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In vitro antifilarial activity, antioxidant potential and phenolic constituents of *Quisqualis indica* L.

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Quisqualis indica L., commonly known as '*Rangoon-ki-bel*' or '*Madhumalti*', has been used by the traditional healers as it is active against some of the commonly occurring diseases like boils, fevers diarrhea and helminthiasis. However, no systematic and scientifically validated studies on antifilarial activity of *Q. indica* are available. In the present study, we report *in vitro* antifilarial activity of ethanolic and hydroethanolic extracts of the leaves (QILE and QILEW) and flowers (QIFE and QIFEW) of this plant on microfilariae (mf) and female adult worms of human lymphatic filariid *Brugia malayi* using motility and or 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT)-reduction assays. The hydroalcoholic extract of flowers (QIFEW) was found effective as it killed adult female worms ($LC_{100:}$ 62.5 µg/mL) and mf (LC_{100}: 125 µg/mL); IC_{50} values for the respective parasite stages were 34.50 and 31.88 µg/mL. SI values recorded with respect to motility of female parasite and mf was more than 20. The active principle(s) responsible for antifilarial activity may thus be present in QIFEW. The antioxidant activity results also indicated QIFEW to possess better antioxidant potential than the other extracts studied. HPLC analysis showed that the 02 keyphenolics present in hydroalcoholic extract of the flowers (QIFEW) were gallic acid and ellagic acid. In the different extracts, the concentration of gallic acid was found to vary from 26.9 mg/g to 2.50 mg/g while ellagic acid ranged between 11.5 mg/g to 6.77 mg/g. It was also observed that the leaves were rich in flavonoids whereas the flowers were rich in phenolics. The findings indicate that active molecule (s) of hydroalcoholic extractfrom *Q. indica* flowers may help in providing new leads for developing antifilarial agents. We believe that this is the first systematically studied report on the *in vitro* antifilarial activity of the hydroalcoholic extract of *Q. indica* flowers.

Keywords: Antifilarial, Antioxidant, *Brugia malayi*, HPLC, *In vitro* assays, Phenolics, *Quisqualis indica*

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Lymphatic filariasis (LF), commonly known as elephantiasis, is a neglected tropical disease. The *malayi* and *B. timori*. It is a painful and profoundly disfiguring disease, causing the body parts to enlarge abnormally. It leads to physical disabilities and the person infected with this disease becomes a social outcast. Filariasis damages the lymphatic system. India, Indonesia, Nigeria and Bangladesh contribute about 70% of the infection worldwide. LF causes a variety of clinical manifestations, including lymphoedema, hydrocele and swelling of the scrotum. The vast majority of infected people from endemic areas are asymptomatic microfilaremics and do not display any clinical manifestations even during their [illegible] are 52 countries with a population of 890 million that require preventive methods for stopping the spreading of this disease. According to WHO, in the year 2000 the number of people found infected was more than 120 million including nearly 40 million who were disfigured and incapacitated[1]. Current treatment is based on combination of a broad spectrum anthelmintic albendazole (ALB) and diethylcarbamazine (DEC)/ivermectin (IVM), or DEC fortified with cooking salt. DEC and IVM principally act on microfilariae (mf; a larval stage of the parasite) and do not exert any appreciable effect on adult parasites[2].

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Although treatment with mass drug administration (MDA) is considerably reducing the incidence of the infection and even eliminating it in some countries[3], unfortunately there are reports of reemergence of infectionin some areas especially in Srilanka[4]. Also, the existing drugs are known to produce adverse effects in treated subjects[5,6]. Therefore, new antifilarials are required to act as macrofilaricidal agents as well as to replace current drugs threatened with development of IVM resistance, as reported in onchocerciasis patients[7].

In recent years, a lot of emphasis is being given to the development of antifilarial agents from plant products. Antifilarials from plants have great potential to be used as medicine because they are generally safer and have lesser side effects than which are often associated with synthetic drugs[8]. India is rich in medicinal plants. In our drug development programme, CSIR Network project NWP-0037, continuous efforts have been made to discover anti-filarial agents through research on medicinal plants[2,9,10]. *Quisqualis indica* Linn. Family: Combretaceae, was one of the plants listed in that project and was selected for detailed studies. *Q. indica* (syn. *Combretum indicum* (L.) De Filipps, popularly called Rangoon-ki-bel or Madhumalti, is a woody climber having vigorous growth that needs sturdy support and can grow up to 8 m tall. Its roots are not very strong nor do they go deep inside the soil. The leaves are arranged in opposite pairs along the stem and are elliptic to elliptic-oblong in shape and measure about 5-18 cm by 2.5-7 cm. The fragrant flowers are borne on a pendant raceme and are white in color, the upper surface of the flower turns pink then red over a period of time. The fruit is a red drupe, turning dark brown when mature. *Q. indica* is an ornamental plant found growing in several countries. Investigations have lead to the isolation and identification of several phytoconstituents such as pelargonidin-3-glucoside, trigonelline, L-proline, L-asparagine, quisqualic acid, rutin and isoenzyme A and isoenzyme B and fixed oil. *Quisqualis* fruits are used for roundworm infection in Traditional Chinese Medicine (TCM)[11]. Preparations from different parts of this plant are used for the treatment of diarrhea, nephritis, rheumatism, headache, boils, ulcers, inflammation, and as anthelmintic[12,13].

The aim of the present study was to investigate the *in vitro* antifilarial activity of flowers and leaves of *Q. indica*, which have been reported to have anthelmintic properties, and evaluate their antioxidant activity in order to evaluate their utility in providing relief from the oxidative stress that is generated as a result of different physiological changes taking place in the body of the patient with lymphatic filariasis. Since it is well known that polyphenols are good antioxidants, different samples were also subjected to HPLC-PDA analysis in order todetermine the different polyphenols present in them.

Materials and methods

Chemicals and reagents

Standards (Ellagic acid, Gallic acid, Protocatechuic acid and Rutin) as well as DPPH, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide, Hanks Balanced Salt Solution (HBSS), Diethylcarbamazine-citrate and Ivermectin were obtained from Sigma-Aldrich. HPLC-grade solvents and other chemicals were procured from Merck Chemicals.

Plant material

The plant *Q. indica* L. (Specimen number NBR/PH/227365) was collected from Lucknow, Uttar Pradesh (India), authenticated and herbarium deposited in the division.

Extraction and sample preparation

The leaves (QIL) and flowers (QIF) (100 g each) of *Q. indica* were dried, coarsely grinded, separately, and both extracted with ethanol and 50% aqueous ethanol (200 mL x 3) at room temperature (25°C) for 24 h on a shaker (60 rpm). The combined extracts were concentrated in a rotatory evaporator (Buchi, Switzerland) under reduced pressure at 45°C and lyophilized to yield dried ethanolic (QILE and QIFE) and 50% hydro alcoholic (QILEW and QIFEW) extracts. Their percentage yields were QILE-3.12%, QILEW- 10.23%, QIFE- 7.30% and QIFEW-12.61%. These extracts were used for evaluation of *in vitro* antifilarial and antioxidant activities as well as for HPLC analysis and determination of total phenolic and total flavonoid contents.

In vitro evaluation of antifilarial activity

For *in vitro* evaluation of antifilarial activity, the maintenance of the animals and strains as well as the isolation of parasites was done according to the methods reported earlier[10,14,15]. Motility and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assayswere carried out for assessing the antifilarial activity[15,16]. The solutions of test samples as well as IVM were prepared in

dimethylsulphoxide (DMSO) while DEC-c was prepared in sterile distilled water. IC_{50} and CC_{50} values were also determined and computed[10,17,18].

Antioxidant assays

Total antioxidant capacity (TAC) by phosphomolybdate antioxidant assay- The phosphomolybdenum method[19] was used to determine the total antioxidant capacity of all the different samples. In this method, the sample under analysis reduces Mo(VI) to Mo(V) with the formation of green compounds, the absorbance of which was measured spectrophotometrically at a wavelength of 695 nm. Ascorbic acid was used as the standard and the results were expressed as mmol ascorbic acid per gram (mmol AA/g) extract.

Ferric reducing antioxidant power (FRAP) assay- The method described by Oyaizu was used to determine the FRAP of all the different samples, but with slight modifications[20,21]. In this method, the sample under analysis reduces potassium ferricyanide (Fe^{3+}) to potassium ferrocyanide (Fe^{2+}). This potassium ferrocyanide (Fe^{2+}) subsequently forms a Pearl's Prussian blue ferric-ferrous complex with ferric chloride. The absorbance is measured at 700 nm using a spectrophotometer which is an indicator of the amount of Fe^{3+} ions present.

Estimation of Total Phenolic content (TPC) and Flavonoid content (TFC)

Folin-Ciocalteu method[22] was used for determining TPC of all the different samples. Different concentrations of gallic acid were used for preparing the standard curve. The total phenolic content of the samples studied was calculated andexpressed as mg GAE g^{-1} of extract. Colorimetric method using aluminium chloride[19] was used for quantification of TFC of the different samples. Quercetin was used at different concentrations for preparing the standard curve which was used to calculate the total flavonoid content of the samples studied, expressed as mg QE g^{-1} of extract.

HPLC analysis for Phenolics and Flavonoids

HPLC analysis was carried out on Waters HPLC system using the same conditions as described earlier by us[23]. The column used for separation was Polaris 5 C18-A (250×4.6 mm i.d.; 5 µm particle size) and PDA at 280 nm was used for detection. Samples of a concentration of 15 mg/mL were used and the chromatograms were visualized under 3 different wavelengths viz. 254, 270 and 350 nm considering the different compounds that may be present. Comparison of the retention times and spectra of the peaks obtained with the known standards was used for identifying the peaks in the chromatograms. Gallic acid, protocatechuic acid, ellagic acid and rutin (0.1 mg/mL) were used as reference standards.

Results

***In vitro* antifilarial activity**

The antifilarial activity of the different ethanolic and hydroethanolic samples was determined *in vitro*. 62.5 µg/mL was set as the cutoff concentration for positive antifilarial activity 'hit' of natural products *in vitro* and 500 µg/mL as the limit concentration for testing. None of the ethanolic extracts (QILE and QIFE) showed any adulticidal or microfilaricidal activity up to the acceptable concentration of 62.5 µg/mL; they showed no activity even up to 500 µg/mL. Results of *in vitro* activity of the hydroalcoholic extract of leaves (QILEW) and flowers (QIFEW) of the plant on adult worms and mf of *Brugia malayi,* evaluated by motility and MTT reduction assays, are shown in Table 1. The hydroalcoholic extract of flowers (QIFEW) killed the female adult worms (LC_{100}: 62.5 µg/mL) and mf (LC_{100}: 125 µg/mL). The extract produced remarkable inhibition (>90%) in MTT reduction potential of the adult worms. IC_{50} values for the respective parasite stages were found to be 34.50 and 31.88 µg/mL. SI values of the extract recorded with respect to motility of female adult worm and mf were 22.89 and 24.78, respectively. QILEW required 250 µg/mL or more than 250 µg/mL concentration to kill the adult worms and failed to affect MTT-reduction potential of the female parasites. Thus, *in vitro* activity of QILEW was not encouraging and it was, therefore, not taken up for further studies.

DEC-c killed the adult worms and mf at 800 µM (LC_{100}) and 500 µM (LC_{100}), respectively: the IC_{50} values of the drug for respective parasite stages were 289.00 µM and 354 µM. IVM required much lower concentrations to kill both adult worms (LC_{100}: 5 µM) and mf (LC_{100}: 2.5 µM) and IC_{50} values were found to be 3.05 and 1.57 µM, respectively. SI values of the reference drugs with respect to motility of the parasites were >25–159.

In summary, only the hydroalcoholic extract of flowers (QIFEW) of the plant remarkably affected viability of female adult worms and mf *in vitro.* SI values of the extract were 23 and 25 with respect to adult worm and mf, respectively.

Table 1 — *In vitro* efficacy of *Quisqualis indica*and the reference drugs ivermectin and DEC citrate on adult worms (AW) and microfilariae (Mf) of *Brugia malayi* evaluated by motility assay (MA) and MTT reduction assay.

Antifilarial agent (Extract)	Macrofilaricidal				Microfilaricidal			CC_{50}
	LC_{100}[a] (µg/mL) for AW in MA	IC_{50}[b] (µg/mL) for AW in MA	Mean % inhibition in MTT reduction by AW	SI in MA	LC_{100} (µg/mL) for Mf in MA	IC_{50} (µg/mL) for Mf in MA	SI in MA	
QIFEW	62.5	34.50	94.52	22.89	125	31.88	24.78	790
QILEW	250	ND	NI	ND	>250	ND	ND	ND
DEC-c*	800	289	64	31.50	500	354	25.71	9103
IVM*	5	3.05	5.80	81.97	2.50	1.57	159.23	250

Control**:** Motility score of parasite+DMSO only wells= 4 (=parasites were motile with highly active); MTT absorbance values (OD_{510nm}) of parasites+DMSO only= 0.64; [a]LC_{100}= Lethal concentration of the test agent which causes 100% irreversible immobility (death) in *B. malayi* adult and mf. [b]IC_{50}= The concentration of the agent at which 50% inhibition in motility of the parasites is achieved; [c]CC_{50}= concentration at which 50% of cells are killed; SI= Selectivity Index (CC_{50}/IC_{50}); DEC-c= Diethylcarbamazine-citrate; IVM= Ivermectin; *µM; Experiments were repeated twice.
NI= No inhibition; ND= Not done.

Table 2 — TPC, TFC and antioxidant activities of leaves and flowers of *Q. indica*

Sample	Total phenolic content* (mg GAE/g)	Total flavonoid content* (mg QE/g)	Total Antioxidant Capacity by Phosphomolybdate Antioxidant Assay (mmol AA/g extract)
QILE	11.49	5.21	0.539
QILEW	10.81	3.75	0.437
QIFE	11.08	2.63	0.715
QIFEW	16.86	1.02	0.732
AA	-	-	-

*Values are means of three determinations each

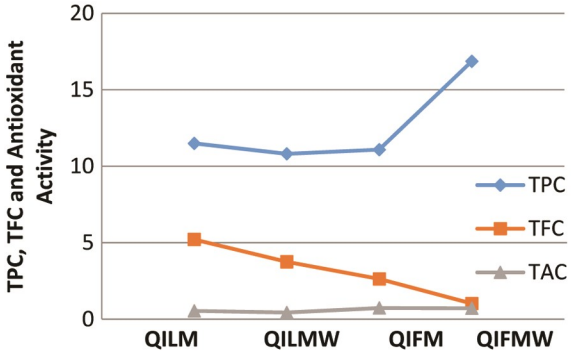

Fig. 1 — TPC, TFC, and TAC (mmol AA/g extract) of methanolic (M) and 50% hydroalcoholic (MW) extracts of different parts of *Q. indica.* (QIL: *Q. indica* leaves; QIF: *Q. indica* flowers)

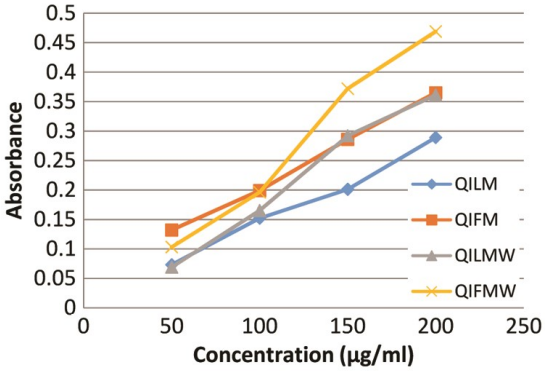

Fig. 2 — Ferric reducing anti-oxidant power of the methanolic (M) and 50% hydroalcoholic (MW) extracts of different parts of *Q. indica.* (QIL: *Q. indica* leaves; QIF: *Q. indica* flowers)

***In vitro* antioxidant activity**

Total antioxidant capacity by phosphomolybdate antioxidant assay– The total antioxidant capacity of the ethanolic and 50% hydroalcoholic extracts of QIL and QIF are given in Table 2 & Fig. 1. The order of total antioxidant capacity was QIFEW>QIFE> QILE>QILEW. It was observed that there was a direct correlation between the phosphomolybdenum complex formed and the amount of the extracts used.

Ferric reducing anti-oxidant power (FRAP) assay-The reducing power of the ethanolic and 50% hydroalcoholic extracts of QIL and QIF was measured using Fe^{3+} to Fe^{2+} reduction assay. The results obtained are shown in Fig. 2. All the samples exhibited reducing power which had a direct correlation with increasing concentration of the samples. However ascorbic acid exhibited a higher reducing power.

Total phenolic and flavonoid contents

TPC and TFC of the ethanolic and 50% hydroalcoholic extracts of QIL and QIF were determined (Table 2, Fig. 1). QIFEW showed the highest TPC with 16.86 mg GAE/g extract while QILE showing the highest TFC with 5.21 mg QE/g extract. The TPC and TFC of the samples were in the order QIFEW>QILE>QIFE>QILEW and QILE> QILEW>QIFE>QIFEW respectively.

HPLC analysis

The ethanol and the ethanol-water extracts obtained from leaves and flowers of *Q. indica* were subjected to HPLC analyses. Different phenolics and flavonoids that may be present were analyzed under the conditions described earlier. The HPLC chromatograms of the standard mixture comprising of gallic acid (Rt: 6.31 min), protocatechuic acid (Rt: 10.33 min), rutin (Rt: 19.73 min) and ellagic acid (Rt: 20.90 min), and the different extracts, monitored at 254 nm, are shown in Fig. 3 & Fig. 4 respectively. Their chemical structures as well as their respective UV spectra are given in Fig. 5. Confirmation of the identity of the individual compounds was done by comparing with the UV spectra and the retention time of the standards and expressed as mg/g of dry extract. The qualitative and quantitative results are summarized in Table 3 & Table 4 respectively. Results indicated that gallic acid and ellagic acid were the two major phenolics found in all the samples. The concentration of gallic acid varied from 26.9 mg/g (QIFE) to 2.50 mg/g (QILEW) while ellagic acid ranged between 11.5 mg/g (QIFEW) to 6.77 mg/g (QILE). Protocatechuic acid was present in all the extracts, although in very minor quantity (0.25-0.56 mg/g extract). Besides these compounds the chromatograms also showed the presence of other peaks, many of them being classified as either phenolics or flavonoids based on the analysis of their UV spectra. Based on the analysis of the spectra of the eluted peaks, QILEW contained other compounds with spectra similar to that of ellagic acid, thereby indicating the presence of ellagic acid related compounds. Although several flavonoids were present in the extracts, none of them corresponded with rutin. Rutin was, therefore, used as reference for determining the presence of other flavonoids. A peak eluting after ellagic acid, at about 21.95 min, was observed in all the samples. Although this compound, tentatively designated as QI-1, was neither isolated nor identified, it appeared to be a flavonoid or its glycoside based on its UV spectrum. Its concentration was calculated with respect to rutin and was found highest in QILE (15.70 mg/g extract) and lowest in QIFEW (1.04 mg/g extract). It was also observed that

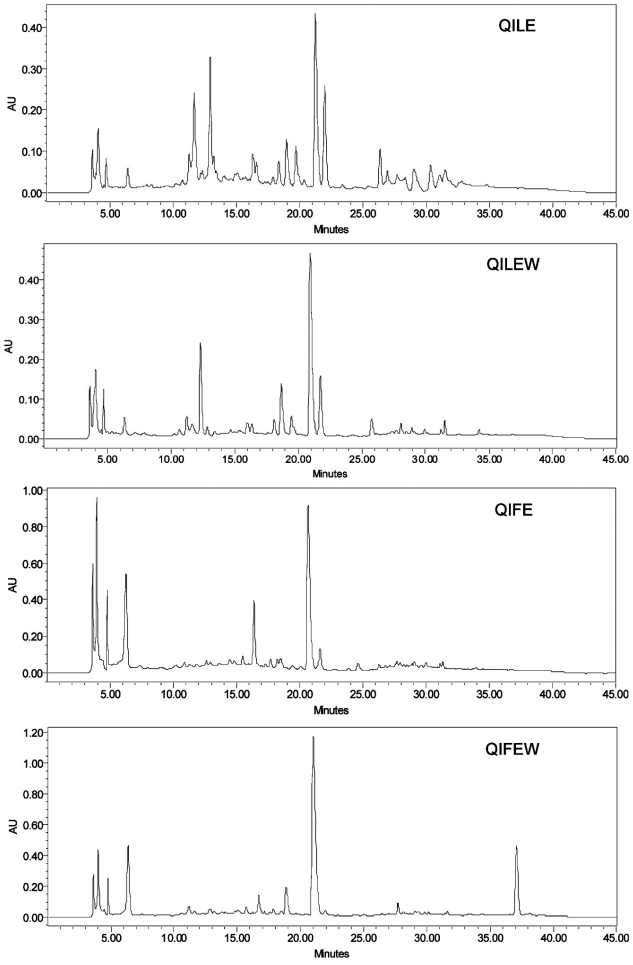

Fig. 4 — HPLC chromatograms of the methanolic and 50% hydroalcoholic extracts of different parts of *Q. indica.* (QIL: *Q. indica* leaves; QIF: *Q. indica* flowers)

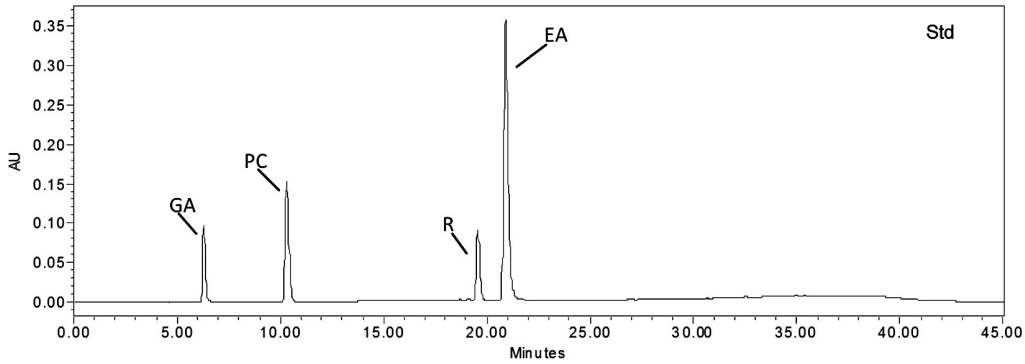

Fig. 3 — HPLC chromatogram of standard mixture (GA-Gallic acid, PC-Protocatechuic acid, R-Rutin and EA-Ellagic acid)

Table 3 — Qualitative HPLC analysis of phenolics and flavonoids present in leaves and flowers of *Q. indica*

Sample	Phenolics and flavonoids					
	Gallic acid	Protocatechuic acid	Ellagic acid	Other phenolics*	Rutin	Other flavonoids*
QILE	+	+	+	+	-	++
QILEW	+	+	+	+	-	++
QIFE	+	+	+	++	-	+
QIFEW	+	+	+	++	-	+

*Unidentified

Table 4 — Quantitative HPLC analysis of phenolics and flavonoids present in leaves and flowers of *Q. indica*

Sample	Phenolics and flavonoids (mg/g ext)			
	Gallic acid	Protocatechuic acid	Ellagic acid	QI-1*
QILE	2.96	0.48	6.77	15.7
QILEW	2.50	0.56	7.62	9.43
QIFE	26.9	0.40	10.2	5.20
QIFEW	17.2	0.25	11.5	1.04

*Unidentified

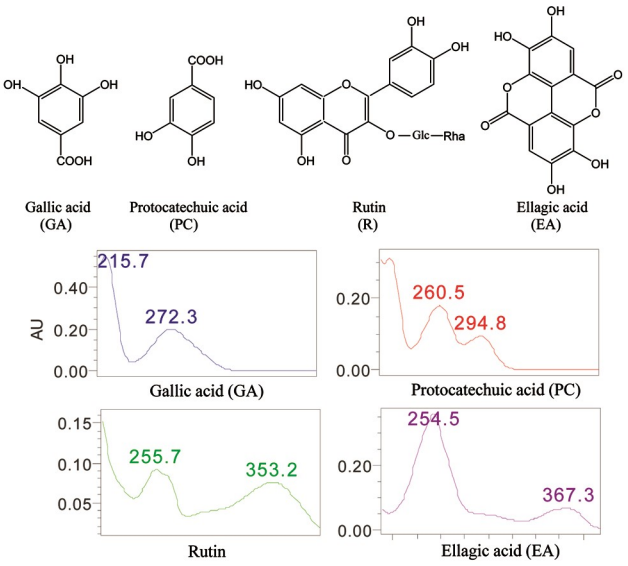

Fig. 5 — Chemical structures and spectral profiles of Gallic acid, Protocatechuic acid, Rutin and Ellagic acid

the leaves were rich in flavonoids whereas the flowers were rich in phenolics.

Discussion

Filariasis damages the lymphatic system, causing the various body parts to enlarge abnormally. It leads to certain disabilities that result not only in physical impairment but also reduced economic productivity, discrimination and serious psychosocial consequences. Thus, a global programme was launched by WHO in 2000 to eliminate lymphatic filariasis. DEC and IVM are available for treatment of filariasis butthey have limitations. They are principally microfilaricidals and their effect on adult worms is doubtful[2]. Their repetitive use may give rise to drug resistance. Considering the limitations of the existing drugs and the huge socio-economic requirements, there is need to search for new antifilarials.

Developing countries mainly use herbal medicines for the primary health care needs of their people. This is because they have been found to be safe, effective, with lesser side effects as well as due to their cultural acceptability. It is believed that the phytoconstituents present in them are more compatable with the human body[24]. As indicated by the results obtained, the hydroalcoholic extract of *Q. indica* flowers possesses promising *in vitro* antifilarial as well as antioxidant activities. QIFEW also contains a good amount of phenolic compounds, some of which are known to exhibit antinematodal activity. HPLC analysis indicated that ellagic acid was the major phenolic acid present in the samples, the content of which was highest in QIFEW. Earlier, the effect of gallic acid, ellagic acid and genistic acid was studied onthe bovine parasite *Onchocerca ochengi* and drug-resistant strains of the free living nematode *Caenorhabditis elegans*[25]. The results indicated that ellgic acid binds differently in the worms and anthelmintic (levamisole, ALB and IVM)-resistant strains of *C. elegans* and could therefore have potential for the treatment even against those nematodal infections which are resistant to anthelmintic drugs. In case of *Q. indica* too, ellagic acid and its related compounds were the major phenolics resent in QIFEW, indicating that they may be responsible for the good level of antifilarial activity exhibited by QIFEW in both motility and MTT reduction assays.

Conclusion

The present studies, therefore, indicate that the hydroalcoholic extract of the flowers of *Q. indica* has the potential to yield compounds that may be used not only for developing novel antifilarial candidates but also for providing relief from the oxidative stress that

is generated as a result of different physiological changes taking place in the body of the patient with lymphatic filariasis.

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