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Original Research Paper

Phytochemical Screening and Antibacterial Properties of *Croton hirtus* L'Her. Plant Against Some Important Pathogenic Bacteria

M. P. Subin and Navya Reghu
Post Graduate Department of Botany, Sree Narayana College, Nattika-680 566, Distt. Thrissur, Kerala, India

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ABSTRACT

The present study is aimed at evaluating the *in vitro* antimicrobial activity of different solvent extracts of *Croton hirtus* L'Her. against ten medically important bacterial strains namely *Escherichia coli, Enterococcus* sp., *Salmonella typhi, Salmonella paratyphi, Bacillus thuringiensis , Bacillus subtili, Staphylococcus aureus, Pseudomonas fluorescens, Proteus* sp. and *Klebsiella pneumoniae.* Phytochemical analysis and antimicrobial properties of shoot, root and whole plant extracts of *C. hirtus* were investigated separately in methanol, ethanol, chloroform, acetone and water. Phytochemical screening reveals the presence of alkaloids, tannins, flavonoids, steroids, phenols, glycosides, terpenoids, anthroquinone, saponins and cardiac glycosides. The methanol extract exhibited higher and wider range of activity against majority of the test organisms. The shoot methanol extracts recorded highest activity against *Bacillus thuringiensis* (13.67 mm ZOI and MIC of 600µg/mL), *Bacillus subtili* (13.83 mm ZOI and MIC of 600µg/mL) and *Salmonella paratyphi* (12.5 mm ZOI and MIC of 800µg/mL). The methanol whole plant extracts demonstrated highest activity against *Salmonella typhi* (13.33 mm ZOI and MIC of 700µg/mL) whereas methanol root extracts demonstrated highest against *Klebsiella pneumoniae* (13.33 mm ZOI and MIC of 800µg/mL) and *Enterococcus sp.* (10.5 mm ZOI and MIC of 1000µg/mL). The performance of methanol extracts was followed by chloroform extracts. The chloroform whole plant extracts showed the highest activity against *Escherichia coli* (14.67 mm ZOI and MIC of 400µg/mL) and *Staphylococcus aureus* (12.67 mm ZOI and MIC of 800 µg/mL), whereas chloroform shoots extracts against *Pseudomonas fluorescens* (12.33 mm ZOI and MIC of 900µg/mL). The highest activity against *Proteus sp.* was recorded in shoot acetone extracts (10.67 mm ZOI and MIC of 1000µg/mL). Water and ethanol extracts demonstrated the least activity against the test bacteria.

INTRODUCTION

The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization. A characteristic feature of plants is their capacity to synthesize and store wide variety of low molecular weight compounds called secondary metabolites. In present days, secondary plant metabolites, previously with unknown biological activities, have been extensively investigated as a source of medicinal agents (Krishnaraju et al. 2005). The major phytochemical substances of interest in this includes alkaloids, steroids and saponins, however, other diverse groups of naturally occurring phytocomponents such as flavonoids, tannins, terpenoids, essential oils, etc., have also been reported (Lozoya & Lozoya 1990). Many of these have been investigated scientifically for antimicrobial activity, and a large number of plant products have been shown to inhibit the growth of pathogenic microorganisms.

The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents have led to the screening of several medicinal plants for their potential antimicrobial activity (Benkeblia 2004). Several plants and herb species used traditionally have potential antimicrobial and antiviral properties (Shelef 1983, Zaika 1988) and this has raised the optimism of scientists about the future of phyto-antimicrobial agents (Das et al. 1999). According to World Health Organization, medicinal plants are the best source to obtain a variety of newer herbal drugs. About 80% of individuals from developed countries use traditional medicines, which have compounds derived from medicinal plants. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. There is currently a large and everexpanding global population base that prefers the use of natural products in treating and preventing medical problems because herbal plants have proved to have a rich resource of medicinal properties. Therefore, such plants should be investigated to better understand their properties, safety and efficacy. The present study is aimed to carry out the preliminary phytochemical analysis and to screen *in vitro* antibacterial activity and minimum inhibitory concentration of plant extracts of *Croton hirtus* L'Her. against ten bacterial pathogens namely *Escherichia coli, Enterococcus* sp., *Salmonella typhi, Salmonella paratyphi, Bacillus thuringiensis , Bacillus subtili, Staphylococcus aureus, Pseudomonas fluorescens, Proteus* sp. and *Klebsiella pneumoniae.*

MATERIALS AND METHODS

The *Croton hirtus* plant was collected from the campus of Sree Narayana College, Nattika, Kerala. The plant was identified, confirmed and authenticated by the taxonomist of Department of Botany, University of Calicut, Kerala, India.

Extraction and sample preparation: *C. hirtus* plant was collected and shade dried. Since certain compounds get denatured in sunlight, it is dried under shade to avoid decomposition. The dried plant material such as shoot, root and whole plant was then powdered with the help of grinding machine separately. The powdered plant material was filled in the thimble and extracted successively with respective solvents used i.e., methanol, ethanol, chloroform, acetone and water in Soxhlet extractor (Pulok 2002) for 48h so that alkaloids, terpenoids and other phytoconstituents if present get dissolved. Each extract was filtered using Whatmann 41 filter paper. The filtrate was then evaporated to dryness using a rotary flash evaporator (Girish & Satish 2008). The dry residue was dissolved in respective solvent to get suitable concentration of the extracts for carrying out *in vitro* antibacterial activity. The concentration of the final extract was 40mg plant residue/10mL solvent.

Collection of test organisms and preparation of stock culture: The media used for bacterial assay were nutrient agar and nutrient broth. Cultures of 10 pathogenic bacteria were used for *in vitro* antibacterial assay. All the microorganisms namely *Escherichia coli, Enterococcus* sp., *Salmonella typhi, Salmonella paratyphi, Bacillus thuringiensis , Bacillus subtili, Staphylococcus aureus, Pseudomonas fluorescens, Klebsiella pneumoniae* and *Proteus* sp. were collected from Microbiology Section, Amala Ayurvedic Hospital and Research Centre, Amalanagar, Thrissur, Kerala. The bacterial cultures were undergone subculturing and maintained on nutrient agar at 4 degree Celsius.

Phytochemical screening: Preliminary phytochemical screening of the shoot, root and whole plant extract was carried out so as to decipher the presence or absence of various phytocompounds. Presence of 10 phytoconstituents namely alkaloids, tannins, flavonoids, steroids, phenols, glycosides, terpenoids, anthroquinone, saponins and cardiac glycosides were tested.

Determination of antimicrobial activity: Antimicrobial activity tests were performed by agar well diffusion method (Cole 1994, Okeke et al. 2001). Mueller Hinton agar medium with 1.5% agar was prepared and plated under aseptic condition. Using 5mm diameter well cutter, three wells each having 5 mm diameter respectively were bored in each plate; wells were made with equal distance. A drop of the soft agar was dropped into the well to seal the bottom. The test bacterial strains obtained from overnight culture of each bacterium were seeded on the respective sterile solidified agar medium plate by swab plate technique using sterile cotton swabs. After allowing for 10min setting, 200 µL of each extract of *C. hirtus* was dispensed into each of the respective wells with the aid of a Pasteur pipette. After holding the plates at room temperature for about 2 hours to

Table 1: Phytochemical screening of whole plant extract of *Croton hirtus.*

S. No	Tests	Different extracts of whole plant				
		Water	Meth-anol	Eth-anol	Chlor-oform	Ace-tone
1	Alkaloids	-	+	-	+	-
2	Tannins	+	+	+	+	+
3	Flavonoids	-	+	-	-	+
4	Steroids	+	+	+	+	+
5	Phenols	+	+	-	+	-
6	Glycosides	+	+	+	-	+
7	Terpenoids	+	-	+	-	+
8	Anthroquinone	-	+	-	-	-
9	Saponins	-	-		-	-
10	Cardiac glycosides	-	-	-	-	-

+ sign represents presence and - sign represents absence

Table 2: Phytochemical screening of shoot extract of *Croton hirtus.*

S. No	Tests	Different extract of shoot				
		Water	Meth-anol	Eth-anol	Chlor-oform	Ace-tone
1	Alkaloids	-	+	-	+	-
2	Tannins	+	+	+	+	+
3	Flavonoids	-	-	-	-	+
4	Steroids	+	+	+	+	+
5	Phenols	+	+	+	-	+
6	Glycosides	+	+	+	-	+
7	Terpenoids	+	+	+	+	+
8	Anthroquinone	+	+	-	-	-
9	Saponins	+	+	+	-	+
10	Cardiac glycosides	-	-	-	+	-

+ sign represents presence and - sign represents absence

Table 3: Phytochemical screening of root extract of *Croton hirtus.*

S. No	Tests	Different extract of root				
		Water	Meth-anol	Eth-anol	Chlor-oform	Ace-tone
1	Alkaloids	-	-	-	-	-
2	Tannins	-	-	+	-	-
3	Flavonoids	-	-	-	-	-
4	Steroids	+	+	-	+	+
5	Phenols	-	-	-	-	-
6	Glycosides	+	+	+	-	+
7	Terpenoids	+	+	+	+	+
8	Anthroquinone	-	-	-	-	-
9	Saponins	-	-	+	-	-
10	Cardiac glycosides	-	-	-	-	-

+ sign represents presence and - sign represents absence.

allow diffusion of the extracts in to the agar, the plates were incubated at 37°C ± 2°C for 24 hours for bacterial activity. The plates were observed for the zone of clearance around the wells. The zone of inhibition was calculated by measuring the diameter (mm) of the inhibition zone around the well including the well diameter. Antibacterial activities of the plant extract were evaluated against both reference and control in the assay. Antibiotic Ampicillin was chosen as the reference and respective pure solvents used for preparing plant extracts were chosen as control for all the bacterial species tested. The readings were taken in two different fixed directions in all 3 replicates and the average values were recorded.

Minimum inhibitory concentration (MIC): The minimum inhibitory concentrations of methanol, ethanol, chloroform, acetone and water extracts of shoot, root and whole plant of *C. hirtus* were determined by broth dilution assay (NCCLS 1993). Stock concentrations of the plant extracts were prepared by using dimethyl sulfoxide (DMSO) and methanol in the ratio of 1:1, which in turn was diluted with equal volume of phosphate buffer saline (pH 7.0). Muller Hinton agar was prepared, sterilized and kept ready in molten condition. Twenty mL of the molten Muller Hinton agar was mixed with known concentrations of different plant extracts, swirled and poured onto the plates. After solidification, the test bacteria were inoculated and incubated at 37°C for 24 hours. MIC was recorded based on the growth of the different bacteria incubated at a range of concentrations 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 µg/mL.

RESULTS AND DISCUSSION

Medicinal plants are important for the study of their traditional uses through the verification of pharmacological effects and can be natural composite sources that act as new anti-infectious agents. Qualitative phytochemical investigation of *C. hirtus* revealed that the extracts contained some phytoconstituents, which are of medicinal importance (Tables 1-3). The screening of whole plant water extracts of *C. hirtus* showed presence of constituents like tannins, phenols, glycosides, terpenoids and steroids. Alkaloids, tannins, flavonoids, steroids, phenols, glycosides and anthroquinone were present in methanol extract; terpenoids, glycosides, steroids and tannins in ethanol extracts; alkaloids, tannins, steroids and phenols in chloroform extracts and alkaloids, tannins, phenols and steroids in acetone extracts. The shoot extracts of *C. hirtus* showed constituents like tannins, steroids and terpenoids in all the extracts. The constituents like phenols, glycosides and saponins were present in all the extracts except in chloroform extract. Besides this, alkaloids in methanol and chloroform; flavonoids in acetone; anthroquinone in water and methanol and cardiac glycosides in chloroform were also present. In root extracts, constituents like glycosides, terpenoids and steroids were present in methanol, acetone and water extracts; tannins, glycosides and terpenoids in ethanol extract, whereas only steroids and terpenoids in chloroform. The study noticed that even though saponins and cardiac glycosides were identified in shoot extracts of *C. hirtus,* the same were not present in the whole plant extracts. It may possibly be due to localization of these constituents in a particular part of the shoot system. The whole plant extracts led to the insufficient concentrations of these constituents to be detected (Stainer et al. 1986). The study also noticed that tannins, steroids, glycosides and terpenoids are the constituents, which were observed as common in the extracts of shoot, root and whole plant. The bioactive components identified in the extracts are known to be bactericidal, pesticidal or fungicidal in nature, thus, conferring the antimicrobial property to the plant (Lutterodt et al. 1999, Pretorius et al. 2001, El astal et al. 2005). It has been widely observed and accepted that the medicinal value of plants lies in the bioactive phytocomponents present in the plants (Veeramuthu et al. 2008). The potent phytochemicals present in the plant extracts might be responsible for the antibacterial activity (Prusti et al. 2008).

The present investigation has selected ten pathogenic bacteria which include four Gram-positive and six Gram-negative bacteria. The study indicates that the different extracts of *C. hirtus* demonstrated antimicrobial activity against the test pathogens. The methanol extracts of shoot, root and whole plant exhibited better antimicrobial activity against majority of the test pathogens compared to other solvent extracts. The data given in Tables 4, 5 and 6 and Figs. 1-3 have clearly revealed that the methanol shoot extracts of *C. hirtus* have demonstrated the highest ZOI against pathogens *B. thuringiensis* (13.67 mm) , *S. paratyphi* (12.5 mm) and *B. subtili* (13.83 mm), whereas methanol root extracts demonstrated the highest ZOI against *K. pneumoniae* (13.33 mm) and *Enterococcus* sp. (10.5 mm). However, in the case of *S. typhi* methanol whole plant extracts demonstrated the highest ZOI (13.33 mm). The pathogen *E. coli* and *S. aureus* exhibited maximum susceptibility in chloroform whole plant extracts (14.67 mm and 12.67mm diameter of ZOI respectively), whereas *P. fluorescens* in chloroform shoots extracts (12.33 mm diameter of ZOI). The pathogen *Proteus* sp. exhibited highest susceptibility in acetone shoot extracts (10.67 mm diameter of ZOI).

The present study revealed differences in the antimicrobial effects of different solvent extracts of shoot, root and whole plant of the same plant against the test pathogens. But it is not surprising because it may be due to various degrees of solubility for different phytoconstituents

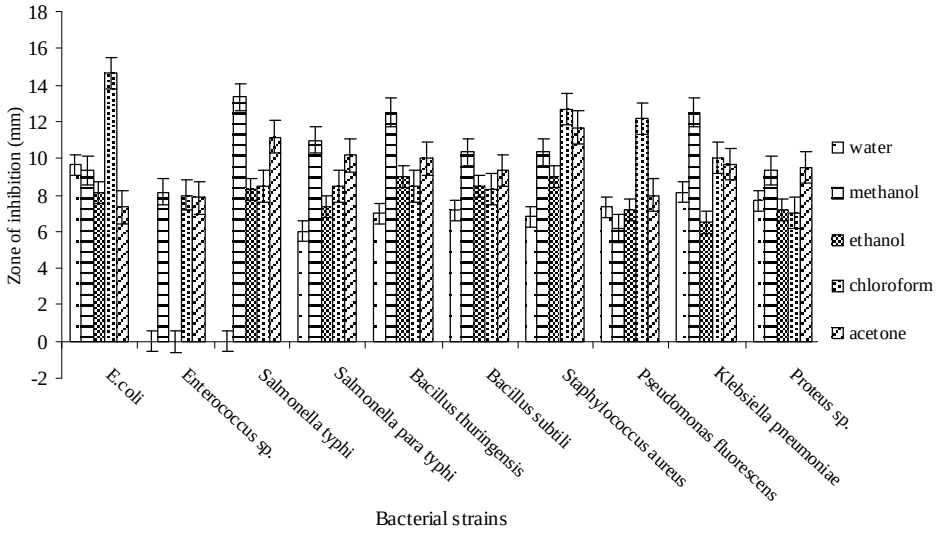

Fig. 1: Antibacterial activity of *Croton hirtus* whole plant extracts.

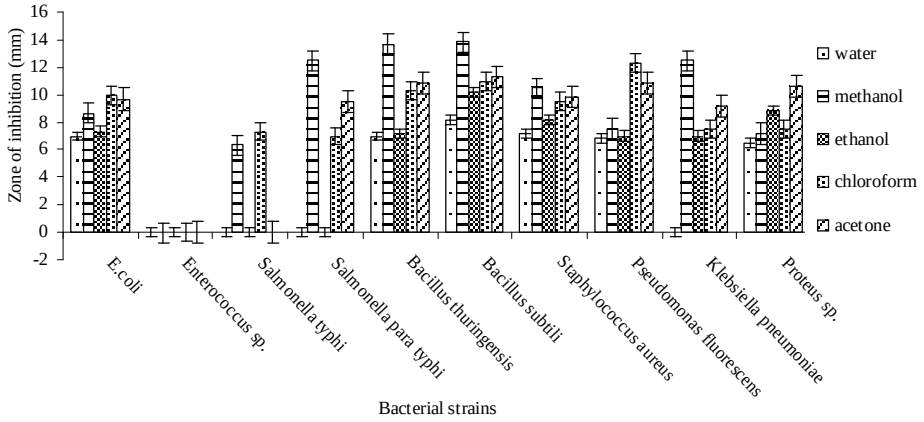

Fig. 2: Antibacterial activity of *Croton hirtus* shoot extracts.

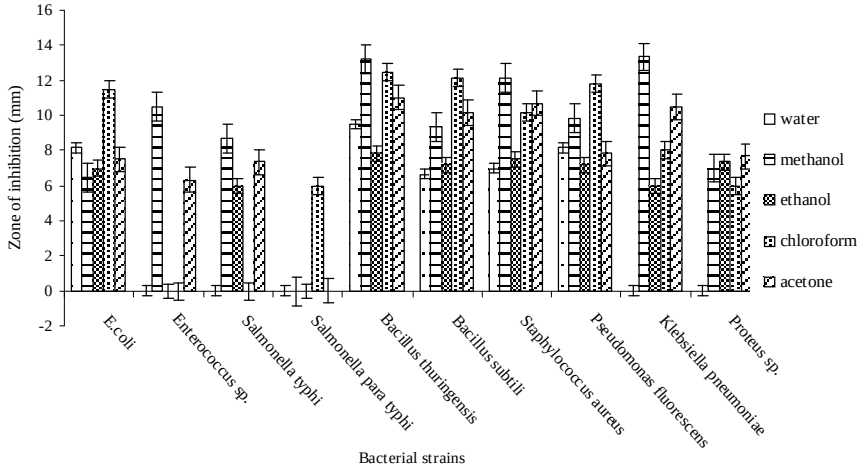

Fig. 3. Antibacterial activity of *Croton hirtus* root extracts.

Table 4: MIC of whole plant extract of C*roton hirtus.*

S. No	Name of Pathogen	MIC of extract against pathogen (µg/mL) Water	Meth-anol	Eth-anol	Chlor-oform	Ace-tone
1	*E.coli*	×	×	×	400	×
2	*Enterococcus* sp.	×	×	×	×	×
3	*Salmonella typhi*	×	700	×	×	1000
4	*Salmonella para typhi*	×	1000	×		×
5	*Bacillus thuringiensis*	×	700	×	×	×
6	*Bacillus subtili*	×	1000	×	×	×
7	*Staphylococcus aureus*	×	1000	×	800	900
8	*Pseudomonas fluorescens*	×	×	×	900	×
9	*Klebsiella pneumoniae*	×	900	×	×	×
10	*Proteus* sp.	×	×	×	×	×

MIC determind up to a range of 1000µg/mL; × = MIC not determined

Table 5: MIC of shoot extract of C*roton hirtus.*

S. No	Name of Pathogen	MIC of extract against pathogen (µg/mL) Water	Meth-anol	Eth-anol	Chlor-oform	Ace-tone
1	*E.coli*	×	×	×	×	×
2	*Enterococcus* sp.	×	×	×	×	×
3	*Salmonella typhi*	×	×	×	×	×
4	*Salmonella para typhi*	×	800	×	×	×
5	*Bacillus thuringiensis*	×	600	×	1000	1000
6	*Bacillus subtili*	×	600	×	1000	1000
7	*Staphylococcus aureus*	×	1000	×	×	×
8	*Pseudomonas fluorescens*	×	×	×	900	1000
9	*Klebsiella pneumoniae*	×	900	×	×	
10	*Proteus* sp.	×	×	×	×	1000

MIC determind up to a range of 1000µg/mL; × = MIC not determined

Table 6: MIC of root extract of C*roton hirtus.*

S. No	Name of Pathogen	MIC of extract against pathogen (µg/mL) Water	Meth-anol	Eth-anol	Chlor-oform	Ace-tone
1	*E.coli*	×	×	×	900	×
2	*Enterococcus sp.*	×	1000	×	×	×
3	*Salmonella typhi*	×	×	×	×	×
4	*Salmonella para typhi*	×	×	×	×	×
5	*Bacillus thuringiensis*	×	700	×	800	1000
6	*Bacillus subtili*	×	×	×	900	×
7	*Staphylococcus aureus*	×	900	×	×	1000
8	*Pseudomonas fluorescens*	×	×	×	900	×
9	*Klebsiella pneumoniae*	×	800	×	×	1000
10	*Proteus sp.*	×	×	×	×	×

MIC determind upto a range of 1000µg/mL; × = MIC not determined

in different solvents and also may be due to differences in the phytochemical properties among different parts of the plant (Majorie 1999). The study also noticed in some cases that the plant extracts show feeble or absence of antibacterial activity against certain pathogens, especially in water extracts. This inference noticed in the investigation may be because of insufficient concentration of antimicrobial constituents contained in the specific extracts so as to be effective or it may be because of insolubility of specific active chemical constituents in the selected solvent or it may be due to the lack of antibiotic properties towards the specific pathogen (Stainer et al. 1986, Majorie 1999).

The minimum inhibitory concentration (MIC) of shoot, root and whole plant extracts of *C. hirtus* was analysed. The concentration of the extracts taken for the study is up to a range of 1000 µg/mL. The results of the present study showed that the different extracts of shoot, root and whole plant of *C. hirtus* demonstrate differences in the MIC value against the test pathogens. Considering the overall analysis, a concentration of 400µg/mL of whole plant chloroform extracts for *E. coli*; 600µg/mL of shoot methanol extracts for *B. subtili* and *B. thuringiensis* ; 700µg/mL of whole plant methanol extracts for *S. typhi*; 800µg/mL of root methanol extracts for *K. pneumoniae,* shoot methanol extracts for *S. paratyphi* and whole plant chloroform extracts for *S. aureus*; 900 µg/mL of shoot, root and whole plant chloroform extracts for *P. fluorescens* and 1000µg/mL of shoot acetone extracts for *Proteus* sp., and root methanol extracts for *Enterococcus* sp. The study also reveals, in many instances, even though the different solvent extracts of *C. hirtus* produced zone of inhibition against test pathogens but failed in the MIC test within the stipulated range of 1000µg/mL, may be due to their feeble activity, and therefore, need to be tested with higher concentrations of extracts as suggested by Kumar et al. (2005).

The higher zones of inhibition and lower MIC value exhibited by methanol or chloroform or acetone extracts of *C. hirtus* against specific pathogens compared to the other solvents extracts used in the study is an indication of their better efficacy. It also indicates higher solubility of specific phytoconstituents present in *C. hirtus* in methanol, chloroform and acetone solvents compared to the other solvents used (Doughari et al. 2008).

The study shows that the control (respective solvents) used in the investigation did not exhibit antimicrobial activity against any of the test pathogenic bacteria, as they failed to produce ZOI around the wells. The inhibition zone formed by the reference ampicillin was higher against all the pathogens (*E. coli*-32mm; *Enterococcus* sp.-25mm; *S. typhi*-25mm; *S. paratyphi*-24mm; *B. thuringiensis* -20mm; *B. subtili*-26mm; *S. aureus*-29mm; *P. fluorescens*-30mm; *K. pneumoniae*-26mm, *Proteus* sp.-22mm). Comparison of negative controls with the solvent extracts of *C. hirtus* generally revealed that the extracts are effective towards pathogenic organisms with few exceptions (Stainer et al. 1986). Comparison with the reference, generally indicates that the

extracts are not much effective towards pathogenic organisms but still they are exhibiting a good activity. Besides, it is also known that by making proper combination of antibiotic agents together with some suitable specific phytoconstituents, there is scope for enhancing activity of the antibiotic agents. This anticipated synergistic effect of some phytoconstituents on antibiotics against some resistant isolates had earlier been reported by Nascimento et al. (2000) and Doughari et al. (2008).

The results of the present study also highlight the fact that the organic solvent extracts exhibited greater antimicrobial activity because the antimicrobial principles are either polar or non-polar, and they were extracted greatly through the organic solvent medium (Mohanasundari et al. 2007, Britto 2001). The present observation suggests that the organic solvent extraction is suitable to verify the antimicrobial properties of the selected plant and same is supported by many investigators (Krishna et al. 1997, Natarajan et al. 2005).

CONCLUSION

The results of the present antibacterial study revealed that methanol and chloroform extracts show more activity towards test pathogenic organisms. On comparing all, the methanol extracts of *C. hirtus* had wider range of activity on the test organisms and this was followed by chloroform extracts. This inference noticed in the present investigation indicates that methanol and chloroform extracts of *C. hirtus* may contain better concentrations of specific potential bioactive components than others. Bioactive substances from this plant can therefore be used in the formulation of antimicrobial agents for the control of various bacterial diseases. Isolation and purification of these phytoconstituents and determination of their respective antimicrobial potencies and toxicological evaluation with the view of formulating novel microbicidal agents should be the future direction for investigations. It is concluded that the traditional plants may represent new sources of antimicrobials with stable biologically active components that can establish a scientific base for the use of plants in modern medicine.

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