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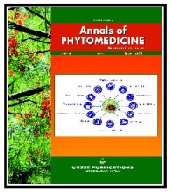

Original article: Open access

Effect of solvents on metallic and phenolics content in buttercup tree bark vis-a-vis in relation to anthelmintic activity

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Abstract

The aim of the present study to evaluate metal ion content and total phenolics in various extracts of *Cochlospermum religiosum* (CR) Alston using two different solvents. The CR is commonly known as Buttercup tree belongs to the family Bixaceae, having many branches. In the present study, ethylacetate, methanol and aqueous solvents are used for the extraction using microwave oven method. All the extracts are tested preliminary for the presence of phytochemicals, followed by TLC for identification. Furthermore, HPLC results estimated the gallic acid content is higher in methanol CR bark extract. The result revealed the presence of carbohydrates, alkaloids, glycosides, tannins, phenolics, flavonoids, and steroidal compounds in aqueous and methanol extracts whereas protein, lipids, and steroids are present in ethyl acetate extract. Content of metal ions (Fe, Mn, Cu, Zn, Co, Ni, As, Hg, Pb, Cd) are determined with atomic absorption spectrophotometer (AAS) and revealed the presence of higher amount of Zn (19.13 mg/g) with lower amount of Mn (0.06 mg/g) in methanol extract. Other heavy metals are present in negligible quantities. Furthermore, total phenolic content was higher in methanol extract (28.23 %) extracted for 10 min than other two extracts. Finally, *in vitro* anthelmintic activity of extracts was evaluated using reference to standard Albendazole and it showed significant results in concentration dependent manner against *Pheretima posthuma*, as test worm. The higher result was obtained with methanolic bark extract followed by aqueous extract (extracted with microwave oven for 10 min with 80°C) and the result was due to presence of polyphenolic compound especially gallic acid (0.38 g), and also showed a positive correlation with yield and metal ion content.

1. Introduction

Plants serve mankind in versatile form and are considered as an important and inevitable source of medicaments in our life. All the parts of plants are playing immense role in herbalism. Therefore, plant secondary metabolites like alkaloids, glycosides, phenolics, flavonoids, phytosterols, saponins, resins, tannins an even terpenoids are important products those are stored in various parts of the plant bodies (roots, stems, barks, woods, leaves, fruits, flowers, *etc*.). Therefore, medicinal virtues of plants are attributed due to presence of the important phytoconstituents such as bioactivities such as antioxidant, anti-inflammatory, antimicrobial, wound healing, anti-Alzheimer, anticancer, *etc*.

Of late, the *Cochlospermum religiosum* (CR) Alston (Family: Bixaceae) is well known medicinal tree in India. The tree is commonly known as golden silk cotton tree or butter cup tree because its flowers are bright yellow colored and look like large-sized buttercups. The plant is used traditionally as sedative, stimulant, antigonorrheal, and in the treatment of jaundice, cough, trachoma, syphilis, *etc*. The bark of CR yields gum, also known as katira. The gum is obtained by stripping the tree bark that contains mainly D-galactose, D-galacturonic acid and L-rhamnose (Vinod *et al*., 2008). All the parts of the CR tree show many therapeutic activities due to the presence of many bioactive constituents. The leaves contain sterols, triterpenes, saponins, alkaloids, tannins, flavonoids, phenols, glycosides (Ponnamma *et al*., 2017) and revealed the antibacterial, antifungal, antioxidant activity (Panda *et al*., 2016; Buch and Arya, 2017). The flowers are powerful insecticide due to presence of flavonoids, and sterols (Swathi *et al*., 2019).The bark contains gum which is economically viable with its potential wound healing activity, cholesterol inhibition activity, bioremediation of toxic metals (cadmium, copper, iron, lead, mercury, nickel), and even elicited immunological response (Vinod and Sashidhar, 2011; Hongsing *et al*., 2012; Girotra and Singh, 2013; Puskuri *et al*., 2017). Powdered roots are used as tonic and to treat jaundice (Satpure, 2017). Fruit juice is used in dysentery and gonorrhea treatment (Savithramma *et al*., 2014). The stem bark is used in sores and fistula, bone fractures (Suneetha *et al*., 2011) with the presence of saponins, tannins, phenolic compounds, alkaloids, phytosterols (Kawde *et al*., 2015). The bark contains high amount of phenolics and flavonoids and reported as broad spectrum antimicrobial activity (Sasikala *et al*., 2015). Hence, it is essential to determine the phenolic content in the bark extracts. Thereafter, heavy metals (Fe, Mn, Cu, Zn, Mg, Cr, Ni, Pb, Hg, As, Co, Cd, *etc*.) also play an immense role in accumulation of the bioactive constituents in the plant body and alternately help in various therapeutic activities. Some heavy metals are essential for plant

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growth and some are toxic in nature and causes hazardous health complications to humans (Ibrahim *et al*., 2017; Das *et al*., 2019). Hence, study on heavy metals is essential to establish complete therapeutic efficacy of plants. But, any literature revealed neither metal ion content nor anthelmintic activity of bark extracts and even any correlation study among them. With the focus of that concept, the present investigation was carried out to establish the potent anthelmintic activity of the CR bark extract and the role of metal ions in the activity by the establishment of the correlation among the phytoconstituents, metal ions and therapeutic activity.

2. Materials and Methods

2.1 Collection and preparation of plant material

The CR bark was collected from Chikmagalur, Karnataka state and was identified and authenticated by Dr. N. Dhatchanamoorthy, Botanist, Foundation for Revitalisation of Local Health Traditions (FRLHT, Bangalore). After collection, the bark was coarsely powdered using hammer mill and further used for the experimental purpose.

2.2 Extraction of CR bark

The coarse CR bark powder (25 g) was extracted separately with three different solvents, *viz*., ethyl acetate, methanol and aqueous using microwave oven at three different time intervals (10, 15 and 20 min), using temperature of 80°C, 70ºC and 60ºC, respectively with microwave input from 300 W to 100 W, respectively with 100 W intervals during the extraction and the volume of solvents were fixed of 30 ml. The extracts were concentrated by rotary evaporator at 40-45ºC for 30-45 min. and stored with proper label in small glass bottles in refrigerator at 5ºC. The percentage yield of extracts were calculated separately.

2.3 Assessment of heavy metals in bark sample

Dried bark extract was pre treated with concentrated nitric acid in a digestion flask, followed by mixing with acid mixtures (H_2SO_4:$HCLO_4$ with ratio of 6:4). Digestion was carried out at 200°C until dense white fumes of gases were evolved and finally white residue was obtained. Subsequently, the digested samples were diluted with demineralized water and the volume made up to 50 ml. Final solutions were analyzed for various heavy metal contents (Fe, Mn, Cu, Zn, Co, Ni, As, Hg, Pb, and Cd) using an AAS (PerkinElmer model: A Analyst 100; Australia). Air acetylene was used and the concentration of the said metal elements was determined using the standard condition. The wavelengths were selected for the analysis based on the concentration ranges of the sample and the linear relation between the absorbance (AU) and concentration of the determining element. All the samples were checked by carrying out triplicate analyses for the reproducibility of the method used against blank preparation (Das *et al*., 2019).

2.4 Pharmacognostical screening

Phytochemical screenings of CR bark extracts were carried out by different chemical tests for presence of various bioactive agents. All the extracts were evaluated for the presence of different phytoconstituents such as alkaloids, glycosides, phytosterols, flavonoids, carbohydrates, and proteins extracted for 10 min at 80º C (based on the yield) (Kokate, 2005).

2.5 Estimation of total phenolics

Total phenolics content in all the extracts was determined by spectrophotometry using the Folin-Ciocalteu assay. First, 1 ml of CR bark extract was mixed in distilled water (9 ml) and then 1 ml of Folin-Ciocalteu reagent was added to the solution, diluted 1:10 with de-ionized water (Alhakmani *et al*., 2013). After 10 min, 7% sodium carbonate solution (10 ml) was added and the final volume was made up to 25 ml. Standard solutions of gallic acid were prepared at various concentrations (50, 100, 150, 200, 250, 300, 350, and 400 µg/ml). The mixed solution was kept for 2 h at 25 ± 2°C and then absorbance was recorded (at 550 nm) for both test and standard solutions. A blank sample was prepared for reading corrections. The phenolics content was estimated and expressed as per cent of gallic acid equivalent of extract.

2.6 Chromatographic identification

Extracts were evaluated for the presence of particular active compounds through TLC method. The present study was aimed for identification and separation of polyphenolic compounds which were responsible for the anthelmintic activity. Toluene, ethyl acetate and formic acid combination was selected for detection and separation of said compounds (Patel *et al*., 2017). Further, HPLC analysis was carried out for determination of content of phenolic acid present in the bark extracts after the method validations (specificity, linearity, accuracy, robustness, *etc*.) as per the standard protocol (ICH, 2005). The mobile phase was prepared by mixing Methanol: Water (60:40). The standard stock solution was prepared by taking 10 mg of gallic acid in 10 ml volumetric flask and made the volume up to 10 ml with methanol (the concentration: 1000 µg/ml). The column Phenomenex Gemini-NX-5 µm C18 (2) 110 Å, LC Column 250 x 4.6 mm, Ea, was used for the estimation using 1 ml/min flow rate of the mobile phase with UV detector at 203 nm wavelength.

2.7 Selection of organism for anthelmintic activity

The present anthelmintic assay was performed on adult earthworm (*Pheretima posthuma*, belong to class Oligochaeta) due to the anatomical and physiological resemblance of the red worms with the intestinal round worm parasites of human beings. They were collected from the medicinal garden of Krupanidhi College of Pharmacy, Bengaluru and washed with normal water to remove the soil parts and other foreign matters from body and further used for the study. The earthworms of 2-8 cm in lengths and 0.2-0.5 cm in width were used for all the experimental protocols. Albendazole (25 mg/ml) was used as a standard solution (prepared by dissolved in DMF), which purchased from local market of Bengaluru and the test solution of extracts (25, 50, 100, 150, 200 mg/ml) were evaluated for anthelmintic activity (Das *et al*., 2017). The parameters such as time taken for paralysis and death of individual worms were observed during the study. When there was no movement of any part of the body, then time noted for the paralysis condition followed by the death time was noted when no movement of any part of the body and also observed with the fading away of their body colors. The whole investigation was carried out as per the guideline of the Institutional Biosafety and Ethical Committee (Chandrashekhar *et al*., 2008).

2.8 Selection of correlation study

The yield of the CR bark extract was correlated with the type of the solvent used along with the method of the extraction conditions. Based on the yield, further study was carried out and also correlated with the metallic ion content that showed higher accumulation of the bioactive constituents especially polyphenolic compounds and alkaloids in the bark.

2.9 Statistical analysis

Data are expressed as mean ± SEM from three replications. For anthelmintic activity, one-way ANOVA test followed by Tukey's post hoc test ($p<0.05$) was used. Correlation coefficient was carried out among the extract and the activities. P values less than 0.05 were considered to be statistically significant.

Further, design of experiments (DOE) is applied to estimate the effect of time, temperature and polarity of the solvent on anthelmintic activity and yield. A custom design using design expert V 13 was employed to estimate the effect of time of extraction, temperature and polarity of solvents affecting the anthelmintic activity and yield. The independent factors as listed in Table 1 varied in two levels, and the design yielded 21 experimental runs (Table 2).

Table 1: Coded variable in two levels

Factors	Low(-1)	Medium(0)	High(+1)
Time (min)	10	15	20
Temperature (°C)	60	70	80
Solvent (Polarity)	Ethyl acetate	Methanol	Water

Table 2: The list of experimental results of the 21 runs

Sr. No.	Temp (°C)	Time (min)	Extracting solvent (Polarity)	Anthelmintic Activity (min)	Yield (%)
1	0	1	1	27.14	9.04
2	1	1	-1	37.02	7.43
3	1	1	0	19.04	16.2
4	1	-1	0	13.14	36
5	-1	1	1	31.28	8.87
6	-1	-1	-1	21.52	18.4
7	-1	1	-1	28.33	17.3
8	0	0	0	15.08	28.8
9	1	0	1	30.28	7.40
10	1	-1	-1	27.02	9.33
11	0	-1	-1	30.11	10.20
12	-1	0	-1	29.20	10.02
13	-1	1	0	18.28	17.2
14	-1	0	1	34.24	12.65
15	1	0	-1	38.24	7.65
16	-1	-1	1	30.02	11.78
17	1	1	1	27.30	12.04
18	0	-1	1	29.21	10.23
19	0	1	-1	33.29	9.44
20	-1	-1	0	18.02	19.13
21	1	-1	1	17.07	24.43

3. Results

3.1 Yield of the extract

The yield of the CR bark was calculated for all the extracts (aqueous, methanol and ethyl acetate extract) extracted by microwave oven with different time intervals in varied temperature. The solvent amount kept constant at 30 ml. The results revealed that the yield of bark was higher in 10 min extraction time at 80^0C temp, followed by 15 min and 20 min for all the extracts. Among them, methanol extract showed highest yield compared to other solvents (Figure 1).

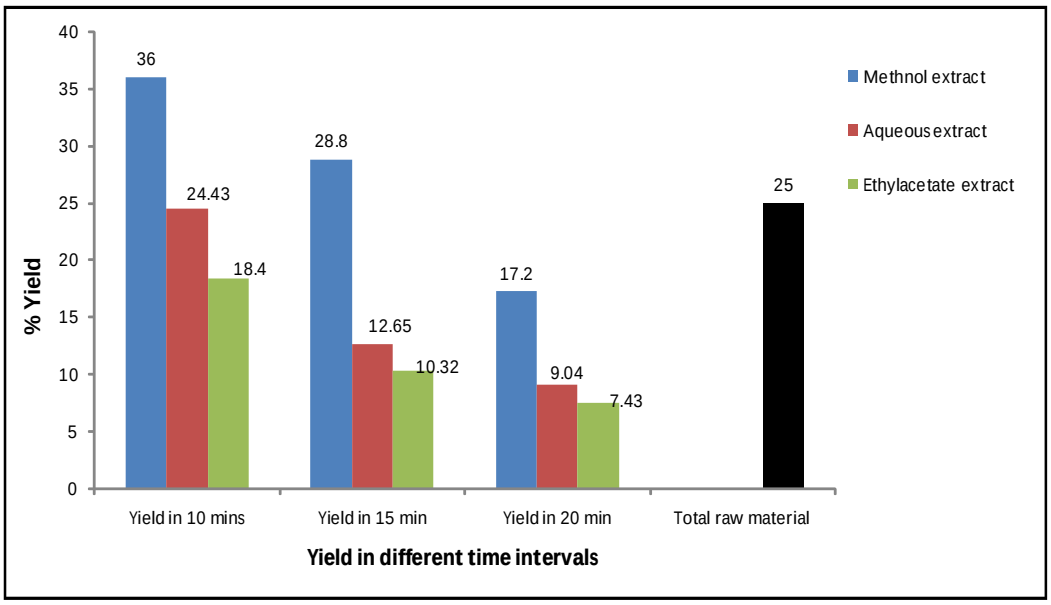

Figure 1: Y-axis: Percentage yield of CR bark extract in different solvents.

Table 3: Metal ion content in CR powdered bark

Metal ions	Wavelength (nm)	Slit	Microwave assisted (mg/g) extract		
			Aqueous	Methanol	Ethyl acetate
Fe	248.3	0.7	07.89 ± 0.21	09.01 ± 0.11	2.31 ± 0.03
Mn	279.3	0.7	00.04 ± 0.10	00.06 ± 0.22	0.01 ± 0.01
Cu	324.8	0.7	00.09 ± 0.04	00.13 ± 0.01	0.02 ± 0.11
Zn	213.9	0.7	17.10 ± 0.20	19.13 ± 0.21	7.22 ± 0.12
Co	240.7	0.7	00.02 ± 0.21	ND	0.03 ± 0.14
Ni	232.1	0.7	ND	ND	0.01 ± 0.02
As	193.7	0.7	ND	ND	ND
Hg	253.6	0.7	ND	ND	ND
Pb	283.3	0.7	ND	ND	ND
Cd	228.8	0.7	ND	ND	ND

• Mean ± Sem (n =3); ND = Not detected

3.2 Assessment of heavy metals

Dried extracts of CR bark were analyzed for uptake of elements separately by AAS and the results revealed the presence of some important metal ions in all three different extracts. Varying concentration of Fe, Mn, Cu and Zn resulted from all the extracts whereas non essential heavy metals such as Ni, As, Hg, Pb and Cd were not detected except minute concentration of Co and Ni in aqueous (0.02 mg/g), ethyl acetate (0.03 mg/g) and solely ethyl acetate extracts (0.01 mg/g) respectively. The overall result was tabulated in Table-3.

3.3 Phytochemical screening

The bioactive compounds varied with the extraction process and also dependent on the type of extracts. Hence, the phytochemical screening tests were carried out for the three different extracts as per the standard method described earlier in methodology section. Based on the higher yield given by all the extracts (10 min at 80° C), the results revealed the presence of maximum secondary metabolites in methanol extract followed by aqueous and ethyl acetate extract (summarized in Table 4).

3.4 Estimation of total phenol

Based on the chemical test and as per the activity planned, the total phenolic content was estimated in terms of GAE (mg of GA/g of extract, standard curve equation: $y = 0.021x + 0.174$, $R^2 = 0.994$). The total phenolic contents were calculated (%) using the following linear equation based on the calibration curve of gallic acid and content of higher amount of phenolic showed by methanol extract followed by aqueous and ethyl acetate extracts. Among the extracts, extraction carried out for 10 min with 80°C showed higher values for all the solvents (Table 5).

Table 4: Various phytochemicals present in different extracts of CR bark

Phytoconstituents	Various tests	Ethyl acetate extract	Methanol extract	Aqueous extract
Alkaloids	Mayer's test	––	++	+
	Dragendorff's test	––	++	+
	Wagner's test	––	++	+
	Hager's test	––	+	+
Flavonoids	Shinado test	––	++	+
	$FeCl_3$ test	––	++	+
	Lead acetate test	––	+	+
Glycoside	Keller killiani test	––	––	+
Steroids	Salkowski test	––	++	+
	Liebermann burchard test	––	++	+
Tannins	$FeCl_3$ test	––	+	+
	Gelatin test	––	––	––
Phenols	FC reagent test	+	++	+
Proteins	Biuret test	+	––	––
Saponins	Foam test	––	+	++
Triterpenes	Salkowski test	––	++	––
	Liebermann burchard test	––	+	––

(– –) = Absent; (+) = present; (++) = Strongly present

Table 5: Total Phenolic content in CR bark extract

Extracts	Phenolic content (%)		
	10 min in 80°C	15 min in 70°C	20 min in 60°C
Methanol extract	$28.23 \pm 0.031^{**a}$	$24.98 \pm 0.021^{**b}$	$18.22 \pm 0.011^{**c}$
Aqueous extract	$17.54 \pm 0.010^{**c}$	$14.23 \pm 0.011^{*b}$	$12.31 \pm 0.002^{*}$
Ethyl acetate extract	$09.11 \pm 0.012^{*a}$	06.03 ± 0.042^{ns}	03.11 ± 0.013^{ns}

Mean ± SD (n =3); One way ANOVA study followed by Tukey's post hoc test. Significant level, $^{*}p<0.05$, $^{**}p<0.01$; Means with different superscript letter (a, b, c) differ significantly ($p<0.05$) from one another. "ns" = non significance.

3.5 Chromatographic identification and estimation

Based on the phytochemical screening, further TLC was performed for the identification and separation of the active components. It was observed that toluene, ethyl acetate and formic acid with the ratio of 7: 5: 1 showed the better separation of phenolic compound, *i.e.*, gallic acid. In case of methanol extract, gallic acid was visible prominently (under UV 366 nm) with the Rf value of 0.42 whereas less visible in ethyl acetate extract (Figure 2).

Further, quantification of the constituents through HPLC was performed. The methanolic extract showed more content of gallic acid (0.38 g/100 g) which were higher than aqueous extract (0.19 g/ 100 g) when experiment was carried out UV at 203 nm. The retention time (Rt) for the gallic acid in methanolic extract showed same as per standards (Figures 3a and b) with the value of 3.105 min (Table 6).

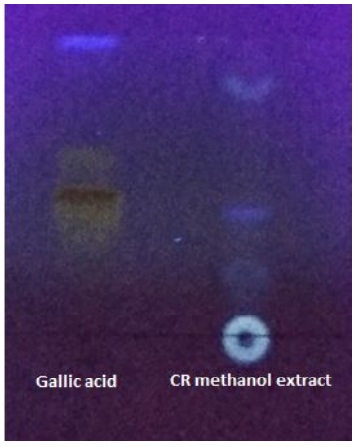

Figure 2: TLC of CR extract with standard gallic acid.

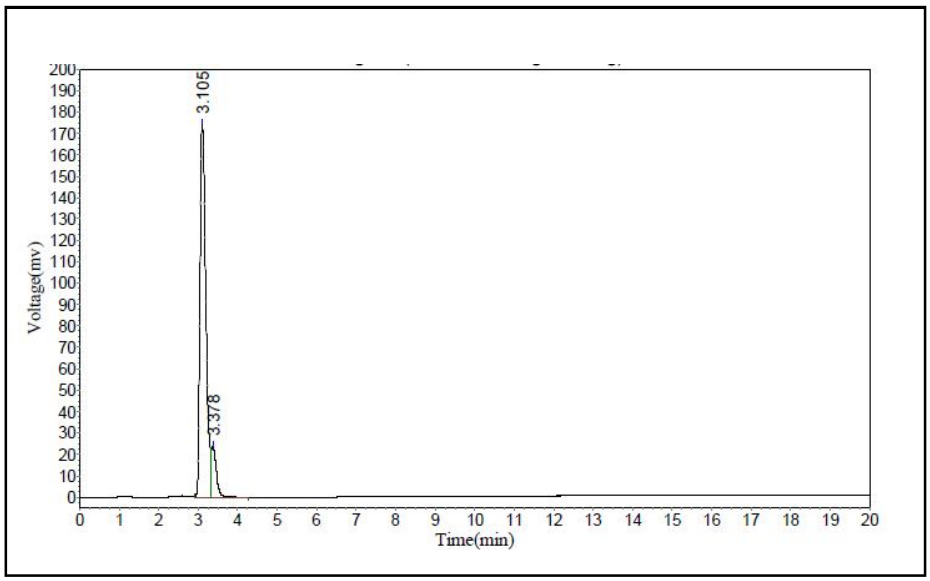

Figure 3a: Standard gallic acid (96% purity).

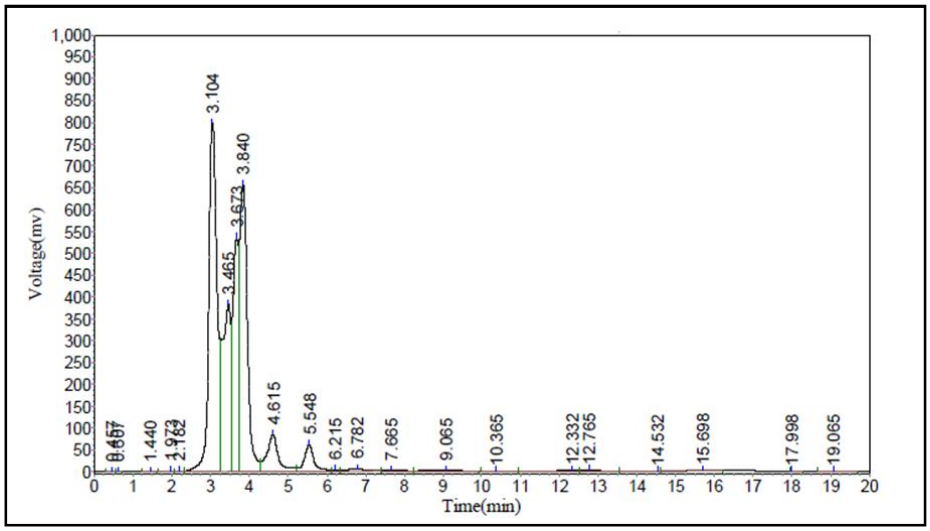

Figure 3b: HPLC of methanol CR bark extract for presence of gallic acid.

Table 6: Gallic acid content in CR bark extract

Extracts	Gallic acid content (g/100 g)		
	10 min in 80°C	15 min in 70°C	20 min in 60°C
Methanol extract	0.38	0.34	0.21
Aqueous extract	0.19	0.12	0.09
Ethyl acetate extract	0.08	0.03	0.01

3.6 Anthelmintic activity

The higher yielded extracts, *i.e.*, methanol, aqueous and ethyl acetate CR bark extracts of 10 min in 80°C, were selected based on the content of phenolic acid and the anthelmintic activity was carried out. The result observed in terms of paralysis and death of the organisms. All the extracts showed activity but methanol extract showed higher activity in lower time than other extracts but the time taken for death of animals was little higher than the standard albendazole (25 mg/ml). Overall the activity showed concentration dependent for all extracts. Among them, concentration at 200 mg/ml (methanol extract) showed higher inhibition of parasite movement (9.24 min), followed by death (13.14 min) and the result showed more significant as compared to control and standard (Figure 4).

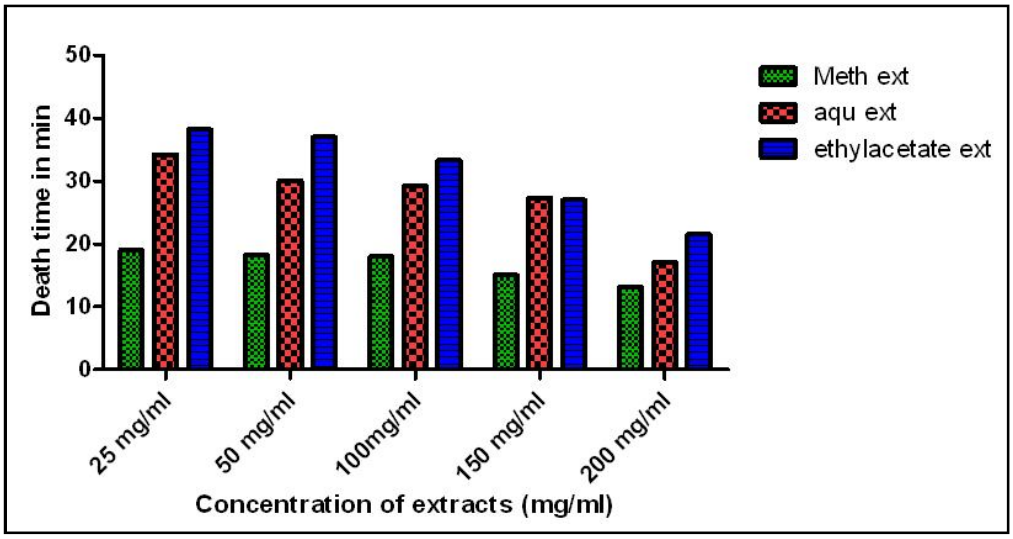

Figure 4: Anthelmintic activity of CR bark extracts (death study shown).

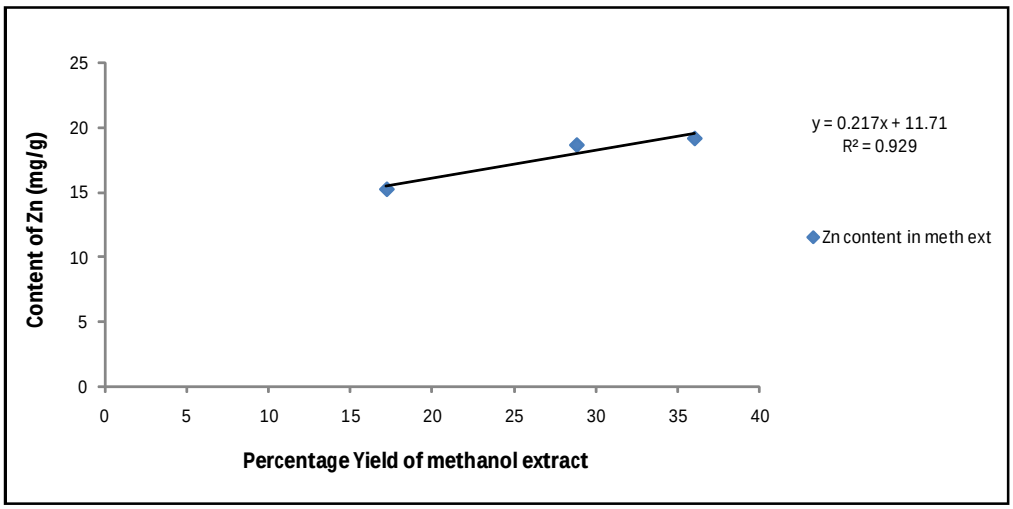

Figure 5a: Zn content in methanol extract.

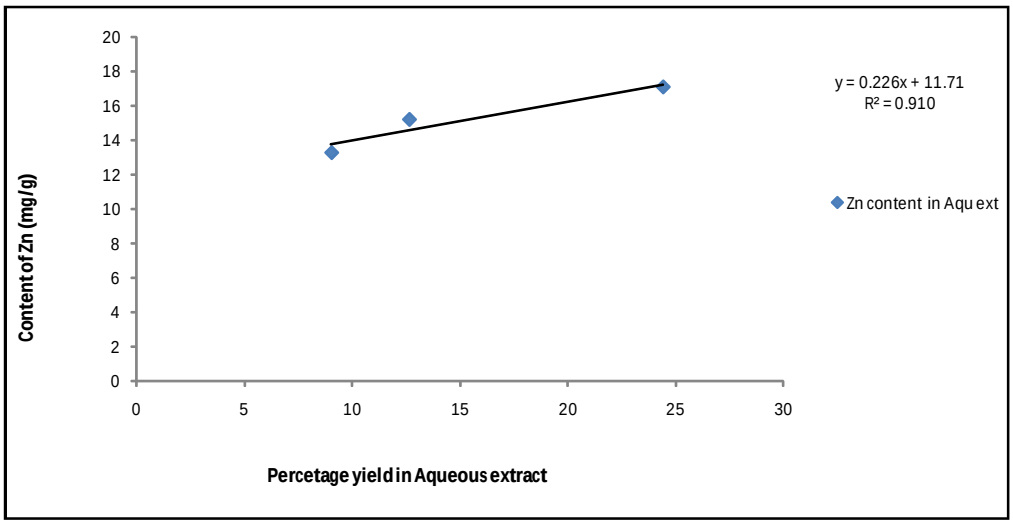

Figure 5b: Zn content in aqueous extract.

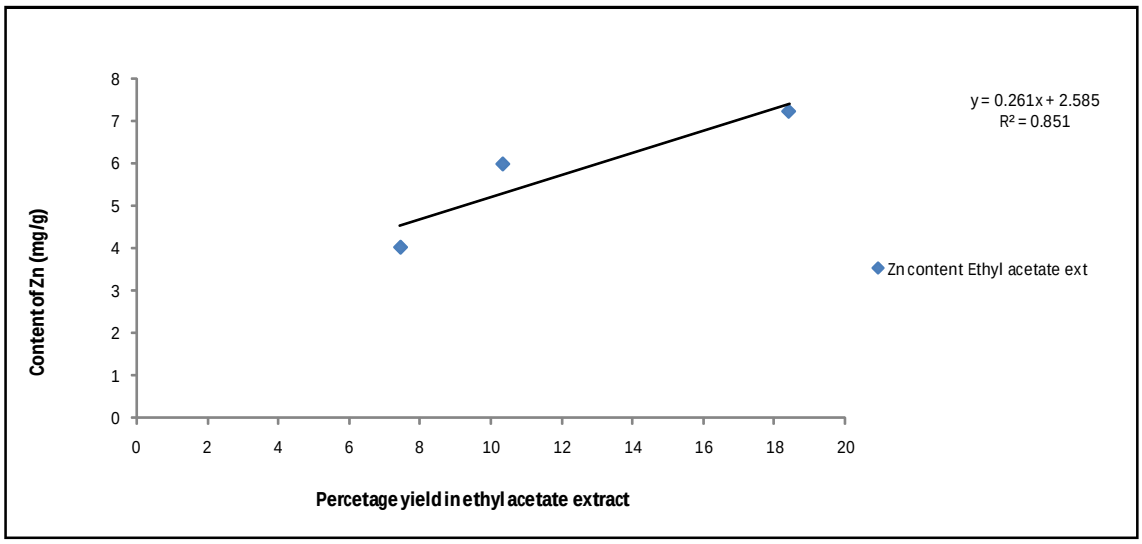

Figure 5c: Zn content in ethyl acetate extract.

3.7 Correlation study

The percentage yield of the extracts from different time intervals were calculated and further correlated with the heavy metals mainly with content of Zn estimated from the same. It was revealed that amount of heavy metals showed positive correlation with the percentage yield of the extracts (Figures 5a, b, and c) but among all methanol extract showed higher correlation.

Further, percentage yield was correlated with percent content of total phenolic and revealed significant positive correlation. Among them methanol extract showed highest correlation with percentage total phenolics (0.984*) than the others two extracts (Table 7), but all were showed significant positive correlation. The leverage graphs are presented in Figure 6 which clearly indicated the responses were affected by the time temperature and type of solvents. The response surface diagram was depicted in Figures 7a and 7b.

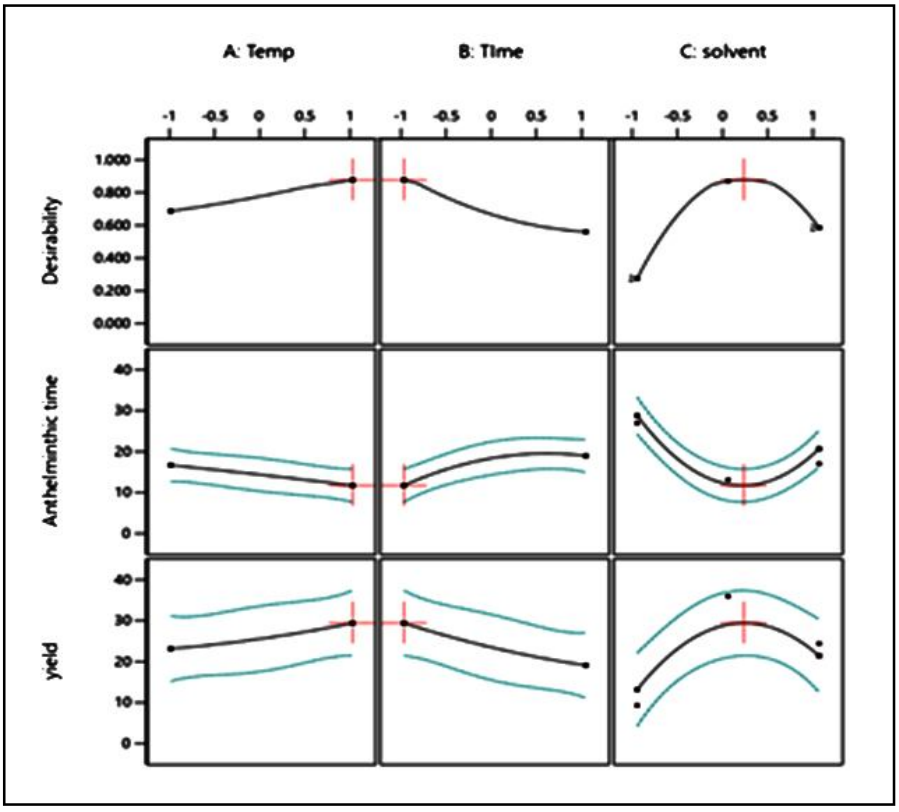

Figure 6: The leverage graphs.

Table 7: Correlation coefficient between percentage yield and percentage phenolic content

	% yield Meth	% phenol content (Meth)	% yield Aqu	% phenol content (Aqu)	% yield Ethyl acetate	% phenol content (Eth Acet)
% yield Meth	1					
% phenol content (Meth)	0.984*	1				
% yield Aqu	0.954*	0.909	1			
% phenol content (Aqu)	0.968*	0.993*	0.918	1		
% yield Ethyl acetate	0.966	0.928	0.999*	0.931	1	
% phenol content (Eth Acet)	0.983*	0.938	0.984*	0.922	0.913	1

Significant at *p<0.05.

Thereafter, a custom design was evaluated at p<0.05 and the model was found to be significant as presented in Table 8.

Table 8: Statistical data analysis of yield Vs anthelmintic activity

Response	Source	Sum of squares	df	Mean square	F-value	p-value	
Anthelmintic time	Quadratic model	952.40	9	105.82	13.46	< 0.0001	Significant
yield	Quadratic model	816.61	9	90.73	2.95	0.0472	Significant

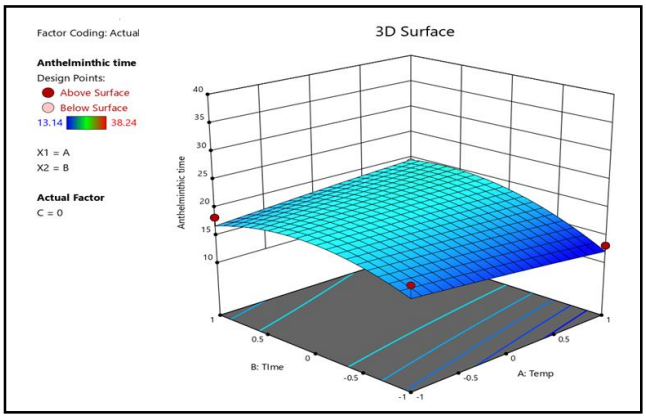

Figure 7a: Response surface diagram for yield variation with respect to time and temperature.

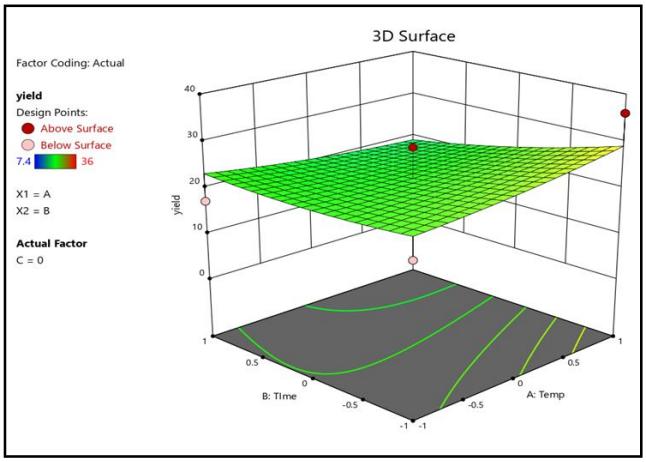

Figure 7b: The response surface diagram for anthelmintic activity with respect to time and temperature.

Optimization of the design at p<0.05 considering fast anthelmintic activity and maximum yield has resulted a prediction of desirability 0.878 as presented in Table 9.

Table 9: Predicted design optimization

Sl.No.	Temp	Time	solvent	Anthelmintic activity with respect to death time	yield	Desirability	
1	1.000	-1.000	0.176	11.758	29.443	0.878	Selected

4. Discussion

4.1 Yield of the extract

Plant bioactive components play important role in various therapeutic activities. The content of phytoconstituents are mainly varied with the extraction procedure and many literatures revealed that microwave oven extraction provides suitable extraction in high selectivity and less time, than any other conventional extraction (Osorio-Tobon, 2020). In the present investigation, methanol extract gave high percentage of yield, followed by aqueous solvent. It was already reported that microwave oven favours polar molecules and solvents with high dielectric constant (Kaufmann and Christen, 2002). Methanol is polar solvent than ethyl acetate and, hence more yield than organic solvent but even more than aqueous solvent. The same result already reported by earlier literature where female Coscinium flower gave higher yield in methanol extract than aqueous when extracted for 25 min with 10 ml of solvent (Roopashree *et al.*, 2021).

4.2 Heavy metal estimation

Determination of heavy metals present in plants are most important aspect for understanding their role in many enzymatic activities in accumulation of bioactive constituents as well as toxicity due to the presence of any unwanted heavy metals. Various elements such as iron (Fe) zinc (Zn), copper (Cu), manganese (Mn), and chromium

(Cr) are essential nutrients and also important for the physiological and biological functions of the human body. Fe is an essential element in haemoglobin and also helps in porphyrin synthesis, transport of oxygen as well as functioning of the immune system (Rout and Sahoo, 2015). Mn helps in bone and tissue formation, and skeletal growth and also acts as lipid and carbohydrate metabolism regulator (ATSDR, 2012). Cu is essential to maintain metabolism of the human body but in high content leads toxic effect. Zn is an essential element that plays a key role in plant growth, stabilizing RNA and DNA structure, in maintaining the activity of DNA synthesizing enzymes, helps in accumulation of bioactive compounds especially phenolic constituents, *etc*. (Kumar *et al*., 2019). Thereafter, some non-essential heavy metals such as As, Hg, Ni, Co, Cd and Pd are causes toxicity in human body beyond the permissible limits as per WHO guideline. In the present study, these toxic heavy metals are reported as not detected which indicated the safe application of the bark extract.

4.3 Phytochemical study

All the extracts were screened for presence of phytoconstituents and revealed methanol extract showed presence of maximum number of phytoconstituents. The identification of phytoconstituents are mainly depends on the solubility of the constituents in the specific solvent. Though, the methanol and aqueous are polar solvents but maximum phytoconstituents are more soluble in methanol than aqueous and other organic solvents (Iloki-Assanga *et al*., 2015; Dieu-Hien *et al*., 2019). The same trend also followed in the present study.

4.4 Total phenolic content

In the present study, methanol extract showed higher phenolic content than other extracts. Many literatures revealed the content of phenolics showed higher in methanol extract than other solvents (Quy-Diem, *et al*., 2014; Esmaeili *et al*., 2015) and the same trend followed in the present investigation. Moreover, it was also evident that microwave oven is more suitable for extraction of phenolic components from the plant parts (Hong *et al*., 2001; Gallo *et al*., 2010; Putnik *et al*., 2015). In this experiment, microwave oven was used for the extraction and estimated high content of phenolics in the methanol extract when extracted for 10 min at 80°C. It was also reported that higher temperature increase yield of the extract (Yaakob *et al*., 2020). The same trend followed in our experiment.

4.5 Chromatographic study

TLC was performed for all the extracts based on percentage yield and phenolic content of the extracts. All the extracts (10 min at 80° C) showed presence of some components but methanol extract showed presence of phenolic acid, *i.e*., gallic acid in the selected solvent system (toluene, ethyl acetate and formic acid with the ratio of 7: 5: 1). The same solvent system was used earlier for HPTLC determination of polyphenolic compound from Fenugreek seeds and HPLC estimation of gallic acid from *Abutilon indicum* leaves respectively, (Laila *et al*., 2014; Das *et al*., 2019). Thereafter, HPLC analysis data revealed methanol bark extract gave higher accumulation of gallic acid than other solvents which was the same as the earlier scientific report (Mehta *et al*., 2017; Das *et al*., 2019).

4.6 Anthelmintic activity

All the extracts were evaluated for anthelmintic activity and resulted higher activity with the methanol extract with respect to death of earthworms in very less time. In the present study, earthworms were selected for the anthelmintic activity due to the physiological similarity between some intestinal round worms infecting man (Awad, 2004). The higher anthelmintic activity was revealed with the increasing in concentration with all the extracts. The plant possesses significant anthelmintic activity due to presence of more polar bioactive phytochemicals such as tannins, phenolics, alkaloids, glycosides, *etc*., in the polar solvents (Stankovic, 2011). Our study also trend the similar results as reported earlier. Commonly alcoholic extracts are reported for their potent biological activities and inhibits the worms by the efficient release of phenols (Das *et al*., 2017). The phenolic acid such as gallic acid was identified in CR bark extract and also reported high content of gallic acid in methanol extract than others. The gallic acid helped in the same where many literatures revealed that gallic acid is responsible for anthelmintic activity (Patel *et al*., 2015).

4.7 Correlation study

All the extracts were correlated with percentage yield and percentage phenolic content. The result showed less time with high temperature provided the high content of both and also showed methanol extract revealed high content as compared to others. The correlation showed significantly positive with respect to extracts. The high content of metals is also positively correlated with the extracts. Thereafter, design expert software resulted the polarity of the solvent nearing zero that indicates selection of methanol as extracting solvent, hence the optimized extraction was carried out at 80°C, for 10 min with methanol and observed that the prediction was correct and significant based on the investigated results.

5. Conclusion

The present study revealed that CR bark has anthelmintic activity. Methanol extract showed higher yield than aqueous and ethyl acetate solvent when extracted with microwave oven extraction using fixed solvent but differed in time and temperature. Thereafter, methanol extract also showed higher content of metals especially Zn which helps in accumulation of phenolics as bioactive agent in CR bark and showed positive correlation with the yield and percentage of phenolic content. Furthermore, phenolic acid was identified with TLC, HPLC studies and revealed the presence of gallic acid that helped in enhanced anthelmintic activity. Finally, the study was confirmed with statistically tabulated a custom design where significant contribution of temperature and time for yield and anthelmintic activity.

Conflicts of interest

The authors declare that there are no conflicts of interest relevant to this article.

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