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A comparative pharmacological and phytochemical analysis of *in vivo* & *in vitro* propagated *Crotalaria* species

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ABSTRACT

Objective: To compare the efficacy for phytochemical, antibacterial and antioxidant activities of petroleum ether, chloroform, ethanol, and aqueous extracts of *in vitro* propagated plants and field grown plants of *Crotalaria* sps., for against five human pathogens. **Methods:** The preliminary phytochemistry, antimicrobial and antioxidant activities were evaluated using disc diffusion and DPPH radical scavenging methods. **Results:** The ethanolic extract of *in vitro* raised *Crotalaria retusa (C. retusa)* was effective on tested microorganisms and optimal ZOI values of 38 mm was obtained against *Pseudomonas aeruginosa (P. aeruginosa)*. The optimal concentration (IC_{50}) required for 50% inhibition of the DPPH radical scavenging was 57.6 μg/mL obtained for ethanolic extract of *in vitro* propagated *C. retusa*. The *in vitro* propagated *C. retusa* has significant pharmacological activities while the *Crotalaria prostrate (C. prostrate)* and *Crotalaria medicaginea (C. medicaginea)* has low pharmacological activites. It was cleared that ethanolic extract of *in vitro* regenerated plants was most effective. **Conclusions:** These findings indicate compounds isolated from ethanolic extracts of *Crotalaria* sps., possesses pharmacological properties and potential to develop natural compounds based pharmaceutical products. The IC_{50} values for ethanolic extracts of *Crotalaria* sps. was evaluated through the Linear regression analysis ($R^2 \leqslant 1$).

1. Introduction

Plants and plant-based medicines are the basis of many of the modern pharmaceuticals we use today for our various ailments[1]. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavonoids and phenolic compounds. The phytochemical research based on ethno-pharmacological information is generally considered to be an effective approach in the discovery of new anti-infective agents from higher plants[2].

The genus *Crotalaria* L. species (family Fabaceae) is considered to be the largest genus, with around 600 species distributed throughout the tropics and subtropic regions of the world, which can be used as antagonistic to nematodes in sustainable crop production systems[3,4]. *Crotalaria* L., species have been reported to contain alkaloids, saponins and flavonoids as a notable chemical markers with basic N-oxides of the genera Leguminaceae having antileukemic, antitumor, antispasmodic, antineoplastic, cardiodepressent, hypotensive properties[5-7]. The leaves are the excellent remedy for ptyalism, diarrhoea, scabies and impetigo. The seeds were powdered and boiled in milk and were used for enhancing body strength, life span and also for curing skin diseases, leprosy, flatulence and fever[8].

The plants are still important for the discovery of new drugs as provider of the drugs based on secondary compounds from plants. Many scientific research has been carried out on these plants and their secondary metabolites of medicinal importance *i.e.*, alkaloids, flavonoids and terpenoids etc. have been reported, pure compound have also been isolated through preliminary phytochemical screening and characterized for their antimicrobial activity against human pathogen cultures[9]. The objective of this research work is to relate antimicrobial and antioxidant activity of extracts and secondary metabolites qualitatively found in this field grown and in vitro propagated *Crotalaria* species to their medicinal properties.

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2. Materials and methods

2.1. Plant Material

The explants for micropropagation of *Crotalaria retusa* (*C. retusa*), *Crotalaria prostrate* (*C. prostrate*) and *Crotalaria medicaginea (C. medicaginea)* were collected from healthy plants growing in Rushikonda area, Visakhapatnam, India and the plant Herbarium was deposited in Andhra University, Andhra Pradesh, India. The voucher numbers were A.U. (B.D.H.) 5527, A.U. (B.D.H.) 2359 and A.U. (B.D.H.) 1850 respectively.

2.2. Micropropagation

The explants were inoculated into the MS medium with growth regulators for their *in vitro* multiplication and regeneration into plants (data not shown). The *in vitro* plantlets of these three species were used for the comparative phytochemical and pharmacological studies.

2.3. Extraction

The field grown and *in vitro* propagated plant materials were shade dried for 7 days. About 50 g powder of the leaves of each *C. retusa*, *C. prostrate* and *C. medicaginea* were powdered separately using a mechanical grinder into a fine powder. These powder were extracted in cold extraction for about 18-20 h successively with petroleum ether, chloroform, 70% ethanol (Merck, India) and finally extracted with water. The percentage extractives of the test samples were performed as per the conventional procedures[10].

2.4. Qualitative chemical analysis

All the extracts were tested with suitable reagents to unfold the diverse classes of chemical constituents present, and then the results were tabulated. Non-polar solvent extracts (petroleum-ether) were tested for the presence of phyto-sterols, triterpenes, and the chloroform fraction were tested for the presence of alkaloids, phenolic compounds such as flavonoids, procyanidine, tannins and phenolic glycosides, saponins and free reducing sugars. The extracts of all the three samples of *Crotalaria* were subjected to preliminary phytochemical analysis using appropriate chemicals and reagents followed by thin layer chromatographic screening[10,11].

2.4.1. Test for alkaloids

About 1 mL of each concentrated extracts was evaporated to dryness at a controlled temperature and then the residue was treated with 5% hydrochloric acid (Merck, India) and filtered. The filterates were tested with different reagents such as Mayer's, Dragendroff's and Wagner's reagents[10].

2.4.2. Test for sterols and triterpenes

10 mL of each of the concentrated extracts were evaporated to dryness under vaccum and the residue was saponified by refluxing with 0.5 N alcoholic potassium hydroxide (Qualigen, India) for two and half hours. Alcohol was evaporated, the residue diluted with excess of water and the contents were extracted with ether several times. The combined ether extracts were washed freely with distilled water, dried over fused calcium chloride (Himedia, India) and filtered. The ether was distilled off completely and the residues were subjected to Salkowski reaction[12].

2.4.3. Test for phenolics

1 mL of each of the concentrated extracts were heated to remove the solvent and the residues were taken in a little of aqueous methanol (Merck, India). 0.5% ferric chloride solution was added to the methanolic solution and the change in color was marked in alcoholic extract indicating the presence of phenolic compounds.

2.4.4. Test for flavonoids

The extracts were added with few magnesium turnings and concentrated hydrochloric acid drop wise, pink scarlet, crimson red or occasionally green to blue color appeared after a few minutes indicates the presence of flavonoids.

2.4.5. Test for saponins

2 mL of test drug was placed in water in a test tube, shaken well, stable froth (foam) appeared and stable for about 30 min[14].

2.4.6. Test for tannins

A few drops of 0.1% ferric chloride solution (Sigma-Aldrich, India) was added to the extracts and observed for brownish green or a blue-black coloration[13].

2.5. Antimicrobial activity disc diffusion method

The extracts of both field grown and *in vitro* plantlets were tested for antibacterial [*Staphylococcus aureus* (*P. aureus*) , *Pseudomonas aeruginosa* (*P. aeruginosa*) , *Proteus vulgaris* (*P. vulgaris*), *Bacillus subtilis* (*B. subtilis*) and *Eschericia coli* (*E. coli*) activities by the disk diffusion method were conducted[15]. Nutrient agar (NA) and Sabouraud dextrose agar (SDA) sterilized in a flask and cooled to 45-50 ℃ were distributed to sterilized petri dishes. The NA plates, containing an inoculums size of 10^6 colony-forming units (CFU)/mL of bacteria or 2×10^5 CFU/mL yeast cells or mold spores on SDA plates, respectively, were spread on the solid plates. The filter paper discs (6 mm in diameter), individually impregnated with 25 μL of extract at concentration of 100 mg/mL., was placed on the agar plates previously inoculated with test microorganisms. Similarly, each plate carried a blank disc by adding methanol solvent alone in the center to serve as a negative control and antibiotic discs (6 mm in diameter) of 30 μg/mL of Rifampicin (Sigma-Aldrich, India) was used as positive controls. All the plates were incubated at 37 ℃ for 24 h for bacteria and 28 ℃ for 48 h for fungi. The diameters for the inhibition zones were measured in millimeters. The sensitivity of the microorganisms to the extract was determined by measuring the size of inhibitory zones on the agar surface around the discs.

2.6. Determination of antioxidant activity

The quantitative measurement of free radical scavenging activities of the extracts of *Crotalaria* species was carried out in a universal bottle[16]. Each reaction mixture contained 50 μL of test sample with concentration ranging from 20, 40, 60, 80 and 100 μg/mL in methanol and 5 mL of 0.004% (w/v) of 2,2- diphenyl-1-picrylhydrazyl (DPPH) radical solution (Sigma-Aldrich, India) in methanol. The Vitamin C (Sigma-Aldrich, India) was used as a positive control[13]. The discoloration as measured at 517 nm after the incubation for 30 min in the dark condition. The 80% (v/v) methanol was used as a blank and DPPH (in 80% MeOH) used as control. Measurements were taken in triplicate. The DPPH radical concentration was calculated using the following equation:

DPPH scavenging effect (%) = A0 − A1/A0 × 100

Where Ao was the absorbance of the control and A1 was the absorbance of the tested sample (crude extracts) in DPPH. The degree of discoloration indicates the free radical scavenging efficiency of the substances.

2.7. Statistical analysis

All the qualitative tests were conducted thrice for each extract and evaluated through the Linear regression analysis ($R^2 \leqslant 1$).

3. Results

The leaves of both field grown and in vitro propagated three species of *Crotalaria (C. retusa, C. prostrata* and *C. medicaginea*) were used for the comparative preliminary phytochemical and pharmacological (antimicrobial and antioxidant) studies.

3.1. Preliminary phytochemical screening

The selected field grown and *in vitro* propagated plants were analyzed for phytochemical screening for the extracts obtained through cold extraction successively using petroleum ether, chloroform, ethanol and water. Each extract of these plants were subjected to various qualitative tests for phyto-constituents such as alkaloids, saponins, tannins, glycosides, steroids and flavonoids. All the qualitative tests were conducted thrice for each extract.

3.2. Screening and qualitative comparison of phytochemicals of Crotalaria species

In the present endeavor, an effort was made to test petroleum ether, chloroform, ethanol and aqueous extracts of *in vivo* and *in vitro* propagated *Crotalaria* species for the presence of secondary metabolites such as alkaloids, sterols, triterpenes, flavonoids, saponins, phenolics and tanins.

Alkaloids: The chloroform and ethanolic extracts of both *in vivo* and *in vitro* propagated *Crotalaria* sps. reacted positively to Mayers, Wagners and Dragendorffs reagent test indicating the presence of alkaloids, while rest of the extracts did not show precipitation indicating the absence of alkaloids.

Steroids: The petroleum ether extracts have shown positive response to Salkowski and Leibermann Burchards test. The ethanolic and aqueous extracts responded negatively to these tests. This indicates the presence of sterols which are present in high concentration in the micropropraged plants of *C. retusa* and *C. prostrate*, where as there is no change in *in vivo* and *in vitro* propagated plants of *C. medicaginae* with regard to these tests.

Phenolics: The chloroform extracts displayed positive response in *C. prostrate* and *C. medicaginae* in micropropagated plants. All the other extracts responded negatively to aforesaid tests.

Triterpenes: The petroleum ether extract responded positively to Salkowski, Leibermann and Tschugajiu tests. The other extracts showed negative response. The triterpene are induced at high level in the micropropagated plants of all the three species of *Crotalaria*, whereas the triterpenes are absent or partially induced in the field grown plants of *C. medicaginea*.

Flavonoids: The chloroform and ethanolic extracts responded positively to flavonoids in in vivo and in vitro propagated plants of *Crotalaria* species. The petroleum ether and chloroform extracts did not respond to flavonoid test showing their absence.

Saponins: The aqueous extracts responded positively to foam tests indicating the presence of saponins. The formation of honeycomb like foam during extraction was seen in micropropaged *C. retusa* compared to other species.

Tanins: These are absent in all the extracts of *in vivo* and *in vitro* propagated *Crotalaria* species.

3.3. Antimicrobial activity by disc diffusion assay

The extracts of both field grown and in vitro propagated plants were experimented for its activity against pathogen cultures with a concentration of 100 mg/mL for each extract. The optimal inhibitory activity was obtained from ethanolic extracts of *in vitro* propagated plants *i.e.*, *C. retusa* against *P. aeruginosa* (Figure 1A), *C.prostrata* and *C. medicaginea* had shown optimal activity against *S. aureus* and *P. vulgaris* respectively (Figure 1B & 1C). The extracts obtained from leaves of field grown *Crotalaria* species had shown comparatively lesser response than *in vitro* plantlets (Figure 2A, 2B & 2C).

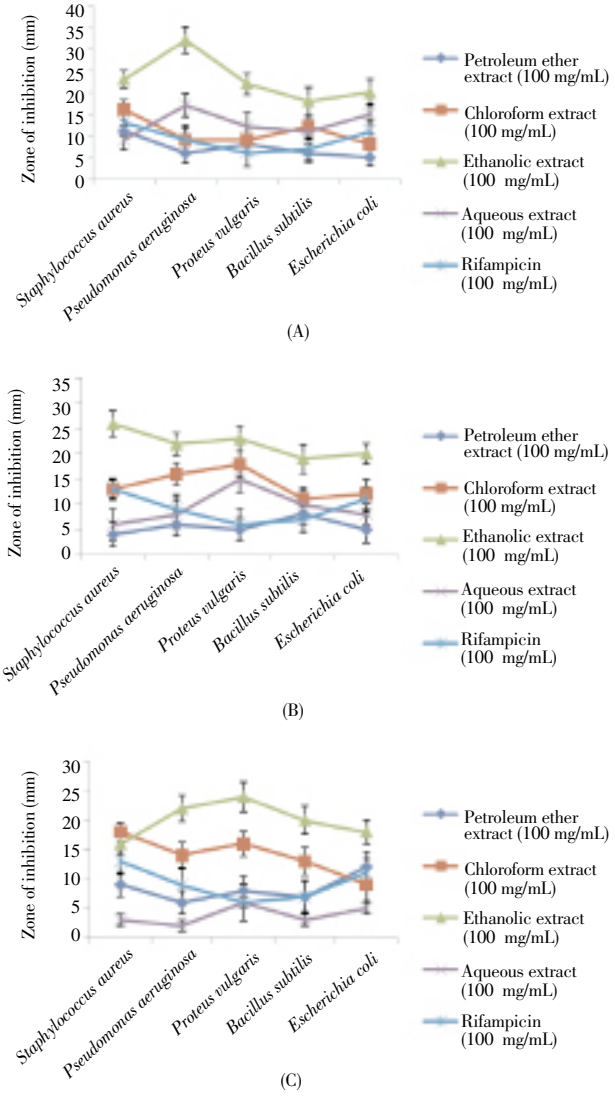

Figure 1. Antimicrobial activity of extracts from *in vitro* propagated *C. retusa* (A), *C. prostrate* (B), and *C. medicaginea*(C).

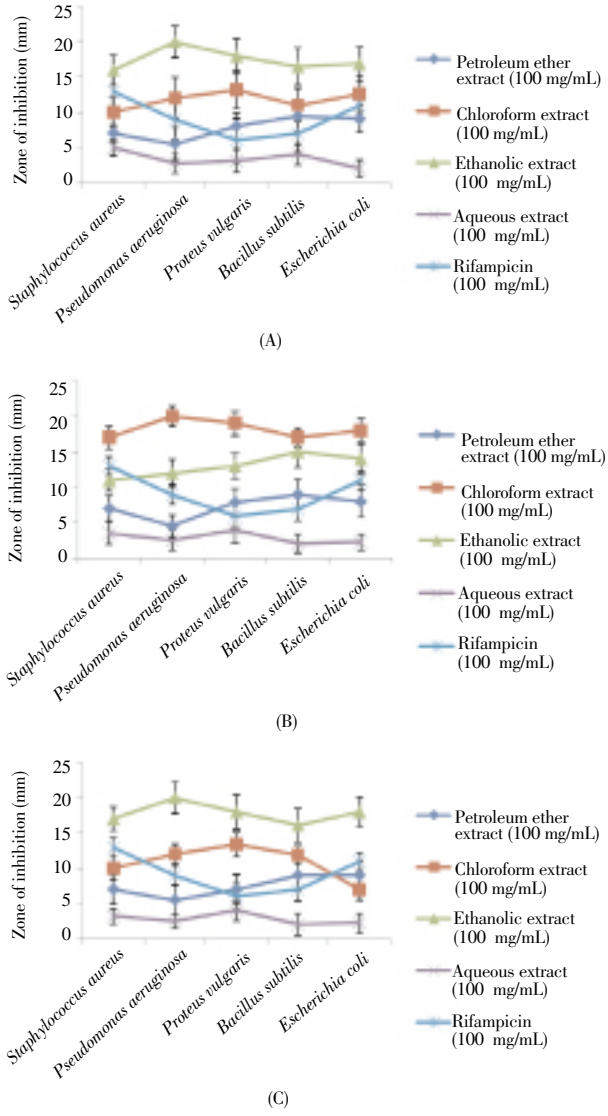

Figure 2. Antimicrobial activity of extracts from field grown *C. retusa* (A), *C. prostrate* (B), and *C. medicaginea*(C).

3.4. Determination of antioxidant activity

The ethanolic extract of in vitro propagated plants of C. retusa exhibited a significant dose dependent inhibition of DPPH activity with 50% inhibition (IC_{50}) at 57.6 μg/mL than *C. prostrate* (IC_{50} – 93.8 μg/mL) and *C. medicaginea* (IC_{50} –74.0 μg/mL) along the standard Vitamin C. The extracts obtained from field grown plants haven't shown a significance anti-oxidant activity (Table 1).

4. Discussion

Plants are the important source of potential compounds for the development of new therapeutic agents. There are several reports available on the antibacterial, antiviral and antifungal properties of plants[17]. Therefore these observations have helped in developing new drugs for the therapeutic use in human beings. However, the reports available on comparative studies are scanty.

In the present study, we have investigated the qualitative phytochemical screening for three field grown and *in vitro* propagated *Crotalaria* species[18]. The phytochemical screening of the plants showed that the leaves are rich in most of the secondary metabolites analyzed using different solvents as shown. These important constituents were extracted from petroleum-ether, chloroform, ethanol and water these extracts were qualitatively tested using standard references[10-13]. The present comparative studies revealed that there was significance in phyto profiles of *C. retusa, C. prostrate* and *C. medicaginea*.

The potential source of antimicrobial agents developing from plant species is an alternative strategy for the production of safe and standardization of phyto medicines against harmful microbes. The plant based antimicrobial agents have enormous therapeutic potential while they don't have any major side affects to the human beings[19]. The plant *Portulaca oleracea* possesses several phytochemicals and has significant antifungal properties against *Aspergillus* species[20-24].

The phytochemicals like alkaloids, saponins, flavonoids and phenolic compounds present in plants are responsible for many biological activities[25]. The ethanopharmacological exploration of plant species derived antimicrobial agents is needed for the production of safe therapeutic drugs. *C. retusa* and *C. medicaginea* extract possess a broad spectrum of antimicrobial activity against a panel of bacteria responsible for the most common bacterial diseases.

The ethanolic extracts of *Crotalaria* species were investigated for antioxidant properties due to the presence of the compounds as revealed in this study. Apart from that, the presence of alkaloids has abundant of medicinal use as antiparasitic and antmicrobial properties. Furthermore, it can be used as natural antioxidant source due to the presence of flavonoids and also phenolic compounds[13]. The DPPH test provides information on the reactivity of the test compounds with stable free radicals. The DPPH assay is

Table 1
Antioxidant activity of in vitro propagated *Crotalaria* species (% inhibition).

Concentration(μg/mL)	Antioxidant activity of ethanolic extracts						
	C. retusa		*C. prostrata*		*C. medicaginea*		Vitamin C (Standard)
	In vitro	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*
20	21.30±0.88	16.20±0.63	10.70±0.81	8.40±0.35	22.20±0.64	18.30±0.63	23.90±0.90
40	31.90±0.75	25.10±0.52	27.30±0.36	16.80±0.24	30.80±0.92	23.60±0.83	40.30±0.40
60	52.00±0.52	46.10±0.66	36.90±0.63	30.50±0.81	45.10±0.51	38.30±0.44	55.70±0.81
80	62.30±0.58	53.60±0.75	40.40±0.43	40.60±0.48	54.00±0.98	52.60±0.40	70.10±0.61
100	77.20±0.66	67.40±0.45	53.30±0.98	50.40±0.76	60.20±0.46	63.10±0.81	80.10±0.78
IC_{50} (μg/mL)	57.6	74.6	93.8	99.2	74.0	76.0	53.8

Each value is presented as mean ± S.E. (n = 3). The IC_{50} was obtained by linear regression equations.

often used to evaluate the ability of antioxidants to scavenge the free radical from test samples, whereby the free radicals cause biological damage through oxidative stress and such process lead to many disorders like neurodegenerative disorders, cancer and AIDS[26]. Therefore, DPPH assay is an effective method to measure their scavenging power. The principle of the DPPH assay is based on the color changes from purple (DPPH solution) to yellow[27]. The color changes can be measured quantitatively by absorbance at 517 nm. *C. retusa* shows significant activity over other two *Crotalaria* sps., thus further confirmed by comparing with standard Vitamin C. Hence this finding will help to develop new drugs for medicinal uses. The plants are still important for the discovery of new drugs as provider of the drugs based on secondary compounds from plants. In the present work, the antimicrobial activity of ethanolic extract of *in vitro* plantlets stands higher, there was less response for field grown species was reported.

The comparative phytochemical and pharmacological studies of both field grown and *in vitro* plantlets of *Crotalaria* species revealed the potency of phytocomponents and antioxidant properties of the ethanolic extracts against pathogenic microorganisms using agar disk diffusion method. The *in vitro* plantlet extracts has high significance than field grown. The present investigation helps in the discovery of plant based new drugs to human welfare.

Conflict of interest statements

We declare that we have no conflict of interest.

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