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Antibacterial activity and preliminary screening of phytochemicals of locally available Green Leafy Vegetables in Batticaloa district, Sri Lanka

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ABSTRACT

Plants and plant products are a better alternative, compared to antibiotics and other synthetic drugs, which show negative side effects, including, sensitization reactions and disruption of the metabolic processes in the body via interaction with the body system. Herbal remedies offer deeper healing benefits, while providing lesser side effects, lower costs, and easy access. This study was aimed to investigate the antibacterial activity and the phytochemical properties of three locally available Green Leafy Vegetables (GLVs), namely, Argyreia pomacea (Convolvulaceae), Coccinia grandis (Cucurbitaceae) and Mollugo pentaphylla (Molluginaceae). All leaf samples were collected from different localities of Batticaloa district, Sri Lanka. The leaf extracts from acetone and ethanol at the concentrations of 25.0, 50.0 and 75.0 mg/100 µL were tested for antibacterial activity against Escherichia coli and Staphylococcus aureus, using well diffusion method and the qualitative phytochemical properties were also determined. The inhibitory effect was observed at all concentrations of acetone and ethanol extracts of C. grandis against both bacteria. The highest inhibitory effect on E. coli and S. aureus was shown by ethanol extract of all samples. The ethanolic extract of C. grandis at the concentration of 75.0 mg/100µL showed highest antibacterial activity against S. aureus (22.7±0.5 mm) and E. coli showed higher susceptibility to C. grandis (20±0.6 mm) in ethanol extract at 75.0 mg/100µL. Phytochemical analysis revealed the presence of alkaloids, saponins, flavonoids and tannins in both extracts of C. grandis. Flavonoids were found in the ethanol extracts of A. pomacea and M. pentaphylla.

Keywords: *Antibacterial activity, Escherichia coli, Green leafy vegetables, Phytochemicals, Staphylococcus aureus*

INTRODUCTION

Plants are producing a diverse range of bioactive compounds which are rich source of different types of medicines (Hussain *et al*., 2010). Also, traditional herbs are famous for their medicinal purpose and to treat a variety of diseases (Muthukrishnan *et al.,* 2014). Large number of research evidences show that the Green Leafy Vegetables (GLVs) are a rich source of nutrition as well as contain high medicinal values. They are also sources of carbohydrates, fats, important proteins, vitamins, minerals, essential amino acids, and fibers (Sharma and Kumar, 2013). The bioactive substances of GLVs have biological functions, including antioxidant and antimicrobial activities and can be helpful in management of oxidative stress and age-related human aliments. GLVs are higher in compounds having antidiabetic and anti-carcinogenic properties and have preventive or curative properties against cardiovascular disease, ageing, obesity, hypertension, insomnia and ageing (Bhat and Al-Daihan, 2014).

There are very limited data available on the antibacterial properties of *M. pentaphylla* and no data available, on the antimicrobial activity or phytochemical properties of *A. pomacea,* even though these two GLVs are very popular among the local community in Sri Lanka, particularly, in Batticaloa district, due to their medicinal properties against several human diseases and disorders,

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including, diabetics, curing deeper skin wounds etc. Whereas, with regard to *C. grandis*, limited reports are available on antimicrobial property in other countries (Sivaraj *et al.,* 2011), yet the geographical condition, particularly the soil composition, may influence and thereby may alter the composition of active compounds and the antimicrobial property of this leaf extract. Hence, *C.grandis* was also considered in this study.

Therefore, the study was aimed to determine the antibacterial activity of some selected GLVs namely, *A. pomacea*, *C. grandis*, and *M. pentaphylla*, by determining the inhibition zone of leaf extracts against *E. coli* and *S. aureus* and to evaluate the phytochemical constituents, qualitatively, of the leaf extracts of these three selected GLVs.

MATERIALS AND METHODS

Collection of GLVs and identification

Fresh and healthy leaves of *A. pomacea*, *C. grandis* and *M. pentaphylla* were collected from the local markets of the Batticaloa. All samples were collected on the same day. The identification of these GLVs were done at the Department of Botany, Eastern University, Sri Lanka.

Preparation of leaf extracts

The leaves were washed thoroughly with tap water to remove associated debris followed by sterile distilled water. Then leaves were dried under shaded condition at room temperature until obtained constant weight. Dried leaves were crushed into fine powder using grinding machine and was stored at room temperature (27^0C) in airtight bottle until further usage. The resulting powders were extracted by two different solvents as described below.

Preparation of acetone and ethanol extract

A 20 g powder of each tested species were separately soaked in 60 ml of ethanol in airtight conical flask for two days on an orbital shaker, and then they were first filtered through double layered Muslin cloth followed by Whatman No 1 filter paper. The filtrates were collected into airtight bottles. Similar process was repeated twice with fresh ethanol and the filtrates were pooled together. Finally, ethanol was removed from the filtrates at 40^oC in an oven. The resulting extracts were then stored into refrigerator until use for further study (Jeyaseelan *et al*., 2012). The similar procedure was followed to obtain the acetone extract as well.

Phytochemical analyses

The phytochemical screening of each sample was carried out using the standard qualitative procedures (Trease and Evans, 1989).

Alkaloids

One milliliter of 1% HCl was added to the 3.0 ml of leaf extract into test tube and was treated with few drops of Meyer's reagent. A creamy white precipitate indicated the presence of alkaloids.

Glycosides

Ten milliliters of 50% H_2SO_4 was added to the 1.0 ml of leaf extract into the boiling tube. The mixture was heated in boiling water bath for 5 minutes. 10.0 ml of Fehling's solution (5.0 ml of each solution A and B) was added and boiled. A brick red precipitate indicated the presence of glycosides.

Flavonoids

A few drops of 1% NH_3 solution was added to the 2.0 ml of leaf extract into the test tube. A yellow coloration was observed for the presence of flavonoids.

Saponins

Five milliliters of leaf extract were shaken vigorously to obtain a stable persistent froth. The frothing was then mixed with 3 drops of olive oil and observed for the formation of emulsion, which indicated the presence of saponins.

Tannins

To 0.5 ml of leaf extract solution, 1.0 ml of distilled water and 1-2 drops of ferric chloride solution were added and observed for brownish green or a blue-black coloration indicated the presence of tannins.

Phlobatannins

Ten milliliters of leaf extract were boiled with 1% HCl in a boiling tube. Deposition of a red precipitate indicated the presence of phlobatannins.

Terpenoids

Five milliliters of extract were mixed with 2.0 ml of $CHCl_3$ into the test tube. Concentrated H_2SO_4 (3.0 ml) was carefully added along the wall of the test tube to form a layer. An interface with a reddish-brown coloration was confirmed the presence of terpenoids.

Cardiac glycosides

Five milliliters of leaf extract were mixed with 2.0 ml of glacial acetic acid containing 1 drop of $FeCl_3$.The above mixture was carefully added to the 1.0 ml of concentrated H_2SO_4. Presence of cardiac glycosides was detected by the formation of brown ring.

Anthraquinones

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

Testing the antibacterial activity

E. coli and *S.aureus* were used to test the antibacterial activity of the leaf samples. Pure cultures were obtained from Microbiology lab, Teaching hospital, Batticaloa. These bacteria were stored on nutrient agar slope at 4 ºC and they were sub cultured before using.

Leaf sample preparation

The test concentrations of 25.0, 50.0 and 75.0 mg/100µL were prepared using the solvent of ethanol and acetone, separately.

Assessment of antibacterial activity

A well diffusion method was used to test the antibacterial activity of ethanol and acetone extracts of the different plant leaves. Nutrient agar plates were prepared for three different types of leaf extracts of each solvent and each bacterium. The experiments were repeated 6 times Totally 72 nutrient agar petri dishes were used (3 plant samples×2 solvents×2 bacteria's ×6 replicates = 72).

Bacterial suspension (1×10^6 cells/ml) was taken from serial dilution using hemocytometer and 0.1 ml of bacterial suspension was spread on the media plates, by sterilized glass spreader. Wells with 8.0 mm diameter were filled with 100.0 µL of each concentration of acetone and ethanol extracts, separately. Streptomycin (25.0µg/100µL) was used as positive control. Acetone and ethanol (100.0 µL) separately were used as respective negative controls. The plates were incubated at 37ºC for 24 hours, and the antibacterial activity was determined by measuring the diameter of inhibition zone around the well (Ragahavendra *et al*., 2010).

Statistical analysis

Diameter of inhibition zone resulted from replicates was expressed as mean ± standard deviation (SD). The data were analyzed by one-way analysis of variance (ANOVA, P value < 0.05) using statistical software, MINITAB 14 system.

RESULTS AND DISCUSSION

Collection of leaf samples

The leaf samples, *A. pomacea*, *C. grandis* and *M. pentaphylla*, collected from the study area are shown in the Figure 1.

Figure 1: The leaf samples, (a) *A. pomacea*, (b) *C. grandis* and (c) *M. pentaphylla* collected from the study area.

Phytochemical screening

The qualitative test for the presence of phytochemicals showed the presence of different types of phytochemicals in ethanol and acetone extracts of selected GLVs (Table 1).

Table 1. Presence of different phytochemicals in each tested GLVs observed by qualitative phytochemical analysis

	A. pomacea		*C. grandis*		*M. pentaphylla*	
Phytochemicals	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol
Glycosides	-	-	-	-	-	-
Alkaloids	-	-	+	+	-	-
Saponins	+	+	+	+	+	+
Flavonoids	-	+	+	+	-	+
Tannins	+	+	+	+	+	+
Terpenoids	-	-	-	-	-	-
Cardiac glycosides	-	-	-	-	-	-
Phlobatannins	-	-	-	-	-	-
Anthraquinones	-	-	-	-	-	-

Note: (+) Presence or (-) Absence of phytochemicals.

Antibacterial activity

The inhibition zone increased with concentration of leaf extract in acetone and ethanol (Table 2). Also, a significant difference ($P<0.05$) was observed in the inhibitory effect among different concentrations.

Acetone and ethanol extracts of leaf samples exhibited significant difference ($P<0.05$) against *S.aureus* among different concentrations (Table 3).

Table 2. Mean diameter of the inhibition zones, caused by acetone and ethanol extract of leaf samples against *E. coli* at different concentrations

Plant samples	Diameter of the inhibitory zone for *E. coli*					
	Acetone extract at different concentrations (mg/100µl)			Ethanol extract at different concentrations (mg/100µl)		
	25	50	75	25	50	75
A. pomacea	0.0	0.0	9.3 ± 0.5^{d}	12 ± 0.6^{l}	14 ± 0.6^{hi}	16.7 ± 0.5^{d}
C. grandis	9.5 ± 0.5^{d}	12.3 ± 0.5^{b}	16.5 ± 0.5^{a}	12.7 ± 0.5^{jk}	16.2 ± 0.7^{de}	20 ± 0.6^{a}
M. pentaphylla	0.0	0.0	10.3 ± 0.5^{c}	12 ± 0.6^{l}	13.8 ± 0.7^{hi}	16.7 ± 0.5^{d}
Streptomycin (25µg/100µl)	28.3±0.5			29.3±0.5		

Values are mean of replicates ±standard deviation. Values with different superscripts are significantly (P<0.05) different.

Table 3. Mean diameter of inhibition zones, caused by acetone and ethanol extract of leaf samples against *S.aureus* at different concentrations

Plant samples	Diameter of zone of inhibition in mm*S.aureus*					
	Acetone extract at different concentrations (mg/100µl)			Ethanol extract at different concentrations (mg/100µl)		
	25	50	75	25	50	75
A. pomacea	10.8 ± 0.4^{lm}	12.7 ± 0.5^{i}	16.2 ± 0.5^{d}	12.7 ± 0.5^{kl}	14.8 ± 0.4^{hi}	17.5 ± 0.8^{de}
C. grandis	11.3 ± 0.5^{k}	16.2 ± 0.4^{d}	18.8 ± 0.7^{a}	12.8 ± 0.4^{k}	17.7 ± 0.5^{de}	22.7 ± 0.5^{a}
M. pentaphylla	10.8 ± 0.4^{lm}	13.8 ± 0.4^{h}	15.8 ± 0.4^{de}	12 ± 0.6^{mn}	14.3 ± 0.5^{ij}	18 ± 0.9^{d}
Streptomycin (25µg/100µl)	34.7±0.5			35±0.8		

Values are mean of replicates ±standard deviation. Values with different superscripts are significantly (P<0.05) different.

Researchers have been involving in searching for the medicinal properties of plants that consist the active constituents as well as their antimicrobial properties (Rathnayaka, 2013; Gopalakrishnan *et al.*, 2016) since the human pathogenic bacteria are becoming resistance to many existing antibiotics (Wingmore *et al*., 2016).

As per the results of this present study, the ethanol and acetone extracts of all three GLVs exhibited the inhibitory effect against *E .coli* and *S. aureus* (Table 2 and 3). Among these GLVs, theantibacterial effect of the leaf extracts of *M. pentaphylla* and *A. pomacea,* are beingreported for the first time.

It was observed that ethanol and acetone extracts of *C. grandis* inhibited the growth of both *E.coli* and *S.aureus* effectively at three concentrations tested. Maximum zones of

inhibition were observed at the concentration of 75.0 mg/100µL against *E. coli* and *S.aureus* (Table 2 and 3). However, the acetone extract of *A.pomacea* and *M. pentaphylla* did not show the inhibitory effect against *E.coli* at 25.0 mg/100µl and 50.0 mg/100µL (Table 2). Whereas, the ethanol as well as the acetone extract of all three GLVs showed the antibacterial effect at all three concentrations against *S.aureus.* However, as per the diameter of the zones of inhibition, the ethanol extracts showed higher inhibitory effect against *S.aureus* than the acetone extract (Table 3). There is no significant difference ($P<0.05$) in the diameter of the zones of inhibition, produced by the ethanol and acetone extracts of *A. pomacea* and *M. Pentaphylla* either against *E.coli* or *S.aureus*.

Sivaraj *et al.* (2011) reported that the acetone extract of *C. grandis* showed intermediate inhibitory activity and the minimum inhibitory concentration was 500µg/ml against *E. coli*, while ethanol extract of *C. grandis* showed higher antibacterial activity. The authors further reported that the acetone extract of *C. grandis* showed no inhibitory activity against *S.aureus* and the ethanol leaf extract of *C. grandis* showed higher antibacterial activity against *S.aureus.* However, as per the results of this study, the acetone extract of *C.grandis* showed inhibition zones against *E.coli* at all three concentrations, 25 mg/100µL and 50 mg/100µL and 75 mg/100µL. Moreover, the ethanol extract of the leaf sample showed the inhibition effect higher than the acetone extract.

According to Bandibas and Roxas (2017), *M. pentaphylla* extract showed average inhibition against *E. coli* and however, the present study indicated that the inhibitory effect of ethanol extract of *M. pentaphylla* is higher.

Secondary metabolites including tannins, flavonoids, terpenoids, saponins, cyanogenic glycosides, nitrogen compounds (example Alkaloids), phenols, and phenolic glycosides, play an important role in reinforcement of plant tissue and thus for the survival of plant (Abdelwahab *et al*., 2010; Chukwuka, *et al*., 2011) against free radicals (Manach, *et al*., 2004; Obeidat *et al*., 2012). Spencer (2008) indicated that the flavonoids show antiallergic, anti-inflammatory, antimicrobial, anticancer, and antidiarrheal activities. Therefore, as per the results of this study, presence of these compounds in the above plant extracts may play an important role in their antimicrobial action. However, further scientific evaluation of these GLVs, including fractionation and quantitative analysis of phytochemicals is needed to identify the active components responsible for the antimicrobial activity and to encourage developing a novel broad spectrum antimicrobial herbal formulation in future.

CONCLUSION

This study revealed that, acetone and ethanol extracts of the Sri Lankan traditional medicinal plants, *A. pomacea, C. grandis* and *M. pentaphylla*, were found to have antibacterial effect against *E. coli* and *S. aureus* and particularly, for the first time, we report the antibacterial and phytochemical properties of ethanol and acetone leaf extract of *A. pomacea* which has been very popular GLV for its medicinal properties, among the rural community of Sri Lanka.

Among the two bacteria, *S. aureus* was more sensitive to all three leaf extracts, tested, than *E. coli*. The ethanol extract of these plants showed stronger antibacterial property, at all three concentrations, examined.

The results of qualitative phytochemical analysis proved the presence of alkaloids in ethanol and acetone extracts of *C. grandis.* Saponins and tannins were found in both extracts of all selected GLVs. Also, flavonoids were found in ethanol extracts of all three leaves samples. Thereby, phytochemicals may responsible and further provide the information regarding the anti-bacterial activity of these test extracts. Hence it can be concluded that these GLVs would direct to invent new and potent anti-microbial drugs of natural origin as natural

remedied against infectious diseases. Yet, further scientific evaluation should be carried out to identify the bio-active compounds, responsible in this antibacterial property of these GLVs. Indeed, this study also supports the traditional uses of these GLVs for various health issues, among the rural community in Sri Lanka, till to date.

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