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In vitro larvicidal potential against *Anopheles stephensi* and antioxidative enzyme activities of *Ginkgo biloba*, *Stevia rebaudiana* and *Parthenium hysterophorous*

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ABSTRACT

Objective: To investigate *in vitro* larvicidal and antioxidant enzymes potential of the medicinal plants *Ginkgo biloba (G. biloba)*, *Stevia rebaudiana (S. rebaudiana)* and *Parthenium hysterophorous (P. hysterophorous)* against *Anopheles stephensi (An. stephensi)* 4th instars larvae. **Methods:** For evaluation of larvicidal potential, the ethanolic, methanolic and dichloromethane leaves extracts of three different plants were used in dose-dependent experiments in two media, while the antioxidant enzymes activities were investigated using four different methods viz., superoxide dismutase, peroxidase, ascorbate and catalase. **Results:** *An. stephensi* has developed resistance to various synthetic insecticides, making its control increasingly difficult. The comparative performance of ethanolic extracts (65%-90%) was found better than the methanolic extract (70%-87%) and dichloromethane extract (60%-70%). Among the three plants extracts tested in two media, *S. rebaudiana* exhibited higher larvicidal activity with LC_{50} (24 h) in methanolic extract than *P. hysterophorous* and *G. biloba*. *G. biloba* and *P. hysterophorous* exhibited the strongest antioxidative enzymes activity and *S. rebaudiana* were less active and no significant difference was observed. **Conclusions:** These three plants exhibit larvicidal potential and can be further used for vector control alternative to synthetic insecticide due to eco-friendly and diseases control, furthermore these plant species have potent antioxidative enzyme activities, therefore, making them strong natural candidate particularly for diseases which are caused due to free radicals.

1. Introduction

Malaria is a parasitic disease from which more than 300 million people suffer yearly throughout the world. Prevalence of mosquito borne diseases are one of the world's most health hazardous problems. Mosquitoes are the principal vectors of malaria[1], and various other diseases like filariasis, Japanese encephalitis, dengue and dengue hemorrhagic fever, yellow fever and chickungunya[2]. Many approaches have been developed to control mosquito menace. One such approach to prevent mosquito borne disease is by killing mosquito at larval stage[3]. Synthetic insecticides are fast acting, highly active and cost effective, yet their continuous application has resulted in gradual deterioration of the environment[4]. Moreover, they are toxic to non-target organisms and their extensive use have created problems like enhancing resistance of mosquito population to synthetic insecticides[5]. Botanical insecticides are now preferred as an ecofriendly alternatives[6], generally pest specific and are relatively harmless to non-target organisms including humans. They are biodegradable and harmless to the environment[7], Many plants produce secondary components that have insect growth inhibitory activity.

Of the principal vector species, *Anopheles stephensi (An. stephensi)* have shown widespread resistance. Thus, the future of vector control mainly relies on the strategies for the management of existing insecticide resistance in malarial vectors and to limit its further spread. One of the possible ways of avoiding development of insecticide

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resistance in field is using nonchemical control method, *i.e.*, biopesticides. Therefore, it is the hour to launch extensive search to explore eco-friendly biological materials for control of *An. stephensi*[1]. Management of disease vector using synthetic chemicals has failed because of resistance, effect on nontarget organisms and environmental pollution. On the other hand, the recent public perception against the vector control using synthetic chemicals has shifted the research effort towards the development of environmentally sound and biodegradable agents. In that way, plant extracts have much attention to control the disease transmitted vectors[1].

Reactive oxygen species (ROS) such as the superoxide radical, hydrogen peroxide and singlet oxygen are constantly produced in plants, However, uncontrolled production of ROS can cause cellular damage directly or through the formation of toxic secondary metabolites[8]. The level and kind of ROS are determining factors for the type of response. ROS induce defense genes and adoptive responses at low concentrations, and trigger a genetically controlled cell death program at higher. But the role of ROS during the normal physiological function of plant is little known. Plants have developed a complex antioxidant system to protect themselves against such oxidative damage. Antioxidant protection system includes enzymes like superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate (ASC), which scavenge both radicals and their associated non-radical oxygen species.

The overall objective of the current study was to evaluate the *in vitro* larvicidal activity against *An. stephensi* for natural and ecofriendly bioinsecticides. The antioxidant enzyme activities in three medicinally important plants were also evaluated to compare with each other.

2. Materials and methods

2.1. Collection of plant materials

The leaves of *Ginkgo biloba (G. biloba)* were collected from Qarshi Research International, Haripur KPK, leaves of *Stevia rebaudiana (S. rebaudiana)* were collected from Islamabad nursery, leaves of *Parthenium hysterophorus (P. hysterophorus)* were collected from Quaid-i-Azam University Islamabad in 2009 and were authenticated by Dr. Lajber Khan, Head; Medicinal Botanical Centre, PCSIR complex Peshawar, Pakistan.

2.2. Insect rearing

An. stephensi larvae were collected from stagnant ponds of Quaid-i-Azam University Islamabad and Haripur KPK and identified by Department of Animal Sciences Quaid-i-Azam University Islamabad. These larvae were kept in 15 L plastic containers containing tap water. They were maintained and reared in the laboratory under controlled conditions.

2.3. Extract preparation

The leaves of *G. biloba*, *S. rebaudiana* and *P. hysterophorous* were shade dried (28±2 ℃), ground and sieved to get fine powder from which the extracts were prepared. Ethanol extract of the plant were obtained by taking 10 g of dried leaf powder in a separate container. With this 50 mL of ethanol were added and kept for 1 week with periodic shaking, then filtered and the filtrate was collected. This procedure was repeated three times with fresh volume of ethanol. The filtrates were pooled. Methanol and dichloromethane extract of the plant material was also prepared in a similar manner with that of ethanol. The pooled ethanol methanol and dichloromethane extracts were concentrated separately by rotary vacuum evaporator at 40 ℃ and evaporated to dryness and stored at 4 ℃ in an air-tight bottle[8]. The extract obtained from each plant were dissolved in each solvent independently to get stock solutions of 15 mg/mL for each solvent. From the stock solution different dilution of different concentration were prepared (15 mg/mL, 1.5 mg/mL, 0.15 mg/mL and 0.015 mg/mL). Different test concentrations for larval exposure were prepared by further diluting these stocks. Each beaker was placed in air to evaporate the solvent, after drying beakers were then filled up to 100 mL.

2.4. Larvicidal activity

Only IV instars larvae were selected for the experiments; they were fed with yeast powder, glucose and small amount of straw as medium 1[6], also their growth were studied in natural water of natural habitat and used as medium 2 for growth, these larvae were maintained at controlled conditions of 28±2 ℃ temperature and 70%-80% relative humidity. For each concentration separate beakers are used. In the experiment 10 larvae were exposed to each extract at each concentration in working volume of 100 mL in 250 mL of glass beaker. Three replicates for each concentration and the control (with water) were tested for larval bioefficacy. In the experiment *An. stephensi* larvae under laboratory condition, were subjected to dose dependent efficacy of each extracts of three plants. The larval mortality at different concentrations and in control was recorded after 24 h continuous exposure. A symptom of treated larvae after 24 h was recorded immediately, without food to each larvae. Mortality and survival were registered after 24 h of exposure period; only the dead larvae data was recorded. The dead larvae failed to move and settled down at the bottom while the living one can freely move in the medium.

2.5. Superoxide dismutase activity

The superoxide dismutase activity was determined using the method of Beauchamp[9]. 0.2 g of fresh leaves was taken grinded in 4 mL of phosphate buffer containing 1% PVP (in ice bath). The solution was centrifuged in chilled stage (4 ℃) at 3 000 rpm for 15 min. The supernatant was collected in fresh tube and again centrifuged to get pure enzymes. Three assays were prepared, reaction mixture, blank and reference assay. Reaction mixture was prepared by adding 2 mL of (10 mL solution containing 0.27 g Na_2EDTA + 1.492 1 g methionine + 0.049 g Nitro blue tetrazolium), 0.5 mL of (20 mL

containing 12 mmol riboflavin in buffer pH 7.8) and 0.5 mL of enzyme (supernatant). The reference solution was placed in dark while blank solutions have no enzymes. Spectrophotometer readings were made at 560 nm using the equation:

$SOD = R_4/A$

$A = R_1 (50/100)$

$R_4 = R_3 - R_2$

R_3 = OD of sample

R_2 = OD of blank

R_1 = OD of reference

Where R_1 is absorbance of the reference solution, R_2 absorbance of blank nothing is added, R_3 absorbance of sample when extract has been added at a particular level.

2.6. Peroxidase activity

Peroxidase activity was measured by using the method of Kar[10]. 0.1 mL of enzyme extract was taken. Add 1.35 mL of 100 mmol/L MES buffer (pH 5.5) to the enzyme solution. Then 0.05% of H_2O_2 was added. Finally 0.1% of phenylene diamine was added to the above solution. Changes in absorbance were recorded at 485 nm for 3 min with the spectrophotometer.

Equation:

$$POD = R_I - R_F/T_M$$

Where R_I is initial reading at zero min, R_F is reading after 3 min and T_M is time intervals.

2.7. Ascorbate activity

Ascorbate activity was determined according to the method of Asada[11]. 2 mL of Phosphate buffer (pH 7) and 0.2 mL of 3% H_2O_2 was taken. Then 0.2 mL of 50 μ mol/L ascorbic acid was added. Finally 0.1 mL enzyme extract was added. Reading on spectrophotometer was taken at 290 nm. Two readings were taken at 0 and 3 min.

Equation:

$$ASC = R_I - R_F/T_M$$

Where R_I is initial reading at zero min, R_F is reading after three min and T_M is time intervals.

2.8. Catalase activity

Catalase activity was determined according to the method of Arrigoni O[12]. 0.3 mL of 3% H_2O_2 and 2.5 mL of 0.05 mol/L phosphate buffer (pH 7) was taken. Finally 0.2 mL of enzyme extract was added. Reading on spectrophotometer was taken at 240 nm. Two readings were taken at 0 and 3 min.

Equation:

$$CAT = R_I - R_F/T_M$$

Where R_I is initial reading at 0 min, R_F is reading after 3 min and T_M is time intervals.

3. Results

On exposure of larvae to each concentration, it was observed that the mortality rate was dose dependent, mortality increased with increase in concentration of each extract. The percent mortality values for 4th instar larvae of *A. stephensi* treated with various concentrations (ranging from 0.015 mg/mL to 15 mg/mL) with leaf extracts of *G. biloba*, *S. rebaudiana* and *P. hysterophorous* are presented in the current experiment. LC_{50}, *Chi* square test, their lower confidence limits and upper confidence limits of the leaf extract for 24 h exposure of *A. stephensi* are given in Table 1. Abbott's formula was used for mortality. LC_{50} confidence interval, upper confidence interval, lower confidence interval and *Chi* square test were analyzed by means of computerized probit analysis program[13].

The ethanolic extracts of leaves of *G. biloba*, *S. rebaudiana* and *P. hysterophorous* showed 65 to 90% results at 15 mg/mL. The best larvicidal activity was shown by *S. rebaudiana* and *P. hysterophorous* ranged from 83% to 90% in ethanolic extract while *G. biloba* showed 65% results (Figures 1-2). The larvicidal activity was dose dependant, as the extract concentration was reduced to 0.015 mg/mL. The activity was recorded as 11% to 36% by *G. biloba*, *S. reboudiana* and *P. hysterophorus*. Mortality was checked in two different media, one medium contain yeast powder, glucose and small quantity of straw (M1) and second media was normal pond water from which they are collected (M2). In both media the results recorded were similar and no significant difference was found[14]. Find *in vitro* activity of 2-methoxy-1,4-naphthoquinone and stigmasta-7,22-diene-3b-ol from

Table 1

Larvicidal activity of three plant extracts in terms of LC_{50} (mg/mL) against *An. stephensi*.

Species	Extract	Growth medium 1				Growth medium 2			
		LC_{50} (mg/mL)	Upper confidence limit(mg/mL)	Lower confidence limit(mg/mL)	χ^2	LC_{50} (mg/mL)	Upper confidence limit(mg/mL)	Lower confidence limit(mg/mL)	χ^2
P. hysterophorous	Ethanol	0.292 26	0.745 09	0.103 05	0.417	0.214 26	0.490 24	0.082 82	1.115
	Methanol	0.185 76	0.508 77	0.051 57	0.183	0.171 88	0.409 87	0.059 56	1.269
	DCM	1.093 90	2.353 04	0.545 46	0.258	1.264 40	2.666 79	0.640 59	0.325
S. rebaudiana	Ethanol	0.196 42	0.385 70	0.094 74	0.029	0.218 09	0.473 19	0.091 20	0.689
	Methanol	0.144 55	0.345 05	0.048 27	2.106	0.098 91	0.221 77	0.034 67	3.786
	DCM	2.418 80	5.225 95	1.268 16	2.816	3.682 10	9.739 19	1.786 60	3.628
G. biloba	Ethanol	2.846 50	6.966 25	1.399 35	5.680	2.034 40	6.454 26	0.851 16	6.683
	Methanol	0.761 52	1.869 98	0.032 54	2.598	0.965 06	1.930 83	0.427 56	11.550
	DCM	1.794 70	3.536 14	0.969 26	0.263	1.112 90	2.179 21	0.589 54	0.304

Impatiens balsamina L. *against* multiple antibiotic-resistant Helicobacter pylori in similar way. In methanolic extracts the overall results recorded at higher concentration ranged from 71% to 87%. The best activity was recorded for *S. rebaudiana* 82% to 87%, and *P. hysterophorous* 80% to 85%, while *G. biloba* had less activity *i.e.*, 71%-78% at 15 mg/mL as shown in (Figures 3-4). At lower concentration 0.015 mg/mL the result was similar for all three plants ranging from 23% to 31%. The dichloromethane extracts comparatively showed lower larvicidal activity at higher concentration. *G. biloba* had 66% to 70% activity, similar activity was also shown by *P. hysterophorous* 65% to 70%, while *S. rebaudiana* having 61% to 63% activity as shown in (Figures 5-6). Same activities of other plants were also found by many researchers[15-16].

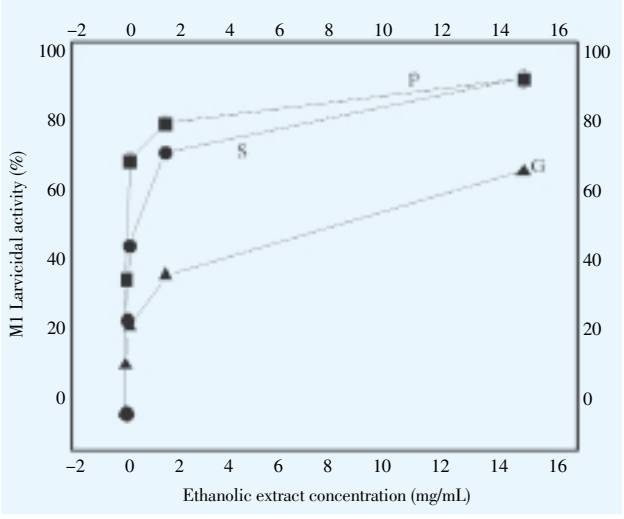

Figure 1. Comparative larvicidal potential in medium 1 in ethanolic extract of *P. hysterophorous* (■), *S. rebaudiana* (●) and *G. biloba* (▲) against *An. stephensi* with highest activity of *P. hysterophorous* followed by *S. rebaudiana* and *G. biloba* in terms of percentage.

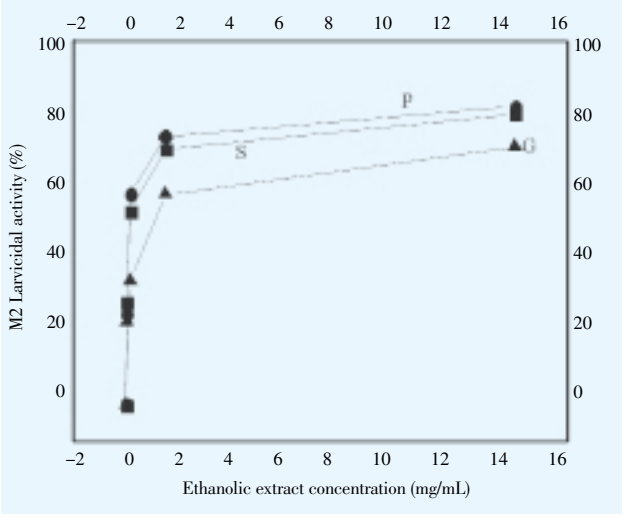

Figure 2. Comparative Larvicidal potential in medium 2 in ethanolic extract of *P. hysterophorous* (●) , *S. rebaudiana* (■) and *G. biloba* (▲) against *An. stephensi* with highest activity of *P. hysterophorous* followed by *S. rebaudiana* and *G. biloba* in terms of percente.

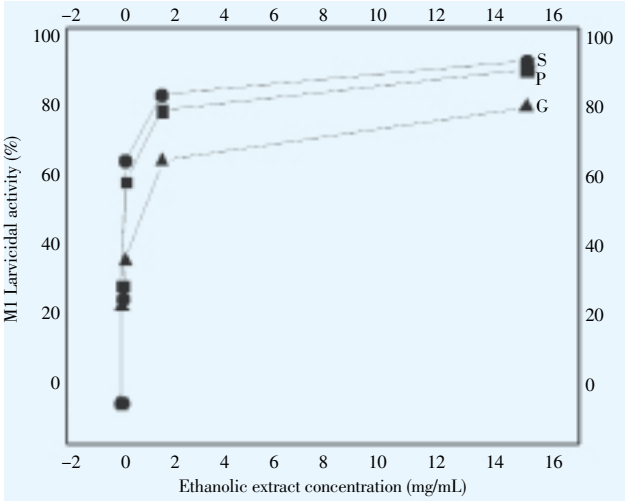

Figure 3. Comparative Larvicidal potential in medium 1 in methanolic extract of *P. hysterophorous* (■), *S. rebaudiana* (●) and *G. biloba* (▲) against *A. stephensi* with highest activity of *S. rebaudiana* followed by *P. hysterophorous* and *G. biloba* in terms of percentage.

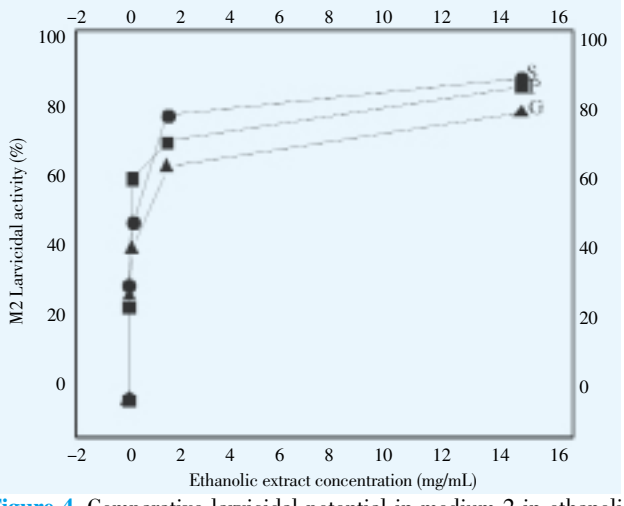

Figure 4. Comparative larvicidal potential in medium 2 in ethanolic extract of *P. hysterophorous* (■), *S. rebaudiana* (●) and *G. biloba* (▲) against *An. stephensi* with highest activity of *S. rebaudiana* followed by *P. hysterophorous* and *G. biloba* in terms of percentage.

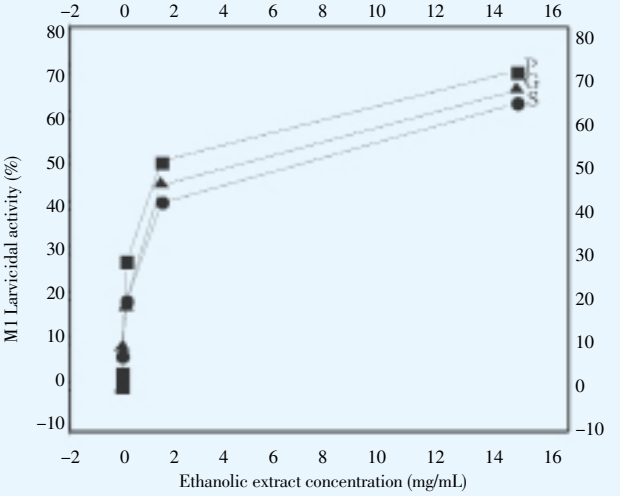

Figure 5. Comparative larvicidal potential in medium 1 in ethanolic extract of *P. hysterophorous* (■), *S. rebaudiana* (●) and *G. biloba* (▲) against *An. stephensi* with highest activity of *P. hysterophorous* followed by *G. biloba* and *S. rebaudiana* in terms of percentage.

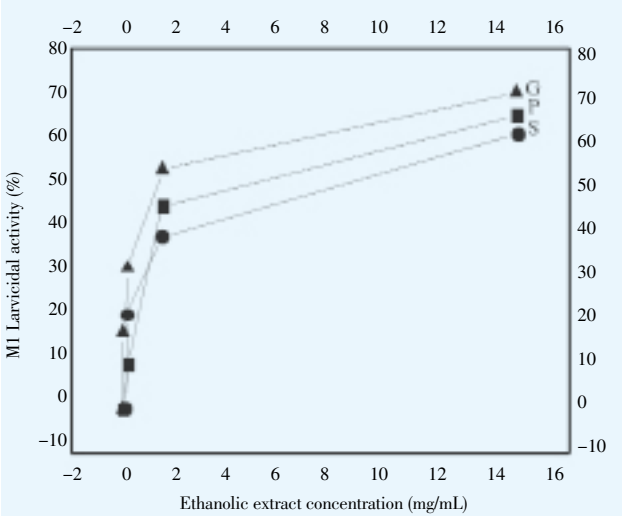

Figure 6. Comparative larvicidal potential in medium 2 in ethanolic extract of *P. hysterophorous* (■) , *S. rebaudiana* (●) and *G. biloba* (▲) against *An. stephensi* with highest activity of *G. biloba* followed by *P. hysterophorous* and *S. rebaudiana* in terms of percentage.

Larvicidal activity was also reported as LC_{50} representing the concentrations in mg/mL that killed 50% of larvae in 24 h respectively. The susceptibility level of *A. stephensi* larvae to the different extracts of three plants was determined. From the results, it appears that ethanolic, methanolic and dichloromethane extracts of the three plant leaves exhibit high activity against *A. stephensi* as shown in Table 1. Estimated LC_{50} for methanolic extracts of leaves of *S. rebaudiana* were 0.098 and 0.144 while in ethanolic extract the results of LC_{50} was 0.144 and 0.218 mg/mL, respectively (Table 1). From the experiment it was concluded that the maximum activity was observed in the case of *S. rebadiana* followed by *P. hysterophorous* which was recorded 0.171 and 0.185 for methanolic extracts, however, the ethanolic extracts was also having activity of 0.214 and 0.292 respectively. In case of *G. biloba* 50% mortality was comparatively lower than other two plants. Different activities of plant extracts were also found[7,17-20].

The second objective of the current study was to compare the activities of antioxidative enzymes. A different trend in the activity of antioxidant enzymes was observed in these three plant species. The changes in antioxidant enzymes activities are directly related to the plants secondary metabolites. As compared to the larvicidal activity the antioxidative enzymes activities showed different results. The best antioxidant activity was observed in the case of *G. biloba*. *G. biloba* showed maximum activity of POD, CAT and ASC than other two plant species, while *S. rebaudiana* showed maximum SOD activity than other plant species as shown in (Figures 7-10). Comparatively *S. rebaudiana* and *P. hysterophorous* showed maximum results against *A. stephensi*, *G. biloba* showed greater antioxidant enzymes activities. Different plants antioxidant enzymes activities were determined by different scientists[3,21-26].

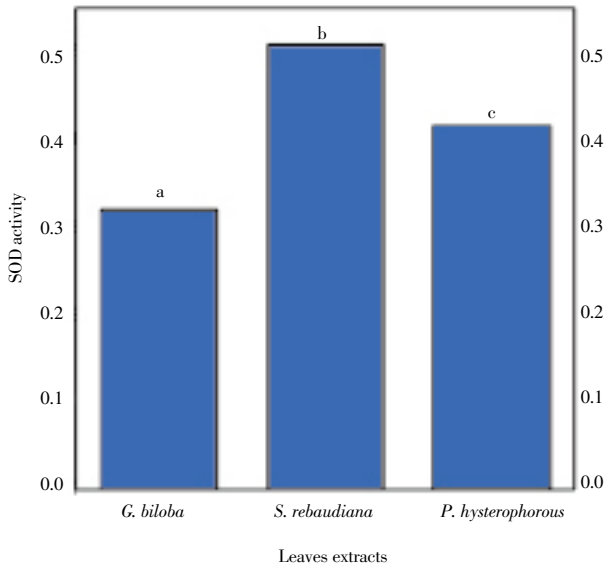

Figure 7. Comparative Superoxide dismutase activity of enzyme extracts of *P. hysterophorous*, *S. rebaudiana* and *G. biloba*, with highest SOD activity of *S. rebaudiana*.

(a) followed by *P. hysterophorous* (b) and *G. biloba* (c). Mean values in each column with common letters are significantly different at $P<0.05$.

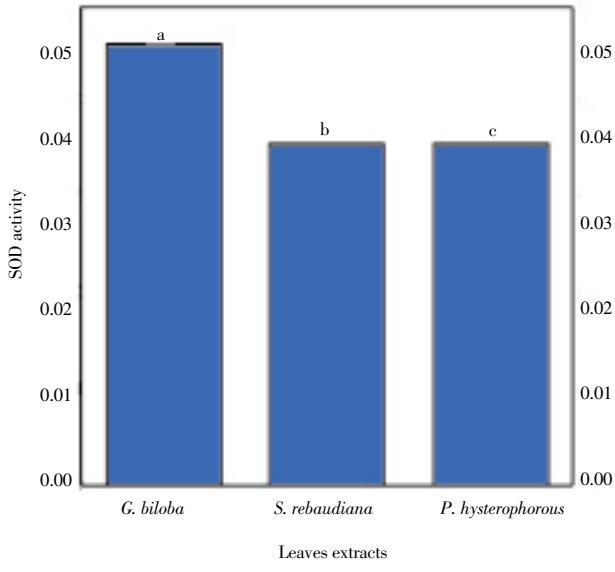

Figure 8. Comparative peroxidase activity of enzyme extracts of *P. hysterophorous*, *S. rebaudiana* and *G. biloba*, with highest POD activity of *G. biloba*.

(a) followed by *P. hysterophorous* (b) and *S. rebaudiana* (c). Mean values in each column with common letters are significantly different at $P<0.05$.

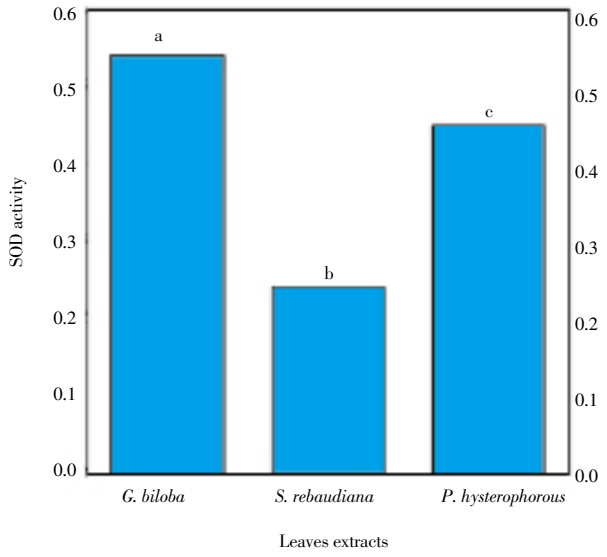

Figure 9. Comparative ascorbate activity of enzyme extracts of *P. hysterophorous*, *S. rebaudiana* and *G. biloba*, with highest Ascorbate activity of *G. biloba*.
(a) followed by *P. hysterophorous* (b) and *S. rebaudiana* (c). Mean values in each column with common letters are significantly different at $P<0.05$.

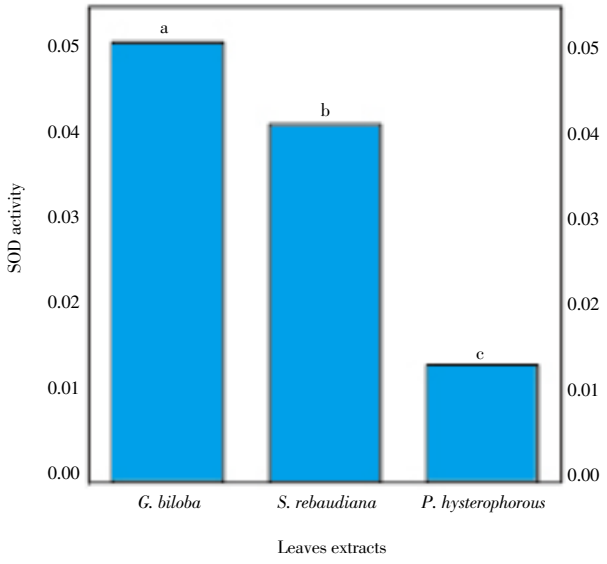

Figure 10. Comparative catalase activity of enzyme extracts of *P. hysterophorous*, *S. rebaudiana* and *G. biloba*, with highest catalase activity of *G. biloba*.
(a) followed by *S. rebaudiana* (b) and *P. hysterophorous* (c). Mean values in each column with common letters are significantly different at $P<0.05$.

4. Discussion

G. biloba contains a particular class of diterpenes and sesquiterpenes, called ginkgolides and bilobalide. Some of the compounds are currently being studied for the treatment of allergic diseases, blood disturbances, and neoplastic and immunological disorders[27-28]. Although recent studies of many plant-derived terpenes have showed that compounds belonging to this class can display antiparasitic, antifungal, antibacterial, antiviral, or immunomodulating activities[28]. One of the potent members of the Asteraceae family is *S. rebaudiana* (commonly referred to as honey leaf, candy leaf and sweet leaf). It is rich in terpenes and flavanoids. The phytochemicals present in *S. rebaudiana* are austroinullin, β -carotene, dulcoside, nilacin, rebaudi oxides, riboflavin, steviol, stevioside and tiamin. *S. rebaudiana* has important industrial uses in beverages, energizers as well as medicinal uses such as low uric acid treatment, vasodilator cardiotonic, anesthetic and anti-inflammatory[29-30]. Antibacterial activity of a large number of growth toxins present in leaf and root of *P. hysterophorouswas* reported[31]. Leaf or inflorescence of *P. hysterophorus* were either algistatic or algicidal to *Chlorella vulgaris* and *Synechococcus elongatus*[32].

In the above literature these three plants containing such secondary metabolites that was used for different uses and diseases like allergic, blood disturbances, immunomodulating, antiparasitic, antifungal, antibacterial, antiviral, neoplastic, immunological disorders, low uric acid treatment, vasodilator cardiotonic, anesthetic, anti-inflammatory algistatic and algicidal. In the experiment it was investigated that these three plants having larvicidal activity and can be further used for vector control alternative to synthetic insecticide due to eco-friendly and diseases control.

It was observed from the results that these plant extracts produced high larval mortality against *An. stephensi*. Out of the nine extracts tested, the maximum activity was observed at higher concentration of the extracts. Among these extracts, the most promising ones are the methanolic extracts of *S. rebaudiana* and *P. hysterophorous* (LC_{50}=0.098, 0.144, 0.171 and 0.185 mg/mL) and *G. biloba* (LC_{50}=0.214 and 0.292 mg/mL). The findings of many researchers are an agreement with our data[5,7,20,33-35]. From the current experiment it was concluded that the best solvent was methanol and ethanol for resolving the larvicidal components, however, dichloromethane was poor solvent comparatively. The best results were obtained in the order of *S. rebaudiana*, *P. hysterophorous* and *G. biloba* at 15 mg/mL the percentages of mortality recorded were 90%, 90% and 65% respectively. In last the plants examined in this study offer great potential as new control agents against *An. stephensi*. Furthermore, *P. hysterophorous* grows wild in uncultivated and cultivated zones in Pakistan and other countries, its leaves which are available throughout the year could be easily collected without any additional cost. Therefore, leaves extracts could be used as a larvicidal agent in an integrated vector control program. Separation of the active principles, research into their mode of action, effect on non target organisms and field evaluation are presently under investigation. It should be also noticed that these three plant species have a potent antioxidative enzymes activities, therefore, could be selected for further studies particularly diseases which was caused due to free radicals.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Kumar SM, Maneemegalai S. Evaluation of larvicidal effect of *Lantana camara* Linn against mosquito species *Aedes aegypti* and *Culex quinquefasciatu* . *Adv Biol Res* 2009; **2**: 39-43.

[2] Mittal PK, Subbarao SK. Prospects of using herbal products in the control of mosquito vectors. *Indian Counc Med Res ICMR Bull* 2003; **33**(1): 1-10.

[3] Kumar BSA, Lakshman K, Jayaveera KN, Shekar DS, Kumar AA, Manoj B. Antioxidant and antipyretic properties of methanolic extract of *Amaranthus spinosus* leaves. *Asian Pac J Trop Med* 2010; **3**(9): 702-706.

[4] Tyagi BK. Future of phyto chemicals against vector mosquitoes. *J Appl Zool Res* 2003; **14**: 201-206.

[5] Agan TU, Ekabua JE, Iklaki CU, Oyo-Ita A, Ibanga I. Prevalence of asymptomatic malaria parasitaemia. *Asian Pac J Trop Med* 2010; **3**(1): 51-54.

[6] Rajmohan D, Ramaswamy M. Evaluation of larvicidal activity of the leaf extract of a weed plant, *Ageratina adenophora*, against two important species of mosquitoes, *Aedes aegypti* and *Culex quinquefasciatus*. *Afr J Biotech* 2007; **5**: 631-638.

[7] Medhi SM, Reza SA, Mahnaz K, Reza AM, Abbas H, Fatemeh M, et al. Photochemistry and larvicidal activity of *Eucalyptus camaldulensis* against malaria vector, *Anopheles stephensi*. *Asian Pac J Trop Med* 2010; **3**(11): 841-845.

[8] Benson EE. Do free radicals have a role in plant tissue culture recalcitrance? *In Vitro Cell Dev Biol Plant* 2000; **36**: 163-170.

[9] Beuchamp C Fridovich. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 1971; **44**: 276-287.

[10]Kar M, Mishra D. Catalase, peroxidase and polyphenol oxidase activities during rice leaf senescence. *Plant Physiol* 1976; **57**: 315-319.

[11]Asada K. Chloroplasts: formation of active oxygen and its ascavenging. *Methods Enzymol* 1984; **105**: 422-429.

[12]Arrigoni O, Gara LD, Tommasi F, Liso R. Changes in the ascorbate system during seed development of *Vicia faba* L. *Plant Physiol* 1992; **99**: 235-238.

[13]Finney DJ. *Probit analysis: a statistical treatment of sigmoid response curve*. 3rd ed. Cambridge: Cambridge University Press; 1971, p. 333.

[14]Wang YC, Li WY, Wu DC, Wang JJ, Wu CH, Liao JJ, et al. *In vitro activity* of 2-methoxy-1,4-naphthoquinone and stigmasta-7,22-diene-3b-ol from *Impatiens balsamina* L. against multiple antibiotic-resistant *Helicobacter pylori*. *eCAM* 2009; **147**: 1-8.

[15]Ng LT, Wu SJ. Antiproliferative activity of *Cinnamomum cassia* constituents and effects of pifithrin-alpha on their apoptotic signaling pathways in Hep G2 cells. *eCAM*, 2009; **220**: 1-6.

[16]Krisanapun C, Lee SH, Peungvicha P, Temsiririrkkul R, Baek SJ. Antidiabetic activities of *Abutilon indicum* (L.) sweet are mediated by enhancement of adipocyte differentiation and activation of the GLUT1 promoter. *eCAM* 2010; **4**: 1-9.

[17]Tangpukdee1 N, Wai1 KM, Muangnoicharoen S, Kano S, Phophak N, Tiemprasert J, et al. Indicators of fatal outcome in severe *Plasmodium falciparum* malaria: a study in a tertiary-care hospital in Thailand. *Asian Pac J Trop Med* 2010; **3**(11): 855-859.

[18]Goel A, Kumar S, Kumar AK. Effect of *Ocimum sanctum* on the development of protective immunity against *Salmonella typhimurium* infection through cytokines. *Asian Pac J Trol Med* 2010; **3**(9): 682-686.

[19]Adiga S, Trivedi P, V Ravichandra, Deb D, Mehta F. Effect of *Punica granatum* peel extract on learning and memory in rats. Asian *Pac J Trop Med* 2010; **3**(9): 687-690.

[20]Govindarajan M. Larvicidal and repellent activities of *Sida acuta* Burm. F. (Family: Malvaceae) against three important vector mosquitoes. *Asian Pac J Trol Med* 2010; **3**(9): 691-695.

[21]Gupta SD, Datta S. Antioxidant enzymes activities during *in vitro* morphogenesis of gladiolus and the effect of application of antioxidants on plant regeneration. *Biologia Plantarum*, 2003; **47**: 179-183.

[22]Meratan AA, Ghafari SM, Niknam V. *In vitro* organogenesis and antioxidant enzymes activity in *Acanthophyllum sordidum*. *Biologia plantarum* 2009; **53**: 5-10.

[23]Newton RJ, Tang W. Peroxidase and catalase activities are involved in direct adventitious shoot formation induced by thidiazuron in eastern white pine zygotic embryos. *Plant physiol Biochem* 2005; **43**: 760-769.

[24]Chen J, Ziv M. The effect of ancymidol on hyperhydricity, regeneration, starch and antioxidant enzymatic activities in liquid-cultured *Narcissus*. *Plant cell rep* 2001; **20**: 22-27.

[25]Tian M, Gu Q, Zhu M. The involvement of hydrogen peroxide and antioxidant enzymes in the process of shoot organogenesis of strawberry callus. *Plant Sci* 2003; **165**: 701-707.

[26]Ahmad N, Fazal H, Abbasi BH, Farooq S. Efficient free radical scavenging activity of Ginkgo biloba, Stevia rebaudiana and Parthenium hysterophorous leaves through DPPH. *Int J Phytomed* 2010; **2**: 231-239.

[27]Oh SM, Chung KH. Estrogenic activities of *Ginkgo biloba* extracts. *Life Sci* 2003; **3**(2): 99-104.

[28]Lehotský J, Kaplan P, Pavlikova M, Urban P, Saniova B. Biological activity of ginkgo. In: Ramawat KG. *Herbal drugs: ethnomedicine to modern medicine*. Heidellberg: Springer; 2009, p. 321-331.

[29]Ibrahim NA, El-Gengaihi S, Motawe H, Riad SA. Ph ytochemical and biological investigation of *Stevia rebaudiana* Bertoni; 1-labdane-type diterpene. *Chem Mater Sci* 2010; **244**(4):483-488.

[30]Abou-Arab EA, Abu-Salem FM. Evaluation of bioactive compounds of *Stevia rebaudiana* leaves and callus. *Afr J Food Sci* 2010; **4**(10): 627-634.

[31]Jayaraman S, Manoharan MS, Illanchezian S. *In-vitro* antimicrobial and antitumor activities of *Stevia Rebaudiana* (Asteraceae) leaf extracts, *Trop J Pharm Res* 2008; **7**(4): 1143-1149.

[32]Maurya S, Kushwaha VB. Effect of ethanolic extract of *Parthenium hysterophorous* on haematological parameters ion rat. *The bioscan* 2010; **5**(3): 437-440.

[33]Mandal S. Exploration of larvicidal and adult emergence inhibition activities of *Ricinus communis* seed extract against three potential mosquito vectors in Kolkata, India. *Asian Pac J Trop Med* 2010; **3**(8):605-609

[34]Hasasan V, Hossein ZA. Responsiveness of *Anopheles maculipennis* to different imagicides during resurgent malaria. *Asian Pac J Trop Med* 2010; **3**(5): 360-363.

[35]Zahir AA, Rahuman AA, Ba-gavan A, Elango G, Kamaraj C. Adult emergence inhibition and adulticidal activities of medicinal plant extracts against *Anopheles stephensi* Liston. *Asian Pac J Trop Med* 2010; **3**(11): 878-883.