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Isolation and characterization of antimicrobial compound from *Chromolaena odorata*

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Abstract

Solvents such as methanol, ethanol, ethyl acetate, hexane and chloroform with extracts of *Chromolaena odorata* were tested against clinical bacteria (*Escherichia coli* and *Staphylococcus aureus*) and phytopathogenic bacteria (*Xanthomonas vesicatoria* and *Ralstonia solanacearum*). Among treatments, maximum *In vitro* inhibition was scored in methanol extracts of *C. odorata* which offered inhibition zone of 8mm, 7mm, 5mm and 7mm against tested bacteria *E. coli, S. aureus, X. vesicatoria* and *R. solanaccearum,* respectively. Further, ethyl-acetate and hexane extracts of *C. odorata* on TLC produced 9 spots with varied level of inhibition. Partially purified compound from TLC (band 2) showed maximum inhibition against tested bacteria. The promising anti-microbial compound from ethyl-acetate and hexane extracts (5:5) of *C. odorata* was further purified using column chromatography which recorded 11mm, 10mm, 9mm and 7mm against *E. coli, S. aureus, X. vesicatoria* and *R. solanaccearum,* respectively. The minimum inhibitory concentration (MIC) value for clinical bacteria was ranged between 0.35 to 4.0 mg/ml and 0.25 to 4.0 mg/ml for phytopathogenic bacteria. HPLC chromatogram showed only one peak, where the retention time was 21.775 min. Structure of the anti-microbial compound was determined by Fourier Transform Infrared Spectra (FTIR) and 2D nuclear magnetic resonance (NMR) and liquid chromatography mass spectra (LC-MS). The structure of anti-microbial compound found to be phenolic groups. LC-MS spectrum of methanolic extract *of C. odorata* compound exhibiting intense peak at m/z 301.3109 and the molecular weight of the compound was probably m/z 437.4129.

Keywords: Chromolaena odorata; clinical and phytopathogenic bacteria; antimicrobial assay

INTRODUCTION

Chromolaena odorata (L.) R. king and Rabinson 1970 (formely: *Eupatorium odoratum* L.), is an herbaceous perennial belongs to the family Asteraceae (=Compositae), is a diffuse, scrambling shrub that is mainly a weed of plantation crops and pastures of southern Asia and western Africa [1]. It grows to a height of 3m in the open situation and upto 8m when assumed a scrambling habitat in the interior forests [2]. It is native to Mexico, the West Indies, and tropical South America; it was spread widely by early navigators. It is commonly called saim weed, triffid weed, christmas bush, bitter bush or jack in the bush. It is a weed competes with 13 major crops in 23 countries [3].

C. odorata commonly known as *Eupatorium,* is an alien, obnoxious and aggressive weed. It grows in pastures, marginal lands, open areas, dry deciduous forests and interior shrub jungles, where it is highly competitive and does not let other flora grow. It is menace in plantations, agriculture and other ecosystems. It suppresses young plantations, agricultural crops and smothers vegetation as it possesses allelopathic potentialities and growth inhibitors [1, 2, 4].

Previous investigations of the leaves and stems of *C. odorata* revealed the presence of essential oils [5-7], steroids, triterpenes [8 and 9], and flavonoids [10-16]. Flowers of this plant have been subjected to investigate for essential oils [17], fats [18] and alkaloids [19]. It has been reported to have antispasmodic, antiprotozoal, antitrypanosomal, antibacterial and antihypersensitive activities. It has also been reported to possess anti-inflammatory, astringent, diuretic and hepatotropic activities [20-23]. In the southern part of Nigeria, the leaves of *C. odorata* are used for wound dressing, skin infection and to stop bleeding. Some specific phenolic compounds have also been isolated from the plant [24]. The medicinal values of plants lie in their component phytochemicals such as alkaloids, tannins, flavonoids and other phenolic compounds, which produce a definite physiological action on the human body [25]. A systematic search for useful bioactivities from medicinal plants is now considered to be a rational approach in neutraceutical and drug research. In this unfolding scenario, it is necessary to develop ways of putting *C. odorata* to beneficial uses. The assessment of nutritive value of *C. odorata* showed that it has good potential for feeding livestock due to its high crude protein (CP), low fibre and low extractable phenolic contents.

In India, numerous studies have been carried out to extract various plant materials for screening antimicrobial compounds but much attention has not been focused on *C. odorata.* Therefore, by

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considering the possibility of having antimicrobial compounds in *C. odorata,* we planned this study to isolate and characterize the antimicrobial compound which has considerable agrochemical properties, environmentally safe, and economically feasible.

MATERIALS AND METHODS

Plant material

Fresh leaves of *C. odorata* were collected from different locations of Mysore (12.18 N– 76.42 E, 770 m above sea level), Karnataka, India, during 2007-2008. The plant was identified taxonomically and authenticated at the Herbarium, in University of Mysore, Mysore. Fresh leaves were washed thoroughly 2-3 times with running tap water and then with sterile water followed by shade-dried, powdered and used for extraction.

Test microorganisms

Human pathogenic bacteria such as *Escherichia coli* and *Staphylococcus aureus* were collected from JSS medical college Mysore, India. Plant pathogenic bacteria such as *Xanthomonas vesicatoria* and *Ralstonia solanacearum* collected from the culture collection of department of applied botany and biotechnology, University of Mysore, India. All the test bacterial species were maintained on nutrient agar media.

Preparation of solvent extraction

Twenty five gram of shade dried, powder of plant materials were filled separately in the thimble and extracted successively with 150 ml of methanol using a Soxhlet extractor for 48 hours. All the extracts were concentrated using rotary flash evaporator. After complete evaporation of solvent, each of these solvent extract was weighed and preserved at 4^{o}C in airtight bottles until further use. One gram of methanol solvent residue was dissolved in 10 ml of respective solvent were used as the test extracts for antimicrobial activity assay.

Activity guided purification of anti-microbial compound

Thin layer chromatography (TLC) plates were prepared by making slurry of 30 gm of silica gel-G with 60 ml of distilled water. Spreading was done manually over glass plates (20x20cm) and air dried. The plates were activated in an oven for 3h at 110 ^{0}C. Methanolic extracts of *C. odorata* (100 mg sample dissolved in 1 ml of respective solvent) were used for TLC spotting. Separation of the TLC spots was done using different solvent system individually and in combinations using ethyl acetate: hexane (5: 5) as mobile phase. Spots developed on TLC plates were observed under UV transilluminator. Spots were eluted separately and isolated fractions were tested for its inhibition against four different micro organisms as described above. Further, the fractions showing maximum inhibition were rechromatographed on TLC plates using the same mobile phase. This process of repeated chromatography on TLC was carried out until a single fluorescing spot was obtained (Fig. 2B). The inhibition of all four different bacteria to all the TLC fractions were compared with negative control as well as positive control (Chloromphenicol standard antibiotic disc) (30mcg/disc).

The partial purified compound obtained from methanolic extract of *C. odorata* by TLC (Described in materials and methods) was loaded into silica gel (60-120 mesh), (SRL, Mumbai) column (35 10mm) and successively eluted with a stepwise gradient of ethyl acetate: hexane (0, 10, 25, 50, 75 and 100%). Fractions were collected at 20-min intervals.

Reverse phase high performance liquid chromatography (RP-HPLC)

The analytical HPLC system with Waters LC 600 pump and 996 Photo diode array detectors, C18 column was used at room temperature. Gradient elution propiles such as 'A' Trifluro acetic acid (TFA) and 'B' was MeOH-H_2O, 1:1. The flow rate was 0.5 ml/min. Absorbance was measured at A_{215} and A_{225} nm 20µl of fraction was injected for separation. Further, minimum inhibitory concentration was checked for TLC purified methanolic extract instead of crude extract of *C. odorata*.

Fourier transform infrared spectra (FTIR)

The infrared spectra were recorded on Shimadzu IR-470 model. The spectra were scanned in the 400 to 4000 cm^{-1} range. The spectra were obtained using paraffin oil technique. One hundred µl of paraffin oil with 1mg of sample was taken and the spectra were plotted as intensity *versus* wave number.

Nuclear magnetic resonance (NMR)

The 2D ^{1}H NMR and ^{13}C NMR spectra were recorded with Brucker AM-500, Switzerland, (500MHz) spectrophotometer with the solvent signal as internal reference and chemical shifts were given in ppm. The compound sample was dissolved (7 mg for ^{1}H NMR and 3 mg for ^{13}C NMR) in 3 ml of $CDCl_3$ and analysed by nuclear magnetic resonance (NMR).

Liquid chromatography mass spectra (LCMS)

LCMS analysis was carried out using API QSTAR pulsar Germany and data dependent acquisition, during which compound precursor ions were detected by scanning from m/z 50 to 2000 with column flow rate of 5µl/min solvent A consisted of 90% (water in 0.1% Trifluro acetic acid) and solvent B consisted of 10% (acetonitrile in 0.1% Trifluro acetic acid) and 30 µl sample were injected onto the column.

RESULTS

On TLC plates, ethyl acetate : hexane (5:5 v/v) with crude extracts of *C. odorata* developed various spots upon elution and tested against four bacteria and varied level of bacterial inhibition was noticed (Fig.1 A-D).

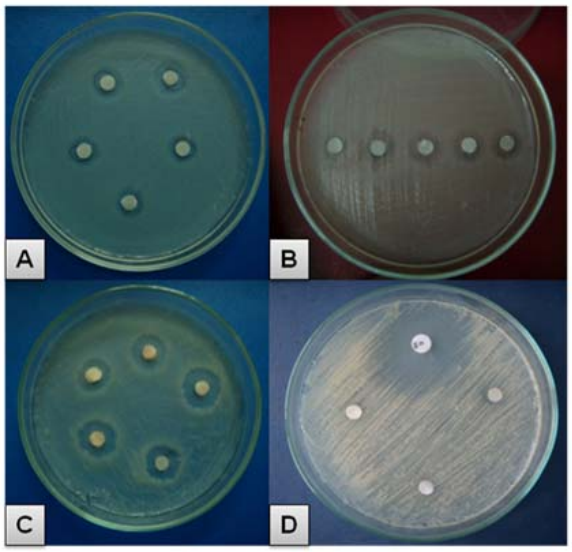

Fig. 1. Effect of methanolic crude extract of *Chromolaena odorata* (L) on against *Escherichia coli* (A), *Staphylococcus aureus* (B), *Xanthomonas vesicatoria* (C) and *Ralstonia solanacearum* (D).

Under UV ethyl acetate : hexane (5:5 v/v) yielded nine spots with variable fluorescing ability consisting Rf values of 0.21, 0.37, 0.46, 0.59, 0.65, 0.71, 0.76, 0.85, 0.98 (Fig. 2).

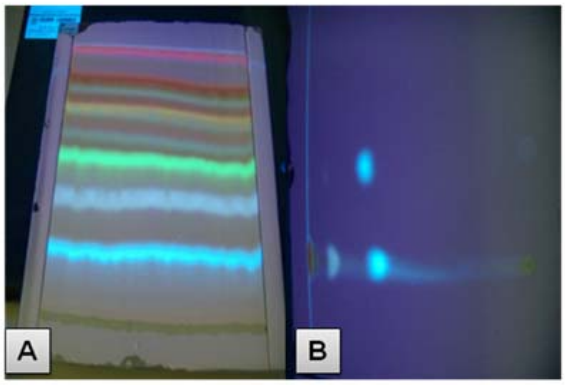

Fig. 2.TLC Fingerprinting of methanol crude extracts (A) and Purified Compound (B) on TLC of *C. odorata*.

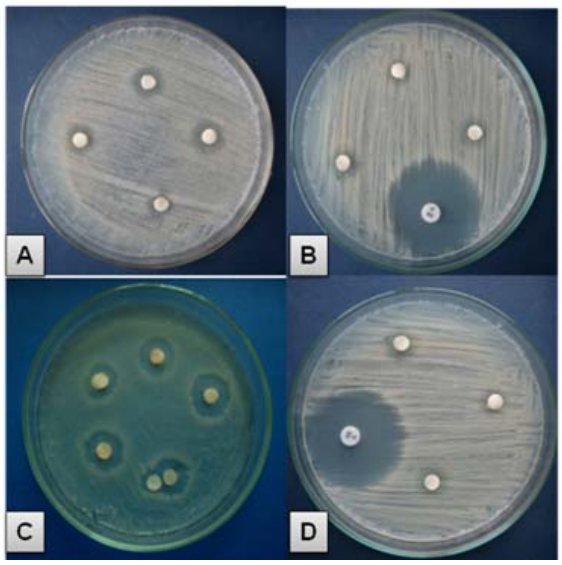

Fig. 3. Inhibition of tested bacteria with purified compund isolated from *C. odorata* against *Escherichia coli* (A), *Staphylococcus aureus* (B), *Xanthomonas vesicatoria* (C) and *Ralstonia solanacearum* (D).

Again the antagonistic activity was rechecked for two bands. Band one showed very good activity (Fig. 3 A-D) compared to band 2. The minimum Inhibitory concentration of purified compound was checked against four different bacteria (as mentioned earlier.) *Escherichia coli, staphylococcus aureus and Xanthomonas vesicatoria* have very good inhibitory effect than and *Ralstonia solanacearum* (Table 1).

Table 1. Minimum inhibitory concentration (MIC) of Methanolic extract of *Chromolaena odorata* Purified compound

Concentration (%)	*E.coli*	*S.aureus*	*X.vesicatoria*	*R.solanacearum*
10	1	3	1	1
20	2	4	2	2
30	3	4	3	2
40	4	5	5	3
60	5	6	6.5	4
80	7	7	7.2	5
100	8.5	7.5	8	6
Chloromphenicol	12	12	15	16

(*E.coli = Escherichia coli*, *S.aureus = Staphylococcus aureus, X.vesicatoria = Xanthomonas vesicatoria, R.solanacearum = Ralstonia solanacearum)*

HPLC analysis

The elutents used in the RP-HPLC analysis of phenolics are mixtures of aqueous pH modifiers with a polar, water-soluble organic solvent : methanol (MeOH), Trifluro acetic acid (TFA). HPLC chromatogram showed only one peak, where the retention time was 21.775 min and the percentage of concentration was 100% (Fig. 4).

Detector A - 2 (225nm)			
Pk #	Retention Time	Area	Area Percent
1	21.775	32031050	100.00
Totals		32031050	100.00

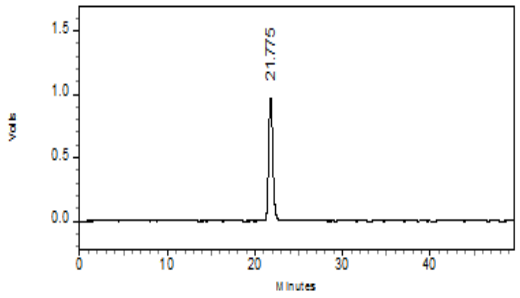

Fig. 4. High Performance liquid chromatogram showing single peak

Fourier transform infrared spectra (FTIR)

The Infra- Red spectrum (Fig. 5) of the isolated compound exhibits following absorptions (Table 2).

Table 2. IR Spectrum peak number, wave number and functional groups

Peak No.	Wave number	Functional Group*
1	3423 cm^{-1}	Intermolecular H bonded O-H stretch
2	2956cm^{-1}	Aromatic C-H stretch
3	2924cm^{-1}	C-H stretching due to methylene group
4	2854cm^{-1}	C-H stretching due to methylene group
8	1644cm^{-1}	Overtone or combination bands
9	1462cm^{-1}	C=C ring stretch
10	1376cm^{-1}	In plane O-H bending
12	1154cm^{-1}	C-O stretching vibrations in alcohol and phenol
13	1076cm^{-1}	C-O stretching vibrations in alcohol and phenol
16	723cm^{-1}	Out of plane aromatic C-H bending

*The above results indicate the presence of phenolic groups.

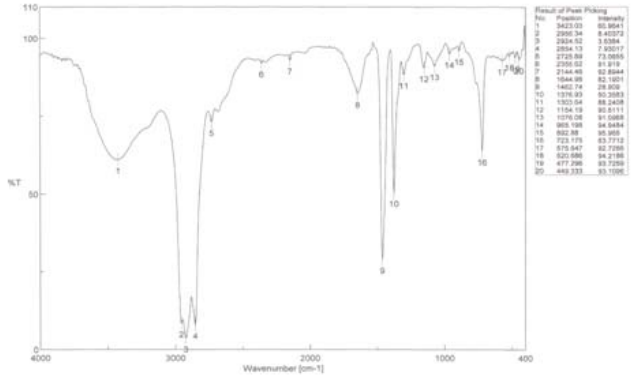

Fig. 5. IR Spectrum of methanolic extract of *Chromolaena odorata* compound.

Nuclear magnetic resonance (NMR) and Liquid chromatography mass spectra (LC-MS)

^{1}H NMR and ^{13}C NMR (500 MHz) spectrum data shows the purified compound to be phenolic compound (Supplementary Fig. 1 and 2). LC-MS spectrum of methanolic extract *of Chromolaena odorata* compound exhibiting intense peak at m/z 301.3109 and the molecular weight of the compound was probably m/z 437.4129 (Fig. 6).

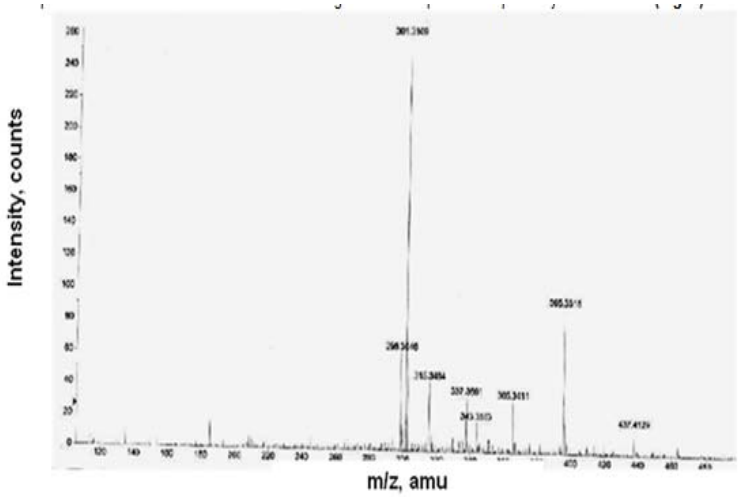

Fig. 6. LC-MS spectrum of methanolic extract of *Chromolaena odorata* compound exhibiting intense peak at m/z 301.3109 and 437.4129 molecular mass of compound.

DISCUSSION

Separation of the active fraction on TLC showed that two bands exhibited the antibacterial activity in the crude methanolic extract of *C. odorata.* Further, detailed investigation of these two bands again confirmed the presence of inhibitory activity of the extract. Remaining seven bands indicated the loss of antibacterial activity suggesting synergistic activity of the extract. There is a possibility of synergism between the compounds in crude extract than in isolated constituents [26].

C. odorata is a prolific producer of novel phytochemicals [25]. In the search for active compounds from *C. odorata*, the majority of research has been performed on extracts of the leaves which inhibited the growth of clinical bacteria such as *Escherichia coli,* and *Staphylococcus aureus.* However, it has not been tested on phytopathogenic bacteria such as *Ralstonia solanacearum* and *Xanthomonas vesicatoria.* It is clear that leaf extract with methanolic solvent showed varied degree of inhibitory effect on both clinical and plant pathogens. This might be due to the solubility of active compounds particular to specific solvents. Various investigators have shown that the use of different solvent influences the ability of the bioactive molecule in demonstrating different degrees of antimicrobial effect [27, 28]. The phenolic compounds of natural origin have the positive property of being soluble in polar solvents. This leads to the possibility of using reversed phase HPLC (RP-HPLC) in their analysis, sufficient retention being achieved by using acidic conditions in order to avoid the presence of ionized forms of the analytes. The purified compound from methanolic extract of *C. odoratum* (3mg ml^{-1}) consistently showed good inhibitory effect on above mentioned bacteria. These results demonstrated that *C. odoratum* has the potential for inhibiting the growth of above mentioned bacteria at low concentrations, even though, FTIR spectral data of the purified *C. odorata* compound showed the presence of phenolic groups. However, from our studies we observed that the mass weight of our phenolic compound is m/z 437.4129.

The need for new, safe and more effective antibacterial is a major challenge to the pesticides industry, especially with the

increase in phytopathogenic strains resistant to bactericides and also fungicides apart from being hazardous to environment and human health. On this background, the biotechnological potential of *C. odoratum* in terms of production of phenolic compound inhibiting both clinical and phytopathogenic bacteria is noteworthy. Results obtained in the present investigation indicated that *C. odoratum* produced a stable phenolic compound with active antimicrobial activity.

Field existences of antibiotic resistant phytopathogenic bacteria are increasing in recent years. WHO banned many agriculturally important pesticides due to wide range of toxicity against non target organisms including humans which are known to cause pollution [29]. Some of the developing countries are still using these pesticides despite their harmful effects. Exploitation of naturally available chemicals from plants, which retards the reproduction of undesirable microorganism, would be a more realistic and ecologically sound method for plant protection and will have a prominent role in the development of future commercial pesticides [30, 31]. Many reports on antibacterial activity of plants extract against human pathogens and their pharmaceutical application are available [32-35], but not much has been done on the antibacterial activity of plants extract against plant pathogens [35]. This is mainly due to lack of information on the screening/evaluation of diverse plants for their antibacterial potential. Thus the present study reveals that *C. odorata* is a potential and promising plant. Therefore it should be successfully exploited for the management of the diseases caused by different plant pathogenic bacteria which are known to cause many diseases in wide variety of crops.

In the present investigation, the antibacterial activities of extracts of *C. odorata* against phytopathogenic bacteria like *R. solancearum* and *X. vesicatoria* has been demonstrated for the first time. A further detailed study is required to know the mode of action of these active ingredients on bacterial cell. However, the present investigation forms a basis for further research in this connection. To date, LC/ (API) MS technique have been widely accepted for the analysis of phenols, flavonoids, including a wide variety of different RP-HPLC conditions. The most frequent application of LC/MS in the analysis of phenols and flavonoids is a full scan over the selected m/z range during the course of an HPLC run. Being a complementary detection method to DVD-UV/Vis, this provides a basis for the identification of compounds for resolving the problem of co-eluted peaks.

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