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Anthelminthic and Bactericidal Activity of Extracts from *Flaveria trinervia* Spring C. Mohr.

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Research Article

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ABSTRACT

Objective: To determine the anthelminthic and bactericidal activity of *Flaveria trinervia*.
Study design: Assessment of Anthelminthic and bactericidal activity.
Place and Duration of Study: Anthelminthic and bactericidal activity of extracts from *Flaveria trinervia* Spring C. Mohr. between August 2010 and May 2011.
Methodology: The methanol and aqueous extracts of *Flaveria trinervia* were screened for antibacterial activity against 20 clinical strains belonging to *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhi, Salmonella paratyphi, Echerichia coli,* and *Staphylococcus aureus* isolated from different infectious sources. Minimum inhibitory concentration (MIC) assay of both the extracts was carried out against clinical isolates using two fold agar dilution method. Ciprofloxacin and piperazine citrate were used as the standard reference for bactericidal and anthelminthic activity respectively.
Results: Evaluation of anti-bacterial activity revealed that both the extracts showed effective activity against all the six bacterial pathogens. Specifically, aqueous extract was more efficient than methanol extract but less potent than standard drug ciprofloxacin. Among the various concentrations of aqueous extract tested, 250 mg/ml showed efficient anthelminthic activity and among all the concentrations methanol extract tested 250 mg/ml gave significant results. This investigation revealed that methanol extract of *F. trinervia* showed significant anthelminthic activity against *Pheretima posthuma* when compared to the aqueous extract.
Conclusion: From the results of this investigation we can conclude that *F. trinervia* is a potent antibacterial and anthelminthic plant.

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1. INTRODUCTION

Natural products are conventionally used as antimicrobial and anthelminthic agents, whose effectiveness is often limited by the resistance that the infectious pathogens have developed against these agents (Ali et al., 1999; Nimri et al., 1999). Plants produce a wide variety of compounds that originate from their secondary metabolism and accumulate in different parts of the plant. Many reports are available on the uses of medicinal plants against pathogenic microorganisms and helminthes with multiple resistances to third and fourth generation drugs (Greenwood, 1998; Struelens, 1998).

New antibiotics that are active against resistant bacteria are required to combat with the present scenario of bacterial pathogenesis. Bacteria have lived on earth since several billion years. During this time, they encountered in nature a wide range of naturally occurring antibiotics. To survive bacteria developed antibiotic resistance mechanism (Raja et al., 2010). Raw meat remains an important and probably the major source of human food borne infection with pathogenic bacteria. In spite of decades of effort it has been difficult to obtain food free of pathogenic bacteria (Purabi et al., 2011).

Anthelminthics are those agents that expel parasitic worms (helminthes) from the body, by either stunning or killing them (Chaturvedi et al., 2009). Various problems have been evolved with chemotherapeutic control practices, such as parasites are developing resistance to several families of chemical anthelminthics (Chartier et al., 2001), chemical residues and toxicity problems (Muhammad et al., 2004), un-economical and nonavailability of drugs in remote areas. Furthermore, it has been recognized recently that anthelmintic substances having considerable toxicity to human beings are present in foods derived from livestock, posing a serious threat to human health (Paras et al., 2009). For these various reasons, interest in the screening of medicinal plants for their anthelminthic activity remains of great scientific significance despite extensive use of synthetic chemicals in modern clinical practices all over the world (Von Bingen, 1974).

Several herbal products are available all over the world with an acclaimed anthelminthic and antibacterial activity, which are considered to be less toxic and free from side effects. *Flaveria trinervia* spring C. Mohr (Asterecae) population grows only in the alkaline soil [pH 7.2-8.2], mainly in the marshy lands near Chitradurga Dist, Karnataka State, India (Manjunatha et al., 2004). This plant is locally referred as Bellary halabu or katthe kivi gida. Traditionally it is used as a promising wound healing drug and as an antimicrobial agent for infectious wounds in Karnataka state, India (Manjunatha et al., 2004). However, anthelminthic activity of *F. trinervia* whole plant extract is not scientifically reported. To justify the ethnomedical claims, methanolic and aqueous extracts of *F. trinervia* were screened for anthelminthic and antibacterial activity.

2. EXPERIMENTAL DETAILS

2.1 Drugs and Chemicals

The standard drug piperazine citrate (SD Fine Chemicals Ltd., Mumbai) was used for evaluation of anthelminthic activity and ciprofloxacin (Usan pharmaceuticals Pvt. Ltd. Maharastra, India) was used for evaluation of bactericidal activity.

2.2 Plant Resource and Extract

Flaveria trinervia herb was collected from the agricultural fields near Chitradurga city of Karnataka, India. Plant was authenticated by Dr. Manjunatha by comparing with the voucher specimen deposited at Kuvempu University herbarium specimen FDD-No. 53 (Manjunatha et al., 2004).

The fresh whole plant material was shade dried, powdered mechanically and was subjected for soxhlet extraction using methanol as solvent system for about 48 h followed by distilled water with 5% ethanol successively. The extracts were filtered and concentrated in vaccum under reduced pressure using rotary flash evaporator (Buchi, Flawil, Switzerland) and allowed it for complete evaporation of the solvent. Methanol and aqueous extracts were vacuum dried.

2.3 Test Organism for Anthelminthic Activity

Indian adult earthworms (*Pheretima posthuma)* collected from the Indian Institute of Horticultural Research, Bangalore, India. The earthworms were maintained under normal vermicomposting medium with adequate supply of nourishment and water, for about three weeks. Before the initiation of experiment the earthworms were washed with normal saline. Adult earthworms of approximately 4 cm in length and 0.2 - 0.3 cm in width were used for the experiment. This organism was selected as model for anthelminthic activity due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings (Thorn et al., 1977; Vigar, 1984).

2.4 Extract Preparation for Experiment

The porously powdered plant material was used for extract preparation. After the extraction process, the crude extract was stored in desiccator until further use. Methanol extract, aqueous extract and standard drugs were dissolved in 0.5% DMSO in normal saline (v/v), and used for evaluation of anthelminthic activity and bactericidal activity.

2.5 Anthelminthic activity

The anthelminthic activity of whole plant extracts of *Flaveria trinervia* were evaluated as per the method reported by Dash et al., 2002. Twelve groups with three earthworms in each groups, each earthworm was separately released into 20 ml of desired formulation in normal saline, Group I earthworms were released in 20 ml normal saline in a clean petri plate. Group II, III, IV, V, VI earthworms were released in 50, 100, 150, 200 and 250 mg/ml of methanol extract in 20 ml of normal saline respectively. Similarly, group VII, VIII, IX, X, XI earthworms were released in 50, 100, 150, 200 and 250 mg/ml of aqueous extract in 20 ml of normal saline respectively. Group XII earthworms were released in normal saline

containing standard drug piperazine citrate (50 mg/ml) in 20 ml of normal saline. Earthworms were observed; the time taken for paralysis and the time taken for death was monitored and documented in minutes. Paralysis time was analyzed based on the behavior of the earthworm with no revival body state in normal saline medium. Death was concluded based on total loss of motility with faded body color (Tambe et al., 2006). The result of anthelminthic activity is depicted in Table 1.

2.6 Determination of Minimum Inhibitory Concentrations (MIC)

The agar dilution susceptibility test was performed based on modified method of NCCLS, 2003 and CLSI, 2009 to determine the MIC. The extracts were dissolved in sterilized 5% dimethyl sulfoxide (DMSO; that enhances compound solubility) (800 mg/ml concentration) and were taken as standard stock. A series of two fold dilutions of each extract with a final concentration of 80, 40, 20, 10 and 5 mg/ml were prepared in nutrient agar. After solidification, the plates were spotted with 100 µl of overnight grown bacterial cultures approximately containing 1×10^4 CFU/ml. The test was carried out in triplicates. The plates were incubated overnight at 37°C. After 18 – 24 h, the MIC was determined.

2.7 Antibacterial Screening

The antibacterial activity of the methanol and aqueous extracts was screened by agar well radial diffusion method against twenty clinical isolates of bacterial strains belonging to *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Salmonella typhi, Salmonella paratyphi* and *Escherichia coli* respectively (Cowen and Steel, 1993). The bacterial strains were collected from different infectious status of the patients with the help of authorized physicians, in district health center of Gulburga, Karnataka state, India. The clinical isolates were identified in Microbiology Laboratory, Gulburga University following the standard method (Islam et al., 2008). The bacterial suspensions were diluted in 10^{-1} to 10^{-8} phosphate buffered saline. Samples were homogenized and then loaded in six aliquots of 20 µl each onto nutrient agar plates. The working cultures were prepared by inoculating a loopful of each test microorganism in 3 ml of nutrient broth (NB) from NA slants. Broths were incubated at 37°C for 24 h. The suspension was diluted with sterile distilled water to obtain approximately 10^6 CFU/ml.

The different infectious sources of pathogen are mentioned in the Tables 3. The methanol and aqueous extracts were dissolved in 5% aqueous DMSO to get stock solutions. Commercial bactericide ciprofloxacin was used as standard (100 µg/100 µl of sterilized distilled water) concomitantly with the test samples. The activity was screened comparatively with the reference ATCC strains (*Pseudomonas aeruginosa*- ATCC-20852; *Staphylococcus aureus*- ATCC 29737), (*Salmonella typhi* – ATCC-19430), (*Salmonella paratyphi* – ATCC-9150), (*E. coli* – ATCC-25922) and MTCC strains (*Klebsiella pneumoniae* – MTCC-618).

Sensitive agar well radial diffusion technique was used for the assessment of antibacterial activity of the test samples. Sterilized nutrient agar medium was poured into sterilized petridishes. Nutrient broth containing 100 µl of 24 h incubated cultures of clinical isolates was spread on the agar medium. Wells were created using a sterilized cork borer in an aseptic condition. 100 µl of crude methanol extract, 100 µl of aqueous extract and 100 µl of standard drug ciprofloxacin were loaded on to their corresponding wells. The plates were incubated at 37°C for 24 h. The diameter of the zone of complete inhibition of the bacteria was measured around each well and readings were recorded in mm. The results of these

experiments were expressed as mean ± S.E.M. of three replicates in each test (Lehrer et al., 1991).

2.8 Statistical Analysis

The data of anthelminthic and bactericidal evaluations were expressed as mean ± S.E.M of three replicates. The statistical analysis was carried out using one way ANOVA followed by Tukey's *t*-test. The difference in values at P< 0.01 was considered as statistically significant. The analysis of variance (ANOVA) was performed using ezANOVA (version 0.98) software to determine the mean and standard errors of the experiments.

3. RESULTS AND DISCUSSION

3.1 Anthelminthic Activity

Earthworms belonging to control group showed paralysis time at 57.33 ± 2.73 min and death time at 190.33 ± 3.18 min. The methanol extract at the concentration of 50 mg/ml showed the time of paralysis and death at 66.33 ± 5.04 and 101 ± 5.69 min respectively. For concentrations at 100, 150, 200 and 250 mg/ml of methanol extract, the paralysis was shown at 64.67 ± 6.94, 35 ± 2.52, 28.33 ± 0.33 and 22.67 ± 3.38 min respectively and death occurred at 80 ± 3.61, 74 ± 5.03, 57.67 ± 8.01 and 55.33 ± 6.69 min respectively. Among all the concentrations of methanol extract tested, concentration at 250 mg/ml gave significant results. On the other hand, aqueous extract at the concentration of 50 mg/ml showed the time of paralysis and death at 103.33 ± 4.33 and 164.33 ± 5.55 min, respectively. For concentration of 100 mg/ml, the paralysis and the death time was found to be 74 ± 0.58 and 159.33 ± 1.45 min respectively. At concentrations 150, 200 and 250 mg/ml, the time taken for paralysis was 56.33 ± 5.93, 52 ± 3.79 and 43.67 ± 1.20 min respectively and death time was 113.67 ± 4.81, 102.33 ± 11.26 and 84.33 ± 3.84 min respectively.

Table 1: *In vitro* anthelminthic activity of methanol and aqueous extracts of *Flaveria trinervia* against *Pheretima posthuma*.

Test samples	Concentration (mg/ml)	Time taken for paralysis (min)	Time taken for death (min)
Control (Normal Saline)		57.33 ± 2.73	190.33 ± 3.18
Methanol extract of *Flaveria trinervia*	50	66.33 ± 5.04^{ns}	101 ± 5.69**
	100	64.67 ± 6.94^{ns}	80 ± 3.61**
	150	35 ± 2.52**	74 ± 5.03**
	200	28.33 ± 0.33**	57.67 ± 8.01**
	250	22.67 ± 3.38**	55.33 ± 6.69**
Aqueous extract of *Flaveria trinervia*	50	103.33 ± 4.33**	164.33 ± 5.55**
	100	74 ± 0.58**	159.33 ± 1.45**
	150	56.33 ± 5.93^{ns}	113.67 ± 4.81**
	200	52 ± 3.79*	102.33 ± 11.26**
	250	43.67 ± 1.20**	84.33 ± 3.84**
Piperazine citrate	50	34.67 ± 7.54*	44 ± 3.61**

Values are the mean ± S.E.M. of three earthworms. Symbols represent statistical significance.
** P < 0.05, ** P < 0.01, ns: not significant as compared to compared to control group.*

Among the various concentrations of aqueous extract tested, 250 mg/ml showed efficient anthelminthic activity (Table 1). Standard drug at 50 mg/ml showed the paralysis time at 34.67 ± 7.54 min and the death time at 44 ± 3.61 min (Table 1). This investigation revealed that methanol extract of *Flaveria trinervia* showed significant anthelminthic activity against *Pheretima posthuma* when compared aqueous extract. Methanol extract also proved to be less efficient than the standard drug.

3.2 Evaluation of Minimum Inhibitory Concentrations (MIC)

The MIC values of both extracts ranged from 5 to 40 mg/ml (Table 2). Aqueous extract of *F. trinervia* showed significant inhibition at MIC 10 mg/ml against *Staphylococcus aureus* and *Salmonella paratyphi.* Whereas, methanol extract showed maximum activity against *Salmonella typhi* and *E. coli* at MIC 10 mg/ml. Lowest MIC was observed by aqueous extract at 5 mg/ml against *E. coli*.

Table 2: *In vitro* minimum inhibition concentrations evaluation of methanol and aqueous extracts of *Flaveria trinveria* against *Staphylococcus aureus, Pseudomonas aureginosa, Klebsiella pneumoniae, Salmonella typhi, Salmonella paratyphi* and *Escherichia coli*.

Test pathogenic bacteria	Strain	*Flaveria trinervia* plant extracts	
		Methanol Extract	Aqueous Extract
Staphylococcus aureus	ATCC-29737	20	10
Pseudomonas aureginosa	ATCC-20852	40	*
Klebsiella pneumoniae	MTCC-618	*	20
Salmonella typhi	ATCC-19430	10	20
Salmonella paratyphi	ATCC-9150	20	10
Escherichia coli	ATCC-25922	10	5

** indicates values more than 80 mg/ml.*
The value of each constituents consisted of ± S.E.M. of 03 replicates. ND – Not Defined.

3.3 Antibacterial Screening

Aqueous extract of *F. trinervia* showed significant results in inhibiting *S. aureus* with 18.67 ± 0.88 mm zone of inhibition when compared to the methanol extract (17.33 ± 0.88 mm). Aqueous extract against *P. aureginosa* produced 11 ± 0.58 mm zone of inhibition whereas, methanol extract showed 18.67 ± 0.33 mm. Methanol extract was potent against *K. pneumoniae* (14 ± 1.73 mm) than aqueous extract (13.33 ± 1.2 mm). Aqueous extract proved to be insignificant against clinical isolates of *S. typhi* and *S. paratyphi*. Methanol extract also showed better inhibition of *S. paratyphi* (8.67 ± 0.33 mm) and *E. coli* (14 ± 1.53 mm). Aqueous extract inhibited the growth of *E. coli* (15 ± 1.15 mm), this inhibition was better when compared to methanol extract. Evaluation of anti-bacterial activity revealed that the methanol and aqueous extracts of *Flaveria trinervia* showed effective activity against all the six bacterial pathogens. Specifically, aqueous extract was more efficient than methanol extract but less potent than standard drug ciprofloxacin. Result of *in-vitro* antibacterial activity is depicted in table 3.

Table 3: *In vitro* antibacterial activity of methanol and aqueous extracts of *Flaveria trinveria* against *Staphylococcus aureus, Pseudomonas aureginosa, Klebsiella pneumoniae, Salmonella typhi, Salmonella paratyphi* and *Escherichia coli*

Pathogens	Bacterial strains tested	Source of collection	Methanol extract	Aqueous extract	Reference drug Ciprofloxacin
			Zone of inhibition (in mm)		
Staphylococcus aureus					
	Sa-1	ATCC-29737	17.33 ± 0.88	18.67 ± 0.88	21 ± 0.86
	Sa-2	Abscess	15 ± 0.58	18 ± 0.58	21 ± 1.63
	Sa-3	Urine	16.33 ± 0.567	19.67 ± 0.88	19.5 ± 0.96
	Sa-4	Wound	18.67 ± 0.33	17.33 ± 0.88	18.67 ± 1.41
	Sa-5	Hospital effluents	ND	15.33 ± 2.03	21 ± 1.81
Pseudomonas aureginosa					
	Pa-1	ATCC-20852	11 ± 0.58	18.67 ± 0.33	22.67± 0.8
	Pa-2	Urine	14.67 ± 1.86	17.67 ± 1.45	21 ± 1.13
	Pa-3	Pus	ND	17 ± 1.53	18.83 ± 1.01
	Pa-4	Stool	11.33 ± 1.45	16 ± 0.58	19.17 ± 0.95
Klebsiella pneumoniae					
	Kp-1	MTCC-618	14 ± 1.73	13.33 ± 1.2	16 ± 0.58
	Kp-2	Urine	12 ± 1	10.67 ± 0.33	16.83 ± 1.4
	Kp-3	Feaces	9.67 ± 0.84	10.67±0.88	14.33 ± 1.31
	Kp-4	Sputum	11 ± 1.55	ND	16 ± 1.32
Salmonella typhi					
	St-1	ATCC-19430	13.33 ± 0.58	15.33 ± 1.2	21 ± 0.58
	St-2	Blood clot	10 ± 0.58	ND	20.83 ± 0.98
Salmonella paratyphi					
	Spt-1	ATCC-9150	8.67 ± 0.33	7.33 ± 0.33	16.83 ± 1.3
	Spt-2	Blood clot	11.33 ± 0.88	ND	20 ± 1.06
Escherichia coli					
	Ec-1	ATCC-25922	14 ± 1.53	15 ± 1.15	17.33 ± 0.49
	Ec-2	Hospital effluents	17 ± 0.58	14 ± 2.52	16.33 ± 1.76
	Ec-3	Urine	14.33 ± 0.33	17 ±0.58	20 ± 1.06

The value of each constituents consisted of ± S.E.M. of 03 replicates. ND – Not Defined.

Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Srivastava et al., 1996). The antibacterial activity of aqueous, different solvent extracts and isolated constituents of leaves of *Acacia nilotica* were evaluated by the cup diffusion method against three phytopathogenic *Xanthomonas pathovars* (Raghavendra et al., 2006). In continuation to our interest in medicinal plants and their bactericidal properties, our efficient attempt to

screen the antibacterial and anthelminthic property of *Flaveria trinervia* based on the traditional claims of *F. trinervia*, revealed that the methanol and aqueous extracts of *F. trinervia* possess efficient antibacterial and anthelminthic property, but they proved to be less potent than the respective standard drugs. This significant effect of the phytoextract is due to the presence of a single active constituent in higher levels or due to the combined effect of more than one phytoconstituent.

4. CONCLUSION

In the present investigation, the methanol and aqueous extracts of *Flaveria trinervia* were evaluated for anthelminthic and bactericidal activity. The results of this investigation revealed that both the extract were significantly effective in paralyzing and killing earthworm (*Pheretima posthuma*), extracts were also effective in controlling the growth of all the bacterial strains under study. This investigation supported the ethnomedical claims of *F. trinveria.*

CONFLICT OF INTEREST

Authors do not have any conflict of interest.

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