In vitro evaluation of different aqueous extracts of *Senna alata* leaves for antibacterial activity

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Abstract

The present study was to enrich the knowledge of antimicrobial activity of aqueous extracts (cold, hot and fresh juice) of leaf of *Senna alata* (L.) Roxb. and to confirm their effect through qualitative phytochemical analysis. The preliminary antibacterial assay was performed against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Proteus vulgaris* by agar well diffusion method. The Minimum Inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the cold and hot extracts were determined by macro broth dilution method. Streptomycin and sterile distilled water were used as the standard and control respectively. Qualitative phytochemical analysis was done to identify the chemical compounds present in the extracts. The antibacterial activity of the test extracts differed significantly ($P < 0.05$); the hot and cold extracts were able to inhibit the growth of all tested bacteria, while the fresh juice inhibited only *B. subtilis* and *S. aureus*. The cold extract revealed significantly ($P < 0.05$) higher inhibition on all test bacteria except *B. subtilis*, which was highly inhibited by fresh juice. The MIC of the cold extract ranged from 5 mg/ml to 40 mg/ml. The lowest value was against *P. vulgaris*. The MBC of the cold and hot extracts ranged from 20 mg/ml to 160 mg/ml. Phytochemical analysis revealed the presence of glycosides, alkaloids, saponins, cardiac glycosides, tannins, phlobatannins, flavonoids, terpenoids and anthraquinones. This study has proved the feasibility of *in vitro* control of tested bacteria by aqueous extracts of *S. alata* leaves.

Introduction

Senna alata (L.) Roxb (formerly known as *Cassia alata* Linn.) (Family-Fabaceae) (Tamil-Vandugolli, Sinhala-Et-tora) is a tropical plant, widespread in South East Asian countries [1]. It is a large shrub with very thick, finely downy branches; leaves large, sub sessile and pinnate; flowers are irregular, bisexual, golden yellow in spiciform pedunculate racemes; pod is long, ligulate with a broad wing down the middle of each valve [2]. This plant is traditionally used for the treatment of various ailments including several infections caused by bacteria, protozoa, fungi and viruses. The aqueous leaf extracts are used in the treatment of ringworm and parasitic skin diseases [3]. In Belgian Congo, the plant is employed as a remedy for leprosy. In Ghona the leaves are crushed, mixed with black peppers and applied on dhoby's itch, craw – craw and ringworm on the head and skin. In the Pacific Island and Mauritius the leaves are used for skin disease [2].

Owoyale et al. (2005) carried out antibacterial and antifungal screening of different organic solvent extracts of *Senna alata*, and they reported that the flavonoid glycoside is an active ingredient of the extracts [4]. Adedayo et al. (1999) demonstrated the antifungal activity of methanolic crude extract and partially purified fractions of flowers of *Senna alata* against standard and local fungal isolates [5]. Sule et al. (2010) reported the *in vitro* control of fungi; *Microsporum canis, Trichophyton jirrucosum, Trichophyton mentagrophytes and Epidermophyton jlorrcosum* using hot ethanol leaf extract of *Senna alata* [6]. The present study is an attempt to enrich the knowledge of antibacterial activity of different forms of aqueous extract of leaf of *S. alata* against some selected bacteria known to be pathogenic in human. The phytochemical components were also investigated as a scientific assessment of the claim of therapeutic potency of the extracts.

Materials and Methods

Preparation of plant extracts

The healthy plant leaves were collected from botanical garden of Department of Botany, University of Jaffna, Sri Lanka.They were dried in shade. Completely dried leaves were ground into fine powder using an electric blender. The powder was used to get cold and hot aqueous extractions as described below.

a). Cold extract

20 g powder was soaked in 60 ml sterile distilled water with intermittent shaking for one hour at ambient temperature. Then the mixture was filtered through doubled layered muslin cloth and the filtrate was further filtered through Whatman no 1 filter paper. The filtrate was completely dried in an oven at 45 °C [7].

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b). Hot extract

20 g powder was soaked in 60 ml sterile distilled water and kept in boiling water bath with intermittent shaking for one hour. Then the mixture was filtered through doubled layered muslin cloth and the filtrate was further filtered through Whatman no 1 filter paper. The filtrate was completely dried in an oven at 45 °C [8].

c). Fresh extract

20 g fresh healthy leaves were crushed with 20 ml distilled water using motar and pestle. The crushed material was filtered through two layered muslin cloth, and the filtrate was further filtered through Whatman no 1 filter paper. The filtrate was immediately used for the study [9].

Test microorganisms

Test bacteria, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris* were obtained from a bacterial culture collection, Department of Botany, University of Jaffna, Sri Lanka.

Phytochemical analysis

The phytochemical analysis of the fresh leaf juice was carried out to determine the presence of the following biomolecules using the standard qualitative procedures as described by Trease and Evans (1989) [10].

a). Test for tannins

1 ml of distilled water and one to two drops of ferric chloride solution were added to 0.5 ml of extract solution and observed for brownish green or a blue black coloration.

b). Test for terpenoids

5 ml of extract was mixed with 2 ml of $CHCl_3$ in a test tube. 3 ml of concentrated H_2SO_4 was carefully added along the wall of the test tube to form a layer. An interface with a reddish brown coloration indicated the presence of terpenoids.

c). Test for steroids

0.5 ml of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated H_2SO_4 was added slowly. Bluish green color was observed for steroids.

d). Test for saponins

5 ml of extract was shaken vigorously to obtain a stable persistent froth. The frothing was then mixed with three drops of olive oil and observed for the formation of an emulsion, which indicated the presence of saponins.

e). Test for flavonoids

A few drops of 1% NH_3 solution was added to the 2 ml of extract in a test tube. A yellow coloration was observed for the presence of flavonoids.

f). Test for cardiac glycosides

1 ml of concentrated H_2SO_4 was taken in to a test tube. 5 ml of extract was mixed with 2 ml of glacial CH_3CO_2H containing one drop of $FeCl_3$.The above mixture was carefully added to the 1 ml of concentrated H_2SO_4. Presence of cardiac glycosides was detected by the formation of a brown ring.

g). Test for phlobatannins

10 ml of extract was boiled with 1% HCl in a boiling tube. Deposition of a red precipitate indicated the presence of phlobatannins.

h). Test for Alkaloids

1ml of 1% HCl was added to the 3 ml of extract in a test tube. Then it was treated with a few drops of Meyer's reagent. A creamy white precipitate indicated the presence of alkaloids.

i). Test for Resins

5 ml of copper solution was added to the 5 ml of extract. The resulting solution was shaken vigorously and allowed to separate. A green precipitate indicated the presence of resin.

j). Test for Glycosides

10 ml of 50% H_2SO_4 was added to the 1 ml of extract in a boiling tube. The mixture was heated in a boiling water bath for 5 min. 10 ml of Fehling's solution (5 ml of each solution A and B) was added and boiled. A brick red precipitate indicated the presence of glycosides.

k). Test for Anthraquinones

Extract was mixed well with benzene, and then half of its own volume of 10% ammonia solution was added. Presence of a pink, red or violet coloration in the ammonial phase indicated the anthraquinones.

Determination of antibacterial activity

Agar well diffusion method was used to determine the antibacterial activity. 20 ml molten nutrient agar media were mixed with 1 ml of 10^6 colony forming units/ml) each test bacterial inoculum and poured into sterile Petri dishes separately. After complete solidification, 8 mm diameter wells were made using sterile cork borer.

The cold and hot test extracts were dissolved in distilled water. The wells were filled with filter sterilized 100 μl of (50 mg) cold extract, (50 mg) hot extract, fresh juice, (50 μg) streptomycin and sterile distilled water. Streptomycin and sterile distilled water were used as standard and control respectively. The plates were incubated at 37°C for 24 hours and antibacterial activity was determined by measuring the diameter of clear zone around the well using Vernier caliper [11]. Each experiment was repeated three times.

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

The minimum inhibitory concentration was determined by the macro broth dilution method [12]. The hot and cold extracts were diluted to 320, 160, 80, 40, 20, 10, 5, 2.5, and 1.25 mg/ml and the streptomycin was diluted from 5.12 mg/ml to 0.02 mg/ml as two fold dilution in nutrient broth. The tubes were inoculated with 1.0 ml (0.5 McFarland standards) of test bacteria and incubated at 37°C for 24 hours. The MIC was taken as the lowest concentration of test samples that did not permit any visible growth. For the determination of MBC, two loops full of culture were taken from each of the broth tubes that showed no growth in the MIC tubes and inoculated onto fresh nutrient agar plates. After 24-hour incubation, the plates were observed for the growth of bacteria. The concentrations of the extracts that showed no growth were recorded as the MBC. Each experiment was repeated three times.

Statistical analysis

Results were expressed as mean ± SD of three experiments. Statistical significance was determined using analysis of variance and Tukey test at $p = 0.05$ using statistical software SPSS Windows version 13.0.

Results

The qualitative tests for the presence of phytochemicals revealed that the fresh juice of *S. alata* possess glycosides, alkaloids, saponins, cardiac glycosides, tannins, phlobatannins, flavonoids, terpenoids and anthraquinones. But, the tests for steroids and resins did not show positive results (Table 1).

The hot and cold extracts were able to inhibit the growth of all test bacteria, while the fresh juice failed to inhibit the growth of *E. coli*, *P. vulgaris* and *P. aeruginosa*. The cold extract showed significantly highest inhibition on all test bacteria except *B. subtilis* compared to other two test extracts and the largest zone of inhibition was produced against *P. vulgaris* (23.1 ± 0.2 mm). There was no significant difference between the inhibitory effects produced by the cold and hot extracts on *S. aureus*. The *B. subtilis* was highly inhibited by the fresh juice of *S. alata* leaf (Table 2).

The antibiotic streptomycin inhibited the growth of all test bacteria except *P. aeruginosa*. In most of the cases the diameter of clear zone produced by the (50 mg/ml) crude test extracts on test bacteria were found to be larger than that produced by the (50 μg/ 100 μl) streptomycin to the respective bacteria (Table 2).

The minimum inhibitory concentration (MIC) of hot and cold extracts ranged between 5 mg/ml and 80 mg/ml. The lowest MIC value, 5 mg/ml was exerted by cold extract on *P. vulgaris*. The required MIC value of the cold extract for *E. coli*, *P. vulgaris* and *P. aeruginosa* were found to be lower than the hot extract. However, for *B. subtilis* and *S. aureus*, the required MIC values were equal in both hot and cold extracts. The MIC value of streptomycin was found between 0.04 mg/ml and 1.28 mg/ml. The minimum bactericidal concentration (MBC) of test extracts ranged from 20 mg/ml to 160 mg/ml, and the hot and cold extracts revealed the lowest MBC against *P. vulgaris* (Table 3).

Table 1: Phytochemical constituents of fresh leaf juice of *Senna alata*

Phytochemicals	*Presence / Absence*
Glycosides	+
Alkaloids	+
Saponins	+
Cardiac glycosides	+
Tannins	+
Phlobatannins	+
Resins	-
Flavonoids	+
Terpenoids	+
Steroids	-
Anthraquinones	+

+ present, - absent

Table 2: Antibacterial activity of different form of aqueous extracts of *S. alata* leaf

Treatment	*Diameter of inhibition zone (mm)**				
	Gram positive bacteria		*Gram negative bacteria*		
	B. subtilis	*S. aureus*	*P. vulgaris*	*E. coli*	*P. aeruginosa*
Fresh extract	20.6±0.6^a	11.6±0.8^b	-	-	-
Cold extract	14.6±0.7^b	14.3±0.3^a	23.1±0.2^a	21.1±0.8^a	19.0±0.7^a
Hot extract	13.8±0.4^b	14.6±0.5^a	21.7±0.6^b	18.8±0.7^b	10.5±0.5^b
Streptomycin	14.5±0.6	13.6±0.7	13.5±0.5	14.8±0.4	-

- No activity; * Zone of inhibition includes the diameter of well (8 mm); Values are mean ± SD, Values with different superscript on the same column are significantly ($P < 0.05$) different.

Table 3: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Senna alata* leaf extracts and streptomycin

Test bacteria	*MIC (mg/ml)*			*MBC (mg/ml)*		
	Hot	*Cold*	*Streptomycin*	*Hot*	*Cold*	*Streptomycin*
B. subtilis	40	40	0.08	80	80	0.32
E. coli	20	10	0.04	40	40	0.08
P. vulgaris	10	5	0.64	20	20	1.28
S. aureus	40	40	0.64	80	160	1.28
P. aeruginosa	80	20	1.28	160	80	2.56

Discussion

The present study was undertaken to determine the feasibility of *in vitro* control of bacteria by using three different forms of aqueous extracts of leaf of *Senna alata*. In indigenous medicine, these three forms of extracts are widely used for the treatment of various diseases. The results revealed that both cold and hot extracts were more effective than fresh juice, and these two extracts were able to inhibit the growth of both Gram negative and Gram positive bacterial species selected for this study.

The activity of the plant extracts against both Gram positive and Gram negative bacteria is an indication of the presence of broad spectrum antibacterial compounds [13]. Generally Gram positive bacteria shows higher sensitivity to plant extracts than Gram negative bacteria [14-17]. This variation is due to the differences in the cell wall structure and composition of Gram positive and Gram negative [18]. In this study, even though the cold extract had broad spectrum of activity, the Gram negative bacteria were highly inhibited than Gram positive bacteria. This suggests that there might be specific substances which inhibit the growth of Gram negative bacteria more.

The lower or absence of bacterial growth inhibition by the fresh juice of the leaf may be due to the lower concentration of active ingredients which are toxic to bacteria. Further study with higher concentration may give better inhibition. The amount of active ingredients in plant extracts depend on the climate conditions where the plants grow. Wandee (2010) reported that the amount of anthraquinone glycosides in the leaves of *S. alata* varied with season. In winter (November-February) and summer (March-May) plants contain the highest amount of total antraquinone glycosides (1.24% dry weight). But, the samples collected in rainy season (June-October) contain only 0.16% dry weight [1]. In the present study, the sample was collected from botanical garden where the plant is irrigated well. Therefore, the amount of active ingredients may be lower than that grow in wild.

The inhibitory effect of a plant extract resulted from the activity of phytochemicals was present in the extract. The type of phytochemical present in an extract depends on the type of solvent used for the extraction and the mode of extraction. In this study, the plant material was extracted in three different methods with water. It can be clearly seen the variation in the inhibitory effect with the variation of extraction method (Table 2).

The result of this experiment correlates with a former study, where the *E. coli*, *S. aureus* and *P. aeruginosa* were inhibited by aqueous leaf extract of *S. alata* in agar well diffusion method. In a previous study done by Okoro et al. (2010) documented that *S. aureus* was susceptible to polyphenol extracts of *S. alata*, while *E. coli* appeared to be resistant to the extracts [19]. But in the present study both bacteria were inhibited by aqueous extracts. In another study, hot (soxhlet) aqueous leaf extract of *S. alata* failed to inhibit the growth of *S. aureus* and *P. vulgaris*, where the antimicrobial screening was performed by agar disk diffusion method [20]. The variation in the results may be due to the variation in the extraction method or method of antibacterial screening or by both. It was already reported that agar well diffusion method is more effective than disc diffusion method for antibacterial screening as filter paper disc composed of cellulose where many free hydroxyl groups present on each glucose residues makes the surface of the disc hydrophilic [21], and therefore if an extract contains cationic active constituents with a good antibacterial activity it will not be expressed in disc diffusion method [22]. In the present study the hot extract showed comparatively lower activity than cold extract. Generally, treatment of plant extracts to high temperature could inactivate volatile compounds, but could also increase the release of active components and free radicals [7].

The result for the qualitative phytochemical analysis also correlates with some previous studies [4,7]. It has been reported that different phytoconstituents have different degree of solubility in different type of solvents depending on their polarity [7]. In traditional preparations water is largely used as the solvent.

The inhibition of tested bacteria by *S. alata* leaf extracts confirmed their antibacterial activity and this is most likely due to the action of different phyto-constituents present in the extract. Owoyale et al. (2005) reported that the antimicrobial activity of *S. alata* is associated with the presence of phytochemicals such as phenols, tannins, saponins, alkaloids, steroids, flavonoids and carbohydrates [4]. Flavonoids act as cytoplasmic poisons and also they have been reported to inhibit the activity of enzymes [23]. Saponins are surface active agents which interfere with or alter the permeability of the cell wall. Therefore, this facilitates the entry of toxic materials or leakages of vital constituents from the cell. Tannins act by coagulating the cell wall proteins [24]. Anthraquinones react irreversibly with amino acids in proteins, often leading to inactivation of the protein and loss of function. The alkaloids have ability to intercalate with DNA [6].

The standard antibiotic streptomycin showed higher activity with lower MIC and MBC values compared to the test extracts. Streptomycin is refined and purified product, whereas the test extracts are a mixture of varies plant constituents. Some of these constituents can interfere within them and this ultimately affects the antibacterial activity of the extract [7]. Therefore, further study with bioassay guided fractionation and isolation of pure compound(s) is necessary to authenticate the effect of these extracts.

Conclusion

The results of the present study have confirmed the long history of the use of aqueous leaf extracts of *S. alata* in traditional medicine for the treatment of microbial infections. Even though the hot and cold extracts had inhibition on all test bacteria, the cold extract showed comparatively better effect. Therefore, cold aqueous leaf extract of *S. alata* can be used for antibacterial treatment and antibacterial drug screening.

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