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EVALUATION OF PHYTOCHEMICAL, ANTIOXIDANT, ANTHELMINTIC AND ANTIMICROBIAL PROPERTIES OF *CRATAEVA NURVALA* BUCH. HAM. LEAVES

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ABSTRACT: The aim of this study was to determine principle phytochemical classes and total phenolic contents and to investigate antioxidant, anthelmintic and antimicrobial activities of ethanolic extract of *Crataeva nurvala* leaves. Standard test methodology suitable for each chemical class was followed to screen out major chemical classes preliminarily. The total phenolic content of ethanolic leaf extract was determined using Folin-Ciocalteu colorimetric assay. DPPH free radical scavenging assay and total reducing power test were carried out to investigate the antioxidant capacity. The conventional earthworm method and the Kirby-Bauer disc diffusion test were also performed to assess anthelmintic and antimicrobial activities of the extracts, respectively. Phytochemical screening of the ethanolic leaf extract showed the presence of significant amount of flavonoids and steroids along with other phyto-constituents in minor amount. Folin-Ciocalteu assay showed that 45.53 mg gallic acid equivalent was present per gram of the dried extract of the leaf. Leaf extract exhibited noticeable antioxidant activity in both of the DPPH free radical scavenging assay and the total reducing power test where ascorbic acid and BHT were used as standard compounds, respectively. The earthworm test demonstrated an excellent anthelmintic activity of the leaf extract like albendazole, especially at the high dose (1.0%). The disc diffusion assay did not show any antimicrobial activity of the leaf extract against different microbes. This study provides the evidence that the ethanolic extract of *Crataeva nurvala* leaf can be a potential source of natural antioxidant and anthelmintic molecules.

INTRODUCTION: Today's medicinal world is enriched with numerous drugs. However, the quest for new drugs with better efficacy and fewer side effects has always been the concern of the scientific community to fight against the diseases that are still incurable.

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In developing countries, more than one-third of the population lacks access to essential medicines [1] due to their inability to afford the expenses of drugs. Moreover, growing number of patients rely on alternative medicines for preventive purpose or for palliative care in wealthy countries [1]. So, the search for cheap but effective drugs is very important and a lot of researchers are working to screen out new drug candidates from traditionally used plants and to explore their mechanism of action.

Crataeva nurvala Buch. Ham. (Capparidaceae) is a high value medicinal plant [2] and it is widely distributed throughout Bangladesh, India and other

tropical regions of the world [3]. The plant is commonly known as 'Barun' or 'Barna' [4] in Bangladesh. Different parts of this plant are used as an ingredient in different formulations of ayurveda to treat prostatitis, prostate enlargement, other inflammatory conditions [4], thyroid problems, paralysis and urinary tract problems [5]. It is also used traditionally in the treatment of urolithiasis [6], carbuncle [7], nephritic disorders [8], breast cancer [9] and as an oral contraceptive [10].

Phytochemical studies of *C. nurvala* revealed the presence of various types of compound in its different parts. The bark of this plant possesses cadabicine, cadabicine diacetate, phragmalin triacetate, lupeol, lupenone, succinic acid, mannitol, lactic acid, betulinic acid, *β*-sitosterol and stigmasterol [11-15]. It's root contains lupeol, *β*-sitosterol and varunol [16], while the leaf investigation showed the presence of dodecanoic anhydride, methyl pentacosanoate, kaemferol-3-*O*-*α*-D-glucoside and quercitin-3-*O*-*α*-D-glucoside [17]. Its fruit possesses pentadecane, octanamide, 12-tricosanone and friedelin [18]. Heneicosane, 1-octadecanol, methyl pentanoate, 1-eicosanol and 9-heptadecanone were also isolated from the flowers of this plant [19].

A constituent of *C. nurvala* bark, lupeol, showed antioxidant [20, 21], cardio-protective [22] chemopreventive [21] and chemotherapeutic [23] properties. The bark part also exhibited antidiabetic [24], antidiarrhoeal [25], antifertility [26] and analgesic [27] properties. The leaves of this plant have sedative [3], cytotoxic [3], antidiarrhoeal [28], analgesic [28] and diuretic [29] activities. Antibacterial [31], anthelmintic [31] and wound healing [32] properties were observed in the root part of this plant. There is no reported study on antioxidant, anthelmintic and antimicrobial properties of *C. nurvala* leaves. Therefore, this study was designed to investigate these activities along with the identification of the major chemical classes present in the leaf of this plant.

MATERIALS AND METHODS:

Collection and Identification of the Plant Material:

Fresh leaves of *Crataeva nurvala* Buch. Ham. were collected from Shreepur, Gazipur, Bangladesh on December 2009. The plant leaves were identified at National Herbarium, Dhaka, Bangladesh and a voucher specimen (Accession no. DACB 35292) was deposited there.

Preparation of the Plant Material:

The leaves were cleaned properly and sun dried for two weeks. After complete drying, the leaves were pulverized into coarse powder using a grinder. The powder was stored in an airtight container and kept in a cool and dry place until it was soaked.

Preparation of the Plant Extract:

About 350 g of the powdered leaves was soaked in 1 L of 95% ethanol in a glass container. The container was sealed and kept in a dry place for a period of 7 days accompanying with occasional shaking and stirring. A coarse filtration was done using cotton and the final filtrate was obtained by filtering through Whatman filter paper No.1. The solid residue of ethanolic extract of *C. nurvala* leaves was then obtained using a rotary evaporator (IKA, Germany) at 50 ˚C and 50 rpm under reduced pressure and air dried process subsequently. Finally the dry extract was kept in a refrigerator for further use.

Experimental Worms and Microorganisms:

For the preliminary anthelmintic study, adult earthworms (*Pheretima posthuma*) were collected from moist soil and washed with normal saline to remove all fecal material. The earthworms of 3 - 5 cm in length and 0.1 - 0.2 cm in width were used for all experimental protocols due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings.

For the antimicrobial assay, two Gram-positive bacteria (*Bacillus cereus* and *Bacillus subtilis),* four Gram-negative bacteria (*Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Shigella dysenteriae)* and one fungus (*Candida albicans)* were selected. These microbes were available in the Microbiology laboratory of the Pharmacy department, East West University, Dhaka, Bangladesh.

Phytochemical Screening:

The ethanolic dry extract was tested qualitatively to screen out the major chemical classes such as

alkaloids, flavonoids, steroids, saponins, tannins, anthraquinones, reducing sugar and cardiac glycosides using standard procedures [32, 33].

a. Dragendroff's test for alkaloids:
About 0.1 g of the plant extract was dissolved in 1% hydrochloric acid (5 ml) on a steam bath. The filtrate (2 ml) was treated with few drops of Dragendroff's reagent (potassium iodide 0.11 M, bismuth nitrate 0.6 M in acetic acid 3.5 M). Turbidity or precipitation was taken as indicative of the presence of alkaloids.

b. Borntrager's test for anthraquinone:
The hydro-extract of the plant material (equivalent to 100 mg) was shaken vigorously with 10 ml of benzene, filtered, and 5 ml of 10% ammonia solution was added to the filtrate. The mixture was shaken and the appearance of a pink, red, or violet color in the ammonia (lower) phase was indicating the presence of free anthraquinone.

c. Keller Kiliani's test for cardiac glycoside:
The extract (about 0.5 g) was dissolved in glacial acetic acid (2 ml) containing 1 drop of 1% ferric chloride followed by the addition of 1 ml concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxy-sugar, characteristic of cardiac glycosides. A violet ring may form just above the ring and gradually spreads through this layer.

d. Test for flavonoids: (1) Free flavonoids test:
Five milliliters of ethyl acetate was added to a solution of 0.5 g of the extract in water. The mixture was shaken, allowed to settle, and inspected for the production of yellow color in the organic layer which is taken as positive for free flavonoids. **(2) Lead acetate test:** To a solution of 0.5 g extract in water about 1 ml of 10% lead acetate solution was added. Production of yellow precipitate is considered as positive for flavonoids. **(3) Reaction with sodium hydroxide:** Dilute sodium hydroxide solution was added to a solution of 0.5 g of the extract in water. The mixture was inspected for the production of yellow color which is also considered as positive test for flavonoids.

e. Test for phlobatannins:
When an aqueous extract of the plant part was boiled with 1% aqueous hydrochloric acid, deposition of a red precipitate was taken as an evidence for the presence of phlobatannins.

f. Test for reducing sugar: Both Fehling's solutions I and II (1 ml each) were added to an aqueous solution of the extract (0.5 g in 2 ml distilled water). The mixture was heated in a boiling water bath for about 2 – 5 min. The production of a brick red precipitate indicated the presence of reducing sugars.

g. Frothing test for saponins: The extract (about 0.5 g) was boiled with 5 ml of distilled water in a water bath for 10 min and then filtered. The filtrate (1 ml) was diluted 5 times with 4 ml distilled water and shaken vigorously for 2 min to form a stable persistent froth. Appearance of frothing indicated the presence of saponin in the filtrate.

h. Lieberman's test for steroidal compounds:
About 0.5 g extract was dissolved in 2 ml of acetic anhydride and cooled well in an ice-bath. Concentrated sulfuric acid was then carefully added. A color change from purple to blue to green indicated the presence of a steroidal compound.

i. Ferric chloride test for tannins: Plant extract (about 0.5 g) was dissolved in 2 ml distilled water and filtered using Whatman No.1 filter paper. The presence of blue color following the addition of 10% ferric chloride solution to the clear filtrate indicated the presence of tannins.

Total Phenolic Content:
Total phenolic content of the extract was estimated using Folin-Ciocalteu colorimetric assay [34] with some modification. About 0.5 ml of the methanolic solution of crude extract at concentration of 1 mg/ml was mixed with 5 ml of 0.2 mol/L Folin-Ciocalteu reagent (1:10 in distilled water) for 4 min. Then 5 ml sodium carbonate solution (7.5%) was added to the reaction mixture. The absorbance was taken at 760 nm after incubation at room temperature for 20 min. The estimation of the

phenolic compounds was carried out in triplicate. Gallic acid was used as standard and the total phenolic content was determined from extrapolation of a calibration curve for gallic acid solution (0 - 100 µg/ml). The result was expressed as milligrams of gallic acid equivalent (GAE) per g of the dried sample.

Antioxidant Activity:

DPPH free radical scavenging assay: The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay is a decolorization assay that measures the capacity of antioxidants. DPPH is a stable organic nitrogen centered free radical with a dark purple color; however, when reduced to its non-radical form by antioxidants, it becomes colorless [35]. The odd electron in the DPPH free radical gives a strong absorption at 517 nm and the absorption is reduced when this odd electron of it is paired with a hydrogen from a free radical scavenger i.e. antioxidant and forms the reduced DPPH-H. The DPPH free radical scavenging assay [36] was carried out by taking 0.05 ml of DPPH methanol solution (0.04% w/v) to different concentration (10 - 100 µg/ml) of extract with final volumes 5 ml and was allowed to react at room temperature in dark for 20 min.

In this test, ascorbic acid was used as a standard. After 20 min, the absorbance of all test samples was taken at 517 nm using UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan). The free radical scavenging effect was calculated as follows:

Scavenging effect (%) = [(control absorbance - sample absorbance) /control absorbance] × 100

Total reducing power: The reducing power assay of the extract was estimated by the method described by Saeed *et al* [37]. It often serves as a significant indicator of its potential antioxidant activity. In this assay, the ability of the extract to reduce Fe^{3+} to Fe^{2+} was determined. The presence of antioxidant(s) in the extract results in the reduction of the ferric (Fe^{3+}) cyanide complex to the ferrous (Fe^{2+}) cyanide form, thereby changing the color of the solution in various shades from green to blue, depending on the reducing power of the compound(s) present in the extract. Strong reducing agents, however, form Perl's Prussian blue color which is absorbed at 700 nm [38]. A definite amount (2.5 ml) of various concentrations (6.25 - 200 µg/ml) of ethanolic extract and standard [butylated hydroxytoluene (BHT)] samples was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (10 mg/ml) and incubated at 50 °C for 20 min. The mixture was cooled and then 2.5 ml of 10% tricholoroacetic acid was added followed by the centrifugation at 3000 rpm for 10 min.

The supernatant (2.5 ml) was collected from the upper layer and mixed with 2.5 ml of distilled water. Then 0.5 ml of freshly prepared 0.1% ferric chloride was added to it and the absorbance was measured at 700 nm against a blank using the UV-visible spectrophotometer after 10 min.

Anthelmintic Activity:

The anthelmintic activity [39] was estimated by placing the earthworms (*Pheretima posthuma*) in Petri dishes containing two different concentrations (0.5% and 1.0%) of aqueous suspension of the dried leaf extract of *Crataeva nurvala*. Two worms were placed in each Petri dish and observed for paralysis or death. The paralysis time was recorded when any sort of movement of worms was not observed for at least 30 seconds except when they were shaken vigorously. The death time was recorded after ascertaining the worms moved neither when shaken vigorously nor when dipped in the warm water (50 ºC). The values of test samples were compared with that of values obtained from the standard drug, albendazole (Square Pharmaceuticals Limited, Bangladesh), treated group.

Antimicrobial Activity:

Disc diffusion method [40, 41] was performed for the screening of antimicrobial property of *C. nurvala* leaves against seven different microorganisms. Various amount of extract (50 - 2000 µg) was impregnated on sterile filter paper (Whatman filter paper No.1) disc using suitable solvent system and kept for air drying. Standard drug, Kanamycin (30 µg/disc) was used as positive control. Two test sample discs, standard antibiotic disc and blank disc were placed in an agar media previously inoculated with 100 µl suspension of specific microorganism. The plates were incubated at 37 °C

for 24 h and the antimicrobial activity was assessed by measuring the diameter of zone of inhibition.

Statistical Analyses:

Data were analysed and the figures were plotted using Microsoft Excel version 2007. Results were expressed as mean ± SD (standard deviation) where applicable. Statistical differences were estimated by one-way analysis of variance (ANOVA) followed by Bonferroni's test. A *p* value of 0.05 or less was considered to be significant.

RESULTS:

Phytochemical Screening:

The ethanolic extract of *C. nurvala* leaves showed the presence of alkaloids, flavonoids, reducing sugar, saponins, steroids, tannins as chemical constituents. Among all these, steroids and flavonoids were present in considerable amount. The results were shown in **Table 1.**

TABLE 1: PHYTOCHEMICAL SCREENING OF ETHANOLIC LEAF EXTRACT OF *CRATAEVA NURVALA*

Phytoconstituents	Tests performed	Observation	Result
Alkaloids	Dragendroff's test	Red precipitation	+
Anthraquinone	Borntrager's test	Pink/violet coloration	-
Cardiac glycosides	Keller Kiliani's test	Brown ring at the surface	-
Flavonoids	Free flavonoid test	Yellow coloration	++
	Lead acetate test	Yellow precipitation	++
	Reaction with NaOH	Yellow coloration	++
Phlobatannin	-	Red precipitation	-
Reducing Sugars	Fehling's test	Red precipitation	+
Saponins	Frothing test	Emulsion formation	+
Steroids	Liberman's test	Green coloration	+++
Tannins	Ferric chloride	Blue coloration	+

Total Phenolic Content

The total phenolic content of ethanolic extract was calculated using the standard curve (y = 0.005x + 0.130; R^2 = 0.996) for gallic acid. The experiment revealed the presence of 45.53 mg gallic acid equivalent (GAE) per gram of the dried extract of leaves (**Table 2**).

TABLE 2: TOTAL PHENOLIC CONTENT OF LEAF OF *CRATAEVA NURVALA*

Absorbance of Extract	Total Phenolic Content (mg GAE/g)	
	Individual value	Mean ± SD
0.369	47.8	45.53 ± 2.2
0.347	43.4	
0.357	45.4	

Antioxidant Activity:

DPPH free radical scavenging assay:

The free radical scavenging activity of the ethanolic extract was found to increase with increasing concentration as shown in **Figure 1**. At higher concentration (100 µg/ml), the extract showed maximum scavenging activity of 98.99% whereas ascorbic acid exhibited almost similar kind of scavenging activity in a wide range of concentration. This is perhaps, small amount (40 µg per ml) of ascorbic acid was enough to neutralize the given quantity of DPPH free radical and the extra amount (more than 40 µg per ml) of ascorbic acid present in standard samples did not show any additional effect as antioxidant. The IC_{50} values of the extract and ascorbic acid were 2.17 and 23.79 µg/ml respectively.

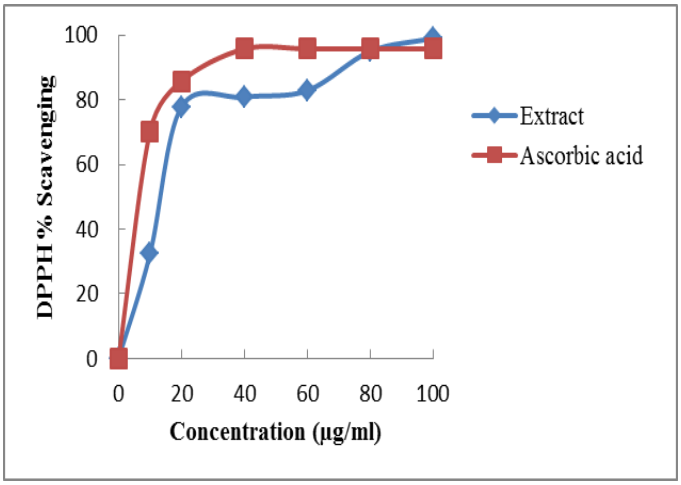

FIG. 1: DPPH SCAVENGING ACTIVITY OF *CRATAEVA NURVALA* LEAVES

Total Reducing Power:

In this method, higher absorbance of the sample mixture indicates strong reducing capability. In **Figure 2**, it was observed that ethanolic extract possessed nearly the same capacity to reduce ferric

ion (Fe^{3+}) to ferrous ion (Fe^{2+}) like BHT at lower concentration (6.25 - 100 µg/ml), but it showed greater reducing potential at high concentration (200 µg/ml) than that of the standard.

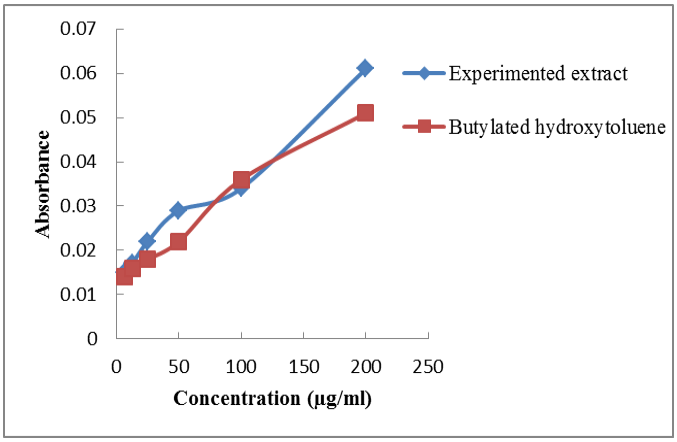

FIG. 2: TOTAL REDUCING CAPACITY OF ETHANOLIC LEAF EXTRACT OF *CRATAEVA NURVALA*

Anthelmintic Activity:

At doses of 0.5% and 1.0%, the ethanolic leaf extract took 79 and 73.25 min and albendazole took 107 and 67.5 min to paralyse the earthworms, respectively (**Figure 3**). The ethanolic leaf extract did not show statistically significant (p>0.05) time difference to paralyse the earthworms as compared to that of albendazole. In case of death of earthworms, the ethanolic leaf extract took 186 min and albendazole took 250 min at the 0.5% dose which were statistically significant (p<0.01). But at the dose of 1.0%, the leaf extract showed no time differences statistically (p value 1.00) to kill the earthworms while compared to albendazole.

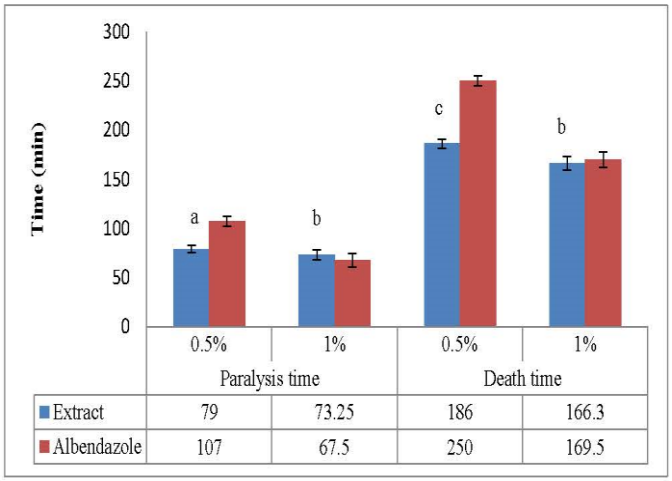

FIGURE 3: ANTHELMINTIC ACTIVITY OF LEAF EXTRACT OF *CRATAEVA NURVALA* ON EARTHWORMS (*PHERETIMA POSTHUMA*).

Values were represented as Mean ± SD; n = 4 in each group. Statistical difference was assessed between standard versus treated group using one-way analysis of variance (ANOVA) followed by Bonferroni's test where, [a]p<0.001; [b]p>0.05; [c]p<0.01

Antimicrobial Activity:

The ethanolic leaf extract of *Crataeva nurvala* demonstrated no antimicrobial activity against different microbes. There was no clear zone around the test disc whereas standard drug, kanamycin, showed clear zone of inhibition. The results were depicted in **Table 3**.

TABLE 3: ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACT OF *CRATAEVA NURVALA* LEAVES

Species	Zone of Inhibition (Diameter in mm)						
	Extract (µg/disc)						Kanamycin
	50	100	200	400	1000	2000	(30 µg/disc)
Bacillus cereus	0	0	0	0	0	0	25
Bacillus subtilis	0	0	0	0	0	0	30
Salmonella typhi	0	0	0	0	0	0	25
Escherichia coli	0	0	0	0	0	0	28
Pseudomonas aeruginosa	0	0	0	0	0	0	27
Shigella dysenteriae	0	0	0	0	0	0	30
Candida albicans	0	0	0	0	0	0	26

DISCUSSION: The ethanolic extract of *Crataeva nurvala* leaves possesses most of the phytoconstituents. But it is rich in steroids and flavonoids. The total phenolic content experiment also revealed the presence of phenolic compounds in terms of gallic acid equivalent (GAE). The bleaching of purple colored solution of DPPH radical is an indicator of electron donation by a radical species or antioxidant. The more the color change, the higher the potency or concentration of the antioxidant(s) present in the test sample [37].

Present study showed that the ethanolic leaf extract have prominent antioxidant activity at the higher dose (100 µg/ml) compared to that of the standard (ascorbic acid). In reducing power assay, the presence of reducing agent(s) in the test sample changes the yellow color of the solution to blue by

converting the ferricyanide complex to ferrous form. The absorbance of the formed ferrous ion was measured at 700 nm. The higher absorbance at this wavelength indicates that more powerful reducing agent was present in the test sample. The potent reductant present in the ethanolic leaf extract may be the contributing factor for the higher absorbance values compared to that of the standard compound.

The presence of phenolic compound(s) in the experimented extract might be responsible for the free radical quenching activity. The hydroxyl groups of the phenolic compound(s) confer scavenging activity [37]. Flavonoids are one of the most important classes of phenolic compounds. The antioxidant activity of flavonoids is associated with multiple features of chemical structures. Usually flavonols with free hydroxyl group at the C-3 position and double bond between C-2 and C-3 show maximum antiradical activity [42].

Previous phytochemical experiment on leaves of *Crataeva nurvala* found the existence of two flavonols named kaemferol-3-*O*-*α*-D-glucoside and quercitin-3-*O*-*α*-D-glucoside [17]. Kaemferol-3-*O*-*α*-D-glucoside showed 20-70% antioxidant activity because of having substituted hydroxyl group at the C-3 position and hydroxyl group at the C-4′ position. On the other hand, Quercitin-3-*O*-*α*-D-glucoside demonstrated strong antioxidant property, comparable to that of free flavonols. The presence of the *O*-dihydroxy system, where C-3-*O*-*α* hydroxyl group accelerates the reactivity of the hydroxyl group at C-4′, in the B-ring of its structure makes it highly effective against free radicals [42].

So it could be concluded that phenols and flavonols present in the ethanolic leaf extract might have greater contribution to the antioxidant activity.

In anthelmintic activity test, the ethanolic extract exhibited excellent anthelmintic activity against earthworms as compared to that of albendazole even at low dose (0.5%). Therefore, extensive screening and proper evaluation of the phyto-constituent(s) of the ethanolic leaf extract may be helpful to find out a promising vermicide. But antimicrobial experiments exhibited the negative outcome in different concentrations of the leaf extract against different microorganisms.

CONCLUSION: It can be summarized that the ethanolic extract of *Crataeva nurvala* leaf showed a very good potential as a source of antioxidant and anthelmintic. Further studies should be carried out to characterize the chemical constituents responsible for these activities and to establish the safety profile of those isolated compounds for *in vivo* experiments.

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