



Original Research Article

Antibacterial, Insecticidal Activity and Cytotoxicity of Methanol, Ethanol, Hot Aqueous and Cold Aqueous Extracts of *Crotalaria juncea* L.

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Abstract	Keywords
<p>Water and alcoholic extracts of <i>Crotalaria juncea</i> L. plant were tested for their antimicrobial properties <i>in vitro</i> against some selected human pathogenic bacteria such as <i>Pseudomonas aeruginosa</i>, <i>Escherichia coli</i>, and <i>Staphylococcus aureus</i> by agar well diffusion method and were subjected to brine shrimp lethality assay to detect their cytotoxic ability. In addition, the anticancer properties of these extracts were tested by cytotoxicity assay on HeLa cell line. In case of the antibacterial assay, hot aqueous extract had an average inhibitory zone of 14 mm while it was 10 mm in case of cold aqueous extract. The Ethanol extract showed the highest activity against the tested microorganisms. For insecticidal assay, in the first 24h, the hatching of eggs was significantly prevented with increasing concentration of both the alcoholic extracts. Brine shrimp lethality assay indicate that after 24h, live <i>Artemia</i> started dying in direct proportion with increasing concentration of alcoholic extracts. Cell toxicity assay indicate its cytotoxic nature on cancer cells. Results of this study show that ethanol and methanol extracts have more potency of antibacterial, antilarvicidal, and anticancer agent than hot and cold aqueous extracts of <i>Crotalaria juncea</i>.</p>	<p>Antibacterial assay Brine shrimp lethality assay <i>Crotalaria juncea</i> Cytotoxicity assay</p>

Introduction

Plants have served as a natural source of antibacterial substances. There are many plants used by primitive people in different countries to control microorganisms. Though many indigenous plants have been shown to have antimicrobial property, *Crotalaria juncea* L. (Syn.: *Crotalaria benghalensis* Lam.) is generally considered to have originated in India, where it has been cultivated since prehistoric times, but is now widely grown

throughout the tropics and subtropics (Chaudhury, 1966; Purohit, 1999; Hemendra et al., 2011). It is a short-day plant generally 1 to 4 m in height. Leaves are simple, spirally arranged along the stem. This plant is extensively cultivated for green manure and in Sri Lanka dried leaves, bark and boiled seeds are fed to cattle. This plant has been extensively used in cultivation. Its seed is used as an infertile purpose. It also has antiulcer, anti-inflammatory activity. *Crotalaria juncea* L. (Papilionaceae-family) is also being used in Indian

Ayurveda medicine as analgesic, astringent, emmenagogue, and abortifacient (Adams and Gianturco, 1956; Chaterjee et al., 1994; Perry, 2012).

Now a days, pathogenic bacteria are become resistant to antibiotic therapies. Incidents of epidemics due to such drug resistant microorganisms are now a common global problem posing enormous public health concerns. The worldwide emergence of multi-drug resistant bacterial strains is increasingly limiting the effectiveness of current drugs and leading to the failure in treatment of infections (Sibanda and Okoh, 2007, Donadio et al., 2010, Ciocan and Bara, 2007, Agarwal et al., 2012, Thota et al., 2012).

In spite of the need for new antibiotic therapies there has been a continuous decline in the number of newly approved drugs (Ho et al., 2009). Antibiotic resistance therefore poses a significant problem to human health.

A few of the resistant pathogens are (i) *Staphylococcus aureus*, (ii) *Pseudomonas aeruginosa* and (iii) *Escherichia coli*. *Staphylococcus aureus* is a major resistant pathogen that is extremely adaptable to antibiotic pressure. *Pseudomonas aeruginosa* is a highly prevalent opportunistic pathogen with low antibiotic susceptibility. *Escherichia coli* come directly from contaminated food and have been documented to be resistant to multiple fluoroquinolone variants. Therefore, amidst growing concern over the overuse of prescription antibiotics, many medical experts are taking a new look at natural, safe antibiotic alternatives. Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being.

Their role is two-fold in the development of new drugs:

- (i) they may become the base for the development of a medicine, a natural blueprint for the development of new drugs, or
- (ii) a phytomedicine to be used for the treatment of disease (Chew et al., 2010).

In the present study, the potency of the ethanolic, methanolic hot and cold aqueous extracts of the leaf of *Crotalaria juncea* an antibacterial agent for three different strains of bacteria, and as an insecticidal agent for inhibiting the hatching of eggs of *Artemia salina* and further the survival rates of brine shrimp in the leaf extracts have been examined.

Materials and methods

Collection of plant material

Fresh and healthy leaves of *Crotalaria juncea* were obtained from West Bengal, India. The leaves were first washed with tap water, followed by distilled water and then moist weight of the total leaf content was taken.

Preparation of plant extract

For preparation of hot and cold aqueous extracts, 5gm of leaf was weighed for each. The leaves were cut into small pieces and grinded using mortar pestle. For the cold aqueous extract, distilled water (30 ml) at normal room temperature was used to make a thick paste. For the hot aqueous extract, distilled water (30 ml) boiled at 60°C was used to make a thick paste. The final concentrations of the extracts were 160 mg/ml. The extracts were kept in tightly stopper bottles until they were to be used for biological testing. The methanol and ethanol dissolved plant metabolites were isolated from these leaves. Dried leaf (22g) was soaked in 350 ml of the respective solvent and kept for 2 days to release the metabolites into the solvents. The solvents were then filtered using separate clean muslin clothes and the filtrates were evaporated to dryness, so that the solvents get evaporated. These metabolite extracts were then dissolved in DMSO (100%) to obtain a final concentration of 85 mg/ml for each of the solvent extract (Mohan et al., 2010).

Antibacterial assay

The antibacterial activity of the leaf extracts of *Crotalaria juncea* were carried out individually on fresh cultures of pathogenic strains namely *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Gram positive and Gram negative). For assay of antibacterial activity 20ml of molten and cooled media (LB agar) were poured into sterilized petri plates. The plates were left overnight at 37°C to check for any contamination. The bacterial test organisms were grown for 12h (Ciocan and Bara, 2007; Agarwal et al., 2012). 200µl of Luria broth culture containing 10⁵cfu/ml of each of the microorganism was used to prepare bacterial lawns on the agar plates. Three plates were prepared corresponding to each of the three bacterial strains. Each plate containing wells were loaded with different concentrations of each of the extraction solvent. Standard antibiotic, cefranas was used as control for

comparison. The plates were then incubated at 37°C for overnight. The assay for antibacterial activity was carried on the basis of diameter of zone of inhibition (in mm) was measured for each extract at the end of incubation period.

Hatching efficiency assay

Brine shrimp-*artemia* cysts were used for testing the percentage of hatching. Experiment was performed in 12-well plates. And in each well containing 2 mL of 33 ppt in saline water along with control (without plant extract), about 20 *artemia* cysts were transferred with the addition of desired concentrations of plant extract (20, 40, 85, and 167 µg). Each test concentration along with the control was carried out in three replicates in 12-well plate. The experimental setup was allowed to remain 48h in shaker for aeration and the experimental set up was exposed to light to provide favorable environment for the cyst to hatch out. Hatched cysts were counted after the incubation period of 48h. Percentages of hatching for various tests were determined. Percentages of hatching were calculated by the following formula:

$$H\% = N / C + N \times 100.$$

Where *H* is the hatching percentage, *N* is the number of

hatched cysts, including the umbrella stage, and *C* is the

decapsulated full cysts (Mohan et al., 2010; Olowa and Nuñez, 2013; Ghosh and Chatterjee, 2003). Cysts were decapsulated using sodium hypochloride. Results were tabulated and plotted as a graph.

Brine shrimp lethality assay

A solution of brine (0.05g/ml) was prepared by dissolving 50g of sea salt in 1 liter of distilled water for hatching the shrimp eggs. The seawater was put in a small container for hatching shrimp eggs with a partition for dark (covered) and light areas. After two days, when the shrimp larvae were ready, 3 mL of the artificial

seawater was added to each test tube and 20 brine shrimps were introduced into each tube. Then the volume was adjusted with artificial seawater up to 5 mL per test tube. The number of surviving shrimps were counted and recorded after 24 h. For the test plate, the test samples were added in varying concentration of plant extract (167 µg, 85 µg, 40 µg and 20 µg) The percentage mortality (%M) was also calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100% (Das and Chandra, 2012; Vanhaecke et al., 1982; Sosan et al., 2001). This is to ensure that the death of the nauplii is attributed to the bioactive compounds present in the plant extracts. Results were tabulated and plotted as a graph. Percentage mortality was calculated by following the formulae % of Mortality = Number of dead *Artemia nauplii* / Initial number of live *Artemia nauplii* x 100.

Cytotoxicity assay

Human Cervical cancer cell line (HeLa) cancer cells were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS), 1% antibiotic. Cultured cells were incubated at 37°C, 5% CO₂ and 95% relative humidity. HeLa cells were seeded in 96-well tissue culture plate. After 24 hrs different concentrations of alcoholic and aquatic plant extract were added to the culture medium and incubated for 24 hrs at 37°C. Non-treated cells were used as control. Some of the wells with cells were kept as DMSO control. Incubated cultured cells were then subjected to tetrazolium salt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay (Goswami et al., 2014a; Goswami et al., 2014b; Goswami et al., 2014c). The MTT is used to determine cell viability and cytotoxicity. MTT was added at a final concentration of 0.5mg/ml and the cells were incubated at 37°C for 3.5 hrs (Goswami et al., 2014a; Goswami et al., 2014b). Then formazon dissolved with 100µl of DMSO in each well. The color changes were measured using a ELISA reader (Robonik, Readwell touch ELISA PLATE analyzer, India). The rate of survival was determined by using the following formulae: Cell viability (%) = (1- ODA₁/OD A₀)/100, where A₀=Absorbency of control cells and A₁=Absorbency of treated cells.

Results and discussion

Antibacterial activity of *Crotalaria juncea*

The *in vitro* antibacterial activities of hot and cold water extracts of the leaves of *Crotalaria juncea* are shown in Fig. 1. Three different bacteria were used for this purpose. Also, the inhibition zones formed by the antibacterial activities of each of the alcoholic and aqueous extracts and the antibiotic have been listed in

Table 1. The results showed that the hot water extract is more efficient in the antimicrobial activity compared to cold water extracts. It has also been noticed that *E.coli* is not susceptible to the alcoholic extracts whereas the other two pathogenic microorganisms are more sensitive to these alcoholic extracts.

Fig. 1: Effect of hot and cold extract of *Crotalaria juncea* on three bacterial cultures. A: stands for antibiotic ciprofloxacin (0.5mg/ml), H: stands for hot water extract, C: stands for cold water extract.

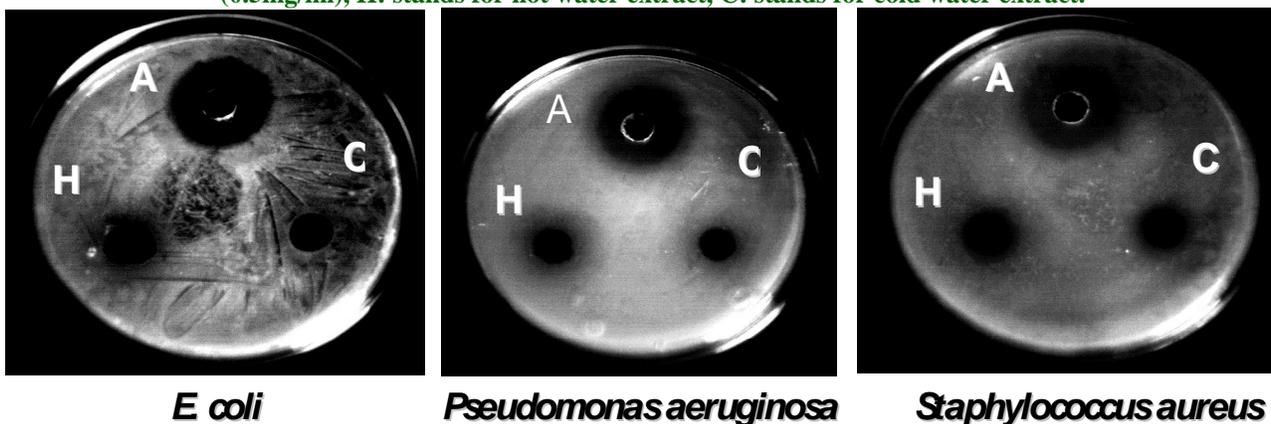


Table 1. Antibacterial susceptibility assay

Extracts	Zone of inhibition (mm)		
	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>
Hot	14	13	15
Cold Aqueous	10	10	12
Ethanol	26	14	11
Methanol	27	15	12
Ciprofloxacin 0.5mg/ml	26	26	25

Hatching efficiency assay of *Crotalaria juncea*

A plot of the hatching efficiency of the eggs of *Artemia salina* with increasing concentration of the leaf extracts has been shown in Fig. 2. At a concentration of 20 µg of alcoholic plant extract, the hatching efficiency of artemia was drastically reduced (almost 50%) compared to the hatching efficiency by aqueous extract of plant parts (~93%). The results thus indicate that the hatching efficiency is inversely proportional to the concentrations of alcoholic extracts.

Fig. 2: Effect of *Crotalaria juncea* plant extract on hatching efficiency of Brine shrimp.

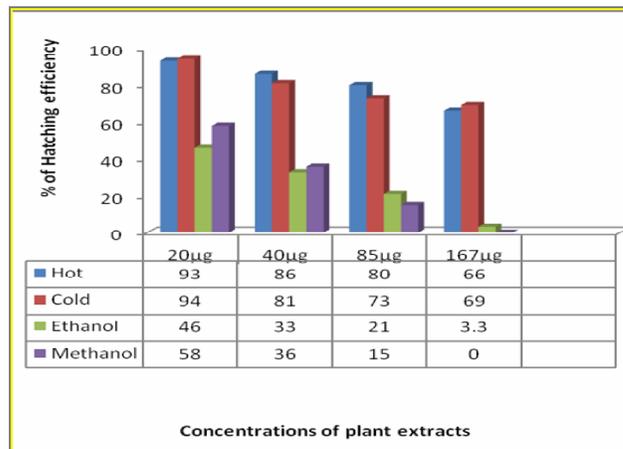
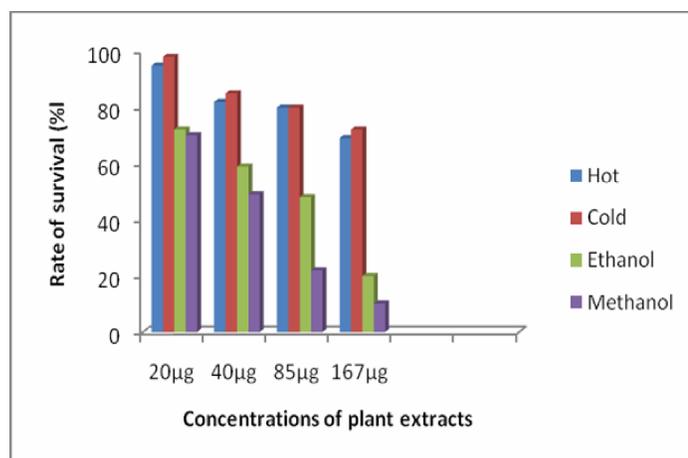


Fig. 3: Effect of *Crotalaria juncea* plant extract on brine shrimp lethality assay.



Brine shrimp lethality assay of *Crotalaria juncea*

The plant extracts of *Crotalaria juncea* showed significant brine shrimp larvicidal activity (Fig. 3). The lethality concentration (LC50) of Ethanolic and Methanolic extract are 85µg, and 40 µg respectively whereas lethality concentration (LC50) of aqueous extracts (both hot and cold) is extremely lesser than alcoholic extract. The degree of lethality was directly proportional to the concentration of the each extract. Based on the results, the brine shrimp lethality of the plant extracts were found to be concentration-dependent. The observed lethality of the plant extracts to brine shrimps indicated the presence of potent cytotoxicity on alcoholic extract.

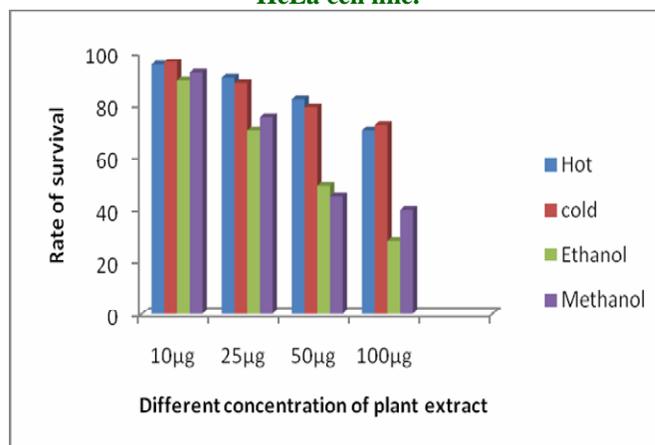
Bactericidal activity of *Crotalaria juncea*

The Hot and Cold aqueous extracts of the leaves of *Crotalaria juncea* show Zone of Inhibition for each of the bacterial strains. However, the Hot Aqueous extract shows a greater antibacterial activity against the same concentration of the cold extract used in the assay. This can be interpreted from the greater Zone of Inhibition obtained in Hot Aqueous extract. In case of alcoholic extract, Ethanolic extract showed more toxicity than methanolic extract.

Cell cytotoxicity assay

The extract of *Crotalaria juncea* was noted as having an anti-proliferative activity against the HeLa cells (Fig. 4). Furthermore, the methanolic and ethanolic extract of leaves showed 50% cytotoxic activity against HeLa cells at concentration of 50µg whereas aqueous extract showed only 10% lethality at this concentration.

Fig. 4: Effect of plant extract on cytotoxicity in HeLa cell line.



Conclusion

Graphical representation of lethality test indicate that in alcoholic extract of plant are more toxic than aqueous extract where 20% shrimp are died in 85 µg/ml hot water extract then 75% shrimp died in same concentration of ethanolic extract. Cell cytotoxicity test on HeLa cell line indicate that alcoholic extract have more cytotoxic than water extract. The study showed that the leaf extracts of *Crotalaria juncea* is a potent source for antibacterial and larvicidal agent. It is effective against both Gram positive as well as Gram negative bacteria. Thus, it can be used as a potential substitute for commercially used antibiotics. Although there is wide-spread recognition of many plants that possess remarkable insecticidal properties, very few pest control products obtained from plants are in use because the commercialization of new botanicals can be hindered by a number of issues. Presently, only 1% of the world insecticide market is constituted of botanicals used as insecticides. Therefore, an effort can be made for the commercialization of the plant extracts as a potent antibacterial and insecticidal agent. It also shows anti-cancer activity as its alcoholic extract killing HeLa cell in culture condition. So, *Crotalaria juncea* may be used as a potent larvicidal agent and antibacterial agent.

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