Table 3: Fungal count (SFU g⁻¹) from sampled junk foods collected from different zones in Akure metropolis

Food samples	A	B	C	D	E
Pop corn	$6.00^a \times 10^2$	$1.00^b \times 10^3$	$1.30^c \times 10^3$	$7.50^a \times 10^2$	$1.90^d \times 10^3$
Egg roll	$4.00^a \times 10^3$	$1.90^c \times 10^4$	$1.60^c \times 10^4$	$6.00^a \times 10^3$	$1.20^b \times 10^4$
Meat pie	$1.00^a \times 10^4$	$2.70^b \times 10^4$	$1.40^a \times 10^4$	$9.60^a \times 10^3$	$2.20^b \times 10^4$
Dough nut	$7.60^a \times 10^2$	$7.60^a \times 10^2$	$1.50^b \times 10^3$	$8.00^a \times 10^2$	$1.90^c \times 10^3$
CNP	$1.00^a \times 10^2$	$1.10^a \times 10^2$	$1.10^a \times 10^2$	$1.10^a \times 10^2$	$1.10^a \times 10^2$
Sausage roll	$1.80^c \times 10^4$	$6.00^a \times 10^3$	$1.20^b \times 10^4$	$8.80^a \times 10^3$	$1.70^c \times 10^3$
HRF	$5.50^a \times 10^3$	$9.00^b \times 10^3$	$8.30^{ab} \times 10^4$	NA	$1.60^c \times 10^4$
FSF	$1.00^{ab} \times 10^4$	$7.60^a \times 10^3$	$1.00^a \times 10^4$	NA	$1.30^b \times 10^4$

Values are mean of replicates (n=3), Values are mean of replicates (n=3), means with different letters within a row, for each count obtained from food sample are significantly different by Duncan test ($P < 0.05$). A =FUTA zone, B= Alagbaka zone, C= King's market zone, D= Highways, E= Motor parks, NA= Samples are not available at zone, SNK: snack produced by a food company, FSF: food samples from fast food joints, HRF: hawk ready to eat foods.

Table 4. Distribution and occurrence (%) of bacterial isolates from examined street foods in different zones

Bacterial Isolates	Zones ^a	Pop corn	Egg roll	Meat pie	Dough nut	SNK	Sausage roll	HRF	FSF	N	% occurrence
<i>S. aureus</i>	A, B, C, D, E	+	+	+	+	-	+	+	+	24	23.30
<i>E. coli</i>	A, B, C, D, E	-	+	+	-	+	+	+	+	18	17.50
<i>S. typhi</i>	A, B, C, D, E	-	+	+	+	-	+	+	+	15	14.60
<i>S. dysenteriae</i>	A, B, C, D, E	-	+	+	-	+	+	+	+	9	8.70
<i>S. lactis</i>	A, B, C, D	+	-	-	+	+	+	-	+	7	6.80
<i>B. subtilis</i>	B, C, D, E	-	+	+	-	-	-	+	+	7	6.80
<i>P. aeruginosa</i>	A, B, C, D	-	+	+	-	-	-	+	-	7	6.80
<i>K. pneumoniae</i>	B, D, E	-	+	+	-	-	+	-	+	5	4.90
<i>P. vulgaris</i>	A, C	-	-	-	+	-	-	+	+	3	2.90
<i>B. cereus</i>	D, E	-	-	+	-	-	-	+	+	3	2.90
<i>E. aerogenes</i>	A, C, E	-	+	-	-	-	+	-	-	3	2.90
<i>V. parahaemolyticus</i>	B, E	-	-	-	-	-	+	+	-	2	1.90

-: organisms are absent; +: organisms are present; N= number of isolates

^aBacterial isolate is present in food examined in the zone, where A =FUTA zone, B=Alagbaka zone, C= King's market zone, D= Highways, E= Motor parks, SNK: snack produced by a food company, HRF: hawk foods, FSF: food samples from fast food joints.

Table 5. Distribution and occurrence (%) of fungal isolates from examined street foods in different zones

Fungi isolates	Zones ^a	Pop corn	Egg roll	Meat pie	Dough nut	SNK	Sausage roll	HRF	FSF	N	% Occurrence
<i>Saccharomyces cerevisiae</i>	A, B, C, D, E	–	+	+	+	–	+	+	+	11	34.30
<i>Candida albicans</i>	A, C, D, E	+	+	+	–	–	–	–	+	4	12.50
<i>Penicillium chrysogenum</i>	A, B, D, E	+	–	–	+	–	–	–	+	4	12.50
<i>Mucor mucedo</i>	A, B, D,	–	–	+	–	–	–	+	+	4	12.50
<i>A. niger</i>	C, E	–	–	+	–	+	–	+	–	3	9.40
<i>Penicillium commune</i>	A, C	–	–	+	+	–	–	–		2	6.25
<i>R. stolonifer</i>	C	–	–	–	–	–	+	+	–	2	6.25
<i>A. flavus</i>	C, E	–	–	–	+	–	–	+	–	2	6.25

–: organisms are absent; +: organisms are present; N= number of isolates

^aFungal isolate is present in food examined in the zone, where A =FUTA zone, B=Alagbaka zone, C= King's market zone, D= Highways, E= Motor parks, NA= Samples are not available at zone, SNK: snack produced by a food company, HRF: hawk foods, FSF: food samples from fast food joints.

Table 6 shows the resistance pattern of the bacteria isolates against commercial antibiotics. The isolated microorganisms exhibited varying degree of resistance (13.3-100%) to the readily available antibiotics. *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Salmonella typhi* and *Escherichia coli* displayed multiple antibiotic resistance.

Table 7 shows the percentage resistance of fungi isolated from street foods against antifungal drugs. The value obtained for the percentage

resistance of the fungi was within 33.3% to 100%. *Mucor mucedo*, *Rhizopus stolonifer* and *Aspergillus flavus* were susceptible to the used antifungal drugs.

The Multiple Antibiotic Resistance (MAR) of the isolated bacteria are shown in Table 8. The MAR index ranged from 0.125 to 0.89. Bacteria, *S. aureus*, *E. coli*, *S. typhi*, and *P. aeruginosa* have the higher value of MAR ranging from 0.75 to 0.89.

Table 6. Percentage of resistance (%) of bacterial isolates from street foods against commercial antibiotics.

Bacteria	N	Antibiotics											
		AUG	AMX	GEN	TET	ERY	COT	OFL	STR	NAL	CHL	NIT	CFX
<i>E. coli</i>	18	66.7	83.3	55.5	83.3	NT	50.0	27.7	NT	66.7	NT	55.5	NT
<i>S. typhi</i>	15	66.7	73.3	53.3	53.3	NT	80.0	13.3	NT	66.7	NT	80.0	NT
<i>S. dysenteriae</i>	9	66.7	77.8	55.6	44.4	NT	33.3	0	NT	55.6	NT	66.7	NT
<i>P. aeruginosa</i>	7	71.4	100.0	71.4	85.7	NT	100.0	28.5	NT	71.4	NT	100.0	NT
<i>K. pneumoniae</i>	5	0	40.0	40	60.0	NT	0	0	NT	100.0	NT	60.0	NT
<i>P. vulgaris</i>	3	100.0	66.7	100.0	0	NT	0	0	NT	0	NT	66.7	NT
<i>E. aerogenes</i>	3	0	0	0	33.3	NT	0	0	NT	100.0	NT	100.0	NT
<i>Vibrio parahaemolyticus</i>	2	100.0	100.0	50.0	100.0	NT	0	0	NT	50.0	NT	100.0	NT
<i>S. aureus</i>	24	83.3	NT	41.7	75.0	62.5	54.1	NT	91.6	NT	62.5	NT	50.0
<i>S. lactis</i>	7	28.5	NT	42.8	71.4	42.8	0	NT	0	NT	0	NT	57.1
<i>B. subtilis</i>	7	71.4	NT	85.7	42.8	0	42.8	NT	28.5	NT	42.8	NT	71.4
<i>B. cereus</i>	3	100.0	NT	100.0	33.3	66.6	0	NT	33.3	NT	66.6	NT	100.0

Key: N= number of isolates, 0 = all isolates are susceptible, NT= Antibiotics are absent in selected disc for Gram positive or Gram negative bacteria, code for antibiotics were stated in materials and methods.

Table 7. Percentage resistance (%) of fungi tested against antifungal drugs

Fungi	N	fluconazole	ketoconazole	Nystatin
<i>Saccharomyces cerevisiae</i>	11	0.0	36.6	18.2
<i>Candida albicans</i>	4	75.0	25.0	50.0
<i>Penicillium chrysogenum</i>	4	50.0	50.0	75.0
<i>Mucor mucedo</i>	4	0.0	0.0	0.0
<i>A. niger</i>	3	33.3	66.7	100.0
<i>Penicillium commune</i>	2	50.0	0.0	50.0
<i>R. stolonifer</i>	2	0.0	0.0	0.0
<i>A. flavus</i>	2	0.0	0.0	0.0

N= number of isolates, 0.0 = all isolates are susceptible

Table 8. Number of isolates and value obtained for multiple antibiotic resistance (MAR) index

Bacterial isolates	A		B		C		D		E	
	N	MAR	N	MAR	N	MAR	N	MAR	N	MAR
<i>S. aureus</i>	5	[0 -0.625]	4	[0 -0.75]	6	[0.25-0.75]	4	[0.25-.87]	5	[0.13-0.87]
<i>E. coli</i>	4	[0.13-0.89]	2	[0.75]	4	[0- 0.75]	3	[0.75]	5	[0.5-0.75]
<i>S. typhi</i>	3	[0.13-0.88]	3	[0.25-0.75]	3	[0.13-0.88]	3	[0.25-0.6]	3	[0.125-0.75]
<i>S. dysenteriae</i>	2	[0.25-0.5]	1	[0.5]	2	[0.5 -0.63]	1	[0.63]	3	[0.5]
<i>S. lactis</i>	2	[0.125]	1	[0.5]	3	[0.125-0.5]	1	[0.5]	-	-
<i>B. subtilis</i>	-	-	2	[0.25-0.5]	3	[0.38-0.63]	1	[0.5]	1	[0.5]
<i>P. aeruginosa</i>	2	[0.5-0.875]	2	[0.625-0.75]	2	[0.5 -0.88]	1	[0.75]	-	-
<i>K. pneumonia</i>	-	-	3	[0.125-0.5]	-	-	1	[0.5]	1	[0.38]
<i>P. vulgaris</i>	1	[0.5]	-	-	2	[0.25-0.5]	-	-	-	-
<i>B. cereus</i>	-	-	-	-	-	-	1	[0.625]	2	[0.5-0.75]
<i>E. aerogenes</i>	1	[0.25]	-	-	1	[0.37]	-	-	1	[0.25]
<i>V. Parahaemolyticus</i>	-	-	1	[0.5]	-	-	-	-	1	[0.75]

Value in parenthesis [*] is the range of Multiple Antibiotic Resistance [MAR] index calculated, while N, the value outside the parenthesis is the number of organisms from the each zones, Values are mean of replicates, -: organisms are absent, A= FUTA zone, B= Alagbaka zone, C= King's market zone, D= Highways and E= Motor parks.

4. Discussion

Street food vendors need to abide by the principles of Good Manufacturing Practices (GMP) in order to produce safe and wholesome foods. Aluko *et al.* (2014) revealed that many street-food vendors do not understand this principle due to lack of knowledge on basic food hygiene. Campos *et al.* (2015) also reported that food-handlers' hygienic status is contributing to the poor microbiological quality and safety of the street foods examined in Porto, Portugal. Thus, the low level of personal and environmental hygiene with the inadequate education of most vendors on the safety of food has discredited the acceptance of most ready-to-eat foods.

The microbiological analysis of the examined foods reveals the number of bacteria and fungi, which has a similar trend to what was reported by Adolf and Azis (2012). These authors have reported varying index from 10^3 to 10^6 for the total aerobic count, coliform count, yeast and mold in foods

served in elementary schools. The higher microbial loads could be a result of the crowded area, environmental pollutants from open places since microorganisms are ubiquitous and most fungal spores disperse uncontrollable (Frazier and Westhoff, 2008). Contaminants from unwashed hands during distribution, materials used for wrapping especially using old newspaper, re-use nylon or polyethylene bags, dirty leaves, continuous use of utensil without cleaning and undercooked methods could also lead to the presence of high microbial loads. The actions highlighted above are directly or indirectly transmitting factors of foodborne pathogens and toxins in foods, which can endanger human health (Proietti *et al.*, 2014). Hence, the presence of a higher microbial load in ready to eat food signifies its potential risk. WHO (2015b) showed concerns on the outbreak of foodborne diseases due to improper use of additives, gross contaminations of foods, poor processing methods and low sanitary quality of the environment.

Some *genera* that belong to the family Enterobacteriaceae, *Staphylococcus aureus*, *Bacillus* spp and *Pseudomonas aeruginosa* were isolated in the present study. Chung *et al.* (2010), Nyenje *et al.* (2012), and Kim *et al.* (2013) reported similar microorganisms in ready to eat foods examined in Korea and South Africa. Baker *et al.* (2015) confirmed the transfer of bacteria from hand while eating popcorn as unhygienic status of the food handlers. Clarence *et al.* (2009) also reported the occurrence of the *S. aureus*, *E. coli*, *Klebsiella* spp, *Pseudomonas* spp, *Bacillus* spp and *Enterococcus* spp in meat pie sold in Benin City, Nigeria. These microorganisms may originate from any of the following sources: raw materials, the use of low quality food materials that is half rotten because they are cheap, the body contact with food during preparation, the poor storage of foods by food sellers, the selling street-foods close to sewage drainage, the exposure to dusts and smokes from vehicles, the use of contaminated water for cooking and the re-distribution of leftover foods. The finding of Taban and Halkman (2011) associated the presence of pathogenic organisms to the potential risk of microbiological contamination due to the usage of untreated irrigation water or sewage, methods of slaughtering of animal, inappropriate organic fertilizers or inadequately composted manure, the harvesting, the handling, processing and distributing during the restaurant service.

The high prevalence of *S. aureus* (Table 4) could be associated with its resistance to heat, drying and radiation (Adam and Moss, 2009), which could make it survive some of the processing stages during food preparation. *S. aureus* has been identified as an important foodborne pathogen due to its ability to produce heat stable and potent enterotoxin; a common food poisoning agent. Also, the occurrence of *E. coli*, *Shigella* spp, *Vibrio* spp and *Salmonella* spp in street food was reported by Nyenje *et al.* (2012) and Mugampoza *et al.* (2013). These microorganisms are responsible for several infections, such as diarrhea, typhoid fevers and gastroenteritis. Therefore the presence of coliforms isolated in the present study reveals the hygienic level under which these foods are prepared. FAO (2009) stated that these organisms are presently used as an indicator of the microbial quality of foods since these microorganisms are a typical component of the fecal microbiota and their detection specify the potential occurrence of other microorganisms.

Most of the snacks and street foods were made from cereal flours, corn, sugar, honey, chocolate and other additives, which could be the source of fungi; *Aspergillus* spp, *Penicillium* spp, *Rhizopus stolonifer*, *Mucor mucedo* in the tested street foods. The findings of Makun *et al.* (2010) revealed the colonization of these fungi in some foods and their mycotoxin producing potentials.

The microorganisms isolated from the ready to eat foods showed significant resistance to commonly used antibiotics. Oluyeye *et al.* (2009)

and Akinyemi *et al.* (2013) reported similar antibiotic resistance percentage of 20% to 100 % for *S. aureus*, *E. coli*, *Salmonella* spp, *Shigella dysenteriae*, *Pseudomonas aeruginosa* against some commercial antibiotics. A marked resistance of microorganisms to commonly used antibiotics, like amoxicillin, tetracycline, cotrimoxazole, gentamicin, nalidixic acid, chloramphenicol, ciprofloxacin, streptomycin were associated with coexistence of resistance genes with mobile elements, such as plasmids, transposons and integrons (Sunde and Nordstrom, 2006; Thong and Modarressi, 2011). The transfer of resistance gene in food products and the environment were directly or indirectly linked to several human activities, such as the use of antibiotics in farming to produce some edible foods (Economou *et al.*, 2013). The emergence of antibiotic-resistant microorganisms from foods raises concern as most of the resistant strain spread into other environments where they can infect man through some conscious or unconscious activities.

5. Conclusion

The presence of bacteria and fungi in the examined street foods shows that prompt attention of public health officers is needed to train food vendors on how to produce safe foods that will reduce the incidence of foodborne diseases and the transfer of antibiotic resistant microorganisms from food to man.

6. Recommendation

Adequate enlighten programme is important for the food vendors on the principle of good manufacturing practice to ensure the production of safe food and prevent the spread of pathogenic microorganisms in the foods serve to the cities.

No conflict of interest

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Assessment of Antioxidant Activity of Ethanol and n-Hexane Seed Extracts of *Annona muricata* Rats

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Abstract

The *in vitro* and *in vivo* antioxidant effects of *Annona muricata* seed extracts (n-hexane and ethanol extracts) were investigated using ascorbic acid as standard. Free radical scavenging activity *in vitro* was evaluated using 2,2-diphenyl-1-picryl-hydrazyl (DPPH). Lipid peroxidation was assayed using TBARS. Reduced glutathione and catalase activity were also investigated. Twenty-four male albino rats, divided into six groups were used for the *in vivo* assay. Group A (control) received olive oil, group B and C received 200mg/kg n-hexane and ethanol extracts, respectively, group D received ascorbic acid, group E and F received 100mg/kg of n-hexane and ethanol extracts, respectively. Ethanol and n-hexane extracts at 100µg/ml and 20µg/ml, respectively, exhibited 49% and 32% inhibition of DPPH radical, respectively. Ascorbic acid (standard) exhibited upto 96.9% inhibition of DPPH radical even at 20µg/ml. The extracts significantly increased catalase activity, glutathione levels and reduced the formation of malondialdehyde in the treated groups compared with the control especially in the heart and liver tissues. The results of the present study suggest that *Annona muricata* seed extracts could be a promising source of potent antioxidants that could inhibit lipid peroxidation in tissues as well as ameliorate oxidative stress.

Keywords: *Annona muricata*, Antioxidants, lipid peroxidation, 2,2-Diphenyl-1-picrylhydrazyl Ascorbic acid, Catalase Glutathione.

1. Introduction

The ability to utilize oxygen has provided humans with the benefit of metabolizing fats, proteins, and carbohydrates for energy; however, oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called “free radicals.” These free radicals are capable of attacking healthy cells of the body, causing them to lose their structure and function (Carr and Frei, 1999).

Cell damage caused by free radicals appears to be a major contributor to aging, degenerative diseases, such as cancer, cardiovascular disease, cataracts, immune system decline, and brain dysfunction (Sies et al., 1992). Free radical formation is however controlled naturally by compounds known as antioxidants (Poongothai et al., 2011). These include α -Tocopherol, ascorbic acid, carotenoids, flavonoids and related polyphenols, α -lipoic acid, glutathione etc. (Devasagayam et al., 2004).

Annona muricata, family (Annonaceae), commonly known as ‘soursop’ or ‘Graviola’, is a deciduous, terrestrial, erect tree of about 5-8 meters

in height. Although a native of America, it has now been naturalized and is established in many tropical countries of the world. The plant is used medicinally in many tropical African countries for an array of human ailments, especially for parasitic infections and cancer (Adewole et al., 2009). Preliminary studies have confirmed the antioxidant activity of *Annona squamosa* – a different species in different *in vitro* models (Baskaret et al., 2007). In continuation of the search for potential free radical scavenging agents (Kokate, 1999), the present study is designed to establish the antioxidant capacity of ethanol and n-hexane seed extracts of *Annona muricata* in albino rats.

2. Materials and Method

2.1. Preparation of Plant Sample

The matured fruits of *Annona muricata* were collected from Abocho in Dekina local government area, Kogi State, Nigeria. The fruits were identified by department of herbarium and ethnobotany, National Institute of Pharmaceutical Research and Development (NIPRD), Abuja Nigeria; with the identification number NIPRD/H/6164.

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The seeds were collected from the ripe fruits and air dried at room temperature, de-shelled to expose the inner fleshy core of the seeds, which were air dried and then blended, with a sterilized iron hand blender and later fine-blended using an electric blender.

2.2. Experimental Animals (albino rats)

Thirty healthy male albino rats weighing 129-250g were purchased from Salem University's animal house. The animals were housed in metallic cages under standard environmental conditions of light, temperature and relative humidity. They were allowed to acclimatize for one week, and were given free access to food (broiler's finisher) and water.

2.3. Animal Groupings

The rats were divided into six groups of five animals each. They were given different doses of the ethanol and n-hexane extracts, reconstituted with olive oil. The different groups are as shown below:

- Group A (control Olive oil)
- Group B 200mg/kg (n-Hexane extract in olive oil)
- Group C 200mg/kg (ethanol extract in olive oil)
- Group D 200mg/kg ('Ascorbic acid' in distilled H₂O)
- Group E 100mg/kg (n-Hexane extract in olive oil)
- Group F 100mg/kg of (ethanol extract in olive oil)

2.4. Preparation of Seed Extracts

About 50g of the blended seed powder was extracted each time using 250ml n-hexane in a Soxhlet extractor. The extraction was allowed to continue for about 2-3 hours, after which the solvent was recovered and the extract further concentrated (using rotary evaporator, Model -ST15 OSA UK) followed by oven drying at 40°C. The bulk extract, 1000mg/ml, was prepared from the dried extract using olive oil. This was stored in sample bottles and kept in a refrigerator for further use. This process was repeated using absolute ethanol as the extraction solvent.

2.5. Administration of Extract

The extracts were administered orally using a cannula (intubator). The animals were treated with different doses of the extracts as shown in the animal groupings (100mg/kg- 200mg/kg) for a period of eight weeks. At the end of this period, the animals were fasted overnight prior to sacrifice. They were weighed and anesthetized using chloroform soaked in cotton wool and sacrificed by humane decapitation.

2.6. Chemicals

All chemicals were of analytical grade. These include, absolute ethanol, n-hexane (Sigma-Aldrich), 2,2-Diphenyl, 1-picryl hydrazine, Ascorbic acid, trichloroacetic acid, thiobarbituric acid and methanol.

2.7. Preparation of Serum

Blood was collected into non-heparinized tubes and centrifuged at 3000 RPM (using a Microfield

centrifuge, Model 90-2) for 10 minutes. The sera were then decanted into another sample tubes and stored in the refrigerator for subsequent use.

2.8. Preparation of Tissue Homogenates

Liver and heart tissues from each animal were rapidly excised during the sacrifice, washed with cold normal saline to remove excess blood, weighed and stored immediately at -4°C. Subsequently, they were homogenized individually (using a Bosch PSB 570-2 homogenizer) in ice-cold phosphate buffer (pH 7.4). The homogenates were centrifuged at 3000 RPM for 15 minutes and the supernatant decanted into sample tubes and kept in the refrigerator for further use.

2.9.0. Assessment of Lipid Peroxidation

Thiobarbituric acid reacting substances (TBARS) in tissue were estimated by the method of (Torres *et al.*, 2004).

Principle

At low pH and high temperature malondialdehyde binds TBARS to form a pink complex that can be measured at 535nm.

Procedure

One milliliter of thiobarbituric acid (TBA) and trichloro acetic acid (TCA) were added to 50µl of the tissue homogenates, respectively. The mixture was incubated for 30 minutes at 80°C. The tubes were allowed to cool immediately under ice and centrifuged at 3000 RPM for 15 minutes. The supernatant was measured using a spectrophotometer at a wavelength of 535nm. The results are expressed as malondialdehyde concentration in µmol/mg protein.

2.9.1. Determination of Catalase (CAT) Activity

The activity of CAT was measured spectrophotometrically as described by (Gott, 1991).

Procedure

About 100µl of liver and heart homogenates, respectively, were mixed with 500µl of hydrogen peroxide at 37°C for a minute. The catalase preparation was allowed to split hydrogen peroxide for different periods of time. The addition of 500µl of ammonium molybdate solution stopped the reaction with a formation of a yellow complex. The absorbance of the yellow complex formed between ammonium molybdate and hydrogen peroxide was then measured at a wavelength of 405nm using a spectrophotometer. One unit of catalase was defined as the amount of enzyme that catalyses a decomposition of 1 micro mole of hydrogen peroxide per min.

2.9.2. Estimation of Reduced Glutathione (GSH) Level

The method of Beutler *et al.*, (1963) was used.

Procedure

An amount of four hundred and fifty microliters (450µl) of distilled water was added to 100µl of test sample and 1.5ml of sulphosalicylic acid added

(deproteinization). The mixture was then centrifuged at 3000RPM for 10mins. 2ml of 0.1M phosphate buffer pH 7.4 and 2.25ml of Ellman's reagent was added to 0.25ml supernatant. Readings were taken within 5mins at 412nm.

2.9.3. Protein Determination

Principle

Under alkaline conditions, substances containing two or more peptide bonds form a purple complex with copper salts in the reagents.

Procedure

An amount of four hundred and fifty milliliter of distilled water was pipette into test-tubes (in duplicate) and 50 μ l of the sample was added after which 1.5ml of biuret reagent was added. The absorbance was read at 540nm.

2.9.4. In Vitro Antioxidant Assay

2,2- Diphenyl-1-picrylhydrazyl Radical Scavenging Activity

This was estimated according to the method described by Mensoret *al.*, 2001.

Procedure

Extracts (40-2000 μ g) in 4ml of distilled water were added to the methanolic solution of DPPH (1mM, 1ml). The mixture was shaken and left to stand at room temperature for 30mins. The absorbance of the resulting solution was measured spectrophotometrically at 517nm for 20, 100, 250, 500 and 1000 μ g/ml of the extracts. The standard used was ascorbic acid dissolved in distilled H₂O.

Calculation

$$\%I = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

where %I is inhibition of the DPPH free radicals in percentage; A_{control} is the absorbance of the control reaction containing all reagents except the test compound and A_{sample} is the absorbance of the test compound.

2.9.5. Statistical Analysis

The results were expressed as mean \pm SD of five animals in each group. The data were evaluated by one way ANOVA using SPSS version 20. $P < 0.05$ was observed to be statistically significant.

3. Results

3.1. Body Weights of Treated Rats

Table 1 shows the effect of ethanol and n-hexane seed extracts on body weight of the treated animals. There was a relative increase in body weights of animals treated with ascorbic acid, and 100mg/kg of the extracts. However, 200mg/kg of both the ethanol and n-hexane seed extract caused a relative decrease in the final body weights of the treated animals.

Table 1. Effect of *Annona muricata* ethanol and n-hexane seed extract on body weights of rats

Groups	Ethanol extracts		n- Hexane extracts	
	Initial weight(g)	Final weight (g)	Initial weight (g)	Final weight (g)
A	157.7 \pm 5.0	164.4 \pm 12.0	157.7 \pm 11.0	164.4 \pm 3.0
D	220.7 \pm 10.0	243.9 \pm 17.0	220.7 \pm 8.0	243.9 \pm 5.0
F	107.9 \pm 9.7	166.7 \pm 12.2	144.7 \pm 13.0	185.4 \pm 15.0
C	212.8 \pm 4.0	165.9 \pm 10.0	212.4 \pm 5.0	202.3 \pm 12.0

Keys: A (control), B-200mg/kg n-hexane extract, C-200mg/kg ethanol extract, D-200mg/kg ascorbic acid, E-100mg/kg n-hexane extract, F- 100mg/kg ethanol extract.

3.2. Effects on Lipid Peroxidation

Figure 1 shows the concentration of thiobarbituric reacting substances (TBARS) in the liver, heart and serum of the treated animals. There was a significant decrease ($P < 0.05$) in TBARS concentration in the liver and serum of the treated animals compared to the control group.

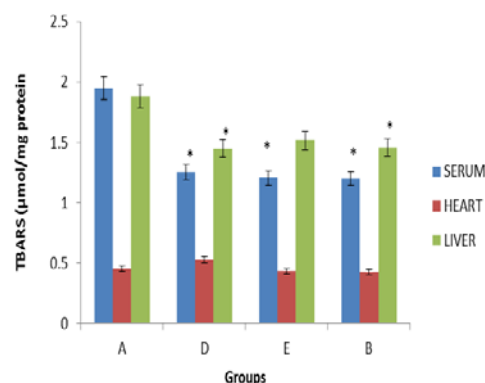


Figure1. Effect of n-hexane extracts of *Annona muricata* seeds on lipid peroxidation in the liver, serum and hearts of rats.

*S.D at ($P < 0.05$) from control.

Keys: A (control), B-200mg/kg n-hexane extract, D-200mg/kg ascorbic acid, E-100mg/kg n-hexane extract.

Figure2 reveals the effect of ethanol extract of *A. muricata* seed on lipid peroxidation in liver heart and serum of rats.

There was a significant decrease ($P < 0.05$) in TBARS concentration in the serum of animals treated with ascorbic acid and 100mg/kg extract compared with the control. There was also an observed decrease in the TBARS concentration in the heart of the animals treated with 100 and 200mg/kg of the ethanol extract compared with control.

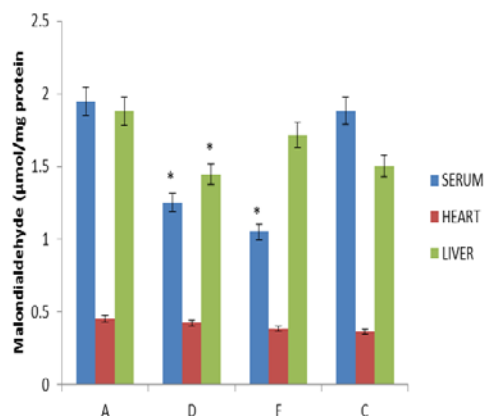


Figure 2. Effect of ethanol extract of *Annona muricata* seeds on lipid peroxidation in serum, liver and heart tissues of rats.

*S.D at ($P < 0.05$) from control.

Keys: A (control), C-200mg/kg ethanol extract, D-200mg/kg ascorbic acid, F- 100mg/kg ethanol extract.

3.3. Effect on Catalase Activity

Figure 3 shows the effect of *Annona muricata* hexane seeds extract on catalase activity of the treated animals.

There was a significant difference ($P < 0.05$) in catalase activity in the serum of animals treated with ascorbic acid and 100mg/kg of the extract compared with the control. There was also an observed increase in catalase activity in the heart and liver of animals treated with 100 and 200mg/kg of the extracts compared to the control.

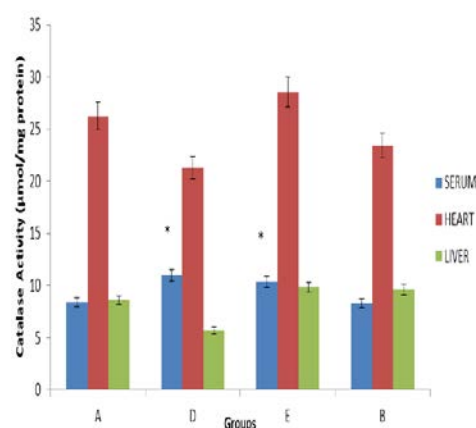


Figure 3. Effect of n-hexane extract of *Annona muricata* seeds on catalase activity of liver, serum and heart of rats.

*S.D at ($P < 0.05$) from control.

Keys: A (control), B-200mg/kg n-hexane extract, D-200mg/kg ascorbic acid, E-100mg/kg n-hexane extract.

Figure 4 shows the effect of ethanol extract of *Annona muricata* seeds on catalase activity. There was a significant difference ($P < 0.05$) in the catalase activity in the serum of animals treated with ascorbic acid and 100mg/kg extract compared with the control. An increase was also observed in the catalase activity in the heart and liver of the animals treated with 200mg/kg of extract compared with the control.

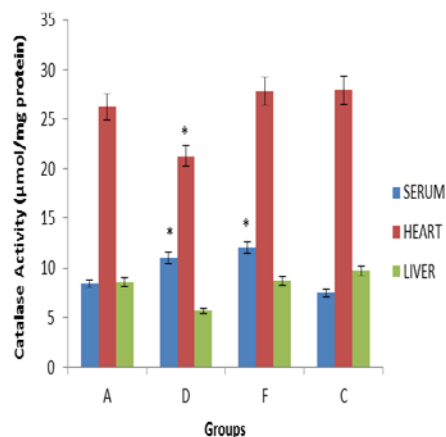


Figure 4. Effect of ethanol extract of *Annona muricata* seeds on catalase activity in the serum, liver and heart of albino rats.

*S.D at ($P < 0.05$)

Keys: A (control), C-200mg/kg ethanol extract, D-200mg/kg ascorbic acid, F- 100mg/kg ethanol extract.

3.4. Assessment of Glutathione

Figure 5 depicts the effect of n-hexane extract of *Annona muricata* seed on glutathione.

The result revealed a significant increase ($p < 0.05$) in glutathione activity in the heart of rats treated with 100mg/kg extracts and ascorbic acid treated with 100mg/kg extracts compared to the control group. There was also a significant increase ($p < 0.05$) in glutathione activity in the liver of animals treated with the standard and extracts compared to control.

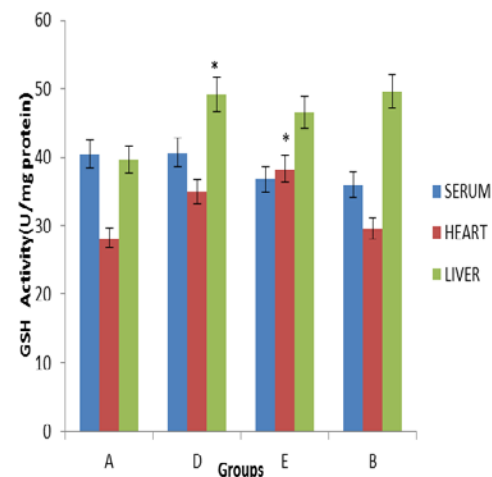


Figure 5. Effect of n-hexane extract of *Annona muricata* seeds on glutathione activity in rats.

*S.D at ($P < 0.05$)

Keys: A (control), B-200mg/kg n-hexane extract, D-200mg/kg ascorbic acid, E-100mg/kg n-hexane extract.

Figure 6 shows the effect of ethanol extract of *Annona muricata* seeds on glutathione. There was a significant increase in glutathione activity in the liver of the rats treated with 200mg/kg extracts and standard and in the hearts of the treated groups compared with the control group.

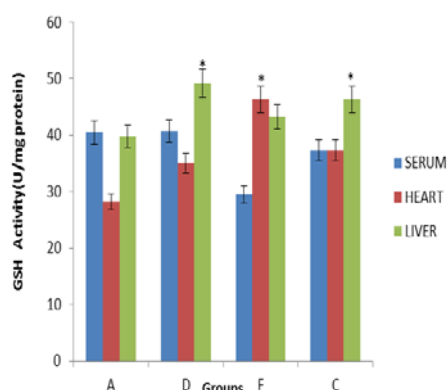


Figure 6. Effect of ethanol extract of *Annona muricata* seeds on glutathione activity in the liver serum and heart of rats.

*S.D at (P<0.05)

Keys: A (control), C-200mg/kg ethanol extract, D-200mg/kg ascorbic acid, F- 100mg/kg ethanol extract.

3.5. In Vitro Antioxidant Activity

Figure 7 shows the percentage inhibition of ethanol extract of *A. muricata* seeds on DPPH. Percentage inhibition was observed to be highest at a concentration of 100µg/ml

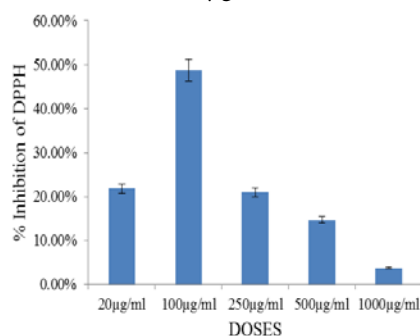


Figure 7. Percentage inhibition of ethanol extract of *Annona muricata* seeds on DPPH

*S.D at (P<0.05)

Figure 8 shows the percentage inhibition of n-hexane extract of *Annona muricata* seeds on DPPH. The percentage inhibition was observed to be highest at a concentration of 20µg/ml of n-hexane extract.

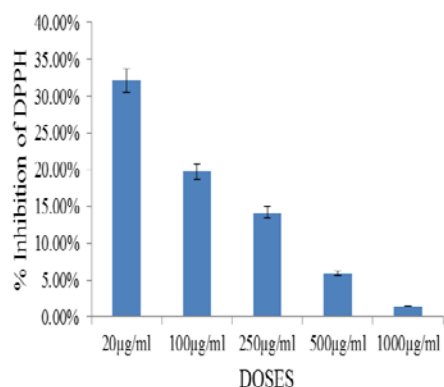


Figure 8. Percentage inhibition of n-hexane extract of *Annona muricata* seeds on DPPH.

*S.D at (P<0.05)

Figure 9 shows the scavenging effect of ascorbic acid on DPPH. The scavenging effect of Ascorbic acid seemed to be decreasing as the concentration was increasing.

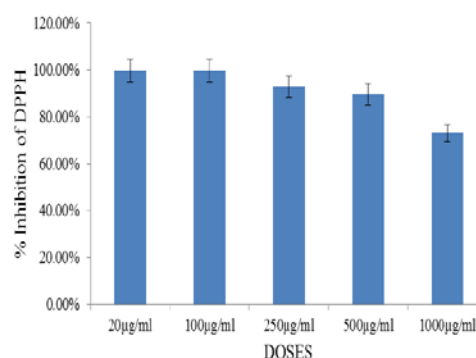


Figure 9. Percentage inhibition of Ascorbic Acid on DPPH

*S.D at (P<0.05)

4. Discussion

Plants constitute a reservoir of potentially useful chemical compounds which serve as drugs, as well as provide newer leads and clues for modern drug design and synthesis (Arunet *al.*, 2011). Experimental evidence suggests that Free Radicals (FR) and Reactive Oxygen Species (ROS) are involved in quite a number of diseases (Sajeedet *al.*, 2011). Many studies investigated the role of antioxidant drugs and plant-derived compounds in the prevention of oxidative stress (Madhavanet *al.*, 2010). Thus the role of plants and plant products cannot be over emphasized in this area.

In the present study, a decrease in the final body weight was observed in rats treated with a higher dose of both n-hexane and ethanol extracts (200mg/kg). This decrease could be a result of the enhancing activities of the bioactive compounds (phenols, flavonoids, saponins alkaloids etc.) which have been found to be present in the administered extracts (yet to be published article) on the lipolytic enzymes. This is an indication that these extracts could be useful as a weight reduction agent especially at higher doses.

Lipid peroxidation involves the formation of lipid radicals which lead to membrane damage (Baskaret *al.*, 2007). Increased lipid peroxidative status in membranes indicates changes in the membrane bilayer as a result of the formation of reactive oxygen species (ROS), thereby impairing membrane functions by decreasing membrane fluidity and changing the activity of the membrane-bound enzymes and receptors.

Research findings (yet to be published) revealed the presence of relatively high levels of phenols, flavonoids and saponins in both the n-hexane and ethanol although much higher in the ethanol seed extracts. Flavonoids are known to be potent antioxidants found in most plant species and accounts for significant percentage of chemical

constituents in vegetables, fruits and seeds (Mamta *et al.*, 2013).

In the present study, there was a significant decrease in the concentration of thiobarbituric acid reactive substances in the serum, heart and liver of the treated animals especially for those treated with 100mg/kg of the extracts. This is a pointer to the protective effect of these extracts on oxidative stress and membrane damage. This result is similar to that obtained by Adewole *et al.* (2009) who reported a similar reduction in nitric oxide (NO) and Malonaldehyde levels in rats treated with *A. muricata* leaf extracts.

Oxidative stress has been shown to be characterized by altered non-enzymatic and enzymatic antioxidant systems (Zima *et al.*, 2001). Oxidative stress also plays a key role in aging and the pathogenesis of many diseases (including kwashiorkor, seizure, alzheimer's disease, parkinson's disease, liver disease, cystic fibrosis, sickle cell anemia, HIV, cancer, heart attacks, stroke and diabetes) (Wu *et al.*, 2004). Catalase activity as observed in the present study was relatively high in the heart and liver of the treated animals; moreover, in the serum of the n-hexane extract treated group. Increased catalase activity could be an indication of the chemopreventive activity of the extracts as observed by (Ojo and Ladeji, 2005) in a similar study of the black tea in rats. Likewise, glutathione activity in the tissues and serum of the treated groups were significantly ($p < 0.05$) high. *Annona muricata* seed extracts therefore could be a good source of potent natural antioxidants that could ameliorate oxidative stress.

2,2-diphenyl-1-picryl hydrazyl (DPPH) stable free radical method is an easy, rapid and sensitive way to survey the *invitro* antioxidant activity of a specific compound or plant extracts (Koleva *et al.*, 2002). In the present investigation, the antioxidants which were present in the seed extracts and ascorbic acid reacted with DPPH which was measured at 517nm. The n-hexane extract at 20µg/ml, ethanol extract at 100µg/ml and ascorbic acid 20µg/ml and 100µg/ml produced percentage inhibition of DPPH, 32%, 49% and 96.9%, respectively. Although, the percentage inhibition of the extracts were lower than that of the standard (ascorbic acid); they are however, significant. This free radical scavenging effect of the extracts could possibly be due to the presence of antioxidant phytochemicals (phenols, flavonoids etc.) present in the extracts.

According to Carr and Frei (1999), antioxidants inhibit the growth of transformed cells decreasing their intercellular communication by the oxidative protective mechanism. *Annona muricata* seed extracts therefore could be a promising source of potent antioxidants and may be efficient as a preventive and management agent in some degenerative diseases caused by oxidative stress.

However, further research is required to fractionate this extracts in order to determine the fraction with the highest antioxidant activity.

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Effect of Thermal Treatment on Microbial load of Faecal Sludge From Some Faecal Sludge Collection Sites in Oyo State, South Western, Nigeria

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Abstract

Faecal sludge which is usually highly laden with pathogenic microorganisms serves as a route of disease transmission especially when used as organic amendment or when it contaminates water sources. Therefore, the present study investigates the effect of thermal treatment on the microbial load of faecal sludge. Faecal sludge samples were collected from two major faecal collection points in Ibadan metropolis, the samples were subjected to chemical (proximate minerals and heavy metals) and microbiological (bacteriological, mycological and parasitological) analysis before and after thermal treatment. The chemical analysis of the faecal sludge samples before and after treatment revealed the presence of heavy metals such as lead, chromium and zinc among others; proximate matter, such as protein, fat and fibre, and minerals contents such as sodium and potassium. Microbiological examination before treatment revealed that the faecal sludge samples had a high microbial load of species such as *Bacillus*, *Salmonella*, *Aspergillus* among others and ova of *Ascaris*. However, after the thermal treatment of the faecal sludge samples, it was observed that the microbial load was reduced as only spore forming organisms such as *Bacillus* and *Aspergillus* could be observed. It was observed that most organisms present in faecal sludge were removed as a result of thermal treatment; this implies that faecal sludge can be made safe for use in agricultural practices by application of thermal treatment. The present study shows the efficacy of thermal treatment in reducing the microbial load of faecal sludge, thereby increasing its chances of been used as organic amendment in agricultural practices.

Keywords: Heavy Metals, Proximate Minerals, Bacteriological, Mycological, Parasitological.

1. Introduction

According to Klingel *et al.* (2002), faecal sludge is made up of sludge of various consistencies accumulating in and evacuated from so-called on-site sanitation systems, such as septic tanks, aqua privies, family latrines and un-sewered public toilets by vacuum trucks. In most cities, faecal sludge collection and haulage are faced with great challenges, such as emptying vehicles having no access to pits, traffic congestion which prevents efficient emptying and haulage, and often times emptying services are poorly managed.

Faecal sludge is a highly variable organic material with considerable levels of grease, grit, hair and debris. In addition to its variable nature, faecal sludge tends to foam upon agitation, resists settling and dewatering and serves as a host for many disease-causing viruses, bacteria, and parasites (USEPA, 1999). The concentrations of helminth

eggs, ammonium, organic compounds and solids in faecal sludge are typically higher by a factor of ten or more than that obtainable in wastewater (Montangero and Strauss, 2002). Major components of faecal sludge include grease, kitchen/solid waste and various materials which have the potential to cause groundwater intrusion. However, the physical characteristics of faecal sludge vary significantly due to factors, such as climate, tank emptying technology and pattern, storage duration (months to years), performance of tank, among others (Montangero and Strauss, 2002).

Faecal sludge management is the collection, transport and disposal/reuse of fresh faecal material (night soil) or digested faecal material (faecal sludge) from non-sewered sanitation facilities, such as latrines and septic tanks. It is a notoriously difficult aspect of sanitation in low-income urban communities, where pits and septic tanks are typically emptied by manual operators without proper hygienic protection, and the sludge is

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dumped haphazardly in the local environment (for example in open drains) (Misaki and Matsui, 1996).

According to Vision 21 of the Water Supply and Sanitation Collaborative Council (WSSCC), sanitation is a basic human right, and one of the major components of poverty eradication (WSSCC, 2000). Globally, at least 2.6 billion people lack access to basic sanitation and the most affected population are those in Africa and Asia continents (WHO, 2004a). More than 90% of the sewage generated in developing countries is discharged untreated (Esrey, 2001; Langergraber and Muellegger, 2005). Consequently, around the world, there is a drive to ensure the provision of proper sanitation and access to clean water supply. In line with this, the Millennium Development Goal (MDG) 7 aims at halving the proportion of the world's population without safe drinking water and basic sanitation between 1990 and 2015 (UN, 2002, Eales, 2005). There have been various documentations on the importance of improved sanitation in safeguarding the health and well being of humans (WHO, 2001; Cairncross, 2003; WHO, 2004a; Moe and Rheingans, 2006). According to Kulabako *et al.* (2007), in societies where sanitation is lacking, human excreta may accumulate around homes, in nearby drains and in garbage dumps, leading to environmental pollution, whereas in some societies where conventional sanitation systems are in use, insufficiently treated excreta from latrines and wastewater systems often end up in deep pits and recipient waters. One way to decrease the environmental pollution in residential areas, as well as in recipient surface waters and groundwater, and thereby decrease the negative impacts of untreated excreta on society is to use the nutrients present in the excreta for plant production (Vinnerås and Jönsson, 2002). The use of excreta-based nutrients closes the nutrient loop, thereby enabling an increase in the sustainability due to recycling of renewable nutrient content (WHO, 2006). The safe use of excreta nutrients can be simplified by collecting the excreta fractions separately, treating them and applying them in plant production. A major challenge in the utilisation of excreta-derived nutrients is that they may contain pathogenic microorganisms, which can be a route of disease transmission that needs to be properly managed. Most of the pathogens of concern are excreted in large concentrations via faeces (WHO, 2006) and only a few of them via urine (Höglund *et al.*, 1998). Fresh faeces are always considered unsafe due to the potential presence of high concentrations of pathogens (Feachem *et al.*, 1983; WHO, 2006). Therefore, both faeces and urine need to be sanitised in order to safely recycle their nutrient content to production of food (Schönning and Stenström, 2004; WHO, 2006). Various techniques used for treatment and sanitation of faecal sludge include storage, composting, incineration and chemical treatment (Schönning and Stenström, 2004; Austin and Cloete, 2008; Jönsson *et al.*, 2004; Nordin *et al.*, 2009a,b). The present study is

therefore designed to study the effect of thermal treatment on the microbial load of faecal sludge collected from two faecal sludge dumping site in Ibadan metropolis, Oyo State, South western Nigeria.

2. Materials and Method

Sample Collection

Composite faecal sludge samples were collected from two different sources in a sterile polythene bag and transported immediately to the laboratory. Sample A was collected from a faecal sludge dump site in Omi Adio, Ido Local Government Area of Oyo State while sample B was collected from the sewage facility of the University College Hospital (UCH), which is located in Ibadan North Local Government Area of Oyo State located in South-western, Nigeria.

Physico-Chemical Analysis of the Faecal Sludge Samples

Physico-chemical analysis was carried out on the faecal sludge samples before and after the thermal treatment to determine the following: concentration of heavy metals, such as lead, chromium, zinc, nickel and manganese present in the sludge. The concentration of the heavy metals was determined by digesting the samples and analysing the digested samples using Buck Scientific 210/211 VGP Atomic Absorption Spectrophotometer (AAS) (APHA, 1992). Concentration of proximate matter such as percentage fat, percentage fibre, percentage ash; mineral content, such as nitrogen, carbon, calcium, magnesium, average phosphorus, sodium, potassium, and physical properties, such as pH, percentage sand, percentage silt, percentage clay, were determined using standard methods as described by AOAC (2012).

Microbiological Analysis

Bacteriological, mycological and parasitological analyses were conducted on the faecal sludge samples before and after thermal treatment. Culture media, such as *Pseudomonas* Centrimide Agar (PCA), Eosin Methylene Blue agar (EMB), *Salmonella-Shigella* agar (SSA), Manitol Salt Agar (MSA) and Nutrient Agar (NA), were used for the bacteriological examination of the faecal sludge samples. Pure cultures of the bacterial isolates obtained were Gram stained and subjected to various biochemical tests, such as catalase test, oxidase test, starch hydrolysis test, citrate utilization test, indole test, methyl red test, oxidative fermentation test etc. after which they identified using Cowan and Steel (1993) and Holt *et al.* (2000).

Potato Dextrose Agar (PDA) was used for the mycological examination of faecal sludge samples. Pure cultures of the fungal isolates were identified by staining them with lacto-phenol cotton blue and were viewed under the microscope. Images

observed under the microscope were compared to those in a compendium (Domsch *et al.*, 1980).

Parasitological analysis was carried out to concentrate the eggs of parasites present in the faecal sludge samples using the formol-ether concentration technique as described by Cheesbrough (2009).

Thermal Treatment of Faecal Sludge Samples

Five kilograms of the faecal sludge collected from each source was put in a clean sterile crucible and oven-dried at standard temperature of 100°C for 1 hour.

3. Results

Physico-Chemical Analysis of the Faecal Sludge Table 1 shows the result obtained for the physico-chemical analysis of the faecal sludge samples before and after thermal treatment. For the pre-treatment analysis, it was observed that the faecal sludge collected from Ibadan North Local Government had a higher concentration of heavy metals, proximate matter and mineral contents present in it than the faecal sludge collected from the sewage facility of UCH. Though there was a reduction in the quantity of heavy metals, proximate matter and mineral contents present in the faecal sludge samples from both Ibadan North Local Government and UCH sewage facility after the treatment exercise, it was however observed that faecal sludge samples collected from Ibadan North Local Government still had a higher concentration of heavy metals and proximate mineral contents present in it than the faecal sludge samples collected from UCH sewage facility.

Table 1: Physico-chemical properties of the faecal sludge samples

	A	B	A	B
pH	6.6	6.76	6.88	7.13
% Sand	80.96	84.96	78.21	82.87
% Silt	8.77	7.24	7.10	6.18
% Clay	7.9	7.8	9.80	10.95
Heavy metals				
Lead	25.7	13.2	18.6	11.9
Chromium	44.2	18.4	37.3	16.8
Zinc	96.3	31.28	84.5	26.74
Nickel	56.3	8.3	47.8	6.5
Copper	14.2	7.6	43.8	11.9
Manganese	61.2	47.36	53.6	43.29
Mineral content				
% Organic Carbon	36.79	26.7	31.38	19.75
% Nitrogen	3.18	3.75	2.69	2.86
Average Phosphorus	1126	987	993	886
% Calcium	1.23	2.4	0.79	1.34
% Sodium	0.31	0.18	0.24	0.25
% Magnesium	0.29	0.64	0.21	0.75
% Potassium	0.78	0.43	0.65	0.51
Proximate matter				
% Crude protein	18.38	12.60	12.76	9.67
% Crude fat	2.94	1.80	3.11	2.10
% Crude fibre	31.86	25.44	25.91	18.11
% Ash	6.28	3.33	7.13	2.43

Key: A- Faecal sludge sample from Ibadan North LG

B- Faecal sludge sample from UCH sewage facility

When microbiological analysis was carried out on the faecal sludge samples before thermal treatment using different agar media, it was observed that faecal sludge samples collected from Ibadan Local Government had higher microbial load compared to the faecal sludge samples collected from UCH sewage facility as seen in Figure 1. Fifty-eight bacterial and twenty fungal isolates were obtained from the untreated faecal sludge samples, parasitological examination revealed dominance of *Ascaris* spp ova in the two samples.

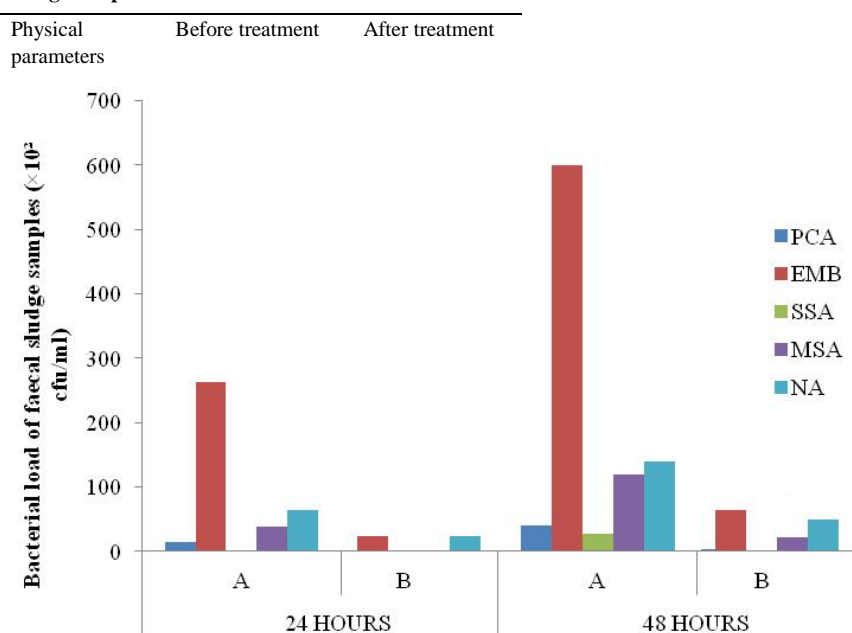


Figure 1. Growth of bacteria on different media

Key : PCA-Pseudomonas centrimide agar; EMP-Enson methulene blue agar; SSA-Samlonella-Shigela Agar; MSA-Manitol salt agar.

Biochemical characterization of the bacterial isolates revealed that the organisms belong to *Bacillus* sp (15), *E. coli* (6), *Enterobacter* sp (10), *Pseudomonas* sp (12), *Salmonella* sp (5) and *Staphylococcus* sp (10). When the fungal isolates were stained and viewed under the 40× objective lens and compared to the Compendium of soil fungi, it was discovered that they were all *Aspergillus* sp viz: *Aspergillus niger*, *A. flavus* and *A. fumigatus*. Figures 2 and 3 show the frequency of occurrence of the bacterial and fungal isolates in the faecal sludge samples collected before thermal treatment. It was observed that *Bacillus* sp had the highest frequency of occurrence among the bacterial isolates while *Aspergillus niger* had the highest frequency of occurrence among the fungal isolates.

No microbial growth was observed on the selective media (PCA, EMB, SSA and MSA) used for culturing the treated faecal sludge samples however some bacteria were observed to grow on nutrient agar medium. When the bacteria were characterized, they were discovered to be predominantly *Bacillus* sp.

Mycological analysis of the treated faecal sludge samples revealed that the treated faecal sludge samples still had some fungal load but was reduced compared to the initial fungal load and the fungi were all *Aspergillus* sp.

Parasitological examination of the treated faecal sludge samples revealed no eggs of parasites in the treated faecal sludge samples.

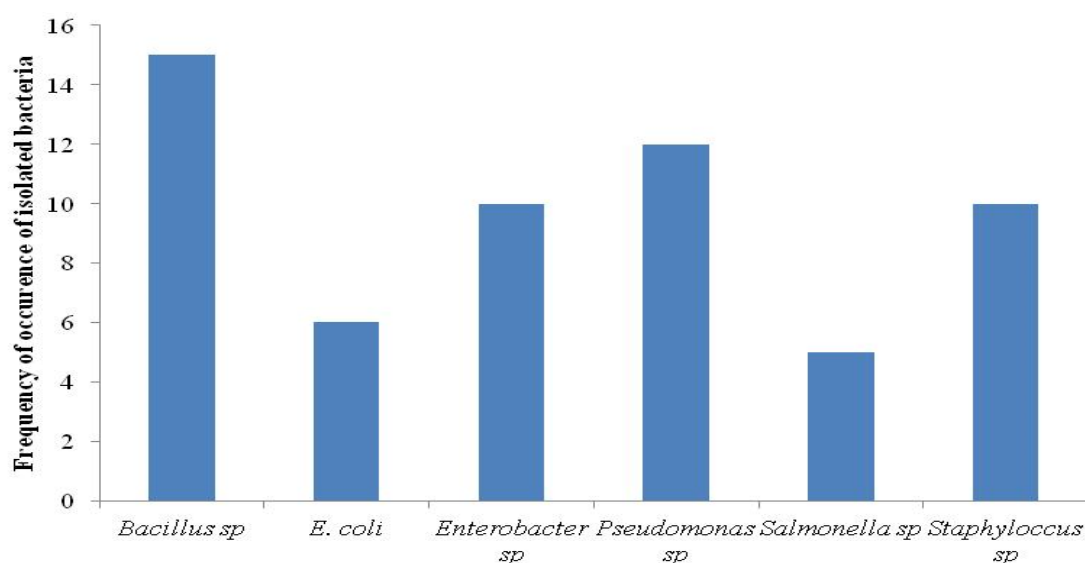


Figure 2. Frequency of occurrence of bacterial isolates from untreated faecal sludge samples

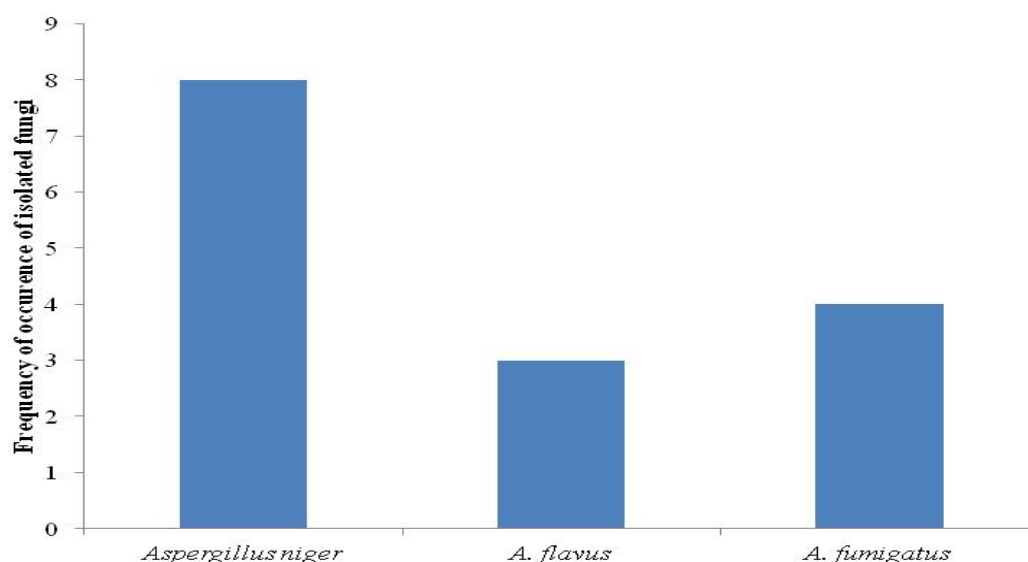


Figure 3. Frequency of occurrence of fungal isolates from untreated faecal sludge samples

4. Discussion

Faecal sludge collected from various points were usually dumped in a dug hole and covered with chemicals, such as izal and kerosene (Ayeni, 2012), though there were some desludgers which empty the faecal sludge into rivers or water bodies, this however is illegal. According to Ayeni (2012), some farmers usually request for faecal sludge to be used on their farms and this is often sold to them either in the raw form, i.e., directly from the emptied soakaways or after it had been dumped in holes; this is a way of turning waste into wealth.

Upon collection of the faecal sludge samples for the study, it was observed that the samples were moist and had varying characteristics; this was supported by an earlier studies by Pescod (1971), Pradt (1971), Um and Kim (1986), Guo *et al.* (1991) and Strauss *et al.* (1997), in which it was noted that the characteristics of collected faecal sludges varied greatly. The characteristics depend, to mention some, on the season, type of the on-site sanitation system (e.g., water closet/septic tank system, "dry" aqua privy, watertight vented pit latrines), the emptying frequency (i.e., the retention time in the facility), the extent of storm water or groundwater infiltration into the sanitation facility and also the users habits.

The treatment methods used in the treatment of faecal sludge by UCH and the municipal desludgers differs, UCH sewage treatment was done using decomposition method while that of the municipal desludgers was done through sun drying. The faecal sludge sample collected from UCH had less microbial load and heavy metal concentration compared to that from the municipal desludgers, this therefore indicates that decomposition might be a better treatment option for faecal sludge than sun drying methods.

During the microbiological examination of the untreated faecal sludge samples, organisms isolated include some bacteria, such as *Bacillus sp.*, *E. coli*, *Enterobacter sp.*, *Pseudomonas sp.*, *Salmonella sp.* and *Staphylococcus sp.*; the fungi isolated all belong to the *Aspergillus* genera, such as *A. niger*, *A. flavus* and *A. fumigatus*. Also ova of worms especially the *Ascaris sp.* were present in the faecal sludge samples. Most of these organisms are commonly found in fresh faeces indicating that microorganisms that can be found in fresh excreta can be invariably found in faecal sludge samples in agreement with earlier studies carried out by Schönning and Stenström (2004).

The chemical analysis of the untreated faecal sludge samples revealed the presence of mineral matter, such as nitrogen, phosphorus, sodium, magnesium, calcium etc. in varying concentrations. Also, heavy metals, such as lead, chromium, zinc, nickel, copper and manganese, were seen to be present in the faecal sludge samples. According to Pehlivan *et al.* (2009), toxic heavy metals, such as lead, cobalt and cadmium, are capable of causing various diseases and disorders even in relatively

lower concentrations in living organisms. Heavy metals, with soil residence times of thousands of years, pose numerous health dangers to higher organisms. They are also known to have an effect on plant growth, ground cover and have a negative impact on soil microflora, since some farmers utilize faecal sludge as organic amendment (Roy *et al.*, 2005).

Faecal sludge samples were subjected to thermal treatment using 100°C for one hour. All pathogens had threshold temperatures beyond which their viability ceases (Madigan and Martinko, 2006). The mechanism of temperature inactivation differs for different types of pathogens. Elevated temperatures irreversibly inactivate enzymes of bacteria, protozoa and helminths, thereby resulting in cellular inactivation (Madigan and Martinko, 2006; Wichuk and McCartney, 2007). The degree of thermal inactivation of pathogens is a function of both the temperature and time of exposure (Feachem *et al.*, 1983; de Bertoldi, 1998; Wichuk and McCartney, 2007). It was observed that there was a reduction in the microbial load of the faecal sludge samples as there were no growth on most of the agars used except a minimal one on nutrient agar and potato dextrose agar. There was also a reduction in the concentration of the heavy metals, proximate matter and mineral content in the faecal sludge samples after treatment even when the treatment temperature was far less than the boiling points of these metals. Hence, it must be that these heavy metals are of organic origin, thus they were volatile and escaped with the organic component of the faecal sludge samples.

In conclusion, the major challenge combating the use of faecal sludge for agricultural purpose is that they may contain pathogenic microorganisms, which can be a route of disease transmission. The present study has shown that thermal treatment can reduce the microbial load and in faecal sludge. Hence ensuring the safe use of excreta nutrients for agricultural purposes as well as serving as a means of recycling waste (faecal sludge) to wealth.

Conflict of Interest

There is no conflict of interest.

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Seasonal Variations of Phytoplankton Community in Relation to Some Physical and Chemical Parameters in a Temperate Eutrophic Reservoir, Turkey

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Abstract

The Çaygören Reservoir is fed by the Simav Stream and the maximum inflow ($1300 \text{ m}^3 \text{ sec}^{-1}$) occurred in spring and the minimum (about $5.2 \text{ m}^3 \text{ sec}^{-1}$) occurred in fall. It has an annual mean water capacity of 392 hm^3 and a total volume of 142.57 hm^3 . A total of 192 taxa in 9 divisions were identified. *Cyclotella meneghiniana* Kützing, *Stephanodiscus neoastraea* Hakansson and Hickel of Bacillariophyta, *Gloeotila subconstricta* (G.S.West) Printz of Chlorophyta, *Mugeotia* sp. of Streptophyta, *Cryptomonas pyrenoidifera* Geitler, *Plagioselmis nannoplantica* (H.Skuja) G.Novarino, I.A.N.Lucas and S.Morrall of Cryptophyta, *Aphanocapsa holsatica* (Lemmermann) G.Cronberg and J.Komárek, *Aphanothece clathrata* West and G.S.West and *Planktothrix* sp. of Cyanobacteria dominated phytoplankton at least for one season during the observation period. Species of Cryptophyta dominated phytoplankton during the winter, while Chlorophyta and Streptophyta species were dominant in the fall. Bacillariophyta species dominated phytoplankton in the spring and Cyanobacteria were dominant in the summer. The maximum phytoplankton biomass and abundance (106.5 mg L^{-1} ; $273154 \text{ individual M}^{-3}$) were recorded in summer 2008 at the third station and the minimum biomass and abundance (0.23 mg L^{-1} ; $799 \text{ individual M}^{-3}$) were recorded in winter 2007 at the second station. The canonical correspondence analysis (CCA) and correlation results showed that water temperature, transparency, phosphate, oxidation-reduction potential and water discharge had significant effects (Monte Carlo test, $p < 0.05$) on the dynamics of dominant phytoplankton of the eutrophic Çaygören Reservoir.

Keywords: Phytoplankton, Temperate Eutrophic Reservoir, Water Discharge, Water Quality Parameters, CCA.

1. Introduction

Çaygören reservoir was built between 1965 and 1968 for the purpose of irrigation and hydropower generation. It is an important source of irrigation water for the towns of Sındırgı and Bigadiç in the province of Balıkesir, Turkey. It is used for flood control as its source stream (Simav Stream) sometimes reaches about $1500 \text{ m}^3 \text{ sec}^{-1}$ debt. The Çaygören Reservoir has a total length of 4.6 km, a surface area of 8.15 km^2 and a maximum depth of 53.5 m. The purpose of the present study is to determine the environmental variables responsible for the seasonal variations of the abundance, species composition and the biomass of the phytoplankton community of the temperate eutrophic Çaygören Reservoir.

Phytoplankton communities play an important role in aquatic ecosystems as they produce food and oxygen, which supports all other life forms (Fathiet al., 2001). Knowledge on their abundance and community composition provided further understanding of ecological interactions in aquatic ecosystems. The seasonal dynamics of phytoplankton have been investigated worldwide (Taveriniet al., 2009; Jeppesenet al., 2011; Elliott, 2012; Caroni et al., 2012; Feuchtmayret al., 2012).

In northern temperate lakes, phytoplankton succession is largely determined by the seasonal cycles of physical, chemical and biological factors, the relative importance of which varies with the different periods of the year (Tiinaet al., 2011). The spatial distribution of phytoplankton in lakes is highly heterogeneous. This heterogeneous distribution is mostly attributed to wind events,

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mixing and the contrasting gradients in the light and nutrient concentrations (Reynolds et al., 2002).

Considering the complexity of phytoplankton dynamics, studying the spatial and seasonal distribution of species and their relationships with the physicochemical parameters can give insights into understanding factors responsible for their dynamics. Arhonditsis et al. (2004) stated that to determine which factors are effective on spatial and temporal distribution of phytoplankton, the system under study should have been sampled for at least two years.

The underwater light climate seems to be one of the selective environmental factors that strongly influence the species composition and biomass of phytoplankton in lakes. Water transparency has received much attention lately because of the self-limitation of photosynthesis imposed by phytoplankton. In turbid environments, algal species with gas vesicles can either move down to avoid the high light intensity at the water surface, or float up when underwater light conditions are poor (Pérez et al., 2007).

Nutrient limitation imposes compositional changes on the phytoplankton community. According to resource competition theory, nitrogen-fixing algae should dominate lakes when nitrogen is limiting. Assuming that phosphorus and nitrogen limit many algae in lakes, blue-green algae should dominate lakes when N:P ratios are low but other types should dominate when they are high (Chaffin et al., 2011).

For a better understanding of the processes affecting phytoplankton dynamics, it is important to study the linkage between changes in environmental variables and phytoplankton abundance, biomass and community composition (George et al., 2004). Multivariate statistical techniques have been proved to be useful for understanding interactions between the ecological factors and plankton communities in aquatic ecosystems (Kruk et al., 2002).

Although few studies have been published on the Çaygören Reservoir (Sevindik, 2010; Sevindik et al., 2011), the present study is the first attempt to describe the seasonal and spatial distribution of environmental variables and their relations with the phytoplankton composition in the temperate eutrophic Çaygören Reservoir using Canonical Correspondence Analysis (CCA) and Pearson's correlation analysis.

2. Materials and Method

2.1. Study Area

The Çaygören Reservoir is located at 39° 17' 24" N; 28° 19' 16" E, 55 km southeast of Balıkesir, Turkey (Fig. 1). It lies at 273 m above the sea level

and has a maximum depth of 28 m, a length of 4.6 km and a surface area of 9 km². The reservoir is fed by the Simav Stream. Its construction started in 1971 and it is used for irrigation and power generation (State Water Works, 2005).

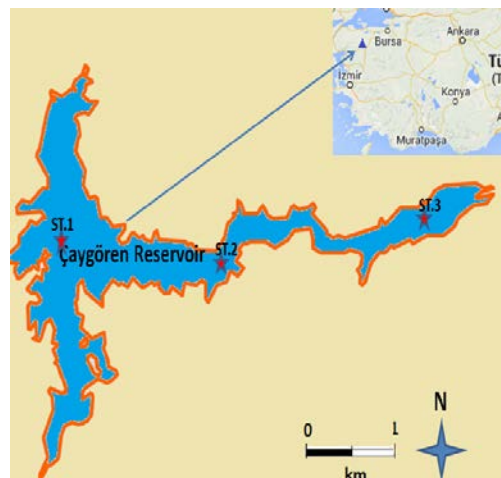


Figure 1. The map of the Çaygören Reservoir and the location of sampling stations (I think this should be placed under the figure above this paragraph or the figure should be moved to above this caption)

2.2. Sampling Procedure and Chemical Analysis

Water sampling was carried out monthly from February 2007 to January 2009 for measurements of physical, chemical and biological parameters. Samples were collected vertically at 5 m intervals using a Kemmerer water sampler. Specific Conductivity (SC), pH, Oxidation-Reduction Potential (ORP) and water Temperature (T) were measured at 1 m intervals using a YSI multi probe. Water transparency was measured using a Secchi disk.

Concentrations of phosphate (PO₄), nitrate-nitrogen (NO₃-N) and ammonium-nitrogen (NH₄-N) were determined spectrophotometrically in samples collected from 1, 5 and 15 meters according to the standard methods (APHA, 1995). Water Discharge (WD) data were obtained from the State Water Works.

For the purpose of minimizing the errors, calibration of apparatus, running of blank and sample at known concentration, measurements in replicate were performed in the laboratory. The accuracy and precision of the used analytical methods were checked by means of standard samples, which were assayed with each series of samples (Table 1). A multipoint calibration of the YSI multi probe was done one day prior to the sampling. Zero and span checks were made regularly as the basic quality assurance procedure for analysis.

Table 1. Mean \pm Standard deviation (SD) physical and chemical water characteristics for water quality parameters in the Çaygören Reservoir

Parameter	Depth	Control	St. 1	St. 2	St.3
Tmp (°C)	1 m	15.2 \pm 6.35	14 \pm 6.8	14 \pm 6.3	15 \pm 6.3
	10 m	16.1 \pm 7.71	15 \pm 7.8	14 \pm 6.4	15 \pm 6.45
	15 m	13.9 \pm 7.65	14.6 \pm 7.1	13.9 \pm 6.39	13.9 \pm 6.38
Cond (mS cm ⁻¹)	1m	0.44 \pm 0.089	0.43 \pm 0.09	0.44 \pm 0.07	0.43 \pm 0.08
	10 m	0.46 \pm 0.081	0.44 \pm 0.08	0.24 \pm 0.04	0.24 \pm 0.07
	15 m	0.45 \pm 0.0074	0.44 \pm 0.075	0.23 \pm 0.05	0.23 \pm 0.06
pH	1 m	9.9 \pm 0.59	9.8 \pm 0.6	9.78 \pm 0.61	9.73 \pm 0.63
	10 m	9.9 \pm 0.579	9.7 \pm 0.59	9.5 \pm 62	9.87 \pm 61
	15 m	9.8 \pm 0.61	9.75 \pm 0.58	10.9 \pm 5.91	10.8 \pm 5.71
ORP (mV)	1 m	102 \pm 43	102 \pm 42	101 \pm 48	101 \pm 43
	10 m	103 \pm 44.5	102 \pm 45	104 \pm 31	104 \pm 32
	15 m	100 \pm 43.12	101 \pm 43	103 \pm 34	103 \pm 33
PO ₄ (mg L ⁻¹)	1 m	0.023 \pm 0.009	0.022 \pm 0.008	0.02 \pm 0.002	0.02 \pm 0.0012
	10 m	0.024 \pm 0.009	0.024 \pm 0.01	0.034 \pm 0.02	0.031 \pm 0.013
	15 m	0.024 \pm 0.01	0.023 \pm 0.011	0.031 \pm 0.0079	0.03 \pm 0.0071
NO ₃ (mg L ⁻¹)	1 m	0.19 \pm 0.051	0.2 \pm 0.05	0.21 \pm 0.05	0.23 \pm 0.045
	10 m	0.21 \pm 0.043	0.2 \pm 0.04	0.18 \pm 0.04	0.19 \pm 0.038
	15 m	0.19 \pm 0.041	0.18 \pm 0.039	0.21 \pm 0.045	0.23 \pm 0.042
NH ₄ (mg L ⁻¹)	1m	0.048 \pm 0.0023	0.05 \pm 0.002	0.025 \pm 0.0012	0.042 \pm 0.0011
	10 m	0.071 \pm 0.0051	0.07 \pm 0.005	0.023 \pm 0.0011	0.041 \pm 0.0013
	15 m	0.072 \pm 0.0025	0.068 \pm 0.002	0.043 \pm 0.0021	0.043 \pm 0.0012
TSS (mg L ⁻¹)	1 m	15.5 \pm 6.56	16 \pm 6.3	14 \pm 4.5	14 \pm 4.5
	10 m	17 \pm 7.69	17 \pm 7.7	14.6 \pm 5.7	15.6 \pm 4.7
	15 m	16.8 \pm 7.25	16 \pm 7.2	15.9 \pm 6.5	15.9 \pm 4.5

2.3. Phytoplankton Sampling and Analysis

In the field, samples for phytoplankton were collected from 1, 5 and 15 meters and placed in 250 ml bottles and fixed with Lugol's solution. In the laboratory, the samples were first agitated, then poured into 50 ml graduated cylinders and were allowed to settle for 24 hours. At the end of the settling period, 45 ml of water was aspirated from each graduated cylinder and the remaining 5 ml was poured into a small glass vial for microscopic analysis. Enumeration and identification of phytoplankton were performed using a Palmer-Maloney counting cell and an Olympus BX 51 compound microscope equipped with water immersion lenses (40X and 60X magnifications) and a phase-contrast attachment.

Phytoplankton species were identified according to Huber-Pestalozzi (1941; 1950; 1969; 1972; 1982; and 1983), Bourrelly (1968), Krammer and Lange-Bertalot (1986; 1991; and 1999), Komarek and Anagnostidis (1986; 1989; 1999; and 2008), Anagnostidis and Komarek (1988), Round et al. (1990), Sims (1996) and John et al. (2003). Phytoplankton biomass was calculated from the biovolume data, assuming a specific gravity of one (Edmondson, 1971). Biovolume was calculated from cell numbers and cell size measurements (Sun and Liu, 2003).

2.4. Statistical Analysis

The CCA was used to determine the relationships between the dominant phytoplankton taxa and the environmental variables. The significance of environmental variables on the dominant taxa was determined with Monte Carlo tests. The CCA and Monte Carlo tests were performed using the CANOCO v.4.5 program (ter Braak and Smilauer, 2002). The relationships between the physicochemical variables and the dominant phytoplankton taxa were further analyzed using the Pearson's correlation coefficients.

The statistical differences in the total phytoplankton abundance and biomass were determined using an ANOVA test. The ANOVA and the Pearson's correlation coefficients were calculated using SAS statistical software. Data were log transformed before the statistical analysis to obtain normal distribution (SAS Institute, 2003).

3. Results

3.1. Physico-Chemical Parameters

The maximum (1300 m³ s⁻¹) and minimum (5.2 m³ s⁻¹) inflows (WD) were recorded in April 2007 and September 2007, respectively (Fig. 2). Secchi disk depth ranged from 0.3 m to 1.5 m at St.1 and it

ranged from 0.6 m to 1.9 m at St.2 and St.3 (Fig. 3). Water temperature ranged from 4.5°C to 27.6 °C at all stations. Maximum surface water temperature values were measured in June and July and minimum values were measured in February (Fig. 4).

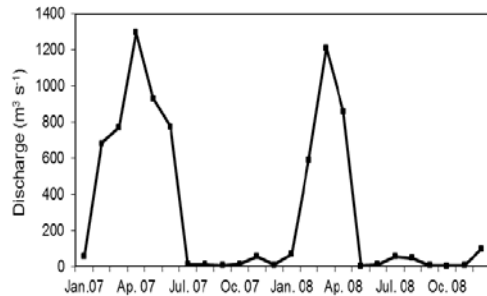


Figure 2. The seasonal variations in the water discharge ($\text{m}^3 \text{s}^{-1}$) of the Çaygören Reservoir

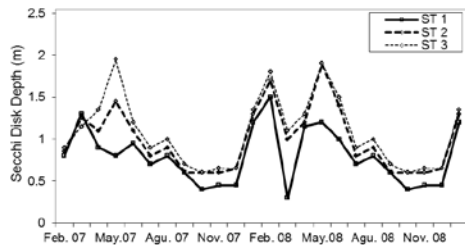


Figure 3. The seasonal variations in the Secchi disk depth (m) of the Çaygören Reservoir

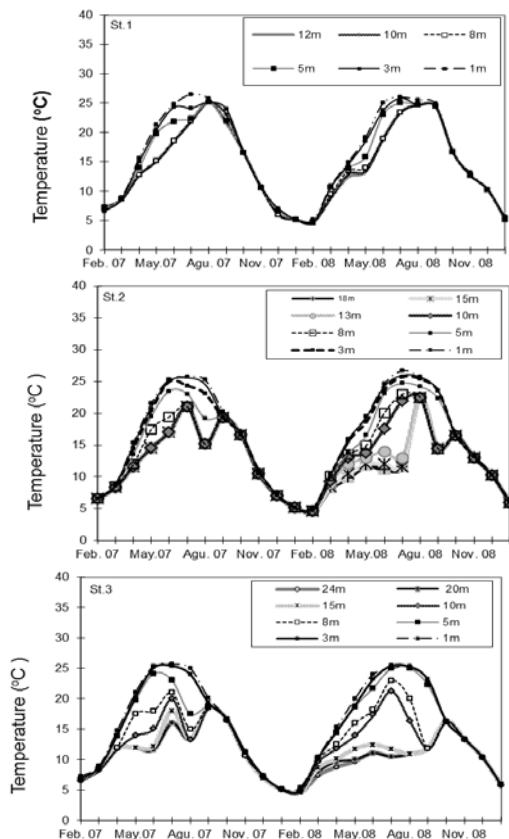


Figure 4. The seasonal variations in the water temperature (°C) of the Çaygören Reservoir.

Specific conductivity ranged from 0.3 mS cm^{-1} to 0.6 mS cm^{-1} at all stations and it was lower in the winter than the other seasons (Fig. 5). pH ranged from 7.4 to 11.6 from February 2007 to September 2008 at all stations. From November 2008 to January 2009, pH fluctuated between 7.7 and 11 (Fig. 6).

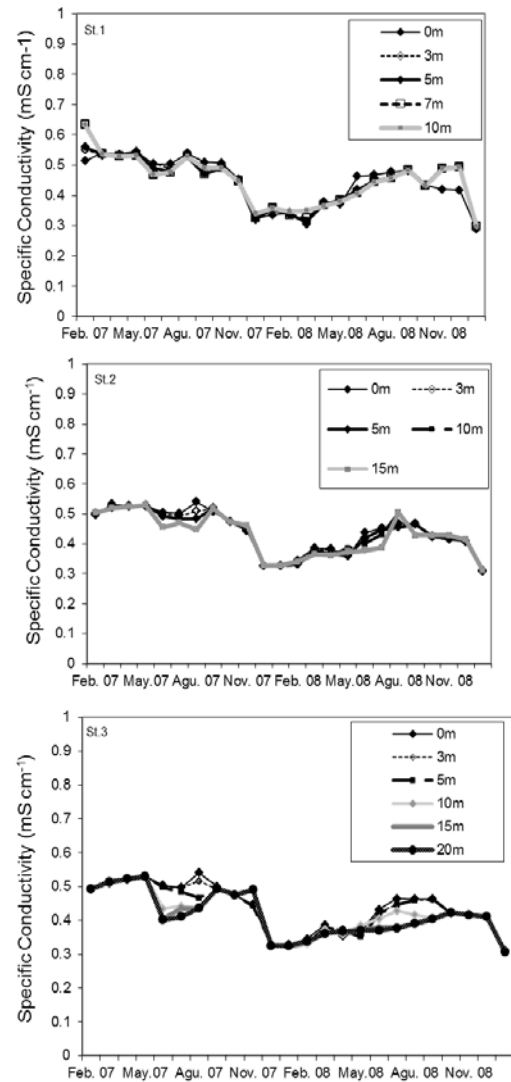


Figure 5. The seasonal variations in specific conductivity (mS cm^{-1}) of the Çaygören Reservoir

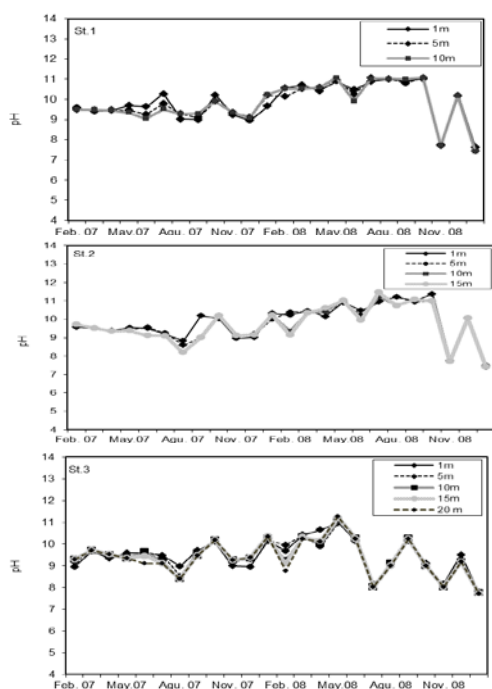


Figure 6 . The seasonal variations inthe pH of the Çaygören Reservoir

ORP ranged from 2 mV to 219.5 mV at all stations and it was lower in the summer than the other seasons (Fig. 7). PO_4 concentrations ranged from 0.005 mg L^{-1} to 0.06 mg L^{-1} , oscillating around 0.02 mg L^{-1} throughout the study period, except for a peak of 0.04 mg L^{-1} in October 2008 at the first station and another one of 0.06 mg L^{-1} in November 2007 at the second station (Fig. 8).

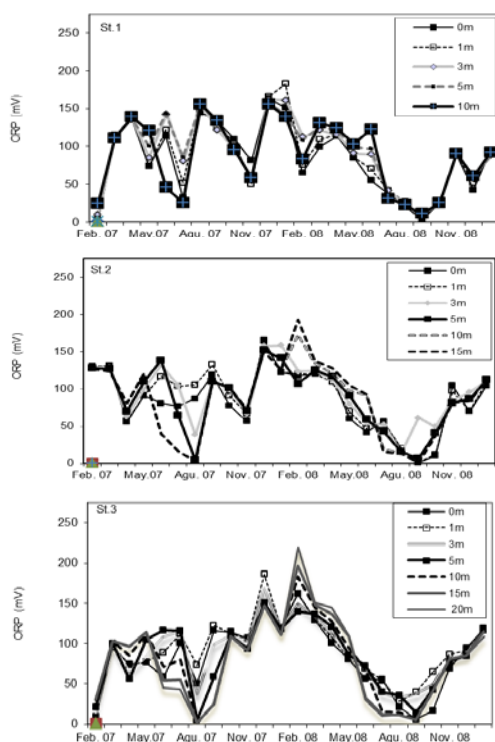


Figure 7. The seasonal variations in the oxidation-reduction potential (mV) of the Çaygören Reservoir

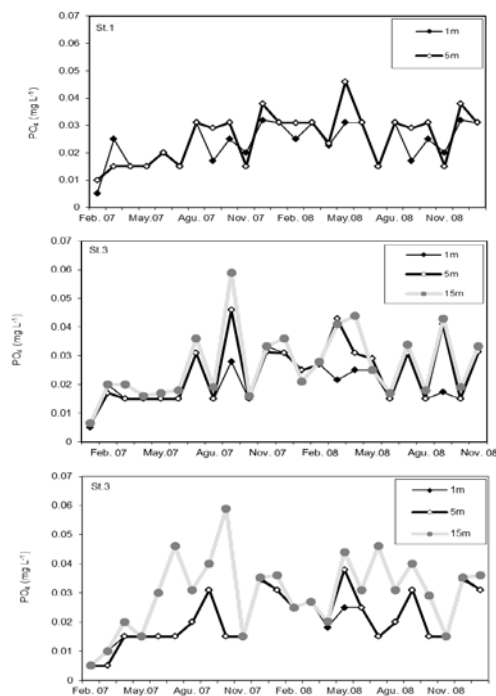


Figure 8 . The seasonal variations inthe phosphate (mg L^{-1}) of the Çaygören Reservoir

$\text{NO}_3\text{-N}$ concentrations ranged from 0.055 mg L^{-1} to 0.3 mg L^{-1} at all stations. A decline of about 0.05 mg L^{-1} in $\text{NO}_3\text{-N}$ occurred in the spring and fall at all stations during the study period (Fig.9). $\text{NH}_4\text{-N}$ concentrations ranged from 0.005 mg L^{-1} to 0.017 mg L^{-1} at the first and second stations and they ranged from 0.001 mg L^{-1} to 0.02 mg L^{-1} at the third station (Fig. 10).

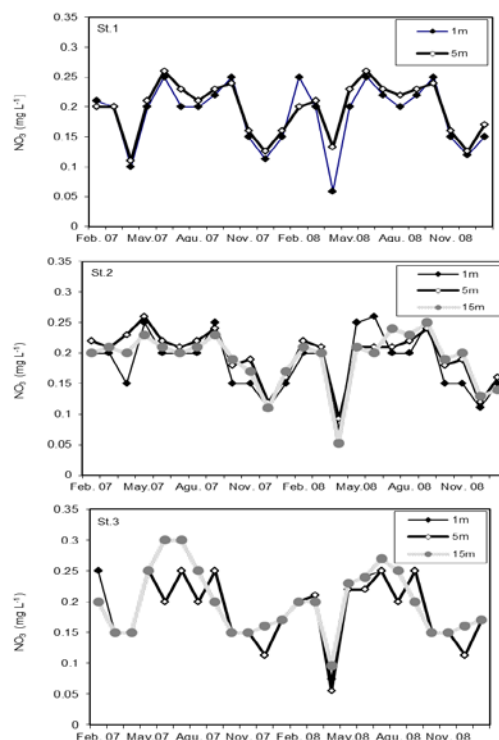


Figure 9. The seasonal variations in nitrate-nitrogen (mg L^{-1})

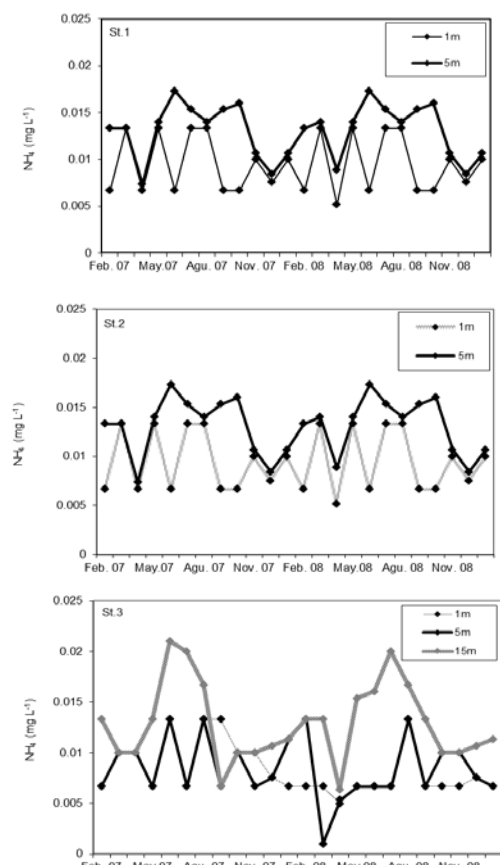


Figure 10. The seasonal variations in the ammonium-nitrogen (mg L^{-1}) of the Çaygören Reservoir

3.2. Phytoplankton Species and Biomass

A total of 192 taxa in nine major taxonomic categories were identified. During the winter, *Plagioselmis nannoplantica* (H.Skuja) G.Novarino, I.A.N.Lucas and S.Morrall, Cryptophyta; 10% of the total biomass) dominated phytoplankton. In the spring, *Cyclotella meneghiniana* Kützing, (Bacillariophyta; 35% of the total biomass) and *Stephanodiscus neoastraea* Hakansson and Hickel (Bacillariophyta; 32% of the total biomass) were dominant. During the summer, *Planktothrix* sp. (Cyanobacteria; 33% of the total biomass), *Aphanocapsa holsatica* (Lemmermann) Cronberg (Cyanobacteria; 12.5% of the total biomass) and *Aphanothece clathrata* West and G.S.West (Cyanobacteria; 30% of the biomass) dominated phytoplankton. In the fall, *Gloeotila subconstricta* (G.S. West) Printz (Chlorophyta; 10% of the total biomass) and *Mougeotia* sp. (Streptophyta; 14% of the total biomass) were dominant in the Çaygören Reservoir (Fig. 11).

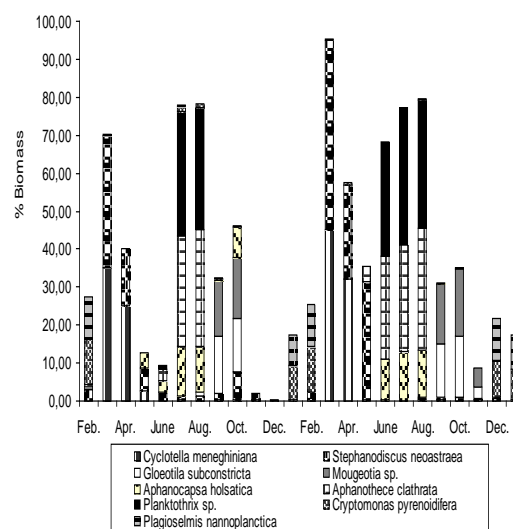


Figure 11. The percent biomass distribution of the dominant phytoplankton taxa in the Çaygören Reservoir

The maximum phytoplankton biomass was recorded in winter 2007 (78 mg L^{-1} at the first station, 99 mg L^{-1} at the second station and 106.5 mg L^{-1} at the third station) and the lowest biomass was recorded in December 2007 (0.31 mg L^{-1} at the first and the third stations and 0.23 mg L^{-1} at the second station; Fig. 12). The phytoplankton biomass was significantly different among the seasons ($F=104$, $P<0.001$), but not among the sampling stations ($F=0.65$, $P>0.01$).

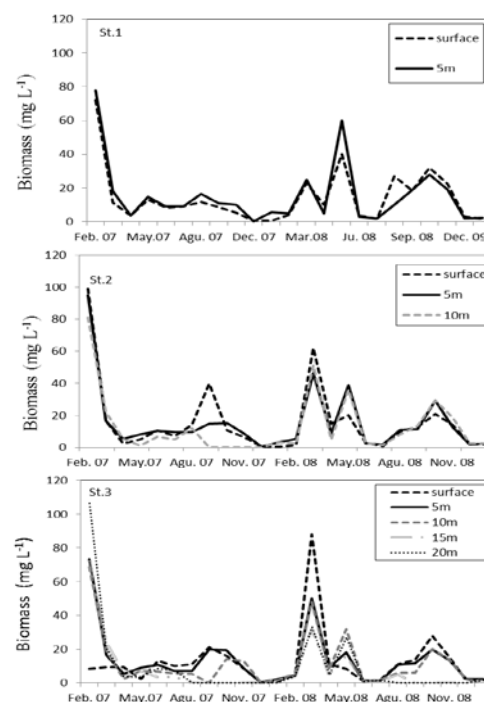


Figure 12. The seasonal distribution of the total phytoplankton biomass (g L^{-1}) in the Çaygören Reservoir

The maximum phytoplankton abundance was measured in summer 2008 because of optimum water temperature and sufficient light and the minimum abundance was recorded in winter 2007 because of low water temperature and insufficient light. The seasonal variations in phytoplankton abundance during 2007-2009 are presented in Fig. 13. The differences in the phytoplankton abundance were significant among the seasons ($F=64$, $P<0.001$), but not among the sampling stations ($F=0.39$, $P>0.05$).

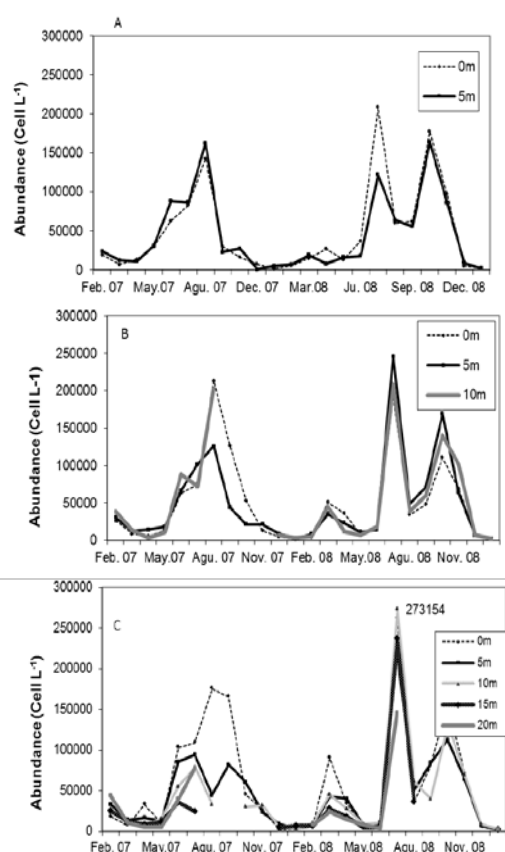


Figure 13. The seasonal dynamics of the total phytoplankton abundance (cell L⁻¹) in the Çaygören Reservoir

3.3. Statistical Analysis of Phytoplankton Species, Biomass and Physico-Chemical Parameters

In the Çaygören Reservoir, from CCA analysis, the first and second axes of CCA explained 77.2% of the total variance in the dominant phytoplankton taxa-environment relationships (eigenvalues, 0.8 and 0.55). The third and fourth axes explained 22.3% of the total variance (eigenvalues, 0.378 and 0.016). Table 2 shows the results of the Monte Carlo tests for the significance of the physicochemical parameters in order of the variance they explain. According to these results, water temperature, water discharge, Secchi disk transparency, oxidation-reduction potential and phosphate had significant effects on the dynamics of the phytoplankton ($p<0.05$).

Table 2. The variance explained by each variable in the Çaygören Reservoir

Variable	Variable number	Variance explained	P	F
T (°C)	1	0.72	0.002*	7.91
WD (m ³ s ⁻¹)	9	0.53	0.002*	7.57
Secc. (m)	2	0.29	0.032*	3.02
Scand. (mS cm ⁻¹)	3	0.07	0.271	1.110
NO ₃ -N (mg L ⁻¹)	6	0.02	0.382	0.154
ORP (mV)	4	0.26	0.038*	2.86
pH	5	0.05	0.584	0.261
NH ₄ -N (mg L ⁻¹)	7	0.03	0.714	0.571
PO ₄ (mg L ⁻¹)	8	0.25	0.039*	2.85

*significant at 0.05 level.

The first axis of CCA was positively related to T, SC, NH₄-N and PO₄ and it was negatively related with Secchi disk transparency, NO₃-N, ORP, WD and pH. The second axis was positively related to Secchi disk transparency, NO₃-N, ORP, pH and NH₄-N and it was negatively related with T, SC, PO₄ and WD (Fig. 14).

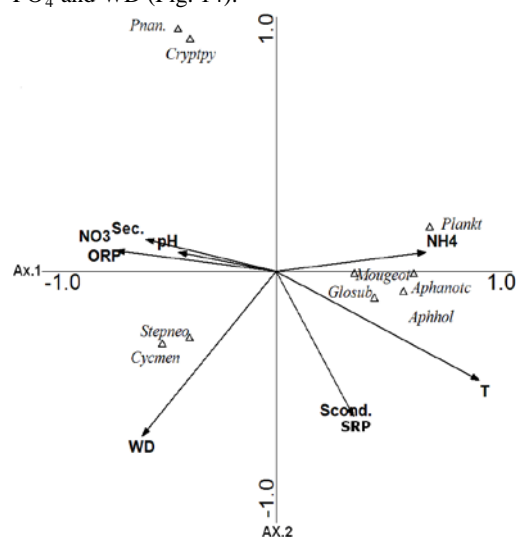


Figure 14. The diagram of Canonical Correspondence Analysis (CCA) showing the relationships between the environmental variables and the dominant phytoplankton taxa in the Çaygören Reservoir. Abbreviation for species: Cycmen, *Cyclotella meneghiniana*; Stepneo, *Stephanodiscus neoastraea*; Glosub, *Gloeotila subconstricta*; Aphhol, *Aphanocapsa holsatica*; Plankt, *Planktothrix* sp.; Cryptpy, *Cryptomonas pyrenoidifera*; Aphanoc, *Aphanocapsa clathrata*; Mougeotia, *Mougeotia* sp.; Pnan, *Plagioselmis nannoplantctica*.

Fig. 14 shows the relationships between environmental variables and the dominant phytoplankton taxa. The distribution of cyanobacteria, *Planktothrix* sp., *A. holsatica* and *A. clathrata*, along the positive side of the first axis of CCA diagram, reflected their occurrence at high temperature. *G. subconstricta* (Chlorophyta) and *Mougeotia* sp. (Streptophyta) were located on the

positive side of the first axis and they were negatively related with water transparency and $\text{NO}_3\text{-N}$. The cryptophytes, *C. pyrenoidifera* and *P. nannoplantica* were located on the positive side of the second axis and they were negatively related with T. The diatoms, *C. meneghiniana* and *S. neoastraea* were located on the negative side of the second axis and they were positively related to WD.

Table 3. The Pearson's correlation coefficients between the physicochemical parameters and the dominant phytoplankton taxa in the Çaygören Reservoir between 2007 and 2009

	T	Secc	SC	ORP	PH	NO_3	NH_4	PO_4	WD
T	1	-0.5	0.6*	-0.6*	0.4	-0.6*	0.6*	-0.6*	0.1
Secc	-0.5	1	0.01	0.2	0.1	0.01	0.01	0.2	0.4
SC	0.6*	0	1	-0.8*	-0.5*	0.1	0.2	0.8*	0.4
ORP	-0.6*	0.2	-0.8*	1	0.5*	-0.8*	-0.4	-0.91*	0
pH	0.4	0.1	-0.5*	0.5	1	-0.5*	-0.1	0.5*	-0.1
NO_3	-0.6*	0	0.1	-0.8	-0.5*	1	0.2	-0.8*	0.4
NH_4	0.6*	0	0.2	-0.4	-0.1	0.2	1	-0.4	-0.1
PO_4	-0.6*	0.2	0.8*	-0.91*	0.5*	-0.8*	-0.4	1	0
WD	0.13	0.4	0.4	0.01	-0.1	0.4	-0.1	0.1	1
<i>Cycmen</i>	-0.2	0.2	0.1	0.2	0.2	0.1	-0.3	0.2	0.7*
<i>Stepneo</i>	-0.1	0.3	0.1	0.2	0.2	0.1	-0.3	0.2	0.7*
<i>Glosub</i>	-0.1	-0.6*	0.2	0.1	0.6*	0.2	-0.2	0.9*	-0.3
<i>Mougeot</i>	0.3	-0.5*	0.2	0.1	0.4	0.2	0	0.9*	-0.4
<i>Aphhol</i>	0.5*	-0.1	-0.2	-0.3	-0.4	-0.2	0.2	-0.3	0
<i>Aphanotc</i>	0.5*	0.1	0	-0.3	-0.6*	0	0.1	-0.3	0.2
<i>Plankt</i>	0.5*	0.2	-0.4	-0.4	-0.6*	-0.4	0.5*	-0.4	-0.3
<i>Cryptpy</i>	-0.7*	0.4	-0.5*	0.3	0.4	-0.45	-0.2	-0.42	-0.3
<i>Pnan</i>	-0.7*	0.4	-0.6*	0.3	0.41	-0.36	-0.2	-0.41	-0.4

*significant at 0.05 level.

Abbreviation: *Cycmen*, *Cyclotella meneghiniana*; *Stepneo*, *Stephanodiscus neoastraea*; *Glosub*, *Gloeotila subconstricta*; *Aphhol*, *Aphanocapsa holsatica*; *Plankt*, *Planktothrix* sp.; *Pnan*, *Plagioselmis nannoplantica*; *Cryptpy*, *Cryptomonas pyrenoidifera*; *Aphanotc*, *Aphanothece clathrata*; *Mougeot*, *Mougeotia* sp.

There were significant correlations between most of the water quality parameters (Table 3). Significant positive correlations were observed between T and SC, T and NH_4 ; pH and ORP, pH and PO_4 ; ORP and PO_4 , while negative correlations were obtained between T and PO_4 , T and $\text{NO}_3\text{-N}$, T and ORP; SC and PO_4 , SC and pH, SC and ORP; ORP and pH, ORP and $\text{NO}_3\text{-N}$; $\text{NO}_3\text{-N}$ and PO_4 .

4. Discussion

4.1. Statistical Analysis and Interpretation of Physico-Chemical Parameters

The results of the present study showed that water temperature was negatively correlated with $\text{NO}_3\text{-N}$ and it was positively correlated to $\text{NH}_4\text{-N}$. The significant positive correlation between ammonium concentrations and water temperature could probably be attributed to the intensified ammonification of $\text{NO}_3\text{-N}$ with the increased water temperature (Liikanen and Martikainen, 2003). Ammonium is often used preferentially by phytoplankton over nitrate when both substrates are available in the water column (Stolte and Riegman, 1996). The negative correlation between the nitrate

There were significant correlations between the following the physicochemical variables and the dominant taxa:

Secchi disk transparency and *G. subconstricta*, Secchi disk transparency and *Mougeotiasp.*; pH and *G. subconstricta*, pH and *A. clathrata*; $\text{NH}_4\text{-N}$ and *Planktothrix* sp.; WD and *C. meneghiniana* and WD and *S. neoastraea* (Table 3).

concentrations and water temperature may also indicate the effective consumption of the winter stock of nitrate by phytoplankton during the summer blooms in the Çaygören Reservoir (Temponeraset al., 2000).

ORP and PO_4 were negatively correlated. ORP is the most important factor influencing the exchange process of phosphorus between the water and sediments (Li et al., 2010). It is well known that a decrease of ORP results in the deoxidization of metal-oxides, which might lead to a release of PO_4 , whereas a rise of ORP helps to cause more PO_4 to be absorbed (House and Deniso, 2000). It could be concluded that high PO_4 concentrations in the summer were attributed to the transfer of PO_4 from the bottom layers due to low ORP values in the Çaygören Reservoir.

Raised temperatures stimulate the overall mineralization and thereby liberate organic- bound phosphorus into the sediment pore water. In addition to this direct effect, increased microbial activity lowers the redox potential in the surface sediment, which may induce release of Fe-bound phosphorus (Wilhelm and Adrian, 2008).

There is a positive correlation between specific conductivity and PO_4 . Specific conductivity is often considered as parameter showing the degree of nutrient loading (Parinet *et al.*, 2004). Intensive agriculture has been practiced in the drainage basin of the Çaygören Reservoir. Agricultural nonpoint sources are a major contributing factor to surface water eutrophication worldwide.

Secchi disk transparency and water temperature were negatively correlated. In standing water bodies, turbidity increases with nutrient levels, which stimulate phytoplankton growth, especially during warm seasons (Scheffer and van Nes, 2007). Low Secchi disk depths in the summer timing are probably due to high abundance of phytoplankton. The eutrophic Çaygören Reservoir is dominated by Cyanobacteria in summer. Cyanobacteria strongly absorb light causing reduced water transparency (LaBounty, 2008).

Nitrate was negatively correlated with pH. It has long been known that the dense populations of phytoplankton deplete the carbon dioxide present in natural waters, resulting in the rise of pH. Jones-Lee and Lee (2005) state that algae take up nitrate and CO_2 from water, causing the increase of pH during daylight in eutrophic lakes. The higher pH values found in the Çaygören Reservoir must accordingly have been a result of the depletion of free CO_2 in the water due to high rate of photosynthesis.

pH is the master variable in the chemistry of aquatic systems and it affects the kinetics of nutrient uptake and controls the chemical species of most of the nutrient ions that algae require. Carbon fixation as a consequence of photosynthetic activity can displace the carbon dioxide-bicarbonate-carbonate equilibrium that is the most common pH-buffering mechanism in freshwater systems. Photosynthesis thus tends to increase pH in lakes (Haande *et al.*, 2011).

4.2. Statistical Analysis and Interpretation of Phytoplankton and Physico-Chemical Parameter Relationships

In the CCA diagram, the cyanobacteria, *Planktothrix* sp., *A. clathrata* and *A. holsatica* occurred near $\text{NH}_4\text{-N}$ and water temperature vectors. *Planktothrix* sp. was the most abundant cyanobacterium that dominated phytoplankton in the summer in the Çaygören Reservoir. The timing of *Planktothrix* bloom in the Çaygören Reservoir appears to be related to high temperature of the eutrophic condition of the reservoir. Temperature is one of the most important factors affecting the biology of phytoplankton species by controlling the rate of enzymatic reactions within the cells. In addition, temperature also regulates the multiplication rate and standing stock of natural phytoplankton populations (Niu *et al.*, 2011).

Padisak *et al.* (2009) state that small colonial non-N-fixing cyanobacteria prefer well-mixed environments at high water temperatures. The high nutrient levels in the Çaygören Reservoir probably

accounted for the development of this cyanobacterium during the summer time.

Water temperature had significant correlations with *A. clathrata* and *A. holsatica*. Komarek and Anagnostidis (1999) point out that *A. clathrata* and *A. holsatica* prefer eutrophic waters. *A. clathrata* and *A. holsatica* are widely collected in eutrophic Turkish lakes during the summer (Sevindik *et al.*, 2010).

C. meneghiniana (Bacillariophyta) and *S. neoastrea* (Bacillariophyta) dominated phytoplankton in the spring. Diatoms are usually common during cooler or windier conditions in freshwater lakes (Munawar and Munawar, 1986). These species were occasionally abundant at the shallower first station. Although *Cyclotella* and *Stephanodiscus* species are widely collected in freshwater phytoplankton, some of them are also benthic (Hutchinson, 1967). They might have been drifted from the bottom due to wind-driven water turbulence at this shallow station.

In the CCA diagram, *S. neoastrea* and *C. meneghiniana* occurred near the water discharge vector. Their relations with the water discharge suggest that these species might have been drifted from the bottom of the feeding river. The highest water discharge occurred in the spring when these species were dominant in the reservoir. The flow rate in rivers is probably the most effective factor controlling the population density of diatoms in the inlets of lakes. Baykal *et al.* (2011) found out that *Stephanodiscus* species were abundant in Melen River, Turkey. They state that *Stephanodiscus* species are well adapted to turbulent and turbid river systems with high nutrient concentrations.

Bere and Tundisi (2011) observed high abundance of benthic *C. meneghiniana* in the eutrophic Monjolinho River, Brazil. They state that certain benthic diatoms are associated with eutrophication and may be used as indicator species of eutrophication in running waters. Although the phytoplankton of the feeding river was not explored during the study, the high nutrient concentration of the Simav Stream might have favored high abundance diatoms in the system (Gunduz *et al.*, 2010).

G. subconstricta (Chlorophyta) and *Mougeotia* sp. (Streptophyta) were dominant during the fall. In the CCA diagram, these species occurred near the water temperature and $\text{NH}_4\text{-N}$ vectors. They had significant negative correlations with Secchi disk transparency and significant positive correlations with PO_4 . The dominance of these filamentous green algae in October seems to be related to the fall overturn in the Çaygören Reservoir. In the fall, nutrients are increased and the transparency is decreased due to the overturn in the reservoir.

C. pyrenoidifera (Cryptophyta) and *P. nannoplantica* (Cryptophyta) dominated phytoplankton during the winter time. These species had significant negative correlations with water temperature. Low water temperatures and low light availability may have acted as selecting factors for

this group during the winter since Cryptophytes are adapted to a low light intensity (Barone and Naselli-Flores, 2003). Various factors may regulate Cryptophyta seasonality in lakes, but it seems that the key factor in the success of Cryptophyta species is their low light requirement. Low Secchi disk transparency, during the winter in the Çaygören Reservoir, might have favored the success of this group during the winter.

The maximum phytoplankton abundance was measured in the summer because of sufficient light and high water temperature and the minimum abundance was measured in the winter because of insufficient light and low water temperature. In temperate lakes, low winter irradiance and water temperature preclude the development of high phytoplankton density in the winter time (Peeters *et al.*, 2007). The high phytoplankton densities in the summer were probably attributed to the dominance of cyanobacteria (over 80%). The high abundance of cyanobacteria during warm seasons in the Çaygören Reservoir can be attributed to the increased water temperature.

Although the maximum phytoplankton density was measured in the summer, the maximum biomass was measured in the spring. This was probably due to the dominance of cyanobacteria in the summer. Cyanobacteria have a smaller cell size than most of the other phytoplankton groups (Ciottiet *al.*, 2002). Therefore, high abundance of cyanobacteria might have not resulted in a high phytoplankton biomass in the Çaygören Reservoir.

5. Conclusions

The present study revealed that the important factors affecting the density, biomass and dominance of the phytoplankton in a temperate eutrophic reservoir were water temperature, underwater light (transparency), water discharge and the relative concentrations of nutrients. High density of cyanobacteria does always not warrant high biomass in the eutrophic freshwater systems. The flow rate of feeding rivers can significantly affect the population density of diatoms in the inlets of reservoirs. Finally, low water temperatures and low light availability may favor Cryptophyta dominance in the eutrophic temperate lakes.

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Glypican- 3 Expression in Primary and Metastatic Neuroblastoma

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Abstract

Glypican-3 is an oncofetal protein found to be overexpressed in different types of tumours, such as hepatocellular carcinoma, malignant melanoma, squamous cell carcinoma of the lungs and testicular yolk sac tumour. Glypican-3 is currently emerging as a tumour marker and/or potential target for therapy of many cancers. However, there are limited studies looking for glypican-3 expression in neuroblastoma, with some evidence for loss of expression. Therefore, we sought to investigate glypican-3 expression in primary and metastatic neuroblastoma and to explore its potential as marker and/or target for development of new therapy for neuroblastoma. A total of 31 archived tissue specimens of neuroblastoma were subjected to immunohistochemical staining using a monoclonal antibody specific for glypican-3. Glypican-3 expression was compared with clinical and histological characteristics for each patient. Immunohistochemical analysis revealed for the first time overexpression of glypican-3 in 33% of neuroblastoma tumours. Its overexpression was surprisingly more predominant in metastatic tumours (71%) than in primary tumours. Glypican-3 expression was significantly correlated with disease clinical stages ($P \leq 0.05$). It was more frequently expressed in the majority of stage 4s patients and connoted poor disease prognosis. On this basis, the biological functions and molecular mechanisms underlying the overexpression of glypican-3 in neuroblastoma warrant further investigations, especially the promising use of glypican-3 for diagnostic and therapeutic purposes.

Keywords: Cancer, Glypican-3, Hepatocellular carcinoma, Immunohistochemistry, Neuroblastoma.

1. Introduction

Neuroblastoma is an embryonal, extracranial solid tumour. It arises from any primitive neural crest of sympathetic nervous system (Ishola and Chung, 2007; Park et al., 2008). It is the second most common paediatric tumour. It accounts for 10% of paediatric tumours and responsible for 15% of childhood fatalities worldwide. Because it originates from the neural crest (sympathogonia), neuroblastoma may occur anywhere in the sympathetic nervous system (Park et al., 2008). Most of primary tumours arise in the abdomen, where the adrenal gland is the most common site.

Other primary locations are neck, chest and pelvis (Maris et al., 2007). Furthermore, neuroblastoma can metastasize to lymph nodes, bone marrow, bones, liver, skin and testis (Humpl, 1995). Despite the recent advances in the management of neuroblastoma, many neuroblastoma patients are dying due to uncontrolled metastasis of disease. Therefore, a new strategic approach for the treatment of neuroblastoma is needed.

Glypicans are proteins bound to external surface of plasma membrane by a glycosyl-phosphatidylinositol anchor. Six family members of glypicans have been identified, namely glypican-1 to glypican-6 in vertebrates (Filmus, 2001; Filmus and Selleck, 2001; Filmus et al., 2008; Fico et al.,

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2011). They play an important role in developmental morphogenesis and their expressions vary in a stage and tissue specific manner. It is believed that glypicans regulate cell signalling through interaction with growth factors, chemokines and structural proteins (Filmus, 2001; Iglesias et al., 2008; Fico et al., 2011).

One of the interesting members of glypicans is glypican-3. It was found to act as an onco-fetal protein in some tumours (Chan et al., 2013; Kinoshita et al., 2015). During malignant transformation, its protein and mRNA levels were abnormally re-expressed and normally silenced in some adult tissues (Filmus, 2001; Filmus and Capurro, 2008). However, the neoplastic role of glypican-3 seems to be dichotomous acting as a tumour suppressor protein in some tumours, like breast and ovary cancers, while an oncogenic protein in development of others, such as liver, colon and embryonal tumours (Lin et al., 1999; Murthy et al., 2000; Saikali and Sinnett, 2000; Zhu et al., 2001; Peters et al., 2003; Chan et al., 2013; Wang et al., 2014; Kinoshita et al., 2015). Moreover, individuals suffering from loss of function mutations for glypican-3 developed a condition called Simpson-Golabi-Behmel Syndrome (SGBS). This syndrome is characterized by malformations of pre- and postnatal overgrowth, and a wide range of abnormalities including macrocephaly, protruding jaw and tongue, widened nasal bridge, and upturned nasal tip (Cottreau et al., 2013; Pilia, Hughes-Benzie et al., 1996). Interestingly, such individuals were found at high risk for developing embryonal tumours most likely neuroblastoma and Wilm's tumour (Hughes-Benzie et al., 1992).

Recently, overexpression of glypican-3 has been reported in many types of tumours, like hepatocellular carcinoma (Zhu et al., 2001), melanoma (Nakatsura et al., 2004), lung squamous cell carcinoma (Aviel-Ronen et al., 2008), and testicular germ cell tumours (Ota et al., 2006). In hepatocellular carcinoma, glypican-3 expression was able to differentiate cases of hepatocellular carcinoma from healthy livers and benign liver lesions (Zhu et al., 2001; Nakatsura et al., 2003; Man et al., 2005; Wang et al., 2008; Zhang et al., 2012; Haruyama and Kataoka, 2016). Moreover, the serum levels of glypican-3 were used to diagnose patients with hepatocellular carcinoma but not in the case of other liver diseases or cancers (Capurro et al., 2003). Therefore, glypican-3 was suggested to be a potential serological and histological marker for the diagnosis of hepatocellular carcinoma. In neuroblastoma, there were few studies examined the glypican-3 expression in primary tumours and none of them had looked for the expression in neuroblastoma metastatic tumours. In these studies, all the neuroblastoma primary tumours demonstrated negative to weak expression of glypican-3 (Chan et al., 2013; Kinoshita et al., 2015). On this basis, the aim of the present study is to investigate glypican-3 expression in both primary

and metastatic neuroblastoma tumours and to explore if glypican-3 could be valuable as a marker or a potential target for development of new therapy for neuroblastoma.

2. Materials and Method

2.1. Tissue Specimens

Archived, formalin-fixed, paraffin-embedded tissue blocks from 31 neuroblastoma and 5 hepatocellular carcinoma patients were obtained from the surgical pathology files of King Hussein Medical Hospital, Royal Medical Services. The neuroblastoma specimens included 14 cases of primary tumours obtained from abdomen, spine, paraspine, sacrum, brain and pharynx. The rest of specimens were metastatic samples of bone marrow and lung. All personal data for specimens were kept anonymous. The present study was ethically approved by the Ethics Committee, Faculty of Medicine, Mutah University.

2.2. Immunohistochemistry

Five μm -thick Formalin-Fixed and Paraffin-Embedded (FFPE) tissue sections were deparaffinised and rehydrated in graded alcohols. Sections were then subjected to heat-induced epitope retrieval in citrate buffer (10 mM citrate buffer, pH 6.0) in a microwave at 600W for 20 minutes. To block the non-specific binding of the antibodies, sections were treated with 1.5% normal goat serum for 20 minutes at room temperature. Sections were then incubated with a primary rabbit monoclonal antibody specific for glypican-3 (Abcam, UK) at concentration of (4 $\mu\text{g}/\text{mL}$) for one hour at room temperature. Following primary antibody treatment, each section was incubated with goat anti-rabbit peroxidase-conjugated secondary antibody (Vector Laboratories Ltd, Peterborough, UK) (7.5 $\mu\text{g}/\text{mL}$) for 30 minutes at room temperature. The colour reaction was developed by incubating sections with 3,3-diaminobenzidine chromogen (DAB) (Vector Laboratories Ltd, Peterborough, UK) solution for 3-5 minutes. Sections were then counterstained in Harris's haematoxylin solution mounted with coverslips using DPX medium.

The resulting slides were viewed and analyzed by using a Leica DMRB microscope (Leica DMRB, Wetzlar, Germany) with the images digitally captured and processed using a Leica MPS52 camera (Q Imaging, Germany) and the AcQuis imaging capture system (Synoptics, Cambridge, UK), respectively.

2.3. Analysis of Glypican-3 Expression

Stained slides were evaluated and scored manually by two independent pathologists. The scoring system used to analyze immunohistochemical labelling of neuroblastoma clinical specimens was based on previously published studies (Gluer et al., 1998; Gluer et al., 1998). For glypican-3 expression, cells were

considered positive if they demonstrated clear membranous and/or cytoplasmic immunolabelling. Immunoreactivity for glypican-3 was scored by evaluating the number of positive tumour cells over the total number of tumour cells. The scoring results were presented as: none (0), weak (1), moderate (2) and high (3). The score 'none' indicated an absence of expression. Sections were allocated a score of 'weak' when less than 33% of cells had expression. A score of 'medium' was applied to cells which had expression on 33% to 66% of the section whilst the score 'high' represented sections which had expression on more than 66% of the cells.

2.4. Statistical Analysis

The data were appropriately coded and subjected to analysis using the Nonparametric Spearman's rank order correlation coefficients using SPSS software (version 16.0, Chicago, IL). Results were considered statistically significant only if $p < 0.05$.

3. Results

3.1. Demographic and Clinicopathologic Features

All the demographic and clinical data were available for 31 children with neuroblastoma Table 1. There was a male predominance (76.7%) while female constituted only 32.3% of the patients. The most common site of primary tumours was the abdomen (61%). The majority of patients were presented with distant metastases including lymph nodes (77%), bone marrow (71%), bone (3%) and lung (3%). Twenty-two patients had metastases to both lymph nodes and bone marrow. One patient had primary tumour in the brain with no evidence of distant metastasis. Based on International Neuroblastoma Staging System (INSS), 58% of patients were clinically presented with low-stage of disease (stage 1, 2, or 4S), whereas the rest of patients were at advanced stage of disease (stage 3 or 4). According to the International Neuroblastoma Pathology Classification (INPC), twenty four patients were presented with unfavourable prognosis of disease.

Table 1. Patient Characteristics. INSS: International Neuroblastoma Staging System, INPC: International Neuroblastoma Pathology Classification

Characteristics	Number/ Percentage (%)
Age(year)	
Mean	5.9
Gender	
Male	21(76.7)
Female	10 (32.3)
Site of primary tumour	
Abdomen	19 (61)
Retropharyngeal	1 (3)
Spine	3 (10)
Paraspine	3(10)
Sacrum	1(3)
Thorax	3(10)
Brain	1(3)
Sites of metastases	
Bone	1(3)
Bone Marrow	22 (71)
Lymph node	24 (77)
Pulmonary	1(3)
INSS stage	
1	5 (17)
2	2 (6)
3	4 (13)
4	9 (29)
4S	11 (35)
INPC classification	
Favorable	7 (23)
Unfavorable	24 (77)
Glypican-3 expression	
0	67%
1	33%
2	0%
3	0%

3.2. Glypican-3 Expression in Primary and Metastatic Neuroblastoma

Hepatocellular carcinoma tissues used as a positive control samples demonstrated a strong glypican-3 staining (Fig. 1A). Glypican-3 was only expressed in 33% of primary and metastatic neuroblastoma clinical specimens (Fig. 1). Glypican-3 staining was predominantly localised to the membrane and/or cytoplasm of tumour cells without any significant staining noted in the nucleus. All tumours expressing glypican-3 showed weak expression with less than 33% of cells expressing glypican-3. Notably, this expression was more predominant in metastatic tissues (bone marrow 71%) than in primary tumour tissues (Fig. 1G).

3.3. Glypican-3 Expression is Correlated with Clinical Stage

INSS clinical staging of neuroblastoma disease was based on the extent of the primary tumour growth, local lymph node involvement, and metastases to distant sites. Glypican-3 expression was compared with INSS clinical stages of neuroblastoma. A significant correlation was found

between glypican-3 expression and clinical stages ($P \leq 0.05$). Glypican-3 was more frequently expressed in patients with low stage of disease (Fig. 1B and F). The majority of tumours from patients presenting with stage 4s neuroblastoma had expression of glypican-3. Only two patients with advanced stage neuroblastoma particularly stage 4 showed glypican-3 expression (Figs. 1E and 2).

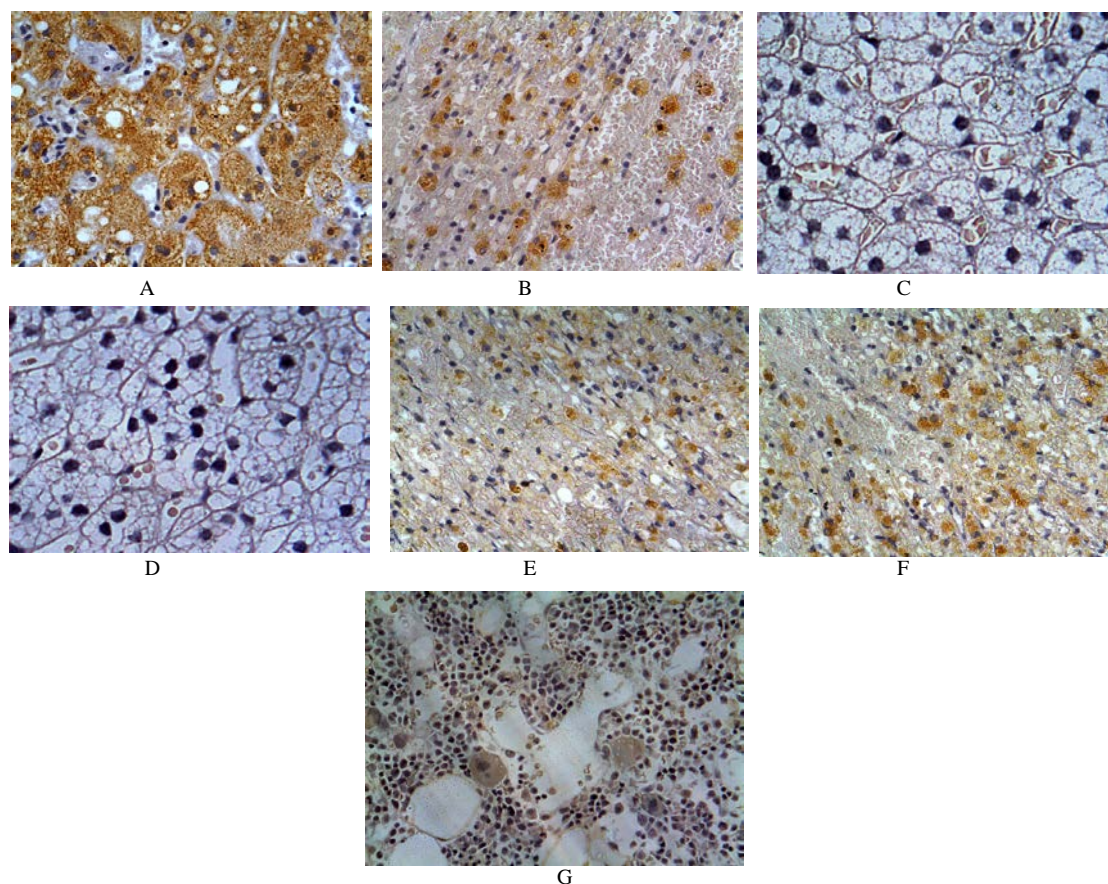


Figure 1. Glypican-3 expression in hepatocellular carcinoma and primary and metastatic neuroblastoma. (A) Distinct glypican-3 labelling of the cell cytoplasm seen for hepatocellular carcinoma (B, E and F) Weak glypican-3 labelling localised to the cell membrane and/ or cytoplasm was seen for neuroblastoma primary tumours in clinical stage of 1, 4 and 4s, respectively. (C and D) Negative glypican-3 expression demonstrated for neuroblastoma primary tumours in clinical stage of 3 and 4, respectively. (G) Weak glypican-3 labelling localised to the cell membrane and/ or cytoplasm was seen for neuroblastoma metastatic tumours of bone marrow a. All images are taken at the same magnification (X400).

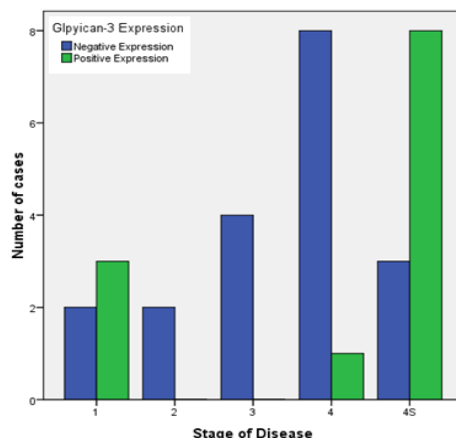


Figure 2. Distribution of glypican-3 expression on primary and metastatic neuroblastoma tumours and INSS clinical stage at presentation

As INPC determines the prognosis of disease in neuroblastoma patients on the basis of tumour histology and patient age, expression of glypican-3 was also compared with the INPC parameters. There was a relationship between glypican-3 expression and INPC parameters but the correlation was insignificant. Glypican-3 expression connoted unfavourable disease prognosis majorly in patients with low stage of disease (stage 4s). No statistical evidence of relationship between glypican-3 expression and age, gender and site of metastasis was detected.

4. Discussion

Several lines of evidence indicate the importance of glypican-3 expression in malignant tumours where it is increasingly recognized as a potential marker or a target for development of new therapy for tumours expressing glypican-3 (Capurro *et al.*, 2003; Aviel-Ronen *et al.*, 2008; Zynger *et al.*, 2008; Tretiakova *et al.*, 2015). There is growing evidence to support the potential impact of glypican-3 that contributes to malignancy in many tumours. In paediatric tumours, high levels of glypican-3 mRNA were detected in Wilm's tumour (Tretiakova *et al.*, 2015), neuroblastoma cell lines (Tretiakova *et al.*, 2015), hepatoblastomas (Zynger *et al.*, 2008) and rhabdomyosarcomas (Thway *et al.*, 2011) while low levels of glypican-3 mRNA were found in medulloblastoma and Ewing sarcoma (Saikali and Sinnott, 2000). Therefore, the oncogenic role of glypican-3 remains unclear in the light of the over-expression in certain tumours and greater risk of developing embryonic tumours secondary to glypican-3 mutations seen in the SGBS syndrome.

In the present study, for the first time we present convincing evidence of glypican-3 expression in neuroblastoma metastatic tumours and we demonstrate generally absent or weak expression in neuroblastoma primary tumours. These results came in accordant with the previous study looking for glypican-3 expression in embryonal tumours (Saikali and Sinnott, 2000). Of these, mRNA expression of glypican-3 was investigated in 10 human neuroblastoma cell lines and 4 primary tumour specimens. Glypican-3 was highly expressed in 70% of neuroblastoma cells lines and several neuroblastoma primary tumour specimens. Further studies are needed to investigate the glypican-3 mRNA and protein level in neuroblastoma metastatic tumours.

Although the glypican-3 expression was weak in neuroblastoma metastatic tumours, it correlated significantly with clinical stage. Most of the metastatic tumours expressing glypican-3 were at low stage of disease (stage 4s). As the sample size of the present study was too small, robust statistically significant correlations between glypican-3 expression and some of clinicopathologic features were not possible. However, some clear trend was apparent, particularly between expression of glypican-3 and

prognosis of disease. According to INPC, all the metastatic tumours expressing glypican-3 had unfavourable disease prognosis. Further investigation is warranted using larger sample size to better delineate the relationships of glypican-3 with clinicopathologic features.

There were limited studies looking for glypican-3 expression in neuroblastoma (Chan *et al.*, 2013; Kinoshita *et al.*, 2015). In these studies, glypican-3 was only examined in neuroblastoma primary tumours. No studies were looking for the expression in neuroblastoma metastatic tumours. The absence of glypican-3 expression in neuroblastoma primary tumours was reported before by Chan *et al.* (2013). In that study, all the neuroblastoma primary tumours (n=136) demonstrated negative glypican-3 expression. Authors explained this observation as a result of low protein expression of glypican-3, protein conformational changes, or protein degradation (Chan *et al.*, 2013). Additionally, Kinoshita and co-workers reported that glypican-3 was weakly expressed only in one patient out of 35 neuroblastoma patients (Kinoshita *et al.*, 2015). This further confirms our results regarding weak or absent glypican-3 expression in neuroblastoma primary tumours.

Despite the growing evidence for the contribution of glypican-3 to tumour malignancy, the underlying mechanisms at the cellular level remain unclear. Glypican-3 seems to play an important role in controlling the functions of other molecules enhancing tumour development and metastasis. Glypican-3 was found to regulate expression of certain types of Matrix Metalloproteinases (MMPs), such as MMP-2, MMP-9 and MMP-14 in different cancers (Akutsu *et al.*, 2010). Moreover, GPC3 reportedly confers oncogenicity by acting as a co-receptor for different types of growth factors, like Fibroblast Growth Factor (FGF) 2 and Insulin-like Growth Factor (IGF) 2 (Song *et al.*, 1997; Cheng *et al.*, 2008). In certain types of cancers, glypican-3 was shown to stimulate growth of tumour cells by regulating autocrine/paracrine canonical Wnt signalling. Regulation of migration, adhesion, and actin cytoskeleton organization in mammary tumour cells through Wnt signalling modulation by glypican-3 expression was reported (Stigliano *et al.*, 2009). Therefore, the impact of GPC3 expression on other molecules functions is interesting to be investigated.

5. Conclusion

Our current investigation revealed for the first time expression of glypican-3 in metastatic neuroblastoma. Despite weak glypican-3 expression, glypican-3 was more predominantly detected in patients with stage 4s of disease and indicated unfavourable disease prognosis. While the present study is the only one exploring the glypican-3 expression in metastatic neuroblastoma specimens, more preliminary data are required on

the potential use of glypican-3 for therapeutic and diagnostic purposes.

Conflicts of Interest

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

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