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In Vitro Antioxidant and Anthelmintic Activity of the Extracts of Whole Plant *Crotalaria Biflora* (L)

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ABSTRACT

Various biological activities of the genus "*Crotalaria*" was reported in several studies. In this study, the antioxidant and anthelmintic activity of the whole plant *Crotalaria biflora* was evaluated. The plant material was collected, identified and authenticated and extracted with different solvents such as petroleum ether, chloroform, ethyl acetate and methanol in soxhlet apparatus. Dried extracts thus obtained were used for the evaluation. Antioxidant activity of the extracts was evaluated by DPPH, reducing power assay, superoxide, nitric oxide and hydroxyl free radical scavenging assay methods. Anthelmintic activity was assessed by using the earthworm, *Pheretima postuma.* From the results of both antioxidant and anthelmintic evaluation, it was found that among the different concentration employed for the evaluation, the highest concentration of test extracts showed their maximum activity. Among these, the methanol extract showed a very significant activity comparing with other tested extracts and standard agents employed for the comparative evaluation followed by ethyl acetate extract showed a significant activity. Findings of this study clearly indicated the potential of methanol extract of the whole plant *C. biflora* in the tested biological activities. Future studies on this extract may give more significant results.

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Introduction

Nowadays, the global market of medicine reaches the worth of about 1.1 trillion US dollars per annum. About 35% of these drugs originated from natural products directly or indirectly that includes plants (about 25%), microbes (13%) and animals (3%) (Calixto, 2019). A survey about the sources of novel drugs revealed that about thirteen drugs of natural origin were approved during the year 2005-2007 (Katiyar *et al.,* 2012) Products of natural origin are one of the important sources for the pharma companies investing on the development of novel therapeutics.

The systematic evaluation of plants used in the traditional healing system may afford more assuring data about their therapeutic value and may be helpful to face the demand of novel drugs to resist the infections and diseases. With this view, the plant *Crotalaria biflora* was selected for our research (Kumar *et al.,* 2020).

The first documentation of the genus *Crotalaria* was done in the year 1753 by Carolus Linnaeus. He documented thirteen species of this genus in his Species Plantarum. Now, the count reaches about 702 species in global distribution particularly in Africa and Madagascar with secondary radiations to the rests of the world (Roux *et al.,* 2013; Samila, 2018; Rokade *et al.,* 2020) The genus *Crotalaria* is highly diverse and widely distributed that includes perennial aquatic herbs, erect herbs and shrubs, terrestrial creepers as well as trees can be found in different habitats viz., open places, forest and grasslands, plains and hill regions. In India, the *Crotalaria* is one of the largest legume genera represented by 116 taxa in which fifty two are endemic to the country with the majority of species concentration in the peninsular region (seventy three species restricted to the peninsular region) (Rokade *et al.,* 2020). Many *Crotalaria* species are under cultivation and consumption by the rural population across the globe for variety of purposes that include medicine, food, green manure, fodder etc (Rouamba *et al.,* 2018). The plant selected for the present study, *Crotalaria biflora* is commonly found in the south India at an altitude up to 300m mean sea level. The seed of this plant is edible and consumed by rural population because of its protein content (Gritto *et al.,* 2015; Sundararajan *et al.,* 2018).

In our previous study the whole plant of *Crotalaria biflora* was collected from the Mekkarai, the village located near the foothills of Western Ghats, Tirunelveli District, Tamil Nadu, India. The collected material was subjected to extraction, preliminary phytochemical

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evaluation and spectral characterization (Kumar *et al.*, 2020). The present study was aimed to evaluate the *in vitro* antioxidant and anthelmintic activity of the extracts of whole plant *Crotalaria biflora.*

Materials and Methods

Plant material collection and extraction

The whole plant, *Crotalaria biflora* was collected from the Mekkarai, a village in the close proximity of Western Ghats foothills in the Tirunelveli District of Tamil Nadu, India. The collected plant material was identified and authenticated, shade dried and powdered by mechanical grinding. The coarse powder thus obtained was extracted by soxhelation using the solvents such as petroleum ether, chloroform, ethyl acetate, and methanol. The dried extracts thus obtained were used for the experiments.

In vitro antioxidant activity

In vitro antioxidant evaluation of all the prepared extracts of *Crotalaria biflora* was evaluated by different approaches such as DPPH, reducing power assay, superoxide, nitric oxide and hydroxyl free radical scavenging assay methods.

DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay

Antioxidant evaluation by this approach was done with the slightly modified procedure of Baba *et al.*, 2015 and Ruskin *et al.*, 2017.

Test extracts (1ml) in different concentration (100, 200, 400 and 800µg/ml) was mixed with methanolic solution of DPPH (1ml; 0.1mM). This reaction mixture was incubated at room temperature in dark condition for 30min. After that, the absorbance of reaction mixtures was measured spectrophotometrically (517nm). Methanol was used as blank; DPPH in methanol was employed as control and the ascorbic acid as standard control. The percentage inhibition of DPPH radical by the *Crotalaria biflora* extracts was determined by

$$\text{Percentage inhibition} = \frac{\text{Control Abs.} - \text{Sample Abs.}}{\text{Control Abs.}} \text{ X } 100$$

Percentage inhibition Vs Concentration was plotted as a graph. The IC_{50} values of the tests were assessed from the regression equation of the graph.

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Reducing power assay

This approach of antioxidant evaluation of test extracts was carried out by using the modified procedure of Moonmun *et al.*, 2017 and Batool *et al.*, 2019. Test extracts (500µl) in different concentration (100, 200, 400 and 800µg/ml) was mixed with sodium phosphate buffer (1.5ml; 0.2M; pH 6.6) and potassium ferricyanide (1.5ml; 1%) This mixture was kept for incubation (50°C; 20min). Then, trichloroacetic acid (5ml; 10%) was added to the incubated mixture and subjected to centrifugation (3000rpm; 6min; 4°C). Upper layer of the centrifuged was collected. Collected upper layer solution (1.5ml) was mixed with equal volume of distilled water and fresh ferric chloride solution (300µl; 0.1%) and kept for 10min. Then, the absorbance of reaction mixture was measured spectrophotometrically (700nm). Ascorbic acid was employed as standard control for comparative evaluation.

Superoxide radical scavenging assay

The superoxide radical scavenging activity of the test extracts was assessed by using the modified procedure of Fazilatun *et al.*, 2004 and Awah *et al.*, 2010. The reaction mixture (3ml) contains test extracts in different concentration (100, 200, 400 and 800µg/ml), Nitro blue tetrazolium (0.1ml; 1.5mM) solution, EDTA (0.2ml; 0.1M), riboflavin (0.05ml; 0.12mM) and phosphate buffer (2.55ml; 0.067M). Control tubes were prepared wherein DMSO was added instead of sample. The reaction mixture was kept (30 min) in front of fluorescent light (34 W) and then the absorbance was measured spectrophotometrically (560nm). Ascorbic acid was used as the standard control. All the tests were performed in triplicate and the results were averaged. The percentage inhibition superoxide radical was calculated by

$$\% \text{ inhibition } = \frac{\text{Control Abs.} - \text{Sample Abs.}}{\text{Control Abs.}} \text{ X } 100$$

The IC_{50} indicated the concentration of the tests that inhibited 50% of radical.

Nitric oxide radical scavenging assay

This method of antioxidant evaluation of test extracts was done by using the modified procedure of Awah *et al.*, 2010 and Habu *et al.*, 2015. 1ml of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthyl ethylene diamine dihydro chloride) was added to the reaction mixture (5ml) which was prepared by mixing different concentration of test extracts (100, 200, 400 and 800µg/ml) with sodium nitroprusside (5mM) in phosphate buffer (pH 7.3). This reaction mixture was kept in 25°C (in front of 25W

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tungsten lamp) for 3h. The nitric oxide radical thus formed was interacted with oxygen to produce nitrite ion which was measured spectrophotometrically (546nm). Normal and standard control (Ascorbic acid) were prepared. The percentage inhibition of nitric oxide radical formation was determined by

$$\% \text{ inhibition } = \frac{\text{Control Abs.} - \text{Sample Abs.}}{\text{Control Abs.}} \text{ X } 100$$

Hydroxyl radical scavenging assay

Evaluation of antioxidant activity by hydroxyl radical scavenging assay was done with the modified procedure of Habu *et al.,* 2015 and Batool *et al.,* 2019. Test extracts (100µl) in different concentration (100, 200, 400 and 800µg/ml) was mixed with deoxyribose (0.1ml; 2.8mM) prepared in potassium phosphate buffer (20mM; pH 7.4), EDTA (0.1mM), ferric chloride (200µl; 100mM), H_2O_2 (100µl; 200mM) and ascorbic acid (100µl; 300mM). This mixture was kept for 1h at 37°C. Then, trichloroacetic acid (1ml; 2.8%w/v) and thiobarbituric acid (1ml; 1%w/v) prepared in NaOH (50mM) were added to it and the whole reaction mixture was kept (15min) in water bath for boiling. After cooling, the absorbance of reaction mixture was measured spectrophotometrically (532nm). Normal and standard control (Quercetin) were prepared. Inhibition of deoxyribose degradation was calculated by

$$\% \text{ inhibition } = \frac{\text{Control Abs.} - \text{Sample Abs.}}{\text{Control Abs.}} \text{ X } 100$$

***In vitro* anthelmintic activity**

Evaluation of anthelmintic activity was carried out in accordance with the procedure of Ajaiyeoba *et al.,* 2001 and Das *et al.,* 2017 with slight modification on adult Indian earthworms, *Pheretima postuma*. Different concentration (25, 50, 100 and 200mg/ml) of petroleum ether, chloroform, ethyl acetate and methanol extracts of the whole plant *C. biflora* was subjected to the evaluation. Normal saline was used as the control and Piperazine citrate (10mg/ml) was used as the standard control. The test and standard drug solution were freshly prepared in carboxy methyl cellulose (1%). Two worms per each group were employed for the evaluation. Observations were made for paralysis and death of individual worms. Time for paralysis was noted when no movement of any sort could be observed except when the worms were shaken vigorously. Time for death of worms were recorded after ascertaining that the worms neither moved when shaken vigorously nor when dipped in warm water at 50°C followed with fading of their body colour. Three sets of experiments were done

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statistical significance. Results are presented as mean ± SEM (Standard error of Mean). The results were analyzed by ANOVA and Dunnet's test.

Results and Discussion

The results of *in vitro* antioxidant evaluation of all the prepared extracts of the whole plant *C. biflora* by DPPH method is shown in Table 1.

Table 1. Antioxidant activity of test extracts by DPPH assay

Con.	% Inhibition				
	Test extracts				
(µg/ml)	Pet. ether	Chloroform	Ethyl acetate	Methanol	Std.
100	12.74±0.12	21.10±0.60	24.73±1.26	45.23±1.58	42.10±0.12
200	17.56±0.31	32.70±0.75	37.38±1.47	58.85±1.75	53.72±1.73
400	23.30±0.55	42.25±0.87	45.29±1.77	70.80±1.90	64.91±1.52
800	36.53±0.69	58.48±1.10	65.83±1.50	85.21±2.03	76.52±2.10
IC_{50}	850.10	720.59	435.80	115.64	285.70

Con. – Concentration; Std. – Standard (ascorbic acid)

From the results, a concentration dependent rise of antioxidant activity was found in all the tested extracts. In the highest test concentration, (800µg/ml), all the extracts showed their maximum activity, however, the methanol followed by ethyl acetate extract of *C. biflora* showed a significantly higher activity comparing with other tested extracts. The methanol extract showed a percentage inhibition of 85.21±2.03 in 800µg/ml concentration, Next to that, the ethyl acetate extract showed a percentage inhibition of 65.83±1.50. The standard agent, ascorbic acid used for the comparative evaluation showed a percentage inhibition of 76.52±2.10 in the same concentration. Thus the results clearly indicated the antioxidant potential of the methanol extract of the *C. biflora*.

The next method employed for the evaluation of antioxidant activity, the reducing power assay method also showed a concentration dependent increase of antioxidant activity in all the tested extracts. In this method also, the methanol extract proved its antioxidant potential by showing a percentage inhibition of 96.50±1.78 followed by the ethyl acetate extract showed a percentage inhibition of 86.80±1.50. Both these two extracts of *C. biflora* revealed a significant antioxidant activity comparing with the standard agent, ascorbic acid, employed for the comparative evaluation (Table 2).

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Table 2. Antioxidant activity of test extracts by reducing power assay

Con. (µg/ml)	% Inhibition				
	Test extracts				
	Pet. ether	Chloroform	Ethyl acetate	Methanol	Std.
100	17.20±0.24	27.80±0.80	39.75±1.30	59.38±1.10	22.64±0.58
200	28.32±0.77	32.85±0.92	48.86±2.05	69.01±1.71	31.50±0.75
400	37.70±1.12	54.96±1.36	65.60±1.65	76.80±1.59	42.14±0.98
800	46.07±1.25	66.20±1.52	86.80±1.50	96.50±1.78	63.53±1.23
IC_{50}	788.86	380.23	296.25	80.27	420.30

A similar kind of results was found in superoxide radical scavenging assay, another one method employed for assessing the antioxidant ability of the extract of the whole plant *C. biflora.* In this method also, comparing with other tested extracts and the standard agent (ascorbic acid), the methanol extract revealed a maximum activity by showing a percentage inhibition of 73.50±0.72 in 800µg/ml, the highest concentration of evaluation (Table 3).

Table 3. Antioxidant activity of test extracts by Superoxide radical scavenging assay

Con. (µg/ml)	% Inhibition				
	Test extracts				
	Pet. ether	Chloroform	Ethyl acetate	Methanol	Std.
100	09.21±0.78	10.63±1.03	12.44±0.15	28.21±0.60	69.57±0.35
200	11.58±0.47	16.32±0.20	18.38±0.20	34.20±0.10	90.60±0.55
400	13.20±0.89	21.65±0.34	27.58±0.86	57.24±0.15	92.18±0.23
800	19.26±0.69	31.20±1.10	43.65±0.09	73.50±0.72	98.08±0.28
IC_{50}	1270	1100	1010	390	80

The other two methods, nitric oxide and hydroxyl radical scavenging assay utilized for the evaluation of antioxidant activity also revealed a same sort of results (Table 4 & 5). Results of these evaluations clearly indicated the antioxidant potential of methanol extract comparing with other tested extracts and standard agent (ascorbic acid in nitric oxide radical scavenging assay and quercetin in hydroxyl radical scavenging assay) used for the comparative evaluation.

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Table 4. Antioxidant activity of test extracts by nitric oxide radical scavenging assay

Con. (µg/ml)	% Inhibition				
	Test extracts				
	Pet. ether	Chloroform	Ethyl acetate	Methanol	Std.
100	12.35±1.10	16.58±0.60	18.69±0.13	40.58±08	25.38±0.90
200	14.25±0.91	19.42±0.50	23.12±0.15	50.21±0.13	30.52±0.40
400	21.49±0.83	26.37±0.97	30.22±0.36	59.50±0.10	60.65±0.32
800	25.53±0.72	32.80±0.63	37.63±0.26	65.60±0.54	55.63±0.30
IC_{50}	1310	1270	1160	260	420

Table 5. Antioxidant activity of test extracts by hydroxyl radical scavenging assay

Con. (µg/ml)	% Inhibition				
	Test extracts				
	Pet. ether	Chloroform	Ethyl acetate	Methanol	Std.
100	08.12±0.74	11.55±0.76	14.96±0.31	36.85±0.08	21.19±0.20
200	10.80±1.16	14.63±0.57	19.22±0.50	46.12±0.31	26.25±0.40
400	13.26±0.80	20.15±0.48	26.44±0.63	55.05±0.08	48.56±0.23
800	19.88±0.55	27.45±0.50	33.36±0.262	61.16±0.45	51.36±0.32
IC_{50}	1160	920	860	220	340

Results of the evaluation of anthelmintic activity are shown in Table 6. In this evaluation, all the tested extracts showed different level of activity based on its concentration. However, all the tested extracts revealed a maximum of their activity in the highest concentration (200mg/ml). But the methanol extract showed a significant activity (58.78 ± 1.0 minutes taken for paralysis and 63.43 ± 0.7 minutes for death) comparing with other tested extracts and the standard drug (Piperazine citrate) for the comparative evaluation.

Table 6. Anthelmintic activity of test extracts

Treatment group	Concentration (mg/ml)	Paralysis time (min)	Death Time (min)
Normal Control	-	-	-

Standard control	10	21.80 ± 0.6	27.51± 0.3
Petroleum ether extract	25	98.32 ± 0.8	105.05 ± 0.5
	50	93.60 ± 1.1	98.30 ± 0.4
	100	89.40 ± 0.3	96.42 ± 1.3
	200	88. 45 ± 0.6	95.55 ± 1.0
Chloroform Extract	25	92.25 ± 1.7	97.15 ± 1.2
	50	86.31 ± 0.3	91.30 ± 0.7
	100	82.07 ± 0.5	87.84 ± 0.8
	200	75.28 ± 0.9	81.48 ± 0.1
Ethyl acetate Extract	25	81.32 ± 1.1	86.10 ± 1.0
	50	80.50 ± 1.2	85.26 ± 0.7
	100	77. 34 ± 1.6	83.03 ± 1.1
	200	75.24 ± 1.3	80.21 ± 0.8
Methanol Extract	25	73.28 ± 1.8	76.52 ± 0.2
	50	69.81 ± 0.8	73. 12 ± 0.2
	100	64.40 ± 1.1	69.40 ± 1.5
	200	58.78 ± 1.0	63.43 ± 0.7

Regarding with antioxidant activity of tested extracts of the whole plant *C. biflora*, a concentration dependent rise of activity was found in the results. A similar type of observation was reported in the studies of Habu *et al.,* 2015 and Sebastin *et al.,* 2021. In the present study it was found that the methanol extract showed a very significant antioxidant activity comparing with other tested extracts. It may be due to the presence of phenolic compounds in this extract which was confirmed in our previous study on preliminary phytochemical evaluation (Kumar *et al.,* 2020). Relation of phenolic compounds and the antioxidant activity was reported by several studies such as Awah *et al.,* 2010, Baba *et al.,* 2015 Ruskin *et al.,* 2017 and Sebastin *et al.,* 2021. A very significant activity of methanol extract was found in the evaluation of anthelmintic activity. It was reported that the presence of secondary metabolites such as alkaloids, flavonoids and terpenoids in the plants significantly contribute to its anthelmintic activity (Das *et al.,* 2017). Our previous study on preliminary phytochemical evaluation (Kumar *et al.,* 2020) found the presence of above said secondary metabolites in the methanol extract. Collectively, our studies on the whole plant, *C. biflora* found diverse secondary metabolites and biological activities.

Conclusion

In the present study, the antioxidant and anthelmintic activity of the *Crotolaria biflora* was evaluated. For that, the whole plant was collected. Properly identified and authenticated material was dried in shade, powdered in mechanical grinder, extracted in soxhlet apparatus using the solvents such as petroleum ether, chloroform, ethyl acetate, and methanol. Dried extracts thus obtained was screened for the *in vitro* antioxidant and anthelmintic activity. Among all the tested extracts, the methanol extract of the whole plant *C. biflora* revealed a very significant activity in both antioxidant and anthelmintic evaluation. Next to that the ethyl acetate extract showed a significant activity. Outcome of the present study is encouraging for further studies in these extracts in the future.

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