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Photo on the cover: *Aconitum napellus* in the Tatry Mountains, Poland. Author: Tomasz M. Karpiński

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BIOLOGY ORIGINAL ARTICLE

Simple models for predicting leaf area of mango (*Mangifera indica* L.)

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ABSTRACT

Mango (*Mangifera indica* L.), one of the most popular tropical fruits, is cultivated in a considerable part of southern Iran. Leaf area is a valuable parameter in mango research, especially plant physiological and nutrition field. Most of available methods for estimating plant leaf area are difficult to apply, expensive and destructive which could in turn destroy the canopy and consequently make it difficult to perform further tests on the same plant. Therefore, a non-destructive method which is simple, inexpensive, and could yield an accurate estimation of leaf area will be a great benefit to researchers. A regression analysis was performed in order to determine the relationship between the leaf area and leaf width, leaf length, dry and fresh weight. For this purpose 50 mango seedlings of local selections were randomly took from a nursery in the Hormozgan province, and different parts of plants were separated in laboratory. Leaf area was measured by different method included leaf area meter, planimeter, ruler (length and width) and the fresh and dry weight of leaves were also measured. The best regression models were statistically selected using Determination Coefficient, Maximum Error, Model Efficiency, Root Mean Square Error and Coefficient of Residual Mass. Overall, based on regression equation, a satisfactory estimation of leaf area was obtained by measuring the non-destructive parameters, i.e. number of leaf per seedling, length of the longest and width of widest leaf (R^2 = 0.88) and also destructive parameters, i.e. dry weight (R^2 = 0.94) and fresh weight (R^2= 0.94) of leaves.

Key words: destructive; leaf area; mango; non-destructive; regression linear models.

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INTRODUCTION

The leaf area measurement is one of the most important parameter in agricultural research especially in plant physiology and nutrition. This parameter is a representative of plant growth and development. Also its relationship with the absorption of light, respiration and photosynthesis is important. The leaf area index is a key structure for forest ecosystems properties and the reason is the roles that green leaves have on controlling physical and biological processes on vegetation cover. Therefore, accurate estimation of leaf area index is necessary for studying ecophysiology, interaction of the atmosphere and ecosystems and global climate change [1]. Leaf area index widely used to describe the vegetation photosynthesis and respiration levels. This index is also used extensively in ecophysiology, and water balance modeling and characterization of vegetation-atmosphere interactions [2].

Determination of the leaf area is necessary for knowing how the energy transfers and dry matter accumulation processes in the vegetation. Leaf area is very important in the analysis of vegetation and also as a factor that makes possible to determinate the light interception, plant growth [3]. Many methods of measuring plants leaf area have been presented but most of them are mix of several measurement models with complex and difficult mathematical equations. For example measuring leaf area by optical methods and image spectroscopy [4] cannot be carried out everywhere. Even in methods such as using digital cameras and calculating the surface by computer programs; although taking photos is fast and very accurate analysis, but because of vast number of leaves this process takes a long time and often equipments are very expensive [5, 6]. Other methods are include, blue printing, photographic and planimeter that they all need to be separated leaves from the plants which cause the destruction of vegetation in order to create special problem in some studies those include plots with limited number of plants to be able to continue alternatively different tests on them [6, 7, 8].

Mango (*Mangifera indica* L.) is one of the worlds oldest and the most popular tropical fruit, because of its wonderful fragrance, flavor, high nutritional value and the beauty of its color variety [9, 10]. Mango as one of the well adapted tropical fruit in south of Iran has been introduced to this region by importing mango seed from India to south of Iran more than 300 years ago. Mango cultivation has been gradually spread to a very narrow costal region of Persian Gulf (geographic coordinates, from 25°, 24´ up to 28°, 57´ in North latitude and from 53°, 41´ up to 59°, 15´ in Eastern longitudes with average rainfall of about 150 to 200 mm/year). At present, mango under grown in Iran is about 4400 hectare and in Hormozgan about 2700 hectare which consists of some old and traditional orchards derived from seedlings and some newly established orchard with grafted new cultivars. Mango cultivation has been considered as one of the most desirable potentials for renovation of the old orchard and development of rural areas in southern provinces of Iran specially Hormozgan, Sistan and Balochestan.

Mango fruit flavor depends on the balance between organic acids (citric acid, malice acid and ascorbic acid) and sugar level solutions (sucrose, fructose and glucose) [11]. Reduction ratio of leaves to fruit could increase the amount of fructose in mango fruit. High value of this ratio increased the rate of sucrose in the fresh and dry weight of the fruit significantly. In general, carbohydrates constitute 60% of fruit dry weight which the main ingredients are sugar and acids. The amount of carbohydrates depends on the rate of the leaves photosynthesis which it also depends on the leaf area and the leaf photosynthetic capacity [12]. According to a recent article we are able to change the mango flavor and the popularity of the fruit by changing or even increasing the number of leaf to fruit ratio.

Estimating the leaf area without separating the leaves of the plant provides a simple, accurate and detailed cost method that can solve many problems of measuring the leaf area, especially in developing countries. Researches in this field results that are using simple regression equations provide a model that includes estimations of the leaf area by measuring the leaf length and the width individually or in combination. In a research [13] to estimate the level of banana leaf area, a model containing both parameters was considered. In both length and width of leaves were shown high correlation coefficient with a banana leaf area ($r = 0.98$). In a study on a cucumber plant [14], it was observed a significant correlation between the length and the width of cucumber leaves, plant fresh weight and

dry weight with leaves area. In another research [15] in order to measure the leaf area of 14 types of fruit trees such as almond, citrus, olives, walnut, pistachio, etc. They reported that regression equations can be used for estimation a branch leaf area with measuring the length of the longest leaf of that branch without separating it and the leave numbers. In other studies [16, 17] observed that using an experimental model could help to estimate the leaf area in the main stem of grapevine based on measuring the number of the leaf in the main stem and the leaf area of the tallest and the smallest leaves. They found that the method is a valid and reliable way to estimate leaf area in the vineyards of Spain (R^2 = 0.94). Other researchers were applied a simple regression model for estimating the leaf area in chestnut successfully [18]. In the present study is followed an attempt to provide a simple model, cheap, fast and without any destroying the leaves (separating leaves from the plant) to measure leaf area in seedling of mango.

MATERIALS AND METHODS

This research was done on the production of mango seedlings in the nursery of Hormozgan province. The first, 50 trees of mango seedlings from nursery of Minab and Roudan city (the two important regions of cultivate mangoes in the Hormozgan province in southern Iran) were selected. Seedlings randomly selected from local varieties (Abassi, Shanai, Klaksorkh, Khooshei, Mikhaki, Sabzanbeh, Halili), those were grown in field conditions. Leaves were sampled during the growing season of plants from February to June 2011 (seedlings age was between 8 to 12 months).Then cut off the seedlings from crown and was transferred to the laboratory. The roots of each plant were carefully brought out of the soil. In laboratory different parts of plants, were separated from each other and the leaves of each plant were counted individually. Fresh weight of leaves per plant was measured. Next, the surface of each separated leaves were measured by the leaf area meter (Li-3100). Also, all figures of the leaves were copied on a paper and the leaf surface was determined with Planimeter device (KP-90 N). Then the length and the width of every seedlings leaves was measured consequently with a ruler (the leaf length, from leaf tip to the junction of the petioles as a leaf length and the width from the widest part of a leaf as a leaf width were measured). Leaves, stems and roots of each seedling separately weighed and then dried at a temperature between 60 to 70°C for 72 hours and at the next stage dry weight of leaves, stems and roots were measured. In this study by measuring some of the parameters of leaves and using conventional regression equations, the leaf area and also the growth in other parts of the plant were estimated. Then the estimated leaf area plotted against the measured values and the models were compared. Also, comparison of quantitative models to calculate the statistic Determination Coefficient (R^2), Root Mean Square Error (RMSE), Maximum Error (ME), Efficiency Factor (EF), Coefficient of Residual Mass (CRM) and the index Mean Square Error (MSE) for each of the models was performed. The calculations of each statistic are listed as follows:

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(E_i - \hat{E})^2}{\sum_{i=1}^{n}(E_i - \bar{E})^2} \qquad RMSE = \sqrt{\frac{\sum_{i=1}^{n}(E_i - \hat{E})^2}{N}}$$

$$ME = \max|E_i - \hat{E}| \qquad EF = \frac{\sum_{i=1}^{n}(E_i - \bar{E})^2 - \sum_{i=1}^{n}(E_i - \hat{E})^2}{\sum_{i=1}^{n}(E_i - \bar{E})^2}$$

$$CRM = \frac{\sum_{i=1}^{n} E_i - \sum_{i=1}^{n} \hat{E}}{\sum_{i=1}^{n} E_i} \qquad MSE = \frac{\sum_{i=1}^{n}(\log E_i - \log \hat{E})^2}{N}$$

Which they show $\hat{E}$ estimated values, E_i measured values, $\bar{E}$ average measured values and n is the number of samples. CRM model tended to estimate higher or lower than the measured values indicate. If CRM value is a positive, indicates an underestimation of the actual amount of leaf area and if CRM is negative, model over estimates leaf area. EF parameter has been used to determine the accuracy of data modeling. The maximum amount is the one that can be achieved when the estimated data and the observed data both shows same amount. The determination coefficient is the indicative extent of the relationship between measured and estimated values. The high and the less amount of R^2 showed the closer or further relationship between estimated and measured values of the leaf area. ME shows the maximum error. RMSE can measure accuracy and validity of training and test data sets. Smaller obtained value the proximity of predicted data with the value measured. If all the estimated and the measured data are the same, the statistics result would be ME = 0, CD = 1, EF = 1 and the CRM = 0 [19, 20, 21].

RESULTS

This study is proposing a simple model to prediction leaf area of various local mango seedlings by measuring of length, width and number of leaves. The regression analysis showed that most of the variation in leaf area values was explained by length and width. Then regression model can be a good alternative method for determining leaf area instead of leaf area meter device (Table 1).

In this experiment the possibility of measuring dry and fresh weight of leaf, stem and also root in mango seedlings by measuring the length and width of leaves were studied. The equations associated with a statistically relevant statistics are given in Table 2.

In some experience that we have to cut the plant for some examination in lab, we can use some simple devices that working with them are simpler than using leaf area meter. Based on, these different regression equations were obtained by using Planimeter and Oven devices. Some of the proposed models with statistically relevant statistics are given in Table 3.

DISCUSSION

Many researchers have been carried out to estimate leaf area through measuring leaf dimensions. In general, leaf length, leaf width, or combinations of these variables have been used as

Table 1. Model of leaf area estimation of mango seedlings in field conditions without destroying the plant, along with relevant a statistically statistics.

Row	Parameter	RMSE	MSE	CRM	R^2	ME	EF
1	$LA=0.2452[(L*W)*N]$	149.12	0.0205	0.17070	0.865	1054.45	0.9428
2	$LA=1.0465[(L*W)*N]^{0.8261}$	243.11	0.0076	0.03266	0.896	990.10	0.8481
3	$LA=0.0848(L^2*N)$	476.48	0.0184	0.07586	0.833	819.06	0.9655
4	$LA=0.6979(L^2*N)^{0.7806}$	236.84	0.0087	0.03124	0.881	1011.76	0.8558
5	$LA=0.259(S_W*N)$	268.62	0.9245	0.05630	0.814	792.98	0.8145
6	$LA=1.6939(S_W*N)^{0.7756}$	271.35	0.0103	0.03799	0.859	1046.80	0.8108
7	$LA=0.2286(S_L*N)+161.56$	271.01	0.0113	0.00011	0.811	1012.31	0.8112
8	$LA=1.4509(S_L*N)^{0.7967}$	275.63	0.0104	0.03393	0.858	1102.74	0.8047

LA: leaf area measured by leaf area meter; SL: leaf area of longest leaf; SW: leaf area of widest leaf; L: length of longest leaf; W: width of widest leaf; N: number of leaf per seedling. Note: Sequence of the models in the table above is according to the preference ($P < 0.01$).

Table 2. Comparison of different models to estimate of leaves dry and fresh weight, fresh weight of stem and root dry weight of mango seedlings in field conditions without destroying the plant.

Row	Parameter	RMSE	MSE	CRM	R^2	ME	EF
9	$DW_{(root)}=0.0017(W^2*N)+1.2146$	1.102	0.01899	-0.00614	0.6472	2.295	0.6470
10	$FW_{(stem)}=0.0145[(L+W)*N]+3.1817$	3.845	0.02387	-0.00591	0.7244	8.395	0.9370
11	$FW_{(leaf)}=0.0051[(W*L)*N]$	2.741	0.02245	-0.02212	0.8637	19.449	0.9651
12	$DW_{(leaf)}=0.0021[(W*L)*N]$	2.236	0.14503	0.03974	0.8391	7.416	0.8386

DW: dry weight; FW: fresh weight, L: length of longest leaf; W: width of widest leaf; N: number of leaf per seedling ($P < 0.01$).

Table 3. Comparison of quantitative different models to estimate leaf area of mango seedlings in field conditions.

Row	Parameter	RMSE	MSE	CRM	R^2	ME	EF
13	LA=0.9932*P	6.727	0.000027	-0.0023	0.9999	8.167	0.9988
14	LA=0.6876*R	35.226	0.000541	-0.0061	0.9968	106.448	0.9968
15	LA=46.97*FW	105.199	0.017207	0.0732	0.9420	373.451	0.9977
16	LA=113.41*DW	143.932	0.007166	0.0192	0.9468	454.160	0.9468

LA: leaf area measured by leaf area meter; P: leaf area measured by planimeter; R: leaf area measured by ruler; DW: dry weight; FW: fresh weight ($P < 0.01$).

parameters of leaf area models. In some researches it is necessary to measure the leaf area of leaves, without damaging the plant and continue testing while the plant is performing its functions.

The non-destruction of the plant (leaves) and consecutive measurements at a desired time period is especially important. In this direction many researches do modeling for estimating the leaf area a garden and crop plants has been done without the destruction of vegetation [7, 8, 13, 14, 15, 22]. In this part of the experiment, in order to minimize the time, facilitate the determination and also to obtain an accurate estimation of the leaf area, we developed simple methods, involving the measurement of some parameters like the length, width and the number of leaves per plant. Based on statistically statics, the reliable models were compared and the best models according to table one was found. All of the length-width models can provide accurate estimations of mango seedlings leaf area, but comparison between models shows that models 1&2 from Table 1 are preferred due to higher R^2, lower RMSE and higher EF, in spite of using more leaf dimension characteristics than other models. Following the discussion with equations 3 and 4 were observed despite the use of

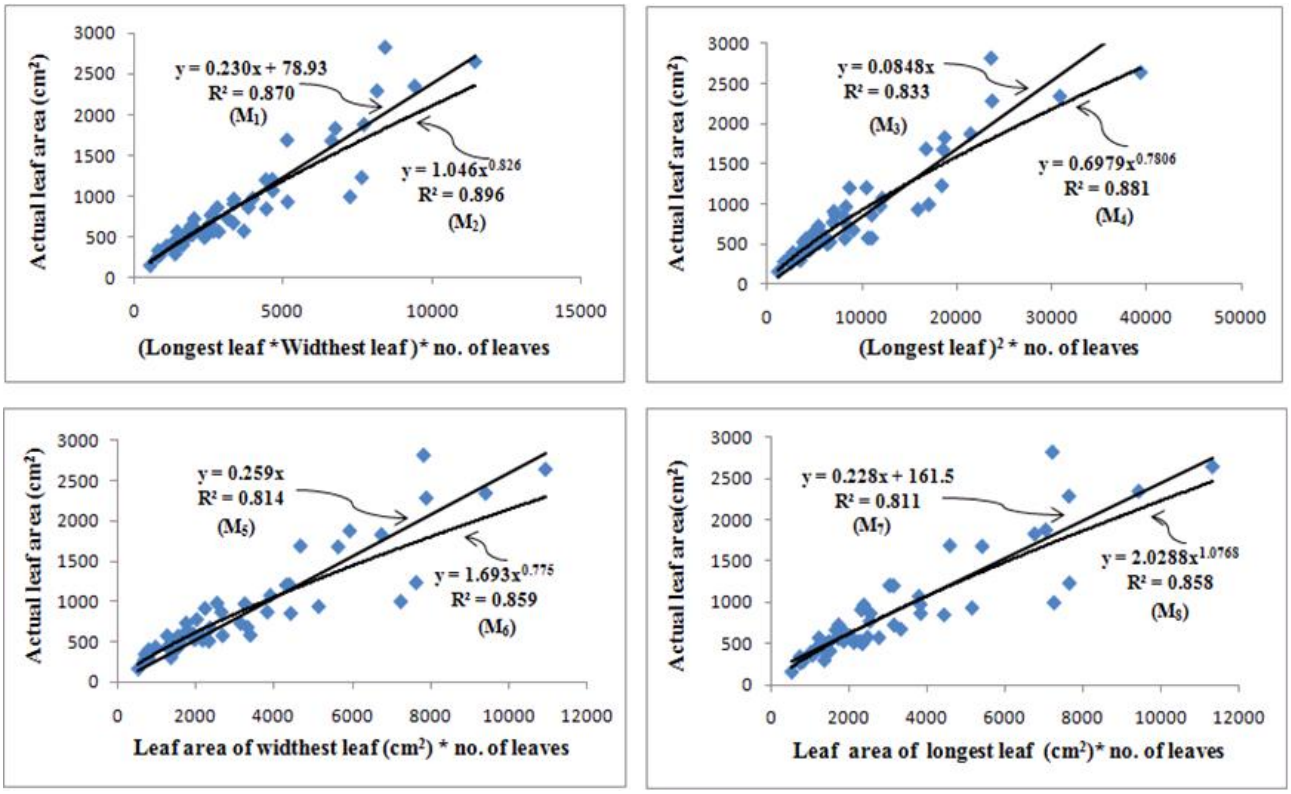

Fig. 1. Relationship between actual leaf area (measured by leaf area meter devices) with leaf area model estimating (by ruler) from table one (parameter from 1 to 8 corresponding models M1 to M8).

a variable measuring (only leaf length) due to lower R^2 and higher RMSE the previous equations in comparison with these equations were preferred. After that sequence of the models (from 5 to 8) in the Table 1 is according to statistically statics preference. The different non-distractive model (the linear and exponential regression) and relationship between actual leaf area and leaf area measured with dimensions of leaves were showed in Figure 1.

Results from the present study were in accordance with some of the previous studies on establishing reliable equations for predicting leaf area through measuring leaf dimensions. Leaf area estimation models in some species of fruit trees and some other plants such as chestnut [18], grapevines [23], peach [24], pecan [25], bergenia purpurascens [26], cabbage and broccoli [27], grapevine [28] and basil [29], were developed using leaf length, width and number of leaf per shoot as performed in this study.

Further research for estimating, the fresh and dry weight of leaves by measuring the length and width of leaf without separating the leaf from the plant, was obtained by several equations that the best of them was selected in based on the comparison statistically statics between the equations. Figure 2 shows the linear relationship between leaf dry and fresh weight, with the width of the widest leaf times the length of the longest leaf leaves times the number of leaves with relatively high determination coefficient (R^2=0.86, R^2=0.84). According to these results, leaf length, width and number of leaf contribute to accurately determine fresh and dry weight of mango. Result of this study is approved by some of the previous studies on cucumber [14], on pistachio [30], and on cotton [31].

To validate the developed models for the estimation of stem fresh weight and root dry weight of seedlings without damaging the plants, measured and estimated data were compared.The fresh weight of stem, with the sum of the width of the widest leaf and the length of the longest leaf times

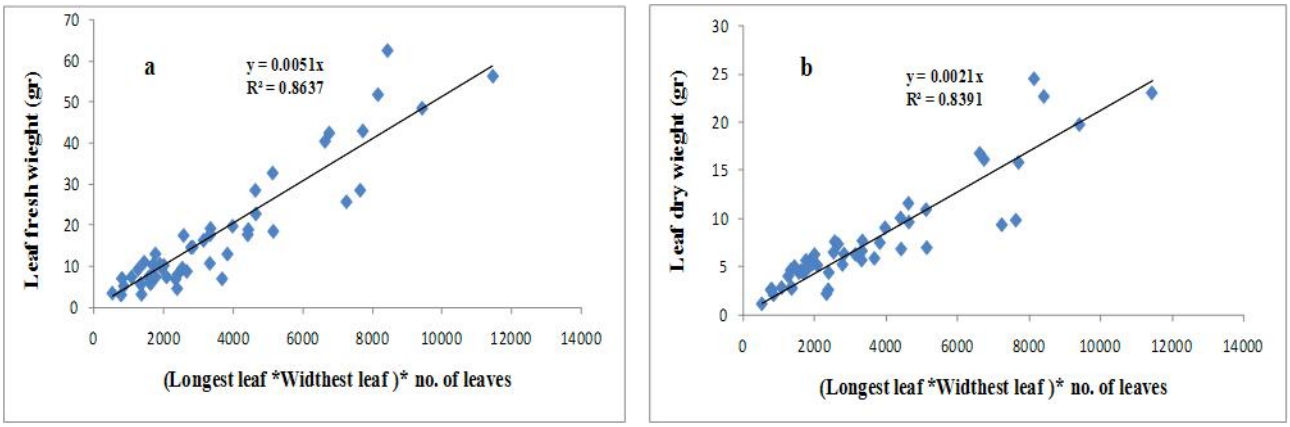

Fig. 2. Relationship between leaf fresh weight (a) and leaf dry weights (b) with leaf area model estimating from table two (parameters 11 and 12).

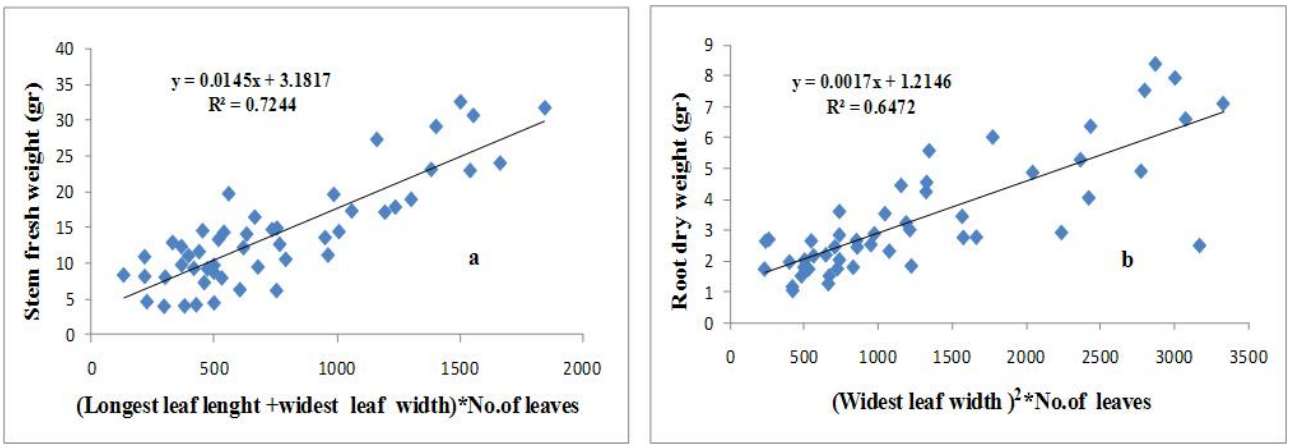

Fig. 3. Relationship between stem fresh weight (a), root dry weight (b) with leaf area model estimating from table two (parameters 9 and 10).

the number of leaves show a significant linear regression relationship (R^2 = 0.72). Root dry weight with the square of the width of the widest times the number of leaves show relatively a good determination coefficient (R^2 = 0.65). Linear relationship can be seen in Figure 3.

However high determination coefficient for estimating the root dry weight was not obtained in this model, but due to the importance of knowing the amount of root growth in physiological and nutritional studies, and considering the difficulties in directly measuring the root, this model can be a good guide to solve this problem. Results obtained from this research are consistent with the research results conducted on cucumber [14].

As can be seen in rows 13 and 14 in Table 3, measuring the leaf area with ruler and planimeter are beneficial methods for determining the leaf area of mango leaves, according to the determination coefficient of regression models (R^2 = 0.99). Other statistics in rows 13 and 14 in Table 3 are also confirmed this. Linear relationship with the leaf area and two methods (planimeter and ruler) is shown in Figure 4.

Each of the recommended methods has some advantages compare to the method using a leaf area meter. Planimeter device, is used in the planimeter method, in comparison with the leaf area meter is much cheaper and more available, due to its light weight, and being small and portable to different locations. Since the leaf area measured by a planimeter had an extremely high correlation with the measurement using a leaf area meter it was considered a suitable alternative device. Another way is measuring the length and the width of the seedling leaves with a ruler. Although it takes a lot of time to measure leaf area but the simplicity and extremely low cost compared to the other methods and considering that the amount of leaf area measured with this method strongly agreed with the actual amount of the leaf area, makes it a good way to determine the leaf area. In particular, it allows measuring without separating the leaves of the seedlings and therefore not destroying it during the measuring procedure.

Other possible proposed methods of predicting the leaf area of mango leaves are measurements of fresh and dry weight of leaves and then using them

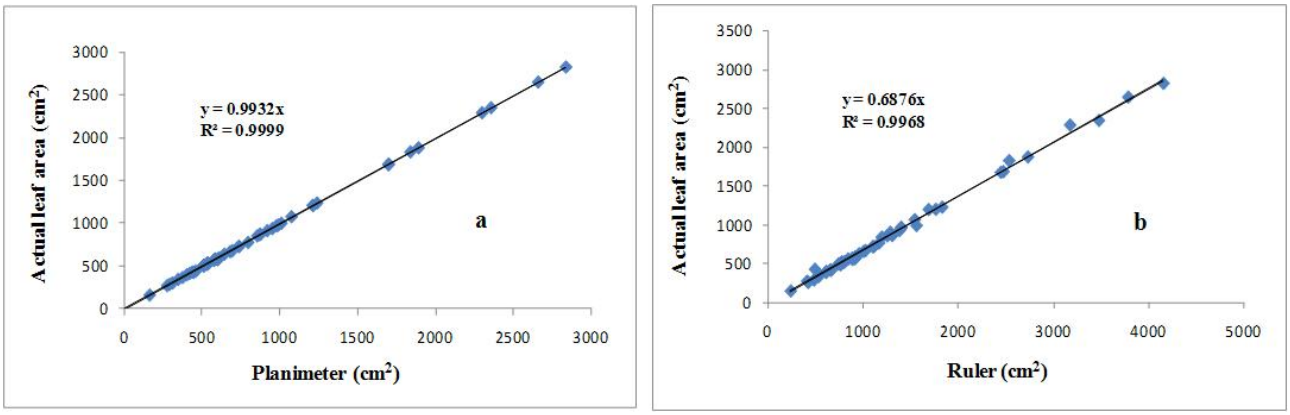

Fig. 4. Relationship between leaf area with leaf area meter and digital planimeter (a) and ruler (b).

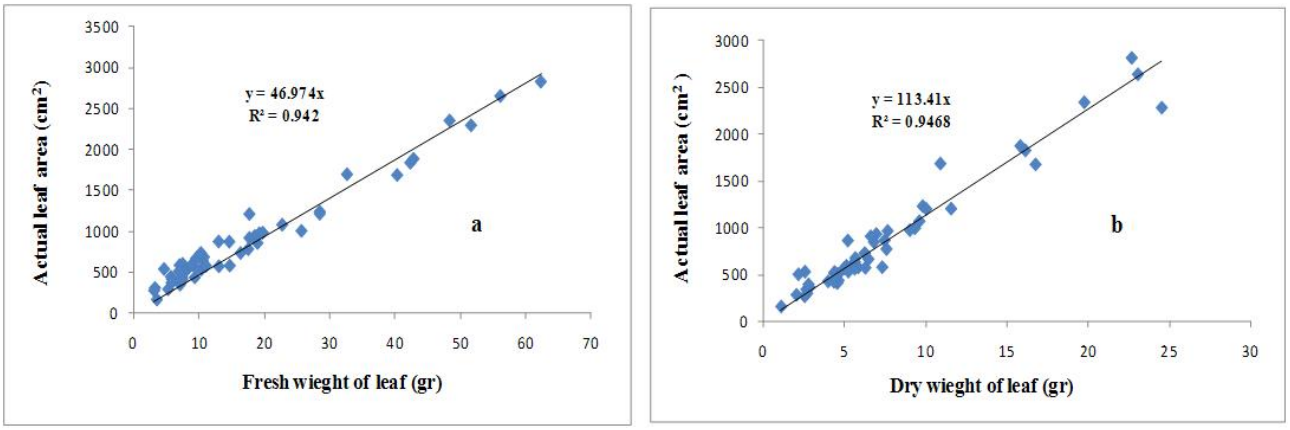

Fig. 5. Relationship between leaf area with leaf area meter with fresh weight (a) and dry weight (b) of mango leaves.

in the regression models, respectively, in rows 15 and 16 are given in Table 3, could be obtained reasonably estimated from the leaf area due to high R^2 and low RMSE, these models were validated with other research on basil [29], and on peanut [32]. Linear relationship between the leaf area and each of the methods of fresh weight, dry weight is shown in Figure 5.

These two methods are much simpler and faster than methods of planimeter and ruler, while the other two methods require measurement of all the leaves separately. However the disadvantage is that, unlike the method of measuring with a ruler (measuring without separating), the leaves of the plant could be separated. As mentioned, the comparison between the two methods of fresh weight and dry weight shows despite the determination coefficient is almost the same, using the fresh weight method is preferred, first, because it is time saving (no need for spending time in the oven for drying leaves) and secondly, as a fresh leaf tissue is required in many experiments (including determination of chlorophyll, sugars, hormones, antioxidants, etc). In general, these methods are usually used in situations where the researchers have to do chemical tests on the elements of plant and the destruction of vegetation is not considered in research.

CONCLUSION

In this case study, the simple regression models were obtained to estimate the leaf area of mango that can be used with high percentage confidence in the physiological and nutritional studies. The results indicate that the leaf area of a mango seedling with high speed and accuracy can be achieved by measuring the length of the longest leaf, width of the widest leaf and also number of leaf without using expensive equipments. Models were time saving and easily predicted in the field conditions. A point to note is the estimation method of the leaf area without destruction; it makes possible to measure of leaf area in reload period of the plant growth. Thus this model can be convenient and quick alternative, especially at places where there is no access to modern equipment or other devices for measuring the leaf area.

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TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Chen JM, Rich PM, Gower ST, Norman JM, Plummer S. Leaf area index of boreal forests: theory, techniques, and measurements. J Geophys Res. 1997; 102(D24): 29429-29443.
2. Rich PM, Chen J, Sulatycki SJ, Vashisht R, Wachspress WS. 1995. Calculation of leaf area index and other canopy indices from gap fraction: a manual for the LAICALC software. Kansas Applied Remote Sensing Program Open File Report.
3. Bhatt M, Chanda SV. Prediction of leaf area in *Phaseolus vulgaris* by non-destructive method. Bulg J Plant Physiol. 2003; 29(1-2): 96-100.
4. Gosa AG, Schaepman-Strub G, Kooistra L, Schaepman M, Pellikka P. 2007. Estimation of leaf area index using optical field intriments and imaging spectroscopy. Proceeding 5th Earsel Workshop on Imaging Spectroscopy. Bruges, Belgium.
5. Bignami C, Rossini F. Image analysis estimation of leaf area index and plant size of young hazelnut plants. J Horticult Sci. 1996; 71: 113-121.
6. Lu HY, Lu CT, Wei ML, Chan LF. Comparison of different models for nondestructive leaf area estimation in taro. Agron J. 2004; 96: 448-453.
7. Cristofori V, Rouphael Y, Gyves EM, Bignami C. A simple model for estimating leaf area of hazelnut from linear measurements. Scient Horticult. 2007; 113: 221-225.
8. Ugese FD, Baiyeri KP, Mbah BN. Leaf area determination of shea butter tree (*Vitellaria paradoxa* C. F. Gaerts). Int Agrophys. 2008; 22: 167-170.
9. Tahir FM, Ibrahim M, Hamid K. Seasonal variation in nutrient concentration of bearing and non-bearing terminals in mango (*Mangifera indica* L.). Asian J Plant Sci. 2003; 2(1): 113-115.
10. Shafique MZ, Ibrahim M, Helali MOH, Biswas SK. Studies on the physiological composition of different mango cultivars at various maturity levels. Bangladesh J Sci Ind Res. 2006; 41(1-2): 101-108.
11. Medlicott AP, Thompson AK. Analysis of sugars and organic acids in ripening mango fruits (*Mangifera indica* L. var Keitt) by high performance liquid chromatography. J Sci Food Agricult. 1985; 36: 561-566.

12. Léchaudel M, Joas J, Caro Y, Genard M, Jannoyer M. Leaf:fruit ratio and irrigation supply affect seasonal changes in minerals, organic acids and sugars of mango fruit. J Sci Food Agricult. 2005; 85: 251-260.
13. Potdar MV, Pawar KR. Non-destructive leaf area estimation in banana. Scient Horticult. 1991; 45: 251-254.
14. Cho YY, Oh S, Oh MM, Son JE. Estimation of individual leaf area, fresh weight and dry weight of hydroponically grown cucumbers (*Cucumis sativus* L.) using leaf length, width, and SPAD value. Scient Horticult. 2007; 111: 330-334.
15. Spann TM, Heerema RJ. A simple method for non-destructive estimation of total shoot leaf area in tree fruit crops. Scient Horticult. 2010; 125: 528-533.
16. Lopes C, Pinto PA. Easy and accurate estimation of grapevine leaf area with simple mathematical models. Vitis. 2005; 44(2): 55-61.
17. Sanchez-de-Miguel P, Junquera P, De la Fuente M, Jimenez L, Linares R, Baeza P, Lissarrague R. Estimation of vineyard leaf area by linear regression. Span J Agricult Res. 2011; 9(1): 202-212.
18. Serdar U, Demirsoy H. Non-destructive leaf area estimation in chestnut. Scient Horticult. 2006; 108: 227-230.
19. Mohammadi A, Mosaede A, Heshmatpour A. Determination of the best model to estimate suspended sediment loads in Gbazaghly gauge station-Gorganrood River, Iran. J Agricult Sci Nat Res. 2007; 14(4): 232-240.
20. Memarian Fard M, Beigi Harchagani H. Comparison of artificial neural network and regression pedotransfer functions models for prediction of soil cation exchange capacity in Chaharmahal-Bakhtiari province. J Water Soil Sci. 2009; 23(4): 90-99.
21. Zare Abyaneh H, Moghaddamnia A, Bayat Varkeshi M, Ghasami A, Shadmani M. Spatial variability of pan evaporation in Iran and its estimation using several empirical models. J Water Soil Sci. 2010; 20/1(4): 113-130.
22. Giuffrida F, Rouphael Y, Toscano S, Scuderi D, Romano D, Rivera CM, Collia G, Leonardi C. A simple model for nondestructive leaf area estimation in bedding plants. Photosynthetica. 2011; 49(3): 380-388.
23. Williams III L, Martinson TE. Nondestructive leaf area estimation of 'Niagara' and 'Dechaunac' grapevines. Scient Horticult. 2003; 98: 493–498.
24. Demirsoy H, Demirsoy L, Uzun S, Ersoy B. Non-destructive leaf area estimation in peach. Eur J Horticult Sci. 2004; 69(4): S.144-S.146.
25. Torri SI, Carla Descalzi C, Frusso E. Estimation of leaf area in pecan cultivars (*Carya illinoinensis*). Cienciae Investigación Agraria. 2009; 36(1): 53-58.
26. Zhang L, Liu XS. Non-distractive leaf area estimating for *Bergenia purpurascens* acrass timberline ecotone, southeast Tibet. Annales Botanici Fennici 2010; 47: 346-352.
27. Olfati JA, Peyvast Gh, Shabani H, Nosratie-Rad Z. An estimation of individual leaf area in Cabbage and Broccoli using non-destructive methods. J Agricult Sci Technol. 2010; 12: 627-632.
28. Beslic Z, Todic S, Tesic D. Validation of non-destructive methodology of grapevine leaf area estimation on cv. blaufränkisch (*Vitis vinifera* L.). S Afr J Enol Viticult. 2010; 31(1): 21-25.
29. Mousavi Bazaz A, Karimian Fariman Z, Bannayan M. Modeling individual leaf area of basil (*Ocimum basilicum*) using different methods. Int J Plant Prod. 2011; 5(4): 439-447.
30. Karimi S, Tavallali V, Rahemi M, Rostami AA, Vaezpour M. Estimation of leaf growth on the basis of measurements of leaf lengths and widths, choosing pistachio seedlings as model. Aust J Basic Appl Sci. 2009; 3(2): 1070-1075.
31. Akram-Ghaderi F, Soltani A. Leaf area relationships to plant vegetative characteristics in cotton (*Gossypium hirsutum* L.) grown in a temperate sub humid environment. Int J Plant Prod. 2007; 1: 63-71.
32. Ma L, Gardner FP, Selamat A. Estimation of leaf area from leaf and total mass measurements in peanut. Crop Sci. 1992; 32: 467-471.

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BIOLOGY ORIGINAL ARTICLE

Molluscicidal activity of *Morus nigra* against the freshwater snail *Lymnaea acuminata*

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ABSTRACT

The molluscicidal activity of *Morus nigra* fruit, bark and leaf powder against the snail *Lymnaea acuminata* was time and concentration dependent. Toxicity of fruit powder (96h LC_{50}: 166.92 mg/L) was more pronounced in comparison to bark powder (96h LC_{50}: 173.17 mg/L) and leaf powder (96h LC_{50}: 173.69 mg/L). Ethanolic extracts of *M. nigra* fruit, bark and leaf was more toxic than their other organic solvent extracts. The molluscicidal activity of ethanolic extract of *M. nigra* fruit powder (24h LC_{50}: 116.23 mg/L) was more effective than the ethanolic extract of bark powder (24h LC_{50}: 154.41 mg/L) and leaf powder (24h LC_{50}: 139.80 mg/L). The 96h LC_{50} of column-purified fraction of *M. nigra* fruit powder was, 10.03 mg/L whereas that of bark and leaf powder was 8.69 mg/L and 4.97 mg/L, respectively. Column and thin layer chromatography analysis demonstrates that the active molluscicidal component in *M. nigra* is quercetin (96h LC_{50}: 1.11 mg/L), apigenin (96h LC_{50}: 1.92 mg/L) and morusin (96h LC_{50}: 2.12 mg/L), respectively. Co-migration of quercetin (R_f 0.49), apigenin (R_f 0.51) and morusin (R_f 0.52) with column-purified fruit, bark and leaf of *M. nigra* on thin layer chromatography demonstrates same Rf value. The present study indicates that *M. nigra* may be used as potent source of molluscicides against the snail *Lymnaea acuminata*.

Key words: *Lymnaea acuminata*, *Morus nigra*, fasciolosis, plant molluscicide.

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INTRODUCTION

Fasciolosis is an important helminth disease caused by two trematodes *Fasciola hepatica* and *F. gigantica*. This disease belongs to the plant-borne trematode zoonoses. Fasciolosis is a zoonosis, i.e. a disease of animals that can be transmitted to humans. Cattle and human fasciolosis is a major public health problem in several areas of the world [1, 2]. In Europe, the Americas and Oceania only *F. hepatica* is a concern, but the distributions of both species overlap in many areas of Africa and Asia [3]. The definitive host range is very broad and includes many herbivorous mammals, including humans. Its life cycle includes freshwater snails (*Lymnaeidae*) as an intermediate host [4]. The worldwide losses in animal productivity due to fasciolosis were conservatively estimated at over US$ 3.2 billion per annum [5]. In addition, fasciolosis is now regarded as an emerging human disease: the World Health Organization (WHO) has estimated that 2.4 million people are infected with *Fasciola*, and a further 180 million are at risk of infection [6].

Studies carried out in recent years have shown human fasciolosis to be an important public health problem [7]. Human cases of fasciolosis have also been reported from India [8]. Human fasciolosis has been reported from countries in Europe, America, Asia, Africa, and Oceania. Humans are infected by ingestion of aquatic plants that contain the infected metacercariae [9]. Because *F. hepatica* cercariae also encyst on water surface, humans can be infected by drinking of fresh untreated water containing metacercariae [7].

The control of snail population is one of the major tools to reduce the incidence of fasciolosis in cattle as well as human being [1, 3, 10]. One of the most efficient methods for preventing the spread of fasciolosis is the use of molluscicides [1, 3]. The molluscicides of plant origin are gaining special importance in comparison to synthetic counterpart, because they are more effective, cheaper and safer to non-target organisms and culturally acceptable [1, 11]. Many plant products have been found to have a high molluscicidal potential [12, 13].

Morus nigra L. (*Moraceae*) belongs to the genus *Morus* and is found in Africa, South America and in Asia. *M. nigra* has been used in Unani medicine as antitussive, diuretic, expectorant and hypotensive. It has wide range of medicinal uses and can be used either as single drug or compound drugs to treat different ailments. The phenolic compounds of *M. nigra* have anti-oxidant and anti-bacterial activities. The bark of *M. nigra* has been used as antihelmintic and its extracts have antibacterial and fungicidal activity [14]. In the present study the molluscicidal activity of the fruit, bark and leaf of *Morus nigra* against the target snail *Lymnaea acuminata* has been evaluated to explore its full potential in control of fasciolosis.

MATERIALS AND METHODS

Morus nigra fruit, bark and leaf were collected from Gorakhpur (India) and were dried separately.

Preparation of fruit, bark and leaf powder

Dried fruit, bark and leaf were pulverized separately in the electric grinder and the crude powders obtained were then used separately for toxicity experiments.

Organic solvent extracts

Five gram of fruit, bark and leaf powder of *Morus nigra* were extracted separately with 100 mL of each solvent viz. ethanol, ether, carbon tetrachloride, acetone and chloroform at room temperature for 24h. Each preparation was filtered separately through sterilized Whatmann No.1 filter paper [15] and the filtered extracts were subsequently evaporated at 40°C under vacuum. The residues thus obtained were used for the determination of molluscicidal activity. Fruit crude powder of *M. nigra* yielded 320 mg of ethanol extract, 310 mg of ether extract, 290 mg of carbon tetrachloride extract, 380 mg of acetone extract, and 335 mg of chloroform extract. Bark crude powder of *M. nigra* yielded 300 mg of ethanol extract, 325 mg of ether extract, 340 mg of carbon tetrachloride extract, 360 mg of acetone extract and 295 mg of chloroform extract. Leaf crude powder of *M. nigra* yielded 305 mg of ethanol extract, 315 mg of ether extract, 345 mg of carbon tetrachloride extract, 400 mg of acetone extract and 350 mg of chloroform extract.

Column chromatography

The ethanolic extract of *M. nigra* fruit, bark and leaf powder was obtained by dissolving 1000 mg of each in 25 mL of ethanol separately. Further the ethanolic extract of *M. nigra* fruit, bark and leaf

powder was subjected to silica gel (60-120 mesh, Qualigens glass, Precious Electrochemindus Private Limited, Mumbai, India) chromatography through a 5×45 cm column. 10 milliliters fractions of 32 elutents (fruit powder), 35 elutents (bark powder), 37 elutents (leaf powder) were eluted with 95% ethanol for each column preparation. Ethanol was evaporated under vacuum and the remaining solids obtained from all the 10 mL elutents were used for the determination of molluscicidal activity.

Pure compounds

Quercetin (3,3,4,5,7-pentahydroxyflavone) was purchased from Sigma Chemical Co. USA. Apigenin (4´,5,7-trihydroxyflavone) was isolated by the method of Liu et al. [16] and morusin 2-(2,4-dihydroxyphenyl)-5-hydroxy-8,8-dimethyl-3-(3-methyl-2-butenyl)-4H, 8H-benzo[1,2-b:3,4-b´] dipyran-4-one was isolated by the method of Tati et al. [17].

Thin layer chromatography

Thin Layer Chromatography (TLC) was performed by the method of Jaiswal and Singh [18] to identify the active molluscicidal component present in the fruit, bark and leaf powders of M. nigra. TLC was done on 20×20 cm pre-coated silica gel (Precious Electrochemical Industry, Pvt. Ltd., Mumbai, India) using benzene/ethyl acetate (9:1, v:v) as the mobile phase. Spots of column-purified fractions of *M. nigra* fruit, bark and leaf along with their respective active components were applied on TLC plates with a micropipette. Further, the TLC plates were developed by I_2 vapor. Copies of chromatogram were made by tracing the plates immediately and retardation factors (R_f) were calculated.

Collection of snails

The adult freshwater snails, *L. acuminata* (2.25 ± 0.20 cm in length) were collected locally from different ponds of Gorakhpur. The collected snails were acclimatized for 72h in the laboratory condition. They were kept in a glass aquarium containing de-chlorinated tap water at 22-24°C. The pH of the water was 7.1-7.3 and dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were 6.5-7.2, 5.2-6.3 and 102-105 mg/L, respectively.

Treatment protocol for concentration-response relationship

The toxicity experiments were performed by the method of Singh and Agarwal [19]. Ten experimental animals were kept in a glass aquarium containing 3L of de-chlorinated tap water. Snails were exposed continuously for 96h to different concentrations of plant products separately (Table 1). Six aquaria were set up for each concentration. The control animals were kept in the equal volume

Table.1. Concentration of different preparations of fruit, bark and leaf powder and active components of *Morus nigra* used in toxicity trial against *Lymnaea acuminata*.

Plant material used	Concentration (mg/L)
Morus nigra fruit powder	150, 200, 250, 300
Ethanol extract	30, 50, 70, 90
Ether extract	30, 50, 70, 90
Carbon tetrachloride extract	30, 50, 70, 90
Acetone extract	50, 70, 90, 110
Chloroform extract	30, 50, 70, 90
Column purified	7, 10, 15, 20
Quercetin	1, 3, 5, 7
Morus nigra bark powder	150, 200, 250, 300
Ethanol extract	30, 50, 70, 90
Ether extract	30, 50, 70, 90
Carbon tetrachloride extract	30, 50, 70, 90
Acetone extract	50, 70, 90, 110
Chloroform extract	30, 50, 70, 90
Column purified	9, 15, 25, 35
Apigenin	1, 3, 5, 7
Morus nigra leaf powder	100, 150, 200, 250
Ethanol extract	30, 50, 70, 90
Ether extract	30, 50, 70, 90
Carbon tetrachloride extract	30, 50, 70, 90
Acetone extract	50, 70, 90, 110
Chloroform extract	30, 50, 70, 90
Column purified	7, 10, 30, 50
Morusin	1, 3, 5, 7

of water under similar conditions without treatment. Mortality of snails was recorded at the interval of 24h each; upto 96h. The dead animals were removed immediately to avoid contamination in aquarium water. The mortality of snails was established by the contraction of body within the shell; no response to needle probe was taken as evidence of death. The LC values, lower and upper confidence limits (LCL and UCL), slope values, t-ratio, g-values and heterogeneity factors were calculated by using POLO computer software of Robertson et al. [20].

The regression coefficient between exposure time and different values of LC_{50} was determined by the method of Sokal and Rohlf [21].

RESULTS

The toxicity of fruit, bark and leaf powder of *M. nigra* and their different organic solvent extracts was time and concentration dependent. The 24h LC_{50} of the fruit, bark and leaf powder were 353.21, 325.19 and 377.90 mg/L, respectively. At 96h, LC_{50} values were 166.92, 173.17 and 173.69 mg/L, respectively (Table 2, 3 and 4). Maximum toxicity among different organic solvent extracts of fruit,

Table 2. Toxicity of *Morus nigra* fruit powder, its different organic solvent extracts, column-purified fractions and active component quercetin against the snail *Lymnaea acuminata* at different exposure periods.

Exposure Period	Tested Materials	LC_{50} mg/l	Limits LCL-UCL	Slope Value	t-ratio	g-value	Heterogeneity
	Fruit Powder	353.21	302.52-505.35	4.05±.92	4.40	0.20	0.23
	Ethanol Extract	116.23	92.12-196.92	2.67±.60	4.47	0.19	0.24
	Ether Extract	168.12	115.27-559.99	2.38±.67	3.58	0.30	0.33
24h	CCl_4 Extract	151.38	106.05-457.94	2.15±.60	3.61	0.30	0.20
	Acetone Extract	148.14	117.17-292.33	2.94±.79	3.74	0.27	0.15
	Chloroform Extract	135.53	113.58-204.58	3.73±.85	4.40	0.20	0.26
	Column Purified	26.81	20.75-49.16	2.57±.59	4.35	0.20	0.21
	Quercetin	10.34	7.10-23.85	1.52±.34	4.46	0.19	0.26
	Fruit Powder	310.62	265.47-462.65	2.96±.79	3.77	0.27	0.12
	Ethanol Extract	101.89	81.26-169.69	2.25±.53	4.28	0.21	0.16
	Ether Extract	109.63	81.22-279.96	1.69±.50	3.39	0.34	0.13
48h	CCl_4 Extract	109.18	84.04-212.91	2.04±.52	3.92	0.25	0.22
	Acetone Extract	105.35	91.03-142.36	2.92±.69	4.23	0.22	0.13
	Chloroform Extract	109.06	93.02-155.100	2.77±.69	4.01	0.24	0.10
	Column Purified	23.18	17.59-50.46	1.85±.51	3.62	0.29	0.13
	Quercetin	6.73	4.56-16.03	1.05±.28	3.82	0.26	0.19
	Fruit Powder	222.92	198.50-252.46	3.46±.75	4.59	0.18	0.19
	Ethanol Extract	59.84	49.66-74.33	2.10±.48	4.43	0.20	0.14
	Ether Extract	75.70	62.96-104.85	2.11±.49	4.33	0.20	0.11
72h	CCl_4 Extract	86.62	64.71-244.93	1.35±.47	2.87	0.47	0.12
	Acetone Extract	74.55	62.15-87.64	2.53±.65	3.87	0.26	0.10
	Chloroform Extract	75.27	62.69-89.04	2.45±.65	3.80	0.27	0.11
	Column Purified	13.36	10.99-17.17	1.98±.49	4.08	0.23	0.11
	Quercetin	2.77	1.70-4.08	1.01±.26	3.88	0.26	0.17
	Fruit Powder	166.92	144.33-183.08	4.86±.82	5.89	0.11	0.29
	Ethanol Extract	36.95	27.74-43.75	2.54±.49	5.18	0.14	0.24
	Ether Extract	50.57	36.21-64.81	1.60±.46	3.45	0.32	0.09
96h	CCl_4 Extract	42.65	34.26-49.52	2.64±.49	5.43	0.13	0.19
	Acetone Extract	53.49	38.76-62.37	2.94±.68	4.32	0.20	0.24
	Chloroform Extract	50.23	36.94-58.46	3.32±.70	4.71	0.17	0.25
	Column Purified	10.03	8.11-11.70	2.47±.50	4.96	0.16	0.30
	Quercetin	1.11	0.49-1.65	1.25±.27	4.61	0.18	0.20

Mortality was determined at every 24h up to 96h. Each set of experiment was replicated six times.

Abbreviation: LCL= lower confidence limit; UCL= upper confidence limit. Significant negative regression ($p < 0.05$) was observed between exposure time and LC_{50} of treatments. T_s= testing significance of the regression coefficient of Morus nigra fruit powder (11.08^{+}), ethanol extract (7.81^{+}), ether extract (11.40^{+}), carbon tetrachloride extract (12.81^{++}), acetone extract (17.76^{+}), chloroform extract (18.00^{+}), column-purified (6.81^{+}), quercetin (4.25^{+}). (+) Linear regression between x and y. (++) Non-linear regression between log x and log y.

bark and leaf powder was observed in the ethanolic extract (Table 2, 3 and 4). The column-purified fraction of *M. nigra* was highly toxic. In fruit elutent Nos. 25-30, in bark 25-30 and in leaf elutent No. 30-37 were toxic against the snail *L. acuminata*. The 24h LC_{50} of column-purified fraction of *M. nigra* fruit, bark and leaf were 26.81, 43.30 and 65.61 mg/L and at 96h, LC_{50} was found to be 10.03, 8.69 and 4.97 mg/L, respectively (Table 2,3 and 4). The LC_{50} of quercetin, apigenin and morusin at 24h was 10.34, 12.57, 13.40 mg/L, respectively (Table 2, 3 and 4). Thin layer chromatography analysis demonstrated that the R_f values of quercetin (0.50), apigenin (0.53) and morusin (0.54) were equivalent to the R_f values of the column-purified fractions of *M. nigra* (fruit, bark and leaf).

The slope values were steep and separate estimation of LC based on each of the six replicates was found to be within 95% confidence limits of LC_{50}. The t-ratio was higher than 1.96 and heterogeneity factor was less than 1.0. The g-value was less than 0.5 at all the probability levels i.e. 90, 95, 99. There was significant negative regression ($p < 0.05$) between exposure time and LC_{50} of the treatments (Table 2, 3 and 4).

Table 3. Toxicity of *Morus nigra* bark powder, its different organic solvent extracts, column-purified fractions and active component apigenin against the snail *Lymnaea acuminata* at different exposure periods.

Exposure Period	Tested Materials	LC_{50} mg/l	Limits LCL-UCL	Slope Value	t-ratio	g-value	Heterogeneity
	Bark Powder	325.19	279.23-466.51	3.39±.82	4.13	0.23	0.15
	Ethanol Extract	154.41	122.61-281.19	2.88±.72	4.03	0.24	0.21
	Ether Extract	157.91	110.28-480.58	2.31±.64	3.63	0.29	0.23
24h	CCl_4 Extract	162.47	106.37-831.89	1.76±.55	3.19	0.38	0.09
	Acetone Extract	166.98	122.03-589.50	2.34±.75	3.12	0.40	0.14
	Chloroform Extract	159.42	124.32-315.92	2.73±.70	3.88	0.26	0.16
	Column Purified	43.30	33.39-73.80	2.22±.46	4.85	0.16	0.25
	Apigenin	12.57	8.05-38.11	1.43±.35	4.15	0.22	0.22
	Bark Powder	268.15	236.72-339.78	3.13±.77	4.08	0.23	0.10
	Ethanol Extract	86.48	70.88-129.60	2.16±.50	4.30	0.21	0.16
	Ether Extract	146.84	96.53-917.50	1.53±.51	2.97	0.44	0.19
48h	CCl_4 Extract	130.90	105.36-237.99	2.31±.63	3.66	0.29	0.13
	Acetone Extract	115.73	96.74-182.30	2.62±.69	3.79	0.27	0.14
	Chloroform Extract	99.72	84.08-140.94	2.23±.60	3.69	0.28	0.11
	Column Purified	29.51	23.79-42.92	1.91±.40	4.81	0.17	0.15
	Apigenin	9.31	5.92-30.68	1.07±.29	3.73	0.28	0.16
	Bark Powder	224.37	200.86-253.19	3.59±.76	4.74	0.17	0.21
	Ethanol Extract	54.86	43.76-68.15	1.92±.47	4.09	0.23	0.09
	Ether Extract	80.61	62.89-152.16	1.56±.47	3.29	0.36	0.13
72h	CCl_4 Extract	80.13	68.02-95.32	2.51±.60	4.17	0.22	0.12
	Acetone Extract	78.28	67.72-91.15	2.83±.66	4.29	0.21	0.11
	Chloroform Extract	75.76	60.82-91.81	2.16±.60	3.63	0.29	0.11
	Column Purified	16.59	12.25-21.20	1.58±.38	4.22	0.22	0.13
	Apigenin	4.60	3.06-9.41	0.92±.26	3.47	0.32	0.11
	Bark Powder	173.17	141.38-194.12	3.57±.77	4.65	0.18	0.20
	Ethanol Extract	36.50	19.86-46.66	1.64±.47	3.50	0.31	0.11
	Ether Extract	59.26	45.17-82.89	1.54±.47	3.31	0.35	0.11
96h	CCl_4 Extract	64.64	52.32-73.99	2.83±.61	4.62	0.18	0.12
	Acetone Extract	62.91	48.92-72.47	2.69±.66	4.10	0.23	0.11
	Chloroform Extract	56.18	35.31-67.87	2.14±.60	3.54	0.31	0.11
	Column Purified	8.69	6.01-10.73	2.59±.46	5.68	0.12	0.38
	Apigenin	1.92	1.20-2.58	1.33±.27	4.98	0.16	0.28

Mortality was determined at every 24h up to 96h. Each set of experiment was replicated six times.

Abbreviation: LCL= lower confidence limit; UCL= upper confidence limit. Significant negative regression ($p < 0.05$) was observed between exposure time and LC_{50} of treatments. T_s= testing significance of the regression coefficient of Morus nigra bark powder (13.44^{+}), ethanol extract (10.22^{+}), ether extract (5.69^{+}), carbon tetrachloride extract (9.33^{+}), acetone extract (10.86^{++}), chloroform extract (15.86^{+}), column-purified (9.37^{+}), apigenin (7.20^{+}). (+) Linear regression between x and y. (++) Non-linear regression between log x and log y.

DISCUSSION

The results of the present study clearly indicate that the fruit, bark and leaf powder of *M. nigra* are the potent molluscicides. Their toxic effects are time and concentration dependent as evident from negative regression between exposure period and LC_{50} of different treatments. The time dependent toxic effect of *M. nigra* plant products may be either due to the uptake of the active moiety which progressively increases the amount of active component in the snails body with increase in exposure duration or it might be possible that the active compound(s) could change into more toxic forms in the aquarium water or in the snail's body due to the action of various enzymes. Among the organic solvent extracts, the higher toxicity of ethanolic extracts of *M. nigra* fruit, bark and leaf powder indicates that the molluscicidal component present in fruit, bark and leaf are more soluble in ethanol than other organic solvents. It is evident from co-migration on thin layer chromatographic plates that the molluscicidal activity of *M. nigra* fruit, bark and leaf may be due to the presence of quercetin, apigenin and morusin.

Morus nigra L. (*Moraceae*) belongs to the genus

Table 4. Toxicity of *Morus nigra* leaf powder, its different organic solvent extracts, column-purified fractions and active component morusin against the snail *Lymnaea acuminata* at different exposure periods.

Exposure Period	Tested Materials	LC_{50} mg/l	Limits LCL-UCL	Slope Value	t-ratio	g-value	Heterogeneity
24h	Leaf Powder	377.90	310.86-650.11	3.39±.88	3.84	0.26	0.13
	Ethanol Extract	139.80	103.22-320.08	2.47±.63	3.93	0.25	0.25
	Ether Extract	140.29	101.55-357.31	2.21±.59	3.76	0.27	0.20
	CCl_4 Extract	143.49	102.50-393.90	2.15±.59	3.67	0.29	0.19
	Acetone Extract	154.74	118.18-383.16	2.57±.59	3.41	0.33	0.15
	Chloroform Extract	146.88	101.87-487.38	1.93±.56	3.47	0.32	0.22
	Column Purified	65.61	41.58-178.51	1.18±.26	4.46	0.19	0.21
	Morusin	13.40	8.33-46.16	1.39±.35	4.02	0.24	0.22
48h	Leaf Powder	310.42	266.35-452.61	3.05±.79	3.86	0.26	0.09
	Ethanol Extract	78.82	64.71-115.44	2.00±.49	4.12	0.23	0.11
	Ether Extract	106.56	80.80-232.17	1.82±.50	3.61	0.30	0.14
	CCl_4 Extract	103.89	79.58-214.13	1.85±.50	3.67	0.28	0.13
	Acetone Extract	134.08	106.04-288.89	2.37±.70	3.37	0.34	0.14
	Chloroform Extract	111.63	85.62-220.98	2.06±.53	3.92	0.25	0.15
	Column Purified	21.75	15.28-33.54	1.09±.24	4.50	0.19	0.22
	Morusin	9.31	5.92-30.68	1.07±.29	3.73	0.28	0.16
72h	Leaf Powder	231.87	202.44-276.11	2.88±.75	3.86	0.26	0.15
	Ethanol Extract	57.10	45.70-72.36	1.87±.47	3.98	0.24	0.11
	Ether Extract	71.85	58.92-101.76	1.90±.48	3.97	0.24	0.11
	CCl_4 Extract	67.54	54.98-94.06	1.82±.47	3.85	0.26	0.13
	Acetone Extract	78.59	64.45-97.81	2.19±.65	3.38	0.34	0.10
	Chloroform Extract	77.97	62.94-121.49	1.81±.48	3.78	0.27	0.14
	Column Purified	7.88	3.14-12.04	0.97±.25	3.94	0.25	0.15
	Morusin	3.79	2.67-5.81	1.11±.27	4.18	0.22	0.17
96h	Leaf Powder	173.69	153.50-188.95	5.10±.82	6.20	0.10	0.29
	Ethanol Extract	30.79	22.24-36.92	2.87±.53	5.44	0.13	0.43
	Ether Extract	35.73	19.63-45.59	1.68±.47	3.58	0.30	0.12
	CCl_4 Extract	45.73	35.19-54.62	2.12±.47	4.49	0.19	0.15
	Acetone Extract	55.60	35.10-66.51	2.29±.66	3.49	0.32	0.11
	Chloroform Extract	50.57	36.21-64.81	1.60±.46	3.45	0.32	0.09
	Column Purified	4.97	2.82-6.76	2.01±.37	5.46	0.13	0.40
	Morusin	2.12	1.38-2.84	1.32±.27	4.95	0.16	0.32

Mortality was determined at every 24h up to 96h. Each set of experiment was replicated six times.

Abbreviation: LCL= lower confidence limit; UCL= upper confidence limit. Significant negative regression ($p < 0.05$) was observed between exposure time and LC_{50} of treatments. T_s= testing significance of the regression coefficient of Morus nigra leaf powder (14.36^{+}), ethanol extract (8.31^{+}), ether extract (29.78^{+}), carbon tetrachloride extract (29.11^{+}), acetone extract (8.50^{+}), chloroform extract (14.30^{++}), column-purified (10.61^{+}), morusin (6.04^{+}). (+) Linear regression between x and y. (++) Non- linear regression between log x and log y.

Morus which is widely distributed in Asia, Europe, North and South America and Africa. Mulberry (genus *Morus*) is an economically important plant used for sericulture, as a feed for the domesticated silkworm, *Bombyx mori* [22] and has a long history of medicinal use in Chinese medicine as a herbal medicine called "Sang Bai-Pi" [23]. The fruits are one of the constituent of Unani medicine named "Tut-i-aswad" which is said to be against cancer [24]. The ripe fruit contains about 9% sugar, with malic and citric acid. The juice forms a grateful drink during convalescence after febrile diseases; it checks thirst and cools the blood. The extracts of *M. nigra* fruit were reported to have a protective action against peroxidative damage to biomembranes and biomolecules [25] while the roots methanolic extract showed mushroom tyrosinase inhibitory activity [26]. The bark is purgative and vermifuge. Root bark contains tannins, phlobaphenes, a sugar, a phytosterol (m.p.132°), ceryl alcohol, fatty acids and phosphoric acid. The bark of *M. nigra* was reputed to be used to expel tape worm. The decoction of the leaves possesses blood purifying properties, reduces fever and is diuretic [27]. Black mulberry genotypes have a higher bioactive content. A study was conducted to investigate the chemical constituents in the barks of *M. nigra*. In this study, nine compounds were isolated and identified as olcancolic acid, apigenin, cyclocommunol, morusin, cyclomorusin, kuwanon C, daucosterol, ursolic acid and 63-sitosterol [28].

Quercetin has been isolated from fruit of *M. nigra*. Quercetin targets cysteine string proteins (CSPα) and impairs synaptic transmission [29]. Apigenin has been isolated from bark of *M. nigra*. Apigenin is a natural product belonging to the flavones class that is the aglycone of the several naturally-occurring glycosides. It is a yellow crystalline solid. Apigenin may contribute to the chemopreventive action of vegetables and fruits [30]. It was recently shown that apigenin induces a process called autophagia (a kind of cellular dormancy) that may well explain its chemopreventive properties, but at the same time it induces resistance against chemotherapy [31]. Apigenin is a potent inhibitor of CYP2C9, [32] an enzyme responsible for the metabolism of many pharmaceutical drugs in the body. Apigenin has been shown to reverse the adverse effects of cyclosporine. Research has been conducted to study the effects of apigenin on reversal of cyclosporine-induced damage, and this was assessed by immunohistochemical estimation of expression of *bcl-2*, and estimation of apoptosis in histopathological sections [33]. Morusin has been isolated from leaf of *M. nigra*. Morusin, a flavonoid has high cytotoxicity against murine leukemia cell P-388, IC_{50} 3.1µg/mL [17].

A comparison of the molluscicidal activity of the active components present in *M. nigra* fruit, bark and leaf powder with synthetic molluscicides clearly demonstrates that these components are more potent against *L. acuminata*. The LC_{50} at 96h of quercetin (1.11), apigenin (1.92) and morusin (2.12) are lower than those of synthetic molluscicides carbaryl (14.40 mg/L), phorate (15.0 mg/L), formothion (8.56 mg/L) and niclosamide (11.8 mg/L) [18 ,19]. LC_{50} at 96h of crude powder of *M. nigra* fruit (166.92), bark (173.17) and leaf (173.69) against *L. acuminata* are lower than the crude powder of *Canna indica* root (359.02 mg/L) [34], *Thuja orientalis* leaf powder (250.55 mg/L), *Thuja orientalis* fruit powder (255.12 mg/L) [35], *Zingiber officinale* rhizome (273.80 mg/L), *Allium cepa* bulb (253.27 mg/L) [36].

It is evident from the steep slope values that a small increase in the concentration of different treatments causes a marked mortality in snails. A t-ratio value greater than 1.96 indicates that the regression is significant. Values of heterogeneity factor less than 1.0 denote that in the replicate tests of random samples the concentration response lines would fall within 95% confidence limit and thus the model fits the data adequately. The index of significance of potency estimating values indicates that the values of the mean are within the limits at all probability levels (90, 95, 99) as it is less than 0.5.

CONCLUSION

In conclusion it can be stated that molluscicidal activity of *M. nigra* is due to quercetin, apigenin and morusin. *M. nigra* can be used as potent molluscicide as it is easily available and ecologically and culturally more acceptable by livestock keepers. Toxicity of active components and crude extracts is more than the other plant extracts and synthetic molluscicides, respectively. Further studies will assess the mode of action of purified molluscicidal components of the plant in snail *L. acuminata*.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Singh A, Singh DK, Mishra TN, Agarwal RA. Molluscicide of plant origin. Biol Agric Hortic. 1996; 13: 205-252.
2. WHO. 2006. Report of the WHO informal meeting on use of triclabendazole in fascioliasis control. 17-18 October, Geneva, Switzerland.
3. Agarwal RA, Singh DK. Harmful gastropods and their control. Acta Hydrochim Hydrobiol. 1988; 16(2): 113-138.
4. Torgerson P, Claxton J. 1999. Epidemiology and control. In: Dalton, ed. Fascioliasis: pp. 113-149. CAB International Publishing, Wallingford, United Kingdom.
5. Spithill TW, Smooker PM, Copeman DB. 1999. *Fasciola gigantica*: Epidemiology, control, immunology and molecular biology. In: Dalton, ed. Fascioliasis: pp. 465-525. CAB International Publishing, Wallingford, United Kingdom.
6. Anonymus. 1995. Control of food borne trematode infections. WHO Technical Series No. 849. WHO, Geneva: pp.157.
7. Chen MG, Mott KE. Progress in assessment of morbidity due to *Fasciola hepatica* infection: a review of recent literature. Trop Dis Bull. 1990; 87: R1-R38.
8. Narain K, Biswas D, Rajguru SK, Mahanta J. Human distomatosis due to *Fasciola hepatica* infection in Assam. Ind J Commun Dis. 1997; 29(2): 161-165.
9. Markell EK, Voge M. 1999. Medical Parasitology. 8th edition: pp. 185-188. Saunders Company Publication.
10. Mas-Coma S, Valero MA, Bargues MD. 2009. *Fasciola*, Lymnaeids and Human fascioliasis with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. In: Rollinson D., Hay S.I. ed. Advances in Parasitology, vol.69: pp. 41-146. Burlington Academic Press.
11. Marston A, Hostettmann K. Plant molluscicides. Phytochem. 1985; 24: 639-652.
12. Hostettmann K, Lea PJ. 1987. Biologically active natural products. pp. 65-84. Oxford Science Publication, Oxford.
13. Silva TMS, Camara CA, Agra MDF, Carvalho MGD, Frana MT, Brandoline SVPB, Paschoal LDS, Braz-Filho R. Molluscicidal activity of *Solanum* species of the northeast of Brazil on *Biomphalaria glabrata*. Fitoterapia 2006; 77: 449-452.
14. Mohiuddin E, Khan U, Akram M, Asif HM, Akhtar N, Shah Pervaiz A, et al. *Morus nigra*. L.A. J Med Plants Res. 2011; 5(20): 5197-5199.
15. Ogundiya MO, Okunade MB, Kolapo AL. Antimicrobial activities of some Nigerian chewing sticks. Ethnobot Leaflets. 2006; 10: 265-271.
16. Liu B, Ning Z, Gao J, Xu K. Preparing Apigenin from leaves of *Adinandra nitida*. Food Technol Biotechnol. 2008; 46(1): 111-115.
17. Tati S, Yandri AS, Sutopo H, Jhons Fatriyadi S. Morusin, a Bioactive Compound from the Root Bark of *Artocarpus dadah*. Eur J Sci Res. 2009; 38: 643-648.
18. Jaiswal P, Singh DK. Molluscicidal activity of *Carica papaya* and *Areca catechu* against the freshwater snail *Lymnaea acuminata*. Vet Parasitol. 2008; 152: 264-270.
19. Singh DK, Agarwal RA. Correlation of the anticholinesterase and molluscicidal activity of the latex of *Euphorbia royleana* on *Lymnaea acuminata*. J Nat Prod. 1984; 47: 702-705.
20. Robertson JL, Russell RM, Preisler HK, Savin NE. 2007. Bioassay with Arthropods POLO Computer Programme for Analysis of Bioassay Data. 2nd edition: pp. 1-224. CRC Press, Taylor and Francis.
21. Sokal RR, Rohlf F.J. 1973. Introduction to biostatistics. pp. 271-273.W.H. Freeman and Co., San Francisco.
22. Awasthi AK, Nagaraja GM, Naik GV, Kanginakudru S, Thangavelu K, Nagaraju J. Genetic diversity and relationships in mulberry (genus *Morus*) as revealed by RAPD and ISSR marker assays. BMC Genet. 2004; 5: 1471-2156.
23. Nomura T. Phenolic compounds of the mulberry tree and related plants. Fortschr Chem Org Naturst. 1988; 53: 87-201.
24. Ahmad J, Farooqui AH, Siddiqui TO. *Morus nigra*. Hamdard Med. 1985; 15: 76-78.
25. Naderi GA, Asgary S, Sarraf-Zadegan N, Oroojy H. Antioxidant activity of three extracts of *Morus nigra*. Phytother Res. 2004; 18: 365-369.
26. Zheng ZP, Cheng KW, Zhu Q, Wang XC, Lin ZX, Wang M. Tyrosinase inhibitory constituents from the roots of *Morus nigra*: Structure-activity relationship study. J Agric Food Chem. 2010; 58: 5368-5373.
27. Kumar A, Gupta PN. 1996. Genetic resources of indigenous tropical fruits having medicinal value. In: Genetic resources of tropical fruits-collection, evaluation and conservation. Ed. Gupta PN, Mathura R, Chandal KPS.: pp. 134-144. National Bureau of Plant Genetic Resources, New Delhi.
28. Wang L, Wang HQ, Chen RY. Studies on chemical constituents from bark of *Morus nigra*. China J Chin Mat Med. 2007; 32(23): 2497-2499.
29. Xu F, Proft J, Gibbs S, Winkfein B, Johnson JN, Syed N, et al. Quercetin targets cysteine string protein (CSPα) and impairs synaptic transmission. PLoS One 2010; 5: e11045. DOI: 10. 1371/journal.pone.0011045.
30. Ferreira CV, Gusto GZ, Sousa AC, Queiroz KC, Zambuzzi WF, Aoyama H, et al. Natural compounds

as a source of protein phosphatase inhibitors; application to the rational design of small-molecule derivatives. Biochemie. 2006; 88(12): 1859-73.

31. Ruela-de-Sousa RR, Fuhler GM, Blom N, Ferreira CV, Aoyama H, Peppelenbosch MP. Cytotoxicity of apigenin on leukemia cell lines: implications for prevention and therapy. Cell Death Dis. 2010;1(e19): 1-11.
32. Dayong S, Wang Y, Zhou Y-H, Guo Y, Wang J, Zhou H, et al. Mechanism of CYP2C9 inhibition by flavones and flavonols. Drug Metab Dispos. 2009; 37 (3): 629-634.
33. Chakravarthi S, Wen CF, Nagaraja HS. Apoptosis and expression of bcl-2 in cyclosporine induced renal damage and its reversal by beneficial effects of 4, 5, 7 – trihydroxyflavone. J Anal Bio-Sci. 2009; 32 (4): 320-327.
34. Tripathi SM, Singh DK. Molluscicidal activity of *Punica granatum* bark, *Canna indica* root. Braz J Med Biol Res. 2000; 33: 1351-1355.
35. Singh A, Singh VK. Molluscicidal activity of *Saraca asoca* and *Thuja orientalis* against the freshwater snail *Lymnaea acuminata*. Vet Parasitol. 2009; 164: 206-210.
36. Singh S, Singh VK, Singh DK. Molluscicidal activity of some common spice plants. Biol Agric Hortic. 1997; 14: 237-249.

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BIOLOGY ORIGINAL ARTICLE

Taxonomic status of *Bupleurum* (*Apiaceae*) in outer hills of Kashmir Himalayas, India

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ABSTRACT

Introduction: The genus *Bupleurum* is perennial rhizomatous herbs, recognized by simple leaves, conspicuous bracts and bractlets, often shows a great deal of variation in morphological characteristics. The genus is well developed in temperate and alpine zones of Kashmir Himalayas and other lesser Himalayan ranges of Jammu and Kashmir State. A key to the species, brief description, flowering and fruiting periods are given. The approximate elevation, distribution of species in the region and illustrations of selected species are provided.

Material and Methods: The present communication is based on the surveys conducted between 1990 and 2007. The speciemens were mounted on the herbarium sheets and studied in the laboratory with the help of floristic literature.

Results: As many as 10 species of genus *Bupleurum* have been recognized, from the different climatic zones of outer hills of Kashmir Himalayas of Jammu and Kashmir State.

Conclusion: The report is first of its kind being communicated from the State of Jammu and Kashmir. Ten species of *Bupleurum* are described.

Key words: Flora, *Apiaceae*, *Bupleurum*, Jammu and Kashmir, India.

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INTRODUCTION

Genus *Bupleurum* L. (*Apiaceae*) comprises of 180-190 species mostly confined to temperate regions of the world [1]. *Bupleurum* L. is represented by 10 species in the outer hills of Kashmir Himalayas of Jammu and Kashmir State (Fig. 1). Most of the species inhabit to the alpine zones of the Himalayas. In past, species of the genus have been enumerated by Royle (1833-39), Hooker (1879), Kaul (1971), Stewart (1972), Rau (1975), Singh and Kachroo (1976), Sharma and Kachroo (1981) and Swami and Gupta (1998) [2-7]. Specific attention has been paid by Hamal et al. [8, 9] to the Umblifers of Kashmir. Hamal et al. [10] made an exploration to district Doda and enumerated some umblifers in addition to other angiosperms. Bhellum and Magotra [11] listed 36 species of Umblifers in the form a contribution of the flora of district Doda of Jammu and Kashmir State - family *Apiaceae*. Apart from this the species of the Himalayan ranges and other areas have been studied by Qureshi et al. [12, 13]. Floristic information and distribution is poorly available therefore, the main focus is inventorization and documentation of the species of this genus as the species are of medicinal value. The lacunae in the knowledge of these species can pose problem to find out the global diversity of this genus. The survey of natural resources plays a vital role in the economic development of at state and global level. The flora of North-west Himalayas is quite rich in terns of floristic diversity of the region. Keeping in mind extensive survey of species and intensive scanning of floristic literature was made in the outer hills of Kashmir Himalayas. All the species of *Bupleurum* L. collected from outer hills of Kashmir Himalayas have been described as under. The voucher specimens have been deposited in the Department of Botany, University of Jammu.

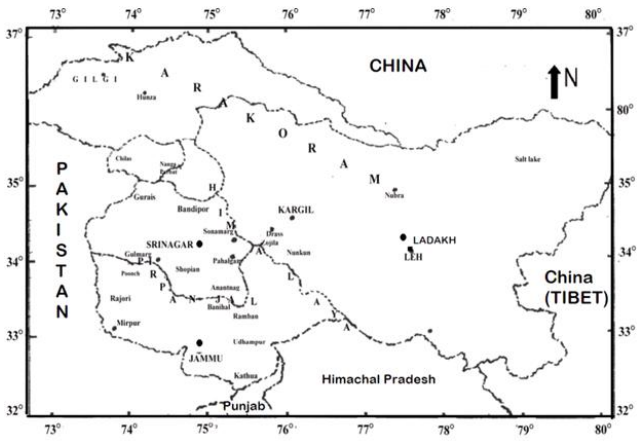

Fig.1. Map of Jammu & Kashmir state showing location.

MATERIALS AND METHODS

The plant explorations were carried out in the area of study from 1990-1998 and later the area was visited occasionally. This paper is based on the collection made and thereafter identified the species in the following years. The size of the flowers of *Bupleurum* L. being smaller the pose problems in identification, however, the species of genus *Bupleurum* L. are very easy to identify in the field from the fresh plant material. The leaves of all the species of *Bupleurum* L. are simple. The flowers were boiled for about 1- 2 minutes to study them under the stereoscope from the dried specimens. The species were identified with the help of floristic literature. Key to species of *Bupleurum* L. is presented in the table 1.

RESULTS

Earlier, a single species of *Bupleurum* from Jammu, four species from Srinagar and Udhampur each had already been reported from this state (Fig. 4). A total of 10 species of *Bupleurum* have been described from the outer hills of Kashmir Himalayas. Key to the species is provided for all these species. The present communication is first of its kind from the state. Based on the observation of morphology, these species are differentiated from each other.

TAXONOMIC DESCRIPTION OF *BUPLEURUM* L.

1. ***B. candollei*** Wall. ex DC., Prodr. **4**: 131. 1830; Clarke in Hooker f., FBI. **2**: 674. 1879; Stewart, l.c. 512. 1972.

Perennial, somewhat tufted, aromatic herbs; stem 20-80 cm in height, fistular, thinly furrowed; leaves simple, entire, basal linear to lanceolate, oblong, sessile to subsessile (Fig. 2A), apex acute, base amplexicaule, margin whitish, entire, upper leaves lanceolate, oblong, shorter; involucral bracts 1-4, foliaceous, unequal; rays 6-10, unequal; involucel 5, lanceolate; umbellets 15-20 flowered; flowers actinomorphic, bisexual; calyx teeth obsolete; petals 5, obovate, apex notched, inflexed,

Table 1.

Key to species of *Bupleurum* L.

1	+	Leaves acicular, rarely linear; umbels uniseriate ---------- 9. ***B. subuniflorum***
	-	Leaves linear laceolate, oblong or ovate; umbels multiseriate ---------- 2
2	+	Plants decumbent ascending ---------- 3
	-	Plants erect ---------- 4
3	+	Basal leaves oblanceolate, upper lanceolate-oblong, sessile or subsessile; fruits furrowed, 1-vittate ---------- ---------- 5. ***B. hamiltonii***
	-	Basal leaves linear, slightly amplexicaule, upper leaves similar, shorter; fruits furrowed, 3-vittate ---------- ---------- 4. ***B. gracillum***
4	+	Stem solid; flowers yellow; involucral bracts 0 to 5, linear to lanceolate ---------- 5
	-	Stem fistular, flowers yellow or blakish-purple ---------- 7
5	+	Basal leaves numerous, linear to linear-lanceolate, veinlets indistinct ---------- 6
	-	Basal leaves broadly lanceolate to ovate, veinlets few distinct ---------- 6. ***B. lanceolatum***
6	+	Leaves linear, stiff, margin white cartilaginous, minutely serruate ---------- 8. ***B. marginatum***
	-	Leaves linear to falcate, not stiff, margin non-cartilaginous, never serrulate -- 3. ***B. falcatum***
7	+	Flowers blakish-purple to black ---------- 7. ***B. longicaule***
	-	Flowers yellow ---------- 8
8	+	Apices of leaves obtuse, acute, or long acuminate; bases of upper leaves amplexicalue or auricled; involucral bractlet 4- 8 ---------- 9
	-	Apices of leaves acute or obtuse, mucronate, never long acuminate, bases of upper leaves never amplexicaule or auricled; involucral bracts 1- 4 ---------- 1. ***B. candollei***
9	+	Involucral bracts absent; involucel bractlets 5- 8, linear; flowers 14- 22 per umbellets ---------- 2. ***B. clarkeanum***
	-	Involucral bracts 1- 3 rarely absent; involucel bractlets 4- 5, ovate or lanceolate; flowers 12- 17 per umbellet ---------- 10. ***B. thomsoni***

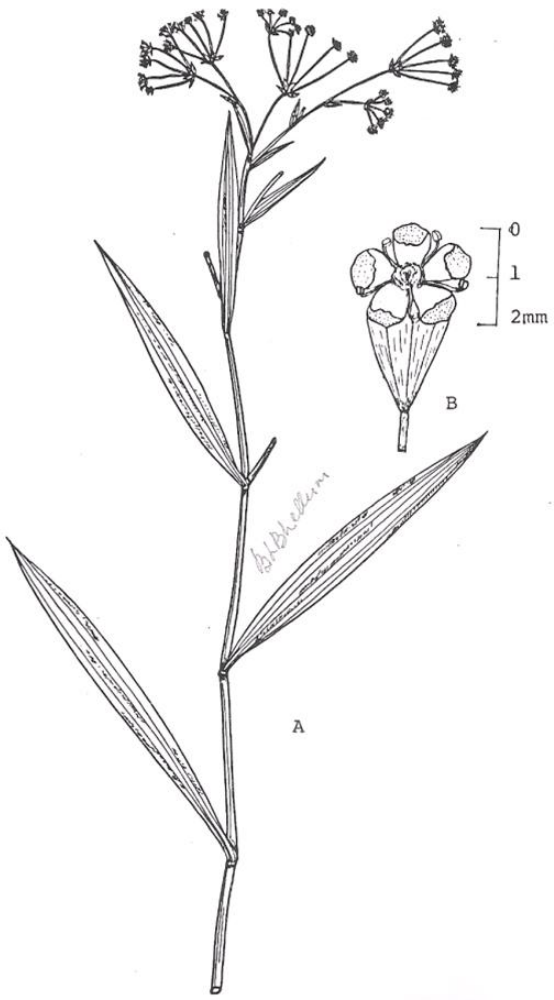

Fig. 2. *Bupleurum candolei* Wall. ex DC.; A, Upper portion of a twig; B, Flower.

yellow; stamens 5, inflexed (Fig. 2B); fruit ovoid, oblong, ridges, furrows 3-vittate.

Rare along the roadsides near Sashu and Karthai (Padder). Specimens collected from Kathrai.
Specimens examined: (1800 m) *Bhellum* 12058
Flrs.: July–August, *Frts*.: August–September

2. ***B. clarkeanum*** (Wolff) E. Nasir in Fl. W. Pak. **20**: 50. 1972. *B. diversifolia* auct. non Koch, Clarke in Hooker f., FBI. **2**: 675. 1879.

Perennial herbs; leaves lanceolate, long-pointed, base ovate, nearly stem clasping; bracts absent or one, small, lanceolate, rays 8–14; flowers yellow, sepals 5; Petals 5, yellow; fruits ridged, and furrowed.

Grows along open and dry slopes at Kailash Parbat.
Specimens examined: Kailash Parbat (3500 m) *Bhellum* 15050
Flrs.: May–June, *Frts*.: June–July

3. ***B. falcatum*** L. Sp. Pl. 237. 1753; Stewart, *l.c.* 512. 1972.

Perennial decumbent herbs; leaves redical linear, half stem clasping or sessile, long pointed; bracts absent, 1-5 when present, lncleolate; umbels with 5-8 rays; fruits distinctly furrowed.

Abudant along the roadsides at Mansar near Batote. Specimens collected from Mansar (1400 m).
Specimens examined: Batote (1400 m) *Bhellum* 13201
Flrs.: May–June, *Frts*.: June–July

4. ***B. gracillimum*** Kl. in Kl. & Garck, Bot. Ergeb. Reise Prinz. Waldemar 148. 1862; *B. virgatum* Wall., (*nom. nud.*); *B. falcatum* var. *nigrecarpa* Jacquem. ex C.B. Clarke in Hooker f., FBI. **2**: 676. 1879.

Perennial, decumbent herb; stem glabrous, green; leaves simple, linear, many at base, amplexicalue, margin entire, apex acuminate, cauline shorter, linear, lanceolate; inflorescence compound umbels; flower actinomorphic, bisexual; involucral bracts 2-5, linear, lanceolate; involucel 5-6, acute; calyx teeth obsolete; petals obovate, notched, inflexed, yellow; stamens 5; ovary bicarpellary surrounded by disc shaped stylopodium; fruit oblong, glabrous, grooved, ridges prominent.

Abundant along the grassy slopes at Kailash Parbat and Jawathatop. Specimens collected from Kailash Parbat (3500 m).
Specimens examined: Kailash Parbat (3500 m) *Bhellum* 13162
Flrs.: May–June, *Frts*.: June–August

5. ***B. jucundum*** Kurz, Seem Journ. Bot. **5**: 240. 1863; Sharma & Kachroo, Fl. Jmu. **1**: 175. 1981, Stewart, *l.c.* 513. 1972.

Perennial herbs, 60-70 cm tall; stem fistular; leaves mucronate, white margined, lower linear, to lanceolate, amplexicaule, upto 10 cm long, upper ones ovate, ovate-oblong, base deeply cordate; umbels 6-10 rayed, rays angled; bracts solitary, foliaceous, bracteoles lacking; flowers minute, calyx obsolete; petals 5, yellow.
Specimens examined: Ramsu (1200 m) *Bhellum* 15070
Flrs.: July–August, *Frts*.: August–September

6. ***B. lanceolatum*** Wall. ex DC. Prodr. **4**: 132. 1830; Clarke in Hooker f., FBI. **2**: 674. 1879; E. Nasir in Univ. Calif. Pub. Bot. **27**: 422. 1954; Stewart, *l.c.* 513. 1972.

Perennial, erect herbs; stem glabrous, furrowed, 0.5-1.5 m tall; leaves simple, basal leaves long petioled, lamina ovate (Fig. 3A, B), lanceolate, veins distinctly 7-9 venation parallel, converging towards apex, cauline leaves smaller and shortly petioled; involucral bracts absent; rays 5-10; involucel 4-5, veins 3; umbellets 10-12 flowered; flowers actinomorphic bisexual (Fig. 3C), yellow; calyx obsolete; petals 5, obovate, apex notched, inflexed; stamens 5, inflexed; stylopodium circular; fruit oblong-lanceolate, compressed, ridges distinct.

Abundant along the slopes between Ramsu and Banihal. Specimens collected from Ramsu and Banihal.

Specimens examined: Banihal (1400 m) *Bhellum* 15125

Flrs.: July–August, *Frts*.: August–September

Fig. 3. *Bupleurum lanceolatum* Wall. ex DC.; A, Upper portion of a branch; B, Middle portion of a branch; C, Flower.

7. ***B. longicaule*** Wall. Cat. n. 557. 1828; DC., Prodr. **4**: 131. 1830; Clarke in Hooker f., FBI. **2**: 677. 1879; E. Nasir in fl. W. Pak. **20**: 47. 1972.

Perennial herbs; stem fistular, 1-many from base; leaves simple, margin entire, white cartilaginous, basal leaves linear-lanceolate, cauline leaves usually shorter, ovate, lanceolate, sessile; involucral bracts 1-4, foliaceous, lanceolate, margin white; rays 14-40 flowered; involucel bractlets 5-8, ovate, lanceolate; flowers actinomorphic, bisexual, blackish-purple; calyx teeth obsolete; petals 5, blackish-purple, apex minutely notched, inflexed; stamens 5; fruit oblong-cylindric, ridges winged.

Abundant along the dry slopes at Kailash Parbat.

Specimens examined: (3500 m) *Bhellum* 12887

Flrs.: June–July, Frts.: July–August

i) *B. longicaule* var. *himalayense*.

ii) *B. longicaule* var. *ramosum*.

8. ***B. marginatum*** Wall. ex DC., Prodr. **4**: 132. 1830; Stewart, *l.c.* 514. 1972; *B. falcatum* var. *marginata* Wall. ex DC.; Clarke in Hooker f., FBI. **2**: 676. 1879.

Perennial, erect herbs; stem glabrous, green, furrowed, ridges acute; leaves simple, basal leaves linear, lanceolate, crowded at base, upper leaves shorter, linear-lanceolate, margin white; involucral bracts 1-5, linear, lanceolate, unequal; ray 3-10; involucels 3-5, lanceolate, linear; flowers actinomorphic, bisexual; calyx teeth obsolete; petals 5, yellow, apex notched, inflexed; stamens 5; fruit oblong, cylindrical, distinctly white, ridged.

Abundant along the grassy slopes near Atholi. Specimens collected from Sanasar.

Specimens examined: Sanasar (1700 m) *Bhellum* 12315

Flrs.: June–July, Frts.: July–August

9. ***B. subuniflorum*** Boiss. et Heldr. in Boiss. Diagn. Ser. 1: 10. 28. 1849; E. Nasir, Fl. Pak. **20**: 41. 1972. *B. setaceum* Clarke in Hooker f., FBI. **2**: 678. 1879. non Fenzl. 1866.

Annual erect herbs; stem dichotomously branched, furrowed; leaves simple, basal leaves linear, lanceolate, 1-3 veined, apex acute, base clasping to stem, upper leaves smaller; involucral bract one, acute, veins inconspicuous; rays 2-4; involucel 4-5, acicular; flower actinomorphic, bisexual; calyx teeth obsolete; petals 5, yellow apex

notched, inflexed; stamens 5, inflexed in bud; fruit oblong, prominently redged.

Abundant along roadsides between Atholi and Machail.

Specimens examined: Machail (3000 m) *Bhellum* 1174.

10. ***B. thomsoni*** Clarke in Hooker f., FBI. **2**: 675. 1879; Stewart, Ann. Cata. Vasc. Pl. W. Pak. & Kashm. 514. 1972.

Perennial, erect herbs; stem branched, fistular, thinly furrowed, 0.5- 1 m tall; leaves simple, lower ones linear-lanceolate, margin white, entire, apex acute, base stem clasping; involucral bacts absent or one or two, cordate; rays 6- 10; involucral bracts as long as or slightly longer than umbellets, apex acute; flower actinomorphic, bisexual; calyx teeth obsolete; petals 5, obovate, yellow, apex notched, inflexed; stamens 5, inflexed in bud; stylopodium brownish; fruit oblong, subquadrate, ridges prominent.

Abundant in dry and gravely soil at Kailash Parbat.

Specimens examined: Kailash Parbat (4000 m) *Bhellum* 1175.

DISCUSSION

Contribution to Umblifers to the flora of Kashmir made [3] subsequently [14] reported four species of Bupleurum from Srinagar Kashmir. Being one of the largest genera of family *Apiaceae* in the flora of Jammu and Kashmir the species of *Bupleurum* are highly variable. Most of the species of this genus occupy the different habitat on the outer hills of Kashmir Himalayas. Most of the species are confined to the alpine zones of the state of Jammu and Kashmir [11]. Species of *Bupleurum* [1] are confined to the temperate regions of the world. Only a single species has been reported from Jammu [6]. Earlier reports indicated four species of *Bupleurum* from Udhampur district [15]. The species of *Bupleurum* are recongised on the basis of vegetative and reproductive chararcters. Among the vegetative characters the most important are leaf shape, margins, colour of margins, size, venation, bracts and barcteoles, The significant reproductive characteristics of the species are number of flowers per umbel and umbellets, petals shape, apex, colour of flowers, shape and size of fruits. Hamal et al. studied the position nucleolar organization in Umblifers [8] and made a systematic study of Centella from Jammu [9]. Koul et al. [16] studied the reproductive biology of wild and cultivated carot (*Daucus carota* L.) from Kashmir. While making the exploration of district Doda of Jammu and Kashmir. Hamal et al. [10] contributed some additions to the flora of district Doda of Jammu and Kashmir. Systematic and evolutionary trends in fruit structure of genus *Torilis*, *Apiaceae* were studied [15]. Thirty six umblifers were recognized from district Doda of Jammu and Kashmir [11]. The present investigation deals with ten species of *Bupleurum* (Fig. 4) which indicates that the genus is well represented in the area of present study as well as the other parts of Jammu and Kashmir. The number of occurrence of species in the higher elevation is more than that of plains.

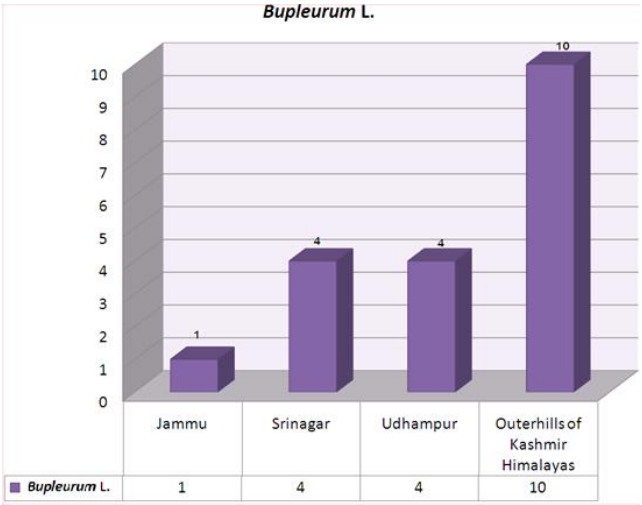

Fig. 4. The numerical size of *Bupleurum* L. in different parts of Jammu and Kashmir State.

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TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

REFERENCES

1. Liu M, Shi L, Wyk BV, Tilney PM. Fruit anatomy of genus *Bupleurum* (*Apiaceae*) in northeastern China and notes on systematic implications. South Afr J Bot. 2003; 69: 151-157.
2. Royle JF. 1833-1839. Illustrations of the Botany and other branches of natural history of the other Himalayan mountains and of the flora of Cashmere. Vol. I & II. Wm. H. Allen, London.
3. Kaul MK. Contribution the Umbilifers of Kashmir. J Bomb Nat Hist Soc. 1971; 72(3): 692-715.
4. Stewart RR. 1972. An Annotated Catalogue of Vascular Plants of West Pakistan and Kashmir. Fakhri Printing Press, Lahore.
5. Rau MA. 1975. High Altitude Flowering Plants of West Himalayas. BSI. Indian Botanic Garden, Howrah, Calcutta.
6. Sharma BM, Kachoo P. 1981. Flora of Jammu and Plants of Neighbourhood. Vol. I Bishen Singh Mahendra Pal Singh, Dehra Dun.
7. Swami A, Gupta BK. 1998. Flora of Udhampur district. Bishen Singh Mahendra Pal Singh, Dehra Dun.
8. Hamal IA, Langer A, Koul AK. Nucleolar Organiser region in the *Apiaceae* (*Umbiliferae*). Plant Syst Evol. 1986; 154: 11-30.
9. Hamal IA, Langer A, Koul AK. Taxonomic position of *Centella asiatica* (L.) Urban. Proc Indian Acad Sci (Plant Sci.). 1989; 363-368.
10. Hamal IA, Karihaloo JL, Wafai BA, Wakhlu AK. Additions to the Flora of district Doda of Jammu and Kashmir State. J Econ Tax Bot. 1990; 14(1): 205-213.
11. Bhellum BL, Magotra R. A contribution to the flora of district Doda of Jammu and Kashmir State - Family Apiaceae. J Econ Tax Bot. 1996; 20(1): 143-147.
12. Qureshi SJ, Awan AG, Khan MA, Bano S. Palynological study of the genus *Tragopogon* from Pakistan. Asian J Plant Sci. 2002; 1: 283-287.
13. Qureshi SJ, Awan AG, Khan MA, Bano S. Study of the pollen fertility of the genus *Launaea* from Pakistan. Asian J Plant Sci. 2002; 1: 73-74.
14. Singh G, Kachroo P. 1976. Forest flora of Srinagar and neighbourhood. Bishen Singh Mahendra Pal Singh, Dehra Dun.
15. Gupta SK, Koul AK, Hamal IA. Evolutionary trends in fruit structure of genus *Torilis* Adans. (*Apiaceae*). Phytomorphol. 1991; 41(3-4): 325-329.
16. Koul P, Koul AK, Hamal IA. Reproductive biology of wild and cultivated carrot (*Daucus carota* L.). New Phytologist. 1989; 112(3): 437-443.

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BIOLOGY ORIGINAL ARTICLE

Bioremediation of chromium ions with filamentous yeast *Trichosporon cutaneum* R57

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ABSTRACT

Heavy metal pollution is a serious environmental issue today. Recognizing the ability of microorganisms and biological materials to remove them from the polluted sites makes it cheaper method of heavy metal remediation. Microorganisms remove metals as a result of biosorption and bioaccumulation.

In the present work, filamentous yeast *Trichosporon cutaneum* R57 was cultivated on batch in Andreev medium containing biotin, thiamine and glucose. Metal chromium was added in concentration ranging from 0-10 mM ($K_2Cr_2O_7 \cdot 7H_2O$) to the culture during the stationary phase of the culture growth. Viability, glucose consumption and removal efficiency of the strain was studied. 1.5 mM of $K_2Cr_2O_7 \cdot 7H_2O$ was found to be lethal to the strain, consumes maximum glucose at 5 mM and removes maximum at 1 mM concentration. Removal efficiency decreases with increase in metal concentration.

Key words: Andreev medium, bioaccumulation, biosorption, chromium, yeast, *Trichosporon cutaneum* R57.

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INTRODUCTION

The contamination of air, water and soil with hazardous and toxic chemicals pose high risks for living things both directly and indirectly. Heavy metals particularly chromium is considered to be more dangerous pollutant even at low concentration. It is more important because of the fact that it cannot be decomposed in situ biologically [1]. The major source of chromium pollutant is effluents of industrial processes like [2] electroplating, metallurgical work, tanning, [3, 4] chemical manufacturing and battery manufacturing. Each pollutant in the environment has its individual effect to the environment and human health. Chromium enters the body through contaminated foods or exposure to chromium contaminated sites in the vicinity of industries using chromium.

Generally, chromium occurs in two oxidation states Cr^{3+} and predominantly Cr^{6+} in air, water and soil [4-6]. Cr^{6+} species are strong oxidants, which are carcinogenic, mutagenic and teratogenic in living systems [6]. It is hundred times poisonous and thousand times mutagenic than Cr^{3+} hence it has been listed as a priority pollutant and a human carcinogen by the US EPA [4]. Cr^{6+} is highly soluble in water and mobile through ecosystem while Cr^{3+} is insoluble and forms precipitate with organics in nature.

Microorganisms play a significant role in bioremediation of heavy metal contaminated soil and wastewater. Many microorganisms like fungi, bacteria and microalgae have been recognized for their ability to either resist the toxic effect of heavy metals or they biotransform so that the heavy metal ions become less toxic or non toxic to them via sequestration mechanisms such as reduction, complexation, alkylation, precipitation, etc. [1, 7]. Through these mechanisms, microorganisms are also able to biosorb and bioaccumulate heavy metals on/in their body with the help of numerous binding sites provided by the functional groups present on their cell wall. Ironically, some of these mechanisms make an environment susceptible to heavy metal toxicity. For example, reduction of toxic heavy metal ions to relatively less toxic (Cr^{6+} to Cr^{3+}) makes the heavy metal ions mobile through the soil water there by chances of its presence to non-polluted sites and the probability of getting into runoff water increases.

Toxic metals classified as environmental pollutants cannot be degraded, but their oxidation state can be changed to another less toxic state by microorganisms. Thus, bioremediation of heavy metals aims at sequestering the metals to make them unavailable to flow in the ecosystem, or extract to mobilizing them for reuse or safe disposal [8]. The properties of microorganisms to adsorp and bioaccumulate heavy metals give potential for cheap alternative method of heavy metal removal from industrial wastewaters. Both living and dead biomaterials are capable of removing heavy metal ions from wastewater through diverse mechanisms collectively known as biosorption [9].

Role of conventional methods in remediating heavy metals has become ineffective and costly in threshold of ever increasing industrial effluents. Conventional methods can remove only up to certain minimum level. Therefore, the bioremediation has come as cost effective, efficient and environmentally friendly alternative of removing heavy metals from industrial effluents. The advantage of bioremediation is that this process does not require using aggressive and concentrated chemicals, and metal ions bound biomass could be reused after elution [10]. Bioremediation is a viable alternative to conventional methods, but metal toxicity at co-contaminated (contaminated with organic and heavy metal) sites may limit its application [11]. Heavy metal remediation using bioaccumulation is promising alternative and the interest in it has grown high in recent time. Microbial systems like fungi, bacteria and microalgae have been successfully used as adsorbing agents for removal of heavy metals. Studies on bacteria, yeast, fungi and microalgae have shown that yeast are better biosorbent for the removal of heavy metal ions from wastewater due to their high growth rate. Yeast can be cultivated with ease in cheap growth media and also yeast biomass cheaply available in good quantities from fermentation industries for remediation of wastewater at lower pH [7, 12].

MATERIALS AND METHODS

Strain, Media and Growth

Trichosporon cutaneum (*Tr. cutaneum*) R57 [13] obtained from Bulgarian National Bank of Industrial Microorganisms and Cell Cultures was used in the experiments. The strain was maintained on Yeast Extract Peptone Dextrose (YEPD) solid medium in test tubes. The composition of YEPD medium and

concentration of each constituent were as described by Giorgieva [13], Yeast extract 10, bacterial peptone 20, dextrose (glucose) 20 and agar 20 gram per liter sterilized for 20 minutes at 0.8 atmospheres in autoclave.

Preculture Cultivation

After the incubation at 28°C for 48 hours in thermostat, the colonies were picked up on a sterile loop and cultivated in Erlenmeyer flasks as described by Giorgieva [13] containing 90 ml Andreev medium, 10 ml (10%) glucose solution, 10 mg thiamine and 10 µl biotin. Glucose solution acts as the source of carbon and the source of energy for the growing cells. Andreev medium contains H_3PO_4 (1:10) 3.75 ml, CH_3COOH (1:10) 18.5 ml, KCl 3 gram, $MgSO_4 \cdot 7H_2O$ 0.15 gram, $Ca(H_2PO_4)_2$ 0.042 gram NaOH (0.1N) 4 ml per liter. The culture containing Erlenmeyer flasks were incubated on a rotary shaker (180 rpm) at 30°C for 24 hours.

Culture Cultivation

After 24 hour incubation, [13] 10 ml preculture culture was taken in 80 ml Andreev medium, 10 ml (10%) glucose solution, 10 mg thiamine and 10 µl biotin. The culture was incubated for 24 hours on a rotary shaker (180 rpm) at 30°C.

After 24 hours, Cr ions were supplied in the form of potassium dichromate ($K_2Cr_2O_7 \cdot 7H_2O$) solutions. A series of concentrations were added into different flasks. 0 mM in control, 1 mM, 1.5 mM, 2 mM, 2.5 mM, 5 mM and 10 mM per liter of $K_2Cr_2O_7 \cdot 7H_2O$.

Viability

The growth and viability of *Tr. cutaneum* R57 was studied by making colony forming units (CFU)-spread plate method, and microscopic observation of the cells. CFU was performed by serial dilution, homogenized on rotary vortex, cultivated in YEPD media and incubated at 30°C for 24 hours [8]. For microscope observations, the cells were washed twice with distilled water, stained with 2% solution of methylene blue for 20 minutes at room temperature, washed again with distilled water and observed using bright field microscope Olympus BX53, Camera SC30 (Japan).

Glucose consumption

Glucose consumption by *Tr. cutaneum* R57 in the presence of Cr ions was analyzed by dinitrosalicylic method [14]. It is a standard method of determining unknown solute concentration in a solution by spectrophotometry of a solution containing known solute concentration. The standard curve was drawn using absorbance values at 540nm and series of glucose solutions containing µg/ml sugar to determine the amount of glucose

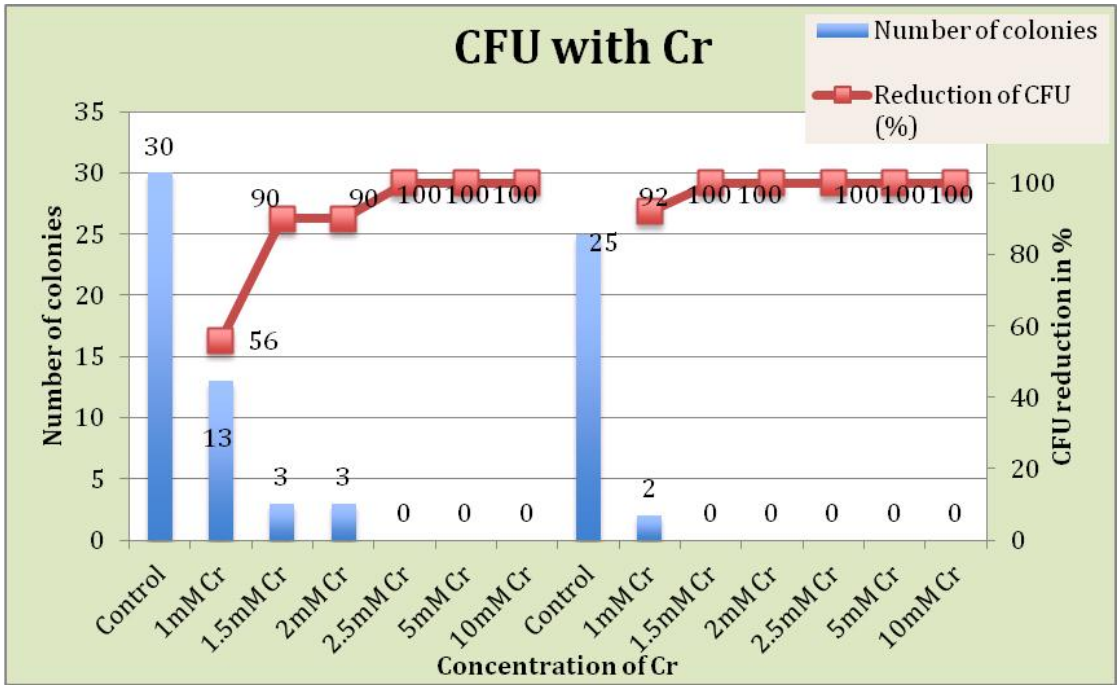

Fig. 1. Viability of *Tr. cutaneum* R57 in different concentrations of Cr ions. Left side: control and concentrations of Cr ions in one hour; Right side: control and concentrations of Cr ions in two hours.

present.

Metals Determination

After supplying Cr ions to the cultivated strain, samples for Inductively coupled plasma mass spectrometry (ICP-MS) analysis were taken at 30, 60, 90 and 120 minutes by separating the solid biomass from the culture by filtration. ICP-MS is a type of mass spectrometry capable of detecting metal ions at very low concentration. The filtrate was analyzed for Cr ions by Prodigy High Dispersion ICP Leeman Labs.

RESULTS

Viability of *Tr. cutaneum* R57 in the presence of different concentration of chromium ions

Cr ions were added in the form of $K_2Cr_2O_7 \cdot 7H_2O$ in different concentrations from 0-10 mM to the culture in separate Erlenmeyer flask incubated for 24 hours in a rotary shaker and the strain cultivated on agar solid media in petri dishes. Viability of *Tr. cutaneum* R57 in different concentration of Cr ions is represented in the figure 1. Viability was checked for 1 hour and 2 hours after adding Cr to the culture. Viability of *Tr. cutaneum* R57 decreases with incubation time in the presence of Cr ions. Lethal dose of Cr ions is higher than 2 mM in one hour and higher than 1.5 mM in two hours. The viability of the strain at 1 mM is 44% and at 1.5 mM and 2 mM is 10% after one hour of incubation. In two hours incubation, the viability of the strain is just 8% in 1 mM and zero in concentration higher than 1 mM. Georgieva et al. [15] reported 0.2 mM as inhibitory threshold value, 0.6 mM as moderate inhibitory value and 1.5 as the lethal dose. The added concentrations of $K_2Cr_2O_7 \cdot 7H_2O$ causes disturbance in the growth phases, abnormal cell morphology with disrupted cell wall and smaller sized cell.

Glucose consumption by *Tr. Cutaneum* R57 in the presence of chromium ions

The rate of glucose consumption by *Tr. cutaneum* R57 in the presence of Cr ions was analyzed first at 24th hour of incubation just before adding $K_2Cr_2O_7 \cdot 7H_2O$ and after 2 hours at 26th hour. Glucose consumption increases with the presence of Cr ions. The maximum amount of glucose is consumed at 5mM concentration of $K_2Cr_2O_7 \cdot 7H_2O$. From microscope study and viability study in the presence of Cr ions, it can be concluded that glucose is consumed for resistance to toxicity of Cr ions although they die ultimately.

Chromium ions removal efficiency of *Tr. cutaneum* R57 from the culture media

Cr removal efficiency of *Tr. cutaneum* R57 was determined by subtracting the amount of Cr ions present in the cultural solution with the initial concentration. Table 1 shows the removal efficiency at every 30 minutes after adding metals to the culture.

It removes 20.89% from 1 mM (104 mg/l) of ions in the cultural medium while only 14.01% from 10 mM (1040 mg/l) in 30 minutes. As the concentration

Table. 1. Cr removal efficiency of *Tr. cutaneum* R57 from cultural media in different concentration of Cr.

Incubation time in minutes	Cells treated with chromium 6^+ ions					
	1mM (104mg)	**1.5mM (156mg)**	**2mM (208mg)**	**2.5mM (260mg)**	**5mM (520mg)**	**10mM (1040mg)**
	Cr removal efficiency from the cultural media %	Cr removal efficiency from the cultural media %	Cr removal efficiency from the cultural media %	Cr removal efficiency from the cultural media %	Cr removal efficiency from the cultural media %	Cr removal efficiency from the cultural media %
0	0	0	0	0	0	0
30	20.89	16.67	17.86	18.42	13.85	14.01
60	21.81	17.20	18.09	19.38	14.87	14.61
90	21.69	18.09	19.54	20.58	14.30	13.90
120	24.08	19.38	20.06	22.18	14.99	14.49

of Cr ions in the cultural medium increases, the removal efficiency decreases. It removed least of 13.85% at 5 mM (520 mg/l) of Cr ions in the cultural medium after 30 minutes.

DISCUSSION

Lethal dose of Cr ions is higher than 2 mM in one hour and higher than 1.5 mM in two hours. The viability of the strain at 1 mM, 1.5 mM and 2 mM is 44%, 10% and 10% respectively after one hour of incubation. In two hours incubation, the viability of the strain is just 8% in 1 mM and zero in concentration higher than 1 mM. Georgieva et al. [15] reported 0.2 mM as inhibitory threshold value, 0.6 mM as moderate inhibitory value and 1.5 as the lethal dose. The added concentrations of $K_2Cr_2O_7 \cdot 7H_2O$ cause disturbance in the growth phases, abnormal cell morphology with disrupted cell wall and smaller sized cell. Initially the cells try to resist the Cr^{6+} toxicity using the optimum energy source in the medium but ultimately all cells die in concentration above 1.5 mM due high toxicity of Cr^{6+} species. Highly toxic hexavalent chromium Cr^{6+} is reduced by the growing organism in the medium to less toxic Cr^{3+}. The biotransformation and bioaccumulation in *Paecilomyces lilacinus* [3] was reported to depend upon the growth phase/state of the fungi in the growth media while considering employing live cells for the removal and detoxification of Cr. In aerobic condition, microbial reduction of Cr^{6+} is catalyzed by soluble enzymes [4]. Optimum growth of the fungus depends on the pH, temperature and residual ions present in the medium. *Mucor* grows maximum at pH 5.5 [16].

The removal of Cr ions happens by two processes of bioaccumulation (active) and biosorption (passive) [10]. Bioaccumulation is the process by which living biomass immobilize metal ions into the cells whereas biosorption process either absorb metal ions on the cell wall of the living biomass or total absorption by the dead biomass [8, 17]. The removal efficiency of *Tr. cutaneum* R57 is higher in lower initial ions concentration. It removes 20.89% from 1 mM (104 mg/l) and 14.01% from 10 mM (1040 mg/l) in medium during the same contact time. Removal efficiency of the strain increases with incubation time in lower concentration and no significant change in percentage removal at higher concentration. Removal efficiency from 1 mM (104 mg/l) is 24.08% and 14.49% from 10 mM (1040 mg/l) in 120 minutes. Research reported *Mucor meihi* removes maximum Cr ions from the solution [8]. The percentage of increase in removal efficiency with time gets decreased with the concentration of ions in the cultural medium. In macrophyte, Cr^{6+} accumulation increases with the contact time [18]. Biomass of *Sargassum wightii* pretreated removed maximum (83%) at pH 3.5-3.8 in a contact time of 6 hours [9]. The toxic effect of Cr^{6+} of paramecium is light dependent and increased body ratio, with average accumulation 1.72 to 15.5 pg Cr/cell [19]. The pH is one of the most important parameters that determines the biosorption of metal ions from aqueous solutions. The removal of Cr^{6+} from the solution depends greatly on the pH of the solution, which also affects the uptake ability [20]. The maximum biosorption takes place at pH between 2.5 to 3. Decrease in pH increase the metal uptake by making surface area of the biomass positively charged [8].

CONCLUSION

The viability study of *Tr. cutaneum* R57 demonstrated that the strain is very sensitive to Cr ions present in the culture medium. The adsorption data obtained from the experiments showed that the applicability of *Tr. cutaneum* R57 in the remediation of heavy metals particularly Cr from solutions. The strength of employing this filamentous fungus lie on its ability to remove higher percentage of heavy metal when it is present in lower concentration which is the main drawback of conventional methods of heavy metal removal. The removal efficiency of *Tr. cutaneum* R57 increases with decrease in the concentration of metal ions present in the solution up to certain minimum level; it removes 20.89% from 1 mM (104 mg/l) of ions in the cultural medium while only 14.01% from 10 mM (1040 mg/l) of Cr ions in 30 minutes. Removal efficiency also increases with the contact time between the metal ions and fungal biomass in lower concentrations. The percentage increase in removal of metal ions with time gets decreased with the concentration of ions in the cultural medium. Taking advantage of the ability of conventional methods to remove higher % in higher initial concentration, this technique using Tr. cutaneum R57 can be integrated with the unit operation of conventional method of metal extraction from the effluent in

series where the stream of effluent treated by conventional method can undergo further extraction of metals in low concentration.

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TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Ilhan S, Nourbakhsh NM, Kilicarslan S, Ozdag H. Removal of chromium, lead and copper ions from industrial wastewaters by *Staphylococcus saprophyticus*. Turk Electr J Biotechnol. 2004; 2: 50-57.
2. Lesmana SO, Febriana N, Soetaredjo FE, Sunarso J, Ismadji S. Studies on potential applications of biomass for the separation of heavy metals from water and wastewater. Biochem Eng J. 2009; 44: 19–41.
3. Sharma S, Adholeya A. Detoxification and accumulation of chromium from tannery effluent and spent chrome effluent by *Paecilomyces lilacinus* fungi. Int Biodeterior Biodegrad. 2011; 65: 309-317.
4. Cheung KH, Gu J. Mechanism of hexavalent chromium detoxification by microorganisms and bioremediation application potential: A review. Int Biodeterior Biodegrad. 2007; 59: 8-15.
5. Coreño-Alonso A, Acevedo-Aguilar FJ, Reyna-López GE, Tomasini A, Fernández FJ, Wrobel K, et al. Cr(VI) reduction by an *Aspergillus tubingensis* strain: Role of carboxylic acids and implications for natural attenuation and biotreatment of Cr(VI) contamination. Chemosphere. 2009; 76: 43–47.
6. Daulton TL, Little BJ, Jones-Meehan J, Blom DA, Allard LF. Microbial reduction of chromium from the hexavalent to divalent state. Geochim Cosmochim Acta. 2007; 71: 556–565.
7. Ertugrul S, San NO, Dönmez G. Treatment of dye (Remazol Blue) and heavy metals using yeast cells with the purpose of managing polluted textile wastewaters. Ecol Engin. 2009; 35: 128–134.
8. Crawford RL, Crawford DL. (Eds). (1995). Bioremediation: Principles and Applications. UK. Cambridge University Press.
9. Vindhan RM. Bioaccumulation of Chromium from Tannery Wastewater: An Approach for Chrome Recovery and Reuse. Environ Sci Technol. 2004; 38: 300-306.
10. Chojnacka K. Bioaccumulation of Cr(III) ions by Blue-Green alga *Spirulina* sp. Part I. A Comparison with Biosorption. Am J Agricult Biol Sci. 2007; 2(4): 218-223.
11. Singh SN, Tripathi RD. (Eds). (2007). Environmental Bioremediation Technologies. Springer-Verlag, Berlin Heidelberg.
12. Sanghi R, Sankararamakrishnan N, Dave BC. Fungal bioremediation of chromates: Conformational changes of biomass during sequestration, binding, and reduction of hexavalent chromium ions. J Hazard Mater. 2009; 169: 1074–1080.
13. Georgieva N. Growth of *Trichosporon cutaneum* R 57 in the presence of toxic concentration of cadmium and copper. Int J Agricult Biol. 2008; 10: 325–328.
14. Nigam A, Ayyagari A. (2008). Lab manual in Biochemistry, Immunology and Biotechnology. New Delhi: Tata McGraw-Hill.
15. Georgieva N, Peshev D, Rangelova N, Lazarova N. Effect of hexavalent chromium of growth of *Trichosporon cutaneum* R57. J Univ Chem Technol Metall. 2011; 46(3): 293-298.
16. Sethi BK, Kanungo S, Rout JK, Nanda P. Sahoo SL. Effect of Chromium on *Mucor* species and optimization of growth conditions. Nature Sci. 2010; 8(4): 29-32.
17. Georgieva N, Peshev D, Rangelova N, Lazarova N, Angelova T. Immobilization of *Trichosporon cutaneum* R57 cells onto SiO_2/HPC hybrid materials and removal of Mn^{2+} ions. Sofia: Adv Bulg Sci. 2011: 33-37.
18. Ramoliya J, Kamdhar A, Kundu R. Movement and bioaccumulation of Chromium in an artificial freshwater ecosystem. Indian J Exp Biol. 2007; 45: 475-479.
19. Mortuza MG, Takahashi T, Ueki T, Kosaka T, Michibata H, Hosoya H. Toxicity and bioaccumulation of hexavalent chromium in green *Paramecium*, *Paramecium bursaria*. J Health Sci. 2005; 51(6): 676-682.
20. Chen G, Zhang WJ, Zeng GM, Huang JH, Wang L, Shen GL. Surface-modified *Phanerochaete chrysosporium* as a biosorbent for Cr(VI)-contaminated wastewater. J Hazard Mater. 2011; 186: 2138–2143.

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BIOLOGY ORIGINAL ARTICLE

Toxicity of *Bauhinia variegata* and *Mimusops elengi* with plant molluscicides against *Lymnaea acuminata*

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ABSTRACT

Molluscicidal activity of binary combination of *Bauhinia variegata* and *Mimusops elengi* with other plant molluscicides *Saraca asoca* and *Thuja orientalis* against snail *Lymnaea acuminata* have been studied. It was observed that toxicity of binary combinations of plant molluscicides with other plant molluscicides were toxic against fresh water snail *L. acuminata*. Among all combinations of toxicity *Mimusops elengi* leaf + *Saraca asoca* bark (24h LC_{50}: 98.25 mg/l; 96h LC_{50}: 40.40 mg/l) and *Bauhinia variegata* leaf powder + *Saraca asoca* leaf (24h LC_{50}: 123.98 mg/l; 96h LC_{50}: 57.91 mg/l) was more toxic than other binary combinations of plant molluscicides. *Mimusops elengi* leaf powder + *Saraca asoca* leaf powder and *Bauhinia variegata* leaf powder + *Saraca asoca* leaf powder are more potent molluscicides.

Key words: Fascioliasis, *Saraca asoca*, *Thuja orientalis*, plant molluscicides, synergist, harmful snail.

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INTRODUCTION

Fascioliasis is one of the most debilitating parasitic diseases in terms of its socioeconomic and public health importance in tropical and subtropical countries [1-3]. In 2006 fascioliasis was reported in 51 different countries from five continents [1]. Singh and Agarwal [4] reported that 94% of buffaloes slaughtered in local slaughter houses of Gorakhpur district, Uttar Pradesh, India carried heavy infection. The disease is transmitted by liver flukes *Fasciola hepatica* and *Fasciola gigantica* through the intermediate host snails *Lymnaeidae* or *Planorbidae*. Human infection normally occurs in areas where animal fascioliasis is endemic [5]. About 2.4 million peoples in rural/agricultural areas to be infected and further, 180 million people at risk of infection [1].

Kumar et al. [6] reported that there is a little interest in snail control due to higher finding properties on SARS, AIDS and malaria. One of the best solutions to eradicate or control this problem by eliminating snail, which is essential of life cycle. Snails can be controlled indirectly by destroying their habitat or directly by killing them. The continuous use of synthetic molluscicide has a long term detrimental effect and create a problem of acute and chronic toxicity to other non-target animals [7]. Plant derived molluscicides are easily biodegradable and safer than their synthetic counter parts. A large number of plant products, which possess molluscicidal activity [8].

Recently, it has been observed that dried powder of *Bauhinia variegata* leaf, bark and seed and *Mimusops elengi* are potent molluscicides [9]. In the present study we have evaluated the toxicity of binary combination of *Bauhinia variegata* (Fabaceae) and *Mimusops elengi* (Sapotaceae) with other plant molluscicides against *Lymnaea acuminata* to explore their synergistic molluscicidal properties.

MATERIALS AND METHODS

Plant used

The leaf/bark/seed of *B. variegata* and *M. elengi* were obtained from the local area in Gorakhpur (Latitude 26º46' N, Longitude 83º22' E). The specimen were identified and authenticated by Department of Botany, DDU Gorakhpur University, Gorakhpur, India.

Preparation of leaf/bark/seed powder

The dried part of leaf/bark/seed of *B. variegata* and *M. elengi* were pulverized separately in the electric grinder and the crude powder obtained, were then sieved with the help of fine meshed cloth by the method of Singh et al. [9]. This fine powder was then used separately for toxicity experiments.

Collection of test animals

The adult fresh water snails, *Lymnaea acuminata* (2.25 ± 0.20 cm in length) were collected locally from different ponds, lakes and low lying submerged fields in Gorakhpur were used as test animals. The collected snails were acclimatized for 72h in the laboratory condition. Experimental animals kept in the glass aquaria containing dechlorinated tap water at 22-24°C. The pH, dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were 7.1-7.3, 6.5-7.3 mg/l, 5.2-6.3 mg/l and 102-105 mg/l, respectively. Dead animals were removed to avoid any spoilage of the aquaria water.

Binary combination

Binary combination of crude powder of *B. variegata* leaf/bark/seed and *M. elengi* leaf/bark/seed and other plant molluscicides *S. asoca* (Caesalpiniaceae) leaf/bark and *T. orientalis* (Cupressaceae) leaf/fruit crude powder of in 1:1 ratio were used for the determination of molluscicidal activity (Table 1).

Toxicity experiment

The binary combination toxicity experiments were performed by the method of [10]. Ten experimental animals were kept in a glass aquarium containing 3L of dechlorinated tap water. Snails were exposed continuously for 96h to different concentrations of *B. variegata* and *M. elengi* with other plant molluscicides. Six aquaria were setup for each concentration. The control animals were kept in the equal volume of water under similar conditions without treatment. Mortality of snails was recorded at interval of 24h each up to 96h. The mortality of snails was established by the contraction of body within the shell, no response to needle probe was taken as evidence of death.

Concentration-mortality data for each group of snails were analyzed using the probit analysis program, POLO-PC (LeOra Software) [11] to estimate the LC_{50} of the 95% confidence intervals

Table. 1. Binary Combination of plant products of *B. variegata* and *M. elengi* with other plant product molluscicides used for the toxicity determination against *Lymnaea acuminata*.

Treatment	Concentration (mg/l) used against *Lymnaea acuminata*
Mimusops elengi leaf + *Thuja orientalis* leaf	30, 50, 70, 90
Mimusops elengi bark + *Thuja orientalis* leaf	30, 50,70,90
Mimusops elengi leaf + *Thuja orientalis* fruit	30, 50, 70, 90
Mimusops elengi seed + *Thuja orientalis* leaf	60, 80, 100, 120
Mimusops elengi leaf + *Saraca asoca* bark	30, 50, 70, 90
Mimusops elengi leaf + *Thuja orientalis* fruit	30, 50, 70, 90
Mimusops elengi leaf + *Saraca asoca* leaf	60, 80, 100, 120
Mimusops elengi bark + *Saraca asoca* leaf	60, 80, 100, 120
Mimusops elengi bark + *Saraca asoca* bark	60, 80, 100, 120
Mimusops elengi seed + *Saraca asoca* leaf	30, 50, 70, 90
Bauhinia variegata leaf + *Saraca asoca* leaf	50,70, 90, 120
Bauhinia variegata seed + *Thuja orientalis* leaf	50,70, 90, 120
Bauhinia variegata leaf + *Saraca asoca* leaf	50,70, 90, 120
Bauhinia variegata seed + *Saraca asoca* leaf	60, 80, 100, 120
Bauhinia variegata leaf + *Thuja orientalis* fruit	50,70, 90, 120

for these concentrations. The slope of the probit line was also estimated. This program ran chi-square test for goodness-of-fit of the data to the probit model. If the model fits, the calculate heterogeneity factor of chi-square is less than the chi-square table value for the appropriate degrees of freedom. If the model does not fit, the LC_{50} for the particular population may not be reliably estimated and is adjusted with the heterogeneity factor (observed chi-square value divided by degrees of freedom).

This program uses the heterogeneity factor as a correction factor when the value of Pearson's chi-square statistic is significant at $P < 0.05$. The index of significance for potency estimation (g-value) was used to calculate 95% confidence intervals for potency (relative potency is equivalent to tolerance ratio). Parallelism of the probit regression lines implies a constant relative potency at all levels of response. POLO-PC was used to test equality and parallelism of the slope of the probit lines.

RESULTS

The plant *B. variegata* and *M. elengi* leaf/bark/ seed powder with other plant molluscicides *S. asoca* leaf/ bark and *T. orientalis* leaf/fruit powder used in toxicity of binary combination (1:1) against snail *Lymnaea acuminata* was time and concentration dependent.

Toxicity of binary combination of ML+SB (24h LC_{50}: 98.25 mg/l) was more effective than the ML+TF (24h LC_{50}: 164.67 mg/l) respectively against *Lymnaea acuminata*. Toxicity order of binary combination of ML+SB > MS+SL > MB+TF > MB+SL > ML+TL > MB+TL > MS+TL > MB+SB > ML+SL > ML+TF, respectively (Table 2).

Other binary combination of *B. variegata* with other plant molluscicides *S. asoca* and *T. orientalis*. Toxicity of binary combination (1:1) of BL+SL (24h LC_{50}: 123.98 mg/l) was more effective than the BS+TL (24h LC_{50}: 175.44 mg/l) against *Lymnaea acuminata*. Toxicity order of binary combination of BL+SL > BL+TF > BL+TL > BS+SL > BS+TL, respectively (Table 3).

The slope values were steep and separate estimation of LC based on each of six replicates was found to be within 95% confidence limit of LC_{50}. The t-ratio was greater than 1.96 and the heterogeneity factor is less than 1.0. The g-values was less than 0.5 at all probability level (90, 95 and 99).

Table. 2. Toxicity of binary combinations (1:1 ratio) of leaf, bark and seed of *M. elengi* crude powder against *Lymnaea acuminata* at different exposure period.

Exposure periods	Treatment	LC_{50} (m/l)	Limit LCL_UCL	Slope value	t-ratio	g-value	Heterogeneity
	ML+TL	123.00	100.39-187.88	2.81±0.64	4.39	0.17	0.50
	MB+TL	125.50	101.07-194.42	2.85±0.63	4.37	0.20	0.37
	MB+TF	119.35	97.60-176.80	3.62±0.83	4.50	0.18	0.36
	MS+TL	128.30	113.41-162.59	3.24±0.61	4.34	0.20	0.16
24h	ML+SB	98.25	85.19-124.54	2.75±0.71	5.25	0.13	0.41
	ML+TF	164.67	131.27-277.23	3.21±0.81	3.85	0.25	0.26
	ML+SL	132.49	114.90-174.22	5.21±0.94	3.93	0.24	0.31
	MB+SL	122.12	112.22-139.66	4.63±0.93	5.50	0.12	0.35
	MB+SB	131.45	118.10-158.78	3.17±0.68	4.96	0.15	0.28
	MS+SL	116.92	97.30-165.73	2.07±0.50	4.46	0.17	0.34
	ML+TL	100.74	84.24-157.96	1.98±0.51	4.07	0.23	0.40
	MB+TL	110.71	87.41-185.74	2.36±0.52	3.84	0.26	0.25
	MB+TF	93.32	78.56-127.06	3.10±0.76	4.52	0.18	0.27
	MS+TL	106.44	95.68-127.10	3.10±0.76	4.06	0.23	0.16
48h	ML+SB	80.88	70.23-100.75	2.53±0.51	4.94	0.15	0.33
	ML+TF	127.05	105.97-188.95	2.34±0.62	3.72	0.27	0.15
	ML+SL	105.28	97.08-136.00	2.83±0.75	3.73	0.27	0.26
	MB+SL	103.73	95.52-116.52	4.03±0.79	5.11	0.14	0.19
	MB+SB	114.82	102.66-133.31	4.10±0.82	4.98	0.15	0.18
	MS+SL	99.35	81.99-143.55	2.24±0.52	4.29	0.20	0.19
	ML+TL	64.07	55.97-75.35	2.31±0.47	4.88	0.16	0.58
	MB+TL	83.00	67.88-124.07	1.71±0.47	3.57	0.30	0.23
	MB+TF	68.90	58.97-86.12	2.03±0.48	4.24	0.21	0.23
	MS+TL	84.87	74.64-95.39	2.83±0.74	3.82	0.26	0.15
72h	ML+SB	56.86	49.20-66.01	2.29±0.47	4.78	0.16	0.44
	ML+TF	92.88	80.78-115.09	2.24±0.60	3.74	0.27	0.13
	ML+SL	87.19	77.10-98.56	2.81±0.74	3.80	0.26	0.26
	MB+SL	87.20	79.77-95.24	3.77±0.76	4.96	0.15	0.24
	MB+SB	97.41	89.05-109.68	3.52±0.76	4.62	0.18	0.16
	MS+SL	71.07	60.51-90.69	1.98±0.54	4.14	0.16	0.19
	ML+TL	36.39	27.94-42.77	2.28±0.47	4.83	0.24	0.98
	MB+TL	52.56	43.03-62.77	1.84±0.46	3.93	0.17	0.20
	MB+TF	48.22	40.22-55.69	2.19±0.47	4.63	0.18	0.18
	MS+TL	68.48	58.07-57.73	3.51±0.76	4.56	0.11	0.39
96h	ML+SB	40.40	34.11-45.65	2.86±0.49	5.77	0.24	0.47
	ML+TF	68.26	56.73-77.87	2.38±0.59	3.97	0.20	0.13
	ML+SL	69.83	58.84-77.47	3.29±0.75	4.34	0.20	0.45
	MB+SL	73.57	66.31-79.45	4.36±0.78	5.57	0.12	0.48
	MB+SB	78.97	71.65-85.46	4.05±0.76	5.27	0.13	0.34
	MS+SL	48.44	39.94-56.40	2.07±0.47	4.40	0.19	0.24

Mortality was determined at every 24 h up to 96 h. Each set of experiment was replicated six times.
Abbreviation: ML - *M. elengi* leaf powder, MB - *M. elengi* bark powder, MS - *M. elengi* seed powder, TL - *T. orientalis* leaf powder, TF - *T. orientalis* fruit powder, SL - *S. asoca* leaf powder, SB - *S. asoca* bark powder; LCL = lower confidence limit; UCL = upper confidence limit. Significant negative regression ($P < 0.05$) was observed between exposure time and LC_{50} of treatments. Ts - testing significant of the regression coefficient: ML+TL - 11.30^{+}; MB+TL - 9.56^{+}; MB+TF - 14.05^{+}; MS+TL - 21.58^{+}; ML+SB - 19.46^{+}; ML+TF - 15.39^{+}; ML+SL - 12.36^{+}; MB+SL - 21.44^{+}; MB+SB - 60.98^{+}; MS+SL - 16.61^{+}.
+: linear regression between x and y; ++: non-linear regression between log x and log y.

Table. 3. Toxicity of binary combinations (1:1 ratio) of leaf, bark and seed of *B. variegata* crude powder against *Lymnaea acuminata* at different exposure period.

Exposure periods	Treatment	Lc_{50} (m/l)	Limit LCL_UCL	Slope value	t-ratio	g-value	Heterogeneity
24h	BL+SL	123.98	106.31-166.45	2.77±0.64	4.27	0.21	0.32
	BL+TF	129.69	112.91-165.96	3.45±0.16	4.86	0.16	0.31
	BS+TL	175.44	140.25-288.51	3.26±0.80	4.03	0.23	0.46
	BL+TL	153.24	128.74-288.40	3.43±0.89	3.84	0.26	0.20
	BS+SL	164.51	133.05-287.32	3.00±0.87	3.43	0.32	0.11
48h	BL+SL	98.07	86.31-119.97	2.54±0.61	4.17	0.22	0.26
	BL+TF	109.62	96.05-137.82	2.77±0.63	4.39	0.19	0.20
	BS+TL	152.53	121.56-263.86	2.36±0.65	3.61	0.26	0.29
	BL+TL	126.80	110.00-172.52	2.94±0.78	3.73	0.27	0.15
	BS+SL	124.05	107.55-170.07	2.74±0.77	3.59	0.29	0.08
72h	BL+SL	75.24	64.89-85.96	2.45±0.59	4.07	0.22	0.29
	BL+TF	86.82	76.62-101.72	2.52±0.60	4.18	0.22	0.16
	BS+TL	116.80	97.85-172.99	2.11±0.60	3.47	0.13	0.21
	BL+TL	101.45	90.15-123.26	2.66±0.74	3.56	0.30	0.11
	BS+SL	100.02	88.52-122.02	2.55±0.74	3.43	0.32	0.08
96h	BL+SL	57.91	46.91-65.75	2.78±0.61	4.49	0.19	0.35
	BL+TF	65.71	55.16-74.16	2.63±0.60	4.32	0.20	0.26
	BS+TL	79.43	65.77-96.83	1.87±0.59	3.17	0.38	0.18
	BL+TL	76.69	68.35-83.60	3.17±0.76	4.86	0.16	0.40
	BS+SL	76.52	65.34-85.17	2.93±0.74	3.94	0.24	0.12

Mortality was determined at every 24 h up to 96 h. Each set of experiment was replicated six times.
Abbreviation: BL - *B. variegata* leaf powder, BS - *B. variegata* seed powder, TL - *T. orientalis* leaf powder, TF - *T. orientalis* fruit powder, SL - *S. asoca* leaf; LCL = lower confidence limit; UCL = upper confidence limit. Significant negative regression ($P < 0.05$) was observed between exposure time and LC_{50} of treatments. Ts - testing significant of the regression coefficient: BL+SL - 16.16^{+}; BL+TF - 60.80^{++}; BS+TL - 13.38^{+}; BL+TL - 95.15^{+}; BS+SL - 9.90^{+}.
+: linear regression between x and y; ++: non-linear regression between log x and log y.

DISCUSSION

Binary combination of *B. variegata* and *M. elengi* with *S. asoca* and *T. orientalis* shows effective molluscicidal activity than single treatment against *Lymnaea acuminata* [9, 12]. The molluscicidal activity of these combinations was time as well as concentration dependent. A number of studied on binary combination of plant derived molluscicides have been conducted against harmful snails [13-20].

Toxicity of *M. elengi* leaf with *T. orientalis* leaf/ fruit (96h LC_{50}: 36.39 mg/l, *M. elengi* leaf + *T. orientalis* fruit LC_{50}: 68.26 mg/l), *M. elengi* leaf with *S. asoca* leaf/bark (96h LC_{50}: 69.83 mg/l and 40.40 mg/l), *M. elengi* seed with *T. orientalis* leaf and *S. asoca* leaf (96h LC_{50}: 68.48 mg/l), *M. elengi* seed with *S. asoca* leaf (96hLC_{50}: 48.44 mg/l) and *B. variegata* leaf with *T. orientalis* leaf/fruit and *S. asoca* leaf (96h LC_{50}: 76.69, 65.71 and 57.91 mg/l) and *B. variegata* seed with *T. orientalis* leaf, and *S. asoca* leaf (96h LC_{50}: 79.43 and 76.52 mg/l) against the snail *Lymnaea acuminata* in the present study is very high in comparison to other plant molluscicides *Polianthes tuberosa* bulb powder + oleoresin of *Z. officinale* (96h LC_{50}: 69.51 mg/l), *Azadirachta indica* oil + *Polianthes tuberosa* bulb powder (96h LC_{50}: 94.42 mg/l), *Lawsonia inermis* + *A. sativum* (96h LC_{50}: 88.21 mg/l), *L. inermis* + *Polianthes tuberosa* (96h LC_{50}: 86.27 mg/l) against the snail *Lymnaea acuminata* [21, 22].

Mortality caused by the binary combination of plants product was time and dose dependent and there was a negative regression between exposure time and LC values. The penetration of the toxicant also have a greater significant for the aquatic environment, because there is whole body is bathed in a diluted solution of toxicant. To have maximum

effect, the binary combination must penetrate the organism and transported to active sites rapidly. It seems the high level of binary combination in snails may be due to rapid penetration of other plant molluscicides through soft foot of snails body and/or it may possible the plant active component may changed in to toxic form in the aquarium water or in snail body which triggered by different enzymes.

The steep slope values indicate that even a small increase in the concentration causes higher snail mortality. Values of t-ratio higher than 1.96 indicate that the regression is significant. Values of heterogeneity factor, less than 1.0, denotes that in the replicates test of random sample the concentration response curves fall with the 95% confidence limit and thus the model fit the data adequately. The index of significance of potency estimate g-value indicate that the value of the mean is within the limits at all probability (90, 95, 99) since it is less than 0.5.

It can be concluded from the present study that the use of these plant in binary combination will be more helpful in controlling the aquatic snails, than their individual components. The effective toxic concentration of each component is lower and would be safer in aquatic environment.

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TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. WHO. Report of the WHO informal meeting on use of triclabendazole in fascioliasis control. 2006, 17-18 October, Geneva, Switzerland.
2. Mas-Coma S, Bargues MD, Valero MA. Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol. 2005; 35: 1255-1278.
3. Mas-Coma S, Valero MA, Bargues MD. Fasciola Lymnaids and human fascioliasis with a global overview on disease transmission, epidemiology, evolutionary genetics molecular epidemology and control. In: Rollinson D, Hay S. (Eds.) Burligton: Academic press. Adv Parasitol. 2009; 69: 141-146.
4. Singh O, Agarwal RA. Toxicity of certain pesticides to two economic species of snail in northern India. J Econ Entomol. 1981; 74(5): 568-571.
5. Robinson MW, Dalton JP. Zoonotic helminth infections with particular emphasis on fascioliasis and other trematodians. Phill Trans R Soc B. 2009; 364: 2763-2776.
6. Kumar P, Singh DK, Singh VK. Combination of molluscicides with attractant carbohydrates and amino acids in bait formulation against the snail *Lymnaea acuminata*. Eur Rev Med Pharmacol Sci. 2011; 15: 550-555.
7. Safer TJ, Mayer DA, Crofton KM. Developmental neurotoxicity of pyrethroid insecticides: Critical review and future research needs. Environ Health Persp. 2005; 113: 123-136.
8. Singh VK, Singh DK. Molluscicidal activity of pre- and post-harvested *Allium sativum* (Garlic). Biol Agric Hortic. 1996; 12: 311-318.
9. Singh KL, Singh DK, Singh VK. Characterization of the molluscicidal activity of *Bauhinia variegata* and *Mimusops elengi* plant extract against the *Fasciola* vector *Lymnaea acuminata*. Rev Inst Med Trop Sao Paulo. 2012; 54 (3): 135-140.
10. Singh DK, Agarwal RA. Correlation of the anticholinesterase and molluscicidal activity of latex of *Euphorbia royleana* on *Lymnaea acuminata*. J Nat Prod. 1984; 42: 702-705.
11. Russell R M, Robertson JL, Savin NE. POLO: A new Computer Program for probit analysis. Bull Entomol Soc Am. 1977; 23: 209-213.
12. Singh A, Singh VK. Molluscicidal activity of *Saraca asoca* and *Thuja orientalis* against the fresh water snail *Lymnaea acuminata*. Vet Parasitol. 2009; 164: 206-210.
13. Singh A, Singh DK. Pestoban, a potent herbal molluscicides. Biol Agric Hortic. 1994; 10: 175-178.
14. Singh K, Singh A, Singh DK. Molluscicidal activity of different combinations of the plant products used in the molluscicides pestoban. Biol Agric Hortic. 1995; 12: 253-261.

15. Singh K, Singh DK. Acute toxicity of neem based pesticide on *Lymnaea acuminata*. Malays Appl Biol. 1997; 26 (1): 15-18.
16. Tripathi SM, Singh DK. Molluscicidal activity of *Punica granatum* and *Canna indica* combination with plant derived molluscicides against harmful snails. Malays Appl Biol. 2001; 30 (1&2): 25-31.
17. Kumar P, Singh DK. Binary combination of the some common spices against harmful snails. J Appl Bio Sci. 2007; 33 (2): 167-170.
18. Jitendra KP, Singh DK. Toxicity of *Piper cubeca*, *Piper longum* and *Tribulus terrestris* against the snail *Lymnaea acuminata*. Malays Appl Biol. 2008; 37(1): 41-46.
19. Singh A, Kumar P, Singh DK, Singh VK. Toxicity of binary combination of *Saraca asoca* and *Thuja orientalis* with synergist piperonyl butoxide and MGK-264 against the fresh water snail *Lymnaea acuminata*. The Bioscan. 2010; 5(1): 13-18.
20. Kumar S, Singh DK, Singh VK. Binary combination of different breeds of freeze- dried cow urine (FCU) with some plant molluscicides against *Lymnaea acuminata*: vector of fasciolosis. Adv in Life Sci. 2011; 1(1): 24-29.
21. Singh A, Singh DK. Molluscicidal activity of *Lawsonia inermis* and its binary and tertiary combination with other plant derived molluscicides. Indian J Exp Biol. 2000; 39: 263-268.
22. Shukla S, Singh VK, Singh DK. Binary and tertiary combinations of plant derived molluscicides against the vector snail *Lymnaea acuminata* and *Indoplanorbis exustus*. Malays Appl Biol. 2005; 34(2): 15-24.

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BIOLOGY ORIGINAL ARTICLE

The protective strategy of antioxidant enzymes against hydrogen peroxide in honey bee, *Apis mellifera* during two different seasons

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ABSTRACT

Introduction: Honey bees collect floral nectar and pollens, for feeding, which rich with allelochemicals and phenols. The oxidation of these materials produce reactive oxygen species (ROS), among them the hydrogen peroxide (H_2O_2) and superoxide. The honey bee antioxidant enzymes are of particular interest in detoxification of these ROS. Superoxide dismutases (SODs) are the first line of defense against oxygen free radicals. In concert with catalase; SODs have strong antioxidant properties. Since catalase is inefficient at removing H_2O_2 because of its high K_m the ascorbate peroxidase (APOX) serves better in H_2O_2 detoxification.

Results: The antioxidant enzymes under investigation showed highly significant variation during the whole duration of the experiment. However, a combined effect of months, race (Hybrid and Carniolan) and type (foraging and nursing) showed similar activity in SOD and CAT.

Conclusion: The correlations between SOD, CAT and APOX, indicating that there must be a specific manner strategy for managing peroxides at safe levels. The increase or decreases of the antioxidants are according to the contents and levels of peroxides.

Key words: ascorbate peroxidase, catalase, Carniolan, foraging, Hybrid, hydrogen peroxide, nursing, superoxide dismutases.

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INTRODUCTION

The free radicals are a part of the normal metabolic process for energy production [1, 2], they are inevitable by-products and once formed, a chain reaction start [3], they are unstable and can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants [4].

Reactive oxygen species (ROS) are generated in mitochondria during cell respiration [5], where more than 90% of oxygen is utilized during metabolic process to produce energy in the presence of cytochrom oxidase, NADPH oxidase and coenzyme Q [6]. Oxygen is essential for aerobic organisms, however, exposure to oxygen at concentrations more than 21% can lead to injury [7]. The exposure of radiation, ultraviolet rays, cigarette smoke, air pollutants and some industrial chemicals may results in the production of a ROS [8]. Currently, a number of free radicals and non radical species derived from molecular oxygen, included, superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet -}$), lipid peroxide (LOOH) and hypochlorous acid (HOCl) [9]. Reactive oxygen species become so harmful when exceeding the normal limit [10]. Reactive oxygen species are directly dangerous to cellular component like proteins and amino acids [11], and attack polyunsaturated fatty acid (PUFA), phospholipids leading to lipid peroxidation, impairment in membrane function and losing cell membrane structure [12]. Also, ROS implicates in mutation and aging [13, 14].

Insects are subjected to increasing loads of oxidative stress due to their life style. First, their tracheal system delivers O_2 directly to tissues via gas diffusion. Second, tissue demand for O_2 during insect flight is exceptionally high [15]. Third, the diet of herbivorous insects may be rich in pro-oxidant compounds that may increase the normal load of oxidative stress [16]. In insects, also the oxidative stress occurs when the formation of free radicals and other pro-oxidants overwhelm the organism's antioxidant defence system [17].

All organisms including arthropod insects have developed many defences to protect themselves from the harmful influences of reactive oxygen species [16]. The most endogenous defensive and protective strategy is the antioxidant systems.

Antioxidant defences typically involve the intervention of endogenous mediators (e.g. enzymes) as well as exogenous elements and substances that are acquired with food [18]. Insects may restrict oxidative radicals and other oxidants from reaching metabolically active tissues [19]. First, antioxidant enzyme activities are high in the tissues of some insect species that feed on some host plants [20-22]. Second, in some species; some antioxidant enzymes are induced by ingested prooxidants [20, 23, 24]. The major components of the honey bee antioxidant system have been identified using the honey bee genome sequence [25].

Elevated expression of several traditional antioxidant-encoding genes occurs in young queens and old workers of *Apis mellifera* [26], suggesting that queen longevity is not related to higher expression of these particular genes, a result consistent with findings for SOD in *Lasius niger* ant queens [27]. Weirich et al. [28] and Collins et al. [29] reported that catalase, glutathione S-transferase (GST) and SOD might contribute to the ability of queens to store sperm in their spermatheca for several years without loss of viability. Insects have evolved a complex antioxidant mechanism to overcome the toxic effects of ROS, based on the enzymatic actions of glutathione peroxidase, catalase, SOD and APOX [30].

Honey bees collect flower nectar and convert it to honey, which provide the energy [31], also collect pollens and propolis in their feeding and activities, it is well known that these substances contain phenol, tannin and alkaloids [32]. The allelochemicals are present in nectar [33]. Propolis contains tannins, polysaccharides [34, 35]. The oxidation of phenols produces ROS [36, 37]; and the ingested phenolic compounds become extensively oxidized [38, 39]. Quinones, the oxidation products of phenols [40] may also be toxic and can cause the formation of gut lesions [41, 42]; and oxidative stress in the midgut tissues of *Lymantria dispar* [43]. Honey bee antioxidant enzymes are of particular interest because of their potential involvement in some of the exceptional biological characteristics of the queen honey bee, especially its longevity relative to worker bees [44].

Honey bee antioxidant enzymes are of particular interest because of their potential involvement in some of the exceptional biological characteristics of the honey bees and affect on their activities as well as the longevity. Thus, the purpose of the present

study was to spot the light and determine of the antioxidant enzymes and total peroxide concentration, and measure the biochemical activities of these honey bee enzymes simultaneously with the meteorological records.

MATERIALS AND METHODS

Apiary construction

Pure, fertilized Carniolan honey bee queens were prepared and each one introduced into single hive its queen was just eliminated. Totally, ten hives were constructed, left for forty five days after introduction of the pure queen. Only the five strongest hives, with similar number of individuals were selected for experiments. Hybrid honey bees (Egyptian X Carniolan) were brought from Faculty of Agriculture apiary, Assiut University. Similarly to that of Carniolan, the strongest five hives were selected for experiments in the case of Hybrid honey bees.

Monthly biochemical experiments concerning antioxidant enzymatic activities of both races were carried out. Meteorological data were obtained from meteorological station, Faculty of Agriculture farm, Assiut University.

Measurements of honeybee activities

Bee population

The number of adult honeybees was determined by a visual method of comparison with standard photographs of known numbers of honeybees on combs [45]. Honey bee activities in both races were classified into active season (June - September) and moderately active season (October - December) according to the weather temperature, photoperiod (Table 1).

Antioxidant enzyme assays

Super Oxide Dismutase

The assay is based on the oxidation of epinephrine by superoxide ($O_2^{\bullet-}$) to adrenochrome is inhibited by superoxide dismutase (SOD) [46]. The absorbance was read at 480 nm using Ultrospec 3300 pro UV/Visible spectrophotometer. One unit of SOD activity was defined as the mass of protein (mg) corresponding to a 50% inhibition in the rate of epinephrine oxidation [46]. Protein concentration for each sample was measured and calculated using total protein kit (Diamond Diagnostics) according to the manual instructions.

Catalase enzyme

Catalase activities were determined by the method of Aebi [28, 47, 48], based on the rate of hydrogen peroxide decomposition measured at 240 nm. The absorbance was read at 240 nm. Catalase activity was expressed as micromoles of H_2O_2 reduced per minute per milligram of protein, using an extinction coefficient (39.4 M^{-1} cm^{-1}) [47].

Ascorbate Peroxidase

The assay is based on the disappearance of ascorbate, as it is oxidized to dehydroascorbic acid by H_2O_2 [19, 48]. The reduction in absorbance was followed at 290 nm. Enzyme activity was expressed as micromolar ascorbate oxidized per minute per milligram protein using a molar extinction coefficient 2.8 mM^{-1} cm^{-1} [49].

Total Peroxide

The total peroxide (H_2O_2) concentrations were determined using FOX2 method [50] with minor modifications [51]. The FOX2 system is based on the oxidation of ferrous iron to ferric iron by the various types of peroxides contained in the samples, in the presence of xylenol orange, which produces a colored ferric-xylenol orange complex whose absorbance can be measured. The absorbance of the supernatant was determined at 560 nm. Blank contained all the reaction mixture components except the ferrous ammonium sulphate [51].

Statistical analysis

Data obtained were statistically analysed by using factorial analysis and F-test. Means were compared according to Duncan. Differences among means were determined by Duncan's Multiple Range Test. Simple and multiple correlations for the relations between studied factors were calculated (SAS) [52].

RESULTS

Apiary seasonal variation of honey bee activities

In this experiment, some factors such as bee activities; bee population was studied, and meteorological factors such as temperature; relative humidity and photoperiod were considered, attempting to explain the seasonal variation activities of some antioxidant enzymes in both Carniolan and Hybrid worker bees under apiary

Table 1. Meteorological factors surrounding the colony during the experiments.

Season		Active Season			Moderately Active Season			
Month		June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Temperature / ˚C		36.2-44.8	37-41.6	37-40.8	34-40.2	28-40.4	26.6-32.6	24-29.2
Relative Humidity/degrees		9-21	13-29	16-30	20-50	15-42	19-33	20-37
Photoperiod	Light	13.4-13.5	13.2-13.4	13.0-13.1	12.1-12.4	11.2-11.1	10.4-10.1	10.2-10.0
	Dark	10.2-10.1	10.4-10.2	11.0-10.5	11.5-11.2	12.4-12.5	13.2-13.5	13.4-14.0

Table 2. Seasonal variation of honeybee activities in Hybrid and Carniolan honeybees.

Season	Active Season			Moderately-Active Season			
Month	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Hybrid Bees (individual/colony)	4520	4170	4600	9480	7980	7830	1750
Carniolan Bees (individual/colony)	3960	4330	5220	10900	9760	7290	2040

Table 3. Probability values from analysis of antioxidant enzymes activities (• P ≤ 0.05, ••P ≤ 0.001).

EFFECT	SOD		CAT		APOX		H_2O_2	
	F. value	P. value	F. value	P. value	F. value	P. value	F. value	P. value
Month	11.752••	0.000	67.386••	0.000	79.291••	0.000	1856.246••	0.000
Races	0.028	0.867	12.373••	0.001	1.267	0.263	81.533••	0.000
Type	0.028	0.867	11.237••	0.001	0.553	0.459	0.880	0.350
Month X Race	0.092	0.997	11.007••	0.000	9.368••	0.000	51.033••	0.000
Month X Type	0.092	0.997	8.348••	0.000	1.737	0.119	3.821••	0.002
Race X Type	0.681	0.411	0.292	0.590	0.006	0.940	27.729••	0.000
Month X Race X Type	0.157	0.987	1.065	0.388	2.745•	0.016	37.597••	0.000

conditions during active and moderately-active seasons (Table 1).

Bee population

Data represented in Tables 1, 2 and Figure 1 shows the number of bees, which reached its peak during September-October for both Carniolan and Hybrid worker bees. The maximum bee number of both races was in September. For Carniolan, the maximum number was 10900 bees per colony, while it was 9480 bees/colony in Hybrid bees.

The biochemical activities of the antioxidant enzymes

The antioxidant enzymes under investigation showed highly significant variation during the whole duration of the experiment [all p ≤ 0.01 (Table 3)]. Similar highly significant variation in the two honeybee races (Hybrid and Carniolan) were observed in CAT and total peroxide concentration (Table 3). However, the two races showed similar activity in SOD, APOX (Table 3).

When the effect of months together with the race was considered, a highly significant variation was

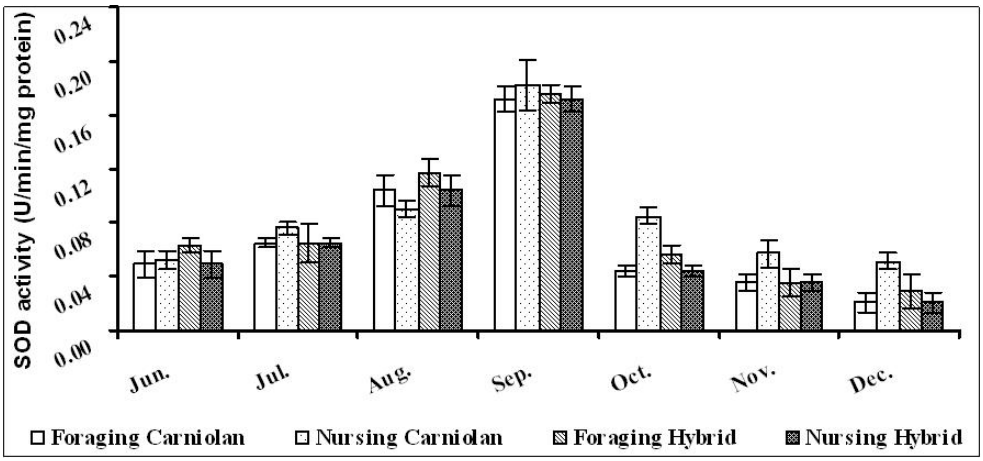

Fig. 1. The changes in superoxide dismutase (SOD) activity during the active and moderately active seasons. The results are means (+ SE) of five replicates for each type of the hive.

Table 4. A comparison between foraging and nursing workers of Hybrid and Carniolan during the active season (June - September).

Month	Race	Type	SOD	CAT	APOX	H_2O_2
June	Hybrid	Foraging	0.063 ± 0.005	0.495 ± 0.039	0.059 ± 0.004	0.296 ± 0.012
		Nursing	0.049 ± 0.010	0.302 ± 0.037	0.070 ± 0.004	0.080 ± 0.025**
	Carniolan	Foraging	0.049 ± 0.010	0.292 ± 0.046#	0.058 ± 0.004#	0.065 ± 0.002##
		Nursing	0.052 ± 0.007	0.167±0.028*#	0.046 ± 0.002	0.061 ± 0.010
July	Hybrid	Foraging	0.065 ± 0.014	0.173 ± 0.024	0.076 ± 0.004	0.948 ± 0.065
		Nursing	0.065 ± 0.003	0.170 ± 0.020	0.077 ± 0.002	1.300 ± 0.161
	Carniolan	Foraging	0.065 ± 0.003	0.147 ± 0.014	0.066 ± 0.003#	0.891 ± 0.030#
		Nursing	0.076±0.005*#	0.141 ± 0.012	0.063 ± .004##	0.835 ± 0.040#
August	Hybrid	Foraging	0.117 ± 0.010	0.099 ± 0.008	0.107 ± 0.008	1.460 ± 0.063
		Nursing	0.104 ± 0.011	0.140 ± 0.012*	0.106±0.007**	2.126 ± 0.037**
	Carniolan	Foraging	0.104 ± 0.011	0.096 ± 0.014	0.076 ± 0.007	2.429 ± 0.062##
		Nursing	0.090 ± 0.006	0.076±0.005##	0.071±0.003*##	1.924±0.024**##
September	Hybrid	Foraging	0.176 ± 0.0064	0.090 ± 0.007	0.107 ± 0.012	2.666 ± 0.038
		Nursing	0.172 ± 0.0095	0.069 ± 0.005*	0.114 ± 0.010	2.426 ± 0.039**
	Carniolan	Foraging	0.172 ± 0.0095	0.057±0.004##	0.176 ± 0.022	2.865 ± 0.030##
		Nursing	0.182 ± 0.019	0.056 ± 0.004	0.133 ± 0.011	2.943± 0.11**##

* Significant against foraging (of the same type) # Significant against hybrid (foraging or nursing)

** Highly significant against foraging (of the same type) ## Highly significant against hybrid (foraging or nursing)

observed with all enzyme activities, except for the SOD. However, a combined effect of months, race and type (foraging and nursing) showed similar activity in SOD and CAT [$p \geq 0.01$ (Table 4)]. A significant variation ($p \leq 0.05$) in the APOX activity observed when the three combined effects tested. Nevertheless, highly significant differences ($p \leq 0.01$) noticed in the total peroxide concentration.

Superoxide dismutases

Superoxide dismutase activity was similar in both Hybrid and Carniolan honey bee races [$p \geq 0.05$, (Table 3)]. In the active season, the activity was gradually elevated from June to September (active season) in both two races; the highest activity was observed in September, while the lowest activity was noticed in June (Table 4 and Figure 1). In July, the SOD activity of nursing Carniolan was significantly increased (0.076 ± 0.005) comparing with both the foraging bees of the same race (0.065 ± 0.003) and the nursing bees of the Hybrid race (0.065 ± 0.003) (Table 4).

In the moderately active season, the SOD activity in both races was dramatically reduced when compared with the active season. Moreover, SOD activity was gradually reduced thought the moderately active season (Figure 1). Yet, in October

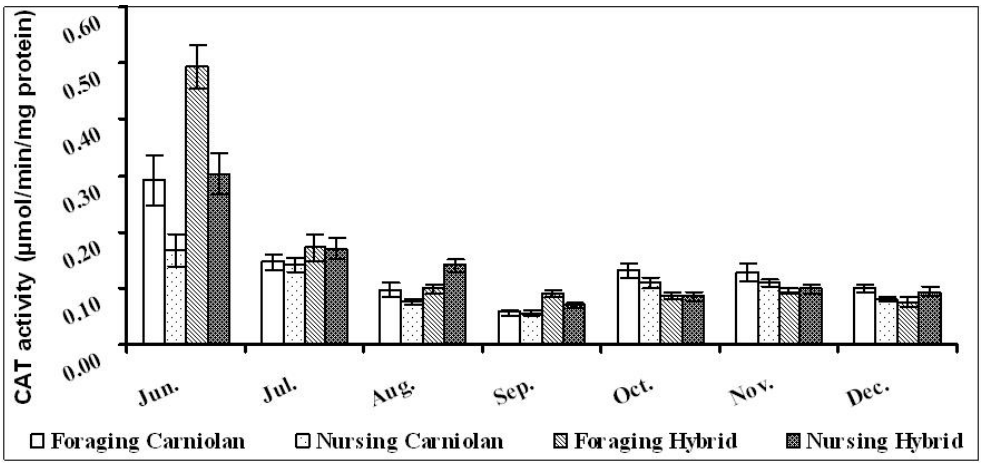

Fig. 2. The changes in catalase (CAT) activity during the active and moderately active seasons. The results are means (+ SE) of five replicates for each type of the hive.

highly significant increase in SOD activity was noticed in nursing Carniolan (0.058 ± 0.006) comparing with both the foraging Carniolan (0.044 ± 0.004) and the nursing Hybrid (0.044 ± 0.004).

Catalase

Catalase activity showed highly significant variations against the three single effects (month, race and type), where the p value was less than/equal 0.01 in all cases. The same highly significant variations noticed in combined effects (month together with race and month together with type). However, CAT activity was similar when tested against the combined effects (race together with type), as well as when tested against the combined effects of month, race and type [$P \geq 0.05$, (Table 3)].

In the active season, in both Hybrid and Carniolan, the highest CAT activity was measured in June, followed by remarkable decline in July and then gradual decrease during August and September (Table 4 and Figure 2). Moreover, in August CAT activity of nursing Carniolan (0.076 ± 0.005) showed highly significant decrease against nursing Hybrid (0.140 ± 0.012).

Furthermore in September, the CAT activity of nursing Hybrid decreased significantly (0.069 ± 0.005) against the foraging Hybrid (0.090 ± 0.07) (Table 4). However, the only significant increase in the CAT activity was noticed in August with foraging Hybrid (0.099 ± 0.008) against nursing Hybrid (0.140 ± 0.012). In June, the CAT activity of the nursing Carniolan (0.167 ± 0.028) decreased significantly comparing with both the foraging Carniolan (0.292 ± 0.046) and nursing Hybrid (0.302 ± 0.037).

In the moderately active season, the CAT activity was distinctly lower than that measured in the active season in the two races (Figure 2). The highest CAT activity was recorded in November and October in the Hybrid and Carniolan honeybee races, respectively. However, the lowest CAT activity in both races was detected in December (Figure 2). Generally, the CAT activity of Carniolan race was higher than that of Hybrid race in the moderately active season. In October, the CAT activity of foraging Carniolan (0.131 ± 0.012) was highly significant increased against the foraging Hybrid (0.086 ± 0.007). In December, a significant increase in the CAT activity was detected in the foraging Carniolan (0.099 ± 0.007) against that of the foraging Hybrid (0.074 ± 0.008).

Ascorbate peroxidase

Ascorbate peroxidase activity didn't affect neither by race (Hybrid and Carniolan) nor type (foraging and nursing), where the p value of both effects was more than/equal 0.05. However, the effect of months highly affected the APOX activity ($P \leq 0.01$). On studying combined effects of months and the races, a highly significant variation was noticed in the APOX activity ($P \leq 0.01$). Moreover, significant variation of the APOX activity ($P \leq 0.05$) was revealed when triple effects (months, races and the type) were studied (Table 3).

In the active season, the APOX activity generally increased through months. The highest activity was measured in September in both of the two races, while the lowest activity was observed in June (Table 4 and Figure 3). In July and August, a highly

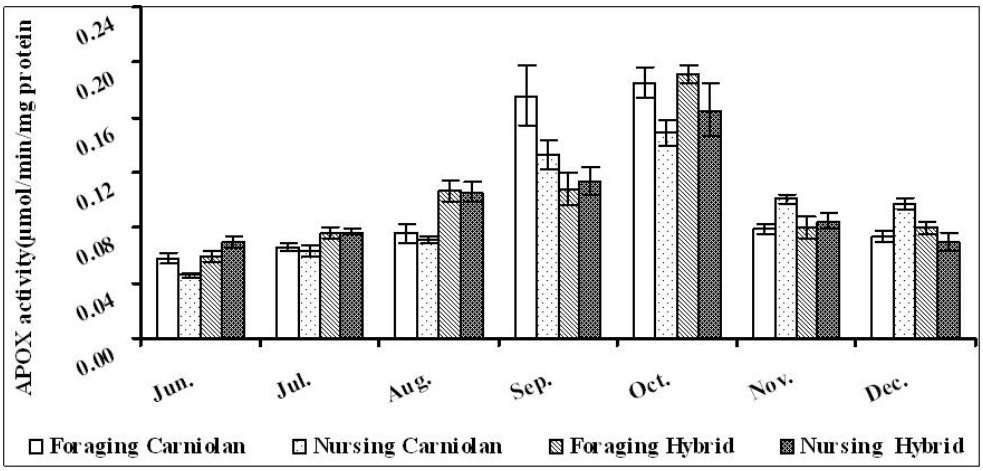

Fig. 3. The changes in ascorbate peroxidase (APOX) activity during the active and moderately active seasons. The results are means (+ SE) of five replicates for each type of the hive.

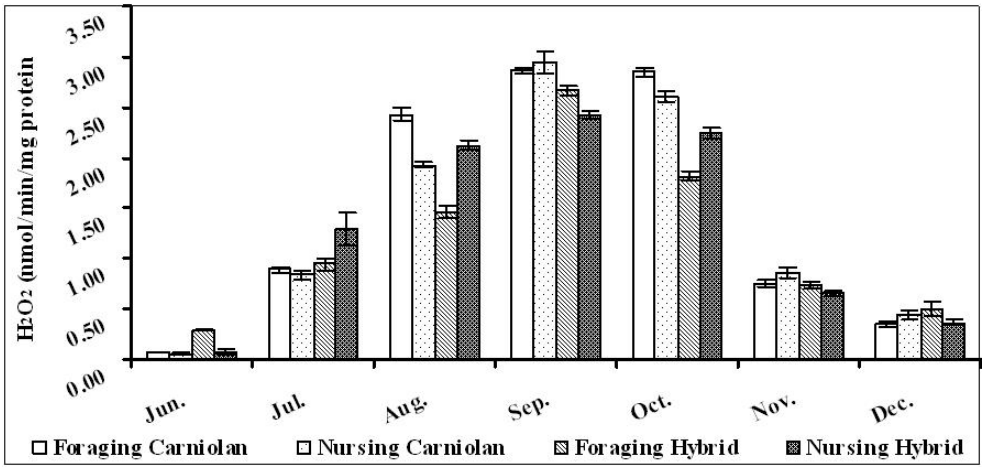

Fig. 4. The changes in the total peroxide concentration (H_2O_2) during the active and moderately active seasons. The results are means (+ SE) of five replicates for each type of the hive.

considerable decrease in APOX activity of nursing Carniolan (0.063 ± 0.004, 0.071 ± 0.003, respectively) was noticed when compared with nursing Hybrid (0.077 ± 0.002, 0.106 ± 0.007) of the two months (Table 4). In June, the APOX activity of foraging Carniolan (0.058 ± 0.004) showed significant reduce against foraging Hybrid (0.059 ± 0.004). Moreover, in August there was a highly significant increase in APOX activity of nursing Hybrid (0.106 ± 0.007) comparing with foraging Hybrid (0.076 ± 0.007) (Table 4).

In the moderately active season, the APOX activity generally declined allover the season, the highest activity was detected in October in both races (Figure 3). In October a highly significant decrease in APOX activity was observed in nursing Carniolan (0.149 ± 0.009), when compared with both the foraging of the same race (0.185 ± 0.011) and the nursing Hybrid (0.165 ± 0.019). Contrary, in November the APOX activity of nursing Carniolan (0.101 ± 0.003) was highly significant increased against the foraging Carniolan (0.079 ± 0.004) (Figure 3).

Total peroxide concentration

Total peroxide was similar only when tested against a single effect of type ($P \geq 0.05$), i.e. both foraging and nursing honeybee within the same race were statistically similar regarding total peroxide concentration (Table 3). However, total peroxide concentration showed highly significant variation, when tested against single effect of

Table 5. Correlation Coefficient between analyzed antioxidant components in the active season in foraging and nursing Hybrid bees (under and above the diagonal, respectively).

	SOD	CAT	APOX	H_2O_2
SOD	—	-0.721*	0.798*	0.755*
CAT	-0.443*	—	-0.603*	-0.816**
APOX	0.095*	-0.689**	—	0.660**
H_2O_2	0.241**	-0.811**	0.731**	—

* P ≤ 0.05, **P ≤ 0.001

months as well as race where P value was less than 0.01 in both cases. Moreover, double effects of months and race, months and type as well as race and type showed great considerable effect on the total peroxide concentration (P ≤ 0.01). Additionally, triple effect of months, race and type had highly significant affected the total peroxide concentration [(P ≤ 0.01, (Table 3)].

In the active season, the total peroxide concentrations were markedly increased from June to September. The minimum concentration of total peroxide was measured in June, while the greatest was in September in both of the two races (Table 4 and Figure 4). In August and September the total peroxide concentration of the nursing Hybrid (2.126 ± 0.037, 2.426 ± 0.039, respectively) showed highly significant increase, when compared with the foraging Hybrid (1.460 ± 0.063, 2.666 ± 0.038) of the same two months (Table 4).

In the same two months, similar highly significant increase in the total peroxide concentration of foraging Carniolan (2.429 ± 0.062, 2.865 ± 0.030, respectively) was observed, when compared with the foraging Hybrid (1.460 ± 0.063, 2.666 ± 0.038). Moreover, in September the total peroxide concentration of the nursing Carniolan (2.943 ± 0.011) showed highly considerable increase against the nursing Hybrid (2.426 ± 0.039).

However, in July the total peroxide concentration decreased significantly in both foraging and nursing Carniolan (0.891 ± 0.030, and 0.835 ± 0.040, respectively), when tested against foraging and nursing Hybrid (0.948 ± 0.065, and 1.300 ± 0.161). Furthermore, in June highly considerable decrease in the total peroxides concentration in both the nursing Hybrid (0.080 ± 0.025) and the foraging Carniolan (0.065 ± 0.002), when tested against the foraging Hybrid (0.296 ± 0.012). In August and September, the total peroxide concentration of nursing Carniolan (1.924 ± 0.024, 2.943 ± 0.011, respectively) showed highly remarkable decrease, when compared with the foraging Carniolan (2.249 ± 0.062, 2.865 ± 0.030) (Table 4).

In the moderately active season, contrary to that observed in the active season the total peroxide concentrations were gradually reduced allover the season, October-December, in both races. The highest total peroxide concentration was detected in October, whilst the lowest concentration was measured in December (Figure 4). In October the total peroxide concentration showed highly significant decrease in nursing Hybrid (2.240 ± 0.050), when tested against foraging Hybrid (1.815 ± 0.043). However, in the same month highly significant increase was noticed in the total peroxide concentration of foraging Carniolan (2.848 ± 0.040) against foraging Hybrid (1.815 ± 0.043). Additionally in November and December, the total peroxide concentration of nursing Carniolan (0.860 ± 0.050, 0.443 ± 0.050, respectively) showed highly significant increase comparing with both the nursing hybrid and foraging Carniolan (0.666 ± 0.025, and 0.353 ± 0.026).

Performing correlation analysis

Correlation analysis between the antioxidant enzymes activities, the total peroxide concentration for foraging and nursing Hybrid and Carniolan honeybees in active season and moderately active season and comparing the calculated correlation matrices could indicate the presence of significant coordinated antioxidant actions.

The results of such analyses are listed in Tables (Tables 5, 6 for the active season and Tables 7, 8 for the moderately active season). In Table 5, under the diagonal, several correlations in the foraging

Table 6. Correlation Coefficient between analyzed antioxidant components in the active season in foraging and nursing Hybrid bees (under and above the diagonal, respectively).

	SOD	CAT	APOX	H_2O_2
SOD	—	-0.721*	0.798*	0.755*
CAT	-0.443*	—	-0.603*	-0.816**
APOX	0.095*	-0.689**	—	0.660**
H_2O_2	0.241**	-0.811**	0.731**	—

* P ≤ 0.05, **P ≤ 0.001

Table 7. Correlation Coefficient between analyzed antioxidant components in the moderately active season in foraging and nursing Hybrid bees (under and above the diagonal, respectively).

	SOD	CAT	APOX	H_2O_2
SOD	—	-0.344*	0.369*	0.302**
CAT	-0.366*	—	-0.667**	-0.803**
APOX	0.175*	-0.510*	—	0.753**
H_2O_2	0.418**	-0.837**	0.627**	—

* P ≤ 0.05, **P ≤ 0.001

Hybrid during the active season were recorded. Statistically, highly significant positive correlation for total peroxide vs. SOD and APOX and significant positive correlation for APOX vs. SOD were recorded. Highly significant negative correlations for CAT vs. total peroxide, significant correlations CAT vs. APOX, SOD were negatively found. In nursing Hybrid bees (Table 6, above the diagonal), significant correlations for total peroxide vs. SOD and APOX were highly positive, significant positive correlation for APOX vs. SOD was recorded. Contrary, two significant negative relations for CAT and SOD; as highly significant negative correlation for CAT vs. APOX and the total peroxide were obtained.

In foraging Carniolan bees, during the active season (Table 6, under the diagonal), there was significant positive correlations between SOD and APOX. Correlations for total peroxide vs. APOX and SOD were highly significant and positive. While, greatly significant negative correlations for CAT vs. APOX and total peroxides were shown, also significant negative one between SOD and CAT. Significant positive correlations in nursing Carniolan bees for APOX and the total peroxide vs. SOD were shown in (Table 6, above the diagonal), while the correlation between APOX vs. total peroxides was found to be greatly significant and positive. Where, highly significant negative relations for CAT vs. GSH and the total peroxides, while significant negative one between CAT vs. APOX and SOD were recorded.

During the moderate active season, the correlation analysis for the antioxidant activities for Hybrid and Carniolan bees was observed as follows: In foraging Hybrid bees (Table 7, under the diagonal), several significant positive correlations were detected, APOX vs. SOD, CAT and total peroxide. However, SOD showed greatly positive correlation against total peroxide. The correlations of nursing Hybrid bees in (Table 7, above the diagonal), indicated the presence of more than significant positive correlations between APOX and total peroxides, in the same for SOD vs. APOX and total peroxide.

Reversely, significant negative correlations for CAT and APOX were noticed. In Table 8 (under the diagonal), the foraging Carniolan bees showed significant positive correlation for SOD vs. APOX and the total peroxides and for APOX vs. CAT and

Table 8. Correlation Coefficient between analyzed antioxidant components in the moderately active season in foraging and nursing Carniolan bees (under and above the diagonal, respectively).

	SOD	CAT	APOX	H_2O_2
SOD	—	0.021	0.107*	0.200**
CAT	-0.293	—	0.663**	0.446
APOX	0.426*	0.412*	—	0.509**
H_2O_2	0.596*	0.371	0.555*	—

* P ≤ 0.05, **P ≤ 0.001

total peroxides. Several correlations were observed for the nursing Carniolan bees in Table 8 (above the diagonal). There were highly significant positive correlations for APOX vs. both CAT and total peroxides; while significant positive one for SOD vs. APOX was recorded.

DISCUSSION

Honey bees obtain all the materials which require for growth and activity from nectar, pollen and store honey, which provides the main carbohydrate source [53, 54]. The floral nectar and pollens are full of allelochemicals and phenolics [55, 56], which contain tannins, phenols, alkaloids [57]; the propolis includes phenols, tannins and aldehydes [34, 35]. The oxidation of phenolics and allelochemicals produce ROS [37, 39]; and caused oxidative stress in the midgut tissues of *Lymantria dispar* [43]. Insects are subjected to increasing loads of oxidative stress by delivering O_2 directly to tissues [16] and tissue demand for O_2 during flight [58, 59].

Insects are continuously exposed to ingested pro-oxidants suffering from oxidative injury, which could be attributed to hydrogen peroxide-mediated effects [39, 60].

Total peroxide concentration

The rapid dismutation of $O_2^{\bullet-}$ to H_2O_2 increased concentrations of hydrogen peroxide and hydroxyl radical, which results in oxidative damage to lipids, proteins and nucleic acids [19]. The oxidation of phenols, peroxides produces reactive oxygen species [36], including hydrogen peroxide. Hydrogen peroxide may also be produced without the intermediacy of an $O_2^{\bullet-}$ radical [16]. Several enzymes are involved in production of H_2O_2, like, glucose oxidase [61]; xanthine oxidase [62]; L-amino acid oxidase [63]; cytochrome P-450 [64]; cytochrome oxidase [65]; cytochrome b [66]; cytochrome c from the honeybee, *Apis mellifera* [67] all of them have been detected and isolated from the honey bee *Apis mellifera* [68], the cytochrome enzymes metabolize hormones, pheromones, detoxifying plant allelochemicals and insecticides; they are key enzymes in many metabolic pathways [69]. Subsequently, we can predict that there are overproduction and generation of H_2O_2 as result of the oxidative stress and the many sources for the generation of H_2O_2. The results in the current work, showed that total peroxide concentrations markedly increased during the active season, this might be due to the increase in activity of honey bees and flight to collect more nectar, the allelochemicals are present in pollen and nectar [33] and the oxidation of phenols produces ROS including H_2O_2 [36, 37]. The increasing of activity in apiary is result in raising the metabolic pathways in which to detoxify the peroxides, allelochemicals by cytochrom (P-450, b, c) [64-67].

In addition, the flight activity raise during the active season, resulted in increasing the tissue demand for more oxygen subsequently, the honey bees are subjected for increasing loads of oxidative stress and producing more ROS. In consistence, Felton and Summers [58] demonstrated that insects are subjected to increasing loads of oxidative stress due to their life style and demanding for O_2 during flight, as well chronic performance of this behavior entails exposure to stressors (e.g., reactive oxygen species) [59]. Increasing the honey bees' activities in apiary met by increasing the bee population, where they showed the peaks in for both races, so

the ingestion of alellochemicales increased and the production and generation of peroxides raised also. Subsequently, increasing the dismutation process by SOD followed by the action of the rest of detoxifing enzymes on H_2O_2, this was reinforced by the highly positive correlation for the SOD and total peroxide. The significant positive correlation between the apiary activities, temperature and the total peroxide concentration supported this observation.

The contrary have been observed in the moderately active season when the activity and flight of honey bees declined, and the results indicated that the total peroxide concentrations were gradually reduced allover the season, where the apiary activity had decreased so the load of oxidative stress and metabolic activities decreased too, therefore the production of peroxides declined. In addition, the activity of flight had decreased as a result the demanding for oxygen decreased too, consequently reduced the production of ROS including H_2O_2. Agree with these observations Yan and Sohal [70] pointed out that, the experimental prevention of flight activity resulted in a decrease in the rate of H_2O_2 generation, attenuated oxidative damage to specific mitochondrial proteins. The apiary records during the present study were in line with biochemical experiment, where bee population was significantly decreased as a result of decreasing the activity and stressor factors.

The more activities increased the more demanding for oxygen as well as more generating peroxides as result of the metabolic activity and of course increasing the leakage of some reactive oxygen species, this must be resulted in elevating the oxidants and increasing the load of the oxidative stress.

Therefore, the antioxidants form an important component of the defense of insects against both exogenous and endogenous oxidative radicals [19]. Honey bee antioxidant enzymes are of particular interest because of their potential involvement in some of the exceptional biological characteristics of the queen honeybee [44].

Superoxide dismutases

Superoxide dismutases are the first line of defense against oxygen free radicals, $O_2^{\bullet-}$ is dismutated by SOD to hydrogen peroxide, $2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2^{\bullet}$ [16]. Superoxide dismutases have strong antioxidant properties and have been shown to protect normal cells as well as a number of pathogens from reactive oxygen species [71] especially the $O_2^{\bullet-}$. Whitten and Ratcliffe [72] gave an evidence for respiratory burst-like $O_2^{\bullet-}$ generative reactions in the haemolymph of the cockroach, *Blaberus discoidalis*, in *Drosophila* [73] and secreted into the midgut lumen of the adult *Aedes aegypti* mosquito [74]. The enzymatic action of superoxide dismutase was detected in insects to overcome the toxic effects of ROS [30]. Superoxide dismutase activity was determined in the midgut tissue of *Rhodnius prolixus* [75] and housefly [76]. Moreover, Weirich et al. [28] detected the activities of SOD in mitochondrial fractions of tissue homogenates; hemolymph plasma of queen honey bee Apis mellifera, also the honeybee has an extracellular Cu/ZnSOD (SOD3) of 178 amino acids [25].

As well, in the current study, determination of SOD activities in honey bee *Apis mellifera*, were carried out, the accomplished results pointed out that in both races of honey bee, Hybrid and Carniolan, the SOD activity was similar. Even so, the activity was evidently increased allover the whole months in both two races in the active season, in consistence Krishnan and Kodrík [19] showed that SOD activity was distinctively elevated in the midgut tissues of *S. littoralis* larvae, which could be more useful in catalyzing the dismutation of superoxide in midgut tissues. It was observed that the highest activity was in September, while the lowest activity was noticed in June, this may be attributed to the overproduction of SOD to overcome $O_2^{\bullet-}$ generation. Accordingly, these findings have been concurred with those obtained by Rojas and Leopold [77], who found that despite the elevated SOD activity in housefly in the most cold-susceptible stage, the $O_2^{\bullet-}$ is generated faster than it can be dismutated, so SODs work continuously to eliminate $O_2^{\bullet-}$ and result in more production of H_2O_2. This may also explain the spectacularly increasing in the H_2O_2 concentrations and agree with Krishnan and Kodrík [19], elevation in the rate of superoxide generation (as in case of oxidative degradation of allelochemicals) can lead to a corresponding enhancement in the production of H_2O_2 and hydroxyl free radical.

The results in the present study demonstrated that, the SOD elevation was analogous with honey bee activities where the bee population considerably increased as well, this might be

related to the hyperactivity and more feeding on pollens and nectar, prooxidants, which rich with allelochemicals and phenols [33], and result in increasing the production of ROS, as a result of oxidation of allelochemicals, including $O_2^{\bullet-}$ and increase the oxidative stress consequently raise in SOD activity in order to accomplish the dismutation process. In agree Krishnan and Kodrík [19] indicated that, a surfeit of oxidants as $O_2^{\bullet-}$ and hydrogen peroxide results in oxidative stress, which is exacerbated by the ingestion of pro-oxidant allelochemicals, this increasing probably raised the oxidative stress, result in overproduction of $O_2^{\bullet-}$ which encounter by SOD elevation, and increasing the dismutation process.

On the contrary, the recorded SOD activities in the moderately active season were reduced by months, this might be due to the lesser production and generation of $O_2^{\bullet-}$ which observed by the declining of the total peroxide concentrations, this reduction was parallel to the honey bee activities, which reduced in the same period.

The hypoactivity of honey bees during this season of course resulted in reduction the ingestion of allelochemicals, indicated by the reduction in stored pollens, consequently reduction in superoxide, the dismutation process reduced either, so there were low concentrations of H_2O_2 observed, in consistence [19]. In addition, it might be taken in consideration that the SOD activity was also affected by the temperature and relative humidity, the elevation was in the active season, where the degrees of both temperature and relative humidity were high, while the opposite has been observed in the moderately active season.

Catalase

During the reduction of oxygen to water in mitochondria, approximately 1–2% of total oxygen consumption gives rise to the potentially cytotoxic species H_2O_2 and $O_2^{\bullet-}$ [78]. Since the product of SOD reaction, H_2O_2 is the substrate of catalase, therefore, catalase activity has been shown to be the highest in the *Rhodnius prolixus* midgut [75]. Significant amounts of H_2O_2 can diffuse out from mitochondria [79], two enzymes defense against H_2O_2, the CAT and glutathione peroxidase (GPx) [75]. Several studies on lepidopteran larvae showed that CAT activity toward H_2O_2 is distributed throughout the cell [80, 81], and tissues of the *Trichoplusia ni* larvae have high specific CAT activity [82]. In the silkworm, *Bombyx mori*, the catalase activity was detected in all homogenates of tissues tested including the fat body [83].

In the same way the CAT activity was observed during the present work on honey bees and unanticipated, our findings showed greatly dissimilarity against the months; the CAT activity was declined allover the months, through the active and moderately active seasons, in both the Hybrid and Carniolan honey bees although the marked increasing of the total peroxide concentrations and this could be returned to the capacity of CAT to eliminate H_2O_2 exceptionally, when they were produced with slightly levels. In agreement with this opinion, Clavaron-Mathews et al. [84] proposed that CAT is inefficient at reducing H_2O_2 to low levels because of its high Km and that APOX better serves this role as well as APOX could compensate the inefficiency of catalase in removing H_2O_2 in Helicoverpa zea larvae. It is possible that CAT could effectively reduce hydrogen peroxide to low levels, despite its high K_m, if high levels of this CAT enzyme were produced [48].

The values of CAT activity in foraging honeybees (Carniolan and Hybrid) were to some extent higher than those observed in nursing bees, this might be due to the flight activity that lead to increase the load of oxidative stress, and the metabolic activity, which produced H_2O_2 in which detoxified by producing more antioxidants. In line with this observation, Corona et al. [26] old workers tended to have the highest levels of antioxidant gene expression; as foragers, these are the individuals that likely had the highest levels of flight activity.

Ascorbate peroxidase

Some investigators believe that the distribution of CAT throughout the cell can compensate for the lack of selenium-dependent GPx in insect larvae [20, 84], others believe that the lack of GPx and the inefficiency of CAT at low H_2O_2 concentrations suggests the need for another enzyme with a higher H_2O_2 affinity [22, 85].

As previously observed from the results, the CAT activities were declining through the two seasons. Since catalase is inefficient at removing H_2O_2 because of its high K_m [85] and it could be done if high levels of CAT enzyme were produced, consequently insects may utilize another strategy for managing peroxides at safe levels, APOX better serves this role [48].

The APOX activity was determined in the gut lumens and midgut tissues of *M. sanguinipes* and *A. ellioti* [48] as well as measured in the Egyptian armyworm, *Spodoptera littoralis*, by Krishnan and Kodrík [19], they observed elevation of APOX activity in the foregut, midgut contents and midgut tissue. Insects encounter ROS from exogenous and endogenous sources consequently detoxify the deleterious effect of ROS by a suite of antioxidant defence enzymes such as SOD, CAT and APOX [86].

As well, the APOX activities of honeybees were measured in the current study, the results showed that APOX activity did not affect neither by race (Hybrid and Carniolan) nor type (foraging and nursing) and nevertheless, it affected by months, where it increased in the active season. Honey bees feeding on plants and they obtain the nectar which contain phenols. The oxidative stress can be exacerbated as a result of the ingestion of oxidizable allelochemicals [16, 58].

Ascorbate peroxidase could be compete with the overproduction of H_2O_2 and compensate the defect of CAT levels, and the increasing of course to eliminate and detoxify the elevating levels of H_2O_2. In contrast, when the oxidative stress reduced, the APOX activity declined allover the moderately active season, where the H_2O_2 concentrations decreased consequently, there were no more need for APOX action.

This might be explained the raise and decline of APOX activity. Moreover, the increasing and decreasing of APOX during the current study might be returned to the existence of other action for APOX, that beside its ability to reduce H_2O_2. It also could have other effect on peroxides and hydroperoxides. In consistence, Clavaron-Mathews et al. [85] detected APOX activity in whole-body of *Helicoverpa zea*. They observed that the enzymatic activity of APOX was not only towards H_2O_2, but also towards two model lipophilic peroxides, cumene hydroperoxide and t-butyl hydroperoxide.

Consequently, in light of this opinion and our findings, the elevation of APOX was to eliminate the hydrogen peroxides, in addition to other hydroperoxides, as result of increasing the activity of SOD, and the decline of APOX activity was due to the decrease in H_2O_2 concentrations and other proxides (not measured).

Performing correlation analysis

The results of correlation analysis between the antioxidant enzymes activities, the total peroxide concentration in active and moderately active seasons could indicate the presence of significant coordinated antioxidant actions.

The results in the present study revealed in both races (Carniolan and Hybrid) and types (foraging and nursing) the elevation of SOD activity during the active season that resulted in increasing the level of total peroxides contrary to the moderately active season, where the SOD activity decreased and the total peroxide either. This is reinforced by the correlation analysis, which has pointed out to the highly significant positive correlation between the SOD and total peroxides allover the experiment. This indicated that the oxidative stress was high during the active season, so there were overproduction of ROS including $O_2^{\bullet -}$, which in turn dismutated by the SOD and produced H_2O_2. This may also explain the spectacularly increasing in the total peroxide concentrations (H_2O_2) and agree with Krishnan and Kodrík [19]. Elevation in the rate of superoxide generation (as in case of oxidative degradation of allelochemicals) can lead to a corresponding enhancement in the production of H_2O_2 and hydroxyl free radical also due to the formation of ROS from various sources, and that antioxidant systems are key to the removal of these reactive species to prevent subsequent damage from their activity [87].

Similarly, when the SOD elevated the H_2O_2 elevated, so there are an increasing in some other enzymes like APOX to mange this increasing. The results indicated a significant positive correlation between the SOD and APOX in the Carniolan and Hybride honey bees, while the opposite occurred in the moderately active season, where the SOD activity declined as well as the APOX, also the positive significance between them were observed. Furthermore, there was positive correlation between oxidative stress resistance and ROS [88].

Other correlations were negative as SOD and CAT; CAT and APOX, indicating that there must be a strategy and specific manner strategy for managing peroxides at safe levels. The increase or decreases of the antioxidants are according to the contents and levels of peroxides. The most endogenous defensive and protective strategy is the antioxidant systems, changes in the activities of

antioxidant enzymes have been shown to correlate with the physiological and metabolic activities [89].

In consistence with our hypothesis that increased oxidative stress leads to an up-regulation of antioxidant enzymes, we found that SOD, APOX were elevated when the total peroxide increased in order to detoxify their dangerous effect and dismutating the most aggressive radical ($O_2^{\bullet -}$) by the action of SOD, while the contrary occurred as the declined when the peroxide decreased. Also, the significant remarkable positive correlation between the SOD, APOX with H_2O_2 as they elevated in the active season as a result of increasing the concentrations of H_2O_2 and the contrary occurred during the moderately active season. This is indicating the coordination and regulation between the enzymes and this synergetic action is in order to maintain the redox status of the cell and do not allow the free radical to exceed the limits of the normal activities.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Ruuge EK, Ledenev AN, Lakomkin VL, Konstantinov AA, Ksenzenko MY. Free radical metabolites in myocardium during ischemia and reperfusion. Amer J Physiol. Suppl. 1991; 261: 81–86.
2. Baskin IS, Salem H. 1997. Oxidants, antioxidants and free radicals. Taylor and Francis, Washington, DC.
3. Pala FS, Tabakçolu K. Free radicals. Adv Mol Biol. 2007; 1: 63–69.
4. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Phcog Rev. 2010; 4: 118–126.
5. Diaz-Albiter H, Mitford V, Genta FA, Sant-Anna MRV, Dillon RJ. Reactive oxygen species scavenging by catalase is important for female *Lutzomyia longipalpis* fecundity and mortality. PLoS One. 2011; 6: e17486.
6. Punchard NA, Kelly FJ. 1996. Free radicals, a practical approach. Oxford University Press Inc. New York.
7. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. Methods in Enzymology. 1990; 186: 1–85.
8. Davies MJ, Dean RT. 1997. Radical-Mediated Protein Oxidation: From Chemistry to Medicine. Oxford Science Publications, Oxford.
9. Bouayed J, Bohn T. Exogenous antioxidants-Double-edged swords in cellular redox state. Oxid Med Cell Long. 2010; 3: 228–237.
10. Halliwell B, Gutteridge JMC. 1999. Free Radicals in Biology and Medicine. (3rd ed.) Oxford University Press, Oxford.
11. Fridovich I. Superoxide dismutases-an adaptation to a paramagnetic gas. J Biol Chem. 1989; 264: 7761–7764.
12. Spickett CM, Wiswedel I, Siems W, Zarkovic K, Zarkovic N. Advances in methods for the determination of biologically relevant lipid peroxidation products. Free Rad Res. 2010; 44: 1172–202.
13. Arking R, Buck S, Hwangbo D, Lane M. Metabolic alterations and shifts in energy allocations are corequisites for the expression of extended longevity genes in *Drosophila*. Ann NY Acad Sci. 2002; 959: 251–262.
14. Barja G. Endogenous oxidative stress: relationship to aging, longevity and caloric restriction. Ageing Res Rev. 2002; 1: 397–411.
15. Krogh A, Weis-fogh T. The respiratory exchange of the desert locust (*Schistocerca gregaria*), before, during and after flight. J Exp Biol. 1951; 28: 342-357.
16. Ahmad S. Biochemical defense of pro-oxidant plant allelochemicals by herbivorous insects. Biochem System Ecol. 1992; 20: 269–296.
17. Rojas RR, Leopold RA. Chilling injury in the housefly: Evidence for the role of oxidative stress between pupariation and emergence. Cryobiol. 1996; 33: 447–458.
18. Saino N, Caprioli M, Romano M, Boncoraglio G, Rubolini D, Ambrosini R, Bonisoli-Alquati A, Romano A. Antioxidant defenses predict long-term survival in a passerine bird. PLoS One. 2011; 6: e19593.
19. Krishnan N, Kodrík D. Antioxidant enzymes in *Spodoptera littoralis* (Boisduval): Are they enhanced to protect gut tissues during oxidative stress? J Insect Physiol. 2006; 52: 11–20.
20. Pritsos CA, Ahmad S, Bowen SM, Elliott AJ, Blomquist GJ, Pardini RS. Antioxidant enzymes of the black swallowtail butterfly, *Papilio polyxenes*, and their response to the prooxidant allelochemical, quercetin. Arch Insect Biochem Physiol. 1988; 8: 101–112.
21. Aucoin RR, Philogene BJR, Arnason JT. Antioxidant enzymes as biochemical defenses against phototoxin-induced oxidative stress in three species of herbivorous lepidoptera. Arch Insect Biochem Physiol. 1991; 16: 139–152.
22. Felton GW, Duffey SS. Ascorbate oxidation reduction in *Helicoverpa zea* as a scavenging system against dietary oxidants. Arch Insect Biochem Physiol. 1992; 19: 27–37.
23. Ahmad S, Pardini RS. Antioxidant defense of the cabbage looper, *Trichoplusia ni*: Enzymatic

responses to the superoxide-generating flavonoid, quercetin, and photodynamic furanocoumarin, xanthotoxin. Photochem Photobiol. 1990; 51: 305–311.
24. Lee K. Glutathione S-transferase activities in phytophagous insects: induction and inhibition by plant phototoxins and phenols. Insect Biochem. 1991; 21: 353–361.
25. Corona M, Robinson GE. Genes of the antioxidant system of the honey bee: annotation and phylogeny. Insect Mol Biol. 2006; 15: 687–701.
26. Corona M, Hughes KA, Weaver DB, Robinson GE. Gene expression patterns associated with queen honey bee longevity. Mech Ageing Dev. 2005; 126: 1230–1238.
27. Parker JD, Parker KM, Sohal BH, Sohal RS, Keller L. Decreased expression of Cu-Zn superoxide dismutase in ants with extreme life-span. Proc Nat Acad Sci USA. 2004; 101: 3486–3489.
28. Weirich GF, Collins AM, Williams VP. Antioxidant enzymes in the honey bee, *Apis mellifera*. Apidol. 2002; 33: 3–14.
29. Collins AM, Williams V, Evans JD. Sperm storage and antioxidative enzyme expression in the honey bee, *Apis mellifera*. Insect Mol Biol. 2004; 13: 141–146.
30. Suh HJ, Kim SR, Lee KS, Park S, Kang SC. Antioxidant activity of various solvent extracts from *Allomyrina dichotoma* (*Arthropoda*: *Insecta*) larvae. J Photochem Photobiol B: Biology. 2010; 99: 67–73.
31. Gross CL, Mackay D. Honey bees reduce fitness in the pioneer shrub *Melastoma affine* (*Melastomataceae*). Biolog Conse. 1998; 86: 169-178.
32. Hagler JR, Buchmann SL. Honey bee (*Hymenoptera*: *Apidae*) foraging responses to phenolic-rich nectars. J Kansas Entomol Soc. 1993; 66: 223–230.
33. Detzel A, Wink M. Attraction, deterrence or intoxication of bees (*Apis mellifera*) by plant allelochemicals. Chemoecol. 1993; 4: 8-18.
34. Gonzalez M, Guzman B, Rudyk R, Romano E, Molina MAA. Spectrophotometric Determination of Phenolic Compounds in Propolis. Lat. Am J Pharm. 2003; 3: 243-248.
35. Farooqui T, Farooqui AA. Beneficial effects of propolis on human health and neurological diseases. Front Biosci. 2012; 4: 779–793.
36. Canada AT, Giannella E, Nguyen TD, Mason RP. The production of reactive oxygen species by dietary flavonols. Free Radic Biol Med. 1990; 9: 441–449.
37. Barbehenn RV, Bumgarner SL, Roosen EF, Martin MM. Antioxidant defenses in caterpillars: role of the ascorbate-recycling system in the midgut lumen. J Insect Physiol. 2001; 47: 349–357.
38. Barbehenn RV, Martin MM. Tannin sensitivity in *Malacosoma disstria*: Roles of the peritrophic envelope and midgut oxidation. J Chem Ecol. 1994; 20: 1985–2001.
39. Barbehenn RV, Jaros A, Lee G, Mozola C, Weir Q, Salminen JP. Hydrolyzable tannins as “quantitative defenses”: limited impact against *Lymantria dispar* caterpillars on hybrid poplar. J Insect Physiol. 2009; 55: 297–304.
40. Barbehenn RV, Jones CP, Karonen M, Salminen JP. Tannin composition affects the oxidative activities of tree leaves. J Chem Ecol. 2006; 32: 2235–2251.
41. Zheng J, Cho M, Jones AD, Hammock BD. Evidence of quinone metabolites of naphthalene covalently bound to sulfur nucleophiles of proteins of *Murine clara* cells after exposure to napthalene. Chem Res Toxicol. 1997; 10: 1008–1014.
42. Thiboldeaux RL, Lindroth RL, Tracy JW. Effects of juglone (5-hydroxy-1,4-naphthoquinone) on midgut morphology and glutathione status in *Saturniid moth* larvae. Comp Biochem Physiol. 1998; 120: 481–487.
43. Pecci CD. 2011. Oxidation of ingested phenolic compounds creates oxidative stress in the midgut tissues of *Lymantria dispar* caterpillars. Cell and Molecular Biology, University of Michigan: 1–22.
44. Page-RE, Jr, Peng CY. Aging and development in social insects with emphasis on the honey bee, *Apis mellifera* L. Exp Control. 2001; 36: 695–711.
45. Jeffree EP. Aphotographic presentation of estimated numbers of honey bees (*Apis mellifera* L.) on combs in 14x8½ inch frames. Bee World 1951; 32: 89-91.
46. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 1972; 247: 3170–3175.
47. Aebi H. Catalase in vitro. In: Packer L, editor. Methods in Enzymology. New York: Academic Press. 1984; 105: 121–126.
48. Barbehenn RV. Gut-based antioxidant enzymes in polyphagous and a graminivorous Grasshopper. J Chem Ecol. 2002; 28: 1329–1347.
49. Asada K. Chloroplasts: Formation of active oxygen and its scavenging. Methods Enzymol. 1984; 105: 422–429.
50. Miyazawa T. Determination of phospholipid hydroperoxides in human blood plasma by a chemiluminescence-HPLC assay. Free Radic Biol Med. 1989; 7: 209-217.
51. Harma M, Erel O. Measurements of the total antioxidant response in preeclampsia with a novel automated method. Eur J Obstet Gynecol Reprod Biol. 2005; 118: 47-51.
52. SAS Institute. SAS/STAT. User's guide: Release 6.04. SAS institute, Inc 1990; Cary, N C.
53. Gheldof N, Engeseth NJ. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. J Agric Food Chem. 2002; 50: 3050–3055.

54. Kesić A, Mazalović M, Crnkić A, Ćatović B, Hadžidedic S, Dragošević G. The Influence of L-ascorbic acid content on total antioxidant activity of bee-honey. Eur J Sci Res. 2009; 32: 95–101.
55. Johnson SD, Hargreaves AL, Brown M. Dark, bitter-tasting nectar functions as a filter of flower visitors in a bird-pollinated plant. Ecol. 2006; 87: 2709-2716.
56. Gegear RJ, Manson JS, Thomson JD. Ecological context influences pollinator deterrence by alkaloids in floral nectar. Ecol Lett. 2007; 10: 375-382.
57. Adler LS, Irwin RE. Ecological costs and benefits of defenses in nectar. Ecol. 2005; 86: 2968-2978.
58. Felton GW, Summers CB. Antioxidant systems in insects. Arch Insect Biochem Physiol. 1995; 29: 187–197.
59. Roberts SP, Elekonich MM. Commentary: behavioral development and the ontogeny of flight capacity in honey bees. J Exp Biol. 2005; 208: 4193-4198.
60. Pritsos CA, Ahmad S, Elliott AJ, Pardini RS. Antioxidant enzyme levels response to prooxidant allelochemicals in larvae of southern armyworm moth. *Spodoptera eridania*. Free Radic Res Commun. 1990; 9: 127–133.
61. Cavener RD. Genetics of male-specific glucose oxidase and the identification of other unusual hexose enzymes in *Drosophila melanogaster*. Biochem Genetics. 1980; 18: 929-937.
62. Atmowidjojo AH, Erickson EH, Wheeler DE, Cohen AC. Regulation of hemolymph osmolality in feral and domestic honeybees, *Apis mellifera* L. (*Hymenoptera*: *Apidae*). Comp Biochem Physiol. 1999; 122: 227–233.
63. Samy RP, Pachiappan A, Gopalakrishnakone P, Thwin MM, Hian YE, Chow VT, Bow H, Weng JT. In vitro antimicrobial activity of natural toxins and animal venoms tested against *Burkholderia pseudomallei*. J Bacteriol. 2006; 18: 2073-2080.
64. Claudianos C, Ranson H, Johnson RM, Biswas S, Schuler MA, Berenbaum MR, Feyereisen R, Oakeshott JG. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Mol Biol. 2006; 15: 615–636.
65. Armengaud C, Jamila A, Causse N, Gauthier M. Nicotinic acetylcholine receptor ligands differently affect cytochrome oxidase in the honeybee brain. Neurosci Lett. 2001; 304: 97-101.
66. Jermiin LS, Crozier RH. The cytochrome b region in the mitochondrial DNA of the ant *Tetraponera rufoniger*: sequence in hymenoptera may be associated with neoclotide content. J Mol Evol. 1994; 38: 228-294.
67. Inoue S, Matsubara H, Yamanaka T. Complete amino acid sequence of cytochrome C from the honeybee, *Apis mellifera*, and evolutionary relationship of the honeybee to other insects on the basis of the amino acid sequence. J Biochem. 1985; 97: 947-954.
68. Alaux C, Ducloz F, Crauser D, Conte LY. Diet effects on honeybee immunocompetence. Biol Lett. 2010; 6: 562-565.
69. Tarès S, Berge JB, Amichot M. Cloning and expression of cytochrome P450 genes belonging to the CYP4 family and to a novel family, CYP48, in two hymenopteran insects, *Trichogramma cacoeciae* and *Apis mellifera*. Biochem Biophys Res Commun. 2000; 268: 677–682.
70. Yan LJ, Sohal RS. Prevention of flight activity prolongs the life span of the housefly, *Musca domestica*, and attenuates the age-associated oxidative damage to specific mitochondrial proteins. Free Radic Biol Med. 2000; 29: 1143–1150.
71. McCord JM, Fridovich I. Superoxide dismutase: the first twenty years. Free Radic Biol Med. 1988; 5: 363–369.
72. Whitten MMA, Ratcliffe NA. In vitro superoxide activity in the haemolymph of the West Indian leaf cockroach, *Blaberus discoidalis*. J Insect Physiol. 1999; 45: 667–675.
73. Miwà S, St-Pierre J, Partridge L, Brand MD. Superoxide and hydrogen peroxide production by *Drosophila* mitochondria. Free Radic Biol Med. 2003; 35: 938–948.
74. Oliveira JH, Goncalves RL, Lara FA, Dias FA, Gandara AC, Menna-Barreto RF, Edwards MC, Laurindo FR, Silva-Neto MA, Sorgine MH, Oliveira PL. Blood meal-derived heme decreases ROS levels in the midgut of *Aedes aegypti* and allows proliferation of intestinal microbiota. PLoS Pathog. 2011; 7: e1001320.
75. Paes MC, Oliveira MB, Oliveira PL. Hydrogen peroxide detoxification in the midgut of the blood-sucking insect, *Rhodnius prolixus*. Arch Insect Biochem Physiol. 2001; 48: 63–71.
76. Allen RG, Oberley LW, Elwell JH, Sohal RS. Developmental patterns in the antioxidant defenses of the housefly, *Musca domestica*. J Cell Physiol. 1991; 146: 270–276.
77. Rojas RR, Leopold RA. Chilling injury in the housefly: Evidence for the role of oxidative stress between pupariation and emergence. Cryobiol. 1996; 33: 447–458.
78. Chance B, Sies H, Boveris A. Hydrogen peroxide metabolism in mammalian organs. Physiol Rev. 1979; 59: 529-605.
79. Turrens JF, Alexandre A, Lehninger AL. The effect of hyperoxia on superoxide production by lung submitochondrial particles. Arch Biochem Biophys. 1985; 127: 401–410.
80. Ahmad S, Pritsos CA, Bowen SM, Heisler CR, Blomquist GJ, Pardini RS. Antioxidant enzymes of larvae of the cabbage looper moth, *Trichoplusia ni*: subcellular distribution and activities of superoxide dismutase, catalase and glutathione reductase. Free

Radic Res Commun. 1988; 4: 403–408.
81. Ahmad S, Pardini RS. Mechanisms for regulating oxygen toxicity in phytophagous insects. Free Radic Biol Med. 1990; 8: 401–413.
82. Ahmad S, Duval DL, Weinhold LC, Pardini RS. Cabbage looper antioxidant enzymes: Tissue specificity. Insect Biochem. 1991; 21: 563–572.
83. Yamamoto K, Banno Y, Fujii H, Miake F, Kashige N, Aso Y. Catalase from the silkworm, *Bombyx mori*: Gene sequence, distribution, and overexpression. Insect Biochem Mol Biol. 2005; 35: 277–283.
84. Clavaron-Mathews M, Summers CB, Felton GF. Ascorbate Peroxidase: A novel antioxidant enzyme in insects. Arch Insect Biochem Physiol. 1997; 34: 57–68.
85. Ahmad S, Beilstein MA, Pardini RS. Glutathione peroxidase activity in insects: a reassessment. Arch Insect Biochem Physiol. 1989; 12: 31–49.
86. Rajarapu SP, Mamidala P, Herms DA, Bonello P, Mittapalli O. Antioxidant genes of the emerald ash borer (*Agrilus planipennis*): gene characterization and expression profiles. J Insect Physiol. 2011; 57: 819-24.
87. Joanisse DR, Storey KB. Oxidative stress and antioxidant in strees and recovery of cold-hardy insects. Insect Biochem Molec Biol. 1998; 28: 23–30.
88. Kaneuchi T, Togawa T, Matsuo M, Fuyama Y, Aigaki T. Efficient measurement of H_2O_2 resistance in *Drosophila* using an activity monitor. Biogerontol. 2003; 4: 157–165.
89. Jovanović-Galović A, Blagojevic DP, Grubor-Lajšić G, Worland R, Spasic MP. Role of antioxidant defense during different stages of preadult life cycle in European corn borer (*Ostrinia nubilalis*, Hubn.): Diapause and Metamorphosis. Arch Insect Biochem Physiol. 2004; 55: 79–89.

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BIOLOGY ORIGINAL ARTICLE

Variant abiotic factors and the infection of *Fasciola gigantica* larval stages in vector snail *Indoplanorbis exustus*

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ABSTRACT

The aquatic environment has numerous physical and chemical parameters that may influence the physiology and maturation rate of parasite found inside the vector snail. It may be possible that abiotic factors (temperature, pH, CO_2, O_2 and conductivity) and higher population density of snails could promote the transmission of parasite and raise their local abundance. In the present paper, we examined that how these environmental factors affect the transmission of cercaria throughout the year 2009-2010. The infection of *Fasciola gigantica* larvae in *Indoplanorbis exustus* in Ramgarh Lake and GIDA pond was maximum in month of October (40%) and minimum in month of November (8.33%). Trend of higher infection in *I. exustus* was observed in July to October. This study conclusively, shows that variant abiotic factors in different months of the year can significantly alter the infection rate and development process of larvae (sporocyst, redia and cercariae) in the snail *Indoplanorbis exustus*. The paper also includes a discussion on the important factors that influence the timing of molluscicide operation for the control of fascioliosis in the Gorakhpur.

Key words: Fasciolosis, Cercaria, Infection rate, *Indoplanorbis exustus*, Abiotic factors.

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INTRODUCTION

Two major trematode disease are transmitted by aquatic snails viz., schistosomiasis caused by *Schistosoma* species and fasciolosis caused by *Fasciola* species [1]. Fasciolosis is a worldwide zoonotic disease [2, 3] caused by liver flukes of the genus *Fasciola* [4]. It has been considered as the veterinary problem, particularly with intensive sheep and cattle production, leading to high economic losses [5]. Freshwater snails are considered to be intermediate host of schistosomiasis/fasciolosis because snail's habour the asexual stage of the parasite while human/herbivores habour the sexual stage of the parasite [6]. They are of importance in medical veterinary practice since they serve as intermediate hosts for certain parasitic worms of man and his domestic animals. *Indoplanorbis exustus* acts as intermediate host for Schistosome [7] and Fasciola [1]. The freshwater snail *Indoplanorbis exustus* [8] (Planorbidae: Bulininae) is the sole member of its genus and is widely distributed across the tropics. Several attempts have been made to reduce the incidence of fasciolosis by using synthetic pesticide and plant derived active components against vector snails [1, 9]. In order to implement control measures, it is important to determine the seasonal transmission pattern of parasite [10, 11] and environmental factors which produce collective effect on intermediate host. It is usually difficult to separate the effect of any one factor from other [12], so it is important to determine the seasonal transmission pattern of parasite [10] to control them in suitable month when cercariae number/snail was higher in the host. No work of this nature has been carried out. The present study was planned to determine the best time for application of molluscicides in Gorakhpur district.

MATERIALS AND METHODS

Snail sampling and determination of abiotic environmental factors of lake water

Indoplanorbis exustus were collected from six places in a standard manner from Ramgarh Lake and GIDA pond by method of Manga-Gonzalez et al. [13]. Gorakhpur is a place located in the Indian state of Uttar Pradesh lies between latitude 26°46' N and longitude 83°22' E, covers the geographical area of 3483.8 km^2. Adult *I. exustus* (0.85 cm ± 0.20 cm in length) were collected locally from Ramgarh Lake and GIDA pond. Temperature, dissolved oxygen, free CO_2, pH, conductivity, and population density of snails were measured in months of 2009-2010. Temperature, pH, and conductivity were measured by thermometer, digital pH meter and conductivity meter, respectively. Dissolved oxygen and CO_2 were estimated according to method prescribed by the APHA [14]. Population density of snails per meter^2 in natural habitat was counted throughout the year 2009-2010.

Laboratory analysis of snail infection

Snails collected were immediately transported to the laboratory for analysis. The collected *I. exustus* were divided into six groups each containing ten snails in each months of year 2009-2010 in Ramgarh and GIDA pond. A stereomicroscope was used to dissect snails in vivo in order to detect, count and extract the different larval stages of the helminthes found in their various organs [13]. Snails were dissected out and total numbers of sporocyst, redia and cercaria larvae of *F. gigantica* in snails were counted with help of microscope. Every experiment was replicated at least six times. Number of larvae (sporocyst, redia and cercaria), pH, temperature, dissolved oxygen and carbon dioxide were expressed as mean ± SE of six replicates. Product moment correlation coefficient was applied between environmental factors and number of sporocyst, redia and cercaria larvae/ snail in six months of the year 2009-2010 [15].

RESULTS

Table 1 and 2 show the seasonal variation in abiotic factors (temperature, pH, conductivity, dissolved oxygen and free carbon dioxide) of Ramgarh lake (1st site) and GIDA pond (2nd site). Seasonal variation in temperature 16–38°C in month of November to October 2010 was higher than rest months. Maximum water temperature was observed in month of May and June (37–38°C). The minimum water temperature was observed in month December and January (16–18°C) (Table 1). There was a significant positive correlation ($p<0.05$) between the temperature and infection rate of *I. exustus* ($r = 0.64$)/population density ($r = 0.98$) in Ramgarh Lake and GIDA pond. Significant

seasonal variation ($p<0.05$) in the number of sporocyst, redia, and cercaria larvae/snail were observed in dissected snails of Ramgarh Lake and GIDA pond (Table 2). The maximum numbers of sporocyst larvae in snails of Ramgarh Lake was observed in month of October (2.81/snail) and minimum (0.22/snail) in November. The maximum numbers of sporocyst larvae in GIDA pond were observed in month of October (1.45/snail) and minimum number (0.69/snail) in July. Significant ($p<0.05$) positive correlation was observed in between number of sporocyst/snail and temperature ($r = 0.67$), free carbon dioxide ($r = 0.76$), cercaria ($r = 0.89$), population density ($r = 0.78$) and % infection of snail ($r = 0.78$) in different months of year 2009-2010 was observed in Ramgarh Lake. While no marked correlation was observed between sporocyst/snail and infection rate, and temperature of snail in GIDA pond.

The maximum (143.72/snail) and minimum (120.30/ snail) number of redia in Ramgar Lake were observed in month of September and August, respectively. Significant ($p<0.05$) positive correlation was observed in between number of redia/snail and cercaria ($r = 0.66$), % infection ($r = 0.65$) and population density ($r = 0.825$) of snail.

The maximum (148.31/snail) and minimum (121.60/ snail) number of redia in GIDA were observed in month of December and September, respectively. Significant ($p<0.05$) positive correlation was observed in between number of redia/snail and dissolved oxygen ($r = 0.55$), number of cercaria/ snail ($r = 0.56$) and population density ($r = 0.825$). Significant ($p<0.05$) negative correlation was observed between number of redia /snail and water temperature ($r = -0.65$), population density ($r = 0.64$), pH ($r = 0.56$). No marked correlation was observed between redia/snail and % infection, conductivity and free CO_2 of water in GIDA pond locality.

In the Ramgar Lake the maximum number of cercaria (2213.34/snail) were observed in month of October, while lowest (1121.43/snail) in month of December. Number of mature cercaria dissected out of the snail tissue significantly ($p<0.05$) increases with increase in temperature ($r = 0.69$), number of sporocyst ($r = 0.89$), redia ($r = 0.66$), snail population density ($r =0.82$) and % infection of snails (0.93) in different months of the year 2009-2010. Number of cercaria/snail significantly decreases ($p<0.05$) with decrease in conductivity ($r = 0.64$), dissolved O_2 ($r = 0.69$) in GIDA pond water in different months of the year 2009-2010.

In the GIDA pond water the maximum number of cercaria (1934.33/snail) were observed in month of September, while lowest (548.76/snail) in month of December. Number of mature cercaria dissected out of the snail tissue significantly ($p<0.05$) increases with increase in water temperature ($r = 0.56$), number of sporocyst ($r = 0.56$), snail population density ($r = 0.75$) and % infection ($r = 0.67$) in different months of the year 2009-2010.

The % infection of *F. gigantica* larvae in snail *I. exustus* varies in between 15 to 36.67 from November 2009 to October 2010 in Ramgar Lake. Maximum infection was observed in October (36.67%) and minimum in December (15%). Percent infection was higher in month of August to October months. The percent infection of *F. gigantica* larvae varies in between 8.33 to 40 from November 2009 to October 2010 in GIDA pond. Maximum % infection was observed in September (40%) and minimum in November (8.33%).

DISCUSSION

It is evident from the result section that temperature, pH, conductivity, dissolved oxygen and carbon dioxide alter the snail density and % larval infection and number of intramolluscan larvae of *F. gigantica* in *I. exustus*. Temperature is considered as an important abiotic factor. Pathogen replication rate and dynamics may be affected by the invertebrate body temperature and as a result, the infection process in the different organs of the invertebrate and their duration cycle is altered [16]. In nature abiotic factors of freshwater bodies vary considerably. However, organisms living in these environments have adopted themselves and live happily despite change in their ambient environment. Abiotic factors of the environment vary from one season to other [17, 18], the aquatic environment has numerous physical and chemical parameters that may influence the physiology of fresh water organism [19]. Like all other animals in the ecosystem, the distribution and abundance of fresh water snails and life cycle of trematodes are also influenced by the environmental components, such as water qualities, aquatic vegetation, soil substratum, depth of water, temperature, pH, turbidity, dissolved oxygen, hardness, carbon dioxide [20, 21].

Table 1. Seasonal changes in the abiotic environmental factors, infection rate and intramolluscan larval stages in *I. exustus* of Ramgarh Lake from November 2009 to October 2010.

Months	Temp+ (°C)	pH+	Conductivity+ (µmhos cm^{-1})	O_2 (ppm)	CO_2 + (ppm)	% Infection+	Population density+ ($meter^2$)	Sporocyst/snail+	Redia/snail +	Cercaria/snail +
Nov.	25± 0.1*Δ	7.54±0.08	34.65±0.03°≠	2.1±0.06°	13.8±0.07	18.33±0.78*	12.67±0.98 *Δ	0.22±0.08*Δ	132.50±2.34*Δ	1232.77±23.28Δ
Dec.	18±0.47*Δ	7.8±0.01	33.45±0.18°≠	2.6±0.09°	14.9± 0.04	15±0.56*	5.00±0.73*Δ	0.50±0.41*Δ	0±0	1121.43±33.19Δ
Jan.	16±0.16*Δ	7.71±0.01	32.34±0.17°≠	1.8±0.07°	15.5±0.04	0±0	0±0	0±0	0±0	0±0
Feb.	21±0.16*Δ	7.75±0.01	31.56±0.22°≠	2.4±0.01°	20.4± 0.03	0±0	0±0	0±0	0±0	0±0
March	28±0.33*Δ	7.77±0.03	29.45±0.05°≠	1.8±0.04°	20.8±0.31	0±0	0±0	0±0	0±0	0±0
April	34± 0.16*Δ	7.87±0.01	26.7±0.17°≠	1.2±0.03°	21.3± 0.85	0±0	0±0	0±0	0±0	0±0
May	37±0.40*Δ	8.02±0.01	26.2±0.01°≠	1.5±0.06°	24.1±0.81	0±0	0±0	0±0	0±0	0±0
June	38±0.30*Δ	9.71±0.02	25.3±0.07 °≠	1.2±0.06°	27.8± 0.63	0±0	0±0	0±0	0±0	0±0
July	34±0.21*Δ	7.96±0.01	26.4±0.07°≠	2±0.06°	18.2±0.47	21.67±0.87*	8.6±0.92*Δ	1.30±0.87*Δ	0±0	1436.00±9.22 Δ
Aug.	33±0.21*Δ	7.91±0.01	27.5±0.09°≠	1.5±0.04°	17.6±0.05	26.67±0.82*	21.80±1.32*Δ	1.98±0.82*Δ	120.30±1.32*Δ	2144.53±33.34 Δ
Sep.	31±0.16*Δ	7.84±0.01	28.1±0.09°≠	2.2±0.05°	15.5±0.04	35.00±0.67*	28.00±2.213*Δ	1.57±0.67*Δ	143.72±2.213*Δ	2145.04±32.19 Δ
Oct.	31±0.20*Δ	7.30±0.01	28.3±0.07 °≠	1.9±0.04°	17.6±0.06	36.67±1.20*	28.6±1.19*Δ	2.81±1.20*Δ	128.70±1.19*Δ	2213.34±11.16 Δ

Each experiment was replicated six times and values are the mean ± SE of six replicates. +, significant ($p<0.05$) when one way ANOVA was applied between temperature, pH, conductivity, Dissolved oxygen and free carbon dioxide in different months of year 2009-2010.

Product moment correlation was applied between cercaria larvae and other abiotic factors indicate positive (*) and negative correlation (°), and infection rate and other abiotic factors indicate positive (Δ) and negative correlation (≠).

Table 2. Seasonal changes in the abiotic environmental factors, infection rate and intramolluscan larval stages in *I. exustus* of GIDA pond from November 2009 to October 2010.

Months	Temp+ (°C)	pH+	Conductivity+ (µmhos cm^{-1})	O_2 (ppm)	CO_2 + (ppm)	% Infection+	Population density+ ($meter^2$)	Sporocyst/snail+	Redia/snail +	Cercaria/snail +
Nov.	25 ± 0.33*Δ	8.02±0.003*	39.58±0.06	1.78± 0.06Δ	19.25±0.38	8.33±0.92*	12.00±0.78 *Δ	1.31±0.08*Δ	132.5±2.34Δ	931.43±13.67 Δ
Dec.	17± 0.42*Δ	7.94±0.004*	38.45±0.04	2.07±0.07Δ	13.25± 0.45	20.00±0.41*	7.33±1.02*Δ	1.02±0.41*Δ	148.31±4.46*Δ	548.76±4.92 Δ
Jan.	16± 0.75*Δ	7.67±0.005*	39.98±0.05	2.72± 0.06Δ	15.63±0.17	0±0	0±0	0±0	0±0	0±0
Feb.	23± 0.36*Δ	7.75±0.006*	29.35±0.08	3.21± 0.09Δ	13.73± 0.15	0±0	0±0	0±0	0±0	0±0
March	27± 0.33*Δ	7.55±0.030*	28.2± 0.06	2.71± 0.04Δ	25.18±0.14	0±0	0±0	0±0	0±0	0±0
April	33± 0.48*Δ	7.91±0.013*	26.55±0.07	1.30± 0.04Δ	23.01±0.28	0±0	0±0	0±0	0±0	0±0
May	34± 0.36*Δ	8.08±0.007*	21.6± 0.06	1.62± 0.05Δ	24.7±0.13	0±0	0±0	0±0	0±0	0±0
June	36± 0.49*Δ	8.06±0.490*	23.45±0.08	1.42± 0.06Δ	25.1± 0.09	0±0	0±0	0±0	0±0	0±0
July	35± 0.49*Δ	8.22± 0.05*	29.67±0.05	1.65± 0.04Δ	21.69±0.23	28.33±0.52*	22.65±0.78*Δ	0.69±0.87*Δ	128.57±3.43*Δ	832.33±10.23 Δ
Aug.	34± 0.31*Δ	8.26±0.009*	27.5± 0.09	2.28± 0.09Δ	22.53± 0.14	30.00±0.91*	24.16±0.86*Δ	1.20±0.82*Δ	132.70±1.08*Δ	1123.29±28.34 Δ
Sep.	33± 0.45*Δ	8.53±0.008*	38.7± 0.08	2.38± 0.08Δ	13.63±0.18	40.00±0.85*	22.50±1.02*Δ	1.22±0.67*Δ	121.60±1.09*Δ	1934.33±11.64 Δ
Oct.	31± 0.43*Δ	8.34±0.004*	42.68±0.03	2.47± 0.06Δ	15.78± 0.08	28.33±0.60*	19.00±0.72*Δ	1.45±1.20*Δ	145.70±0.78*Δ	1634.34±62.47 Δ

Each experiment was replicated six times and values are the mean ± SE of six replicates. +, significant ($p<0.05$) when one way ANOVA was applied between temperature, pH, conductivity, Dissolved oxygen and free carbon dioxide in different months of year 2009-2010.

Product moment correlation was applied between cercaria larvae and other abiotic factors indicate positive (*) and negative correlation (°), and infection rate and other abiotic factors indicate positive (Δ) and negative correlation (≠).

Indoplanorbis exustus is planorbid snail which, if the environment dries out, may undergo a prolonged state of aestivation, during which transmission of the parasite is suspended. When the rains return, however, there can be rapid recolonization of the environment. *I. exustus* an important intermediate host in India also undergoes aestivation during summer drought. Temperature is considered as a critical environmental factor in the ecology of most organisms [22, 23]. It can act as both a trigger for the commencement of a biological process and as a threshold essential for its continuation. Vector snail *Indoplanorbis exustus* found in Ramgarh lake/GIDA pond were observed during six months (July to December) of year. December onward up to June there was no *I. exustus* in pond water. This is due to the hibernation/aestivation of the snail during these periods. During the dry/cold period snails retract into their shells and cover a fine membrane across the shell opening which may help them to resist from adverse condition [24]. There was significant positive correlation between temperature and number of intramolluscan larval stages, % infection of *F. gigantica* and population density of snail. Most infectious agent do not replicate below a certain temperature threshold level and their growth significantly increased above this level. In invertebrate vector, pathogen replication rate and dynamics may be affected by the temperature of minimum, consequently the infection process and larval development in the invertebrate and duration of the cycle will also be affected [16, 18].

In *I. exustus* the larval stages as well as % infection were higher in the month of August to November, when temperature was 34-25°C. Temperature directly impact on ecology, life cycle, behaviour and survival of invertebrate vectors [25], and therefore, vector population dynamics is related with disease transmission. Higher temperature increases the invertebrate metabolic rate, egg production amount and feeding frequency, reducing the duration of the development periods and impacting on the number of generations per year and population abundance [26]. Temperature seems to exert a powerful dispersal effect on snail population, growth rate of snail and development of trematode from egg to cercariae [27]. Temperature of water varied seasonally throughout the year and alters the number of intramolluscan larvae (sporocyst, redia, and cercaria) in the vector *I. exustus* in Ramgarh Lake/GIDA pond in different months of the year 2009-2010. In the tropics, water temperature variations are governed by climatic conditions. Rainfall and solar radiations are the major climatic factors that influence the physicochemical hydrology of water bodies [28]. Water temperature is dependent on duration and intensity or daily iridescence received by the water body. The intensity of solar radiation may be modified by variations in cloud cover, water flow, phytoplankton species composition and diversity, surface area, depth, wind velocity, solid matter suspension, altitude, etc. resulting in fluctuations in water temperature [29]. The variations in water temperature of Ramgarh Lake/GIDA pond are in accordance with atmospheric temperature, more pronounced in the summer due to clear skies and that produce intense solar radiation in northern hemispheres. Cool dry north east trade winds of winter resulting low temperature. Temperature influences the parameters like dissolved oxygen, solubility, pH, conductivity etc. influencing water chemistry. Solubility of oxygen in the water increases when water temperature decreases [3, 30-32]. Aquatic organisms depend on certain temperature range for optimal growth [14]. Dreyfuss et al. [33] and Rondelaud et al. [34] reported that there was no development of parasite in snail, nor even emergence of cercariae from snail at low temperature. The climatic conditions of Gorakhpur are favourable for the development and growth of freshwater snails. These snails are found in abundance in water streams and ponds almost throughout the year with the exception of small period of dormancy in winter and spring. Summer is generally dry so development of flukes outside the final host takes place from autumn to early winter. It is clear from the result section that high % infection of *F. gigantica* larvae in snail *I. exustus* in July to October in both localities. Persistance of rain throughout the month of June to August provide favourable period for the liberation of cercariae.

An increase in CO_2 concentration in water is known as hypercapnia, which is not appropriate for the aquatic organisms. Carbon dioxide is readily soluble in water. The carbon in all the materials comes from the CO_2 in water. When the oxygen concentration in water containing organic matter is reduced, the CO_2 concentration rises. The rise in carbon dioxide makes it more difficult for fish and other animals to use the limited amount of oxygen

present. The direct effect of high CO_2 exposure can be reduced in metabolic, protein synthesis, growth rate and reproduction in marine animals [35]. The availability of dissolved oxygen in the water is determined by water temperature, salinity, and water quality [36]. Low levels of dissolved oxygen are responsible for more aquatic organism mortalities in culture than any other parameter [37]. During present work the pH of Ramgarh/GIDA pond was observed slightly alkaline. Ambient pH is an important factor in determining the distribution limits of aquatic species [38]. Fluctuation in snail population density can be of major importance in the transmission of fasciolosis [18]. Since under apparently favourable conditions for infection, only a small proportion of the snails are infected. Since the presence of the snail is essential to the completion of the life cycle, an appreciation of the ecology of the snail is required before considering the development phase of the fluke within the snail. The many factors involved in the ecology, particularly those influencing the presence or absence of the snail intermediate host, have been thoroughly reviewed elsewhere [39, 40], and summarized by Boray [36].

CONCLUSION

This study conclusively shows that abiotic factors such as temperature, pH, dissolved oxygen, carbon dioxide and electrical conductivity in surrounding water and population density of snails are crucial factor, which can significantly alter the larval infection of *F. gigantica* in snail *I. exustus* life cycle characteristics of *Fasciola* sp. is greatly influenced by local condition and ethology of intermediate host molluscs and meteorological factors.

Strategies used to control fasciolosis mostly depend on the extent and seasonality of disease transmission, the intermediate host's ability to survive climatic condition. Most suitable period for the control of fasciolosis in this region is in the month of July to October. As a corollary, it is suggested that the treatment of water body with molluscicide for the control of *I. exustus* and ultimately fasciolosis, is not only more potent and cost effective during these months than spending more money by using higher concentration of the molluscicide during the remaining months of year.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Agarwal RA, Singh DK. Harmful gastropods and their control. Acta Hydrochim Hydrobiol. 1988; 16: 113-138.
2. W H O. 2007. Report of WHO informal meeting on use of triclabendazol in fascioliasis control. Meeting: 17-18 October 2006, Geneva, Switzerland. World Health Organization, Headquarter.
3. Singh V, Singh DK. The effect of abiotic factors on the toxicity of cypermethrin against the snail *Lymnaea acuminata* in the control of fascioliasis. J Helminthol. 2009; 83: 39-45.
4. Dalton JP. 1999. Fasciolosis. CAB International. Wallingford, UK.
5. Torgerson, Claxton. Epidermiology and control. In: J.P. Dalton. Fasciolosis. Wallingford, Oxon, UK: CABI Pub. 1999: 113-149.
6. Njoku-Tony RF. Effect of some physico-chemical parameters on abundance of intermediate snails of animal trematodes in Imo state, Nigeria. Researcher. 2011; 3(4): 1-20.
7. Saxena OP, Parashari A. Comparative study of six heavy metals to *Chana punctatus*. Ibid. 1983; 4(2): 91-94.
8. Deshayes GP. In: Belanger C. Voyage aux Indes-Orientales. Zool. 1934; 3: 417.
9. Kumar P, Singh DK. Molluscicidal activity of *Ferula asafoetida*, *Syzygium aromaticum* and *Carum carvi* and their active components against the snails *Lymnaea acuminata*. Chemosphere. 2006; 63: 1568-1574.
10. Kaplon RM, Dame JB, Reddy GR, Courtney CH. The prevalence of *Fasciola hepatica* in its snail intermediate host determined by DNA probe assay. Int J Parasitol. 1997; 27: 1585-1593.
11. Cruz-Mendoza I, Ibarra-Velarde F, Quintero-Martínez MT, Naranjo-García E, Lecumberri-López J, Correa D. Seasonal transmission of *Fasciola hepatica* in cattle and *Lymnaea* (*Fossaria*) *humilis* snails in central Mexico. Prasitol Res. 2005; 95(4): 283-286.
12. Berrie AD. Snail problems in African Schistosomiasis. Advances in Parasitology. Dawes, Ben; Academic Press, New York. 1970; 8: 43.
13. Manga-Gonzalez Y, Gonzalez-Lanza C, Otero--Merino CB, Natural infection of *Lymnaea truncatula* by the liver fluke *Fasciola hepatica* in the Porma Basin, Leon, NW Spain. J Helminthol. 1991; 65: 15-27.
14. APHA. Standard Methods for the Examination of Water and Wastewater. 20th edition, American Public

Health Association, Washington, DC. 1998.
15. Sokal RR, Rohlf FJ. Introduction to Biostatistics. W.H. Freeman and Co., San Francisco. 1973; 271-273.
16. Reiter P. Climate change and mosquito-borne disease. Environ. Health Perspect. 2001; 109 (1): 141-161.
17. Mas-Coma S, Valero MA, Bargues MD. Effect of climate change on animal and zoonotic helminthiases. In: De La Rocque S, Hendrickx G, Morand S. (Eds.). Climate change: Impact on the epidemiology and control of animal diseases. World Organization for Animal Health (OIE), Paris. Sci Tech Rev. 2008; 27: 443-452.
18. Mas-Coma S, Valero MA, Bargues MD. Climate change effects on trematodiases, with emphasis on zoonotic fascioliasis and schistosomiasis. Vet Parasitol. 2009; 163: 264-280.
19. Khangarot BS, Ray PK. Zinc sensitivity of a freshwater snail, *Lymnaea luteola* L., in relation to seasonal variation in temperature. Bull Environ Contam Toxicol. 1987; 39: 45-49.
20. Jahan MS, Rahman MR. Breeding biology of freshwater snail *Lymnaea* (*Radix*) *acuminata* Lamarck (Gastropoda; Lymnaeidae). J Zool Rajshahi Univ. 1996; 14&15: 47-54.
21. Sarkar SK, Saha M, Takada H, Bhattacharya A, Mishra P, Bhattacharya B. Water quality management in the lower stretch of the river Ganges, east coast of India: an approach through environmental education. J Clean Prod. 2007; 15: 1559-1567.
22. Magnuson JJ, Crosder LB, Medvick PA. Temperature as an ecological resource. Zool. 1979; 19: 331-343.
23. Raut SK, Rahman MS, Samanta SK. Influence of temperature on survival growth fecundity of the freshwater snail *Indoplanorbis exustus* (deshayes). Mem Inst Oswaldo Cruz. 1992; 87: 15-19.
24. Frederico S, Barbosa MD, Louis O. Studies on the snail vectors of *Bilharziasis mansoni* in North-Eastern Brazil. Bull Org Mond Sante. 1958; 18: 895-908.
25. Rodhain F. Modifications climatiques: impact potential sur la santé, I exemple des maladies a vecteur. Bull Assoc Anc Eleves Inst Pasteur. 2000; 162: 8-14.
26. De La Rocque S, Rioux JA, Slingenbergh J. Climate change: effects on animal disease systems and implications for surveillance and control. In: De La Rocque S, Hendrickx G, Morand S. (Eds.). Climate Change: Impact on the Epidemiology and Control of Animal Diseases. World Organization for Animal Health (OIE), Paris. Sci Tech Rev. 2008; 27 (2): 309–317.
27. Appleton CC. Review of literature on abiotic factors influencing the cycles of schistosomiasis intermediate host snail. Malacol Rev. 1978; 11: 1-25.
28. Kadiri MA. Seasonal trend in the chemical limnology of shallow Nigerian man-made lake. Acta Hydrobiol. 2000; 421(2): 29-40.
29. Atoma OC. Ph.D Thesis. University of Benin, Benin City. 2004; 220.
30. Joshi PC, Singh A. Analysis of certain physic-chemical parameter and planktons of freshwater hill stream at Nanda Devi Biosphere reserve. Uttar Pradesh J Zool. 2001; 21: 177-179.
31. Awasthi U, Tiwari S. Seasonal trends in abiotic factors of a lentic habitat (Govindgarh lake) Rewa, M.P, India. Eco Env Cons. 2004; 10 (2): 65-170.
32. Jigyasu HV, Singh VK. Effect of environmental factors on the fecundity, hatchability and survival of snail *Lymnaea* (*Radix*) *acuminata* (Lamarck): vector of fascioliasis. J Water Health. 2010; 8: 109-115.
33. Dreyfuss G, Novobilsky A, Vignoles P, Bellet V, Koudela V, Rondelaud D. Prevalence and intensity of infections in the lymnaeid snail *Omphicola glabra* experimentally infected with *Fasciola hepatica*, *Fascioloides magna* and *Paramphistomus daubneji*. J Helminthol. 2007; 81: 7-12.
34. Rondelaud D, Fousi M, Vignoles P, Moncef M, Dreyfuss G. Optimization of metacercarial production for three digenean species by the use of Petri dishes for raising lettuce-fed *Galba trumeatula*. Parasitol Res. 2007; 100: 861-865.
35. Portner HO, Langenbuch M, Reipschlager A, Biological impact of elevated ocean CO_2 concentration. J Oceanogr. 2004; 60: 705-718.
36. Boray JC. Studies on experimental infection with *Fasciola hepatica*, with particular reference to acute fascioliasis in sheep. Ann Trop Med Parasit. 1967; 61: 439-450.
37. Timmons MB, Ebeling JM, Wheaton JM, Summerelt ST, Vinci BJ. (Eds.). 2002. Recirculating Aquaculture Systems. 2nd ed. Cayuga Aqua Ventures, Ithaca, N.Y. 2002: 757.
38. Weisse T, Stadler P. Effect of pH on growth, cell volume, and production of freshwater ciliates, and implications for their distribution. Limnol Oceanogr. 2006; 51(4): 1708-1715.
39. Ollerenshaw CB, Rowlands WT. A method of forecasting the incidence of fascioliasis in Anglesey. Vet Res. 1959; 71: 591-598.
40. Boray JC. Experimental fascioliasis in Australia. Adv Parasitol. 1969; 7: 95–210.

Journal of Biology and Earth Sciences

MEDICINE ORIGINAL ARTICLE

Role of nitric oxide and reduced glutathione and their implication on typhoid

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ABSTRACT

Typhoid one of the most important causes of illness and death, particularly among children and adolescents in south-central and Southeast Asia, due to poor sanitation and unsafe food and drinking water. Nitric oxide (NO) is a versatile molecules produced in a biological system. Reduced glutathione (GSH) is the most abundant cytosolic thiol that easily reacts with NO and forms S-nitrosoglutathione (GS-NO) these thiol compounds might decrease the levels of free forms of NO, thereby affecting its fate and biological activity. Infection with bacteria to control mice resulted in significant decrease in the GSH level by 10% at day 8 of infection and after treatment with formulated drugs significant increment was observed.

Key words: Nitric oxide, Reduced glutathione (GSH), Typhoid.

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INTRODUCTION

Glutathione (GSH) is an important antioxidant, which is synthesized by glutathione synthetase with the help of two molecules of adenosine triphosphate (ATP). The high steady-state levels of glutathione in E. coli and in *S.typhimurium* that maintain a strong reducing environment in the cell [1]. The GSH provides protection against oxidative damage and detoxification of reactive chemicals [2, 3]. Nitric oxide (NO) is an endogenous free radical reactive species, produced from the amino acid L-arginine by the enzymatic action of nitric oxide synthase (NOS) [4]. NO is stabilized by carrier molecule like reduced thiol species that preserves its biological activity. This molecule readily reacts in the presence of NO to yield biologically active S-nitrosothiols that is more stable and potent than NO itself. S-nitrosothiols are unstable in aqueous solution. GS-NO is a bioactive intermediary that may regulate cellular functions and inhibits the sarcoplasmic reticulum bound creatine kinase [5]. Generally S-nitrosoglutathione (GS-NO) has been found endogenously in neutrophils and human airways at μM concentrations [6]. S-nitrosothiols may play the same role in the mechanism of action of Endothelium-Derived Relaxing Factor (EDRF) as NO [7]. Some Authors have assumed that the biological effects of these compounds are due to the spontaneous release of NO. However, this hypothesis is not fully supported yet [8-10]. The aim of this study was to determining the effect of formulated drugs on GSH against *S.typhimurium*.

MATERIALS AND METHODS

Animals

Swiss albino mice (25-30 g) of 6-8 weeks old were obtained from the central animal house of Hamdard University, New Delhi, India. The animals were kept in Poly-propylene cages in an air-conditioned room at 22°/25°C and maintained on a standard laboratory feed (Amrut Laboratory, rat and mice feed, Navmaharashtra Chakan Oil Mills Ltd, Pune) and water *ad libitum*. Animals were allowed to acclimatize for one week before the experiments under controlled light/dark cycle (14/10h). The studies were conducted according to ethical guidelines of the "Committee for the purpose of control and supervision of Experiments on Animals (CPCSEA)" on the use of animals for scientific research.

Bacteria

Standard strain of *Salmonella typhi* and *Salmonella typhimurium* (wild), were obtained from National Salmonella Phage Typing Centre, Lady Harding Medical College, New Delhi, India, and was used for these studies. The bacterial strains were characterized further at the microbiology Department of Microbiology Majeedia Hospital Hamdard University to confirm their identity. Briefly, *S. typhimurium* was grown at 37°C as stationary overnight cultures in nutrient broth. The innoculum was diluted in PBS (Phosphate buffer saline) and injected into peritoneum of mice. Dilution and pour plating onto agar plate was done to check the number of viable bacteria in each innoculum.

Dose and Dosage

Animals were divided into eight groups. Each group comprised of six animals. The study comprised of following treatment schedules.

Groups and Treatments

Group 1. Negative control (Normal Saline)
Group 2. Positive control (*S. typhimurium* (0.6xLD50) + Saline
Group 3. *S. typhimurium* (0.6xLD50) + Arginine (1000 mg per kg b. wt)
Group 4. *S. typhimurium* (0.6xLD50) + Ciprofloxacin (400mg per kg b. wt)
Group 5. *S. typhimurium* (0.6xLD50) + Arginine (500mg per kg b. wt) + Ciprofloxacin (200mg per kg b. wt)
Group 6. *S. typhimurium* (0.6xLD50) + Arginine (250mg per kg b. wt) + Ciprofloxacin (200 mg per kg b. wt)

Effects of above drugs on infected mice by *S. typhimurium* were analyzed. Mice were divided into eight groups having six mice in each group. Post-treatment of drugs was done at above dose orally to the experimental animals, first group was considered as control which received only saline, second group was treated as positive control which was challenged with sub lethal dose of *S. typhimurium* along with saline.

Third group only received full dose of ciprofloxacin. Fourth group was challenged with sub lethal dose of *S. typhimurium* and then mice were treated with standard drug ciprofloxacin. Fifth group

received full dose of Arginine only. In sixth group after infection with *S. typhimurium* animals were treated with full dose of Arginine. In seventh and eight group animals were challenged with *S. typhimurium* and then half and one fourth dose of Arginine was administered along with half dose of Ciprofloxacin respectively.

RESULTS

Reduced glutathione (GSH)

To study the effect of L-arginine, ciprofloxacin and their combination on liver damage, hepatic GSH levels in mice were measured. The mice were challenged with sublethal dose ($0.6xLD_{50}$) of *S. typhimurium* and then treated with drugs. The results have been summarized in Figure 1. Infection with bacteria to control mice resulted in significant decrease in the GSH level by 10% at day 8 of infection.

On day 8, the treatment of mice with L-arginine, ciprofloxacin and their combination, the GSH level was slightly increased by 10%, 28.88%, 33.33% and 24.4% in *S. typhimurium* infected mice as compared with control.

DISCUSSION

Oxidative stress can be defined as an increase in oxidants and/or a decrease in antioxidant capacity, and various oxidants and antioxidants have additive effects on oxidative status. Glutathione (GSH) is an important thiol antioxidant which reduces disulfide bridges caused by oxidative stress in proteins and act as a protector in various cytotoxic conditions [11-13]. GSH reacts with oxide of nitrogen to form GSNO. It should be noted that iNOS is expressed in activated macrophages particularly at the site of inflammation. For example, iNOS activities in Kupffer cells and hepatocytes of septic animals are high. However, it is known that the metabolism of GSH occurs via inter- and intraorgan cycles, which include hepatic secretion of GSH, its degradation by tissues that have γ-glutamyl transferase and peptidases, and reabsorption of its constituent amino acids [14]. Hence, the local concentrations of cysteine might be fairly high in tissues that are enriched with these enzymes, such as renal proximal tubules and ascites hepatoma cells [15, 16]. Nitrosoglutathione (GS-NO) molecule, formed by a reaction between

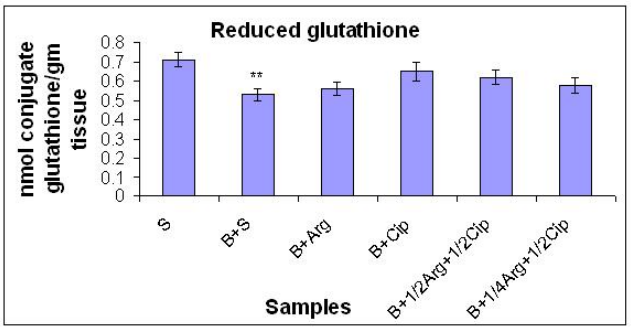

Fig. 1. Hepatic reduced glutathione levels in mice: drugs were given and study was made on day 8 with arginine, ciprofloxacin and their combination.
S = Saline,
B+S = *S. typhimurium* + Saline,
B+Arg = *S. typhimurium* + 1000mg per kg b. wt L-Arginine,
B+Cip = *S. typhimurium* + 400mg per kg b. wt Ciprofloxacin,
B+1/2Arg+1/2Cip = *S. typhimurium* + 500mg per kg b. wt Arginine + 200 mg per kg b. wt ciprofloxacin,
B+1/4Arg+1/2Cip = *S. typhimurium* + 250mg per kg b. wt Arginine + 200mg per kg b. wt Ciprofloxacin.
Values are significantly different **p<0.01

GSH and NO, plays role in many physiologic and pathologic processes through a mechanism similar to that of NO via EDRF [17].

In conclusion, formulated drugs drug exhibits therapeutic induction by reducing oxidative stress.

TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

REFERENCES

1. Loewen PC. Levels of glutathione in *Escherichia coli*. Can J Biochem. 1979; 57: 107-111.
2. Wang W, Nazerrano B. Endogenous glutathione conjugates: occurrence and biological functions. Pharmacol Rev. 1998; 3: 335-356.
3. Akkus I. 1995. Effects of free radicals and pathophysiological. Konya, Turkey: Mimoza.
4. Aldetron WK, Cooper CE, Knowles RG. Nitric oxide synthase: structure, function and inhibition. Biochem J. 2001; 357: 593-615.
5. Wang W, Nazerrano B. Endogenous glutathione conjugates: occurrence and biological functions. Pharmacol Rev. 1998; 3: 335-356.
6. Clancy RM, Levartovsky D, Leszczynska-Piziak J, Yegudin J, Abramson SB. Nitric oxide reacts with intracellular glutathione and activates the hexose

monophosphate shunt in human neutrophils: evidence for S-nitrosoglutathione as a bioactive intermediary. Proc Natl Acad Sci. 1994; 91: 3680-3684.

7. Kowaluk EA, Fung HL. Spontaneous liberation of nitric oxide cannot account for in vitro vascular relaxation by S-nitrosothiols. J Pharmacol Exp Ther. 1990; 255: 1256-1264.
8. Rockett KA, Aburn MM, Cowden WB, Clark IA. Killing of Plasmodium falciparum in vitro by nitric oxide derivatives. Infect Immun. 1991; 59: 3280-3283.
9. Clancy RM, Miyazaki Y, Cannon BJ. Use of thionitrobenzoic acid to characterize the stability of nitric oxide in aqueous solutions and in porcine aortic endothelial cell suspensions. Anal Biochem. 1990; 191: 138-143.
10. Gaston B, Drazen JM, Jansen A, Sugarbaker DA, Loscalzo J, Richards W, Stamler JS. Relaxation of human bronchial smooth muscle by S-nitrosothiols in vitro. J Pharmacol Exp Ther. 1994; 268: 978-984.
11. Nkamura Y, Shiemtani A, Fujii H, Amano O, Sakagami H, Takashi K. Glutathione can efficiently prevent direct current-induced cytotoxicity. J Endod. 2008; 34: 693-697.
12. Jones DP. Radical-free biology of oxidative stress. Am J Physiol Cell Physiol. 2008; 295: 849-868.
13. Banerjee A, Trueblood MB, Zhang X, Manda KR, Lobo P, Whietfielfd PD, Hagen DE, Ercal N. N-acetylcysteineamide (NACA) prevents inflammation and oxidative stress in animals exposed to diesel engine exhaust. Toxicol Lett. 2009; 187: 187-193.
14. Inoue M. Minamiyama Y, Yu H, Yamamasu S, Inoue K, Sato EF. 1995. Biochemical and clinical aspects of extracellular glutathione and related thiols. In: Biothiols in Health and Disease. Eds. Packer L, Cadenas E. pp: 287-304.
15. Horiuchi S, Inoue M, Morino Y. Evidence for the presence of a latent active site in the large subunit of rat kidney g-glutamyl transpeptidase. Biochem Biophys Res Commun. 1978; 80: 873-878.
16. Inoue M, Horiuchi S, Morino Y. γ-Glutamyl transpeptidase in rat ascites tumor cell LY-5. Eur J Biochem. 1977; 78: 609-615.
17. Zeng H, Spencer NY, Hogg N. Metabolism of S-nitrosoglutathione by endothelial cells. Am J Physiol Heart Circ Physiol. 2001; 281: H432-H439.

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EARTH SCIENCES ORIGINAL ARTICLE

Dynamics of metamorphism processes by the fractal textures analysis of garnets, amphiboles and pyroxenes of Lapland Granulite Belt, Kola Peninsula

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ABSTRACT

About thousand analyzes of garnet, amphibole and pyroxene crystals from selected samples of amphibolite and granulite rocks from Lapland Granulite Belt in Kandalaksha region (Kola Peninsula) has been made. Indicated fractal-box dimension of studied minerals has a good correlation with tectonic zones, lead to a new insight in the dynamics of processes, which has modified the examined region. Fractal-box dimension makes the textural analysis more precise, because it consents for the mathematic and repeated review of crystals topology depending directly on processes which had created them.

Key words: Lapland Granulite Belt, Kandalaksha, Kola Peninsula, amphibolites, granulites, fractal dimension, box-counting method, petrology, metamorphism.

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INTRODUCTION

The research area is located in the East of the Kandalaksha on the White Sea, Kola Peninsula (Fig. 1). There are mainly exposures of the metavolcanic and metaintrusive rocks metamorphosed in conditions of amphibolite and granulite facies (Fig. 1). These rocks belong to the Lapland Granulite Belt [1]. They represent granulites and amphibolites mainly [2].

Granulite rocks have a grano-lepidoblastic, massive structure, disordered, rare directional texture, they are rich with garnets piroxenes (diopside and hyperstene), quartz and plagioclases (andezine-labradore). They are accessory minerals accompanied with a rutile, chromite, amphiboles rich in common hornblende unit (in diaphtoretic zone), and an untransparent minerals - ilmenite, ülvite, magnetite and pyrite mainly (Fig. 2). Garnets in these rocks constitute 30-45% vol.

Amphibolites have a grano-lepido-nematoblastic, massive structures, disordered textures, constructed usually with common hornblende (older – rich in Ti, and younger – rich in Mg), garnets, plagioclases (labradore – bytownite) quartz and rare biotite. They are accessory minerals accompanied with rutile, titanite, ilmenite and pyrite (Fig. 3). Amphiboles in these rocks contribute to 30-60% vol., pyroxenes <5% vol. and garnets 25-30% vol. [1-7].

MATERIALS AND METHODS

Experimental methods:

The estimates of fractal-box dimension method [8] were assigned on the selected photographed minerals. Microphotographs of studied minerals are covered by a square netting with the “a” side, and next step is to rank meshes from the netting through which a border of studied crystals transfixes (jointly with inclusions). The square must be equivalent to the size of studied crystals. Next, the length of “a” side of the squares is diminished. Than one should rank the squares and a border of crystals is going through this diminished squares (this cycle was

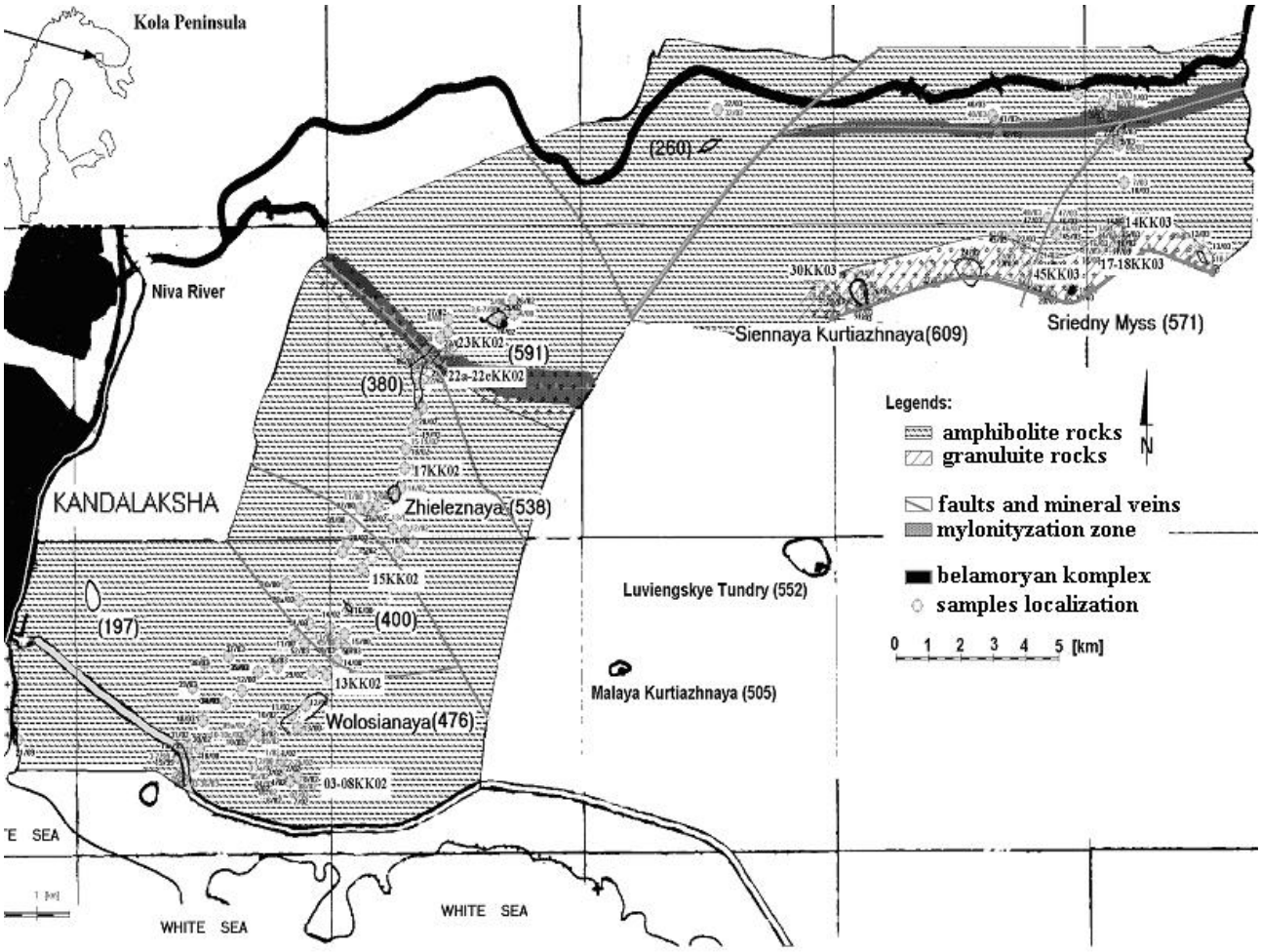

Fig. 1. The sketch of geological map of the investigated area, 1:100 000. Amphibolites marked with the dark, granulites marked with the light.

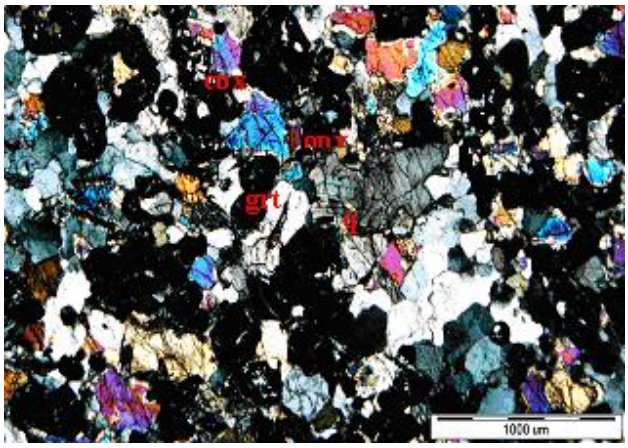

Fig. 2. Microphotograph of granulite from Kandalaksha (transmitted light, crossed polars).

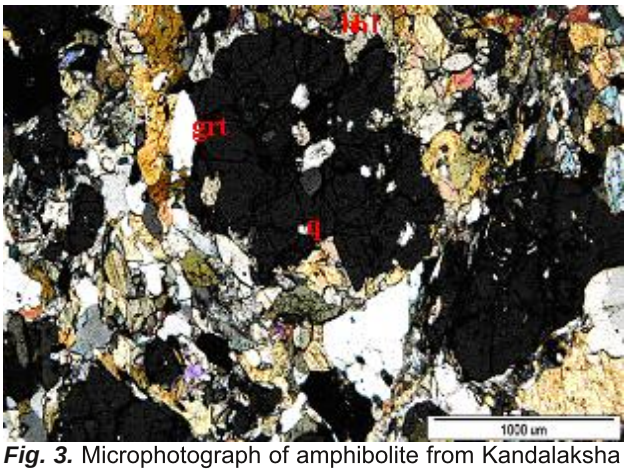

Fig. 3. Microphotograph of amphibolite from Kandalaksha (transmitted light, crossed polars).

repeated several times). After these calculations, on the "Y" axis there is marked a logarithm "a" and on the "X" axis is marked an adequate logarithm of number "N" of these squares from netting, through which a border of studied crystals is going. Calculating a gradient of a slant to this line (1) was obtained a "D" parameter, which is a fractal-box dimension of studied crystals structure.

$$D = \frac{(\log N_2 - \log N_1)}{(Loga_2 - \log a_1)} (1)$$

Study materials:

There were made fifty field sections from chosen rocks to make a fractal-box dimension analysis of studied minerals. Then the microphotographs of selected areas in these sections were taken (on the polarization microscope in a cross light). There were made 355 analysis of garnets, 300 analysis of pyroxenes and 300 analysis of amphiboles, with varying morphological forms. Several analyses were made on selected samples of whole rocks, in case of varying fractal-box dimension. Arithmetic average of the fractal-box dimension was calculated for those samples.

RESULTS

A strict relation with topological character of crystals border is a significant attribute of fractal-box dimension. Moreover, it is not depending on the highest of them. This method permits a precise and repeatable analysis of crystals texture.

The distribution of variation of the fractal-box dimension in studied minerals (Fig. 4A-C) was made after the attributing of fractal values in any individual point of localization of sample in the study area.

The Fig. 4A shows the fractal dimension in the pyroxenes of rocks in the studied area. The lowest value of fractal dimension usually have the pyroxenes from the granulites, which were exposured in the northern slopes of Sriednyj Myss, Siennaya Kurtiazhnaya Mt, and in the px-ga xenolithes from the over thrust zone. In these rocks the pyroxenes have granoblastic structure. The highest value of fractal dimension have the pyroxenes from amphibolites near Zhielieznaya and Volosianaya Mts. in Kandalaksha Tundra. High value of fractal dimension have also the pyroxenes from granulites with advanced secondary processes to amphibolite facies, of north western part of the studied area (Siennaya Kurtiazhnaya Mt.) These pyroxenes have a frayed structure.

Studied amphiboles (Fig. 4B) showed a high diversity of the fractal dimension. The highest dimension is characteristic for amphiboles from tectonic amphibolized granulites of Sriedny Myss Mt. in the northern slopes mostly. They have a frayed structure. High fractal dimension have also the amphiboles from strongly metamorphosed in high P,T conditions amphibolites from northern slopes of Zhielieznaya Mt. Amphiboles from amphibolites in Volosianaya and Zhielieznaya Mt region have an extended edges and inclusions of quartz. A specially low fractal dimension have the after-pyroxene amphiboles (xenomorphic, oval minerals), present in metagabro amphibolites of the northern slopes, northern Zhielieznaya Mt and from metavolkanite amphibolites of the southern slopes

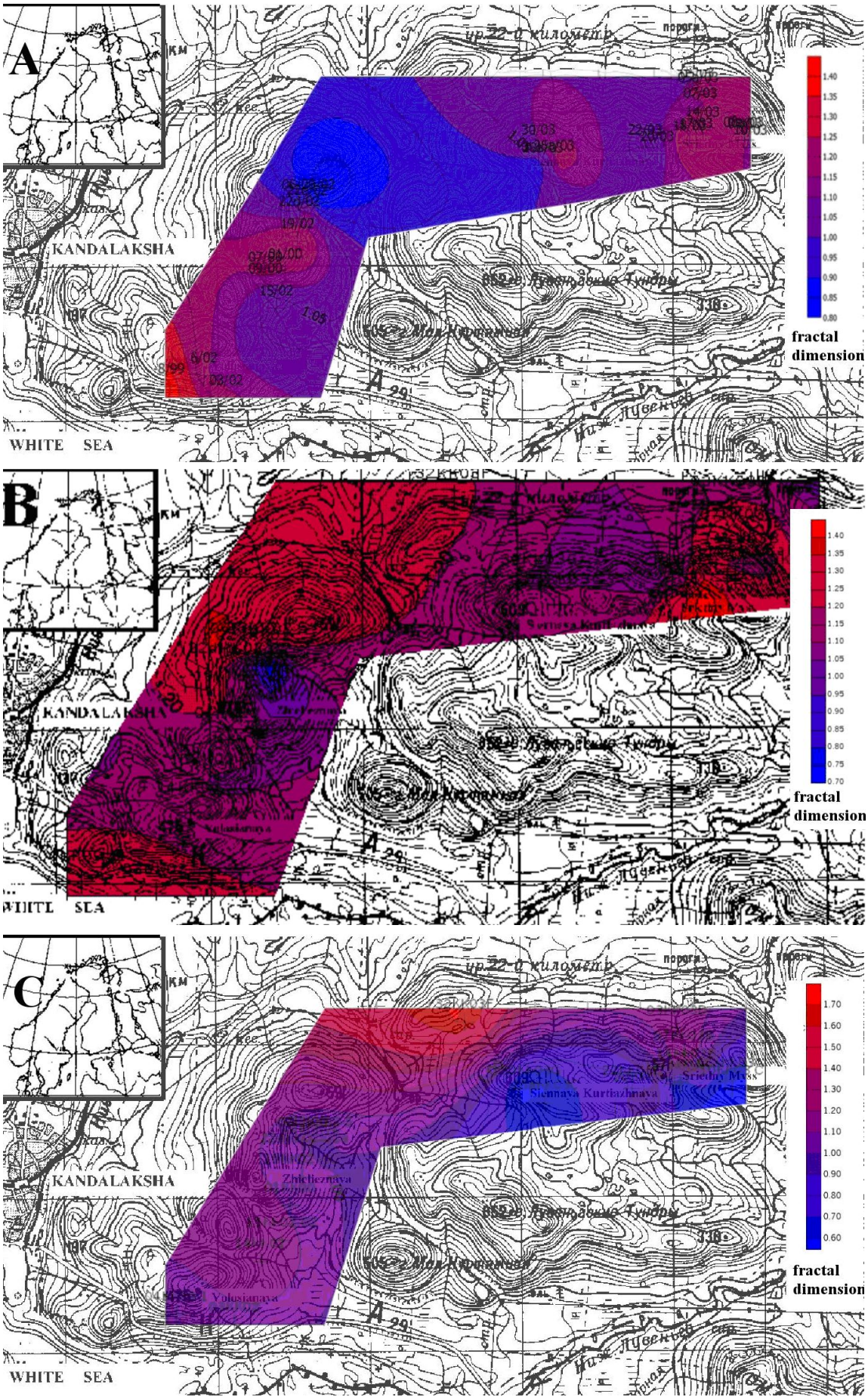

Fig. 4. A, B, C. Map of variation of the fractal-box dimension for pyroxenes (A), amphiboles (B) and garnets (C) in studied area (scale ~1:200 000).

of Zhielieznaya Mt. Similar value of fractal dimension have the amphiboles from metavolcanic amphibolites of the northern slopes of Sriednyj Myss Mt. These minerals have granoblastic, oval structure.

Fig. 4C shows the variation of fractal dimension in garnets. The highest fractal dimension is characteristic for garnets from metavolcanic (metabasement) amphibolites of the Zhielieznaya Mt., which are exposured in the cutting the road from Kandalaksha to Umba and in the granulites from Siennaya Kurtiazhnaya Mt. These garnets usually have a very extended boards, diablastic, atolitic, suture structures. The were crystallized in the regional metamorphism conditions Fig. 5C [9, 10]. The lower value of fractal dimension have garnets from amphibolized granulites in diaphtoretic zone (Volosianaya and Zhielieznaya Mt.). The same fractal value have a garnets of some metavolcanic rocks and metamylonites from overthrust zone (northern slopes of northern Zhielieznaya Mt., northern slopes of Siennaya Kurtiazhnaya and Sriednyj Myss Mt.). These minerals have a granoblastic structures. It was metamorphosed in high dynamics conditions, Fig. 5A, B [9, 10]. Lowest value have the garnets in metagabbro amphibolites (oval structures), which are exposured in the northern part of Kandalaksha Tundra and in the granulites in Sriedny Myss Mt. These minerals were crystallized in a high metamorphism conditions.

DISCUSSION

Variation of calculated fractal dimension of studied minerals' textures from collected samples shows dynamics of metamorphic processes and crystallization. Interpretation of the fractal dimension of each mineral, needs an individual investigation. They are precisely connected with different crystallization of minerals at the metamorphic condition. The immediate comparison of the structure of different minerals is not possible. On Fig. 4A-C areas with higher and lower values of fractal dimension were marked. The areas with higher fractal dimension for pyroxenes are characteristic for the high metamorphosed amphibolite rocks. In these rocks the pyroxenes have a numerous inclusions of amphiboles, quartz and other minerals with the frayed structure. Similar, but a bit smaller value have the amphibolized pyroxenes. Usually in their close vicinity the amphiboles crystallize strongly raising the edge of pyroxenes. The lowest value have the granoblastic pyroxenes from highly metamorphosed rocks (granulite).

Amphiboles, however, have the lowest value in the rocks which were crystallized at the expense of pyroxenes, adapting the oldest structure (pseudomorphism). The high fractal value is characteristic for amphiboles from amphibolites with numerous inclusions of quartz, titanite and others phases. The highest value have the amphiboles, with frayed structure, crystallized in high dynamic surroundings (metamylonites).

The areas with lower value are often related with faults activity in the same time of crystallization garnets processing (see Fig. 1 and Fig. 4A-C). In these areas, where the dynamic of these processes was the highest, the neoblastic crystals had a good conditions to create and displace unnecessary substance by the force of crystallization. These crystals have often automorphic textures. Areas, where the value of fractal dimension is the highest, are often related with lower dynamics of metamorphic processes. The minerals with many inclusions and very extended boards were crystallized in restricted areas in rocks between other minerals.

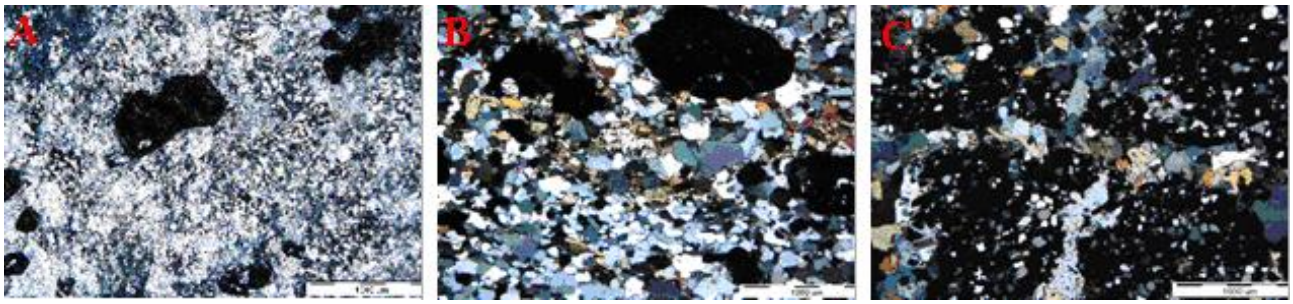

Fig. 5. A,B,C Microphotograph of garnets in rocks samples (transmitted lights, crossed polars): A- garnets crystals in mylonitic rocks, B- garnets in gneiss, C –garnets in massive amphibolite.

CONCLUSION

During the preparation of material for analyzes should be paid attention to the condition of investigated minerals. Very important is proper preparation of the sample and a good understanding of the processes that lead to such and no other formation of minerals in the rock. The mechanical application of this method without adequate preparation can leads to erroneous conclusions. Therefore, this method has considerable restrictions and can be used as a complement to the optical microscope analyzes. A particularly convenient situation is the study of rocks with similar genesis in which on the basis of previous observations have already been selected the most important types and mineral phases. In this case, the types of rocks can be determined as a standard for the investigated massif and using this method can be look for transitions between two types of rocks. In this approach, this method can be very helpful. Method of fractal analysis permits to make a precision analysis of texture of studied minerals and describe the dynamics of blastesy processes.

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TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

REFERENCES

1. Kozlov NE, Ivanov AA, Nyerovich LI. 1990. The Lapland Granulite Belt – the primitive nature and development. Russian Academy of Science, Apatity Branch Press: pp. 166 (in Russian).
2. Huber M. Geologická a petrografická charakteristika granulitového Laplandského pásma z lokality Kandalaksha nad Bielym Morom (polostrov Kola, severné Rusko). Acta Mineral Slov. 2002; 2: 17-31, (in Slovak).
3. Huber M. Geological and petrographic characteristics of the Lapland Granulite Belt near Kandalaksha at the White Sea, Kola Peninsula, Northern Russia. Mineralia Slovaca. 2001; 2: 17-31.
4. Huber M, Bonarski JT. Investigation on the texture of Lapland Granulite Belt using X-ray diffraction technique. Mineralogical Society of Poland – Special Papers. 2003; 22: 73-77.
5. Skublov SG, Huber M. Ossobyennosti geochimi REE v metamorficheskych mineralach S-E chas'ti Laplandskovo Granulitovovo Poyasa. Viestnik SPBU 2005; 7: 87-91.
6. Huber M, Hałas S, Sikorska M. Evolution of prehnitealbite-calcite veins in Metamorphic rocks from the Lapland Granulite Belt (Kandalaksha region of Kola Peninsula). Geologija 2007; 57: 1-7.
7. Huber MA, Heflik W, Pattek-Janczyk A, Pitak M, Stadnicka K, Skublov SG. Investigations of garnets from polymetamorphic rocks of the Lapland Granulite Belt of the Kandalaksha Region. J Biol Earth Sci. 2012; 2(1): E34-E44.
8. Petigen H-O, Jürgens H, Saupe D. 1992. Fractals for the Classroom. Springer-Verlag N.Y. Vol.1: pp.546.
9. Heflik W. 1993. Metamorphizm at the light of some physic-chemistry problems, AGH Branch Press: pp.187 (in Polish).
10. Myiashiro A. 1994. Metamorphic Petrology. N.Y. University Press: pp.404.

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