Volume 23 2015 ISSN 1023-8654

JOURNAL OF BIO-SCIENCE

Institute of Biological Sciences
University of Rajshahi, Bangladesh
http://www.banglajol.info/index.php/JBS/index

THE
JOURNAL OF BIO-SCIENCE
(J. Bio-Sci.)

ISSN 1023-8645

The Institute of Biological Science (IBSc), University of Rajshahi established in 1989 has been publishing the Journal of Bio-Science (J. Bio-Sci.) from 1993. The J. Bio-Sci. is a peer-reviewed journal publishes only one issue per year (June). However, we have intended to publish two issues annually (June and December) from coming year (2018).

Scientific papers in any fields of Biological Sciences from anywhere in world are being considered for publishing in the Journal of Bio-Science as per reviewer's comments and recommendation.

All manuscripts intended for publication in the journal should be sent by postal addressed to the Chief Editor, Journal of Biological Science, C/o: Institute of Biological Science, University of Rajshahi, 3rd Science Building, Rajshahi-6205, Bangladesh or as word file (MS Word 2007- onwards) through e-mail to Executive Editor, jbio@ru.ac.bd with cc. to the Chief Editor, director_ibsc@ru.ac.bd.

Copies are also available from the Director, Institute of Biological Sciences, University of Rajshahi, 3rd Science Building, Rajshahi - 6205, Bangladesh. Tel. +880-72-750928, Fax: +880-721-750064, E-mail: jbio@ ru.ac.bd, director_ibsc @ ru.ac.bd, Website: www.ru.ac. bd/ibsc, <http:// www. banglajol. info/ index. php/ JBS/index>

Price of the Journal	SARC countries	Other Countries
Each issue	US$ 25.00 + 5.00 (Postage)	US$ 25.00 + 8.00 (Postage)
Each volume	US$ 50 .00+ 5.00 (Postage)	US$ 25.00 + 8.00 (Postage)

In Bangladesh Tk. 300.00 for one issue and Tk. 500.00 each volume.

Published by the Chief Editor, Journal of Bio-Science, Institute of Biological Sciences, University of Rajshahi, Bangladesh, June 2018.

Printed by: Uttoran Offset Printing Press, Greater Road, Rajshahi- 6000, Bangladesh. Tel: +88-0721-773782

Editorial Board

Author Instruction

Paper size, margins, fonts and line spacing: Paper size - A4, margins: top, bottom, left, right - 2.5 cm, headers and footers - 1.5 cm, fonts - Times New Romans (TNR), line spacing: text - double between paragraphs spacing 6 pt.

Article length: (a) For Full Paper - 12 pages maximum (Review Article 18 pages max). (b) For Short Communication - 6 pages maximum and 4 pages minimum.

Sections: (a) For full paper -Title, Author(s) name and affiliation, Abstract, Key words, Introduction, Materials and Methods, Results and Discussion (clubbed or separate), Conclusion (if any), Acknowledgements (if any), References. (b) For short communication -Title, Author(s) name and affiliation, Acknowledgements (if any) and References.

Title page, authors name and affiliation: The title page should contain the title of the paper, full name(s) of the author(s) and their institutional address(s). Papers title should be 14 TNR, all caps, bold, single space and centered. Authors name and affiliation should be 12 TNR bold and 11 TNR italics, respectively, centered; authors for correspondence must be indicated by an asterisk (*). Please use superscripts of 1,2,3 etc. for multiple authors from different Institutions.

Example:

COLCHICINE-INDUCED POLYPLOIDY IN *PHALAENOPSIS* ORCHIDS

MA Salam[1], MS Hossain[2], MA Haq[1] and MAK Parvez[3*]

[1]Department of Botany, University of Rajshahi, Bangladesh; [2]Bangladesh Council of Scientific and Industrial Research (BCSIR), Bangladesh; [3]Department of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh*

Abstract (12 TNR): The abstract should be a single paragraph (150 - 250 words) that summarises the content of the article.

Key words (12 TNR): Please give five to six keywords separated by comma in alphabetical order.

References (12 TNR): References at the end of the text should be arranged alphabetically. Please follow the styles of the references given below:

Journal	:	Khatun R, Islam W and Mondal KAMSH (2009). Performance of cypermethrin deltamethrin and nimbicidine on larval development of *Tribolium castaneum* (Herbst), Journal of Bio-Science 17: 45-50.
Book	:	Watson JD (1976). Molecular Biology of Genes. 3rd Ed., WA Benjamin, Inc. Menlo Park, California: 1-739.
Book Chapter	:	Islam SMS and Tuteja N (2012). Production of abiotic stress tolerant fertile transgenic plants using androgenesis and genetic transformation methods in cereal crops. *In*: Tuteja N and Gill SS (Eds.). Crop Improvement Under Adverse Conditions. Springer New York Heidelberg London, 213-229 pp.
Symposium/ Proceedings paper	:	Mamun ANK, Islam SMS and Bari MA (2002). Micropropagation of some wood legumes through tissue culture technique. *In:* BIO-REFOR-Sustainable Forest Management System and Biodiversity, Katsuaki I, Masaya M and Kazuo S (Eds.) Proceedings of the International Workshop of BIO-REFOR, Tokyo, Japan, 139 144 pp.

Thesis/ Dissertation	:	Faruki MSI (1993). Effect of *Bacillus thuringinesis* var. Kurstaki, synthetic pyrethroid and ionizing radiation on *Cadra cautella* (Walker) (Lepidoptera: Phycitidae). PhD Thesis, Institute of Biological Sciences, University of Rajshahi, Bangladesh.
Electronic Resources	:	FAO (2009) Global Agriculture Towards 2050. http://www.fao.org/

Tables, figures and photographs: Number of tables, graphs and figures should be minimum. The same data should not be presented in both tables and graphs. Please give figure caption below the figure, and table caption above the table. All figures and tables must be placed in appropriate places in the text. Please use 'Fig.' when referring to a figure in the body of the text. The photographs should be submitted by JPEG format with good resolution (at least 300 pixles).

Declaration: All authors must sign a declaration certifying that the work was carried out by them and the contents of the paper were neither published before nor submitted for publication in any other journal, and that they have submitted their articles for consideration by the Editorial Board of the Journal of Bio-Science.

The research article should be submitted as attached file in MS Word (2007- onwards) through e-mail to Executive Editor (jbio@ru.ac.bd) with cc. to the Chief Editor (director_ibsc@ru.ac.bd). In case of direct submission or submission by post the article should be submitted in two hard copies along with a soft copy in a CD/DVD.

Postal address:

Chief Editor
Journal of Bio-Science (J Bio-Sci.)
Institute of Biological Sciences
University of Rajshahi, Rajshahi-6205, Bangladesh
E-mail: jbio@ru.ac.bd (For all corresponding)
Tel. : +88-0721-750928 (Office)
Mob. : +88-01716288356

JOURNAL OF BIO-SCIENCE (J. Bio-Sci.)

CONTENTS

	VOLUME 23 2015	Pages
1	N Hindryawati, GP Maniam, CK Feng and MR Karim. TREND IN ENZYME IMMOBILIZATION ON NANO MATERIALS FOR TRANSESTERIFICATION TO PRODUCE BIODIESEL: A REVIEW	1-17
2	J Eshaghi-Rad, A Motallebpour and A Alijanpour. EFFECT OF PHYSIOGRAPHIC FACTORS ON WOODY SPECIES DIVERSITY IN OAK FORESTS (CASE STUDY: SARDASHT FOREST - IRAN)	19-27
3	Ashaduzzaman, MJ Hossain and S Akther. PHYSICO-CHEMICAL PROPERTIES OF WATER OF RAMSHAGAR DIGHI, THE LARGEST MAN MADE HISTORICAL RESERVOIR IN NORTHERN BANGLADESH	29-37
4	S Andriyono, N Masruroh, ED Masithah, J Triastuti and D Winarni. EFFECT OF THERMAL STIMULATION ON GONAD MATURATION IN SEA CUCUMBER *PHYLLOPHORUS* SP. (GRUBE, 1840)	39-46
5	K Biswas and SN Sinha. ANTIBACTERIAL ACTIVITY OF *TAMARINDUS INDICA* L. AGAINST BACTERIA CAUSING URINARY TRACT INFECTION	47-55
6	SHA Mahdi, M Hasan, I Mahfuz and M Khalequzzaman. USE OF DIFFERENT NON-CHEMICAL METHODS FOR THE MANAGEMENT OF ADULT *CALLOSOBRUCHUS MACULATUS* (F.) (COLEOPTERA: BRUCHIDAE) IN STORED CHICKPEA	57-65
7	I Ahmad, S Ahmed, B Yadav, PP Sah, R Ara, MJ Alam and W Zzaman. MICROBIAL, PHYSICOCHEMICAL AND SENSORY CHARACTERISTICS ANALYSIS OF SELECTED ALCOHOLIC BEVERAGES OF FROM BANGLADESH, INDIA AND NEPAL	67-75
8	SMM Ali, HM Zakir, SMS Shahriar, MH Sarkar, HP Nur and M Jesmin. *IN VIVO* ANTICANCER ACTIVITIES OF Ni (II)-BENZOIN THIOSEMICARBAZONE COMPLEX [$Ni(BTSC)_2$] AGAINST EHRLICH ASCITES CARCINOMA CELLS	77-88
Short Communication		
9	T Yeasmin, J Puttaradder and HC Lakshman. EFFECT OF SINGLE AND MULTIPLE AM FUNGAL INOCULANTS ON THE GROWTH PARAMETER OF *OCIMUM BASILICUM* VAR. *THYSIFLORA* BENTH	89-91

J. Bio-Sci. 23: 01-17, 2015 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

-Review Article

TREND IN ENZYME IMMOBILIZATION ON NANO MATERIALS FOR TRANSESTERIFICATION TO PRODUCE BIODIESEL: A REVIEW

N Hindryawati[1], GP Maniam[1], CK Feng[1] and MR Karim*[1,2]

[1]Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang, Kuantan, Malaysia
[2]Departent of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh

Abstract

Biodiesel is a non-toxic and very low sulfur containing, renewable and biodegradable diesel fuel substitute with low volatility and high cetane number. It is derived from fresh or waste vegetable oils and animal fats transesterified with short chain alcohol such as ethanol or methanol in the presence of a catalyst. Several new types of carriers and technologies have been adopted in the recent past to improve the ability of a catalyst in transesterification. One of the new trends is nanoparticles-based immobilization of enzyme as a catalyst. The combination of the precise physical, chemical, optical and electrical properties of nanoparticles with the specific recognition site or catalytic properties has led them to appear in a myriad of novel nanomaterial application. Enzyme immobilized on nanoparticles showed a broader working temperature and pH range and thermal stability than the native enzyme. Enhancement in the reactivity of nanocatalysts is associated with their increased surface area, greater concentrations of highly reactive edge, unusual and stabilized lattice planes. The greater activity of nanomaterial immobilized biocatalyst affords operational simplicity, low energy consumption, and greater safety, in the process of transesterification. This review article highlights the issues including the exploration of the ability of nanomaterial to immobilize biocatalyst and factors that influence the activity of biocatalysts upon immobilization.

Key words: Biodiesel, Immobilization, nanomaterial, transesterification

Introduction

The issue of worldwide energy consumption has steadily increased, claiming higher budget for living, increasing transportation, industrial and petrochemicals. Fig. 1 shows that the majority of the consumed energy is provided by fuel in the world. Global energy consumption in 2010 rebounded strongly in all regions, driven by economic recovery. The world energy consumption growth reached 5.6% in 2010, the highest rate since 1973. After falling for two consecutive years in 2008 - 2009, global oil consumption grew by 2.7 million barrels per day (b/d), or 3.1%, to reach a record level of 87.4 million b/d. Although this was the largest percentage increase since 2004, the worldwide oil consumption growth rate started declining and remained the weakest among all fossil fuels till 2011 and grew slowly again in 2016 - the third consecutive year in which demand has grown by 1% or less - much weaker than the rates of growth world had become used to over the previous 10 years or so (Dudley 2017). As a result, global crude oil production increased by 2.8 million b/d in 2015, led by a 1 million b/d increase in U.S. production. The bulk of the rest of the world's oil production increase came from OPEC, which cumulatively boosted production by 1.6 million b/d over 2015. BP's definition of crude oil "includes crude oil, shale oil, oil sands and NGLs (natural gas liquids - the liquid content of natural gas where this is recovered separately)."

*Author for correspondence: drmrkarim@yahoo.com

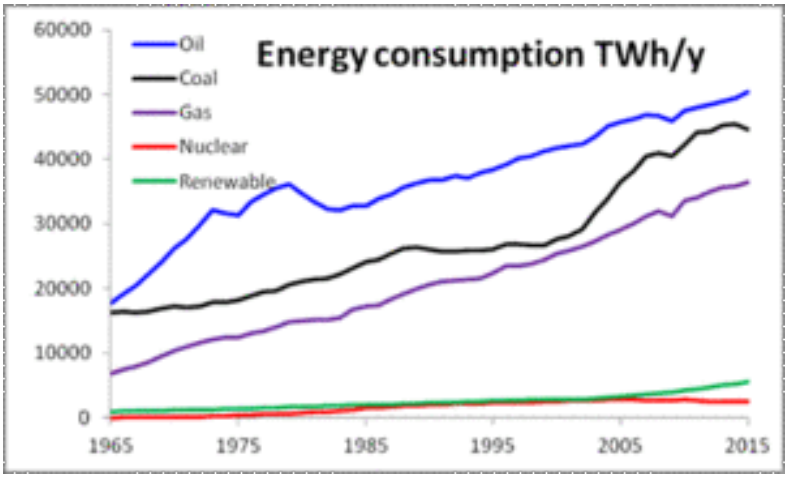


Fig. 1. Global primary energy consumption by fuel 2015.

However, the petroleum is a limited source for fuel that is quickly becoming insufficient and more expensive. In addition, petroleum-based product is one of the main causes of emission of carbon dioxide (CO_2) to the atmosphere. The transportation sector worldwide is almost dependent on petroleum-derived fuels (Fig. 1). The major causes of global CO_2 emissions are transport and industrial sector, which accounts for millions of barrels of oil consumption per day. As in 2015, the strength in oil demand was most pronounced in consumer-led fuels, such as gasoline. CO_2 emission goes up by 0.7%, being trivial in relation to concerns about energy security or climate change (EIA 2011). As a result, there has been a growing interest in alternative source.

Biodiesel is one of the energy sources as an alternative fuel for diesel engines. Biodiesel is defined as a mono alkyl ester, which is produced by transesterification of oil from renewable biological source like vegetable or animal fats. It is an alternative diesel fuel because of its environmental benefits such as being biodegradable, nontoxic and with low carbon dioxide emission profiles (Jegannathan et al. 2010).

In general, there are two prospective methods to produce biodiesel in industrial scale: chemical and enzyme catalyzed method. In chemical-catalyzed method, acid and alkali catalysts can be used (acidic or bases solution) for the production of biodiesel, but there are several drawbacks to this approach, including difficulties in the recovery of the glycerol and potassium and/or sodium salt, and the wastewater treatment problem (Jegannathan et al. 2010, Li et at. 2011). On the contrary, the enzymatic reaction by lipase is a clean technology due to its non-toxic and environment friendly nature. In addition, the process produces high purity grade product and enables easy separation from the by-product, glycerol (Winayanuwattikun et al. 2008).

Lipase is a hydrolytic enzyme, which catalyzes a reversible reaction to hydrolyze triglycerides to free fatty acids and glycerol and the esterification of free fatty acids and alcohol to ester (e.g. biodiesel). So lipase is

used as industrial biocatalyst (Sangeetha et al. 2011). Much research is needed to overcome problems such as enzyme inhibition by methanol, exhaustion of enzyme activity and high cost of enzymes, which may contribute to the global effort on industrial implementation of the enzymatic production of biodiesel in the near future (Iso et al. 2001, Nordblad and Adlercreutz 2008, Fjerbaek et al. 2009). Immobilization improves the enzyme stability under the reaction conditions, and enhances enzyme activity, thus, makes the repeated use of the enzyme feasible, permits the use of enzyme for diverse applications and thus lowers the production cost (Bajaj et al. 2010, Tan et al. 2010, Sangeetha et al. 2011). Immobilization provides a better environment for enzyme to act and also offers better product recovery (Lee et al. 2006).

Lipase can be immobilized on different porous support materials. Immobilized enzymes are defined as biocatalysts restrained and localized into microenvironment to retain their catalytic properties. Immobilization usually can increase stability and makes the reuse of the enzyme preparation very simple (Twyman et al. 2005). Immobilization is studied using covalent bonding, cross-linking, entrapment, adsorption, and encapsulation. Selection of an immobilization strategy greatly influences the properties of biocatalyst (Iso et al. 2001, Yagiz et al. 2007, Meunier et al. 2010, Xie and Ma 2010).

Nanomaterials constitute novel and interesting matrices for enzyme immobilization. While their high surface to volume ratio is an obvious advantage, their Brownian motion can impact the behavior of enzymes immobilized on these matrices. Such immobilized enzyme systems have been used in both aqueous and low water media for bio catalysis and resolution of race mates (Xie and Ma 2010, Yiu and Keane 2012). This overview examines the behavior of enzymes immobilized on nanomaterials and discusses the results reported with such biocatalyst preparations.

Biodiesel in general

Biodiesel is mono alkyl esters of long chain fatty acids contained in the renewable natural resources, such as plant, oils and fats (Moser 2009). Biodiesel is obtained from the transesterification of triglycerides with alcohol using an acid or base catalyst. Biodiesel contains no nitrogen or aromatic component and has sulfur content less than 15 ppm. Biodiesel has efficiency as a fuel similar to diesel oil and at the same time it is non-toxic, biodegradable, and has a low emission value (Jegannathan et al. 2010). The use of biodiesel can reduce emissions of HCs, CO, PM, sulfates, polycyclic aromatic hydrocarbons and nitrated polycyclic aromatic hydrocarbons. Also NO_x emissions increase with the concentration of biodiesel in the fuel (Balat and Balat 2010).

Generally, vegetable oil or animal fats are esters of saturated and unsaturated monocarboxylic acids with the trihydric alcohol glycerol. These esters are called triglycerides, which can react with alcohol in the presence of a catalyst, a process known as transesterification. The simplified form of its chemical reaction is presented in equation:

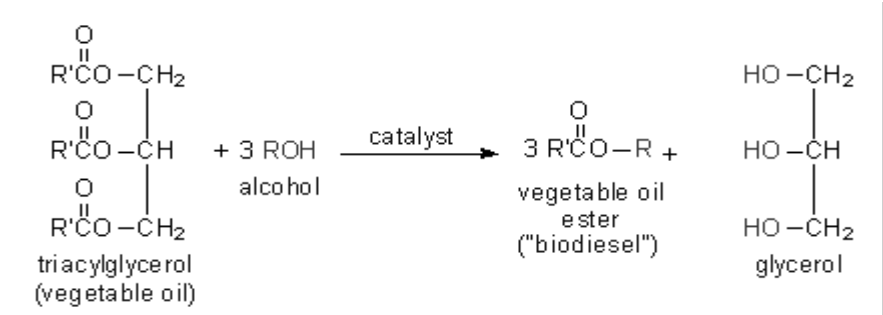


Fig. 2. Transesterification of Triglyceride.

Where, R in triacylglycerol is long-chain hydrocarbons, sometimes called fatty acid chains. Normally, there are five main types of chains in vegetable oils and animal oils: palmitic, stearic, oleic, linoleic, and linolenic. When a triglyceride is converted stepwise to diglyceride, monoglyceride, and glycerol - one mol of fatty acid is liberated at each step. Usually, methanol is the preferred alcohol for producing biodiesel because of its low-cost (Semwal et al. 2011).

Transesterification, also known as alcoholysis is the reaction of vegetable oil or fat with an alcohol to form esters and glycerol. Stoichiometrically to complete a transesterification reaction a 3:1 molar ratio of alcohol to triglycerides is needed. In practice, to have a maximum yield of esters, this ratio must be higher than the stoichiometric ratio (Leung et al. 2010). A catalyst is usually used to improve the reaction rate and yield. Because the reaction is reversible, excess alcohol is used to shift the equilibrium to the products side.

In transesterification, the alcohol is deprotonated with base to make stronger nucleophile. Commonly, ethanol or methanol is used. As can be seen, the reaction has no other inputs than the triglyceride and the alcohol. Transesterification consists of sequence of three consecutive reversible reactions. The first step is the conversion of triglyceride to diglyceride, followed by the conversion of diglyceride to monoglyceride, and finally monoglyceride to glycerol, yielding one ester molecule for each glyceride at each step. The basic mechanism of transesterification is shown in Fig. 3.

Triglyceride + ROH ⇌ Diglyceride + R'COOR

Diglyceride + ROH ⇌ Monoglyceride + R'COOR

Monoglyceride + ROH ⇌ Glycerol + R'COOR

Fig. 3: General equations for transesterification on triglyceride.

The catalyst used for biodiesel production can be grouped as follows base-catalyzed, acid-catalyzed, lipase-catalyzed, heterogeneous catalyzed process.

Homogeneous acid-base catalyzed transesterification

The homogeneous acid-base catalysis is used if the feedstock contains high FFA. The feedstock first treated with H_2SO_4 to reduce the level of FFA to below 1 wt%, followed by transesterification process catalyzed by homogeneous base catalysis. In this method, the yield of FAME is very high but the rate of FFA esterification

reaction is relatively slow. The drawback to this two-step process is even more pronounced due to the requirement of extra separation steps to remove the catalyst in both stages. Although the problem of catalyst's removal from the first stage can be avoided by using a catalyst from the second stage through the neutralization process, the use of extra base catalyst will add to cost of biodiesel production (Lam et al. 2010).

Homogeneous base catalyzed transesterification

The base catalyzed transesterification of vegetable oils proceeds faster than the acid-catalyzed reaction. Industrial processes usually favor base catalysts, such as alkali metal alkoxides and hydroxides as well as sodium potassium carbonates. The mechanism of the base-catalyzed transesterification is the reaction of the base with the alcohol, producing an alkoxide and the protonated catalyst (1). The nucleophilic attack of the alkoxide at the carbonyl group of the triglyceride generates a tetrahedral intermediate (2) from which the alkyl ester and the corresponding anion of the diglyceride are formed (3). The latter deprotonates the catalyst, thus regenerating the active species (4), which is now able to react with a second molecule of the alcohol, starting another catalytic cycle (Dias et al. 2008).

Alkali metal alkoxides (as CH_3ONa for the methanolysis) are the most active catalysts, since they give very high yields (>98%) in short reaction times, even if they are applied at low molar concentrations. Alkali metal hydroxides (KOH and NaOH) are cheaper than metal alkoxides, but less active. The presence of water gives rise to hydrolysis of some of the produced ester, with consequent soap formation and reduces the ester yield and considerably difficult to recover the glycerol due to the formation of emulsions. On the other hand, using potassium carbonate gives a high yield of fatty acyl alkyl ester and reduces the soap formation (Chanakya et al. 2013).

Heterogeneous base-catalyzed transesterification

Base catalysts have been developed for biodiesel production, such as basic zeolites, alkaline earth metal oxides and hydrotalcites. CaO had attracted much attention due to their relatively high basic strength, low solubility in methanol and can be synthesized from cheap sources like limestone and calcium hydroxide. The yield of FAME was up to 90% after 1 h reaction time at methanol reflux temperature and methanol to oil ratio 12:1 (Liu et al. 2008, Son and Kusakabe 2011, Kouzu and Hidaka 2012).

The other research, findings proved CaO as a potential solid catalyst in transesterifying triglycerides to methyl ester. CaO requires a thermal activation to remove CO_2 and moisture. The research reported that CaO acts as a heterogeneous catalyst in transesterifying the adsorbed waste palm oil on spent bleaching clay. Compared to the conventional catalysts (NaOH and KOH), the CaO-catalyzed reaction yielded much higher biodiesel (about 90%) from the waste cooking oil (6.6 - 6.8% FFA content) compared to only 46 and 61% yield using NaOH and KOH, respectively (Boey et al. 2011a,b).

The reaction mechanism for CaO-catalyzed transesterification is described as the methoxide ion that is attached to the catalyst surface attack the carbonyl carbon of the triglyceride molecule. This results in the formation of tetrahedral intermediate. Then the intermediate is rearranged to form a diglyceride anion and a mole of methyl ester. The charged-anion is then stabilized by a proton from the catalyst surface to form

diglyceride and at the same time regenerates the catalyst. The cycle continues until all three carbonyl centers of the triglyceride have been attacked by the methoxide ions to give one mole of glycerol and three moles of methyl esters (Boey et al. 2011c).

However, the yield of FAME dropped when waste cooking oil with high FFA was used under the same reaction condition. It is obvious that the basic sites of CaO were poisoned by strong adsorption of FFA on the surface catalyst. However, the catalytic activity of CaO can be regenerated if CaO is subjected to an activation treatment at 700°C in order to remove the main poisoning species (the carbonate groups) from the surface. Leaching of the catalyst was still observed in the transesterification reaction, although prior thermal treatment was employed (Lam et al. 2010).

Heterogeneous acid-catalyzed transesterification

Biodiesel research is focused on exploring new and sustainable solid acid catalyst for transesterification reaction. The advantages of using a solid catalyst are (1) they are insensitive to FFA content, (2) esterification and transesterification occur simultaneously, (3) eliminate the washing step of biodiesel, (4) easy separation of the catalyst from the reaction medium, resulting in lower product contamination level, (5) easy regeneration and recycling of catalyst, (6) reduced corrosion problem, even with the presence of acid species (Semwal et al. 2011).

The ideal solid acid catalyst for transesterification reaction should have characteristics such as an interconnected system of large pores, a moderate to high concentration of a strong acid site, and a hydrophobic surface. The overviews of various solid acid catalysts used (ZrO_2, TiO_2, SnO_2, zeolite, sulfonic ion-exchange resin, sulfonic modified mesostructure silica, sulfonated carbon-based catalyst, and heteropolyacids) in transesterification reaction that give the high conversion yield of FAME are discussed (Enweremadu and Mbarawa 2009, Petchmala et al. 2010).

Lipase as biocatalyst in transesterification

Lipase (EC.3.1.1.3, triacylglycerol acylhydrolases) are a group of enzymes, which can hydrolyze triacylglycerols at an oil-water interface to release fatty acids ester and glycerol. The substrates of lipase are triacylglycerols, which have very low solubility in water (Fig. 4). Lipase is present in microorganisms, plant and animals. Lipases catalyze a wide range of reactions, including hydrolysis, trans-esterification, alcoholysis, acidolysis, esterification and aminolysis (Joseph et al. 2008).

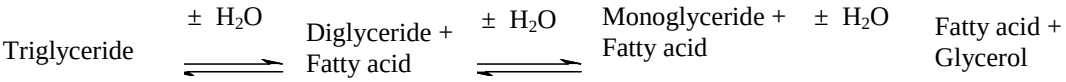


Fig. 4. Lipase catalyzed de-esterification reaction.

Generally, bacterial lipases are glycoproteins but some extracellular bacterial lipases are lipoproteins. The production of extracellular lipase from bacteria is usually dependent on the carbon and nitrogen source, metal salt, chemical reagents, presence of lipids or oil, availability of oxygen, and temperature. The genera of bacteria are *Streptomyces* sp*., Achoromobacter* sp*., Alcaligenes* sp., *Arthrobacter* sp*., Pseudomonas* sp*.* Other than bacteria, many researchers have exploited fungi as valuable sources of lipase due to the following properties: thermal stability, pH stability, substrate specificity and activity in organic solvents. The fungi that can produce commercial lipase are *Aspegillus niger, A. terrus*, *A. carneus*, *Candida cylindracea*,

Humicola lanugirosa, *Mucor miehei*, *Rhizopus arrhizus*, *R. delemar*, *R. Japonicus*, *R. niveus* and *R. oryzae* (Ghosh et al. 1996, Sangeetha et al. 2011).

Lipase from bacteria and fungi are most commonly used for transesterification, and optimal parameters for the use of a specific lipase depend on the origin as well as the formulation of lipase. Based on the result of existing research, lipase from different sources has different properties suitable for the process. Thus, there has been a search for an ideal enzyme (Chen et al. 2009), with screened lipase producing from *Candida* sp. 99-125 from sewage water in north China has a very high conversion rate in lipase-catalyzed esterification and hydrolysis after several induced mutations. Sangeetha et al. (2011) isolated and screened a total of 360 strains of lipase producing bacteria, yeasts and fungi from the samples of oil-contaminated soil and waste water. Among all the screened microbes, the potential lipolytic bacterium, *Staphylococcus warneri*, unicellular yeast, *Candida rugosa* and filamentous fungus*, Fusarium solani* were selected because of their high specific activities. Table 1 enlists some recently screened lipase producing microorganisms with their substrate and optimum parameters.

Table 1. Different sources of microbial lipase.

Type	Source	Substrate	Parameter Activity	References
Bacteria	*Pseudomonas aeruginosa*	Olive oil	T = 45°C, pH = 8, max activity = 0.760 U/ml	Mobarak-Qamsari (2011)
	Burkholderia multivorans	Olive oil	T = 30°C, pH = 9, max activity = 33 U mg^{-1} protein	Gupta et al. (2005)
	Pseudomonas fluorescens	Olive oil	T= 45°C, pH = 8.5, max activity = 9854 U/mg	Kozima and Shimizu (2003)
	Staphylococcus warneri	Palm oil	T= 40°C, pH = 8, max activity = 1.20 U/mg	Sangeetha et al. (2011)
	Burkholderia cepacia	Palm oil	T= 45°C, pH = 7, max activity = 160 U/mg	Rathi et al. (2002)
	Pseudomonas sp.	Groundnut oil	T= 37°C, pH = 7, max activity = 0.54 U/ml	Ghosh et al. (2005)
	Pseudomonas aeruginosa	Olive oil	T= 35°C, pH = 9, max activity = 1098 U/ml	Bisht et al. (2012)
Fungi	*Fusarium oxysporum*	Olive oil	T= 50°C , pH = 8, max activity = 60 U	Prazeres et al. (2006)
	Rhizopus oryzae	Palm oil	T= 55°C, pH = 8 - 8.5	Kharrat et al. (2011)
	Fusarium Solani	Palm oil	T= 40°C, pH= 8, max activity = 1.48 U/mg	Sangeetha et al. (2011)
	Geotrichum sp.	Waste cooking oil	T= 45 - 50°C, pH = 8	Kharrat et al. (2011)
	Aspergillus fumigates	Olive oil	T= 30°C, pH = 8.5 - 10	Rajan et al. (2011)
Yeasts	*Candida rugosa*	Olive oil	T= 50°C , pH = 4 - 9, max activity = 430 U/g	Minovska et al. (2005)
	Candida sp.	Waste cooking oil	T= 50°C , max activity = 222.5 U/mg	Chen et al. (2009)
	Candida cylindracea	Palm oil mill effluent (POME)	T= 30°C, pH= 6.8, max activity = 4.02 U/ml	Salihu et al. (2011)

For the production of enough lipase the media can be optimized by using palm oil as an inducer and lipase activities for both hydrolytic and synthetic catalysis can be compared. *Candida rugosa* lipase, which exhibited the highest potential for catalyzing the biodiesel production, was further purified and immobilization on various hydrophobic support materials and was found to be the most promising for further development as a biocatalyst for biodiesel synthesis.

Lipase transesterification of triglycerides with an alcohol (alcoholysis) involves a two-step mechanism when looking at a single ester bond. The first step is the hydrolysis of the ester bond and release of the alcohol moiety followed by an esterification with the second substrate. The two steps are represented in equation (1) and (2) (Fjerbaek et al. 2009, Gog et al. 2012).

$$E + Es_s \leftrightarrow E \,.\, Es_s \leftrightarrow F \,.\, B_p \leftrightarrow F + B_p \qquad (1)$$

Followed by

$$F + A_s \leftrightarrow F \,.\, A_s \leftrightarrow E \,.\, Es_p \leftrightarrow E + Es_p \qquad (2)$$

Subscripts s and p indicate substrate and product, respectively. For biodiesel, A_s = alcohol substrate, B_p = product with an alcohol moiety (di or monoglyceride or glycerol), E = free enzyme, Es_s =ester substrate (triglycerides) Es_p = FAAE, F = fatty acid (Fjerbaek et al. 2009).

This mechanism conforms to a ping-pong bi bi mechanism as each product is released between additions of the substrate and is the widely accepted mechanism for alcoholysis of triglycerides, although simplifications such as Michaelis-Menten kinetics are applied when fitting to experimental result. An example of an initial rate equation for a ping pong bi bi mechanism can be seen in equation (3)

$$v_i = \frac{V_{max}[TG][A]}{K_{m,TG}[A]\left(1 + \frac{[A]}{K_{i,A}}\right) + K_{m,A}[TG] + [TG][A]} \qquad (3)$$

Where v_i=initial rate; V_{max}, $K_{m,TG}$, $K_{i,A}$, and $K_{m,A}$ = kinetic constants; [TG] and [A] = concentration of triglycerides and acyl acceptor, respectively.

In order to have full image of the kinetics of enzymatic alcoholysis of triglycerides, other parameters must also be included such as lipase type, amount of reactant, mass transfer limitations, presence of organic solvent, formation and conversion of intermediates, the temperatures influence on enzyme deactivation or the equilibrium limitation for conversion. Thus, when trying to evaluate or determine the kinetics in such systems all these aspects become important (Gog et al. 2012). In general, lipases perform their catalytic activity in more gentle condition and with a variety of triglyceride substrate, including waste oils and fats with high level of FFA. Furthermore, biodiesel separation and purification is much easier, resulting in a more environmentally friendly process. Fig. 5 shows the transesterification process using lipase as catalyst.

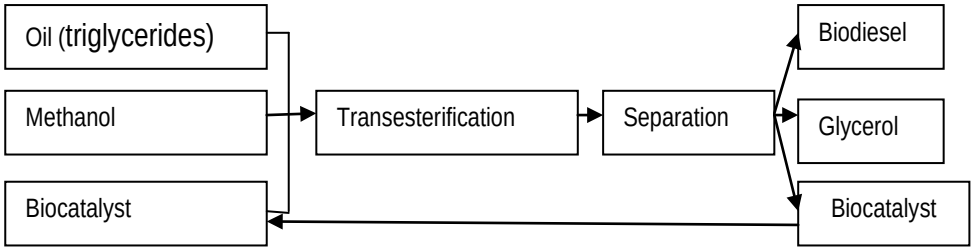


Fig. 5. Transesterification by using lipase as catalyst.

Immobilized lipase in support material

Use of enzymes as industrial catalysts serve to be beneficial if the whole process is economical and the cost of any process involve the production of the biocatalyst also. Hence recovery of the catalyst for repeated use becomes necessary. Free enzymes are labile and vulnerable to degradation during the process of recovery of the used enzyme. Also, most lipases exhibit low stability and activity in organic media (Lee et al. 2006). The disadvantage could be overcome by the use of immobilization of enzyme. Immobilization improves the stability of the enzyme under the reaction conditions, enhances enzyme activity thus, makes the repeated use of the enzyme feasible, permits the use of enzyme for diverse applications and thus lowers the production cost (Sangeetha et al. 2011). Immobilization provides a better environment for enzyme to act and also offers better product recovery (Lee et al. 2006).

Enzyme immobilization methods are classified as chemical or physical. Chemical methods involve the formation of covalent bonds between functional groups on the enzyme. Chemical methods are sub classified as either non-polymerizing or cross-linking methods. Non-polymerizing methods involve the formation of both enzyme-support bonds only between enzyme and support, but not between individual enzyme molecules, while cross-linking methods allow the formation of both enzyme-support bonds as well as enzyme-enzyme cross-links (Mikkelsen and Corton 2004, Twyman 2005).

Physical immobilization methods do not involve covalent bond formation with the enzyme, so that the native composition of the enzyme remains unaltered. Physical immobilization methods are sub classified as adsorption, entrapment, or encapsulation methods. Adsorption of protein to the surface of a carrier is, in principle, reversible, but careful selection of the carrier material and the immobilization conditions can render desorption negligible. Entrapment of enzyme in a cross-linking polymer is accomplished by carrying out the polymerization reaction in the presence of enzyme; the enzyme becomes trapped in interstitial spaces in the polymer matrices (Winayanuwattikun et al. 2005, Yagiz et al. 2007, Meunier and Legge 2010). Encapsulation of enzymes results in regions of high enzyme concentration is being separated from the bulk solvent system by a semi-permeable membrane, through which substrate, but not enzyme, may diffuse (Li et al. 2011).

The new method of enzyme immobilization should be able to provide high enzyme loading (close to that of carrier-free enzyme), high retention of activity, and broad reactor configuration. The development of carrier with a predetermined chemical and physical nature, especially suitable geometric properties and binding chemistry, which can bind (or hold) enzyme directly under mild conditions and thus can be used in different reactor configurations. Lipase from different sources has been investigated for their transesterification activity on different support material in Table 2.

Table 2. Yield in transesterification reaction using various support materials and different lipases.

Support material	Alcohol	Source of enzyme	Feedstock	Yield (%)	References
Polyacrylonitrile (PAN) nanofibrous	Methanol	*P. cepacia*	Soybean Oil	90	Winayanuwattikun et al. (2008)
Textile cloth with co-fixing agents	Methanol	*Candida's lipase*	Waste cooking oil	91.08	Chen et al. (2009)
Hydro calcite	Methanol	*Lipozyme-TL IM*	Waste cooking oil	92.8	Yagiz et al. (2007)
Microprou's polymeric matrix (MPPM)	Methanol	*Thermomyces lanuginous*	Sun flower	97.0	Dizge et al. (2009)
			Waste cooking oil	90.2	
Sepabeads EC-OD	Methanol	*C. rugosa*	Palm oil	70.0	Sangeetha et al. (2011)
Chitosan-Glu	Ethanol	*C. antartica B*	Oleic acid	75.0	Foresti and Ferreira (2007)
Polyurethane Foam	n-hexane	*C. rugosa*	Oleic acid	80.0	Awang et al. (2007)
Zeolite (delaminated zeolite-ITQ)	Methanol	*Rhizomucor miehei*	Olive oil	92.0	MacArio et al. (2007)
Silicate-1(S-1)				99.0	
Phyllosilicate sol-gel matrix	Methanol	*P. cepacia*	Palm	65.0	Winayanuwattikun et al. (2008)
			Menhaden	80.0	
			Corn	71.0	
			Grease	78.0	
		Thermomyces lanuginosa	Palm	62.0	
			Menhaden	88.0	
			Corn	83.0	
			Grease	89.0	
Protein-coated micro-crystals (PCMCs)	*Tert*-butyl alcohol	*Geotrichum sp. lipase*	Waste cooking oil	64.0	Yan et al. (2011)
Ceramic beads	Methanol	*P. cepacia*	Waste cooking oil	40.0	Al-Zuhair et al. (2009)
SiO_2-PVA	Ethanol	*Burkholderia cepacia*	Babassu oil	100.0	Da Rós et al. (2010)
			Beef tallow	89.70	
		Burkholderia cepacia	Babassu oil	74.13	
Nb_2O_5	Ethanol		Beef tallow	40.20	

Nanoparticles as support material in immobilized lipase

Currently, nanosized magnetic particles used widely in the immobilization of enzyme have received considerable attention. Based on the research of Lee et al. (2007), magnetic Fe_3O_4 nanoparticles treated

with (3-aminopropyl) triethoxysilane were used as immobilization material. The lipase from *T. lanuginosa* was covalently bound to the amino-functionalized magnetic nanoparticles by using glutaraldehyde as a coupling reagent with the activity recovery up to 70% and the enzyme binding efficiency of 84%. The optimal condition for immobilized lipase was dependent on the immobilization time, temperature, the concentration of glutaraldehyde, and the ratio of lipase to magnetic carrier.

Table 3 shows the nanoparticles used as support material in transesterification. Enzymes immobilized on support materials could catalyze the transesterification of vegetable oils with over 90% conversion to biodiesel being achieved.

Table 3. The nanoparticles used as support material in transesterification.

Nanoparticle utilized	Material loaded	Feedstock	Parameter process	Yield	References
Fe_3O_4 nanoparticles	Lipase from *T. lanuginosa*	Soybean oil	50°C, 30 h, (M:O = 1:1), 40% catalyst	90%	Xie and Ma (2009)
Fe_3O_4 magnetic nanoparticles	*T. lanuginosa*	Soybean oil	45 °C, 8 h, (M:O = 1.5:1), 3% catalyst	94 %	Xie and Ma (2010)
Magnetic nanoparticles	*Pseudomonas cepacia*	Soybean oil	40°C, (M:O = 3:1), 72 h	93%	Mak et al. (2009)
Fe_3O_4 nanoparticles biocomposite	*Pseudomonas cepacia*	Soybean oil	40°C, (O:DW:M:H = 6:3:1:0.2), 24 h, 0.4g catalyst	>99%	Wang et al. (2011)
Chitosan microspheres	*Candida rugosa*	Soybean oil	35°C, (M:O = 4:1), 30 h	87%	Xie and Wang (2012)
Ferric silica nanocomposite	*Bulkholderia* sp.	Olive oil	40°C, (M:O = 4:1), 30 h, 11%	92%	Tran et al. (2012a)
Ferric silica nanocomposite	*Bulkholderia* sp.	Microalgal oil	40°C, (M:O = 61.75), 48 h, 1203.1 U g^{-1}	97.25%	Tran et al. (2012b)

Note: M = methanol, O = oil, DW = distilled water, H = n-hexane

Immobilization of lipase as a catalyst has a great potential for achieving the design and operation of enzymatic biodiesel production on the industrial scale. By using a packed-bed reactor system with lipase-Fe_3O_4 nanoparticles bio composite catalyst was successfully developed for biodiesel production. The nanoparticles bio composite showed elevated activity and stability in the four-packed-bed reactor with conversion of biodiesel was maintained at the high rate of over 88% for 192 h. The efficient reuse of the enzyme was realized via a simple and effective immobilization procedure that resulted in a high initial activity without inactivation or inhibitor. The packed-bed reactor system has a great potential for achieving the design and operation of enzymatic biodiesel production on the industrial scale (Wang et al. 2011).

In another study, porcine pancreas lipase was covalently immobilized on the surface of silica-coated magnetite nanoparticles. The diameter of silica-coated magnetite was about 17 nm, and the immobilization process did not change the phase of Fe_3O_4. The results showed that the covalent immobilization of lipase on support material improved the thermal, pH and storage stability. Moreover, kinetic study showed the activation of immobilized enzyme. The enzyme recovery represents the establishment of about 64% of residual activity after six cycles of washing (Ranjbakhsh et al. 2012).

The lipase-coated magnetic nanostructures were applied in a reactive extraction process that allowed separation of the products formed during transesterification. It is expected that reactive extraction can directly produce 77% ethyl oleic (biodiesel) using lower ethanol/triolein ratios compared to 35 - 40% purity in the normal stirred reactor with a higher ethanol/triolein ratio. This approach implies a novel and efficient location and use of lipase in column reactors for production of biodiesel (Dussan et al. 2010).

Physicochemical properties of nanomaterial and immobilized lipase

Immobilization is normally considered to be an important method to improve the stability of enzyme. The morphology of nanoparticles observed using SEM micrograph of coated magnetic particle showed agglomerations because the non-coated magnetic particles were not dispersed in an appropriate substance (Dussan et al. 2010). The pure magnetite was observed to be spherical with nano size (Xie and Wang 2012). The particles diameter is an important factor for support material. Smaller particles have larger surface-to-volume ratios and larger capacity to bind more substance on their surface and product will give less restriction for diffusion.

BET analysis for immobilized *Burkholderia* sp. shows that the surface area of pure magnetite, silica magnetite, silica-magnetite nanocomposite and alkyl grafted silica-magnetite nanocomposites has a different surface area. Silica-magnetite nanocomposite have a surface area (202 m^2g^{-1}) and after grafting with the alkyl group at the surface of Fe_3O_4-SiO_2, the surface area decreasing (128 m^2g^{-2}). That would be due to the alkyl group may enter the Fe_3O_4-SiO_2 pores, thereby decreasing surface area and smaller pore size (Tran et al. 2012a).

The hydrolytic activity of free and immobilized lipase were measured at various temperatures (35 - 65 °C). The activity of bound and free lipase showed the highest activity at approximately 45°C. However, the immobilized lipase has higher stability than free lipase. The relative activity of immobilized lipase is 82% at 55 °C. The optimum hydrolytic activity was observed at pH 7.0 for both lipase. It indicated that immobilization did not change the activity of lipase. Immobilized lipase retained activity when the pH was higher than pH optimum. Immobilization method can improve the pH stability of the lipase, until pH 8.5 with the relative activity 60% (Xie and Ma 2010).

Furthermore, immobilized lipase can improve the storage stability and catalyst recycling than free lipase. The immobilized lipase conserved more than 64% of its activity after 21 days while free lipase only 47%. Also in reusing catalyst after 6 cycles, immobilized lipase retained 63% of its initial activity (Ranjbakhsh et al. 2012).

Kinetic parameters

Kinetic parameters of free and immobilized lipase were determined from Lineweaver-Burk plots. The measurement of Michaelis-Menten parameters also revealed a considerable improvement of the immobilized lipase. In a study (Ranjbakhsh et al. 2012), K_m value of immobilized lipase was lower than free lipase, which represents a higher affinity of immobilized lipase to substrates. Certainly with the nano size, magnetic nanoparticles could be imagined to offer lipase molecules a porous surface with a better orientation leading to higher affinity for substrate and more available sites. The result also demonstrated an increase in V_{max} due to immobilization of lipase. The improvement of V_{max} may also be due to more efficient conformation of

immobilized lipase with respect to free lipase. The improvement of kinetic parameters of immobilized lipase can be a good feature for possible industrial application.

Kinetic study showed a dependence of alkyl-grafted-Fe_3O_4-SiO_2-lipase on the substrate, the V_{max} and K_m values were estimated at 6251 U g^{-1} (132.4 U mg^{-1} protein) and 3.65 mM, respectively. The K_m value of alkyl-grafted-Fe_3O_4-SiO_2-lipase is higher than free lipase but smaller than celite-lipase. The V_{max} of alkyl-grafted-Fe_3O_4-SiO_2-lipase is smaller than free lipase and larger than celite-lipase. It indicated that the structure of enzymes could be rigidified on the surface of celite, thus the blocking of the active site of lipase would probably decrease lipase activity. However, with alkyl-grafted-Fe_3O_4-SiO_2-lipase, the binding of lipase on the surface of nanocomposite is multipoint hydrophobic interaction, which may cause the same phenomena as multipoint covalent bonding. The maximum reaction of alkyl-grafted-Fe_3O_4-SiO_2-lipase is much higher than celite lipase. This result indicates that hydrophobic interaction is the better approach for immobilization of the lipase (Tran et al. 2012a).

Conclusion

Nanocrystals and nanomaterials are serving as a novel supports for a catalyst in transesterification reaction. Currently, the use of nanoparticles has emerged as a versatile tool for generating excellent material for the catalyst due to their small size and large surface area and high catalytic activity. Nanoparticles strongly influence the mechanical properties in the material. The nano-magnetic biocatalyst also exhibits as a good catalytic property. Recently, immobilized lipase on nano-magnetic support showed high catalytic activity and advantages of easy separation and reuse. Moreover, nanocomposite grafted with a long alkyl group in order to create affinity for lipase bound on the surface of nanoparticles are shown to be a good matrix for lipase immobilization with high yield of biodiesel and high reusability. Support nanomaterials were prepared by co-precipitation method and also creating a functional group is one of the new trends in immobilization of lipase. Nanocatalyts is a potential material for transesterification reaction and for commercial applications.

Acknowledgment

The authors are thankful to the Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang, for funding the research project on biodiesel production.

References

Al-Zuhair S, Dowaidar A and Kamal H (2009). Dynamic modeling of biodiesel production from simulated waste cooking oil using immobilized lipase. Biochemical Engineering Journal 44: 256-262.

Awang R, Ghazuli MR and Basri M (2007). Immobilization of lipase from *Candida rugosa* on palm-basedpolyurethane foam as a support material. Journal of Biochemistry and Biotechnology 3: 163-166.

Bajaj A, Lohan P, Jha PN and Mehrotra R (2010). Biodiesel production through lipase catalyzed transesterification: An overview. Journal of Molecular Catalysis B: Enzymatic 62: 9-14.

Balat M and Balat H (2010). Progress in biodiesel processing. Applied Energy 87: 1815-1835.

Bisht D, Yadav SK and Darmwal NS (2012). Enhanced production of extracellular alkaline lipase by an improved stran of *Pseudomonas aeruginosa* MTCC 10,055. American Journal of Applied Sciences 9: 158-167.

Boey PL, Ganesan S, Maniam GP and Ali DMH (2011a). Ultrasound aided *in situ* transesterification of crude palm oil adsorbed on spent bleaching clay. Energy Conversion and Management 52: 2081-2084.

Boey PL, Saleh MI, Sapawe N, Ganesan S, Maniam GP and Ali DMH (2011b). Pyrolysis of residual palm oil in spent bleaching clay by modified tubular furnace and analysis of the products by GC–MS. Journal of Analytical and Applied Pyrolysis 91: 199-204.

Boey PL, Maniam GP and Hamid SA (2011c). Performance of calcium oxide as a heterogeneous catalyst in biodiesel production: A review. Chemical Engineering Journal 168: 15-22.

Chanakya HN, Durga MM, Sarada R and Abitha R (2013). Algal biofuel production and mitigation potential in India, 18 (1): 113-136.

Chen Y, Xiao B, Chang J, Fu Y, Lv P and Wang X (2009). Synthesis of biodiesel from waste cooking oil using immobilized lipase in fixed bed reactor. Energy Conversion and Management 50: 668-673.

Da Rós PCM, Silva GAM, Mendes AA, Santos JC and De Castro HF (2010). Evaluation of the catalytic properties of Burkholderia cepacia lipase immobilized on non-commercial matrices to be used in biodiesel synthesis from different feedstocks. Bioresource Technology 101: 5508-5516.

Dias JM, Alvim-Ferraz MCM and Almeida MF (2008). Comparison of the performance of different homogeneous alkali catalysts during transesterification of waste and virgin oils and evaluation of biodiesel quality. Fuel 87: 3572-3578.

Dizge N, Aydiner C, Imer DY, Bayramoglu M, Tanriseven A and Keskinler B (2009). Biodiesel production from sunflower, soybean, and waste cooking oils by transesterification using lipase immobilized onto a novel microporous polymer. Bioresource Technology 100: 1983-1991.

Dudley, B (2017). BP statistical review of world Energy. https://www.bp.com

Dussan KJ, Cardona CA, Giraldo OH, Gutiérrez LF and Pérez VH (2010). Analysis of a reactive extraction process for biodiesel production using a lipase immobilized on magnetic nanostructures. Bioresource Technology 101: 9542-9549.

EIA (2011). Anual Energy Outlook 2011 with projections to 2035. US Energy Information Administration, p. 246. Enweremadu CC and Mbarawa MM (2009). Technical aspects of production and analysis of biodiesel from used cooking oil- A review. Renewable and Sustainable Energy Reviews 13: 2205-2224.

Fjerbaek L, Christensen KV and Norddahl B (2009). A review of the current state of biodiesel production using enzymatic transesterification. Biotechnology and Bioengineering 102: 1298-1315.

Foresti ML and Ferreira ML. 2007. Chitosan-immobilized lipases for the catalysis of fatty acid esterifications. Enzyme and Microbial Technology 40: 769-777.

Ghosh M, Bhattacharyya S and Bhattacharyya DK (2005). Production of lipase and phospholipase enzymes from *Pseudomonas sp.* and their action on phospolipids. Journal of Oleo Science 54: 407-411.

Saxena RK, Ghosh PK, Gupta R, Yadav RP and Davidson S (1996). Microbial lipases: production and applications. Science Progress 72: 199-257.

Gog A, Roman M, Toşa M, Paizs C and Irimie FD (2012). Biodiesel production using enzymatic transesterification – Current state and perspectives. Renewable Energy 39: 10-16.

Gupta N, Rathi P, Singh R, Goswami VK and Gupta R (2005). Single-step purification of lipase from *Burkholderia multivorans* using polypropylene matrix. Applied Microbiology and Biotechnology 67: 648-653.

Iso M, Chen B, Eguchi M, Kudo T and Shrestha S (2001). Production of biodiesel fuel from triglycerides and alcohol using immobilized lipase. Journal of Molecular Catalysis B 16: 53-58.

Jegannathan KR, Jun-Yee L, Chan ES and Ravindra P (2010). Production of biodiesel from palm oil using liquid core lipase encapsulated in κ-carrageenan. Fuel 89: 2272-2277.

Joseph B, Ramteke PW and Thomas G (2008). Cold active microbial lipases: Some hot issues and recent developments. Biotechnology Advances 26: 457-470.

Kharrat N, Ali YB, Marzouk S, Gargouri YT and MahaKarra C (2011). Immobilization of rhizopus oryzae lipase on silica aerogels by adsorption. Process Biochemistry 46: 1083-1089.

Kouzu M and Hidaka JS. 2012. Transesterification of vegetable oil into biodiesel catalyzed by CaO: A review. Fuel 93: 1-12.

Kozima Y and Shimizu S (2003). Purification and caracterization of the lipase from *Pseudomonas fluorescens* HU380. Journal of Bioscience and Bioengineering 96: 219-226.

Lam MK, Lee KT and Mohamed AR (2010). Homogeneous, heterogeneous and enzymatic catalysis for transesterification of high free fatty acid oil (waste cooking oil) to biodiesel: a review. Biotechnology Advances 28: 500-518.

Lam MK, Lee KT and Mohamed AR (2010). Homogeneous, heterogeneous and enzymatic catalysis for transesterification of high free fatty acid oil (waste cooking oil) to biodiesel: A review. Biotechnology Advances 28: 500-518.

Lee DH, Parli CH, Yeo JM and Kim SW (2006). Lipase immobilization on silica gel using cross-linking method. Journal of Industrial and Engineering Chemistry 12: 777-782.

Leung DYC, Wu X and Leung MKH (2010). A review on biodiesel production using catalyzed transesterification. Applied Energy 87: 1083-1095.

Li SF, Fan YH, Hu JF, Huang YS and Wu WT (2011). Immobilization of *Pseudomonas cepacia* lipase onto the electrospun PAN nanofibrous membranes for transesterification reaction. Journal of Molecular Catalysis B: Enzymatic 73 (1-4): 98-103.

Liu X, He H, Wang Y, Zhu S and Piao X (2008). Transesterification of soybean oil to biodiesel using CaO as a solid base catalyst. Fuel 87: 216-221.

MacArio A, Giordano G, Setti L, Parise A, Campelo JM, Marinas JM and Luna D (2007). Study of lipase immobilization on zeolitic support and transesterification reaction in a solvent free-system. Journal Biocatalysis and Biotransformation 25: 328-335.

Mak K-H, Yu C-Y, Kuan I-C and Le S-L (2009). Immobilization of *Pseudomonas cepacia* lipase onto magnetic nanoparticles for biodiesel production, ISESCO Journal of Science and Technology 5: 19-23.

Meunier SM and Legge RL (2010). Evaluation of diatomaceous earth as a support for sol–gel immobilized lipase for transesterification. Journal of Molecular Catalysis B: Enzymatic 62: 53-57.

Mikkelsen SR and Corton E (2004). Bioanalytical Chemistry: Jhon Wiley and Sons, Inc. 61-85 pp.

Minovska V, Winkelhausen E and Kuzmanova S (2005). Lipase Immobilized by different techniques on various support materials applied in oil hydrolysis. Journal of the Serbian Chemical Society 70: 609-624.

Mobarak-Qamsari E, Kasra-Kermanshahi R and Moosovi-Nejad Z (2011). Isolation and Identification of a novel, lipase-producing bacterium, *Pseudomonas aeruginosa* KM110. Iranian Journal of Microbiology 3: 92-98.

Moser BR (2009). Biodiesel production, properties, and feedstocks. *In Vitro* Cellular and Developmental Biology - Plant, 45: 229-266.

Nordblad M and Adlercreutz P (2008). Efficient enzymatic acrylation through transesterification at controlled water activity. Biotechnology and Bioengineering, 99: 1518-1524.

Petchmala A, Laosiripojana N, Jongsomjit B, Goto M, Panpranot J and Mekasuwandumrong O (2010). Transesterification of palm oil and esterification of palm fatty acid in near- and super-critical methanol with SO_4-ZrO_2 catalysts. Fuel 89: 2387-2392.

Prazeres JNd, Cruz JAB and Pastore GM (2006). Characterization of alkaline lipase from *Fusarium Oxysporum* and the effect of different surfactants and detergents on the enzyme activity. Brazilian Journal of Microbiology 37: 505-509.

Rajan A, Kumar DRS and Nair AJ (2011). Isolation of a novel alkaline lipase producing Fungus *Aspergillus fumigatus* MTCC 9657 from aged and crude rice bran oil and quantification by HPTLC. International Journal of Biological Chemistry 2: 116-26.

Ranjbakhsh E, Bordbar AK, Abbasi M, Khosropour AR and Shams E (2012). Enhancement of stability and catalytic activity of immobilized lipase on silica-coated modified magnetite nanoparticles. Chemical Engineering Journal 179: 272-276.

Rathi P, Goswami VK, Sahai V and Gupta R (2002). Statistical medium optimization and production of a hyperthermostable lipase from *Burkholderia cepacia* in a bioreactor. Journal of Applied Microbiology 93: 930-936.

Salihu A, Alam MZ, abdulkarim MI and Salleh HM (2011). Suitability of using palm oil mill effluent as a medium for lipase production. African Journal of Biotechnology 10: 2044-2052.

Sangeetha R, Arulpandi I and Geetha A (2011). Bacterial lipases as potential industrial biocatalysts: An overview. Research Journal of Microbiology 6: 1-24.

Semwal S, Arora AK, Badoni RP and Tuli DK (2011). Biodiesel production using heterogeneous catalysts. Bioresource Technology 102: 2151-2161.

Son SM and Kusakabe K (2011). Transesterification of sunflower oil in a countercurrent trickle-bed reactor packed with a CaO catalyst. Chemical Engineering and Processing: Process Intensification 50: 650-654.

Tan T, Lu J, Nie K, Deng L and Wang F (2010). Biodiesel production with immobilized lipase: A review. Biotechnology Advances 28: 628-634.

Tran DT, Chen CL and Chang JS (2012a). Immobilization of *Burkholderia* sp. lipase on a ferric silica nanocomposite for biodiesel production. Journal of Biotechnology 158: 112-119.

Tran DT, Yeh KL, Chen CL and Chang JS (2012b). Enzymatic transesterification of microalgal oil from *Chlorella vulgaris* ESP-31 for biodiesel synthesis using immobilized Burkholderia lipase. Bioresource Technology 108: 119-127.

Twyman RM (2005). Immobilized Enzymes. Enzymes: Elsevier Ltd., 523-529 pp.

Wang X, Liu X, Zhao C, Ding Y and Xu P (2011). Biodiesel production in packed-bed reactors using lipase–nanoparticle biocomposite. Bioresource Technology 102: 6352-6355.

Winayanuwattikun P, Kaewpiboon C, Piriyakananon K, Tantong S, Thakernkarnkit W, Chulalaksananukul W and Youngvanich T (2008). Potential plant oil feedstock for lipase-catalyzed biodiesel production in Thailand. Biomass and Bioenergy 32: 1279-1286.

Xie W and Ma N (2009). Immobilized lipase on Fe_3O_4 nanoparticles as biocatalyst for biodiesel production. Energy Fuels 23 (3): 1347-1353.

Xie W and Ma N (2010). Enzymatic transesterification of soybean oil by using immobilized lipase on magnetic nano-particles. Biomass and Bioenergy 34: 890-896.

Xie W and Wang J (2012). Immobilized lipase on magnetic chitosan microspheres for transesterification of soybean oil. Biomass Bioener 36: 373-380.

Yagiz F, Kazan D and Akin AN (2007). Biodiesel production from waste oils by using lipase immobilized on hydrotalcite and zeolites. Chemical Engineering Journal 134: 262-267.

Yan J, Yan Y, Liu S, Hu J and Wang G (2011). Preparation of cross-linked lipase-coated micro-crystals for biodiesel production from waste cooking oil. Bioresource Technology 102: 4755-4758.

Yiu HHP and Keane MA (2012). Enzyme–magnetic nanoparticle hybrids: new effective catalysts for the production of high value chemicals. Journal of Chemical Technology and Biotechnology 87: 583-594.

J. Bio-Sci. 23: 19-27, 2015 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

EFFECT OF PHYSIOGRAPHIC FACTORS ON WOODY SPECIES DIVERSITY IN OAK FORESTS (CASE STUDY: SARDASHT FOREST -IRAN)

J Eshaghi-Rad*, A Motallebpour and A Alijanpour

Faculty of Natural Resources, Urmia University, Iran

Abstract

Research on species diversity in different gradients of altitudes, aspect and slope is attempting to understand the interactions of vegetation and the non-living environment. The aim of this study was to examine the impact of altitude, slope and aspect variation on the woody species diversity in the Oak forests of Zagros (northern of Iran). 178 samples were taken by using of transect method with a fixed length of 50 m. Altitude, slope, aspect, woody species and diameter at breast height of all trees was recorded in each transect. Margalef richness index, Shannon and Simpson diversity indices and Shannon evenness index were calculated. The Means of the different diversity indices were compared with Kruskal- Wallis test. Results showed that altitude had significant impact on the diversity, richness and evenness of woody species and the middle elevation class (1400 - 1600 m) allocated maximum values of indices. Also, the highest species richness was observed in the (0 - 20%) and (20 - 40%) slope classes but the slope hadn't effected on the woody species evenness and diversity. The lowest amount of species richness was observed in the east aspect than other aspects. In general, it can be concluded that the altitude had a large proportion of diversity variation than slope and aspect in the research area.

Key words: Iran, Oak, physiographic factors, species diversity, Zagros forest

Introduction

Zagros forest with area of 5 million hectares is one of the largest biome in Iran. The importance of these forests is not for the production of the industrial woods but also play role in soil and water resources conservation, production of various by-products and preventing air pollution, so it must be managed and maintained properly (Bazyar et al. 2013). Since the degradation and destruction of these forests will follow irrecoverable negative effects, it should be preceded based on a correct and efficient management to the conservation, restoration and achievement to sustainable development by maintaining the natural diversity, because decreasing the natural diversity has led to interference in the natural order and reduce the environmental capacity of these valuable forests. The natural diversity is used as one of the important and fast indicator for determining ecosystem condition because the distribution of species can be investigated by measuring diversity and presented the management recommendations needed with an emphasis on the ecosystem dynamics (Maarel 1988).

The changes of the physiographic factors (altitude, slope and aspect) are parameters affecting the distribution and variety of plants that cause the different species distribution in ecosystems by affecting the characteristics of soil and habitat (Solon et al. 2007). Altitude impacts the climate of region, led to the formation of the climatic areas by affecting the amount and type of precipitation, temperature, evaporation, transpiration and the solar radiations intensity, and therefore the plant types form with the specific natural

*Author for correspondence: javad.eshaghi@yahoo.com

diversity (Maguran 2004, Abarghuie et al. 2010). Research conducted in Iran and other parts of the world show that the physiographic factors (especially the altitude factor) will determine the distribution of different plant species. The scale of diversity in the biological form of plants usually decreases with increasing altitude and one or two of the biological form leaves in high altitudes (Paven et al. 2000). The slope is also one of the factors influencing the soil depth and its fertility rate. Soil has greater depth in areas with low slope due to the accumulation of sediments and is more fertile but drainage is more in the steep areas because of less soil erosion. So slope has the important role in the presence or absence of species and their coverage rate (El-Ghani 1998, Alhamed 2006). The aspect plays the important role on the distribution and transmittal of species by affecting amount of receiving solar energy, the evaporation rate, precipitation, the soil moisture and fertility.

Many researchers have investigated the diversity of the plant species in relation to physiographic factors (altitude, slope and aspect). Sharma et al. (2009) investigated the altitude effects on the richness, diversity and the dispersal patterns of different tree species in the temperate forests of the Himalayas concluded that all richness indices and diversity had the highest values at low altitudes (1850 to 2250 m above sea level). Furthermore, Kharakwal et al. (2005) have pointed out that the altitude and climatic variables such as temperature and rainfall determines the richness and diversity of species. Ellu and Obua (2005) have suggested that the different altitudes and slope affect the richness and spatial patterns of the tree species. Mi et al. (2012) illustrated that there was a significant relationship between the aspect and slope and changes in the plant diversity. It was proved by study of Gong et al. (2008) that the aspect was effective on the site productivity and the species composition and diversity in the way that the northern slopes had higher fertility and greater diversity in comparison with the southern slopes. Panthi et al. (2007) stated that *Betula utilis* and *Salix spp* distributed in the northern humid aspect with more species richness, but *Juniperus indica* found in the southern dry conditions with low species richness. It was also observed that species with different ecological amplitudes to the physiographic factors did not give the same niche. Aghaei et al. (2009) studied the effect of altitude factor on plant diversity in temperate forests and concluded that the highest species richness and diversity were found in the low altitudinal range (100 - 400 m). Shirzad and Tabari (2010) illustrated that with increasing slope and altitude, the species diversity reduced. It was observed that physiographic factors directly and indirectly were effective on the distribution and species diversity. Therefore understanding the relationship between vegetation and physiographic factors for estimating type of species for ecological management in different ecosystems is essential (Tamartash et al. 2010).

According to role of physiographic factors in species diversity of woody plants and necessity of investigation of this issue in the Zagros Oak forests which was rich in diversity of Oak species. The aim of this study is to investigate effects of aspect, slope and altitude variation on the diversity of woody species in the northern Zagros forests in Iran.

Materials and Methods

Study area: The studied area is located in the oak forests of Zagros forests (Iran) and in the south western of Azerbayjan province with area of 25000 hectares and with the coordinates 45° 33' 25" longitude and 36° 12' 13" latitude. Altitudinal range of the study area is 1200 - 2000 m above sea level. Parent rock often is the Cretaceous limestone. Average annual rainfall of this region is 724 mm. Average of the maximum temperature is 21°C and the average of the minimum temperature is 6°C. The region has the semi-arid and

Mediterranean climate (Mohajer 2006). *Quercus brantii* Lindl and *Quercus infectoria* Oliv and *Quercus libani* Oliv and *Pyrus communis* L. and *Pistacia mutica* F and M and *Crataegus* spp. are the tree species in study area.

Method of research: The total number of 178 sample plots were taken using of transect sampling method with fixed length of 50 m for sampling of tree and shrub cover (Badano et al. 2005). Altitude, percentage of slope and azimuth of aspect were measured in each transect. Then type of species and DBH or diameter at breast height (larger than 5 cm) of all trees and shrubs that somehow the crown or trunk intercepting along with the sample line were measured. In order to analyze the data, initially the DBH was transformed to basal area in square meter. Altitude was classified into four categories (1200 - 1400, 1400 - 1600, 1600 - 1800 and 1800 - 2000 m), slope into three level (0 - 20%, 20 - 40% and more than 40%) and aspects (North, South, East and West). Margalef species richness, Shannon-Wiener evenness, Shannon-Wiener species diversity, and Simpson species diversity were calculated in each transect according to the following formula in Table 1.

Table1. Biodiversity Indices used in this paper.

Indices	Equation
Shannon (H)	$H= -\sum_{i=1}^{s} p_i \ lnp_i$
Simpson (D)	$D=1-\sum(p_i)^2$
Evenness of Shannon (E)	$E= \frac{-\sum_{i=1}^{s} p_i \ lnp_i}{Ln\ S}$
Margalef (R)	$R= \frac{S-1}{Ln(N)}$

S and Pi refer to total number of species in the sample and proportion of each species to total, respectively.

Normality of the data was examined by using Kolmogorov-Smirnov test and since none of the data did not follow of the normal distribution, Kruskal- Wallis test was used for comparison of average different species diversity indices amongst the slope, aspect and altitude categories and to compare pair comparison of indices in different classes of altitude, slope and aspect, Mann-Whitney test was applied. To calculate species diversity indices, PC-ORD ver.5 software and for statistical comparisons, SPSS 18 software were used respectively.

Results

Results of Kruskal-Wallis test represent the different effects of altitude, slope and aspect on diversity, richness, and evenness. The results showed that the effects of altitude on the diversity, richness and evenness are significant (p <0/05). While effect of aspect is significant on the richness and evenness but isn't meaningful on the diversity. The results also showed that the effect of slope is significant on the richness but isn't significant on the evenness and diversity (Table 2).

Table 2. The result of Kruskal-Wallis test for comparing the mean species diversity indices in altitude, slope and aspect classes.

Physiographical factors	Diversity indices	Chi-square	df	Sig.
Altitude	Shannon (H)	24.24	3	0.000**
	Simpson (D)	25.96	3	0.000**
	Evenness (E)	31.29	3	0.000**
	Margalef (R)	17.54	3	0.001**
Slope	Shannon (H)	2.62	2	0.27ns
	Simpson (D)	2.61	2	0.27ns
	Evenness (E)	3.79	2	0.15ns
	Margalef (R)	8.14	2	0.017*
Aspect	Shannon (H)	3.83	3	0.28ns
	Simpson (D)	3.57	3	0.31ns
	Evenness (E)	16.61	3	0.001**
	Margalef (R)	10.66	3	0.014*

*, ** significant differences at 5% and 1% level, respectively.

Effect of altitude on woody species diversity

The species diversity indices initially increase with increasing altitude and then gradually decrease. The highest values of all calculated indices were observed in the middle altitudinal class (1400 - 1600 m) and their lowest ones were observed in the high altitudinal class (1800 - 2000 m) (Fig. 1). The pair comparison of the species diversity indices in different altitudinal classes showed that there was statistically significant difference between the Shannon-Wiener, Simpson diversity and Margalef indices in altitudinal classes (1200 - 1400 m) with (1400 - 1600 m) and (1600 - 1800 m) with (1800 - 2000 m). There wasn't significant difference between (1400 - 1600 m) and (1600 - 1800 m) in none of the indices (Fig. 1).

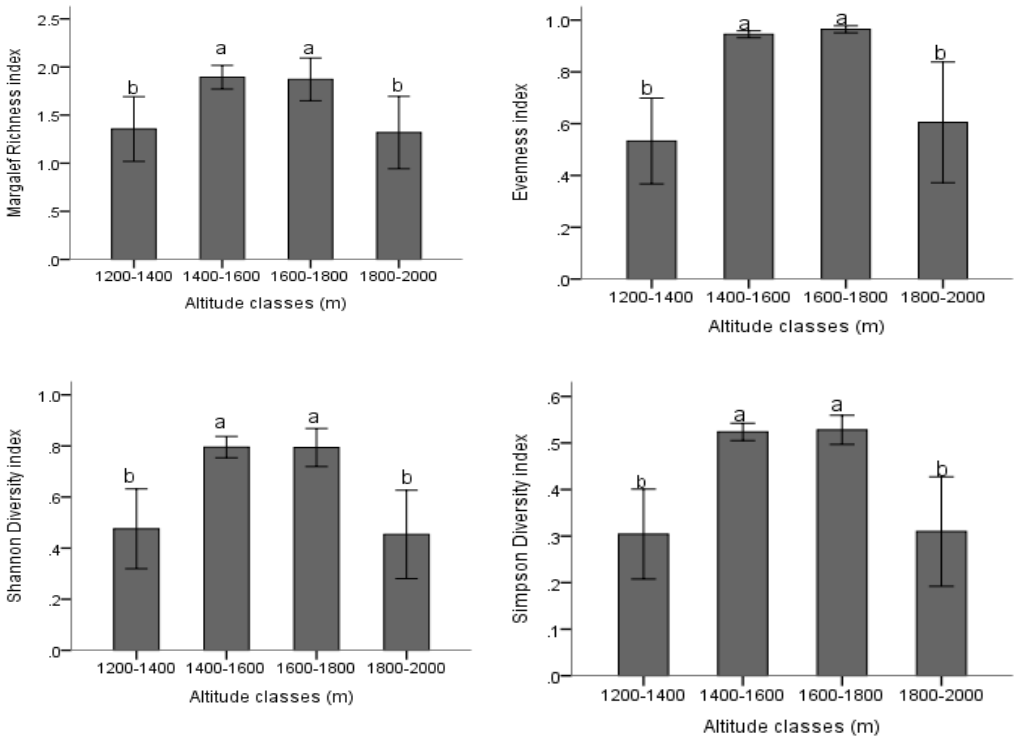


Fig.1. Average indices of richness, Shannon and Simpson species diversity and the Shannon evenness in 4 altitudinal classes (different letters indicate significant difference between the averages).

Effect of slope on woody species diversity

The highest species richness were observed in the (0 - 20%) and (20 - 40%) slope classes. This index was decreased in the upper slope class (40% - 60%). The comparison test of species indices richness means amongst different slope classes illustrated that there were significant differences between the (0 - 20%) and (20 - 40%) slope classes and (40% - 60%) but the result was similar between the first and second slope classes. The results also showed the Shannon and Simpson species diversity and the Shannon evenness were similar with increasing the slope and there were no significant differences in these indices amongst different slope classes (Fig. 2).

Effect of aspect on woody species diversity

The lowest amount of species richness was observed in the east aspect. This index was the highest in the north, south and west aspects. The comparison test results showed that there was significant difference between east and other aspects in terms of species richness but this index were the same in the north, south and west aspects. The highest amount of species evenness was observed in the east aspect. The north, south and west had similar values less that east aspect. The comparison test results depicted that there was

significant difference between east and other aspects. Furthermore the Shannon and Simpson species diversity indices were similar in different aspects and there were no significant differences in these indices amongst different aspects (Fig. 3).

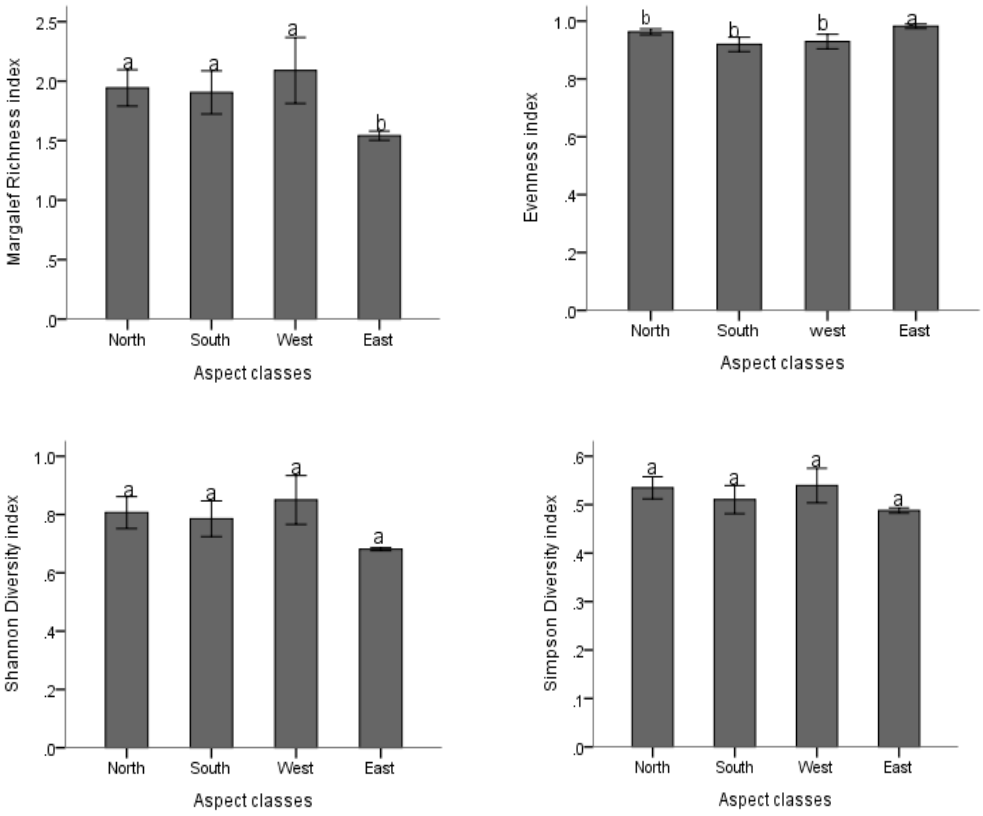


Fig. 3. Average indices of Margalef richness, Shannon and Simpson diversity and Shannon evenness index in four different geographical aspects (different letters indicate significant differences between the averages).

Discussion

Physiographic factors have caused change in micro-climate and edaphic factors and various ecological niches provide for plants. In fact, research on species diversity in different gradients of altitudes, aspect and slope is attempting to understand the interactions of vegetation and the non-living factors of environment (Hua 2002). Conducted research in the field of vegetation dynamics introduces variation in elevation as one of effective factors in vegetation structure as obvious role on the presence or absence of plant species (Aghaei et al. 2009). The results of present study also showed that the altitudes influences on the diversity, richness and evenness of woody plant species in the region. In this way that the species diversity, richness and evenness increases till middle elevation and then reduces with increasing altitude. This result corresponds with Theory's Grime (1973) based on maximizing diversity in terms of average altitude. Hegazy et al. (1998) also investigated the diversity and abundance of vegetation along the altitudinal gradient dna

concluded that the middle altitudes were encompassed greater diversity and evenness eras great compared to other classes. Furthermore, Habib et al. (2011) indicated that the tree species diversity and richness increased in the middle part of altitudinal gradient and decreases upward and downward, respectively. They justified this phenomenon because of human activities and deforestation at low altitudes and soil erosion and extreme climatic conditions at high altitudes. The results of Abarghuie et al. (2010) research also illustrated that the altitude had a significant influence on diversity, richness and evenness of plant species and middle altitudinal range had higher diversity, richness and evenness. The present study also is consistent with the abovementioned research results and its reason might be expressed favorable climatic conditions existence and lack of human activity. On the other hand, the aspect by affecting the amount of received solar energy and soil moisture rate always has controlled the type of vegetation (Small et al. 2005, Fontaine et al. 2007). Based on the present research, the aspect had significant effect on the Margalef's richness and evenness of the Shannon-Wiener. *Quercus infectoria* and *Quercus brantii* and *Quercus libani* distributed over eastern aspect while *Quercus infectoria, Quercus brantii, Quercus libani, Crataegus spp., Pistacia mutica* and *Pyrus communis* were present in other aspects. With this reason species richness was decreased in the east aspect. The aspects had not a significant effect on species diversity calculated based on species basal area. Shirzad and Tabari (2011) concluded indiscriminate uses and grazing in the past had caused disrupt the balance of diversity in *Juniperus excels* habitat of Hezar masjed mountains. In this regard, Saberian (2001) also showed that the aspect hadn't a significant relationship with plant diversity and percentage of canopy cover in Semnan province (Iran). But the research of Maranon et al. (1999) conducted in investigating biological diversity of woody shrubs species in the genus *Quercus* in Strait of Gibraltar and illustrated that the species richness in the southern slope is less than northern slopes. Kabrickt and Shifley (2004) showed that the species diversity was greater in southern aspects in South East Missouri. A study was conducted by Shoshany and Strenberg (2001) on the influence of Mediterranean woody formations in the dry and semi-arid habitats and found that the composition, structure, density and diversity of plant communities alter with the aspect. It can be regarded that the influence of aspect on species diversity were different because of different ecological conditions, types of exploitations, grazing, types of damages, etc. Several studies had represented percentage of slope as one of the effective factors on natural diversity and richness (Maguran 1996, Ellu and Obua 2005, Sohrabi and Akbarinia 2006). The results of the present study showed that slope had significant impact on the Margalef natural richness and there was significant difference between slopes of 20 - 40 class and 40 - 60 class in the study area. Low species richness in the high slopes might be justified by erosion and reducing depth of soil due to slip and drift, reduction in water and humidity holding capacity and loss of soil fertility in areas of steep (El-Ghani 1998, Alhamed 2006). Slope hadn't impact on the diversity and evenness of woody species in the study area. Mirzaei et al. (2008) also achieved similar results. Proctor (1971) and Peet (1981) during their investigation have pointed out that richness and plant species diversity reduction in the steep slope. Takyu et al. (2002) also indicated that the richness and biodiversity rate would increase significantly in the gentle slopes than steep slopes. In general, it can be concluded that among physiographic factors, the altitude had a large proportion of diversity variation than slope and aspect in the area studied. Optimal combination of environmental resources, good thermal conditions and loss human activities had been provided for occurrence of more species in the middle altitudes. Finally, it can be stated that variety study of Zagros forest habitats according to ecological and social special limitations governing on it finds day-to-day more important and recognition of the diversity of plant species information in these habitats and its relation to physiographic factors is issue that requires further investigation and studies.

References

Abarghuie EF, Mesdaghi M, Gholami P and Nasrabad NH (2011). The effect of some topographical properties in plant diversity in Stepped Rangelands of Nodushan, Yazd Province, Iran. Iranian Journal of Range and Desert Research 3: 408-419.

Aghaei SHM, Jalilvand H, Kooch Y and Poormajidian MR (2011). Plant diversity with respect to ecological factor of altitude in Sardabrood forests of Chalous, N. Iran, Iranian Journal of Biology 24: 400-411.

Alhamad MN (2006). Ecological and species diversity of arid Mediterranean grazing land vegetation, Journal of Arid Environments 66: 698-715.

Badano EI, Cavierse LA, Molina-Montenegro MA and Quiroz CL (2005). Slope aspect influences plant association patterns in the Mediterranean Motorral of central Chile, Journal of Arid Environment 62: 93-108.

Bazyar M, Haidari M, Shabanian N and Haidari H (2013). Impact of physiographic factors on the plant species diversity in the North Zagros forest (case study, Kurdistan province, Marivan region), Annals of Biological Research 4: 317-324.

Ellu G and Obua J (2005). Tree condition and natural regeneration in disturbed sites of Bwindi Impentrable forest national park, Tropical Ecology 49: 99-111.

Ghani EMM (1998). Environmental correlates of species distribution in arid desert ecosystems of eastern Egypt Governal, Arid Environments 38: 297-313.

Fontaine M, Aerts R, Ozkan K, Mert A, Gulsoy S, Suel H, Waaelkens M and Muys B (2007). Elevation and exposition rather than soil type determine communities and site suitability in Mediterranean mountains forests of southern Anatolia, Turkey, Forest Ecology and Management 247: 18-25.

Gong X, Brueck H, Giese KM, Zhang L, Sattelmacher B and Lin S (2008). Slope aspect has effect on productivity and species composition of hilly grass land in the Xilin River Basin, Inner Mongolia, China, Journal of Arid Environment 72: 483-493.

Grime JP (1973). Competitive exclusion in herbaceous vegetation, Nature 242: 344-347.

Habib T, Malik ZH and Khan MQ (2011). Plant species diversity along the altutidinal gradient at Garhi Dopatta Hills, Muzafarabad, Journal of Medicinal Plants Research 5: 5194-5196.

Hegazy AK, Demedesh MA and Hosni HA (1998). Vegetation, species diversity and floristic relations along an altitudinal gradient in South-West Saudi Arabia, Journal of Arid Environment 3: 3-13.

Hua Y (2002). Distribution of plant species richness along elevation gradient in Hubei Province, China, International Institute for Earth System Science. Nanjing, China 1-140 pp.

Kabrickt MJ and Shifley RS (2004). Oak forest composition, site quality and dynamics in relation to site factors in the southeastern Missouri Ozarks, USDA Forest Service, 1-311 pp.

Kharakwal G, Mehrotra p and PangteyYS (2005). Phytodiversity and growth form in relation to altitudinal gradient in the Central Himalayan (Kumaun) region of India, Current Science 89: 873-878.

Maguran AE (1996). Ecological diversity and its measurement. Chapman and Hall, 1-256 pp.

Maguran AE (2004). Measuring biological diversity. Blackwell Publishing, UK, 1-256 pp.

Maranon T, Ajbilou R, Ojeda F and Arroya J (1999). Biodiversity of woody species in oak woodland of southern Spain and northern Morocco, Forest Ecology and Management 115:147-156.

Maarel VE (1998). Species diversity in plant communities in relation to structure and dynamics, SPB Academic publishing, Hague, The Netherlands, 1-114.

Mohajer MR (2007). Silviculture. University of Tehran press, Tehran, 1-387.

Mi Z, Hua KZ, Zhu JY and Bin G (2012). Analysis on slope revegetation diversity in different habitats, Earth and Planetary Science 5: 180-187.

Mirzaei J, Akbarinia M, Hosseini SM, Sohrabi H and Hosseinzade J (2008). Biodiversity of herbaceous species in relation to physiographic factors in forest ecosystems in Central Zagros, Journal of Iranian Biology 20: 375-382.

Panthi MP, Chaudhey RP and Vetaas OR (2007). Plant species richness and composition in a trans- Himalayan inner valley of Manang district, Central Nepal, Himalayan Journal of Science 4: 57-64.

Paven NP, Hernandez Trejo H and Rico Gray V (2000). Distribution of plant life forms along an altitudinal gradient in the semi-arid valley of Zapotitnal, Mexico, Journal of Vegetation Science 11: 39-42.

Peet RK (1981). Forest vegetation of the Corolado Front Range, Vegetation 45: 3-75.

Perma R and Joybari S (2010). Effect of physiographic and human on canopy cover and woody species diversity in Zagros forest (case study: protected forests, Ghalajeh, Kermanshah), Iranian Journal of Forest and Poplar Research18: 539-555.

Proctor J (1971). The plant ecology of surpertine II. Plant response to serpentine soils, Journal of Ecology 50: 397-410.

Sharma CM, Suyal S, Gairola S and Ghildiyal SK (2009). Species richness and diversity along an altitudinal gradient in moist temperate forest of Garhwal Himalaya, Journal of American Science 5: 119-128.

Shirzad MA and Tabari M (2011). Effect of some environmental factors on diversity of woody plants in *Juniperus excelsa* habitat of Hezarmasjed mountains, Iranian Journal of Biology 6: 800-808.

Small CJ and McCarthy BC (2005). Relationship of understory diversity to soil nitrogen, topographic variation, and stand age in an eastern Oak forest, USA, Forest Ecology and Management 217: 229-243.

Sohrabi H and Akbarinia M (2006). Study on plant species diversity in relation to physiographic factors in Dehsorkh, Javanroud, Iranian Journal of Forest and Poplar Research 13: 294-279.

Solon J, Degorski M and Roo Zielinska M (2007). Vegetation response to a topographical-soil gradient, Catena Journal 71: 309-32.

Strenberg M and Shoshany M (2001). Influence of slope aspect on Mediterranean woody formations, Comparison of a semi-arid and arid site in Israel, Ecological Research 16: 335-345.

Takyu M, Aibas SI and Kanehiro K (2002). Effects of Topography on tropical lower montage forests under different geological conditions on Mount Kinabalu, Borneo, Plant Ecology 159: 35-49.

Tamartash R, Yousefian M, Tatian MR and Ehsani M (2010). Vegetation Analysis in Rangeland of Lasem, Iran, Agriculture and Environment Science 7: 397-401.

J. Bio-Sci. 23: 29-37, 2015 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

PHYSICO-CHEMICAL PROPERTIES OF WATER OF RAMSHAGAR DIGHI, THE LARGEST MAN MADE HISTORICAL RESERVOIR IN NORTHERN BANGLADESH

Ashaduzzaman, MJ Hossain and S Akther*
Department of Zoology, University of Rajshahi, Bangladesh

Abstract

Ramshagar dighi is a larger historical man made reservoir (Dighi, Bengali meaning) situated at Tajpur village in Dinajpur, Bangladesh. This study was aimed to estimate current status of physico-chemical variables of water of Ramshagar dighi at Dinajpur District, Bangladesh. Monthly average changes in physico-chemical parameters such as water temperature, total dissolved oxygen, air temperature, humidity, rainfall, water depth and pH of water were analyzed for the period of 11 months from May 2011 to March 2012. The average air temperature (°C) at the study area of Ramshagar dighi at Dinajpur District was determined as 24.97 ± 4.92. Our present study showed that physico-chemical properties of water in Ramshagar dighi were monthly average of water temperature (°C) as 24.68 ± 4.77, air temperature (°C) 24.97 ± 4.92, humidity as 82.075 ± 4.14, rainfall as 1534.5 mm, water depth as 9.10 m ± 1.286, pH as7.67± 0.48 and carbon dioxide as 0.85 ± 0.92 as well as dissolved oxygen as 4.65 ± 0.62 respectively during the period of May 2011 to March 2012. Therefore, present study was conducted to assess physico-chemical properties of water of Ramshagar dighi, Dinajpur, Bangladesh.

Key words: Northern Bangladesh, physico-chemical properties, Ramshagar, reservoir, water

Introduction

Bangladesh is a fertile land for aquaculture. It has a wide variety of dynamic ecosystem, *viz*. rivers, canals, mangrove forests, natural lakes, man-made reservoirs, freshwater marshes, oxbow lakes, freshwater depressions and seasonally inundated extensive floodplains (Akonda 1989, IUCN 1993). Rivers, ponds and lakes are the waterways of strategic importance across the world, providing main water resources for domestic, industrial and agricultural purposes (Faith 2006). Water is essential for the survival of any form of life. Of the total water present on earth, only 33,400 m^3 are available for drinking, agriculture, domestic and industrial consumption (Dara 2007). Surface waters are vital and vulnerable freshwater resources that are critical for the sustenance of all life. Water quality parameters are the crucial elements for aquaculture. These water reservoirs that contained water are the main sources of fisheries production. A successful aquaculture is completely dependent on the water quality parameters that arise from a magnitude of physical, chemical and biological interactions. The physico-chemical characteristics of the aquatic system have a direct influence on the types and distribution of aquatic biota (De 2007). Water quality is patho-physiological condition of fish. It is not only the suitability of water for the survival and growth of fish but also is the indicator of aquatic pollution and diseases in fish which is normally governed by only a few variables. Patho-physiological condition of fish depended on the physico-chemical properties of water. Fishes are more dependent on water temperature, p^H, dissolved oxygen, free carbon dioxide, alkalinity and some other salts

*Author for correspondence: sarminhamim@gmail.com

for growth and development (Nikolsky 1963). Mollah and Haque (1978) reported that the physico-chemical factors of water and soil have some effects on plankton periodicity. The study of physico-chemical properties included the physical, chemical and biological parameters of a water body and these are interrelated and have direct effect on the productivity of a water body (Welch 1952). These physico-chemical parameters refer to the temperature, turbidity, odour, colour, total solid, total dissolved solid, total suspended solid, pH, conductivity, iron content, acidity, total hardness, and chloride content (FAO 1984). The quality of water in any ecosystem provided significant information about the available resources for supporting life in that ecosystem. The key feature of an ecosystem is the interaction between the biotic and abiotic components. Good quality of water resources depended on a large number of physico-chemical parameters and biological characteristics. All the vital functions of fish like feeding, digestion, assimilation, growth, response to stimuli and reproduction are depended on water quality. Thus healthy aquatic ecosystem is depended on the physico-chemical and biological characteristics (Venkatesharaju et al. 2010).

Nowadays the physico-chemical properties of water is altered due to the accumulation of large quantities of hazardous contaminants such as heavy metals and organic micro pollutants in the sediments of lakes, rivers and marine areas world-wide (Tuncer et al. 1993). Ramshagar dighi as a pond is highest and largest historical man made pond in the district of Dinajpur, Bangladesh. It bears some exceptional historical facts of natural heritage. As a historical reservoir the significance of the study is the demand of time. Therefore the objective of present study is to assess the physico-chemical properties of water of Ramshagar dighi. Because there is a very little work has been done on the study of physico-chemical properties of water of Ramshagar dighi, Dinajpur, Bangladesh. So it is important to study the physico-chemical properties of water of Ramshagar dighi for the sake of profitable aquaculture and for the upliftment of socio-economic condition of the general people.

Materials and Methods

The present investigation was conducted through a period of 11 months from May 2011 to March 2012 in the study area of Ramshagar dighi, Dinajpur, Bangladesh. The sampling and physical test was done monthly during the study period.

Meteorological data: During this study period meteorological data of the study area were collected from meteorological regional station, Dinajpur.

Air and water temperature: Air temperature of the study area was taken from Meteorological Regional Station Dinajpur. Water temperature was taken by using a centigrade mercury thermometer with a range of 0° to 120°C at the time of sampling. The bottom temperature of water was recorded by descending the thermometer until reaching to the bottom.

Water quality measurement: Sampling at the study area was carried out fortnightly from May 2011 to March 2012. Water samples were collected from the depth of 20 - 30 cm below the surface and also from the bottom. Physical data of the study area were recorded immediately.

Depth of water: Water depth of the study area at the time of sampling was noted by monthly. Depth of water was measured by the help of a meter scale with the ranges of 0 - 20 m. The depth was also measured by a graduated rope at various places of the study area.

p^H (*potenz hydrogen*): p^H refers to the amount of hydrogen ions in a solution. The pH value of water was determined by using a digital pH meter (Model HI, Hanna 96107).

Free Carbon dioxide (fCO_2): Free CO_2 (mg/l) was determined by titration of the water sample with NaOH solution (sodium hydroxide) using Phenolphthalein as an indicator (Welch 1948). The test was done immediately after collecting the sample.

Dissolved oxygen (DO): The dissolved oxygen (mg/l) was estimated by using the Winkler's Method (APHA 1976). Manganese sulphate, sulfuric acid, starch solution and sodium thiosulfate were used as reagents for the determination of dissolved oxygen. A sample of 300 ml glass biological oxygen demand (BOD) stopper bottle brim was filled with sample water which carefully added to 2 ml of manganese sulphate so that no bubble can be introduced. Alkali-iodide-azide and 2 ml of concentrated sulphuric acid were added respectively. If oxygen is present, a brownish-orange cloud of precipitate or flock will appear where the floe was settled down by turning the upside down at several times. Carefully stopper and invert several times to dissolve the floe. At this point, the sample was fixed and stored for 8 hours in a cool and dark place by the lid off with aluminum foil and rubber band. The sample was titrated with sodium thiosulfate up to pale straw color. Next 2 ml of starch solution was added, which imparted blue color to the solution. Thus the titration indicated the culminating point of the experiment and the data were recorded.

Results and Discussion

Mean values of the water parameters such as air temperature, humidity, rainfall, water depth, water temperature, total dissolved oxygen, free carbon dioxide and pH in Ramshagar dighi, Dinajpur, Bangladesh were represented below.

Air temperature: Data regarding the air temperature at the study area during May 2011 to March 2012 was observed as a monthly average maximum and minimum variation of temperature of 33.03°C to 10.88°C in June 2011 and January 2012 (Table 1 and Fig. 1) respectively throughout the study period. Monthly average variation of maximum air temperature (°C) was recorded as 29.63 ± 3.93 and minimum was recorded as 20.30 ± 6.03. Air temperature showed fluctuation as lower in winter and higher in summer, spring and autumn. Thus the impact of air temperature on the fluctuation of water temperature was reported by Ismail et al. (1984) and Begum et al. (1989) in Bangladesh. In pond, bottom water temperature is slightly lower than or equal to surface water temperature (Rahman et al. 1982). Air temperature may fluctuate in the study area due to solar radiation, season, length of the day, geographical position and other meteorological conditions, which were not considered in this study.

Table 1. Monthly average fluctuation of air temperature, humidity, water depth, rainfall, water temperature, pH, free carbon dioxide and total dissolved oxygen in Ramshagar dighi, Dinajpur, Bangladesh from May 2011 to March 2012.

Parameters		Months											Mn ± SD
		2011								2012			
		May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	
AT	Max	32	33.03	32.7	32.1	32.8	32.7	28.7	23.5	22	27.3	29	29.6±3.90
	Min	23.2	25.4	26.2	25.9	26.1	23.7	16.6	12.3	10.1	13.4	19.3	20.3±6.03
	Av	27.6	29.2	29.5	29	29.5	28.2	22.6	17.9	16.4	20.4	24.2	24.9±4.99
RH	Max	93.3	94.3	93.6	94.6	94.1	95.4	95.7	97.4	97.1	93.1	93.3	94.7±1.50
	Min	68.9	64.6	69.4	73.7	77.5	75.7	67.4	75	76.5	55.6	59.2	69.4±7.30
	Av	81.1	79.5	81.5	84.1	85.8	85.5	81.5	85.2	86.8	74.3	76.2	82±4.14
WD		9.5	10.1	10.4	10.9	10.4	9.2	8.7	7.8	7.5	7.2	8.2	9.1±1.30
Rf		249	348.7	283.7	383.3	231.2	1.2	2.3	0.0	5.1	5	25	139.50
WT	Up	32.2	32.5	31.6	30.1	31.7	30.9	27.5	22.3	20.3	27.7	28.5	28.8± 4.1
	Md	30.5	28.9	29.6	27.9	28.1	27.7	24.3	20.2	16.6	21.2	25.4	25.5±4.4
	Lw	24.5	23.6	24.3	23.2	25.6	24.7	19.7	10	9.9	15.2	19.9	20.0± 5.8
	Av	29.1	28.3	28.5	27.1	28.5	27.7	23.8	17.5	15.6	20.7	24.6	24.6±4.76
	p^H	7.3	6.9	7.9	8.2	7.8	7.1	7.7	7.2	7.9	8.1	8.3	7.7±0.48
	fCO	2.2	1.8	0.0	0.0	1.2	0.0	0.7	2.2	0.0	0.0	1.3	0.85±0.92
	DO	4.9	5.8	4.2	3.9	4.4	5.6	5.1	4.3	4.5	4.1	4.4	4.6 ±0.6

AT = air temperature, HD = humidity, WD = water depth, Rf = rainfall, WT = water temperature, p^H = potenz hydrogen, fCO = free carbon dioxide, DO = dissolved oxygen, Max = maximum, Min = minimum, Up = upper, Md = middle, Lw = lower and Av = average.

Humidity: During the study period Relative Humidity was recorded throughout the study period. The maximum humidity of 97.37% was recorded in December 2011 and the minimum one was 55.63% in February, 2012 (Table 1 and Fig. 1). The maximum relative humidity was determined as 94.73 ± 1.51 and minimum relative humidity was as 69.42 ± 7.26. Monthly average relative humidity was recorded as 82.075 ± 4.14. This study is supported by a similar observation made by Islam and Mendes (1976)

Rainfall: The rainfall during the study period in Ramshagar dighi showed a distinct seasonal trend of fluctuation. It was recorded maximum as 383.3 mm in August 2011 and the minimum was as 1.2 mm in October 201l and no rain fall during the month of December 2011 (Table 1 and Fig. 1). The average rainfall over the 11 months was found as 139.5 mm. Rainfall had a cooling effect on air temperature; the hot summer wind took a moderate trend of variation when frequent rainfall began to take place in the month of May and continued till September (Miah et al. 1981). Michael (1968b) reported that the rainfall and air temperature had the direct influences on the variation of water temperature. Bhuiyan et al. (1997) also observed the seasonal variation of rainfall.

Physical condition

Water depth: Maximum depth of water was recorded in August 2001 as 10.90 m where as the minimum one was recorded in February 2012 as 7.3 m. The average depth of Ramshagar dighi was 9.10 m from May 2011 to March 2014 (Table 1 and Fig. 1). There is a seasonal variation in the depthness of water in water body and rise in water level during monsoon and winter rains has been found. Rahman (1982) stated that ponds should not be shallower than 1.1 m and not deeper than 5 m, and the optimum should be 2 m.

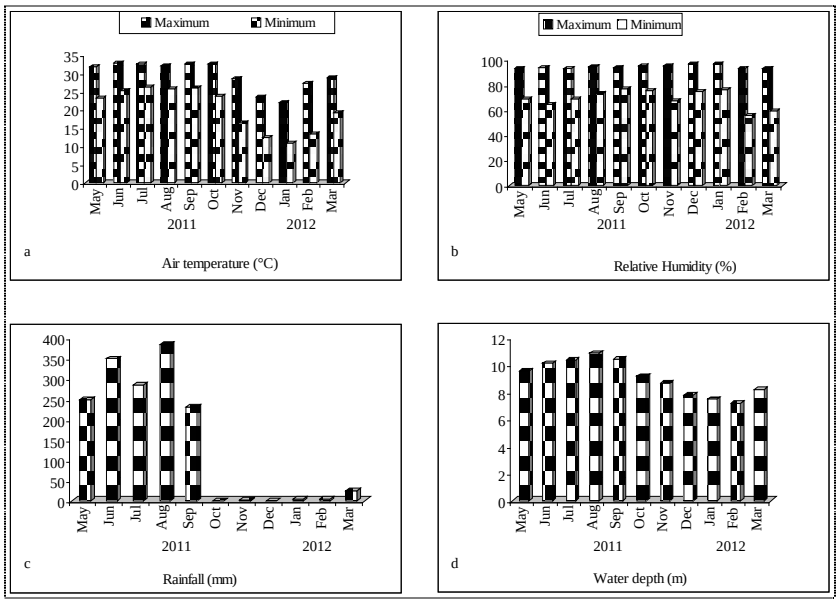


Fig. 1. Fluctuation of air temperature (a), relative humidity (b), rainfall (c) and water depth (d) of water of Ramshagar dighi from May 2011 and March 2012.

Water temperature: Throughout the study period water temperature was found to fluctuate from the maximum of 32.5°C at the month of June to the minimum of 9.9°C at the month of January (Table 1 and Fig. 2). Average mean of water temperature was determined as 24.68 ± 4.77. Many workers observed similar trends while working on different water bodies (Dwivedi and Pandey 2002). The highest water temperature was recorded in summer (Patra and Azadi 1987) and low water temperature was found in winter months (Das and Bhuiyan 1974) in Bangladesh. The fluctuation in water temperature usually depended on the season, geographic location, sampling time and temperature of effluents entering the stream (Ahipathy and Puttaiah 2006). The standard limit of temperature of water is 20 - 30°C (ECR 1997). All the values were within the standard limit. So, the water bodies are suitable for aquatic life. Therefore, the fluctuation of water temperature in the study area of Ramshagar dighi may be due to the variation of excess CO_2, rainfall and air temperature.

Redox characteristics: P^H of water of Ramshagar dighi was measured monthly where the pH value showed a slightly alkaline in nature during the study period. The pH value of water was found to be fluctuated from a minimum of 6.9 in the month of June, 2011 and maximum of 8.3 in the month of March, 2012 (Table 1 and

Fig. 2). During the study period mean of the pH value of water was recorded as 7.67 ± 0.48. Islam et al. (1974) reported the fluctuation of water in Buriganga river, Bangladesh from 7.8 to 6.9 in the months of July and March respectively. P^H value of Ramna lake water showed maximum variation as 9.8 in July and 7.5 in March (Islam and Saha 1975). Ahmed et al. 2005 recorded the maximum p^H value of water of Meghna river, Bangladesh as 8.00 in the month of September and minimum as 7.5 in the month of May. The pH value in alkaline condition in pond water was supposed to be helpful for proper growth and development of fishes and other aquatic organisms (Nikolsky 1963). Jhingran (1985) has shown that pH range 7-8 is suitable for fish culture as well as most of aquatic organisms. In most raw water sources, pH lies within the range of 6.5 - 8.5 (Ahmed and Rahman 2000). So, these aquatic bodies are suitable for aquatic life. Fluctuation in water p^H level found in the present study may due to the change of water temperature and CO_2.

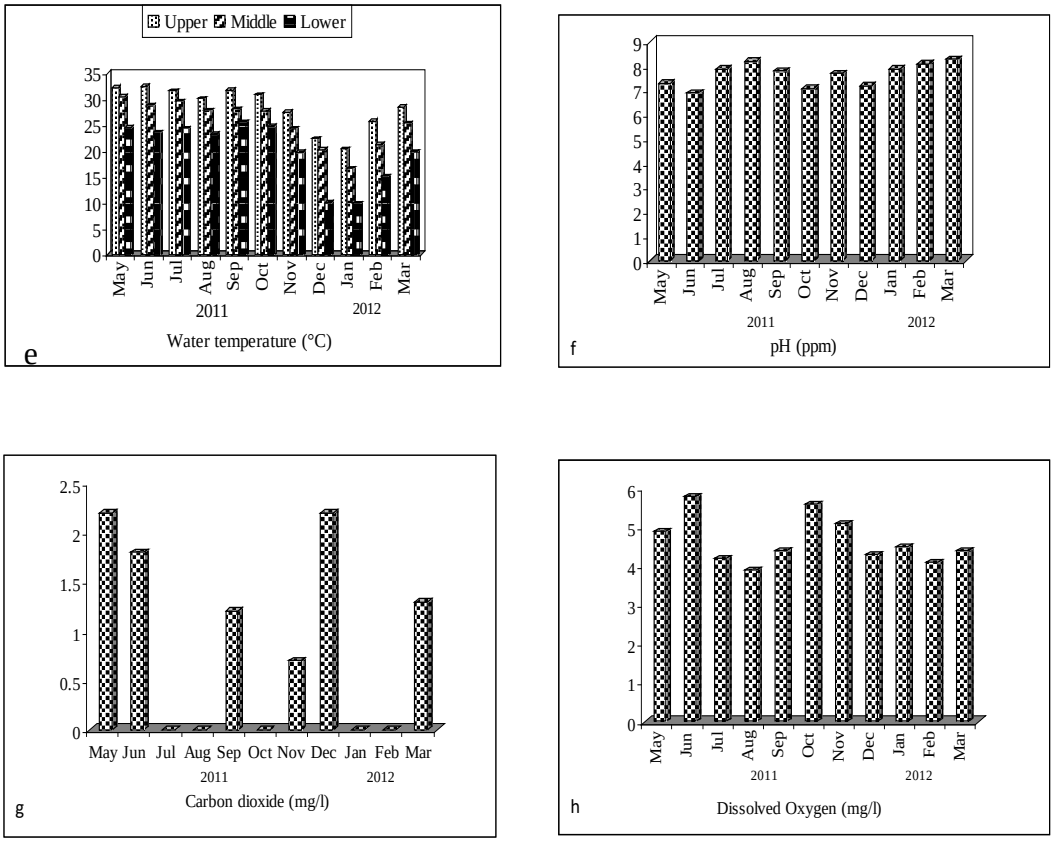


Fig. 2. Fluctuation of water temperature (e), p^H (f), carbon dioxide, (g) and dissolved oxygen (h) of water of Ramshagar dighi from May 2011 and March 2012.

Free carbon dioxide (CO_2): Free carbon dioxide is also a determining factor for aquatic ecosystem. Free CO_2 value of the water ranged from 0.00 to 2.2 mg/1 in the months of July and December respectively during the period of May 2011 to March 2012. During the study period, mean of free carbon dioxide value of the water was estimated at 0.85 mg /l ± 0.92 (Table 1 and Fig. 2).

Bhuiyan and Nessa (1996) found that free CO_2 was absent in May, June and July whereas the highest value (7.35 mg/l) was found in August. Bhuiyan and Nessa (1998a) informed that the free CO_2 fluctuated between 0.0 mg/l (January and March) to 15 mg/l (September). So it was found that low free CO_2 content during winter and spring and high free CO_2 content during autumn. The increase in carbon dioxide level during summer may be due to decay and decomposition of organic matter. This is strengthened by the observations of Joshi et al. (1995) who have observed the addition of drainage was the main causal factor for increase in carbon dioxide in the water bodies.

Dissolved oxygen (DO): Dissolve oxygen (mg/l) is the determining factor for all of the aquatic organisms. The dissolved oxygen value of water of the study area ranged from 3.9 mg/l to 5.80 mg/l during the period of May 2011 to March 2012. The maximum DO was recorded 5.80 mg/l in the month of June and the minimum of DO was recorded 3.90 mg/l in the month of August (Table 1 and Fig. 2). Mean value of the DO (mg/l) was remained 4.65 ±0.62 during the period of this study. The dissolved oxygen content of pond water was found maximum during the month of June in Bangladesh (Chowdhury and Mazumder 1987, Khan et al. 1990). Islam et al. (1979) reported minimum dissolved oxygen in September whereas maximum in February. Ali et al. (1989) found high value during winter and low value in summer and a decline in rains. Bhuiyan and Nessa (1996) noted maximum dissolved oxygen during autumn and minimum value in winter. The quantity of DO in water is directly or indirectly dependent on water temperature, partial pressure of air etc. Similar results were observed by Chaurasia and Pandey (2007). The standard DO value of surface water for Bangladesh is 6 or more (ECR 1997). Huq and Alam (2005) mentioned that water with DO value ranging 4 - 6 mg/l is suitable for drinking purpose. In respect of DO, the water body can be used for fish culture and other aquatic organisms. Our results are in close conformity with those reported above. However, more comprehensive works are to be solicited.

Acknowledgement

The authors are thankful to the Meteorological Station, Dinajpur and Chairman, Department of Zoology, University of Rajshahi, Bangladesh for their sincere co-operation during the study.

References

Ahipathi MV and Puttaiah ET (2006). Ecological Characteristics of Vrishabhavathi River in Bangalore (India). Environmental Geology 49: 1217-1222.

Ahmed KKU, Ahmed SU, Haldar GC, Hossain MRA and Ahmed T (2005). Primary Production and Fish Yield Estimation in the Meghna River System, Bangladesh. Asian Fisheries Science. Asian Fisheries Society, Manila, Philippines, 18: 95-105.

Ahmed MF and Rahman MM (2000). Water Supply and Sanitation. ITN-Bangladesh, Centre for Water Supply and Waste Management, BUET, Dhaka, Bangladesh.

Akonda AW (1989). A Directory of Asian Wetlands. IUCN, Gland, Switzerland and Cambridge, Bangladesh. 541-581 pp.

Ali S, Shaha S and Mahmud N (1989). Studies on the physico-chemical aspects of Maheshkhali Channel, Bay of Bengal. Dhaka University Studies 33 (1): 43-49.

APHA (1976). Standard methods for the examination of water (4th ed.) American Public Health Association, Washington, 1193 pp.

Begum A, Mustafa G, Ali S and Ahmed KH (1989). Studies on limnology in a mini pond and growth of *Tilapia (=Oreochromis) nilotica.* Bangladesh Journal of Zoology 17(1): 34-45.

Bhuiyan AS and Nessa Q (1996). Study on Physico-chemical parameters of a fish pond, Bangladesh. Journal of Bio-Science 4(1): 171-173.

Bhuiyan NIMAS, Nahar Q and Islam MN (1997). Physico-chemical condition in relation to meteorological condition of fish pond in Rajshahi. University Journal of Zoology Rajshahi University 16(1): 85-88.

Bhuiyan AS and Nessa Q (1998). Seasonal variation in the occurrence of some zooplankton in a fish pond. Bangladesh Journal of Fisheries Research 2(2): 201-203.

Mahima C and Pandey GC (2007). Study of Physico-chemical characteristics of some water ponds of Ayodhya.-Faizabad. International Journal of Environmental Pollution 27(11): 1019-1023.

Chowdhury SH and Mazumder A (1987). Limnology of Lake Kaptai 1, Physico-chemical features. Bangladesh Journal of Zoology 9(2): 59-72.

Dara SS (2007). Environmental Chemistry and Pollution Control. 7th edition. S. Chand and Company Ltd. Ram Nagar, New Delhi. 65 p.

Das NG and Bhuiyan AL (1974). Limnoplankton of some inland waters of Dhaka city. Bangladesh Journal of Zoology 2(1): 27-42.

De AK (2007). Environmental Chemistry. 6th edition. New Age Int. (P) Ltd., New Delhi. 169-170 pp.

Dwivedi BK and Pandey GC (2002). Physico-chemical factors and algal diversity of two ponds, (Girija Kund and Maqubara pond), Faizabad. Pollution Research Society 21: 361-370.

ECR (1997). The Environment Conservation Rules. Government of the People's Republic of Bangladesh. Ministry of Environment and Forest. 205-207 pp.

Faith N (2006). Water Quality Trends in the Eerste River, Western Cape, 1990-2005. A mini thesis submitted in partial fulfillment of the requirements for the degree of Magister Scientiae, Integrated Water Resources Management in the Faculty of Natural Science, University of the Western Cape. 41 pp.

FAO (1984). Physical and chemical methods of soil and water analysis. vol. 10.

Huq SMI and Alam MD (2005). A Handbook on Analysis of Soil, Plant and Water. BACER-DU, University of Dhaka, Bangladesh. 1-246 pp.

Islam AKMN and Mendes F (1976). Limnological studies of a Jheel in Sher-E-Bengla Nagar. Dhaka University Studies 24(2): 63-71.

Islam AKMN, Rahman M and Chowdhury AR (1979). Hydrological studies of Dhanmondi lake in Dhaka. Journal of Asiatic Society, Bangladesh 5(1): 59-75.

Ismail M, Rahman H, Ali S and Ahmed Khan (1984). Studies on limnology and some aspects of biology of *Oreochromis nilotica* (L) in a pond Juran, Dhaka. Proceedings of Frourth National Zoological Conference, Bangladesh 97- 105 pp.

IUCN (1993). Freshwater Wetlands in Bangladesh: Issues and Approaches for management. International Union of Nature and Natural Resources. Pub. Services. Unit, Huntingdon road, Cambridge, UK.

Jhingran VG (1985). Fish and Fisheries of India. Hindustan Publ. Corp., Delhi.

Joshi M, Shishodia SK, Kumar SN and Saikia DK (1995). Ecosystem studies in upper region of Ganga River, Environmental monitoring and assessment 35(1): 181-206.

Khan SM, Munzurul H, Aziz KMS, Morshed MG and Shafi M (1990). Seasonal variations in physico-chemical conditions of Dhanmondi lake water. Bangladesh Journal of Zoology 8(1): 61-66.

Miah MI, Bhuiyan NI and Dewan S (1981). A comparative study of the rate of growth of major in relation to physico-chemical and biological factors. Proceedings of Third Zoological Conference, Bangladesh 215-223 pp.

Michael RG (1968). Seasonal trends in physico-chemical factors and plankton of a freshwater fish pond and their role in fish culture. Hydrobiologia 33(2): 144-158.

Mollah MFA and Haque AKMA (1978). Studies of monthly variations of plankton in relation to the physico-chemical conditions of water and bottom soil of two ponds. II. Zooplankton. Bangladesh Journal of Fisheries 1(2): 99-103.

Nikolsky GV (1963). The Ecology of Fishes. Academic press. London. New York.

Patra RWR and Azadi MA (1987). Ecological studies on the planktonic organisms of the Halda River. Bangladesh Journal of Zoology 15(2): 109-123.

Rahman MS, Chowdhury MY, Haque AAKM and Haq MS (1982). Limnological studies of four ponds. Bangladesh Journal of Fisheries 25(1-2): 25-35.

Tuncer GT, Tuncel SG, Tuncel G and Balkas TI (1993). Metal pollution in the Golden Horn, Turkey: Contribution of natural and anthropogenic components: ER Christensen, DN Edgington and JP Giesy (Eds.), Water Science and Technology: Contaminated aquatic sediments, Oxford: Elsevier Science, Ltd., 59-65 pp.

Venkatesharaju K, Ravikumar P, Somashekar RK and Prakash KL (2010). Physico-chemical and bacteriological Investigation on the river Cauvery of Kollegal Stretch in Karnataka, Journal of Science Engineering and Technology 6(1): 50- 59.

Welch PS (1952). Limnology (2nd ed.). Me Graw-Hill Book Company Inc., New York. 538 pp.

Welch PS (1948). Limnological Methods. Me Graw-Hill Book Company Inc., New York. 381 pp.

J. Bio-Sci. 23: 39-46, 2015 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

EFFECT OF THERMAL STIMULATION ON GONAD MATURATION IN SEA CUCUMBER *PHYLLOPHORUS* SP. (GRUBE, 1840)

S Andriyono[1,2*], N Masruroh[3], ED Masithah[2], J Triastuti[2] and D Winarni[4]

[1]Interdisciplinary Program of Biomedical, Mechanical and Electrical Engineering, Pukyong National University, Busan, 48513, Republic of Korea; [2]Department of Marine; [3]Aquaculture Program and [4]Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Campus C UNAIR Jl. Mulyorejo, Surabaya, 601145, Indonesia

Abstract

Sea cucumbers, marine animals from the class Holothuroidea, have been widely known as fishery products for consumption in Indonesia. The Madura Strait is productive waters for sea cucumbers in eastern Indonesia, including sea cucumber species *Phyllophorus* sp. The exploitation of it mainly for consumption even though there are pharmaceutical beneficial of sea cucumber already known. The study of temperature effect is ideal inducement method for sea cucumber, but less research about this stimulation for *Phyllophorus* sp. for domestication purposes. The research showed that thermal stimulation treatment at the temperature of 30°C, 32°C and 34°C were in contrast to the that of control histological analysis proof that some of *Phyllophorus* sp. showed altered levels of gonadal maturation toward growth and advanced growth phase after thermal stimulation, respectively. The ideal stimulation treatment for gonad maturation was shown with stimulated temperature treatment of 32°C.

Key words: Frequency, histology, *Phyllophorus* sp., sea cucumbers, thermal stimulation

Introduction

As one of the popular invertebartes fishery products, sea cucumbers from the class Holothuroidea have long been consumed by Indonesians (Pangkey et al. 2012). The marine ecosystem along Madura Strait, including Surabaya, is productive for sea cucumbers species in eastern part of Java Island in Indonesia. One of the highly abundant species is *Phyllophorus* sp. which is not included in the list of commercial sea cucumber in the global market (Purnama and Winarni 2017) and *Paracaudina australis* (Widianingsih et al. 2018). Intensive exploitation of sea cucumbers *Phyllophorus* sp. only used as food in the form of sea cucumber chips. The demand for this product is quite high so that the population of sea cucumbers *Phyllophorus* sp. in nature is very limited in certain seasons. Some research found active compound and the pharmaceutical ingredients from this sea cucumber such as triterpene glycoside, chondroitin sulfates, and others active compound (Revianti et al. 2016).

Regarding the potential value of sea cucumbers, the cultivation and domestication activities of some sea cucumbers need to be conducted. The sea cucumber culture especially *Phyllophorus* sp. species, not yet done because of the lack of biological information and various efforts in the conservation strategy. The use thermal stimulation for sea cucumber reproduction is not a novelty (James et al. 1994), but for this species are still very important in providing information related to the breeding characteristics of *Phyllophorus* sp. Various spawning stimulation techniques on sea cucumber have been applied and thermal stimulation techniques done by raising the temperature for 3-5°C from the optimal temperature of 28°C is regarded as

*Author for correspondence: sapto.andriyono@fpk.unair.ac.id

the most effective (Pitt 2001). The thermal stimulation (ambient temperature of ± 3-5°C) has gained success in initiating spawning on *Holothuria scabra* (Kumara et al. 2013). Accordingly, this study conducted thermal stimulation on sea cucumbers of *Phyllophorus* sp. aimed at revealing the ideal temperature affect to the gonad maturity by observing the sea cucumber gonadal histology.

Materials and Methods

Samples collection

This study utilized sea cucumber *Phyllophorus* sp. from the East Coast of Surabaya, Indonesia. Sea cucumbers were taken freely during low tide in order to avoid currents and waves and facilitate sampling with some modification (Conan 1993). Sea cucumbers collected were put into 60 × 80 cm^2 plastic bags with each containing of 15 individuals. Further handling for brood stock, mud from habitat was added to cover the entire body of sea cucumbers and a rubber band was then tied around each bag without addition of oxygen. The method of packing sea cucumbers packing was adapted from preliminary study. Subsequently, sea cucumbers were transported to the Educational Laboratory of the Faculty of Fisheries and Marine Science, Universitas Airlangga. Morphological gonads between male and female sea cucumber were analyses directly based on color and some granules (Fig. 1). Female sea cucumbers *Phyllophorus sp.* gonad was moss green in color with bulging and branching tubules, than male sea cucumbers *Phyllophorus* sp. gonad showed a yellowish white color with branching tubules.

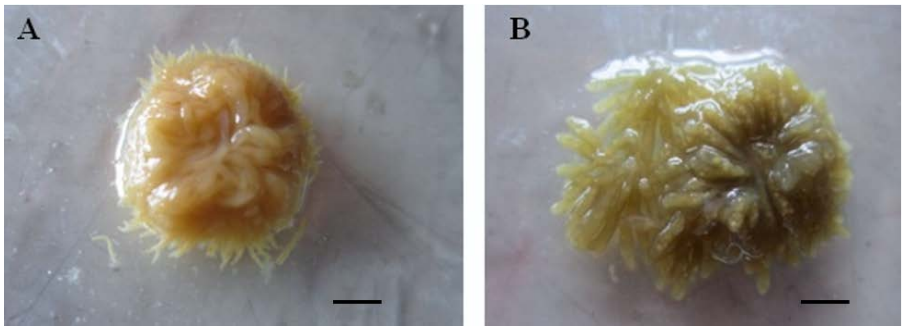


Fig. 1. Morphology of sea cucumber *Phyllophorus* sp. male gonad (A) and female gonad (B). Scale bar 5 mm.

Thermal stimulation

Before getting thermal stimulation difference treatment, sea cucumber keep in the culture 10 liter medium for 15 days until SR more than 80% with sea cucumber density is 1 individual/2 liter water. The water in the plastic container was changed everyday and added aeration for dissolved oxigent maintain in suitable condition. The fresh marine micro algae was given for sea cucumber food. Ten sea cucumber were kept in aech container. This survival rate as indicator for sea cucumber adaptation to the new habitat. The treatments used in this study is thermal stimulation with ambient water temperature at 28°C, 30°C, 32°C and 34°C. Increasing 3-5°C for thermal stimulation was enough to artificial sea cuumber spawning in tank (James et al. 1994, Laxminarayana 2005, Dabbagh and Sedaghat 2012). Twelve plastic containers with a capacity of 10 liter were prepared and then filled with sea water. In order to obtain the temperature of 30°C, 32°C and 34°C, each container was equipped with a heater that has been set with specified temperature. Furthermore, sea cucumbers were inserted into the plastic container. The length of thermal stimulation

ranging from 30-60 minutes each is deemed sufficient to affect the gonads of sea cucumbers (Agudo 2006, Ivy and Giraspy 2006). In this study, thermal stimulation was conducted for 60 minutes (Battaglene et al. 2002). Upon stimulation, sea cucumbers were placed into a plastic container with sea water at normal temperature (28°C) for 1-1.5 hours until they show changes in behavior as characterized by the upward movement of the male parent to the tub wall (Laxminarayana 2005).

Histology

Gonadal histology was performed by means of fixating the gonads in a liquid solution of neutral buffered formalin fixative for more than 24 hours. Gonad preserved in buffer formalin were rinse in aquades and than sore in 70% etanol. For further histology, five tubules of each gonad were taken for further processing that involved several phases. The first step are dehydrated, embedded in paraffin, sectioned to get 6µm thick every layer then affixed, and at last stained with haemotoxylin and eosin (H/E) (Romafafia et al. 2000, Mumford 2004). Five longitudinal cuts were made acros tubules, and results of gonadal histology were further observed under a microscope with 100x magnification and then documented.

Data analysis

The data were analyzed descriptively to illustrate the effect of thermal stimulation on the gonadal histology of sea cucumbers *Phyllophorus* sp. Gonad maturity index (GMI) are comparison between body tissue and gonad ratios commonly use for measure reproctuctive periodicity (Morgan 2000) or in the other word that GMI is a value obtained from the ratio of gonad dimensions (weight, volume or area) with a specific organ, such as the integument either complete or without visceral organs and body-wall weight (Tuwo 1999). Water quality was measured to ensure water condition along thermal stimulation and sea cucumber rearing periods including salinity, disolved oxygent and water acidity (pH level).

Results

Sea cucumbers *Phyllophorus* sp. of GMI

Thermal stimulation at the temperature of 30°C, 32°C and 34°C have significant than control temperature of 28°C based on GMI. Gonadal maturity index of sea cucumbers *Phyllophorus* sp. is highest in treatment C (32°C) with an average index of 12.29%, while the lowest was treatment D (34°C) with an average index of 6.61%. While the treatment of A (28°C) and B (30°C) did not differ significantly that is 10.0% to 10.5% respectively.

Gonadal histology of sea cucumbers of *Phyllophorus* sp.

Histological examination of the gonads of sea cucumbers was conducted to determine the level of gonad maturity of each individual. Some sea cucumbers *Phyllophorus* sp. given thermal stimulation treatment showed altered levels of gonadal maturation toward an advanced growth phase (Table 1).

Table 1. Gonad maturity level based on gonadal histology of sea cucumbers *Phyllophorus* sp.

Gonad maturity level (%)	Thermal stimulation temperature				Survival rate
	28°C	30°C	32°C	34°C	(%)
Growth	66.67	33.33	0	33.33	100
Advanced growth	33.33	66.67	100	66.67	100

Thermal stimulation treatment at 28°C (as the Control) indicated growth phase with a percentage of 66.67% and advanced growth phase of 33.33%. Thermal stimulation treatment 30°C showed advanced growth

phase of growth higher than control with the percentage of 66.67% and growth phase of 33.33%. Thermal stimulation treatment on 32°C suggested advanced growth phase of 100% and 0% growth phase. Meanwhile, thermal stimulation treatment on 34°C signified advanced growth phase with the percentage of 66.67% and growth phase of 33.33%. As a result, thermal stimulation treatment at the temperature of 30°C, 32°C and 34°C was in contrast to thermal stimulation treatment at control temperature in terms of the level of gonad maturity. In all treatments, the survival rate reached 100%. This signified that the media at the time of treatment is in a viable condition for holding sea cucumber *Phyllophorus* sp. The male and female ratio in every container cannot be determined because of the difficulty of distinguishing males and females based on morphology. At the control treatment with temperature 28°C, found 3 males and 6 females. While the treatment temperature of 30°C and 32°C, mostly dominated by females as much as 9 and 8, while the rest are male. At the last treatment at 34°C, male and female compositions were found to be approximately equal to 6 females and 4 males, respectively.

The males in growth phase still had a fairly thick tubular wall, and spermatogenic cells appeared to progress towards the lumen. Meanwhile, the females in growth phase had a very thick tubular wall, and a few pre-vitellogenic oocytes detected near the walls of the tubules and the lumen was filled with vitellogenic oocytes and post vitellogenic oocytes. The males had a fairly thick tubular wall, and the lumen contained spermatozoa in advanced growth phase, than female gonads had a curved and thin tubular wall with a pre-vitellogenic oocyte near the walls of the tubules, and vitellogenic as well as post vitellogenic oocytes in the lumen (Fig. 2-3).

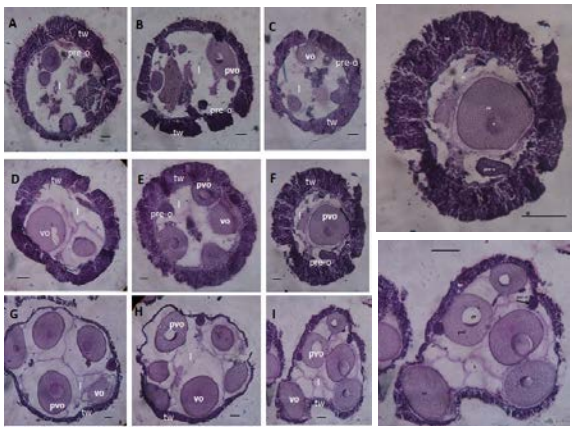


Fig. 2. Female gonad in different maturity level, growth (A-F) and advanced growth (G-H) information symboln: nucleus; ro: relict oocyte; zr: zona radiata; gv: germinal vesicle; pre-o: previtellogenic oocyte; vo: vitellogenic oocyte; pvo: post-vitellogenic oocyte; tw: tubular wall (bar 200 μm).

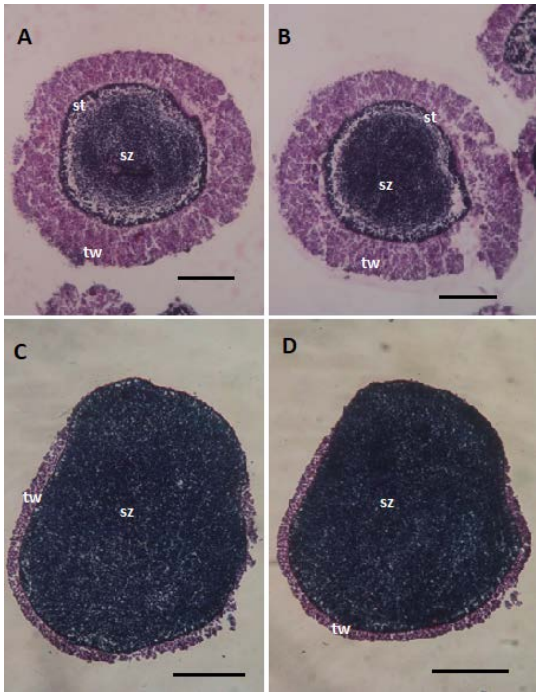


Fig. 3. The male gonad in growth pase (A-B) and advanced growth (C-D). tw: tubular wall; sz: spermatozoa; st: spermatid.

Water quality maintenance on captive medium

Water quality parameters measured include pH, salinity, and dissolved oxygen (DO). Water quality measurement during the study was conducted at 07.00 pm. Data on the water quality during maintenance of sea cucumbers *Phyllophorus* sp. showed a similar average value for each container with pH value of 7.00, salinity is 30 ppt, and DO at 7 ppm.

Discussion

The sea cucumbers *Phyllophorus* sp. is dioceous in nature or having separate sexes. Mostly the tropical sea cucumber are often high in fecundity (Laximinarayana 2005). Male and female individuals cannot be distinguished by external morphology but by observing the shape and color of the gonads, instead. Reproductive organs of sea cucumbers *Phyllophorus* sp. are very simple and consist of many tubules. The gonads of sea cucumbers are in the form of tubules (a tubular body), either plain, notched or forked (Purwati and Luong-Van 2003). They are mostly located in the anterior part of the body cavity. Sexual development generally results in lengthening the tubules, multiplying the branches and thinnning the tubular wall. This gonad morphological almost same to the other of Holothurians (Romafafia et al. 2000). In this study, histological analysis also revealed that thermal stimulation on sea cucumber *Phyllophorus* sp. higher than ambient water themperature resulted in better level of gonad maturity of broodstock *Phyllophorus* sp. However, this is not supported by gonad maturity index value. There are two common problems in using gonad maturity index. Firstly, there is a possibility that during the growth phase of an animal, the size of the

gonads is no longer proportional to its body size and the secondly, difficulties in obtaining accurate data on body weight often arise due to the damage at the time of collection (Purwati and Luong-Van 2003).

Histological studies on the gonads denoted some variations in gonadal maturity level among individuals. Thermal stimulation treatment is commont methode for artificial breeding in hatchery (James et al. 1994). However, this type of sea cucumber *Phyllophorus* sp. hatchery activity has never been done due to face various biological and technical obstacles (Tuwo 2005). Research on the development of gonad sea cucumbers indicates that there are monthly changes. The most common type of oocytes in the gonadal tubule lumen of *Phyllophorus* sp. in April was pre-vitellogenic oocytes with the percentage of 48.13% (Winarni et al. 2015). Therefore, it was predicted that in April sea cucumbers *Phyllophorus* sp. are in growth phase Variation in the level of gonad maturity histologically was also supported by gonad maturity index calculations for each treatment. This affirmed that stimulation at different temperatures affected the level of gonad maturity of *Phyllophorus* sp. In addition, variation in the level of gonad maturity of sea cucumbers *Phyllophorus* sp. also shows that the population of *Phyllophorus* sp. has an asynchronous pattern of reproduction. Population with an asynchronous pattern reproduction may spawn throughout the year. *Holothuria scabra* was present of maturity level (stage IV) and spawns continosly along a year (Tuwo 1999), with the peak of spawning period occurs at certain times and may vary depending on environmental changes (Conan 1993). The temperature is the most influential factor in the sexual reproduction of sea cucumbers act since it plays a role in controlling gonad maturity during spawning (Battaglene et al. 2002). External stimuli from the environment received by radial nerves will be responded by controlling the release of internal stimuli in the form of radial nerve factor (also called gonad-stimulating substance), which is similar to gonadotropin releasing factor in vertebrates (Dubois et al. 2002). In dry season, water themperatur increase and stimulating reproduction of several sea cucumber (Tuwo 1999).

In accordance with our finding that thermal stimulation at 32°C is the suitable thermal stimulation, supported another finding on other sea cucumbers *Holothuria edulis* to spawn more than 90% (Yudiati et al. 2001). Stimulation conducted by raising water temperature 3-5°C above the normal water temperature is also an effective method to induce spawning on sea cucumber *Stichopus japonicus*, which is characterized by the spawning of *Stichopus japonicus* egg and sperm in the water column (Chen 2003). Conducting this kind of stimulation for an hour is regarded as the most common artificial spawning stimulation technique used in the sea cucumber *Holothuria scabra*, which is characterized by the spawning of sea cucumbers *Holothuria scabra* in Solomon Island. Mature gonads of sea cucumbers will also spawn spontaneously during collection or during transport due to stress (Battaglene 1999). Thermal stimulation treatment suggested the best stimulation temperature at 32°C, which was regarded as the optimum temperature for the growth of sea cucumber gonad parent *Phyllophorus* sp. The growth phase in male gonads is denoted by relatively thick walls of the tubules and curved germinal epithelium (Morgan 2000). Spermatogonia is along the surface of the germinal epithelium, and there is a layer of spermatocytes and spermatozoa cells in the middle of the lumen. The histology of the gonads on growth phase is characterized by relatively thick gonadal tubule wall in *Phyllophorus* sp., and spermatogenic cells that can be differentiated into spermatocytes, spermatids, and spermatozoa. In the advanced growth phase, male gonads have a thick tubular wall and lumen containing spermatozoa (Winarni et al. 2015). The wall in male gonads is thinning. The male tubules were filled with spermatozoa cells, yet spermatocyte cells may also be detected around the tubular wall. In the female gonads, the growth phase is characterized by a very thick tubular wall, and the existence of a pre-vitellogenic oocytes, vitellogenic oocytes and post vitellogenic oocytes. Ovary development is characterized by an increase in oocyte diameter as a result of the accumulation of vitellogenic during the vitellogenesis process (Romafafia et al. 2003). Vitellogenesis in female may have positip correlation with the metabolic activity and oxygent consumtion and demand (Hamel et al. 1993). Combination of environment paramater such as

photoperiod, water themperatur and food availability may generate the process of vitelogenesis and gamatogenesis during reproduction cycles of *Holothuria scabra* (Morgan 2000).

Due to this activity is relatifly new in Indonesia especially for sea cucumber *Phylophorus* sp. This is very different from the characteristics of the Holothurian sea cucumber that has been successfully breed in Indonesia such as *Holothuria scabra* (Darsono 1999, Hartati 2001). One, limited reference and biological information regarding *Phylophorus* sp. in Indonesia coupled with a low interest in research on unknown species. Utilization of sea cucumber is still limited as food in the form of chips that are easy to process as well as the demand by the community. However, we think that information related to biological reproduction of *Phylloporus* sp. will be very useful especially to increase the value added of sea cucumber product not only as food but for the pharmaceutical resource.

Conclusion

We can be concluded that thermal stimulation on sea cucumber *Phyllophorus* sp. affected the level of maturity of the gonads and the ideal temperature to give the best effect on the gonad maturation of sea cucumbers *Phyllophorus* sp. was 32°C. The thermal stimulation can be conducted to increase the level of gonad maturity within 60 minutes for stimulation. This research will be helpful for further research on sea cucumber domestication and reproduction biology, so that further related research still needed such as endocrinology during given of the thermal stimulation and stress level.

Acknowledgement

We would like to express our gratitude to the Ministry of Education, through the Directorate General of Higher Education that provides research grants with the scheme of Decentralisation of Higher Education Research 2014. We also grateful for the Indonesian Endowment Fund for Education (LPDP) scholarship under Beasiswa Unggulan Dosen Indonesia-Luar Negeri (BUDI-LN) batch I 2016 (Number: PRJ-3635 /LPDP.3/2016).

References

Agudo N (2006). Sandfish Hatchery Techniques: Secretariat of the Pacific Community.1-43 pp.

Battaglene S (1999). Culture of tropical sea cucumbers for stock restoration and enhancement. Naga, The ICLARM Quarterly 22(4): 4-11.

Battaglene SC, Seymour JE, Ramofafia C and Lane I (2002). Spawning induction of three tropical sea cucumbers, *Holothuria scabra*, *H. fuscogilva* and *Actinopyga mauritiana*. Aquaculture 207(1): 29-47.

Chen J (2003). Overview of sea cucumber farming and sea ranching practices in China. SPC Beche-de-Mer Information Bulletin 18: 18-23.

Conand C (1993). Ecology and reproductive biology of *Stichopus variegatus* an Indo-Pacific coral reef sea cucumber (Echinodermata: Holothuroidea). Bulletin of Marine Science 52(3): 970-981.

Dabbagh AR and Sedaghat MR (2012). Breeding and rearing of the sea cucumber *Holothuria scabra* in Iran. SPC Beche-de-Mer Information Bulletin (32): 49-52.

Darsono P (1999). Perkembangan pembenihan teripang pasir, Holothuria scabra Jaeger, di Indonesia: Oseana. Vol. XXIV (3): 35-45.

Dubois E, Zandbergen M, Peute J and Th HG (2002). Evolutionary development of three gonadotropin-releasing hormone (GnRH) systems in vertebrates. Brain Research Bulletin 57(3-4): 413-418.

Hartati R (2001). Teknologi pembenihan teripang putih (*Holothuria scabra*).

Hamel JF, Himmelman JH and Dufresne L (1993). Gametogenesis and spawning of the sea cucumber *Psolus fabricii* (Duben and Koren). The Biological Bulletin 184(2): 125-143.

Ivy G and Giraspy DAB (2006). Development of large-scale hatchery production techniques for the commercially important sea cucumber *Holothuria scabra* veriscolor (Conand, 1986) in Queensland, Australia. SPC Beche-de-Mer Information Bulletin (24): 28-34.

James D, Gandhi A, Palaniswamy N and Rodrigo JX (1994). Hatchery techniques and culture of the sea-cucumber *Holothuria scabra*. CMFRI Special Publication 57: 1-40.

Kumara PA, Jayanatha J, Pushpakumara J, Bandara W and Dissanayake D (2013). Artificial breeding and larval rearing of three tropical sea cucumber species–*Holothuria scabra*, *Pseudocolochirus violaceus* and *Colochirus quadrangularis*–in Sri Lanka. SPC Beche-de-Mer Information Bulletin 33: 30-37.

Laxminarayana A (2005). Induced spawning and larval rearing of the sea cucumbers, *Bohadschia marmorata* and *Holothuria atra* in Mauritius. SPC Beche-de-Mer Information Bulletin 22: 48-52.

Morgan AD (2000). Aspects of the reproductive cycle of the sea cucumber *Holothuria scabra* (Echinodermata: Holothuroidea). Bulletin of Marine Science 66(1): 47-57.

Mumford SL (2004). Chapter 13 Histology of Finfish. USFWS, Olympia Fish Health Center, Olympia, Washington, 1-12 pp.

Pangkey H, Lantu S, Manuand L and Mokolensang J (2012). Prospect of sea cucumber culture in Indonesia as potential food sources. Journal of Coastal Development 15(2): 114-124.

Pitt R (2001). Review of sandfish breeding and rearing methods. SPC Beche-de-Mer Information Bulletin 14:14-21.

Purnama ER and Winarni D (2017). Fagocytosis of tuberculosis on mice after *Phyllophorus* sp. Treatments as Immunomodulator. BIOMA 1 (2): 105-114.

Purwati P and Luong-Van JT (2003). Sexual reproduction in a fissiparous holothurian species, *Holothuria leucospilota* Clark 1920 (Echinodermata: Holothuroidea). SPC Beche-de-Mer Information Bulletin 12: 11-14.

Ramofafia C, Battaglene S, Bell J and Byrne M (2000). Reproductive biology of the commercial sea cucumber *Holothuria fuscogilva* in the Solomon Islands. Marine Biology 136(6): 1045-1056.

Ramofafia C, Byrne M and Battaglene C (2003). Reproduction of the commercial sea cucumber *Holothuria scabra* (Echinodermata: Holothuroidea) in the Solomon Islands. Marine Biology 142(2): 281-288.

Revianti S, Soetjipto RPR and Parisihni K (2016). Protective role of *Sticophus hermanii* ethanol extract supplementation to oxidative stress and oral hyperkeratosis in smoking exposed rats. International Journal of Chemtech Research 9(05): 408-417.

Tuwo A (1999). Reproductive cycle of the holothurian *Holothuria scabra* in Saugi Island, Spermonde Archipelago, Southwest Sulawesi, Indonesia. INFOFISH International 6(97): 23-29.

Tuwo A (2005). Status of sea cucumber fisheries and farming in Indonesia. FAO Fisheries Technical Paper, 49-56 pp.

Widianingsih W, Zaenuri M, Anggoro S, Kusumaningrum HP and Hartati R (2018). Preliminary study on gonad maturity stages of the sea cucumber *Paracaudina australis* from Kenjeran Water, Surabaya, Indonesia. IOP Conference Series: Earth and Environmental Science, 1-8 pp.

Winarni D, Khaleyla F, Damayanti H, Nugraha YF, Utami CS, Oktafiani D and Hayati A (2015). Reproductive peak, gonadal cycle and gametogenic cells determination of sea cucumber *Paracaudina autralis* from Madura Strait, East Java, Indonesia. International Journal of Academic Research 7(3A): 112-115.

Yudiati E, Hartati R and Suryono CA (2001). Kejut suhu dan salinitas pada spawning teripang hitam *Holothuria edulis* sebagai upaya peningkatan stock alami di perairan jepara, 1-17 pp.

J. Bio-Sci. 23: 47-55, 2015 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

ANTIMICROBIAL ACTIVITY OF *TAMARINDUS INDICA* L. AGAINST BACTERIA CAUSING URINARY TRACT INFECTION

K Biswas and SN Sinha*

Environmental Microbiology Research Laboratory, Department of Botany, University of Kalyani, West Bengal, India

Abstract

Urinary tract infection (UTI) is a common bacterial infection known to affect the different parts of the urinary tract of human which is a great menace for the last decade. Various medicinal plants have been reported for treating infectious diseases including UTI due to their fewer side effects and reduced toxicity. The present study was to investigate the antibacterial activity of *Tamarindus indica* against UTI causing pathogens. Bacteria were isolated from the UTI infected women patients and characterized by using biochemical and morphological methods. Acetonic, methanolic and chloroform leaf extracts were used to evaluate their antibacterial activity against the bacteria isolated from UTI infected patients and the zones of inhibitions were measured. Four bacteria *viz*. *Escherichia coli, Pseudomonas aeruginosa, Klebsiella* sp. and *Enterococcus* sp. were isolated from 60 urine samples infected with UTI. Regarding antibacterial susceptibility test ampicillin showed lowest degree of MIC against *Pseudomonas aeruginosa* and highest degree of MIC against *Klebsiella* sp. Acetone extract of *Tamarindus indica* leaves showed highest antibacterial activity against *E. coli* (22.5 mm). Methanol extract showed highest activity against *Pseudomonas aeruginosa* (21.4 mm) and chloroform extract showed highest activity against *Klebsiella* sp. (6.2 mm). Different phytochemicals found in the plant extract were alkaloids, tannins, flavonoids, sesquiterpenes, carbohydrates, saponins, phlobatannins and anthocyanins.

Key words: Antibacterial activity, *Tamarindus indica,* urinary tract infection

Introduction

Apart from respiratory infections, urinary tract infection (UTI) is most common type of infection which begins in the urinary system. Kidneys, ureters, urinary bladder and the urethra are part of the urinary tract system (Geetha et al. 2011). Women are infected more to UTIs than men for anatomical reasons because woman's urethra is shorter, so bacteria quicker access to the bladder and the urethral opening of women is very close to the anus and vagina for sources of bacteria (Schappert and Rechtsteiner 2008). Nearly 95% of UTI infections are caused by *E. coli* (Kebira et al. 2009). Other microorganisms responsible for UTI infections are *Klebsiella, Pseudomonas, Enterococcus, Enterobacter, Proteus, Staphylococcus, Mycoplasma, Chlamydia, Serratia* and *Neisseria* sp. (Sethi and Gupta 2013). Some potent antibiotics are available for the treatment of UTI, but increasing drug resistance among microbes has made therapy of UTI difficult because bacteria have the genetic potentiality to transfer and acquire resistance to antibiotic, as well as drugs (Srinivasan et al. 2001a).

For the last few decades it is an important job for researchers to find out alternative medicine to prevent UTI infections. Medicines from plant origin have enormous therapeutic potential against microorganisms. Throughout the history, plants have been a valuable resources of medicine (Thomson 1978, Stockwell 1988) and 70-90% of the rural population of the world still use herbal remedies for mental and physical health by

*Author for correspondence: sinhasn62@yahoo.co.in

preventing or treating illness (Lai and Roy 2001). Plants provide a good deal of bioactive components which have benefited human in various ways, including remedy of diseases (Elaine et al. 2002).

Tamarindus indica L. (Tamarind) is a member of the family leguminosae with subfamily caesalpiniaceae (Khanzada et al. 2008). It is found in almost all tropical countries, like India, Africa, Caribbean, South America etc. Tamarind has been used as a medicinal plant from long time; fruits of this plant are the most important part which has often been reported as therapeutic in several pharmacopoeias. The leaves have preventive activity which is due to the presence of polyhydroxylated compounds with majority possessing flavonolic nature (Joyeux et al. 1995). Good levels of fat, protein, fiber and few vitamins such as niacin, thiamine, riboflavin, ascorbic acid and β-carotene are present in the leaves of this plant (El-Siddig et al. 2006). Phenols were, separated from aqueous extract of *T. indica* leaf as active compounds against *B. subtilis* cultures, but not against other microorganisms. On the other hand, the essential oil exhibited a good antimicrobial spectrum when pure but its relative low concentrations in common folk preparations do not allow for any good activity in these extracts (Escalona-Arranz et al. 2010). Ethanolic and aqueous (hot and cold) extracts of *Tamarindus indica leaves* were tested *in vitro* antibacterial activity against 13 Gram negative and 5 Gram positive bacterial strains using agar well diffusion and macro broth dilution techniques, simultaneously. The cold water extract against 95.5% of the test bacterial strains; and the hot water and ethanolic extracts against 90.9% and 86.4%, respectively showed antibacterial activity. The minimum inhibitory concentrations (MIC) ranged from 7.81 mg/mL against *Bacillus subtilis* ATCC 6051 to 31.25 mg/ml against *Escherichia coli* ATCC 11775; and the minimum bactericidal concentration (MBC) ranged from 125 mg/ml against *Pseudomonas aeruginosa* ATCC 10145 to 250 mg/ml against *Bacillus subtilis* ATCC 6051 (Nowdo et al. 2011). Flavanoidal glycosides identified as major compound and using the total ion chromatography (TIC) two major compounds were identified as orientin and vitexin (Gumgumjee et al. 2012). The objective of the present work to isolate bacteria from urinary tract infected patient and also evaluate the antibacterial activity of *T. indica* against isolated UTI pathogens.

Materials and Method

Isolation and identification of bacteria from UTI infected patients

The bacteria present in urine samples of UTI infected women patients were cultured in the HiCrome UTI Agar. It is a differential medium recommended for presumptive identification of microorganisms mainly causing urinary tract infections. The isolated bacterial species was identified by morphological, physiological and biochemical tests (Holt et al. 1994).

Detection of susceptibility to antibacterial agents

From the identified causative agent, highly resistant species was selected by performing antibiotic susceptibility test against 5 different antibiotics that are ampicillin, chloramphenicol, ciprofloxacin, streptomycin and amoxicillin (Doughari 2006). Isolates were inoculated in peptone water and incubated in 37°C for 18-24 h. Next, they were re-cultured in broth and their turbidity compared to 0.5 Mcfarland standard solutions. More ever, new cultures were plated on Mueller-Hinton agar by swabbing. After drying for about 5-10 min, Plates were incubated for about 10-15 min at 37°C. Furthermore, interested antibiotic discs were adjusted on cultured plates using sterile forceps and incubated as inverted for 24 h at 37°C.

Collection of plant materials

Leaves of *Tamarindus indica* were collected from Kalyani, West Bengal, India. Leaves of the plant was washed with running water, dried in shade at room temperature, ground to powder and stored in air tight bag after drying at low temperature.

Preparation of plant extracts

Fifty grams of air-dried leaves of *Tamarindus indica* was grinded to powder and 5 g of plant powder was dissolved in known amount of various organic solvents (chloroform, methanol, acetone and aqueous) and kept for 24 h at room temperature with continuous shaking. The content was filtered to obtain clear decoction. Each preparation was filtered through a Whatman No. 1 filter paper and filtrate evaporated to dryness in a steady air current after which all extracts were stored in a sterile container and stored at room temperature (Saeed and Tariq 2008).

Assaying extracts for antibacterial activity

Antibacterial activity assay of different solvent extracts of the plants were done using the standard agar well diffusion technique. The test bacterial inoculam was standardized by McFarland Nephelometry (NCCLS 1993). The counts of Gram positive bacteria were adjusted to 1.0×10^6 CFU/ml and counts of Gram negative bacteria to 5×10^5 CFU/ml (NCCLS 1993). A 100 µl volume of the standardized test bacterial suspension was seeded and spread uniformly onto each sterile Mueller Hinton agar (MHA) plate so that a confluent growth of bacteria was obtained. The petri dishes were allowed to dry and a sterile 6.0 mm diameter cork borer was used for making wells in the agar plates. The extracts were reconstituted with sterile distilled water to obtain a concentration of 62.5 mg/ml; and 100 µl of this was introduced in wells triplicate on the MHA plates. The plates were allowed to stand for 2 h at room temperature for diffusion and finally incubated at for 24 h at 37°C. The inhibition zone diameter was measured.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration of different extracts of leaf of *T. indica* L. was determined against selected bacteria separately. Concentration ranging from 300 µg/ml to 1 mg/ml of leaf extracts was prepared and 500 µl of each dilution was incubated with 5 ml of Mueller Hinton Broth containing 0.1 ml of bacterial suspension at 37°C for 24 hours. After incubation the tubes were examined for bacterial growth by observing turbidity. The MIC was determined as minimum concentration that showed no visible growth. The experiment was carried out in triplicates. For the macro-broth dilution technique, a 100 µl volume of each dilution of the extract was introduced into duplicate tubes of 2.0 ml Mueller Hinton broth (MHB) seeded with 100 µl of the standardized suspension of the test bacterial strain. Incubation was at 37°C for 24 h; and MIC was taken as the lowest concentration of the extract that made the culture show no visible growth.

The minimum bactericidal concentration was measured by 2 mm diameter agar disc cut out from the inhibition zone of the last three consecutive wells in each dilution showing inhibition was inoculated into a fresh sterile nutrient broth medium. The broth cultures were incubated at 37°C for 24 h after which 100 µl was spread over a fresh sterile MHA. The MHA culture was in turn incubated at 37°C for 24 h and the least concentration of the extract showing no growth was taken as the MBC.

Phytochemical screening

Preliminary phytochemical analysis was carried out using standard protocol for determination of phytoconstituents: alkaloids, tannins, saponins, reducing sugars, anthocyanins, flavonoids, carbohydrates, terpenoids, cardiac glycosides, sesquiterpenes and phlobatannins as directed by references (Harborne 1998).

Results

Sixty urine samples were collected from women patient who are suffered for UTI from JNM Hospital, Kalyani, West Bengal, India. *E. coli* was responsible 39 patient followed by *Pseudomonas aeruginosa* (18.18%) and Klebsiella sp. (11.95%). UTI causing gram positive bacteria, *Enterococcus* sp., is found only in 1 patient out of 60 patients (Fig. 1).

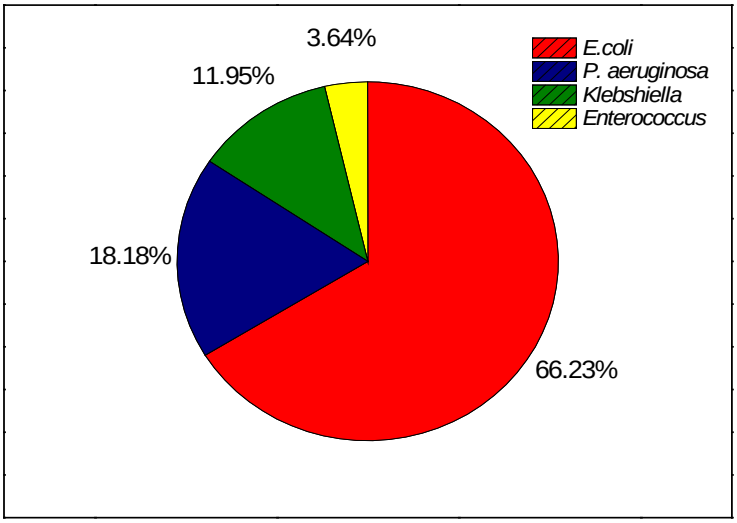


Fig. 1. Distribution of UTI causing pathogens.

All the tested bacteria of same species gave similar characteristics. Urine samples were cultured in UTI agar media. Then it is incubated at 37°C for 24 hrs and isolated by seen their colony characteristics (Table 1).

Table 1. Cultural characteristics of UTI pathogens.

Name of bacteria	Type of growth	Colour of colony
Escherichia coli	Luxuriant	Purple
Pseudomonas aeruginosa	Do	Colourless
Klebsiella sp.	Do	Blue to purple, mucoid
Enterococcus sp.	Do	Blue, small

All of the presumptive isolates were sub-cultured and assessed for their morphological, physiological and biochemical characteristics (Table 2).

Table 2. Identification of isolated microbe using biochemical test.

Tests/Characteristics	*Escherichia coli*	*Pseudomonas aeruginosa*	*Klebsiella* sp.	*Enterococcus* sp.
Cell shape	Rods	Rods	Rods	Coccus
Gram reaction	-	-	-	+
Motility	+	+	-	-
Lipid hydrolysis	-	+	+	+
Starch hydrolysis	-	+	-	+
Casein hydrolysis	-	-	-	+
Catalase test	+	+	+	-
Oxidase test	-	+	-	-
Urease test	-	-	+	-
Growth on TSI	+	-	-	+
Nitrate reduction test	+	+	+	+
Indole production test	+	-	-	+
Methyl red test	+	+	-	-
Voges-proskauer test	-	-	+	+
Citrate utilization test	-	+	+	+
Carbohydrate utilization tests				
Glucose	+	+	+	+
Fructose	+	+	+	+
Lactose	+	-	+	+
Mannitol	+	-	+	+
Sorbitol	-	-	+	+

- Indicates negative results and + indicates positive results

Based on the antimicrobial susceptibility test, ampicillin showed lowest degree of MIC value against *Pseudomonas aeruginosa* but highest degree of MIC value against *Klebsiella* sp (800 μg/ml). In the other hand, *E. coli* was inhibited by ciprofloxacin (300 μg/ml). In case of streptomycin showed highest inhibition activity against *Klebsiella* sp (300 μg/ml). *Enterococcus* sp is inhibited by chloramphenicol (Table 3).

Table 3. Antimicrobial susceptibility test against isolated pathogens.

Antibiotics	MIC value of tests bacteria (µg/ml)			
	Escherichia coli	*Pseudomonas aeruginosa*	*Klebsiella* sp.	*Enterococcus* sp.
Ampicillin	500	50	800	500
Amoxicillin	500	500	800	500
Chloramphenicol	500	800	500	300
Ciprofloxacin	300	800	500	800
Streptomycin	500	500	300	800

Leaves of *Tamarindus indica* was extracted for evaluation of antimicrobial activity against UTI pathogens. Antibacterial activity of different solvent extract of *T. indicus* leaves on the different bacteria is given in Table 4. Results showed that Acetone extracts has greater antibacterial activity among all the extracts its maximum value of the zone of the inhibition is noted against *E. coli (*22.5 mm). Only methanol extracts of leaves showed activity against *P. aeruginosa* (14.7 mm). Chloroform extracts was showed activity against *Klebsiella* sp. (6.2 mm).

Table 4. Diameter of zone of inhibition of various solvent extracts of *Tamarindus indica* leaves shown against UTI causing bacteria.

Tested bacteria	Zone of inhibition (mm)			
	Chloroform	Methanolic	Acetone	Aqueous
Escherichia coli	0	0	22.5	10
Pseudomonas aeruginosa	0	14.7	0	8
Klebsiella sp.	6.2	17.5	18	0
Enterococcus sp.	0	21.4	16	0

Fig. 2a. Zone of inhibition of acetone extracts of *T. indica* leaves; Fig. 2b. Zone of inhibition of methanol extracts of *T. indica* leaves.

MIC value of plant extracts of different solvents is showed in Fig. 3. Highest MIC value of acetone extracts of the plant is showed 1000 µg/ml against *Klebsiella* sp. Lowest degree of MIC value of methanol extracts is showed against *Enterococcus* sp.

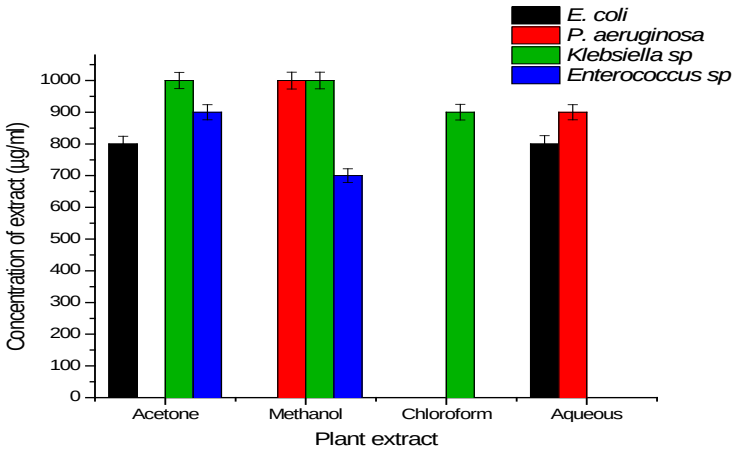


Fig. 3. MIC value of plant extracts.

Phytochemical constituents present in the plant extract included alkaloids, tannins, flavonoids, sesquiterpenes, carbohydrates, saponins, phlobatannins and anthocyanins (Table 5).

Table 5. Phytochemical screening of *T. indicus* leaves extract.

Secondary metabolites	Methanolic leaves extracts
Alkaloids	+
Tannins	+
Steroids	-
Cardiac glycosides	-
Flavonoids	+
Terpenoids	-
Sesquiterpenes	+
Carbohydrates	+
Saponins	+
Phlobatannins	+
Anthocyanins	+

Discussion

Bioactive constituents such as flavonoids, alkaloids, tannins and several other aromatic compounds serve as defense mechanisms in the plant against microorganisms (Lutterodt et al. 1999, Bonjar et al. 2004). The presence of antibacterial activity against Gram positive as well as Gram negative bacteria might be indicative of the occurrence of broad spectrum antibiotic compounds (Srinivasan et al. 2001b, Vaghasiya and Chanda 2007). This will be advantageous to fight the menace of antibiotic refractive pathogens which are very much prevalent in recent times. The results showed that acetone extracts of leaves are more effective both Gram positive bacteria and Gram negative bacteria. Different organic solvents have been reported to have the capability to extract different phytochemicals based on their polarity or solubility in the solvent (Marjorie 1999). Same results had been reported by Doughari (2006). Acetone extracts of leaves might have higher solubility for more phytochemicals, consequently the highest antibacterial activity. The occurrence of antimicrobial activity by aqueous extracts provides the scientific basis for using this plant in the traditional treatment of diseases. The highest MIC values of *Enterococcus* sp. is an indication that either the methanol plant extracts are more effective on Gram positive bacteria or that the organism possesses the capability of acquiring antibiotic resistance, while the low MIC values for other bacteria indicates the potentiality of the plant extracts.

Conclusion

From the above results we can conclude that plants have remarkable antimicrobial activity as compare to antibiotic activity. Organisms are gaining resistance day by day towards the antibiotics, so that some natural product should be needed to overcome these antibiotic resistant organisms. Moreover plants have no side effect. The antibacterial activity of leaf extract of *T. indica* might help to discover new antimicrobial phytochemical which might serve as selective agents for controlling infectious diseases. This work has opened up the possibility of using this plant in the treatment of urinary tract infections in near future. However, the chemical nature of bioactive compounds and their further purification needs to be carried out.

Acknowledgement

This project is supported by a SoRF fellowship internship mode (NASI/SoRF-I/2014-2015/70) from Department of Science and Technology, Govt. of India, New Delhi. We are grateful to Prof. Chandan Sengupta, Head, Department of Botany, University of Kalyani for providing facilities to perform this work.

References

Bonjar GHS, Aghighi S and Nik AK (2004). Antibacterial and antifungal survey in plants used in indigenous herbal medicine of south east regions of Iran, Journal of Biological Science 4(3): 405-412.

Doughari JH (2006). Antimicrobial activity of *Tamarindus indica* Linn, Tropical Journal of Pharmaceutical Research 5(2): 597-603.

El-Siddig K, Gunasena HPM, Prasad BA, Pushpakumara DK, Ramana KVR, Vijayanand P and Williams JT (2006). Tamarind. *Tamarindus indica.* Southampton, UK.

Escalona-Arranz JC, Péres-Roses R, Urdaneta-Laffita I, Camacho-Pozo MI, Rodríguez-Amado J and Licea-Jiménez I (2010). Antimicrobial activity of extracts from *Tamarindus indica* L. leaves, Pharmacognosy Magazine 6(23): 242-247.

Geetha RV, Roy A and Lakshmi T (2011). Nature's weapon against urinary tract infections, International Journal of Drug Development and Research 3(3): 85-100.

Gumgumjee N, Khedr A and Hajar A (2012). Antimicrobial activities and chemical properties of *Tamarindus indica* L. leaves extract, African Journal of Microbiology Research 6: 6172-6181.

Harborne JB (1998). *Phytochemical methods - a guide to modern techniques of plant analysis.* Chapman and Hall, London.

Holt JG, Krieg NR, Sneath PHA, Stanley JT and Williams ST (1994). Bergey's Manual of Determinative Bacteriology. 9th ed., Williams and Wilkins, Baltimore, USA.

Joyeux M, Mortier F and Flurentin J (1995). Screening of antiradical, antilipoproxidant and hepatotoprotective effects of nine plant extracts used in Caribbean folk medicine, Phytotherapy Research 9: 228-230.

Kebira AN, Ochola P and Khamadi SA (2009). Isolation and antimicrobial susceptibility testing of *Escherichia coli* causing urinary tract infections, Journal of Applied Bioscience 22: 1320-1325.

Khanzada SK, Shaikh W, Sofia S, Kazi TG, Usmanghani K, Kabir A and Sheerazi TH (2008). Chemical constituents of *Tamarindus indica* L. medicinal plant in Sindh, Pakistan Journal of Botany 40(6): 2553-2559.

Lai PK and Roy J (2004). Antimicrobial and chemopreventive properties of herbs and spices, Current Medicinal Chemistry 11(11): 1451-1460.

Lutterodt GD, Ismail A, Basheer RH and Baharudin HM (1999). Antimicrobial effects of *Psidium guajava* extracts as one mechanism of its antidiarrhoeal action, Malaysian Journal of Medical Science 6(2): 17-20.

Marjorie MC (1999). Plant products as antimicrobial agents, Clinical Microbiology Review 12(4): 564-582.

National Committee for Clinical Laboratory Standards (1993). Methods for dilution in antimicrobial susceptibility tests: approved standard M2-A5, National Committee for Clinic: Laboratory Standards, Vilanova, P.A.

Nwodo UU, Obiiyeke GE, Chigor VN and Okoh AI (2011). Assessment of *Tamarindus indica* extracts for antibacterial activity, International Journal of Molecular Sciences 12(10): 6385-6396.

Saeed S and Tariq P (2008). *In vitro* antibacterial activity of clove against Gram negative bacteria, Pakistan Journal of Botany 40(5): 2157-2160.

Schappert SM and Rechtsteiner EA (2008). Ambulatory medical care utilization estimates for 2006. National Health Statistics Reports; No. 8. Hyattsville, MD: National Center for Health Statistics.

Sethi S and Gupta S (2013). A review: urinary tract infection and its treatment naturally, Phytomedicine 1(1): 34-39.

Souza-Fagundes EM, Queiroz AB, Martins Filho OA, Gazzinelli G, Corrêa-Oliveira R, Alves T and Zani CL (2002). Screening and fractionation of plant extracts with antiproliferative activity on human peripheral blood mononuclear cells, Memórias do Instituto Oswaldo Cruz 97(8): 1207-1212.

Srinivasan D, Nathan S, Suresh T and Lakshmanaperumalsamy P (2001a). Antibacterial activity of neem (*Azadirachta indica*) and tamarind (*Tamarindus indica*) leaves. Asian Journal of Microbiology Biotechnology 3(1): 67-73.

Srinivasan D, Nathan S, Suresh T and Perumalsamy PL (2001b). Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine, Journal of Ethnopharmacology 74(3): 217-220.

Stockwell C (1988). Nature's Pharmacy, Century Hutchinson Ltd., London, UK.

Thomson WAR (1978). Medicines from the earth, McGraw-Hill Book Co. Maidenhead, UK.

Vaghasiya Y and Chanda SV (2007). Screening of methanol and acetone extracts of fourteen Indian medicinal plants for antimicrobial activity, Turkish Journal of Biology 31: 243-248.

J. Bio-Sci. 23: 57-65, 2015 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

USE OF DIFFERENT NON-CHEMICAL METHODS FOR THE MANAGEMENT OF ADULT *CALLOSOBRUCHUS MACULATUS* (F.) (COLEOPTERA: BRUCHIDAE) IN STORED CHICKPEA

SHA Mahdi*, M Hasan, I Mahfuz and M Khalequzzaman

Department of Zoology, University of Rajshahi, Bangladesh

Abstract

The efficiency of physical factors (dry heat, low temperature and UV-radiation), leaf powders of Neem (*Azadirachta indica* A. Juss.), Bichuti (*Flacourtia* indica Merr.) and Parthenium weed (*Parthenium hysterophorus* L.) and two insecticides (Salvo and Amithrin plus) was evaluated against *Callosobruchus maculatus* (F.). For dry heat treatment, the calculated LD_{50} values for *C. maculatus* were 56.92, 54.26, 52.27, 50.76, 49.09, 50.55 and 29.59°C after 50, 60, 70, and 80 minutes, 24, 36, 48 h of treatment respectively, and the LD_{50} values were 58.61 and 5.18°C at 1 and 2 h for low temperature treatment respectively. LT_{50} values of the treatment of UV-radiation were 48.63, 29.89, 17.54, 11.11, 5.93 and 1.35 °C for the exposure period of 24, 36, 48, 60, 72 and 48 h respectively. The calculated LD_{50} values of leaf powder were 3.38, 3.15, 2.88, 2.45 and 2.40 mg cm^{-2} for *A. indica*, 3.91, 3.80, 3.55, 3.30, and 3.08 for *F. indica*, 12.11, 4.35, 1.86, 1.49, and 1.36 for *P. hysterophorus* after 12, 24, 36, 48 and 60 h of treatment respectively. The calculated LD_{50} values were 0.64, 0.33, 0.23, 0.04 and 0.008 mg cm^{-2} for Salvo and 0.35, 0.23, 0.09, 0.08 and 0.01 mg cm^{-2} for Amithrin plus at 12, 24, 36, 48 and 60 h respectively. The order of effectiveness of physical factors was dry heat >low temperature >UV-radiation. On the other hand, the order of toxicity of plant powders was *F. indica* >*A. indica* >*P. hysterophorus*. In case of insecticides it was Amithrin plus >Salvo. The findings suggest that physical factors and plant leaf powders can be used in integration with other bio rational approaches.

Key words: *C. maculatus*, dry heat, low temperature, non-chemical methods, plant powders, toxicity, UV-radiation

Introduction

Callosobruchus maculatus (F.) (Coleoptera: Bruchidae) is a major pest of several stored pulses and usually found in Southeast Asia including Bangladesh, and the loss caused to stored grain was 55-69% weight loss and 45.6-66.3% loss in protein substance for chickpea (Alam 1971, Gugar and Yadav 1978). Pest management through temperature application (dry heat and low temperature) is receiving renewed interest as a non-chemical method with lack of residue problem (Hallman and Denlinger 1999). It is also one of the most promising bio-rational insect management tools for farm stored grain and grain processing industries (Fields 1992, Dosland et al. 2006, Phillips and Throne 2010). Low temperature control is also currently used along with other pest management techniques to kill insects within a store. Low temperatures have been used to successfully control insect populations in the fur and food industries for over a century. There are a few studies that have examined the management of bruchids by dry heat and low temperatures (Hallman and Denlinger 1999, Dosland et al. 2006).

The possible use UV-radiation as an alternative treatment method in storage premises was used in the laboratory. Experiments with UV-rays, for the management of coleopteran pests have shown to be very promising. Calderon et al. (1985) reported that the egg-hatching in *Tribolium castaneum* was negatively

*Author for correspondence: mahdi_ru@yahoo.com

affected by UV-radiation, whereas Sharma and Dwevedi (1997) observed adverse influences of UV-rays on the egg-to-adult development of *C. chinensis* L. Many of the plants use as protectants have a strong smell whichrepels or kills insect. Plant powders reduce oviposition in bruchids under laboratory conditions (Iqbal and Poswal 1995). The plants, *A. indica* kernel powder, *Tridax procumbens* (L.) and *A. squamosa* seed powder control *C. chinensis* and *C. maculatus* (Ali et al. 1981, Sowunmi and Akinnusi 1983, Bhaduri et al. 1985). *A. indica* seed kernel applied to pea seeds reduced damage by *C. chinensis* over a three month storage period by reducing F1 adult progey (Kumari et al. 1990). The efficacy of diatomaceous earth in mixed formulation with other dusts and an insecticide against *C. chinensis* and *C. maculatus* was reported by Mahdi and Khalequzzaman (2012).

Different non chemical control methods against pulse beetle were thus evaluated. The present research was designed to find out the potentiality of some non-chemical methods *viz.*, dry heat, low (cold) temperature, UV-radiation, leaf powders of *Azadirachta indica* A. Juss., *Flacourtia indica* Merr. and *Parthenium hysterophorus* L. and two insecticides as reference for the control of *C. maculatus*.

Materials and Methods

Test insect: *C. maculatus* used in the experiments was collected from a private store house of Rajshahi, Bangladesh. The cultures were maintained in the Crop Protection and Toxicology Laboratory, Department of Zoology, University of Rajshahi. For continuous and huge supply of the beetles, mass cultures were maintained in earthen pots (3000 ml) and sub-cultures in glass jars (500 ml) or beakers (500 ml) with the food medium.

Food medium: The chickpea, *Cicer arietinum* seeds were used as food medium for *C. maculatus* throughout the experiment. The seeds were kept in an oven and/or incubator for sterilization, about 24 h at 60°C to disinfest them. Then the seeds were thoroughly washed with tap water to remove dusts and carefully sun-dried having 13-14% moisture content. The sterile foods were then preserved in air tight glass jars (1000 ml) in order to impede further infestation.

Dry heat: One hundred chickpea seeds and 30 adults (1-2 days old) of *C. maculatus* were kept in a petri dishes (90 mm) in the dry air oven at 50, 55 and 60±1°C for exposure periods of 50, 60, 70 and 80 minutes, 24, 36 and 48 h for dry heat treatment. The treated and control batch at 29±1°C were maintained in 3 replications. Adult mortality was recorded after the exposure period of each treatment.

Low temperature: One hundred chickpea seeds and 30 adults of *C. maculatus* (1-2 days old) in a petri dish (90 mm) were exposed to low temperatures at 5, 1 and –4±1°C for 1 and 2 h of treatment while control was kept at 29±1°C. Three replicated trials were made, and adult mortality was recorded after each exposure period.

Ultra-violet radiation: A 15W germicidal lamp (GE1578) that emitted a wavelength of 254 nm (1 nmb = 1× 10-9 m) and installed at the Genetics and Molecular Biology Laboratory, Department of Zoology, Rajshahi University, was used as a source of UV radiation. Time-mortality response tests were conducted at a series of irradiation exposure periods *viz*., 5, 10, 15, 20 and 25 minutes. For irradiation, 1-2 day old 30 *C. maculatus* were kept in each 90 mm petri dishes, and placed on table surface 12 cm below the lamp at above time periods. Petri dishes were then exposed to UV-rays for estimating their respective mortalities at 24, 36, 48, 60, 72 and 84 h after post-irradiation. The same number of non-irradiated insects was maintained as controls in room temperature in three replications.

Plant leaf powders: Fresh leaves of Neem (*Azadirachta indica* A. Juss.), Bichuti (*Flacourtia indica* Merr.) and Parthenium weed (*Parthenium hysterophorus* L.) were collected from the surroundings of the campus of

University of Rajshahi, Bangladesh. Afterwards they were washed in running water. The plant materials were kept in shade for air-drying and then dried at room temperature (25 - 35°C) until they become crisp dry. Powdered samples were prepared by pulverizing the dried leaves using a blender. All the plant materials were sieved repeatedly to obtain the fine dust particles. The ground samples were passed through a 25 mesh sieve to obtain fine and uniform dust. The resulting dusts were used as direct admixture to the chickpea seeds at different doses. The dust was preserved in airtight condition in polythene bags till their use. Plant leaf powder was tested individually by mixing in w/w with 10 g of chickpea seed at the doses of 2.594, 2.672, 2.750, 2.830, and 2.908 gm cm^{-2} for Neem, 3.301, 3.379, 3.458, 3.537 and 3.615 gm cm^{-2} for Bichuti, and 1.415, 1.493, 1.572, 1.650 and 1.730 gm cm^{-2} for *Parthenium* weed. Three replications and a control (without insecticide) batches were made and 30 adult beetles were released in each petri dishes. The mortality of the beetles was recorded after 12, 24, 36, 48 and 60 h of treatment.

Dust formulation insecticides: The commercial dust formulation "Salvo 20 sp" and "Amithrin plus 3% WDG" of Bayer Crop Science, Germany was used. Insecticide was tested individually by mixing in w/w with 10 g of chickpea seed at the doses of 0.031, 0.049, 0.063, 0.079 and 0.094 gm cm^{-2}, 0.094, 0.129, 0.157, 0.289 and 0.220 gm cm^{-2} for Salvo and Amithrin plus respectively. Three replications and a control (without insecticide) batches were made and 30 adult beetles were released in each petri dishes. The mortality of the beetles was recorded after 12, 24, 36, 48, and 60 h of treatment.

Analysis of the data: The mortality percentage was corrected using Abbott's formula (Abbott 1925, Busvine 1971): where, $Pt = [(Po - Pc) / (100 - Pc)] \times 100$, where Pt is the corrected mortality (%), Po is the observed mortality (%) and Pc is the control mortality (%). The observed data were then subjected to probit analysis according to Finney (1947) and Busvine (1971) LT_{50} for lethal temperature and lethal period, and LD_{50} for lethal dose were considered through the experiment. Heterogeneity is tested by chi-squared test.

Results

Effect of dry heat, low temperature and UV-radiation

The maximum LT_{50} was 56.95°C at 50 minutes while the minimum LT_{50} was 29.59°C at 48 h of treatment period for dry heat treatment to *C. maculatus* (Table 1). For low temperature treatment, the minimum LT_{50} was 5.18°C at 2 h; however, the maximum LT_{50} was not effective at 1 h treatment period (Table 1). For UV-radiation, the maximum LT_{50} was 48.63 minutes at 24 h while the minimum LT_{50} was 1.35 minutes at 84 h after post-irradiation to *C. maculatus* (Table 1). The result shows that the LT_{50} values of dry heat, low temperature and UV-radiation were decreased gradually with the increase of treatment period. Table 1 also shows the results of 95% confidence limits, regression equations (Y) and χ^2 of dry heat, low temperature treatment and UV-radiation on *C. maculatus*. The order of effectiveness of physical factors was dry heat > low temperature >UV-radiation.

Effect of plant leaf powders

The maximum LD_{50} was 3.38, 3.91and 12.11 mg cm^{-2} at 12 h while the minimum LD_{50} was 2.40, 3.08 and 1.36 mg cm^{-2} at 60 h of treatment period for leaf powders of *A. indica*, *F. indica* and *P. hysterophorus* respectively (Table 2). The result shows that these plant powders were effective against this test insect. With the increasing treatment period, the LD_{50} values of leaf powders were reduced remarkably. The results of LD_{50} (mg cm^{-2}), 95% confidence limits, regression equations (Y) and χ^2 of two insecticides against *C. maculatus* are presented in Table 2. The order of toxicity of plant powders was *F. indica* >*A. indica* >*P. hysterophorus*.

Table 1. LT_{50}, 95% confidence limits and regression equations of physical factors (dry heat, low temperature and UV-radiation) to adult *C. maculates.*

Treatment period	LT_{50} (°C)	95% confidence limits		Regression equation	χ^2 (df)
		Lower	Upper		
Dry heat					
50 min	56.92	55.29	58.59	Y = –39.45881 + 25.33171X	0.33 (1)
60 min	54.26	52.66	55.90	Y = –32.38392 + 21.53968X	2.57 (1)
70 min	52.27	50.64	53.95	Y = –34.25336 + 22.85801X	0.93 (1)
80 min	50.76	49.21	52.36	Y = –41.04586 + 27.00517X	0.57 (1)
24 h	49.09	42.38	56.87	Y = –11.82663 + 9.939746X	2.70 (1)
36 h	50.55	35.96	71.05	Y = –2.861599 + 4.614365X	1.15 (1)
48 h	29.59	18.87	46.40	Y = –1.1125 + 4.154708X	0.42 (1)
Low temperature					
1 h	58.61[ne]	5.02	6.84	Y = 3.778285 + 0.6910058X	31.39 (1)
2 h	5.18	2.53	10	Y = 4.630652 + 0.5164818X	67.26 (1)
UV-radiation					
24 h	48.63	16.17	146.22	Y = 3.40967 + 1.800528X	0.12 (2)
36 h	29.89	16.09	55.51	Y = 3.842401 + 1.722514X	4.69 (2)
48 h	17.54	13.79	22.30	Y = 1.841425 + 2.192907X	1.87 (3)
60 h	11.11	7.99	15.44	Y = 3.121617 + 1.511519X	4.78 (3)
72 h	5.93	3.27	10.78	Y = 3.694252 + 1.343494X	1.49 (3)
84 h	1.35	1.38	8.17	Y = 3.775727 + 1.686104X	1.29 (1)

ne: not effective

Effect of two insecticides

The maximum LD_{50} was 0.64 and 0.35 mg cm^{-2} at 12 h while the minimum LD_{50} was 0.008 and 0.01 mg cm^{-2} at 60 h of treatment period for insecticide Salvo and Amithrin plus respectively (Table 3). The result shows that the LD_{50} values of insecticides were decreased progressively with the increase of treatment period. Table 3 also shows the results of 95% confidence limits, regression equations (Y) and χ^2 of two insecticides against *C. maculatus*. In case of insecticides, the order of toxicity was Amithrin plus >Salvo.

Table 2. $LD_{50,}$ 95% confidence limits and regression equations of three leaf powders to adult *C. maculates.*

Leaf powders	Treatment period (h)	LD_{50} (mg cm^{-2})	95% confidence limits		Regression equation	χ^2 (df)
			Lower	Upper		
	12	3.38	16.17	146.22	Y = 3.40967 + 1.800528X	0.12 (2)
	24	3.15	16.09	55.51	Y = 3.842401 + 1.722514X	4.69 (2)
A. indica	36	2.88	13.79	22.30	Y = 1.841425 + 2.192907X	1.87 (3)
	48	2.45	7.99	15.44	Y = 3.121617 + 1.511519X	4.78 (3)
	60	2.40	3.27	10.78	Y = 3.694252 + 1.343494X	1.49 (3)
	12	3.91	4.38	146.22	Y = -7.750948 + 21.49975X	1.58 (3)
	24	3.80	4.21	55.51	Y = -4.006943 + 15.50508X	7.21 (3)
F. indica	36	3.55	3.72	22.30	Y = -2.303605 + 13.25912X	4.97 (3)
	48	3.30	3.49	15.44	Y = -2.328491 + 14.10567X	0.26 (3)
	60	3.08	3.56	10.78	Y = -1.06144 + 12.38746 X	0.52 (3)
	12	12.11	2.66	55	Y = 3.338045 + 1.534327 X	2.47 (3)
	24	4.35	3.26	57	Y = 4.037036 + 1.507658 X	4.51 (3)
P. hysterophorus	36	1.86	0.90	3.86	Y = 4.538531 + 1.70042 X	1.77 (3)
	48	1.49	1.35	1.65	Y = 4.024939 + 5.600217 X	1.05 (3)
	60	1.36	1.20	1.55	Y = 3.897927 + 8.102146 X	2.63 (3)

Table 3. $LD_{50,}$ 95% confidence limits and regression equations of two insecticides to adult *C. maculates.*

Insecticides	Treatment period (h)	LD_{50} (mg cm^{-2})	95% confidence limits		Regression equation	χ^2 (df)
			Lower	Upper		
	12	0.64	0.01	29.96	Y = 3.372366 + .8990858 X	1.77
	24	0.33	0.02	4.54	Y = 3.635021 + .894236 X	0.35
Salvo	36	0.23	0.01	4.53	Y = 4.173747 + .6000106 X	3.60
	48	0.04	0.02	0.07	Y = 4.315999 + 1.023755 X	1.27
	60	0.008	0.0004	0.18	Y = 5.04595 + .7742266 X	0.41
	12	0.35	0.15	0.77	Y = 2.024741 + 1.925898 X	0.72
	24	0.23	0.15	0.35	Y = 2.24921 + 2.007077 X	0.52
Amithrin plus	36	0.09	0.04	0.20	Y = 3.927226 + 1.079516 X	0.36
	48	0.08	0.04	0.15	Y = 3.483278 + 1.667075 X	1.61
	60	0.01	0.0006	0.55	Y = 4.686913 + 1.087835 X	0.01

Discussion

Temperature is one of the principal factors delimitating survival and reproduction of insects. The application of hot air is an easy, simple and environment friendly method in grain processing industries. Pest management through temperature manipulation is receiving renewed interest as a non-chemical method with lack of residue problem (Hallman and Denlinger 1999). The dry heating of chickpea for 10 minutes at 120°C is used as one of the methods for reducing the anti-nutritional factors and reduced 46% of α-galactosides and 27% of tripsin inhibitor activity (Frias et al. 2000). Adult mortality of *C. maculatus* increased with increased duration of solarisation (exposure period to sun) in Nigeria (Lale 1998). The maximum LT_{50} rate was 56.92°C at 50 minutes and minimum was 29.59°C at 48 h in this experiment agrees with the report of the previous works.

Mullen and Arbogast (1979) investigated the time-mortality relationships for eggs of five species of store-product insects, and found that the *C. maculatus* eggs to be among the most cold tolerant, with LD_{50} values of 2.7, 1.3 and 0.3 h at -10, -15 and -20°C, respectively. The efficacy of microwave radiation and cold storage on *T. castaneum* Herbst and *Sitophilus oryzae* L. was also examined by Gasemzadeh et al. (2010). There was complete egg mortality after 14 days of cold storage (-18°C) and highest survival of eggs located at the centre of the bin. The present finding indicates that LT_{50} value of low temperature was 5.18°C at 2 h. The present study also suggested the temperature-exposure time combinations required for the control of *C. maculatus* in chickpea, which are sometimes different than previous studies.

Faruki et al. (2007) noted that UV treatment on the *T. castaneum*, *T. confusum* and the almond moth *Cadra cautella* decreased egg-hatching and reduced adult emergence. The growth and development in the lesser mealworm *Alphitobius diaperinus* has been shown to be manipulated by UV treatments on eggs, larvae, pupae and adults by a number of workers (Parween et al. 2004, Faruki et al. 2005, Begum et al. 2007). These results nicely corroborate with the findings of the present study. Adult mortality and reduced longevity in the UV irradiated insects might result from structural changes in the haemolymph as well as reduction in the total haemolymph count as demonstrated in the flesh fly *Parasarcophaga ruficornis* by Krishna and Srivastava (1991). The most likely explanation of the UV irradiation effects on the adult insects is that UV-C at 254 nm causes adjacent thymine (T) molecules of the DNA strands to dimerize, and further accumulation of these defects inhibits DNA replication, thereby rendering its harmful impacts on the exposed organism (Allen 2001). The present study thus clearly demonstrated the UV irradiated adult time-mortality response.

Pandey and Singh (1995) found that seeds of black gram could be effectively protected from damage by *C. chinensis*, by mixing the seed with dried powder of *A. indica* leaves at a rate of 100 - 400 mg $^{-50\ gm}$ seed. Rajapakse et al. (1998) observed that *A. indica* gave significant reduction of oviposition and adult emergence of *C. maculatus*. The leaf powder of *A. indica, V. negundo* and *P. hydropiper* and their combinations were tested against *C. chinensis* on *Lens esculenta* seeds by Rouf et al. (1996) who reported that *P. hydropiper* leaf powder at $4g^{-50g}$ lentil seeds was the most effective in reducing oviposiotion and adult emergence of *C. chinensis*. Lakshmi and Venugopal (2000) tested six plant products *viz.*, *V. negundo, A. squamosa* (leaf and seed). *Annona calamus, Curcuma longa* L., *A. indica* seed kernel dusts for their effectiveness against *C. maculatus* in chickpea seeds. It has also been reported that powders of clove and black pepper were most effective within eleven spices powders on *C. maculatus* in black gram seeds (Mahdi and Rahman 2008).

Pulse beetles are not advisable to mix insecticides with food grains. Nevertheless, the potential hazards for mammals from synthetic insecticides and the increase of insect resistance to pesticides has led to explore for new classes of insecticides with lower mammalian toxicity and a lower persistence in the environment (Roger and Hamraoui, 1993). In this experiment, the maximum LT_{50} was 3.38, 3.91 and 12.11 mg cm^{-2} and minimum LT_{50} was 2.40, 3.08 and 1.36 mg cm^{-2} for Neem (*A. indica*), Bichuti (*F. indica*) and Parthenium

weed (*P. hysterophorus*) respectively. The findings of the present study also confirmed the toxic effects of leaves powders of *A. indica*, *F. indica* and *P. hysterophorus* and two insecticides Salvo and Amithrin plus (used as reference) on adult mortality of *C. maculatus* as admixtures in pest management strategies, especially by small scale farmers who store small amounts of pulses for consumption and planting.

These results concluded that LT_{50}/ LD_{50} values were dose and exposure time dependent. Mortality of pulse beetle was increased with the increase in exposure period which may clear the dose-time-mortality relationship in different treatment methods. These data also indicate the potential use of different non-chemical methods for the management of *C. maculatus* in stored chickpea. These physical factors and plant products can be used in integration with other bio-rational approaches. However, further research may be required on these aspects.

Acknowledgment

Thanks are due to Professor Dr. ASM Shafiqur Rahman, Department of Zoology, University of Rajshahi for help in preparation of the manuscript and to the Chairman, Department of Zoology, University of Rajshahi, Bangladesh for providing laboratory facilities.

References

Abbott WS (1925). A method of computing the effectiveness of an insecticide, Journal of Economic Entomology 18: 265-267.

Alam MZ (1971). Pests of stored grain and other stored products and their control. Agricultural Information Service 3 R. K. Mission Road, Dhaka, Bangladesh. 361 pp.

Ali SI, Rawat RR and Sing OP (1981). Effectiveness of various forms of caused apple seed powder as seed protectant against the pulse beetle *Callosobruchus chinensis* (Linn.), Bulletin of Grain Technology 19(1): 53-56.

Allen J (2001). Ultraviolet radiation, how it affects life on earth. Earth Observatory, NASA, USA.

Begum M, Parween S and Faruki SI (2007). Combined effect of UV-radiation and triflumuron on the progeny of *Alphitobus diaperinus* (Panzer) (Coleoptera: Tenebrionidae) at different storage period, University Journal of Zoology Rajshahi University 26: 45-48.

Bhaduri N, Ram S and Patil BD (1985). Evaluation of some plant extracts as protectants against the pulse beetle *Callosobruchus maculatus* (F.) infesting cowpea seed, Journal of Entomological Research 9: 183-187.

Busvine JR (1971). A critical review of the techniques for testing insecticides. Commonwealth Agricultural Bureau, London 345 pp.

Calderon M, Bruce WA and Leesch LG (1985). Effect of UV radiation on eggs of *Tribolium castaneum*, Phytoparasitica 13: 179-244.

Dosland O, Subramanyam BH, Sheppard K and Mahroof R (2006). Temperature modification for insect control. *In*: Heaps J (Ed.), Insect Management for Food Storage and Processing. American Association for Cereal Chemistry, St. Paul, MN, 89-103 pp.

Faruki SI, Das DR and Khatun S (2005). Effect of UV-radiation on the larvae of the lesser mealworm, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) and their progeny, Journal of Bio-Science 5(4): 444-448.

Faruki SI, Das DR, Khan AR and Khatun M (2007). Effects of ultraviolet (254nm) irradiation on egg hatching and adult emergence in the flour beetles, *Tribolium castaneum*, *T. confusum* and the almond moth *Cadra cautella*, Journal of Insect Science 7: 1-6.

Fields PG (1992). The control of stored-product insects and mites with extreme temperatures, Journal of Stored Products Research 28: 89-118.

Finney DJ (1947). Probit Analysis: a statistical treatment of sigmoid response curve. Cambridge University Press, London, 333 pp.

Frias J, Vidal-Valverde C, Sotomayor C and Diaz-Pollan C (2000). Influence of processing on available carbohydrate content and anti-nutritional factors of chickpeas, European Food Research and Technology 210: 340-345.

Gasemzadeh S, Pourmirza AS, Safarligadeh MS and Maroufpoor M (2010). Effect of microwave radiation and cold storage on *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) and *Sitophilus oryzae* L. (Coleoptera: Curculionidae), Journal of Plant Protection Research 50(2): 140-145.

Gugar GT and Yadav TD (1978). Feeding of *Callosobruchus maculatus* and *Callosobruchus chinensis* Linn. in green gram, Indian Journal of Entomology 40: 108-112.

Hallman GJ and Denlinger DL (1999). Temperature sensitivity and integrated pest management. *In*: Hallman GJ and Denlinger DL (Eds.), Temperature sensitivity in insects and application in integrated pest management. West View Press, Boulder, Colorado, 1-5 pp.

Iqbal J and Poswal MAT (1995). Evaluation of certain spices for the control of *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchidae) in cowpea seeds, African Entomology 3: 87-89.

Krishna K and Srivastava US (1991). Effects of ultraviolet rays on the haemocytes of flesh fly, *Parasarcophaga ruficornis* F., Journal of Entomological Research 15(1): 378-381.

Kumari K, Sinha MM, Mehto DN and Hammed SF (1990). Effect of some vegetable oils as protectants against pulse beetles *Callosobruchus chinensis*, Bulletin of Grain Technology 28(1): 58-60.

Lakshmi LG and Venugopal MS (2000). Effectiveness of powdered plant products as seed protectants against the pulse beetle, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) in storage. *In*: Proceeding Entomocongress, Trivandrum, Kerala, India, 54-55 pp.

Lale NES (1998). Preliminary studies on the effect of solar heat on oviposition, development and adult mortality of the cowpea bruchids *Callosobruchus maculatus* (F.) in the Nigerian savanna, Journal of Arid Environments 40: 157-162.

Mahdi SHA and Khalequzzaman M (2012). The efficacy of diatomaceous earth in mixed formulation with other dusts and an insecticide against the pulse beetles, *Callosobruchus chinensis* L. and *Callosobruchus maculatus* (F.), University Journal of Zoology Rajshahi University 31: 73-78.

Mahdi SHA and Rahman MK (2008). Insecticidal effect of some spices on *Callosobruchus maculatus* (Fabricius) in black gram seeds, University Journal of Zoology Rajshahi University 27: 47-50.

Mullen MA and Arbogast RT (1979). Time temperature-mortality relationships for various stored product insect eggs and chilling time for selected commodities, Journal of Economic Entomology 72: 476-478.

Pandey NK and Singh SC (1995). Effect of neem leaf powder on survival and mortality of pulse beetle, *Callosobruchus chinensis* (L.) infestation gram, Uttar Pradesh Journal of Zoology 3: 162-164.

Parween S, Faruki SI and Akhter R (2004). Growth and development of *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) developing from pupae irradiated with ultra-violet ray, University Journal of Zoology Rajshahi University 23: 23-26.

Phillips TW and Throne JE (2010). Biorational approaches to manage stored product insects, Annual Review of Entomology 55: 375-397.

Rajapakse R, Senanayakae SGJN and Ratnasekera D (1998). Effect of five botanicals on oviposition, adult emergence and mortality of *Callosobruchus maculatus* Fabr. (Coleoptera: Bruchidae) infesting cowpea, Journal of Entomological Research 22(2): 1-6.

Roger RC and Hamraoui A (1993). Efficiency of plants from the south of France used as traditional protectants of *Phaseolus vulgaris* L. against its bruchid *Acanthoscelides obtectus* (Say), Journal of Stored Products Research 29: 259-264.

Rouf FM, Sardar MA and Ahmed KS (1996). Individual and combined effect of some plant materials for protection of lentil seeds against pulse beetle, *Callosobruchus chinensis* L., Bangladesh Journal of Entomology 6: 13-21.

Sharma MK and Dwevedi SC (1997). Investigation on the effects of ultraviolet and infra-red light on the life cycle of *Callosobruchus chinensis* Linn., Journal of Advanced Zoology 18: 27-31.

Sowunmi OE and Akinnusi OA (1983). Studies on the use of the neem kernel in the control of stored cowpea beetle *Callosobruchus maculatus*, Tropical Grain Legume Bulletin 27: 28-31

J. Bio-Sci. 23: 67-75, 2015 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

MICROBIAL, PHYSICOCHEMICAL AND SENSORY CHARACTERISTICS ANALYSIS OF SELECTED ALCOHOLIC BEVERAGES OF FROM BANGLADESH, INDIA AND NEPAL

I Ahmad[1*], S Ahmed[2], B Yadav[1], PP Sah[1], R Ara[1], MJ Alam[3] and W Zzaman[1]

[1]Department of Food Engineering and Tea Technology, Shahjalal University of Science and Technology Sylhet-3114, Bangladesh; [2] Department of Microbiology and Hygiene, Sylhet Agricultural University, Sylhet-3100, Bangladesh; [3] Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology, Sylhet-3114, Bangladesh

Abstract

Alcoholic beverage is a drink containing ethanol, commonly known as alcohol. Beer is the most widely used alcoholic beverage in the world. To analyze the microbial, physicochemical and sensory characteristics of selected alcoholic beverages, several selected alcoholic beverages (power, strong, crown, hunter, power horse, god father, thunder, bag piper, commando and star gold) of Bangladesh, India and Nepal were used in this study for their analysis. Microbial analysis, physiochemical analysis, and sensory analysis were performed by using standard methods. The results of microbial analysis showed that the standard plate counts of alcoholic beverages were less than 300 colonies. The fungi were present in Nepal and Bangladesh sample but not in Indian samples. In all samples, the presence of coliform was about 0.03/ ml to 2.4/ ml. In nutrient agar, creamy smooth colony was found in most of the Nepal and Bangladesh beverages but not in Indian beverages. Hunter contained the highest pH (4.6) whereas power and bag piper showed the lowest pH values (3.3), strong contained maximum total solid (19.2%) but bag piper had only 2% total solid, big piper contained the highest amount of alcohol (18.25%) although power contained only 3.15% alcohol. Sensory evaluation of all the samples was quite acceptable as an alcoholic beverage. Among the alcoholic beverages it might be concluded that Nepal beverages are the best product in respect of sensory evaluation, but considering microbial analysis Indian beverages are the best.

Key words: Alcoholic beverage, microbial quality, physicochemical analysis, sensory test

Introduction

Beer is perhaps the most widely used alcoholic beverage. It is the third largest drink consumed all over the world after water and tea. Moderate use of beer in the daily diet can be good but drinking beer exceeds a certain limit would be harmful (Gaetano et al. 2016). Wine is another common alcoholic beverage and everybody is familiar with it. The spirits are also used in making alcohol beverages. The liqueur is the best example of a spirit which is used widely. Beer and wine are the things which are widely used and have the most popularity. Among the beer lager and all are quite popular. There are still many more varieties of alcohols (Roger 2001). Wine is a principally fermented juice. It may also be produced by fermentation of juice of fruit such as apple, peaches, apricots, plums, pears, cherries and berries. Wine is red or white depending on the skin of purple (Lourenço 2012). Fruit wines have traditionally been popular with home winemakers and in areas with cool climates such as North America and Scandinavia. Most fruits and berries have the potential to produce wine. However, the amount of fermentable sugars is often low and

*Author for correspondence: iftekharfet.sust@yahoo.com

need to be supplemented by a process called capitalization in order to have sufficient alcohol levels. Sucrose is often added so that fruits having excessive levels of acids (usually citric or malic acid) can split the sucrose into fermentable fructose and glucose sugars (Chiva-Blanch et al. 2015). Many fruit wines suffer from a lack of natural yeast nutrients needed to promote or maintain fermentation. Winemakers can counter this with the addition of nitrogen, phosphorus and potassium (Yasui et al. 1991). Unlike some grape-based wines, fruit wines often do not improve with bottle age and are usually meant to be consumed within a year of bottling. Fortified wines are made in the Republic of South Africa and North Africa (Dawson et al. 2005).

Fortified wines are made by adding spirits to wines, either during or after fermentation, with the result that the alcohol content of the wines is raised to around 20%, i.e. approximately double that of the table wines. Jack-fruit (*Artocarpus heterophyllus*) wine is an alcoholic beverage made by ethnic groups in the eastern hilly areas of India. As its name suggests, it is produced from the pulp of jack-fruit, ripe fruit is peeled and the skin was discarded (Dawson 2008a). During fermentation, the pH of the wine reaches a value of 3.5 to 3.8, suggesting that an acidic fermentation takes place at the same time as the alcoholic fermentation (Poli et al. 2013). Final alcohol content is about 7 to 8% within a fortnight. The term wine can sometimes include alcoholic beverages that are not grape-based. This can include wines produced from fruits like apples and elderberries, starches like rice, as well as flowers and weeds like dandelion and marijuana (Poli et al. 2013). The most common, narrow definition of wine relates to the product of fermented grape juice, though it is sometimes broadened to include any beverage with a fermentation based on the conversion of a sugar solution into alcohol (fermented beverages based on hydrolyzed barley such as beer are often excluded). Some drinks such as cider, mead and perry are also excluded from this broad definition of wine for historical reasons (Castellar et al. 2003). There were no scientific studies found regarding microbial, physicochemical and sensory analysis of those beverages. Therefore the present study was carried out to evaluate the microbial, physicochemical and sensory analysis of mentioned alcoholic beverages.

Materials and Methods

This study was conducted during the period of February - June 2011, Department of Food Engineering and Tea Technology, Shahjalal University of Science and Technology, Sylhet and Department of Microbiology and Hygiene, Sylhet Agricultural University, Sylhet.

Microbial analysis of alcoholic beverages

Microbial analysis were carried out by the pour plate/spread plate method using plate count agar, sabouraud dextrose agar and nutrient agar, lactose broth for total aerobic bacteria and total yeast count respectively. Colony counts were done after the appropriate period of incubation.

The standard plate count was done by the pour-plate/ spread plate technique (Breed and Dotterer 1916). Colony counts were considered for 30- 300 colonies/plate and others were discarded. In total fungal count technique, the sample was approximately diluted (0.5 ml) and transferred to a SDA plate. Then the sample was distributed evenly over the surface by a spreader. After colonies were grown, those were counted and the numbers of microbes in the original sample were calculated. The most probable number technique, counting the number of tubes showing a positive result and comparing with standard chart, a statistical estimation of the most probable number (MPN) of bacteria were made. This was an estimate of lactose fermenting bacteria.

Physicochemical characteristics of alcoholic beverages

Determination of pH of alcoholic beverages

10 ml of the alcoholic beverage was shaken with 100 ml of water and allowed to stand for a period of 30 min. Then the material was filtered and the pH of the filtrate was determined with a pH meter.

Determination of acidity in alcoholic beverages

Water extracts method (AOAC 1990) was used in the determination of acidity. Eighteen milliliter of alcoholic beverage was measured and shaken with 200 ml of CO_2 free water in a conical flask and placed in a water bath at 40°C for 1 h with the flask loosely stopped. It was filtered and 100 ml of the clear filtrate was titrated with 0.05M of NaOH solution with phenolphthalein indicator. The acidity of water extract increases during storage and is calculated as lactic acid or potassium dihydrogen phosphate (1 ml of 0.05 M of NaOH = 0.0068 g of KH_2PO_4).

Determination of total solid inalcoholic beverages

5 g of alcoholic beverage was weighed into a flat-bottomed metal dish (or small beaker) and placed on boiling water for about 30 min. until the liquid evaporated leaving the solid. It was then transferred into an oven maintained at 100°C for 2½ h as W_2. It was then transferred to desiccators, cooled and weighed. It was heated in the oven again for 1 h, cooled and weighed. The process was continued until constant weight W_3, was obtained (AOAC 1990). The total solid was calculated from the following equation:

$$\text{Total solid} = W_3 - W_1 / W_2 - W_1 \times 100$$

Determination of protein in alcoholic beverages

The mole titration method (Pirie 1975, Lourenço et al. 2012) was used for the determination of protein contents in alcoholic beverages. 10 ml of alcoholic beverage was added to 0.05 ml of 0.5% phenolphthalein indicator. It was mixed and allowed to stand for a few minutes and neutralized with 0.1M NaOH to the standard pink color. 2 ml of formalin was added, mixed and allowed to stand for few minutes. The new acidity produced was titrated with 0.1M NaOH to the same pink color. Then 2 ml of the formalin + 10 ml of H_2O were titrated separately with 0.1M NaOH as blank.

Determination of ash in alcoholic beverages

The crucible dish was cleaned, dried ignited, cooled and weighed as W_1. 24.4 g of the alcoholic beverage was weighed accurately and directly in the dish i.e. W_2. The substance was dried on a boiling water bath and the charred over a bursen flame or hot plate in fume cupboard until no more soot was given out. It was then ashed with a muffle furnace at 500°C to obtain W_3 (AOAC 1990). Percentage of ash was calculated from the following equation:

$$\text{Ash (\%)} = W_3 - W_1 / W_2 - W_1 \times 100$$

Determination of moisture content in alcoholic beverages

This method is based on loss on dry at an oven temperature at 105°C. Besides water the loss will include other matter volatile at 105°C (AOAC 1990). 5 g of alcoholic beverage was weighed into a pre-weighted flat dish (W_1) and dried at an oven temperature of 105°C for 3 h as W_2. It was allowed to cool in airtight desiccators and reweighed. It was heated in the oven again for half an hour, cooled and weighed. The process was repeated until constant weight was obtained W_3 (AOAC 1990). The percentage moisture was calculated from the following equation:

Moisture (%) = $W_2 - W_3 / W_2 - W_1 \times 100$.

Determination of alcohol contents in alcoholic beverages

Alcohol contents of the sample beverages were determined using ABV method as described elsewhere (Tapson 2004).

Sensory evaluation of alcoholic beverages

Sensory attributes (such as colour/appearance, flavour, texture, and overall acceptability) were evaluated using a 9 point hedonic scale (where 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much, 1 = dislike extremely) by 16 panelists (gender: 8 men: 8 women; age group: 20 - 40) selected from teachers, students and staff of several departments. Samples were served in clean transparent glasses. Questionnaires and water for mouth rinsing between each tasting were provided. Prior to evaluation, a session was held to familiarize panelists with the products. Panelists were asked to read through the questionnaires and the meaning of each attribute (colour/appearance, flavour, texture, and overall acceptability) was explained to the panelists to avoid any misinterpretation. Tasters were not allowed to discuss their scores with one another during the evaluation session. We have taken one sample of each country for sensory evaluation, from India (thunder), Nepal (commando) and Bangladesh (hunter).

Results and Discussion

Microbial analysis of alcoholic beverages

The microbial analysis for different parameters like the standard plate count (SPC), fungal count (FU), most probable number (MPN) count, *Escherichia coli* (*E. coli*) count of the alcoholic beverages were shown in Table 1, 2 and 3. The SPC of alcoholic beverages was more than 30 colonies but less than 300 colonies. When the colony forming unit is more than 30 colonies and less than 300 colonies then the SPC was suitable for plate counting. The fungi were present in Nepal and Bangladesh samples but not in Indian samples. In all the samples, the presence of *E. coli* form was about 0.03/ml to 4.6/ml (Tables 1-3). In nutrient agar (NA), creamy smooth colony was found in most of the alcoholic beverages which were collected from Nepal and Bangladesh but out of three Indian samples, two did not form any colony.

Table 1. Microbial analysis of alcoholic beverages of Bangladesh.

Sample name	SPC CFU/ml	FC CFU/ml	*E. coli* count MPN/ml	Colony in NA
Power	7×10^6	Fungus present	0.13	Creamy smooth colony
Strong	>30 colony	Fungus absent	0.03	Do
Crown	74×10^4	Fungus present	2.10	Do
Hunter	98×10^3	Fungus present	0.44	Do
Power horse	43×10^4	Fungus absent	0.09	Do

Table 2. Microbial analysis of alcoholic beverages of India.

Sample name	SPC CFU/ml	FC CFU/ml	*E. coli* count MPN/ml	Colony in NA
God father	>30 colony	Fungus absent	0.03	No colony found
Thunder	52×10^6	Fungus present	2.4	Creamy smooth colony
Bag piper	>30 colony	Fungus absent	0.03	No colony found

Table 3. Microbial analysis of alcoholic beverages of Nepal.

Sample name	SPC CFU/ml	FC CFU/ml	*E. coli* count MPN/ml	Colony in NA
Commando	6×10^7	Fungus present	4.60	Creamy smooth colony
Star gold	32×10^6	Do	0.93	Do

Physicochemical characteristics of alcoholic beverages

The pH of the 10 samples was found in the range of 3.3 to 4.6 (Tables 4-6). The pH of the samples were 3.3, 4.3, 4.4, 4.6, 4.5, 4.2, 4.5, 3.3, 4.5, 3.8 for power, strong, crown, hunter, power horse, god father, thunder, bag piper, commando and star gold, respectively; where hunter showed the highest pH (4.6) but power had the lowest pH (3.3). All the samples collected from Bangladesh, India, and Nepal had almost similar pH values (Tables 4-6). Ortiz-Laurel et al. (2000) observed that pH of the alcoholic beverages before and after storage around similar and within the range of 3.8 to 4.3.

The total solid of alcoholic beverages were found 17.6, 19.2, 2.6, 3.0, 12.4, 3.8, 3.8, 2.0, 3.6 and 3.0% for power, strong, crown, hunter, power horse, god father, thunder, bag piper, commando and star gold, respectively (Tables 4-6). The highest solid content in Bangladesh sample was 17.6% in power and the lowest was 2% (Tables 4-6) in Indian sample bag piper. From Germany beer institute, alcoholic beverages (beer) contain 11 to 14% of total solid (Rehm et al. 2003).

The percentage of total protein in alcoholic beverages were observed as 0.23, 0.42, 0.81, 0.91, 0.13, 3.45, 2.3, 3.6, 2.75 and 3.06% for power, strong, crown, hunter, power horse, god father, thunder, bag piper, commando and star gold, respectively (Tables 4, 5 and 6). The protein contents of alcoholic beverage made it to be more nutritious than any of the alcoholic beverages (beer), which seems to have contained less protein content (Dawson et al. 2008b).

The acidity of alcoholic beverages were found as 1.26, 1.53, 1.52, 1.63, 0.88, 3.26, 0.97, 1.36, 1.90 and 1.18% for power, strong, crown, hunter, power horse, god father, thunder, bag piper, commando and star gold, respectively (Tables 4-6). The acidity in alcoholic beverages was calculated as potassium dihydrogen phosphate (KH_2PO_4).

Table 4. Physicochemical characteristics of alcoholic beverages of Bangladesh.

Sample name	pH	Total solid (%)	Protein (%)	Acidity (%)	Ash (%)	Moisture content (%)	Alcohol (%)
Power	3.3	17.6	0.23	1.26	1.06	76.70	3.15
Strong	4.3	19.2	0.42	1.53	2.50	73.13	3.22
Crown	4.4	2.6	0.81	1.52	0.08	90.28	4.70
Hunter	4.6	3.0	0.91	1.63	0.04	89.62	4.80
Power horse	4.5	12.4	0.13	0.88	0.73	81.32	4.54

Table 5. Physicochemical characteristics of alcoholic beverages of India.

Sample name	pH	Total solid (%)	Protein (%)	Acidity (%)	Ash (%)	Moisture content (%)	Alcohol (%)
God father	4.2	3.8	3.45	3.26	0.08	82.45	6.95
Thunder	4.5	3.8	2.30	0.97	0.12	85.69	7.12
Bag piper	3.3	2.0	3.60	1.36	0.04	74.75	18.25

Table 6. Physicochemical characteristics of alcoholic beverages of Nepal.

Sample name	pH	Total solid (%)	Protein (%)	Acidity (%)	Ash (%)	Moisture content (%)	Alcohol (%)
Commando	4.4	3.6	2.75	1.90	0.80	85.36	5.55
Star gold	3.8	3.0	3.06	1.18	0.16	86.15	6.45

The value of ash contents of the alcoholic beverages were ranges from 0.04 to 2.50% and were estimated as 1.06, 2.50, 0.08, 0.04, 0.73, 0.08, 0.12, 0.04, 0.8 and 0.16% for power, strong, crown, hunter, power horse, god father, thunder, bag piper, commando and star gold, respectively (Tables 4-6). The lower amount of ash was found samples in Indian compared with Nepal and Bangladesh (IARC 1988).

The moisture contents of the alcoholic beverages were in the range of about 73 to 91% (Tables 4-6). The moisture contents of the different samples were measured as 76.70, 73.13, 90.28, 89.62, 81.32, 82.45,

85.69, 74.75, 85.36 and 86.15% for power, strong, crown, hunter, power horse, god father, thunder, bag piper, commando and star gold, respectively (Tables 4, 5 and 6). The moisture contents of the experimental alcoholic beverages were almost similar to that of the values as suggested previously ranging from 80 to 90%.

The alcohol contents of alcoholic beverages were found to be 3.15, 3.22, 4.70, 4.80, 4.54, 6.95, 7.12, 18.25, 5.55 and 6.45 for power, strong, crown, hunter, power horse, god father, thunder, bag piper, commando and star gold, respectively (Tables 4-6). The highest alcohol contents was observed samples in Indian (bag piper 18.25%, Table 5) while the lowest alcohol was present in Bangladeshi samples (power 3.15%, Table 4).

Sensory evaluation of alcoholic beverages

Sensory evaluation of the alcoholic beverages is shown in the Fig 1. Regarding the data, it was observed that samples in Nepal were the best beverages in all aspects e.g. color, flavor, texture, and overall acceptability, whereas samples in Bangladeshi and Indian showed almost similar sensory acceptability

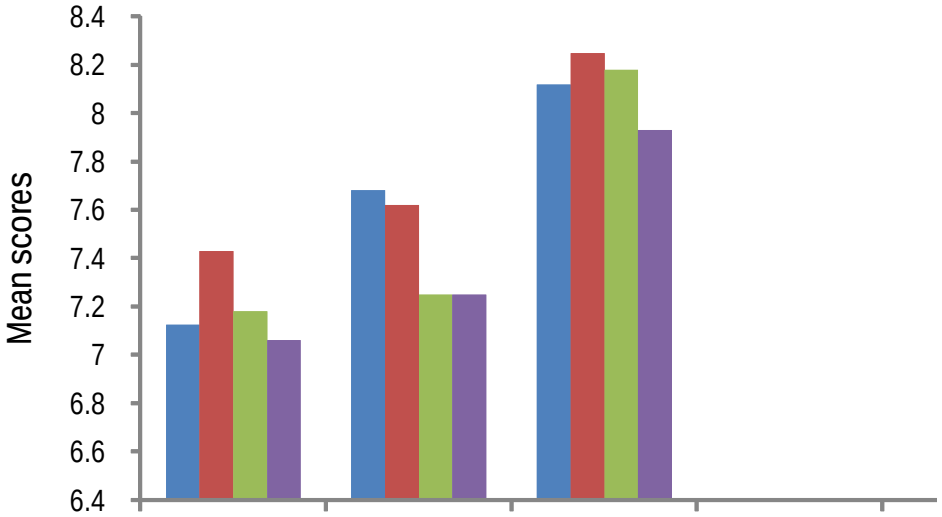


Fig. 1. Frequency score of sensory characteristics of alcoholic beverages.

Chowdhury and Ray (2007) reported that preliminary sensory evaluation analysis of “Jamun wine” is inferior (except colour/appearance) to commercial grape wine ($p < 0.05$) but the attributes like aroma, taste, after taste and colour/appearance were scored at about 3.0 (like much). However, the panelists rated flavour scores between 2.0 - 3.0 (like moderately- like much) probably because of high tannin content in jamun wine which imparted somewhat an astringent flavour. Nevertheless, the jamun wine was acceptable to all the panelists. Adebayo et al. (2010) studied that sensory attributes of “Kunu” samples with respect to taste, odour, colour, texture, flavour and general acceptability. All the samples remain acceptable only within the first 48 hrs of storage at all temperatures and it can be stored in the refrigerator for five days without spoilage. This is also supported by the fact that the pH has fallen to acidic level within the same period. The colour and taste became unattractive after 48 hrs of storage at room temperature.

Conclusion

From the above studies it may be concluded that Indian sample is good in compare to Nepal and Bangladesh samples. Indian samples contain small amount of microorganism and the physicochemical characteristics of Indian samples showed that those contain low amount of total solid, ash and acid; which is good for human health. Among the alcoholic samples, Nepal beverages are the best product in respect to sensory evaluation.

References

Adebayo GB, Otunola and Ajao TA (2010). Physicochemical, microbiological and sensory characteristics of kunu prepared from millet, maize and guinea corn and stored at selected temperatures, Advanced Journal of Food Science and Technology 2(1): 41-46.

AOAC (1990). Official Methods of Analysis, Association of Analytical Chemistry. 15th Ed. Washington DC: 1-1546 pp.

Breed RS and Dotterer WD (1916) The number of colonies allowable on satisfactory agar plates, New York Agricultural Experimental Station Technology Bulletin, 53-63 pp.

Castellar MR, Obón JM, Alacid M and Fernández-López JA (2003). Color properties and stability of betacyanins from Opuntia fruits, Journal of Agricultural and Food Chemistry 51: 2772-2776.

Chiva-Blanch G, Magraner E, Condines X, Valderas-Martínez P, Roth I and Arranz S (2015). Effects of alcohol and polyphenols from beer on atherosclerotic biomarkers in high cardiovascular risk men: a randomized feeding trial, Nutrition, Metabolism and Cardiovascular Diseases 25: 36-45

Chowdhury P and Ray RC (2007). Fermentation of Jamun (*Syzgium cumini* L.) fruits to form red wine, ASEAN Journal of Food 14(1): 15-23.

Dawson DA, Grant BF, Stinson FS, Chou PS, Huang B and Ruan WJ (2005). Recovery from DSM-IV alcohol dependence: United States 2001-2002. Addiction 100(3): 281-292.

Dawson DA, Goldstein RB, Chou SP, Ruan WJ and Grant BF (2008a). Age at first drink and the first incidence of adult onset *DSM-IV* alcohol use disorders Alcoholism, Clinical Experimental Research 32(12): 2149-2160.

Dawson DA, Stinson FS, Chou SP and Grant BF (2008b). Three-year changes in adult risk drinking behavior in relation to the course of alcohol use disorders, Journal of Studies on Alcohol and Drugs 69(6): 866-877.

Gaetano G, Costanzo S, Castelnuovo AD, Badimon L, Bejko D, Alkerwi A, Chiva-Blanch, Estruch R and Vecchia CL (2016). Effects of moderate beer consumption on health and disease: A consensus document, nutrition, Metabolism and Cardiovascular Diseases 26(6): 443-467.

IARC (1988). Alcohol drinking. IARC monographs on the valuation of carcinogenic risk of chemicals to humans, International Agency for Research on Cancer, Lyon, France 44: 416.

Lourenço S, Oliveira A and Lopes C (2012). The effect of current and lifetime alcohol consumption on overall and central obesity, European Journal of Clinical Nutrition 66(7): 813-818.

Ortiz-Laurel H and Mendez-Gallegos SJ (2000). Production of melcocha and queso de tuna from cactus pear fruit in the centre of Mexico, Food Chain 26: 20-21.

Poli A, Marangoni F, Avogaro A, Barba G, Bellentani S, Bucci M, Cambieri R and Catapano AL (2013). Moderate alcohol use and health: A consensus document Nutrition, Metabolism and Cardiovascular Diseases 23(6): 487-504

Pirie NW (1975). Food protein source rothameted experimental station harpenter, UK. 4th Edn. 1-121 pp.

Rehm J, Room R, Graham K, Monteiro M, Gmel G and Sempos CT (2003). The relationship of average volume of alcohol consumption and patterns of drinking to burden of disease: an overview, Addiction 98: 1209-28.

Roger B, Vernon L, Singleton, Linda F, Bisson and Kunkee RE (2001). The Principles and practices of winemaking. Frederick, MD: Aspen Publishers, pp 1999.

Tapson F (2004). Determination of Alcohol Content in Alcoholic Beverages Using 45 MHz Benchtop NMR Spectrometer, International Journal of Spectroscopy 6: 1-8.

Yasui M, Yase Y, Ota K and Garruto RM (1991). Evaluation of magnesium, calcium and aluminum metabolism in rats and monkeys maintained on calcium-deficient diets, Neurotoxicology 12(3): 603-614.

J. Bio-Sci. 23: 77-88, 2015 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

IN VIVO ANTICANCER ACTIVITIES OF NI (II)-BENZOIN THIOSEMICARBAZONE COMPLEX [NI(BTSC)$_2$] AGAINST EHRLICH ASCITES CARCINOMA CELLS

SMM Ali[1], HM Zakir[1*], SMS Shahriar[1], MH Sarkar[2], AK Dey[2], HP Nur[2] and M Jesmin[1]

[1]*Department of Applied Chemistry and Chemical Engineering, University of Rajshahi, Bangladesh*
[2]*Bangladesh Council of Scientific and Industrial Research, Rajshahi, Bangladesh*

Abstract

Cancer is a class of diseases in which a group of cells display uncontrolled growth, invasion and sometimes metastasis. In order to find out a new anticancer drug, Ni(II) complex with benzoin thiosemicarbazon was synthesized and characterized. Anticancer activities of Ni(BTSC)$_2$ has been studied against Ehrlich Ascites Carcinoma (EAC) cells in Swiss albino mice by monitoring tumor cell growth inhibition, tumor weight measurement, survival time of tumor bearing Swiss albino mice. Hematological parameters were also studied in both normal and EAC bearing treated mice. The results were compared with those obtained with a standard anticancer drug *bleomycin* and the compound was found to possess pronounced anticancer effect. Maximum cell growth inhibition was found to be 77.15% after treatment with Ni(BTSC)$_2$ at the dose of 8 mg/kg (*i.p*). About 69.56% enhancement of life span was found at 8 mg/kg (*i.p*).With the same dose Ni(BTSC)$_2$ reduced the tumor weight by 52.17% at day 20. The hematological parameters (WBC, RBC, hemoglobin content and differential counts) were found to be significantly changed as compared to those of the normal mice. These parameters restored more or less towards normal when treated with the test compound.

Key words: Antineoplastic activity, EAC cells, hematological parameters, nickel-(II) benzoin thiosemicarbazone complex, survival time

Introduction

Cancer is a diverse class of diseases which differ widely in their causes and biology. Using the new techniques of molecular biology, the causes of cancer have been searched. It seems to be the inappropriate activation of one or more proteins that regulate cells division, transforming cells to state of cancerous growth. Multidisciplinary researchers are involved to find out the causes of cancer and also developed many treatment procedures. Among them, chemotherapy is a major option. In this case schiff bases and schiff base metal complexes can create the attention of the scientist as one of the major research items to find out new, cheaper, more effective and easily available with less host toxic effects.

Schiff bases are condensation products of aldehyde and ketones with primary amines and containing imine or azomethine (-C = N-) functional group. Schiff bases are found to be a versatile pharmacophore for design and development of various bioactive led compounds. Schiff bases as well as schiff base complexes with transition metals form an important class of the most widely used organic and organometallic compounds and have a wide variety of applications in many fields including analytical, biological and inorganic chemistry. In recent times, schiff bases and schiff base metal complexes have drawn the attention of many researchers in medicinal and pharmaceutical fields due to a broad spectrum of biological activities like anticancer (Ali

*Author for correspondence: hmzakir04acct@gmail.com

2012, 2009; Zakir 2016), antimicrobial (Jesmin 2008, Zakir 2016), anti-tubercular, anti-inflammatory and analgesic (Pandey 2011), antiviral and pesticidal (Ali 2007, Zakir 2016) activities.

In the present paper we have reported anticancer activities of $Ni(BTSC)_2$ against *Ehrlich ascites* carcinoma cells in Swiss albino mice.

Materials and Methods

Chemicals

All chemicals and reagents used to carry out the research work were of reagent grade.

Experimental animal

Swiss albino mice of 5-7 weeks old, weighing 25-30 g were selected for the work as test animals, which were collected from International Centre for Diarrheal Disease Research, Bangladesh (ICDDR'B), Mohakhali, Dhaka.

Animal care

Mice were kept in iron cages with saw dust and straw bedding which was changed once a week regularly. Standard mouse diet (recommended and prepared by ICDDR'B, Mohakhali, Dhaka) and water were given in adequate.

Ethical clearance

Protocol used in this study for the use of mice as animal mode for research was approved by the University Animal Ethical Committee (27/08/RUBCMB).

Synthesis of the compound

Synthesis of Ni (II)-benzoin thiosemicarbazone complex

The compound was synthesized according to the method as described in the literature (Frederick 1974, El-Shahawi 2013). For benzoin thiosemicarbazone (BTSC), benzoin and thiosemicarbazide (1:1 molar ratio) were mixed together and refluxed for a period of 3-4 hours and then distilled to half of the total volume. A saturated solution of nickel(II) acetate in ethanol was added to the condensed solution. Within a few minutes black crystals of Ni(II)-benzoin thiosemicarbazone were obtained. The crystals were then recrystallized twice, dried in an oven at 50°C and stored in a desiccator.

Characterization of the compound

The synthesized compound was characterized by taking melting point by using an electro thermal melting point apparatus, elemental analytical data were determined by using Perkin Elmer 2400 CHNS/O elemental analyzer at BCSIR Laboratory, Dhaka. The amount of metal were determined by using Atomic Absorption Spectrometer at Dhaka University and IR spectra data were obtained from Rajshahi University central laboratory as KBr disc by using a Shimadzu FTIR spectrometer.

Cell lines

EAC cells were obtained by the courtesy of Indian Institute of Chemical Biology (IICB), Kolkata, India. The cells were maintained as ascites tumor in Swiss albino mice by intraperitoneal inoculation (*i.p.*, by weekly) of 2×10^6 cells/mouse.

Toxicity study

An acute toxicity study relating to the determination of LD_{50} was performed by the conventional method (Litehifield 1949). For that purpose, the compound was dissolved in 2% dimethyl sulfoxide (DMSO) and injected intraperitoneally to six groups of mice (each containing six in number) at different doses. LD_{50} values were evaluated by recording mortality after 24 hours. The toxicity of the compound, $Ni(BTSC)_2$ has evaluated by measuring LD_{50} values and was found to be 88 mg/kg (*i.p.*).

Cell growth inhibition

In vivo tumor cell growth inhibition was carried out by the method as described earlier (Sur 1994). For this study, five groups of mice (six in each group) were used. All the mice were inoculated with 2×10^6 EAC cells intraperitoneally. Treatment was started after 24 hours of tumor inoculation and continued for 6 days. Groups 1 to 3 were treated by $Ni(BTSC)_2$ at the doses of 2 mg/kg (*i.p.*), 4 mg/kg (*i.p.*) and 8mg/kg (*i.p.*), respectively per day per mouse. Group 4 received standard drug *bleomycin* (0.3 mg/kg, *i.p.*). Treatment with only normal saline (0.98%) was considered as untreated control (Group 5). The mice of all the groups were sacrificed on the 6th day after transplantation and tumor cells were collected by repeated intraperitoneal wash with 0.98% saline. Viable tumor cells per mouse of the treated groups were compared with those of the control. The cell growth inhibition was calculated by using the following formula:

$\%$ Cell growth inhibition $= (1- T_w / C_w) \times 100$ where

T_w = Mean of number of tumor cells of the treated group of mice

C_w = Mean of number of tumor cells of the control group of mice.

Average tumor weight and survival time

The antitumor effects of $Ni(BTSC)_2$ was assessed by measuring average tumor weight, mean survival time (MST) and percentage increased of life span (% ILS) (Abbot 1976). These parameters were measured under similar experimental conditions as stated in the previous experiment (cell growth inhibition). Treatment was continued for 10 days. Tumor growths were monitored daily by measuring weight change. MST of each group (6 in each) was monitored by recording the survival time. MST and % ILS were calculated by using the following equations.

$$\text{MST} = \frac{\text{survival time (days) of each mouse in a group}}{\text{Total number of mice}}$$

$$\%\ \text{ILS} = \frac{\text{MST of treated group}}{\text{MST of control group}} \times 100$$

Bioassay of EAC cells

The procedure was a modification of the methods as used in literature (Fernades 1979). Two groups of mice (4 in each) were inoculated with 2×10^6 EAC cells. Group 1 was treated with $Ni(BTSC)_2$ at the doses of 2 mg/kg (*i.p.*), 4 mg/kg (*i.p.*) and 8 mg/kg (*i.p.*), respectively per day per mouse for six consecutive days. The group 2 served as control. On day 6, mice of all groups were sacrificed and tumor cells from each group

were harvested in cold saline (0.98%), pooled and centrifuged. These cells were re-inoculated (2×10^6 cells/mouse *i.p*) into two fresh groups of mice (n = 4) as before. No further treatment was done on these mice. On day 5, mice from each group were sacrificed and tumor cells per mouse were counted and compared with that of control.

Hematological studies

The hematological parameters *viz*. WBC, RBC and Hb content were determined by the standard methods using cell dilution fluids and hemocytometer (Ruisa 1988). For this purpose, blood was collected from the mouse by tail puncture. Five groups of mice (n = 4) were taken for this test. Groups 1 to 3 were treated by $Ni(BTSC)_2$ at the doses of 2 mg/kg (*i.p*.), 4 mg/kg (*i.p*.) and 8 mg/kg (*i.p*.), respectively per day per mouse. Treatment was started after 24 hours of tumor transplantation and continued for 10 consecutive days. On days 5, 10, 15 and 25 the blood parameters were assayed.

For normal mice 5 groups (n = 4) were taken for the purpose of hematological studies. The blood from the mice of group 1 was assayed on day 0 (without any treatment). Groups 2-4 were treated with $Ni(BTSC)_2$ at the doses of 2 mg/kg (*i.p*.), 4 mg/kg (*i.p*.) and 8 mg/kg (*i.p*.), respectively per day per mouse. Group 5 received standard drug *bleomycin* (0.3 mg/kg, *i.p*.).

Determination of the effect of schiff base complex $Ni(BTSC)_2$ on normal peritoneal cells

Effect of schiff base complex on normal peritoneal cells was determined by counting total peritoneal cells and number of macrophages (Hundson 1989). One group of mice (4 in each) was treated with $Ni(BTSC)_2$ at the dose of 8 mg/kg (*i.p*.) for three consecutive days. The untreated group was used as control. After 24 hours of last treatment, each animal were injected with 5 ml of normal saline (0.98%) into peritoneal cavity and then sacrificed. Intraperitoneal exuded cells and number of macrophages were counted with 1% neutral red by hemocytometer. Effect of $Ni(BTSC)_2$ complex on enhancement of normal peritoneal cells in normal mice were shown in Fig. 4.

Statistical analysis

The experimental results have been expressed as the mean ± SD. Data were calculated by ANOVA followed by Dunnett “t” test using SPSS software of 20 versions.

Results and Discussion

The synthesized compound was characterized by taking melting point and determining elemental analytical data Table 1 and 2. The structure of $Ni(BTSC)_2$ complex can be assumed to be octahedral (Fig. 1). This view is supported from the IR spectral data presented in Table 3. The ligand BTSC binds to metal ions in a mononegative tridentate fashion through C = S, C = N and OH groups (with deprotonation of OH). It is evident that the ν(C = S), ν(C = N) and ν(OH) have been shifted to lower frequency regions after bonding as compared to those for BTSC (Nakamoto et al.1971, Parashar et al. 1989). ν(C = S) band is shifted from 1263 cm^{-1} to 1130 cm^{-1}, ν(C = N) band is shifted from 1682 cm^{-1} to 1566 cm^{-1} and another coordinating site ν(OH) is also shifted from 3379 cm^{-1} to 3357 cm^{-1} after complexation (not clearly shown due to a broad spectrum)

suggesting involvement of S from C = S, N from C = N and O from OH in coordination with Ni(II) ion. Further the new bonds M-O, M-N and M-S have been detected at 617 cm^{-1} for ν (M-O), 481cm^{-1} for ν (M-N) and 422cm^{-1} for ν (M-S) respectively, which confirmed the formation of the complexes. Both the bonding and structure presented here are very much similar to those obtained earlier (El-Shahawi 2013, Ali 2011).

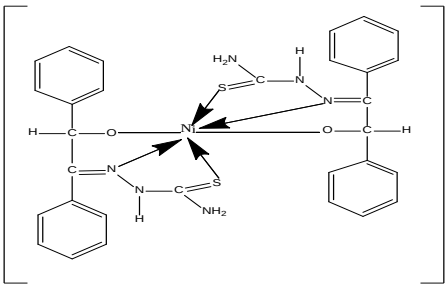


Fig. 1. Structure of $Ni(BTSC)_2$ complex.

Table 1. Yield percentage and physical characteristics of the compound.

Test compound	Yield %	Melting point °C	Physical form	Solubility
$Ni(BTSC)_2$ complex	50	Stable up to ~155°C	Black crystalline	Ethanol, Methanol, DMSO and Acetone

Table 2. Elemental analytical data of the compound.

Compound		Elemental analytical data found (calculated) in %					
		C	H	N	O	S	Metal (Ni)
$Ni(BTSC)_2$ complex	Found	52.36	4.39	12.21	4.65	9.31	16.90
	Theoretical	52.93	4.11	12.44	4.67	9.98	17.08

Table 3. IR spectral data of the compound.

Compound	*v*(OH)	*v*(C = N)	*v*(C = S)	*v*(NH-C = S)	*v*(M-O)	*v*(M-N)	*v*(M-S = C)
$Ni(BTSC)_2$ complex	3357 w	1566 s	1130 s	1029 s	617 s	524 s	422 w

[s = strong, w = weak]

In vivo tumor cell growth inhibition was observed with $Ni(BTSC)_2$ at the doses of 2 mg/kg (*i.p.*), 4 mg/kg (*i.p.*), 8 mg/kg (*i.p.*) per mouse per day. The percentage of cell growth inhibition is found to increase noticeably, with increase the doses. Maximum cell growth inhibition (77.15%) was found after treatment with $Ni(BTSC)_2$ at the dose of 8 mg/kg (*i.p*). Which is quite comparable to that of *bleomycin* at the dose of 0.3 mg/kg *(i.p.)*, when 88.20% inhibition of cell growth was observed (Table 4). The mean survival time (MST) of the untreated tumor bearing mice was 23 days. With the treatment of $Ni(BTSC)_2$, the value was found to be increased. About 69.56% enhancement of life span was found at the dose of 8 mg/kg (*i.p*), whereas the *bleomycin* showed 87.25% increase of life span at the dose of 0.3 mg/kg (*i.p.*) (Table 5).

The treatment with $Ni(BTSC)_2$ complex showed that the average tumor weight for EAC cell bearing treated mice increases at a slower rate than those of untreated EAC cell bearing mice. At day 20, $Ni(BTSC)_2$ at the dose of 8 mg/kg (*i.p.)* reduced the tumor weight by 52.17% whereas the standard drug *Bleomycin* shows 68.33% (at 0.3 mg/kg) when compared with that of control (Fig. 2).

The hematological parameters of both EAC cell bearing mice and normal mice were examined. In EAC cell bearing mice, all parameters (WBC, RBC and hemoglobin content) were found to be significantly changed as compared to those of the normal mice. These parameters restored more or less towards normal when treated with $Ni(BTSC)_2$ (Fig. 3 a-f). In case of parallel treatment of normal mice, these parameters were found to be slightly changed from normal values. After 25 days of the initial treatment, they were found to be restored to almost normal values.

The effect of $Ni(BTSC)_2$ on the loss of transplant ability of EAC cells were observed by the reduction of intraperitoneal tumor growth in mice, reinoculatated with test compound treated EAC cells (Table 6) with respect to control. Maximum reduction (52.86%) of tumor growth was observed with $Ni(BTSC)_2$ at the dose of 8 mg/kg, (*i.p.*).The compound at higher dose also enhanced both the peritoneal cells and the number of macrophages to some extent in normal mice (Table 7).

Table 4. Effect of the $Ni(BTSC)_2$ and *bleomycin* (antitumor drug) on cell growth inhibition *in vivo.*

Experiment	Dose, mg/kg (*i.p.*)	No. of EAC cells in mice on day 6 after tumor cell inoculation × 10^7	% Cell growth inhibition
Control (untreated EAC cell bearing mice)	-	2.180±0.082	-
EAC + *Bleomycin*	0.3	0.257±0.010***	88.2
	2	0.990±0.028 **	44.59
EAC + $Ni(BTSC)_2$	4	0.884±0.036 **	59.45
	8	0.498±0.051***	77.15

Mice were inoculated 2×10^6 EAC cells/mouse (*i.p.*) on days 0. Treatment was started after 24 hours of tumor cell transplantation. Number of mice in each experiment were six (n = 6); the results were shown as mean ± SEM (Standard error of mean). Treatment was continued for 6 consecutive days. Where significant values are *p <0.05, **p <0.01, and ***p <0.001 when compared with control.

Table 5. Effect of $Ni(BTSC)_2$ and *bleomycin* on survival time and increase of life span of EAC cell bearing mice.

Treatment	Dose, mg/kg (*i.p.*)	Mean survival time mean ± SEM (days)	% Increase of life span
Control (untreated EAC cell bearing mice)	-	23±0.98	-
EAC + *Bleomycin*	0.3	43±0.86***	86.95
	2	27±1.52*	17.39
EAC+ $Ni(BTSC)_2$	4	32±1.76**	39.13
	8	39±2.20***	69.56

Data are expressed as the mean of results in 6 mice ± SEM. Treatment was continued for 10 consecutive days. Where significant values are *p <0.05, ** p <0.01 and *** p <0.001 when compared with control.

Table 6. Bioassay of $Ni(BTSC)_2$ compound.

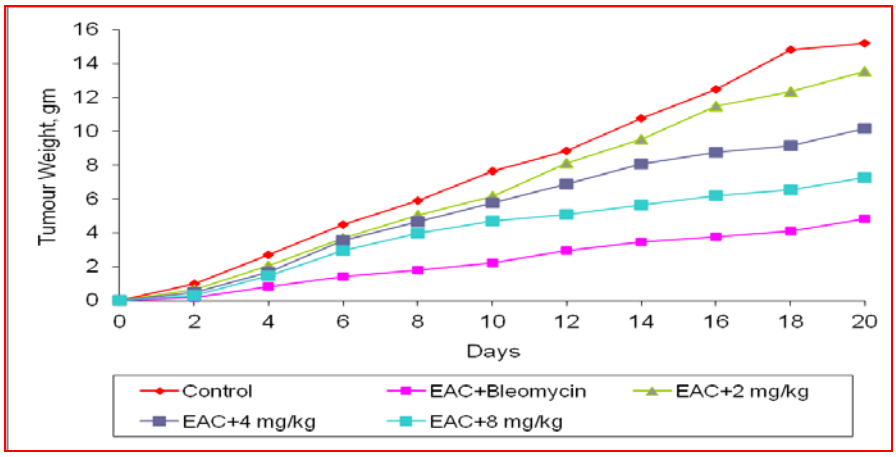
Treatment	Dose mg/kg (*i.p.*)	No. of EAC cells $\times 10^7$	Cell growth inhibition also inoculation with drug treatment EAC cell
Control (untreated EAC cell bearing mice)	-	3.14±0.04	-
	2	2.32±0.05**	26.11%
EAC+ $Ni(BTSC)_2$	4	1.90±0.09**	39.60%
	8	1.48±0.08**	52.86%

Data are expressed as the mean of results in 4 mice ± SEM Where significant values are **p <0.01 and ***p <0.001 when compared with control.

Table 7. Effect of Ni $(BTSC)_2$ on the enhancement of normal peritoneal cells in mice.

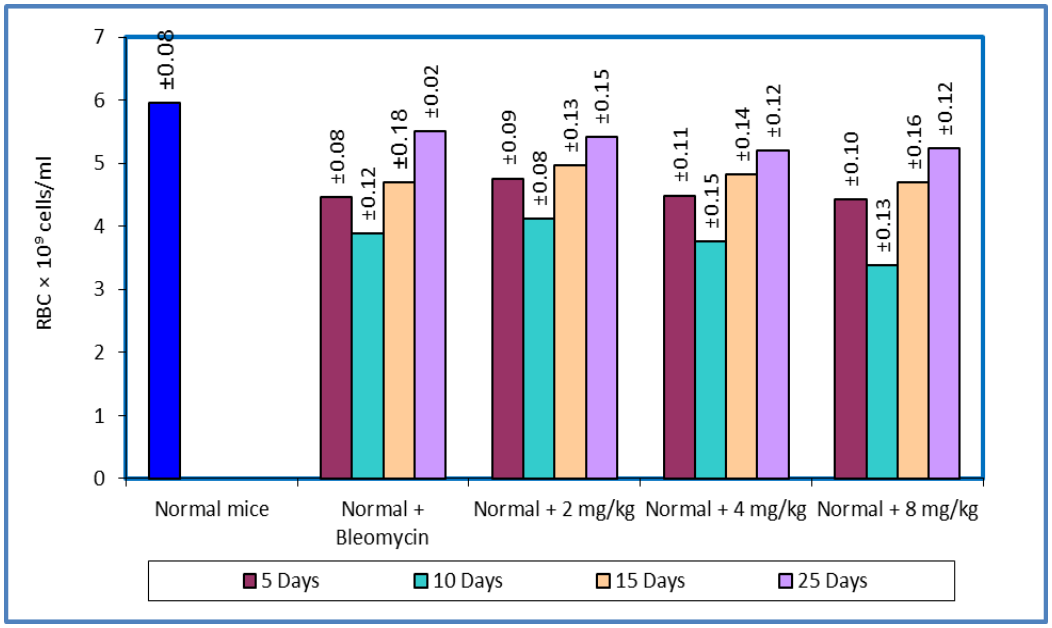
Treatment	Dose mg/kg (*i.p.*)	Macrophages (cells/mL) $\times 10^6$	Total Peritoneal cells $\times 10^6$
Control (normal)	-	1.20 ± 0.42	6.82±0.29
	2	1.90±0.28***	8.54±0.34
Normal + $Ni(BTSC)_2$	4	2.16±0.19***	9.62±0.31
	8	2.98±0.33***	10.86±0.38

Data are expressed as the mean of results in 4 mice ± SEM. Treatment was continued for 3 consecutive days. ***P <0.001 and **P <0.01 when compared with control.



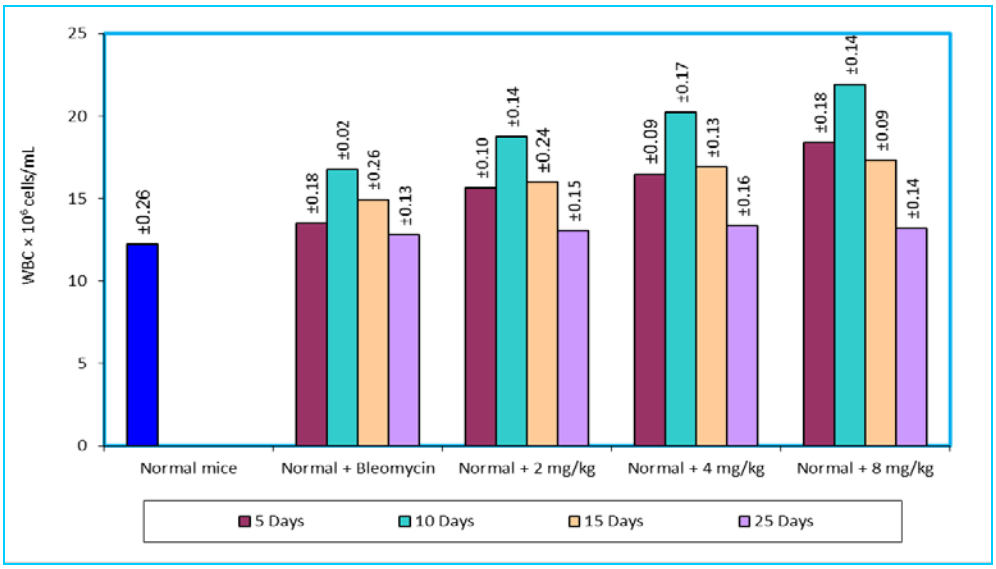
Data are expressed as the mean of results in 6 mice. Treatment was continued for 10 consecutive days.

Fig. 2. Effect of $Ni(BTSC)_2$ on average tumor weight.



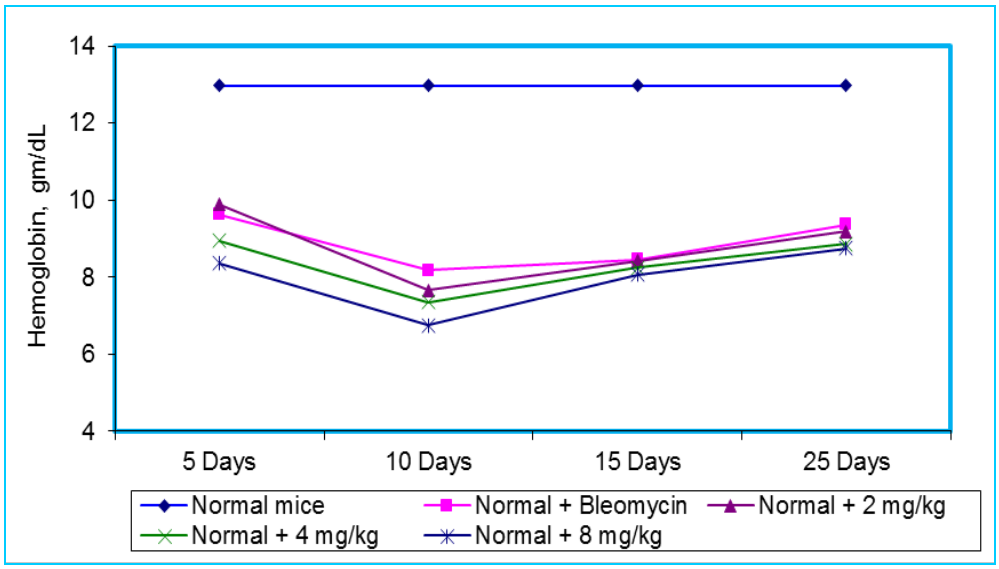
Data are expressed as the mean of results in 4 mice ± SEM. Treatment was continued for 10 consecutive days.

Fig. 3a. Effect of $Ni(BTSC)_2$ on RBC in normal mice.



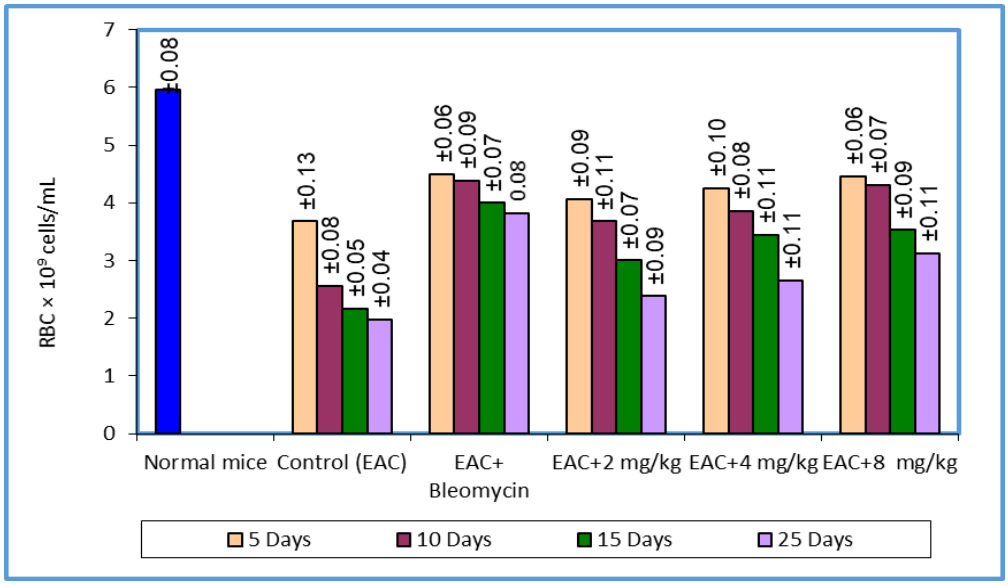
Data are expressed as the mean of results in 4 mice ± SEM. Treatment was continued for 10 consecutive days.

Fig. 3b. Effect of $Ni(BTSC)_2$ on WBC in normal mice.



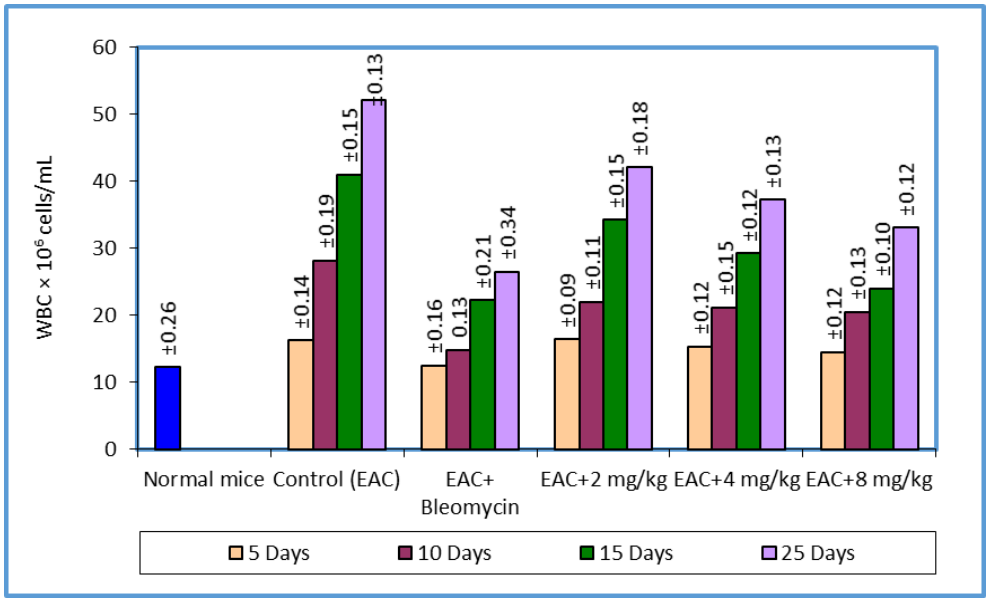
Data are expressed as the mean of results in 4 mice ± SEM. Treatment was continued for 10 consecutive days.

Fig. 3c. Effect of $Ni(BTSC)_2$ on hemoglobin content in normal mice.



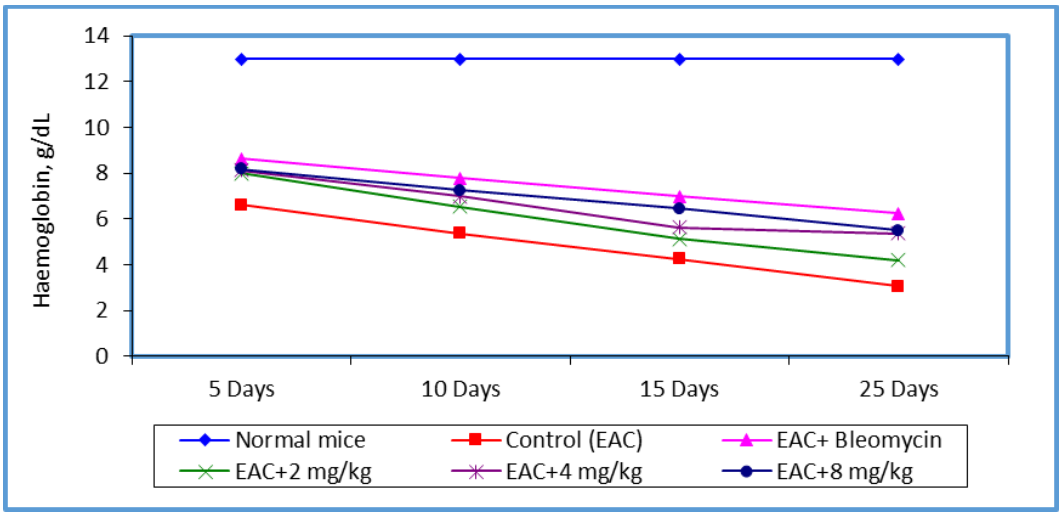
Data are expressed as the mean of results in 4 mice ± SEM. Treatment was continued for 10 consecutive days.

Fig. 3d. Effect of $Ni(BTSC)_2$ on RBC in EAC cell bearing mice.



Data are expressed as the mean of results in 4 mice ± SEM. Treatment was continued for 10 consecutive days.

Fig. 3e. Effect of $Ni(BTSC)_2$ on WBC in EAC cell bearing mice.



Data are expressed as the mean of results in 4 mice ± SEM. Treatment was continued for 10 consecutive days.

Fig. 3f. Effect of $Ni(BTSC)_2$ on hemoglobin content in EAC bearing mice.

Conclusion

The results presented in this study showed that the schiff base complex $Ni(BTSC)_2$ is capable of reducing average tumor weight and increasing life span of tumor bearing mice. It also inhibits the cell growth very successfully and restored all the hematological parameters (RBC, WBC, Hemoglobin and differential counts) more or less to normal. It also found that, Ni(BTSC)2 enhanced the number of macrophages and other peritoneal cells remarkably, so it is speculated that $Ni(BTSC)_2$ kill or destroy tumor cells by boosting cell mediated tumor immunity of the host and expected to be an effective anticancer agent with negligible toxicities. However, the information obtained from the present investigation is insufficient for $Ni(BTSC)_2$ to be used as novel anticancer drug in clinical practice. Many more investigations have to be carried out with this compound using various other cancer cell lines and higher test animals in order to confirm this as potent anticancer agents.

References

Ali MM, Azad MAK, Jesmin M, Ahsan S, Rahman MM, Khanam JA, Islam MN and Shahriar SMS (2012). *In vivo* anticancer activity of vanillin semicarbazone, Asian Pacific Journal of Tropical Biomedicine 2(6): 438-442.

Ali MM, Jesmin M, Islam MN, Shahrair SMS, Habib MR and Islam MF (2009). Anticancer activities of some transition metal complexes of a schiff base derived from salicylaldehyde and glycine, Chemical Research Communications 23: 13-22.

Abbot BJ (1976). Bioassay of plant extract for anticancer activity, Cancer Treatment Reports 60: 1007-1017.

Ali MM, Khalequezzaman M, Taher MA, Ara J, Jesmin M and Khanam JA (2007). Toxicity of some metal cystine complexes against confused flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae), Bangladesh Journal of Life Sciences 19(1): 115-119.

Ali MAA, Mohamad AMS and Hadi MA (2011). Preparation and characterization of some transition metal complexes with new Sshiff base ligand derived from benzophenone, Journal of Kufa for Chemical Science 2(1): 64-71.

Ali MM, Jesmin M, Islam MK, Fatema K and Azad KA (2013). Antineoplastic activities of acetone thiosemicarbazone against Ehrlich ascites carcinoma cells bearing mice, Medical Journal of Islamic World Academy of Sciences 21(3): 97-104.

El-Shahawi MS, Al-Jahdali MS, Bashammakh AS, Al-Sibaai AA and Nassef HM (2013). Spectroscopic and electrochemical characterization of some schiff base metal complexes containing benzoin moiety, Acta Part A: Molecular and Biomolecular Spectroscopy 113: 459-465.

Frederick GM (1974). Elementary Practical Organic Chemistry Part-2: Qualilative Organic Analysis, 2nd Ed., Longman group limited, London: 118.

Fernades DJ and Klubes P (1979). A biochemical and pharmacological study of therapeutic synergism with 5-fluonounacil plus cyclophosphamide in murine L_{1210} Leukemia, Cancer Research 39: 1396-1404.

Hundson L and Hay FC (1989). Isolation of normal peritoneal macrophages. In practical Immunology. 3 rd Ed., Black well science publisher, Oxford London: 26 p.

Jesmin M, Ali MM, Salahuddin MS, Habib MR and Khanam JA (2008). Antimicrobial activity of some schiff bases derived from benzoin, salicylaldehyde, aminophenol and 2, 4 dinitrophenyl hydrazine, Microbiology 36(1): 70-73.

Liu MC, Lin TS, Penketh P and Sartorelli AC (1995). Synthesis and antitumor activity of 4- and 5- substituted derivatives of isoquiniline-1-carboxaldehyde thiosemicarbazone, Journal of Medicinal Chemistry 38(21): 4234-43.

Litchfield JT and Wilcoxon FA (1949). A simplified method of evaluation dose effects experiment, Journal of Pharmacology and Experimental Therapeutics 96: 99-113.

Nakamoto K (1971). Infrared and raman spectra of inorganic and coordination compounds. vol. 222, Wiley Interscience, New York.

Parashar RK, Sharma RC and Mohan G (1989). Biological activity of some schiff bases and their metal complexes. Biological Trace Element Research 23(1): 145-50.

Pandey A, Dewangan D, Verma S, Mishra A and Dubey R.D (2011). Synthesis of schiff bases of 2-amino-5-aryl-1, 3, 4-thiadiazole and its analgesic, anti-inflammatory, antibacterial and antitubercular activity, International Journal of Chemical Technology Research 3(1): 178-184.

Ruisa V and Sood SK (1988). Routine hematological tests In Medical Laboratory Technology, Mukherjee K J (ed.) Tata McGraw Hill Comp. Ltd. New Delhi, 218 pp.

Shahriar SMS, Ali MM and Jesmin M (2015). *In vivo* anticancer activities of vanillin thiosemicarbazone complexes with Co (II) and Ni (II), Medical Journal of Islamic World Academy of Sciences 23(3): 90-95.

Sur P and Ganguly DK (1994). Tea plant root extract (TRE) as an antineoplastic agent, Planta Medica 60: 106.

Zakir HM, Islam MK, Hasan MR, Ali MM and Jesmin M (2016). *In vivo* anticancer activities of Co(II)-Benzoin Thiosemicarbazone Complex $[Co(BTSC)_2]$, International Journal of Biosciences 9(4): 209-220.

Zakir HM, Ali MM and Jesmin M (2016). Antibacterial activities of benzoin thiosemicarbazone and its complexes with Co (II) and Ni (II), Asian Journal of Medical and Pharmaceutical Researches 6(4): 32-40.

Zakir HM, Islam AM, Nurtaj K, Ali MM and Jesmin M (2016). Pesticidal activities of benzoin thiosemicarbazone and its complexes with Co (II) and Ni (II), International Journal of Biosciences 9(6): 92-100.

J. Bio-Sci. 23: 89-91, 2015 ISSN 1023-8654
http://www.banglajol.info/index.php/JBS/index

-Short communication

EFFECT OF SINGLE AND MULTIPLE AM FUNGAL INOCULANTS ON THE GROWTH PARAMETER OF *OCIMUM BASILICUM* VAR. *THYSIFLORA* BENTH

T Yeasmin[1], J Puttaradder*[2] and HC Lakshman[3]

[1]*Institute of Biological Sciences, University of Rajshahi, Bangladesh*
[2,3]*Microbiology Laboratory, P.G. Department of Studies in Botany, Karnataka University, Karnataka, India*

Most land plants form associations with mycorrhizal fungi. Mycorrhizas form mutualistic associations between fungi and plants roots. They are described as symbiotic, because the fungus receives photosynthetically derived carbon compounds and the plant has increased access to mineral nutrients and sometimes water. The symbiosis is characterized by branched fungal structures, arbuscules, which grow intercellularly without penetrating the host plasmalemma (Lakshman 2009). The main function of arbuscular mycorrhizal (AM) fungi is to enable Phosphorus for plants and increase uptake of N, K, Zn, S, Fe, Cu, Mg, Ca and Mn. Extrametrical mycelium of AM fungi access phosphorus and translocate to cortical cells of plants. (Jone and Jakobsen 1995) and so increase the supply of slowly diffusing ions, such as phosphate to the plant (McArther and Knowles 1993).

Pure cultures of single species of AM fungus are being assessed for appreciable plant growth and crop production. AM fungi increased biomass production of sustainable agricultural crops. Multiple inoculations of the plants with AM fungi have often yielded increased biomass production. Therefore, the present investigation was carried out to understand the influence of single or multiple AM fungal inoculation on *Ocimum basilicum* Var. *thysiflora* Benth. Plants were raised in earthen pots measuring (25 × 30) with soil (sandy clay loam, pH 6.8, organic matter 0.72%, and indigenous spore population of *Glomus* spp.) was surface sterilized with 2% streptomycin. Before sowing the seeds, mixed inoculum (AM fungi colonized root bits plus chlamydospores) of different AM fungi was added to 4 cm below the soil of each experimental pots at the rate of 50 gm soil inoculum/pot having 250 - 300 spores. Following treatments with three replications were included in the study:

1. Soil without inoculum (Control)
2. Soil + *Glomus bagyarajii* VS Mehrotra (50 gm)
3. Soil + *Glomus macrocarpum* Tulasne and *C. tulasne* (50 gm)
4. Soil + *Rhizophagus fasciculatus* (Thaxt.) C. Walker and A. Schüßler (50 gm)
5. Soil + *Glomus bagyarajii* (25 gm) + *G. macrocarpum* (25 gm)
6. Soil + *Glomus bagyarajii* (25 gm) + *R. fasciculatus* (25 gm)
7. Soil + *Glomus macrocarpum* (25 gm) + *R. fasciculatus* (25 gm)
8. Soil + *Glomus bagyarajii* (16.66 gm) + *G. macrocarpum* (16.66 gm) + *R. fasciculatus* (16.66 gm)

Pots of all the treatments were maintained under greenhouse conditions. Plants were watered on alternate days. 15 ml of minus P Hoagland nutrient solution was given once in 15 days for experimental pots. Plants

*Author for correspondence: j.puttaradder@gmail.com

were uprooted periodically and per cent colonization of mycorrhizal roots was recorded by methods of Phillips and Hayman (1970). At the same time the spores were extracted from the root washings by the method of the wet sieving decanting technique of Gerdemann and Nicolson (1963) and spore count of rhizosphere soil was recorded. Shoot dry biomass was recorded in terms of dry weight of shoot and root/plant at the harvest. Phosphorus content in the shoot was determined calorimetrically by the phosphoric yellow colour method outlined by Jackson (1973).

The AM fungal inoculants were evaluated for *Ocimum basilicum* Var*. thysiflora* Benth individually as well as in different combinations caused an improvement not only in mycorrhizal colonization in root, spore count in rhizosphere soil, and shoot biomass, yield, and phosphoric content in shoots. However, as expected the magnitude of improvement varied with the AM inoculants and their combinations. While maximum improvement in spore count was caused by an inoculum of *G. macrocarpum*. On the other hand combined inoculum of all the three AM fungi was most effective in improving mycorrhizal colonization, shoot and root biomass, and P content in shoots (Table 1). It was also very effective in improving the yield. The results of the present investigation are in conformity with the results reported earlier by (Sivaprasad et al. 1990, Yao 1996, Atti et al. 2010, Lakshman and Kurandwad 2014). By giving importance to plant biomass and phosphorus content, but not neglecting the other characteristics, *Glomus macrocarpum* was found to be the most promising fungus for inoculating *Ocimum basilicum* Var*. thysiflora* Benth in nursery. The next best fungus was *Rhizophagus fasciculatus*. Daft and Nicolson (1972) reported earlier that higher root colonization allows more fungal host contact and more exchange of nutrients, hence better plant growth. In conclusion repeated observations of this kind with other plants will confirm the superiority of multiple inocula and open a new avenue to achieve better productivity.

Table 1. The effect of *G. bagyarajii* VS Mehrotra*, G. macrocarpum* Tul and Tul, *R. fasciculatus* (Thaxt.) C. Walker and A. Schüßler, on growth characteristics of *O. basilicum* Var*. thysiflora* Benth after 90 days of sowing.

Treatments	SDW	RDW	PMC	SN	P content in shoot
Control	1.5±0.2f	0.20±0.01d	33±1.01e	68±0.30f	0.04±0.02f
Glomus bagyarajii (GB)	1.6±0.9e	0.25±0.01d	76±0.10a	129±0.05b	0.08±0.00c
Glomus macrocarpum (GM)	2.1±0.0d	0.45±0.0c	85±0.00b	158±0.02a	0.10±0.01b
Rhizophagus fasciculatus (RF)	1.8±0.0e	0.33±0.01d	80±0.05a	116±0.10c	0.08±0.00c
GB + GM	3.9±0.0c	0.71±0.0b	71±0.10c	113±0.03d	0.13±0.00a
GB + RF	3.7±0.0d	0.52±0.02	76±0.20b	109±0.04d	0.12±0.00c
GM + RF	5.7±0.0b	0.68±0.0b	67±0.01d	102±0.06e	0.11±0.00d
GB + GM + RF	9.7±0.0a	0.93±0.0a	90±0.00a	100±0.00e	0.20±0.00a

Data represents means ± SE of 3 replicates; each experiment was repeated thrice. Mean separation within column by Duncan's multiple range test (DMRT) at P<0.

SDW-Shoot Dry Weight, RDW-Root Dry Weight, PMC- Percent Mycorrhizal Colonization, SN- Spore Number, RF- *Rhizophagus fasciculatus*, GM- *Glomus macrocarpum,* GB- *Glomus bagyarajii*.

References

Atti T, Danny C, Fabien H, Louis L, Andres W and Fritz O (2010). Efficacy ofindigenous arbuscular mycorrhizal fungi for promoting white yam (*Dioscorea rotundata*) growth in West Africa. Applied Soil Ecology 45: 92-100.

Daft MJ and Nicolson TH (1972). Effect of endogone mycorrhiza on plant growth IV. Quantitative relationship between the growth of the host and the development of the endophyte in tomato and maize, New Phytologist 71: 287-295.

Gerdemann JW and Nicolson TH (1963). Spores of mycorrhizal endogone species extracted from the soil by wet sieving and decanting. Transactions of the British Mycological Society 46: 235-244.

Jackson (1973). Soil chemical analysis. Prantice Hall of India. Pvt. Ltd. New Delhi, 284 pp.

Jone E and Jakobsen I (1995). Growth and extracellular phosphatase activity of arbuscular Mycorrhizal hyphae as influenced by soil organic matter. Soil organic matter. Soil Biology and Biochemistry 27: 1153-1159.

Lakshman HC (2009). Interaction between AM fungi and *Rhizobium* and their effect on *Cassia occidentalis.* National Journal of Plant Science 15(1): 25-30.

Lakshman HC and Kurandawad JM (2014). Selection of suitable AM fungi for *Capsicum annum* L. Bulletin of Basic and Applied Biology 3(2): 89-95.

McArther DAJ and Knowles NR (1993). Influence of VAM and phosphorus nutrition on growth, development and mineral nutrition of potato. Plant Physiology 102: 771-782.

Phillips M and Hayman DS (1970). Improved procedure for clearing roots and staining, parasitic and VAM fungi for rapid assessment of infection, Transactions of the British Mycological Society 55: 158-160.

Sunder SK, Palavesam A and Parthipan B (2011). AM fungal diversity in selected medicinal plants of Kanyakumari district, Tamil Nadu, India. Indian Journal of Microbiology 54(3): 259-265.

Sivaprasad P, Sulochana KK and Nair SK (1990). Comparative efficiency of different VAM fungi on cassava (*Manihot esculenta*). Journal of Root Crops 16(1): 39-40.

Yao KM (1996). Influence de difference especes de champignons endomycorrhiziens sur la croissance et le rendement de cultivars d'oignon (*Allium cepa* L.) soumis a differentes conditions culturales. Memoire de maitrise No. 15508, Universite de Laval, Quebec, Canada.

JOURNAL OF BIO-SCIENCE (J. Bio-Sci.)

CONTENTS

VOLUME 23	2015	Pages
1	**N Hindryawati, GP Maniam, CK Feng and MR Karim.** TREND IN ENZYME IMMOBILIZATION ON NANO MATERIALS FOR TRANSESTERIFICATION TO PRODUCE BIODIESEL: A REVIEW	1-17
2	**J Eshaghi-Rad, A Motallebpour and A Alijanpour.** EFFECT OF PHYSIOGRAPHIC FACTORS ON WOODY SPECIES DIVERSITY IN OAK FORESTS (CASE STUDY: SARDASHT FOREST - IRAN)	19-27
3	**Ashaduzzaman, MJ Hossain and S Akther.** PHYSICO-CHEMICAL PROPERTIES OF WATER OF RAMSHAGAR DIGHI, THE LARGEST MAN MADE HISTORICAL RESERVOIR IN NORTHERN BANGLADESH	29-37
4	**S Andriyono, N Masruroh, ED Masithah, J Triastuti and D Winarni.** EFFECT OF THERMAL STIMULATION ON GONAD MATURATION IN SEA CUCUMBER *PHYLLOPHORUS* SP. (GRUBE, 1840)	39-46
5	**K Biswas and SN Sinha.** ANTIBACTERIAL ACTIVITY OF *TAMARINDUS INDICA* L. AGAINST BACTERIA CAUSING URINARY TRACT INFECTION	47-55
6	**SHA Mahdi, M Hasan, I Mahfuz and M Khalequzzaman.** USE OF DIFFERENT NON-CHEMICAL METHODS FOR THE MANAGEMENT OF ADULT *CALLOSOBRUCHUS MACULATUS* (F.) (COLEOPTERA: BRUCHIDAE) IN STORED CHICKPEA	57-65
7	**I Ahmad, S Ahmed, B Yadav, PP Sah, R Ara, MJ Alam and W Zzaman.** MICROBIAL, PHYSICOCHEMICAL AND SENSORY CHARACTERISTICS ANALYSIS OF SELECTED ALCOHOLIC BEVERAGES OF FROM BANGLADESH, INDIA AND NEPAL	67-75
8	**SMM Ali, HM Zakir, SMS Shahriar, MH Sarkar, HP Nur and M Jesmin.** *IN VIVO* ANTICANCER ACTIVITIES OF Ni (II)-BENZOIN THIOSEMICARBAZONE COMPLEX [$Ni(BTSC)_2$] AGAINST EHRLICH ASCITES CARCINOMA CELLS	77-88
Short Communication		
9	**T Yeasmin, J Puttaradder and HC Lakshman.** EFFECT OF SINGLE AND MULTIPLE AM FUNGAL INOCULANTS ON THE GROWTH PARAMETER OF *OCIMUM BASILICUM* VAR. *THYSIFLORA* BENTH	89-91