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Full Length Research Paper

Comparative antimicrobial activities of Neem, Amla, Aloe, Assam Tea and Clove extracts against *Vibrio cholerae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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This study was undertaken to identify medicinal plants that are effective against multiple human pathogens and to partially purify the active component through thin layer chromatography. Antibacterial activity of selected plant extracts were assayed by agar cup diffusion. Minimum inhibitory concentrations were determined against all the pathogens. Sensitivity of the pathogens was also checked with four standard antibiotics. In addition, the stabilities of the active compounds were checked at different temperature and pH conditions. Extracts were separated using TLC and relative mobilities of bioactive components were determined by contact bioautography. Ethanolic extracts of Amla (*Emblica officinalis*) fruit, Neem (*Azadirachta indica*) leaves, Aloe (*Aloe vera*) leaves, Assam Tea (*Camellia sinensis assamica*) leaves and Clove (*Syzygium aromaticum*) buds were found to inhibit the growth of methicillin resistant *Staphylococcus aureus*, *Vibrio cholerae* and *Pseudomonas aeruginosa*. Bioactive components were stable over a range of pH values and temperatures.

Key words: *Azadirachta indica, Aloe vera, Camellia sinensis assamica, Syzygium aromaticum, Staphylococcus aureus, Vibrio cholerae, Pseudomonas aeruginosa.*

INTRODUCTION

Infectious diseases are the leading cause of untimely death world-wide and it has become a global concern (Kumar et al., 2008; Mahady, 2005; Sakata et al., 2009). The clinical efficacy of many existing antibiotics is being threatened by rapid emergence of multidrug-resistant pathogens (Penner et al., 2005; Westh et al., 2004). Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind (Mitscher et al., 1987; Wadud et al., 2007). Natural products, either as pure compounds or as formulated with measured constituents of plant extracts, provide unlimited opportunities for emergence of new drug leads (Mukherjee and Wahile, 2006). There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases (Barbosa et al., 2009; Hazni et al., 2008; Kumar et al., 2008). Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with many side effects (Mukherjee and Wahile, 2006) and have an enormous therapeutic potential to treat many infectious diseases.

The present study was carried out to identify traditional plants that are effective against the human pathogens, *Staphylococcus aureus*, *Vibrio cholerae* and *Pseudomonas aeruginosa.* Even though these are very serious human pathogens and often associated with nosocomial infection (John, 1996; Prabuseenivasan et al., 2006), medicinal plants effective against all these pathogens put together and a systematic study thereafter towards purification of bioactive components is still scanty. In this study, we report that five traditional Indian medicinal plants (i) Amla (*Emblica officinalis*) fruit, (ii)

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Neem (*Azadirachta indica*) leaves, (iii) Aloe (*Aloe vera*) leaves, (iv) Assam Tea (*Camellia sinensis assamica*) leaves and (v) Clove (*Syzygium aromaticum*) are effective in controlling all the above mentioned human pathogens and that the bioactive components could be separated by thin layer chromatography (TLC). The plants were selected based on an earlier report of antibiotic activity and their common use as traditional medicinal plants (Das et al., 1999; Kumar et al., 2008; Shetty et al., 1994; Srikumar et al., 2007; Thakurta et al., 2007).

MATERIALS AND METHODS

Collection and pre-extraction of plant materials

Neem and Aloe leaves were collected from the Amity Institute of Organic Agriculture Farm, Noida, UP, India. Amla fruits were collected from Bharmar village, district Kangra in Himachal Pradesh. Assam Tea leaves were collected from a local tea farm in Assam, India. Clove buds were purchased from a local market in Noida, UP, India. All the plant materials were identified by the senior horticulturist, Archaeological Survey of India, Government of India, Agra. Plant materials were washed with distilled water and dried in an oven at 35 - 40°C for 4 - 5 days till the weight became constant. Plant materials were regularly examined to check any fungal growth or rotting.

Preparation of plant extracts

Ethanolic extracts were prepared as described previously (Ghoshal et al., 1996) with the following modifications. Ten grams of the plant materials were pounded manually with mortar and pestle and soaked in 40 ml absolute ethanol in 250 ml sterile conical flasks incubated at 37°C incubator with shaking at 120 rpm for 24 h. The content was filtered with Whatman No. 1 filter paper and the residue was again treated with 40 ml of absolute alcohol and incubated as mentioned earlier. It was repeated 3 times. The pooled up filtrates were evaporated to dryness under vacuum using a distillation unit. The dried extract was finally reconstituted in 5 ml of absolute ethanol and then packed in separate sterile glass vials and stored at 4°C until use.

Pathogens

V. cholerae strain was obtained from National Institute of Cholera and Enteric Diseases, Kolkata, India. Methicilin resistant, *S. aureus* and *P. aeruginosa* were procured from NuLife Consultants and Distributors Pvt. Ltd., Lajpat Nagar, New Delhi. Strains of bacteria were maintained at 4°C on LB plates and were sub-cultured (24 h, 37°C) prior to use. Purity of the cultures was checked at regular intervals as described by Acheampong et al. (1988).

Determination of antimicrobial activity of extracts

The agar well diffusion method was used for the primary screening of the plant extracts against target pathogens as described earlier (Perez et al., 1990) with slight modifications. Standardized inoculum (100 µl) of 0.5 McFarland turbidity standard, that is, equivalent to 5 x 10^8 cfu/ml (Lopez-Brea et al., 2008) of each test bacterial strains was spread using a sterile glass spreader onto sterile LB solid media plates so as to achieve even growth. The plates were allowed to dry and then a sterile cork borer (8.0 mm diameter) was used to bore wells in the agar plates. The extracts (50 µl/well) were loaded in the wells and absolute ethanol (50 µl/well) was taken as negative control. The plates were then incubated at 37°C for 24 h. Antimicrobial activity of the extracts was determined by measuring the diameter of inhibition zone in millimeter produced against the pathogens. The experiment was done three times and the mean values were calculated. To determine the minimum inhibitory concentration (MIC), serial dilutions of the extracts were done and assayed by agar well diffusion. The extracts were made out of 10 g dry weight sample and dissolved in the final volume of 5 ml ethanol leading to the concentration of plant extract as 2 µg/µl and calculations were made accordingly after observing hairline inhibition in the plates.

Antibiotic disc assay

The plates were prepared as mentioned above. The antibiotic discs of tetracycline, ampicillin, vancomycin and kanamycin each of 7.0 mm diameter (Hi- media) were placed using sterile forceps on the agar plates. The plates were then incubated at 37°C for 24 h. Susceptibility of the antibiotics against the test strains was determined by measuring the diameter of zone of inhibition (mm) produced against the test strains. The experiment was performed three times and the mean values were calculated.

TLC separation, contact bioautography and pH stability

Plant extracts were separated using pre-coated silica plates (Merck 60F-254) and running buffer composition as mentioned with the results. The TLC plates were cut into thin strips and placed with silica side down on the bacterial plate and growth inhibition was monitored. For pH stability assay, the TLC strips were treated with 100 mM of citrate buffer (100 µl) having pH 2.0, pH 7.0 and pH 8.0 separately for 1 h, after which bioautography was performed. Rf values were measured as the ratio of mobility for bioactive zone to the total length of the run.

RESULTS AND DISCUSSION

Antibiotic activity of plant extracts

In the present study, we identified five plants such as *Azadirachta indica* (Neem), *Aloe vera* (Aloe), *Emblica officinalis* (Amla), *Camellia sinensis assamica* (Assam tea) and *Syzygium aromaticum* (Clove) that are effective against all the three target pathogens *S. aureus*, *V. cholerae* and *P. aeruginosa*. Ethanolic extracts of these plants were serially diluted and the MIC values were determined (Table 1). As shown, all these five plants have the potential to control the growth of all the three pathogens. Extracts of Amla pulp and Clove buds were found to be highly efficient in controlling the growth of all tested pathogens with MIC values of 0.025 µg/µl; whereas, MIC of Neem, Aloe and Assam tea extracts ranged from 0.1 to 0.5 µg/µl (Table 1).

In terms of sensitivity against standard antibiotics, as shown in Table 1, *S. aureus* and *V. cholerae* strains were resistant to 30 µg of ampicillin, but were sensitive to 30 µg of kanamycin, vancomycin and tetracycline. The *P.*

Table 1. MIC of plant extracts and antibiotic sensitivity assay.

	MIC (µg/µl)		
Plant extracts	**Pseudo**	**SMR**	**Vibrio**
Neem	0.25	0.1	0.3
Aloe vera	0.35	0.1	0.3
Amla pulp	0.025	0.025	0.025
Assam tea	0.5	0.1	0.25
Clove bud	0.025	0.025	0.025
Kanamycin (30 µg)	Sensitive	Sensitive	Sensitive
Vancomycin (30 µg)	Sensitive	Sensitive	Sensitive
Tetracycline (30 µg)	Sensitive	Sensitive	Sensitive
Ampicillin (30 µg)	Sensitive	Resistant	Resistant

Table legend: MIC of plant extracts was obtained by agar cup diffusion assay and antibiotic sensitivity was obtained by disc assay. Pseudo - *P. aeruginosa*, SMR – *S. aureus*, Vibrio – *V. cholerae.*

Table 2. Stability of plant extracts at different temperatures.

Plant extract	**Neem**				**Aloe**				**Amla**			
Temperature (ºC)	4	25	60	100	4	25	60	100	4	25	60	100
Pseudo	15	15	15	15	16	13	16	15	17	17	16	16
SMR	24	24	23	24	14	14	16	14	30	31	29	29
Vibrio	15	15	15	15	14	14	15	15	22	21	21	20
Plant Extract	**Assam tea**						**Clove**					
Temperature (ºC)	4	25	60	100	4	25	60	100				
Pseudo	19	18	17	17	13	13	12	12				
SMR	20	15	15	15	28	27	26	27				
Vibrio	15	15	15	15	23	24	26	24				

Table legend: Heat stability of plant extracts was determined by treating the plants extracts for one hour in the indicated temperature followed by measuring zone of inhibition by agar cup diffusion assay. Pseudo -*P. aeruginosa*, SMR – *S. aureus*, Vibrio – *V. cholerae.*

aeruginosa pathogen showed sensitive response to all of the antibiotics tested.

Extreme temperature stability of plant extracts

The plant extracts were placed in a thermal cycler at 4, 25, 60 and 100°C temperature for one h and antibiotic assay was performed by agar well diffusion. The zone of inhibition with 50 µl extracts (Table 2) indicates that the bioactive components were very stable over the wide range of temperatures. The experiment was repeated three times with similar results and a representative one is taken for generating Table 2. Zone of inhibition of *V. cholerae* with Amla extract treated at various temperatures is shown in Figure 1.

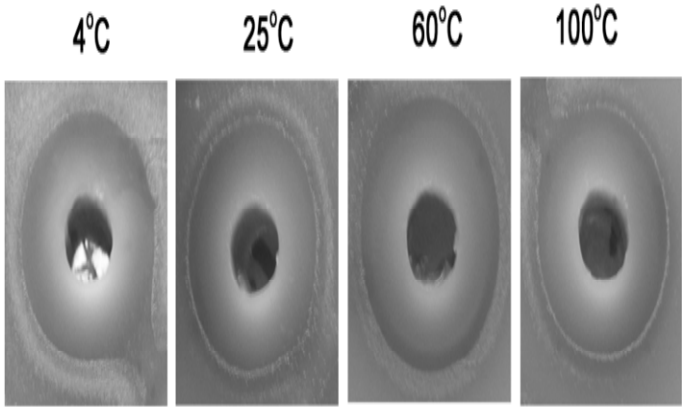

Figure 1. Zone of inhibition of *V. cholerae* with Amla extract after different temperature treatment. Amla extracts were treated at indicated temperature in a thermal cycler for one hour before placing the extract in agar well. Photograph was taken after 24 h of growth at 37ºC.

TLC separation of bioactive components

Plant extracts were separated by TLC after spot loading of 100 µl in pre-coated silica plates (25 x 10 cm Mark 60F-254). Amongst different solvent systems tried,

Table 3. Rf values of bioactive components in TLC strip.

TLC solvent	Toluene : chloroform : acetone: (40:25:35) (TCA)			Methanol : formic acid : (1:1) (MF)		
Pathogen	**Pseudo**	**SMR**	**Vibrio**	**Pseudo**	**SMR**	**Vibrio**
Neem	-	0.8	0.3, 0.8	0.8	0.16, 0.8	-
Aloe vera	-	-	-	0.8	0.7	0.8
Amla	0.13, 0.8	0.13	0.13	-	-	-
Assam tea	-	-	-	0.8	0.7	0.8
Clove	-	0.2, 0.8	0.8	0.8	-	-

Table legend: Rf values of bioactive spots as obtained in TLC under different solvent systems. Pseudo - *P. aeruginosa*; SMR – *S. aureus*; Vibrio – *V. cholerae*.

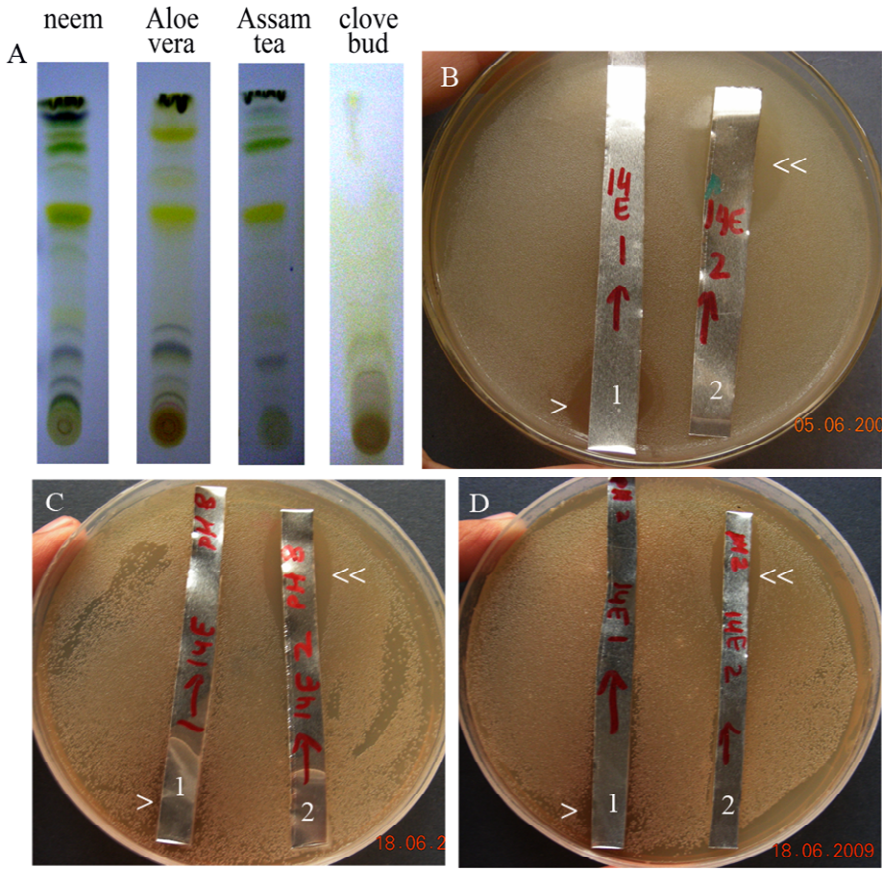

Figure 2. TLC separation of plant extracts and contact autobiography. (A) TLC strips of indicated plant extracts. Photographs were taken without staining. The bands are of natural pigments present. (B) Antibiotic zones detected from TLC strip of Clove bud against *S. aureus*. (C and D) pH stability study after treating the strips with buffers of pH 8.0 and 2.0, respectively. The strip was cut into two pieces to fit in the Petri dish as indicated by 1 and 2. > indicates the slower moving zone and << indicates the faster moving zone of inhibition.

toluene: chloroform: acetone (45:25:15, TCA) and methanol : formic acid (1:1, MF) were found to be differentially suitable (Table 3). TLC plates were cut along the run (Figure 2A) and bioactivity of the TLC separated plant extracts were performed by contact autobiography as shown for Clove extract against *S. aureus* (Figure 2B). The TLC strip was cut into pieces so that it can be accommodated in the 100 mm diameter Petri dish and placed silica side down over the culture plates. Plant extracts showed one or more bioactive components against the test pathogens. As shown for Clove extract (Figure 2B), there are at least two different bioactive component present in the sample, one resistant to movement in the TLC (>) and retained close to the

loaded region and the other one moved faster (<<). To refer the relative position of the migration of active component, the Rf values of the centre position of zone of inhibition was calculated (Table 3). As shown in the table, Assam tea and Aloe extracts showed only one active band against all the pathogens. On the contrary, Clove, Amla and Neem are apparently having at least two bioactive components migrating distinctly and are differentially effective against these pathogens.

Stability of TLC separated components in different pH range

By keeping in mind the potential use of these plant extracts as oral consumption to treat pathogen infection, stability of the plant extracts were studied in acidic and alkaline pH ranges. TLC strips were drenched with citrate buffer solution of pH 2.0, 7.0 and 8.0, respectively and incubated for one hour before testing the antibiotic activity assay through contact autobiography. It was observed that the slower moving zone of inhibition was reduced after treatment with pH 2.0 and 8.0 buffers, whereas the faster moving bioactive component retained activity (Figures 2C and D).

This study identified several novel roles of plant extracts and efficacies in controlling the growth of very challenging human pathogens. For example, *Aloe vera* is being used for decades as a medicinal plant (Lorenzetti et al., 1964; t Hart et al., 1990) against bacterial infection, but to our knowledge, this is the first ever report of its efficacy in controlling growth of *V. cholerae*. Similarly, the role of Amla extract in *V. cholerae* is also a novel finding of this study. However, the most important finding of this study is the identification of bioactive components present in multiple plants having similar mobility in TLC indicating the similar kind of component to be effective against multiple pathogens. This study may help in formulating a wide spectrum plant based antibiotic effective against common infection caused by these pathogens.

Conclusion

Extracts of Neem, Amla, Aloe, Assam tea and Clove showed that they are effective against all the tested human pathogens *P. arugenosa, S. aereus, and V. cholerae* in controlling their growth *in vitro* in culture condition. Bioactive component present therein is highly stable over extreme range of temperature and pH and can be separated out in TLC plate. The slower moving band from Amla extract in TCA solvent is very potent in inhibiting growth of all the pathogens tested. Similarly, faster moving band from Assam tea with MF solvent is effective against all the pathogens tested. Further research may be carried out to purify these components leading towards developing effective measure against bacterial infections.

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