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Research Article

PHYTOCHEMICAL STUDIES AND ANTIMICROBIAL ACTIVITY OF *Cheilanthes bicolor* (Roxb.) Griff. ex Fraser Jenkins

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ABSTRACT

The present investigation was carried out to screen the phytochemical and antimicrobial properties of *Cheilanthes bicolor* collected from Agumbe area, Karnataka, India. The dried whole plant material was extracted by soxhlet method using acetone, chloroform, methanol, ethanol and aqueous solvent systems. The phytochemical screening for the presence of carbohydrates, proteins, phenols and tannins were carried out with standard protocols. Antimicrobial activity of plant was carried out with 50, 100, 200, 500 and 1000 µg/ml concentration against four bacterial strains i.e., *Salmonella typhii, Klebsiella pneumoniae, Staphylococcus aureus and Escherichia coli* with Streptomycin as positive and respective solvents as negative controls. Antifungal activity was carried out against *Aspergillus niger* and *Sclerotium rolfsii.* Among different solvent systems used aqueous yielded good quantity of extract followed by methanol and acetone. The phytochemical study showed the presence of biomolecules such as carbohydrate, protein, tannin and phenols. The chloroform extract exhibited better antimicrobial activity against *Escherichia coli* and *Salmonella typhii* and the ethanolic extract showed better activity against *K. pneumoniae* and *S. aureus.* Ethanolic extract at 50µg, 500µg, and 1000µg exhibited antifungal activity against *A.niger* and acetone extract at 50µg showed activity against *A.niger*.

INTRODUCTION

Mother Nature has a good source of medicinal agents for thousands of years and since the beginning of man. Medicinal plants are the richest source of antimicrobial agents. Many of the plants are used as traditional medicines for many different diseases over several years. Along with angiosperms several pteridophytes also employed in various traditional and ayurvedic preparations from ancient periods. Even though they are lower class plants they possess potential medicinal properties. Ferns and their allies are one of the oldest major divisions of the Pteridophyta, and comprise over 12000 species spread among 250 different genera [1]. Traditionally, the biomedical system and Ayurvedic systems of medicine, named Sushruta (*ca*. 100 AD) and Charka (*ca*. 100 AD), suggested the use of some ferns in the *Samhita* texts. Pteridophytes are also used by physicians in the Unani system of medicine [2]. In the traditional Chinese system of medicine, several ferns are recommended by native doctors [3, 4]. In more recent times, ethnobotanical and advanced pharmacological studies have been carried out on ferns and their allies by several investigators [5-14].

In an invention and documentation by Baskaran *et al*. [15], the medicinal uses of thirty different families of pteridophytes revealed their uses in various treatments. The lycophyte *Selaginella* sp. to have multiple pharmacological activity, such as antioxidant, anti-inflammatory, anti-cancer, antidiabetic, antiviral, antimicrobial, and anti-Alzheimer properties. Among all the pteridophytes examined, taxa from the Pteridaceae, Polypodiaceae, and Adiantaceae exhibited significant medicinal activity. Based on this, many pteridophytes have properties that could be used in alternative medicine for treatment of various human illnesses. Biotechnological tools can be used to preserve and even improve their bioactive molecules for the preparation of medicines against illness. The extracts of some ferns especially Adiantaceae were also used against uropathogens [16-18].The genus *Cheilanthes* is with about 180 to 200 species worldwide [19-21]. Of which about 26 species are found in India [22] and 13 species reported from Kumaon [23]. *Cheilanthes bicolor* is reported from localities like Sikkim, Darjeeling hills, Arunachal Pradesh, South and Central India - Rajasthan and Madhya Pradesh [24-26]. The aim of the present study was to evaluate the phytochemical constituents and potential antimicrobial activities of *Cheilanthes bicolor*.

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MATERIALS AND METHODS

*Cheilanthes bicolor (*Roxb.) Griff. ex Fraser Jenkins [Syn: *Aleuritopteris farinose* (Forssk.) Fée/ *Aleuritopteris bicolor* (Roxb.) Fraser Jenk]. Samples were collected from Agumbe forest, Shimoga district during August-September, identified using fern key books and herbariums were also prepared as authentic specimens. The fern was air dried and later dried in oven at 80°C for 6-8 hours. Then its course powder was prepared using mixer grinder and stored for further work. Sample extract was prepared using solvent like acetone, chloroform, ethanol, methanol and water (aqueous) by soxhlet apparatus. Twenty five grams coarse powder of the fern was extracted by soxhlet process using 150ml of respective solvent for 4-6 hours. This was followed by distillation and the extract obtained was stored at 4°C until further use. For each solvent triplicate were made. Qualitative tests for phytochemicals like carbohydrate, protein, tannin and phenols were carried out by following standard methods. Quantitative estimation of carbohydrate by DNS method, protein by Lowry's method, phenols by Catechol method and tannins by Folin-Denis method [27]. Antibacterial activity by agar well diffusion method and antifungal activity [28]. Pure cultures of bacterial stains like *Escherichia coli*, *Salmonella typhii, Klebsiella pneumoniae* and *Staphylococcus aureus* and fungal species such as *Aspergillus niger* and *Sclerotium rolfsii* were maintained in the slants used as test organisms. A loop full of culture was inoculated into nutrient broth and incubated at 37°C for 24 hours to obtain a bacterial culture. This procedure was carried for the selected bacterial cultures to obtain inoculums of particular broth culture. Petri dishes were plated with Mueller Hinton Agar media and allowed to solidify for 30 min. The test organisms were then spread on the surface of the media using sterile swab stick. Cork borer was used to bore wells in media. The aqueous and other solvent extracts of different concentrations (50µl, 100µl, 200µl, 500µl and 1000µg/ml) were separately dispensed into the wells of different plates using a micropipette. A negative control of respective solvent and a positive control of streptomycin were kept and the extract was allowed to diffuse for 30 min at room temperature. The plates were incubated at 37°C for 24 hours and the zones of inhibition were measured.

RESULTS

Solvent extraction method was used to extract the phytoconstituents from the whole plants of *Cheilanthes bicolor.* The extracts of *C.bicolor* was semi-solid to jelly liquid, brown to black in colour with respect to solvent system, aqueous and methanol yielded good quantity compared to other solvent systems (Table 1).

Table 1 Physical nature and yield* of *Cheilanthes bicolor* extracts in different solvents

xtracts	Consistency	Colour	Yield (%)
Acetone	Semi-solid	Black	10.9+0.24
Chloroform	Pellet	Brownish black	8.37+0.42
Ethanol	Greasy	Dark brown	9.5+0.53
Methanol	Greasy- solid	Greenish-black	15.7+0.29
Aqueous	Jelly-liquid	Brown	15.8+0.17

N=3. *Mean+SE

The phytochemical analysis of extracts of *Cheilanthes bicolor* showed the presence of carbohydrate, protein, phenols and tannins in all the solvent extracts (Table 2)

Table 2 Results for phytoconstituents of *Cheilanthes bicolor* extracts

Phytoconstituents	Acetone	Aqueous	Chloroform	Ethanol	Methanol
Carbohydrates	+	+	+	+	+
Proteins	+	+	+	+	+
Phenol	+	+	+	+	+
Tannins	+	+	+	+	+

+ indicates positive

The quantitative estimation of carbohydrate by DNS method showed the presence of higher carbohydrate content in methanol extract followed by ethanol (Fig.1). The protein estimation by Lowry's method showed the presence of higher protein content in methanol extract followed by ethanol (Fig 2). The phenol and tannin contents are also found to be higher in methanol extract compared to ethanol extract (Fig. 3-4). The standard graphs showed in (Fig. 1-4).

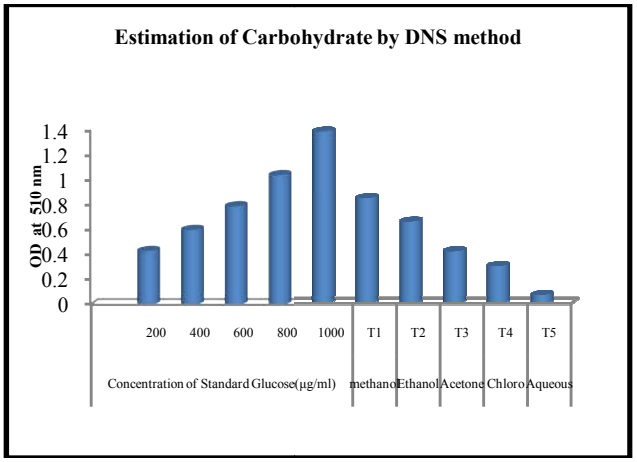

Fig 1 Estimation of carbohydrate by DNS method for *C. bicolor* extract

The concentration of carbohydrate present in methanol extract was 670µg/ml, ethanol was 450µg/ml, acetone was 340µg/ml, chloroform was 250µg/ml and aqueous was 60µg/ml. the carbohydrate content was more in methanol (Fig.1)

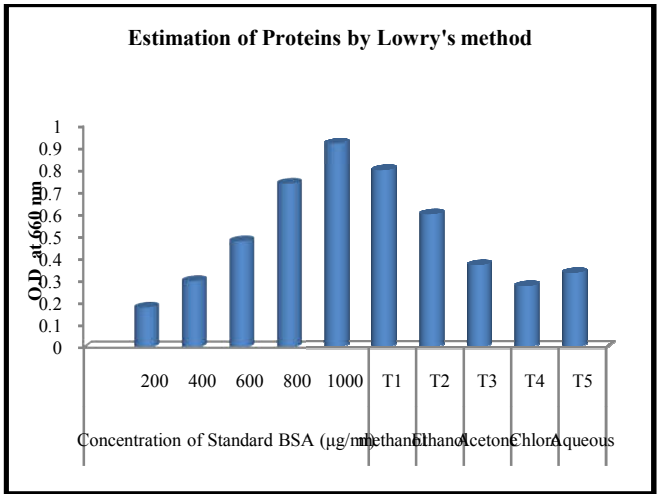

Fig 2 Estimation of protein by Lowry's method for *C. bicolor* extract

The concentration of protein present in methanol extract was 980µg/ml, ethanol was 600µg/ml, acetone was 650µg/ml, chloroform was 300µg/ml and aqueous was 400µg/ml. The protein content was more in methanol (Fig. 2).

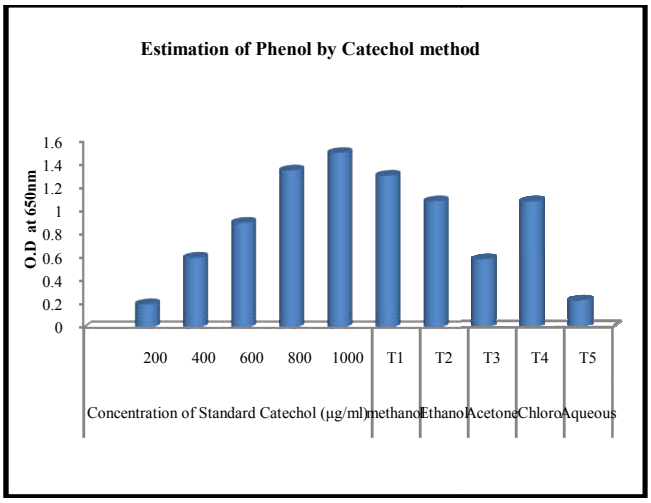

Fig 3 Estimation of phenol by Catechol method for *C.bicolor* extract

The concentration of phenol present in methanol extract was 940µg/ml, ethanol was 750µg/ml, acetone was 380µg/ml, chloroform was 680µg/ml and aqueous was 150µg/ml. The protein content was more in methanol (Fig. 3).

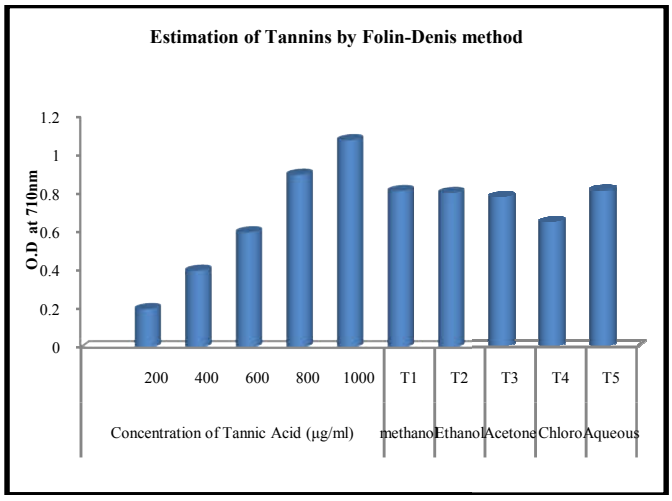

Fig 4 Estimation of tannins by Folin-Denis method for *C.bicolor* extract

The concentration of tannin present in methanol extract was 950µg/ml, ethanol was 820µg/ml, acetone was 750µg/ml, chloroform was 700µg/ml and aqueous was 800µg/ml. The protein content was more in methanol (Fig. 4).

The results for antibacterial activity of aqueous, ethanol, methanol, acetone and chloroform extracts of *Cheilanthes bicolor* were tested against four pathogens *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus* and *Salmonella typhii* showed varied level of sensitivity in terms of zone of inhibition as given in table 3. Aqueous extract was inactive against all the strains tested. The methanolic extract was apparently active against *E.coli, S.aureus* and *S.typhii*. The maximum zone of inhibition (9mm) was observed in 1000µg concentration against *K.pneumoniae.* The ethanolic extract was active against all the bacterial strains, inactive against *E.coli* and *S.typhii*. Maximum zone of inhibition (7mm) was observed in 1000µg concentration against *K. pneumoniae*. The acetone extract was active against *K.pneumoniae* and *S.aureus*, apparently active against *S.typhii*, inactive against *E. coli*. The maximum zone of inhibition (10mm) was observed in 1000µg concentration against *K.pneumoniae*. The chloroform extract was active against *E.coli* and *S.typhii,* apparently active against *K. pneumoniae* and inactive against *S.aureus*. The maximum zone of inhibition (19mm) was observed in 100µg and 200µg concentration against *E.coli*, and maximum zone of inhibition (20mm) was observed in 100µg, 200µg, 500µg, 1000µg concentration against *S.typhii*. The maximum zone of inhibition was observed in chloroform extract with a diameter of 19 mm with *E.coli*. The other organisms showed low to medium activity with other solvents and concentrations.

All five extracts of plants were further subjected to monitor antifungal activities against two fungal agents *Aspergillus niger* and *Sclerotium rolfsii* (Table 4). The antifungal activity of aqueous, ethanol, methanol, acetone and chloroform were recorded. The aqueous extract of plant was inactive against both the organisms. The maximum percentage of inhibition (68.0) was observed in 200µg concentration against *A.niger* and maximum percentage of inhibition (52.0) was observed in 200µg concentration against *S. rolfsii.* The methanolic extract was inactive against *A. niger* and active against *S. rolfsii*. The maximum percentage of inhibition (72.0) was observed in 100µg concentration against *A.niger* and the maximum percentage of inhibition (97.0) was observed in 50µg and 1000µg concentration against *S. rolfsii*. The ethanolic extract was active against both the organisms. The maximum percentage of inhibition (99.0) was observed in 500µg concentration against *A.niger* and the maximum percentage of inhibition (97.0) was observed in 50µg concentration against *S. rolfsii*. The acetone extract was active against both the organisms. The maximum percentage of inhibition (85.0) was observed in 50µg concentration against *A. niger* and the maximum percentage of inhibition (98.0) was observed in 200µg concentration against *S. rolfsii*. The chloroform extract was inactive against both the organisms. The maximum percentage of inhibition (64.0) was observed in 1000µg concentration against *A. niger* and the maximum percentage of inhibition (86.0) was observed in 500µg concentration against *S. rolfsii*.

DISCUSSION

The recent years, although technology and medicine have developed extensively, some countries have made it obligatory to use natural products for many different purposes due to decrease in natural products for many different purposes due to decrease in natural richness and drawbacks. The plants known by people with health benefits are picked up and used for the treatment of various diseases.The aqueous solvent yielded better yield followed by methanol. The extracts of *C. bicolor* were semi-solid to jelly liquid with respect to solvent system and intermediate.

The phytochemical analysis of extracts of *Cheilanthes bicolor* showed the presence of carbohydrate, protein, phenols and tannins in all the solvent extracts. The qualitative phytochemical analysis of methanolic extract of fern *Hemionitis arifolia* by Rakkimuthu *et al*. [29] revealed the presence of phenols, saponins, tannins, steroids, flavonoids, glycosides and carbohydrates. In another study by Irudhayaraj *et al*. [30] in the methanol extract of *Selaginella inaqualifolia* where they showed the presence of steroids, tannin, triteropenoids and saponins. Similarly, Ishaq *et al.* [31] in methanol extract of *Adiantum capilus* and Herin *et al*. [32] in

methanol extract of *Pteris argyreae, P.confuse, P.vittata, P.biaurita* and *P.multiauriita* reported phytochemicals like steroids, alkaloids, saponins, flavonoids, triteropenoids and phenolic. Nair *et al*. [33] reported the presence of phenolics, tannin, carbohydrates, steroid and saponin, xanthoprotein, flavonoid, protein and carboxylic acid in different extracts of *Azolla pinnata*, *Marsilea minuta* and *Salvinia molesta.* There are few other reports also deals the phytoconstituents of ferns [34, 35].

The methanol extract of *in vivo* frond of *Hemionitis arifolia* was analysed by Rakkimuthu *et al.* [29] for antibacterial activity against *Enterococcus faecalis, E.coli, P.aeruginosa, Bacillus* sp. and *K.pneumoniae*. They compared the results with antibiotic ampicillin, which was used as positive control the DMSO served as negative control.

Table 3 Antibacterial activity of *Cheilanthes bicolor* extracts

Extracts	Concentration (µg/ml)	Zone of inhibition (mm)			
		Escherichia coli	*Salmonella typhii*	*Klebsiella pneumoniae*	*Staphylococcus aureus*
Aqueous	Streptomycin	15.0	15.0	17.0	17.0
	Water	-	-	-	-
	50	-	-	-	-
	100	-	-	-	-
	200	-	-	-	-
	500	-	-	-	-
	1000	-	-	-	-
Methanol	Streptomycin	17.0	17.0	17.0	12.0
	Methanol	-	-	-	-
	50	-	-	3.0	-
	100	-	-	4.0	-
	200	-	-	1.0	1.0
	500	3.0	3.0	3.0	3.0
	1000	5.0	-	9.0	-
Ethanol	Streptomycin	13.0	6.0	13.0	11.0
	Ethanol	-	-	-	-
	50	-	-	2.0	3.0
	100	-	-	4.0	3.0
	200	-	-	1.0	3.0
	500	-	-	5.0	5.0
	1000	-	-	7.0	2.0
Acetone	Streptomycin	5.0	3.0	15.0	11.0
	Acetone	-	-	-	-
	50	-	-	1.0	2.0
	100	-	-	1.0	1.0
	200	-	1.0	2.0	2.0
	500	-	-	1.0	2.0
	1000	-	-	10.0	1.0
Chloroform	Streptomycin	5.0	7.0	8.0	5.0
	Chloroform	-	1.0	-	-
	50	1.0	5.0	-	-
	100	19.0	20.0	-	-
	200	19.0	20.0	-	-
	500	15.0	20.0	-	-
	1000	5.0	20.0	5.0	-

Table 4 Antifungal activity of *Cheilanthes bicolor* extracts against *Aspergillus niger* and *Sclerotium rolfsii*

Extracts	Concentration (µg/ml)	Percentage of inhibition	
		Aspergillus niger	*Sclerotium rolfsii*
Aqueous	50	64	20
	100	63	10
	200	68	52
	500	62	24
	1000	61	20
Methanol	50	28	97
	100	72	89
	200	28	85
	500	20	95
	1000	15	97
Ethanol	50	88	97
	100	50	92
	200	67	89
	500	99	92
	1000	86	91
Acetone	50	85	71
	100	36	80
	200	73	98
	500	36	90
	1000	77	72
Chloroform	50	40	18
	100	38	33
	200	50	47
	500	28	86
	1000	64	71

Zone of varying diameter were obtained with various organisms at different concentrations of extract tested. The maximum zone of inhibition was observed in *Enterococcus faecalis* (21 mm) followed by *Bacillus* sp.(20 mm), *E.coli* (19mm) *K.pneumoniae* (19 mm) and *P.aeruginosa* (19 mm) at 100 mg/ml concentration. The minimum zone of inhibition was observed in *P.aeruginosa* (12mm) at 12.5 mg/ml concentration. The highest antibacterial activity was observed against Gram positive bacteria *Enterococcus faecalis* at 100 mg/ml concentration. The qualitative analysis revealed the presence of phenols, saponins, tannins, steroids, flavonoids, glycosides and carbohydrates.

In recent years antibiotic resistance has become a global concern. This drives the need to screen medicinal plants for novel bioactive compounds as they are biodegradable, safe and have fewer side effects [36,37] studied the antibacterial activity in *Diplazium esculentum* (Retz) by disc diffusion method and observed the highest zone of inhibition for *Sarcina lutea* (18.67mm) followed by *Salmonella typhimurium* (16.33mm). The extracts showed relatively lower antibacterial activity against *Klebsiella pneumoniae* (15.33mm) followed by *Bacillus* sp.(15.33mm) and *E.coli* (12.33mm) at 300µg/disc. The highest zone of inhibition was observed with *Klebsiella pneumoniae, Bacillus* sp. and *E.coli* at 12.5 mg/ml concentration.

Methanolic leaf extract of *Lygodium flexuosum* was showed the zone of inhibition in *K. pneumoniae* (22 mm), *P. aeruginosa* (19 mm) and *E.coli* (14 mm) [16]. Similar results were also observed in *Hemionitis arifolia*. Bahadori *et al*. [38] showed the antibacterial activity of some ferns. The maximum activity was exhibited by the extract of *Dryopteris affinis* with MIC value of 2 µl/ml. *Polystichum aculeatum* showed the same antibacterial potential against *S. aureus*. In methanolic extract of *Hemionitis arifolia* showed maximum antibacterial potential against *Enterococcus faecalis*. Antibacterial properties of frond-extracts using the solvent mixture, methanol: dichloromethane at 1:1 with 5 fern species, *L. flexuosum, Selaginella bryopteris, Adiantum philippense, Dryopteris eochleata* and *Tectaria coadunate* had been recorded against human pathogen *P. aeruginosa* [39]. Similar result was observed in methanol extract of *Hemionitis arifolia* which inhibit *P. aeruginosa*. Antimicrobial activity of crude extracts of the epiphytic fern, *Arthromeris himalayensis* had been recorded against *B. subtilis* and *E. coli* by the agar well diffusion method [40]. Similar results were observed in the in methanol extract of *Hemionitis arifolia.*

Johnson *et al*. [18] studied the bio-efficacy of medicinally important ferns viz., *Adiantum caudatum* L., *Adiantum latifolium* Lam. and *Adiantum lunulatum* Burm against the selected UTI pathogens viz., *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* by disc diffusion method. Acetone and methanol extracts were employed in which methanol extracts exhibited good antibacterial activity with all three bacterial species. maximum degree of antibacterial activity was observed in *A. latifolium* followed by *A. caudatum* whereas *A. lunulatum* showed comparatively less degree of antibacterial activity. Shagufta *et al*. [17] also reported antibacterial activity of *Adiantum latifolium, Blechnum orientale* and *Christella parasitica* extracts on uropathogens.

Aspergillus niger causes black mould disease on certain fruits and vegetables. The methanol extract was inactive against *A. niger* except 100µg concentration. The ethanol extract was excellently active against *A.niger. Sclerotium rolfsii* causes rots of the lower stem, root and crown on vegetables and fruits. The chloroform and aqueous extract were inactive against *Sclerotium rolfsii.* The ethanol and methanol extracts were excellently active against *Sclerotium rolfsii.* The antifungal activities might be due to the presence of phenolics, acids, ponasteroside-A, procyanidin, pteridine, ptersinss, quercetin, thaminas-I, tiliroside and xylose.

Several authors reported these secondary metabolites as antimicrobial metabolite like tannins [41]. The aqueous extract of plant was inactive against all the bacterial strains. The chloroform extract of plant was excellently active against *E.coli* and *S.typhii* this may be due to the presence of flavonoids and saponins present in their extracts [42].

This may be the first report regarding this plant against pathogenic bacteria and fungi. The effect of plant extracts, uses as antimicrobial agents to control pathogens were proved. The ethanol, chloroform, acetone, methanol and water extracts of *Cheilanthes bicolor* were subjected to a preliminary screening for antimicrobial activity against *Salmonella typhii, Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus* and antifungal activity against *Sclerotium rolfsii* and *Aspergillus niger*. Thus, the study ascertains the value of plants used in Ayurveda, which could be of considerable interest to the development of new drugs, to control fungal and bacterial pathogens.

CONCLUSION

In the present study, preliminary phytochemical screening has been done in methanol, ethanol, aqueous, acetone and choloroform solvents the extractions were carried out using the soxhlet. The methanolic extract was more when compared to other extracts. The chloroform extract can be used an excellent antimicrobial agent against *Escherichia coli* and *Salmonella typhii* and the ethanolic extract can be used as antimicrobial agent against *K.pneumoniae* and *S.aureus.* Ethanolic and methanolic extract of all the concentration can be used as antifungal agent against *S. rolfsii* and acetone extract of 200µg and 500µg concentration can be used as antifungal agent against *S.rolfsii.* Ethanolic extract of 50µg, 500µg, and 1000µg can be used as fungicide against *A.niger* and acetone extract of 50µg can be used as fungicide against *A.niger*.

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