



Studies on the antibacterial and nucleic acid degradation property of *Cassia alata*

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Abstract:

This study aimed to screen preliminary phytochemical profile of the leaf extract of *Cassia alata* (L), one of the ethnomedicinal plants used by the Kani tribes of the Western Ghats, Tamilnadu, India. To evaluate the antimicrobial potential of multiple solvent based crude leaf extracts of the selected plant species *C. alata* against the few target human pathogenic microorganisms. Multiple solvents were used for the extraction of allelochemicals from the dried leaf material. Crude extracts were subjected to evaluate their efficacy in controlling the target microorganism's growth at *in vitro* level. Variation in the total nucleic acid content was also assessed in both control and treated bacterial samples. *C. alata* leaf extract chemical profile revealed the presence of secondary metabolites like tannins, phlobatanins, saponins, flavonoids, steroids, terpenoids and alkaloids. Crude extracts prepared in the Soxhlet apparatus ceased the growth of *Salmonella typhi* and *Pseudomonas aeruginosa*. *Proteus vulgaris* and *Klebsiella pneumoniae* were resistant even while fortified with 3 mg/ml extract concentration. Extract treated bacteria lack the total nucleic acid content, which proves and supports the presence of antimicrobial potential. Multiple solvent based *C. alata* leaf crude extracts have the potential to control one of the most important human pathogens *S. typhi*. This plant could be exploited further for the analysis of active principles towards the optimization of drug, as an evidence for sustainable utilization of the traditional knowledge on medicinal plants used by the Kani tribes herbal healers.

Keywords: *Cassia alata*, Phytochemistry, Antibacterial, Nucleic acid, MIC and MBC

Introduction

Natural products and secondary metabolites formed by living systems, notably from plant origin, have shown great potential in treating human diseases such as cancer, coronary heart diseases, diabetes and infectious diseases (1). In the recent years, research on medicinal plants has attracted a lot of attentions globally. Large body of evidence has accumulated to demonstrate the promising potential of medicinal plants used in various traditional, complementary and alternate systems of treatment of human diseases. The plants are rich in many pharmaceutical active ingredients like flavonoids, carotenoids, glycosides, phenols, alkaloids, terpenoids and

steroids etc., which have been found *in vitro* to have antimicrobial properties (2). Antimicrobial activities of many plants have been reported by the researchers. According to World Health Organization, 65 - 80% of the world populations rely on traditional medicine to treat various diseases (3). The beneficial health effects of many plants, used for centuries as seasoning agents in food and beverages, have been claimed for preventing food deterioration and as antimicrobials against pathogenic microorganisms (4). The plants have been found useful in the cure of a number of diseases including bacterial, fungal and viral diseases. Medicinal plants are a rich source of antioxidant and antimicrobial agents (5). Due to a rapid increase in the rate of

infections, antibiotic resistance in microorganisms and due to side effects of synthetic antibiotics, medicinal plants are gaining popularity over the drugs (6). Although medicinal plants produce slow recovery, the therapeutic use of medicinal plant is becoming popular because of their lesser side effects and low resistance in microorganisms (7).

Cassia alata L. (Family: Fabaceae) is an erect tropical, annual herb with leathery compound leaves and yellow flowers. The leaves, flowers and seeds have been reported to contain high levels of anthraquinones, crysophanic, naphthoquinone, or hennotannic acids which have been demonstrated traditionally to treat ringworm, eczema, itching skin infections in humans and very effective inhibitors of mite infestations, bacterial and microbial diseases (8). *Cassia spp* is known to contain some secondary metabolites like resin, saponins, phenols, flavonoids, anthraquinone glycosides and alkaloids. It is extensively used in folklore medicine for the treatment of skin diseases (9). The leaves are known for their antigonorrheal and purgative properties as well as a guinea worm and sore healing remedy among the Kanikaran and Paliyar tribes of Tamilnadu, India (10). Alam *et. al.*, (11) identified that the extracts in organic solvents (namely methanol, ethanol, ethyl acetate and chloroform) of two medicinal plants - *Achyranthes aspera* and *Cassia alata* were evaluated for their antibacterial activities against *Escherichia coli*, *Bacillus subtilis*, *Vibrio cholerae*, *Salmonella typhi* and *Staphylococcus aureus*. In the recent years, research on medicinal plants has attracted the stakeholders and they pay lot of attentions globally. Perusal of literature revealed that the promising potential of medicinal plants used in various traditional, complementary and alternate systems of treatment of human diseases. The antimicrobial activities of medicinal plants

can be attributed to the secondary metabolites are present in plants (12). With this backdrop information we had carried out preliminary phytochemical screening, effect of crude extract on the total nucleic acid content and antimicrobial bioassay test for methanol, ethyl acetate and acetone solvents based leaf extract of *Cassia alata* against *Salmonella typhi*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

Experimental

Collection of sample

The plant material used for this research work (*Cassia alata*) was harvest in August, 2009 from plains of Thanjavur, Tamilnadu, India where they were found growing naturally.

Extraction

The leaves were collected, shade dried for a week, ground to fine powder and sieved. Exactly 30 g of the finely grounded leaves were soaked in methanol, ethyl acetate and acetone (1: 1: 1 ratio) for 72 h. The solution was then sieved using first, muslin cloth and then number one Whatman filter paper. The filtrate was collected and concentrated using soxhlet apparatus. The crude extract was kept in the dessicator to dry. The aqueous & solvents (methanol, ethyl acetate and acetone, (1: 1: 1 ratio) hot extracts of sample (30g) was prepared by using Soxhlet apparatus and were concentrated using the same. All the crude extracts were kept in the dessicator to dry.

Phytochemical screening

Phytochemical screenings were carried out on the powder and extracts (wherever required) using standard procedure described by Odebiyi and Sofowora (13) to identify the constituents such as

Tannins, Phlobatannins, Saponins, Flavonoids, Steroids, Terpenoids and Alkaloids.

Collection and maintenance of Test organisms

Bacterial strains examined in this study were obtained mainly from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh. The bacterial strains used in this work were as follows *Salmonella typhi* MTCC (733), *Proteus vulgaris* MTCC (1771), *Pseudomonas aeruginosa* MTCC (3542), *Klebsiella pneumoniae* MTCC (432), and all bacterial strains were cultured in Luria-Bertani broth per liter; 10 g Bacto-tryptone, 5 g NaCl, 5g yeast extract (Difco Laboratories, MI, USA) at 37°C.

Antimicrobial activity assay

The antimicrobial activity of the crude extract (*C. alata*) on the test organism was done using the disc diffusion method (14, 15). About 100µl of 12 h broth culture of each test organisms was introduced onto sterile Mueller–Hinton agar plate prepared in sterile petri dishes and labeled accordingly. Inoculated petri dishes were incubated for an hour at 37°C. Meanwhile the crude extract impregnated discs (Whatman No.1 filter papers were used to prepare discs.) were prepared and air dried well. Discs were placed on the organism inoculated MH agar plate aseptically and incubated overnight at 37°C. Discs soaked in solvents mixture and dried were used as control for the experiments. The relative susceptibility of the organisms to the crude extract is indicated by clear zones of inhibition around the discs, which were observed, measured and recorded in millimeters.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC is the lowest concentration of a drug that prevents growth of a particular pathogen

(16). Different concentrations (1, 2 and 3mg/ml) of the crude extract of *C. alata* leaf were prepared and introduced into the 100 ml nutrient broth seeded with test organism. The flasks were incubated at 37°C. Mueller–Hinton broth inoculated with test organism without crude extract was treated as control for the experiment. Optical density was measured at 600 nm for control and treated samples against the respective blank prepared parallel with specific time interval. Data were interpreted to calculate the MIC of crude extract against test organism. The MBC was determined by sub culturing the test dilution (used in MIC) on to a fresh solid medium and incubated further for 24 h. The concentration of plant extract that completely controlled the organisms was taken as MBC (17).

Effect of *Cassia alata* crude extract on nucleic acid content of target microorganisms

50ml of overnight cultures of target microorganism in LB broth was fortified with crude extract at the concentration of 3mg/ml. Similarly for solvent control Methanol: Ethyl acetate: Acetone (1:1:1) mixture was added at a concentration of 3000 ppm, instead of plant extract. Experimental control flask also maintained which lacks neither plant extract nor solvent's mix. The set up was incubated in shaker with 120 rpm at 37°C. To analyze the effect on Nucleic acid 3.0 ml of sample was collected at two different time intervals like 30 min and 3 hrs.

Isolation of whole genomic DNA

Sample was centrifuged at 5000 rpm for 5 min to get bacterial pellet. Bacterial pellet was resuspended in 50 µl of 0.1% v/v Triton X-100 in sterile water and boiled for 4 min and cooled in ice water for 5min. Finally sample was centrifuged at high speed for 10 min to collect dissolved DNA in supernatant (18). Whole genomic DNA was

subjected to electrophoresis on 0.8% Agarose (Medox, India) gel at 100 V as constant. Gel was stained with ethidium bromide solution (10mg/ml) and documented with the help of Gel Doc system (Orbitec, India).

Results

C. alata leaf crude extracts were subjected to preliminary phytochemical analysis, antibacterial activity against selected pathogens, Antibacterial activity, MIC and effect of crude extract on total nucleic acid content are given in the table 1, figure 1 and figure 2 respectively. Preliminary phytochemical screening of *C. alata* leaf extracts revealed the presence of tannins, phlobatanins,

saponins, flavonoids, steroids, terpenoids and alkaloids in the solvent extract. The antibacterial activity of extracts of *C. alata* was observed against the tested pathogens. Among the tested plant leaf extracts of *C. alata*, multiple solvent based hot extract showed profound antibacterial activity compared to cold extract. The maximum zone of inhibition 16mm, MIC (3 mg / ml) and MBC (<3 mg / ml) were observed for *S.typhi*. Effect on nucleic acid content in *S.typhi* and *K. pneumoniae* was checked when treated with multiple solvent crude extract. It showed significant degradation of nucleic acids in *S.typhi* within 3hrs and there was no change in the total nucleic acid of *K. pneumoniae*.

Table 1: Effect of extraction mode on Antibacterial potency against target pathogens

S. No.	Pathogens	Zone of Inhibition (mm) Solvent – RT *	Zone of Inhibition (mm) Solvent – Hot **
1	<i>S. typhi</i>	8.0	16.0
2	<i>P. vulgaris</i>	-	-
3	<i>P. aeruginosa</i>	-	6.0
4	<i>K. pneumoniae</i>	-	-

* – Room Temperature, ** – Soxhlet based extraction

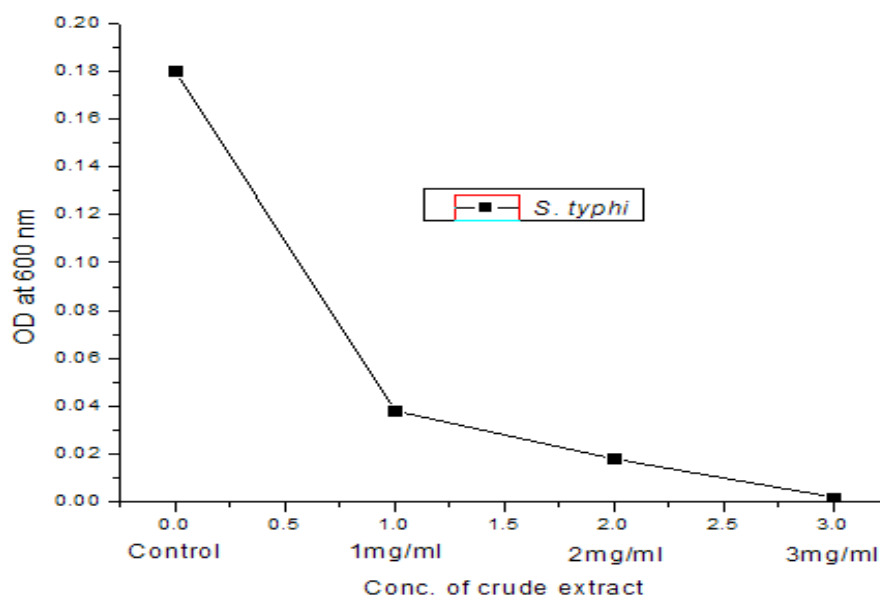


Figure 1: Determination of Minimal Inhibitory Concentration of crude extract against *S.typhi*

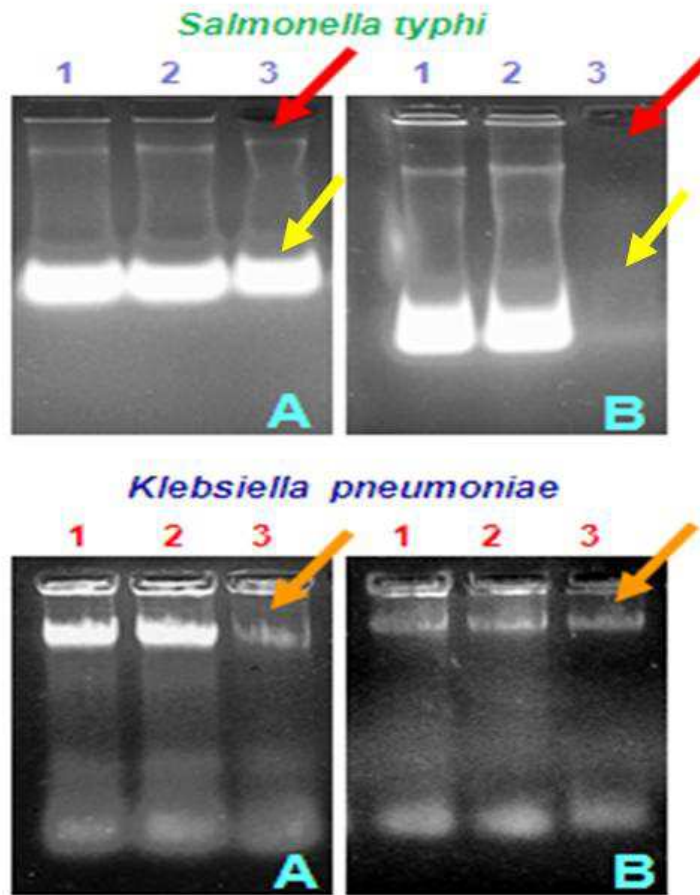


Figure 2: Effect of crude extract on total nucleic acid content of *S. typhi* and *K. pneumoniae* (1-Experimental control; 2-Solvent control; 3-Extract treatment. A – After 30 minutes from incubation; B – After 3 hrs from incubation)

Discussion

Phytochemical screening of the leaves of *C. alata* revealed the presence of tannins, phlobatanins, saponins, flavonoids, steroids, terpenoids and alkaloids, similar to the results presented by Usha *et al.*, (19) and Elmahmood *et al.*, (20). Drugs present in plants are known as active principles and these serves to protect the plants themselves against microbial (bacteria, fungi, viruses) infection as well as predation by pests and animals (21). The inhibitory activities exhibited by the extracts tend to agree with the reports of Jayaveera *et al.*, (22) and Elmahmood *et al.*, (23) all of whom linked antimicrobial properties of plants to the presence of bioactive secondary metabolites. For example, Sadiq *et.*

al., (24) investigated the antibacterial activity of leaf extracts of *Cassia occidentalis* (L.) and reported that the bioactive compounds found in the plant inhibited the growth of *S. typhi*, *E.coli*, *P.aeruginosa*, *Staphylococcus aureus*, and *Shigella* spp, and that the ethanol extract was most active.

A major contribution of higher plants to both traditional and biomedicine healthcare systems is the limitless capability of the plants to produce a large number of these organic compounds of high structural diversity. The accumulation of these bioactive organic compounds in large proportions in plant cells had, over the last 5 decades attracted the attention of the academic and research community, and the

knowledge so generated had helped in the inclusion of herbal medicines as a vital component of the health care systems, as well as identification of native medicinal plants in indigenous pharmacopeias (25). All the crude extracts of *C. alata* leaves were subjected to bioassay test against *S. typhi*, *P. vulgaris*, *P. aeruginosa* and *K. pneumoniae* (Table 1). As shown in the Table 1 multiple solvent systems based hot extract showed significant antimicrobial activity against *S. typhi* (zone of inhibition 16 mm), followed by the same extract obtained by soaking in solvent at room temperature which ceased the growth of *S. typhi* moderately (zone of inhibition 8 mm). Similarly, *P. aeruginosa* was also sensitive to the crude extracts but not up to the level (zone of inhibition 6 mm). Of the two types of extract tried, hot extract (Soxhlet based) was found to be efficient one rather than the extract obtained by simply soaking the leaf powder in the solvent. Since the later one failed to control the growth of *K. pneumoniae*, *P. vulgaris* and *P. aeruginosa* (26). Both the extracts did not control the growth of the *K. pneumoniae* which is one of the multidrug resistant strains (Data not shown). Highlight of the present study is significant level of sensitivity of *S. typhi* to the crude extract. The earlier report of Doughari *et. al.*, (27) revealed that methanol extract of *Senna alata* leaf control the growth of *S. typhi* moderately (Zone of inhibition 8 mm). So these results are reconfirming the use of multiple solvents for the extraction of chemical compounds from the natural materials may help to derive of one or more active principles from the crude extracts. The activity of the plant extracts against bacteria is an indication of the presence of broad or narrow spectrum antibiotic compounds or simply

metabolic toxins in the plant (28). The activity of extracts also varied based on the mode of preparation of the extracts either simple soaking the plant powder in respective solvents at room temperature or extracted by using soxhlets at solvent dependent temperature.

The multiple solvent system used in this study showed more potential than the aqueous extract, similar to the reports of Chavan *et. al.*, (29) that is the ethanol and aqueous extracts of the leaves of *Cassia tora* showed significant *in vitro* antibacterial activity against *P. aeruginosa*, *S. aureus*, *P. vulgaris*, *E. coli*, *S. typhi*, *Bacillus subtilis*, *Lactobacillus* spp, *Enterobacter* spp and *Streptococcus pneumoniae*. Perusal of literature revealed that the present study on the antibacterial activity of multiple solvent leaf extract, against *S. typhi* was found high throughput while compared with the previously published data on the same concept. And also this indicates the effective release of compounds like alkaloids, steroids, terpenoids and tannins, saponins from the plant powder could be achieved only by Soxhlet based hot extracting method. It has been reported that various group of phytoconstituents have different degrees of solubility in diverse types of solvents depending on their polarity nature and the extracting temperature (30). The result obtained in this study for the target organism *K. pneumoniae* was coincided with the findings of Anushia *et al.*, (31) and Duraipandiyar *et. al.*, (32) which showed that methanol and hexane extracts could not control the growth of *K. pneumoniae*. As far as *S. typhi* in concerned Alam *et. al.*, (33) found antibacterial activity of *C. alata* methanol extract against *S. typhi* and failed to control the same when used ethyl acetate extract separately. Antimicrobial activity of the *Cassia*

auriculata extract prepared in ethanol and aqueous crude extract showed 12 and 11 mm inhibition zone diameter against *S. typhi* (34), which is comparatively very less bioactivity rather than obtained in the present study and this helps to quote the justification value for application of multiple solvent systems for plant extraction. It is believed that fractionation of the multiple solvent based extract may yield quite number of high value and low volume active principles, because of the synchronized activity of various polarity properties bearing solvents in a mixture.

The results obtained in this study has shown that the leaf extract of *Cassia alata* possess antibacterial property. The susceptibility of *S. aureus*, *E. coli*, *B. subtilis*, and *K. pneumoniae* to the *Cassia fistula* plant extract justifies the traditional use of this plant in sore-healing and is also in agreement with the findings of Arulpandi *et al.*, (35) which says that the fresh leaves are used as poultices for swellings and wounds. The phytochemical compounds detected in the leaf extract included alkaloids, flavonoids, tannins, phlobatannins, saponins, anthraquinones terpenoids and Steroids. According to Pham-Huy *et al.*, (36) the presence of saponins, tannin, alkaloids, anthraquinone and phlobatannins are responsible for bioactivity of the plant extract.

The Minimal Inhibitor Concentration (MIC) of the leaf extracts were analyzed within the range between 0.1 – 10 mg/ml. In this experiment the MIC was accounted by measuring *S. typhi* bacterial growth in terms of OD₆₀₀. The MIC was confined at 3 mg/ml concentration of the crude extract by the experiments of MBC (Minimum Bactericidal Concentration), since the *S. typhi* growth was completely ceased (Figure. 1). Followed by the *S. typhi* growth was moderately affected when supplemented the crude extract

at 2 mg/ml concentration. And there was a significant effect found at 1 mg/ml concentration of extract against control where there was no extract amendment. The effects of the crude leaf extract correlates with the reports that microorganisms vary widely in their degree of susceptibility to agents (37). High MIC values are indication of low activity while low MIC values are indication of high activity. In this study, *S. typhi* had low MIC values, thus suggesting higher susceptibility to the efficacy of the crude extracts thus showed higher activity against the corresponding organisms. In all of the experiments conducted, water and solvents were used as control which did not show any appreciable activity. Also, the standard antibiotics, Erythromycin, Streptomycin and Kanamycin except Ampicillin consistently displayed superior potency when compared with the crude extracts against *S. typhi*. As far as *K. pneumoniae* is concerned among the four antibiotics only Streptomycin alone displayed superior potency and other three antibiotics were overwhelmed because of the presence of respective resistant genes. From the study it is obvious that the crude leaf extract of *C. alata* altered the nucleic acid content of *S. typhi* and *K. pneumoniae*. Incubation of *S. typhi* with optimum amount of crude extract (3 mg/ml of culture) for 30 min and 3 hrs showed non-degradation and degradation of nucleic acids respectively. This was confirmed and counter checked by running the experimental control and solvent control in parallel mode. Similarly there was no degradation of the nucleic acids in both short and long term incubation of *K. pneumoniae* with crude extract (Figure 2). This may be because of the effect of secondary metabolites present in the extract on the target

microorganisms by means of oxidative nucleic acids degradation. From this experiment, it is obvious that how the crude extracts ceased the growth of *S. typhi* and showed contrary effect on *K. pneumoniae*. This parameter added hypothetical evidence to conventional disc diffusion method and minimal inhibitory concentration experiments of both target microorganism.

Conclusion

The present research work evidently point out that the antibacterial activity of plant extract varies with the type of bacterial pathogens. Here we have compared two types of plant extraction methods and analyzed its potential to be used as antibacterial agent. It seems that soxhlet based hot extraction is better than the simply soaking the plant powder at room temperature. And also we have found that multiple solvent system based plant extractions showed significant biological properties. The phytochemical screening revealed that the presence of tannins, phlobatanins, saponins, flavonoids, steroids, terpenoids and alkaloids in the *C. alata* extracts. Multiple solvent soxhlet extract of *C. alata* showed promising activity against *S. typhi* but failed to control *K. pneumoniae*. Similarly multiple solvent based hot extract significantly degrade the total nucleic acids content in the sensitive *S. typhi* and not degrading the total nucleic acids of extract resistant *K. pneumoniae*. However, it determines the scientific importance of the medicinal plants used in traditional system of medicine by the Kani tribes of the Western Ghats, Tamilnadu, India, and gives a promising lead to the pharmaceutical industry sectors to formulate new antimicrobial drugs.

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