



RESEARCH ARTICLE

INVESTIGATION OF ANTIBACTERIAL ACTIVITY AGAINST METHICILLIN RESISTANT
STAPHYLOCOCCUS AUREUS (MRSA) AND SELECTED PHARMACOLOGICAL
PROPERTIES OF PANCHAWALKALA: AN AYURVEDIC FORMULATION

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ABSTRACT

Panchawalkala is a poly-herbal formulation comprising of equal parts of the dried stem barks of five medicinal plants named, *Ficus benghalensis*, *Ficus racemosa*, *Ficus religiosa*, *Ficus tsiela* and *Garcinia cambogia*. The direct aqueous and sequential organic extracts of Panchawalkala were obtained by 3 extraction procedures to represent hot and cold conditions. The preliminary antibacterial screening was carried out using Cut-well diffusion method and minimum inhibitory concentration (MIC) was determined using micro-broth dilution assay. The antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The highest antibacterial activity was demonstrated by the hot aqueous extract obtained by the reflux method. Both sequential methanol extracts (Soxhlet method and Bottle-shaker method) and hot aqueous extract demonstrated high DPPH radical scavenging activity compared to L-ascorbic acid and other tested solvent extracts.

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INTRODUCTION

During the last few decades, the incidence of microbial infections has increased dramatically (Livorsi et al., 2012). Continuous deployment and misuse of antimicrobial drugs in treating infections has led to the emergence of resistance among almost all strains of microorganisms. Antimicrobial resistance is associated with high mortality rates and increase of health care costs. In addition, there is a significant impact on the effectiveness of currently available antimicrobial agents. Multi-drug resistance (MDR) provokes obstruction in disease control by intensifying the possibility of spreading of resistant pathogens, thus, declining efficacy of treatment and, hence, resulting in prolonged time of infection in patient (Sengupta et al., 2013). Due to the increase of resistance to available antibiotics, there is an urgency of developing alternative antimicrobial agents to combat them. Among the potential sources of new antimicrobial agents, medicinal plants have long been investigated (Cragg and Newman, 2013). Because, they containing many bioactive secondary metabolites that can

be of interest in therapeutics (Schultes, 1978). Ayurveda is known to be a life science and most of its therapies are undertaken by using medicinal plants individually or with combination of several plants parts as a formulation (Subramani et al., 2014). Panchawalkala is such poly-herbal formulation with combination of equal parts of the bark of five medicinal plants named, *Ficus benghalensis*, *Ficus racemosa*, *Ficus religiosa*, *Ficus tsiela* and *Garcinia cambogia*. Panchawalkala is one of the great poly-herbal formulation and used since thousand years for the treatment of infected wounds (Peiris et al., 2015). Some of the previous investigations have been carried out using Panchawalkala which is a combination of five medicinal plants named, *Ficus benghalensis*, *Ficus glomerata*, *Ficus religiosa*, *Thespesia populanea* and *Ficus lacor* Buch.-Ham. It shows properties like antiseptic, anti-inflammatory, immune-modulatory, antioxidant, antibacterial, antimicrobial wound purifying and astringent properties (Gajarmal et al., 2014) and which is differ from the five plant species investigated in the current study. In addition, another report showed that, the decoction of Panchawalkala was demonstrated a zone of inhibition (ZOI) against

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Staphylococcus aureus ATCC 25923 strain in disc diffusion assay (Peiris *et al.*, 2015). However, far too little attention has been paid to the following areas of Panchawalkala: 1) the antibacterial activity against drug resistant bacteria, such as methicillin resistant *Staphylococcus aureus* (MRSA), 2) the comparison study of antibacterial activity of extracts obtained by different extraction procedures and solvents, 3) comparison of antioxidant activity for different solvent extracts of Panchawalkala. Therefore, current investigation was designed, in the aim of, fulfillment of the above mentioned gaps in the scientific literature of Panchawalkala.

MATERIALS AND METHODS

Sample Collection and Preparation

The barks of *F. benghalensis*, *F. racemosa*, *F. religiosa*, *F. tsiela* and *G. cambogia* were obtained from a drugstore in Gampaha. The collected samples were washed with de-ionized water and shade dried. Dried samples were then stored in air tight bags at -20°C until further use.

Sample Authentication

The plant materials were authenticated by the plant taxonomist at the Herbarium of Gampaha Wickramarachchi Ayurveda Institute.

Preparation of extracts

Direct Aqueous extracts: The dried bark of each plant was ground using an electric grinder to obtain powdered plant material. Aqueous extracts were obtained from the powdered plant material using two methods: an aliquot of the powdered material (50 g) was suspended in distilled water (500 mL) and was heated under reflux for 6 h; a second aliquot of the powdered material (50 g) was also extracted into distilled water (500 mL) at ambient temperature using a bottle shaker (GFL 3016, Germany) for 24 h. Each aqueous extract was centrifuged and the supernatant was freeze-dried to obtain a crude powder.

Sequential extracts: The powdered plant material (50 g) was packed in a cellulose thimble, placed in the extraction tube of the Soxhlet apparatus and extracted for 6 h until there was no colour with hexane (500 mL) followed by dichloromethane (DCM, 500 mL), ethyl acetate (EtOAc, 500 mL) and methanol (MeOH, 500 mL). Another aliquot of powdered plant material (50 g) was also extracted into hexane (500 mL) followed by DCM (500 mL), EtOAc (500 mL) and MeOH (500 mL) at ambient temperature for 24 h using a bottle shaker. Solvent of each DCM, EtOAc and MeOH extracts were removed under reduced pressure using a rotary evaporator (Heidolph, Laborota 4000, Germany).

Phytochemical analysis of the hot aqueous extracts of constituent plants of Panchawalkala

Test for alkaloids

The test was carried out according to the method described by Julian *et al.* (2005). Method in briefly, 1.27 g of iodine and 2 g of potassium iodide were dissolved in 5 mL of water and the solution was diluted to 100 mL with water. A few drops of this

reagent were added to the extract, and observed for a brown color precipitate.

Test for saponins

The test was carried out according to the method described by Harborne (1973). Method in briefly, small quantity of the extract was shaken with 2 mL of water.

Test for phenolic compounds/tannins

The test was carried out according to the method described by Allen (1974). Method in briefly, to 3 mL of extract, 3 mL of 5% w/v ferric chloride solution was added.

Test for Terpenoids

The test was carried out according to the method described by Harborne (1973). Method in briefly, 2 mL of chloroform was added to the extract. For that mixture conc. sulphuric acid was carefully added (3 mL) to form a layer.

Test for flavonoids

The test was carried out according to the method described by Sofowora (1993). Method in briefly, 5 mL of dilute ammonia solution was added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated sulfuric acid.

Test for Triterpenoids

The test was carried out according to the method described by Harborne (1973). Method in briefly, to 2 mL of extract 5 drops of concentrated sulphuric acid was added, shaken and allowed to stand.

Test for sterols

The test was carried out according to the method described by Allen (1974). Method in briefly, 5 mg of residue of the extract was taken in 2 mL of chloroform and in it 2 mL of concentrated sulphuric acid was added from the side of the test tube. The test tube was shaken for few minutes.

Test for proteins

The test was carried out according to the method described by Meera and Nagarjuna (2009). Method in briefly, to 3 mL of extract 1 mL of 4% w/v sodium hydroxide and 1mL of 1% w/v copper sulphate was added.

Test for reducing sugars

The test was carried out according to the method described by Meera and Nagarjuna (2009). Method in briefly, equal volume (2 mL each) of Benedict's solution and extract was mixed in a test tube and heated in boiling water bath for 10 min.

Preliminary screening for antibacterial activity by Cut-well diffusion method

Screening of the aqueous and organic extracts of Panchawalkala was performed using the Cut-well diffusion method (Okeke *et al.*, 2001). Briefly, bacterial suspensions of

test and control organisms were adjusted to McFarland turbidity of 0.5 (approximately $1.5 \times 10^{7-8}$ cfu/mL) and inoculated onto Muller-Hinton agar (MHA) (SRL, India). The inoculated plates were swirled evenly to distribute the organisms and excess broth removed using a sterile pipette. The plates were left at room temperature for 30 min after which 8 mm diameter wells were bored in the agar and the bottom sealed with molten MHA. The dissolution of organic extract was aided by 10% (v/v) dimethylsulfoxide (DMSO). Using a prepared template, aliquots of each reconstituted extract (10 mg/mL) were pipetted into the wells and the plates incubated at 35 °C for 24 h. The diameter of the zone of inhibition (ZOI) around the well was measured. Each screening was carried out in triplicate and the mean diameter of the ZOI was recorded.

Determining the minimum inhibitory concentration by micro-broth dilution assay

Determination of MIC of the direct aqueous and sequential methanol extracts obtained from Panchawalkala was performed using the micro-broth dilution method (Mattana *et al.*, 2010). Briefly, the 96-well plates were prepared by dispensing into each well 95 µL of Muller-Hinton broth (MHB) and 5 µL of the inoculum (McFarland turbidity of 0.5). An aliquot (100 µL) from each of the 8 serial dilutions prepared from each of the stock solutions of the extracts was transferred into eight consecutive wells in the microplate. The final volume in each well was 200 µL. The plates were incubated at 37 °C for 18 h. Controls were included. Negative control; 95 µL MHB + 5 µL organisms + 100 µL distilled water (DW), Positive control; 95 µL MHB + 5 µL Organism + 100 µL antibiotic (vancomycin) Blank; 95 µL MHB + 5 µL DW + 100 µL extract respectively. Sterility control; 200 µL of MHB.

Determination of antioxidant activity of Panchawalkala

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Sigma Aldrich, USA) scavenging activity was determined according to the method of Liyana-Pathirana and Shahidi (2005) with slight modifications. Briefly, 100 µL of each extract at various dilutions (2500-4 ppm) were mixed with 100 µL of 0.2 mM DPPH solution. The mixture was vortexed for 1 min, kept for 30 min in the dark and the absorbance measured at 490 nm in an automated microplate reader. All determinations were performed in triplicates. L-ascorbic acid was used as a positive control.

The percentage scavenging effect was calculated as:

$$\% \text{ Scavenging rate} = (A_0 - (A_1 - A_2) / A_0) \times 100\%$$

Where, A_0 is the absorbance of the control (without sample) and A_1 is the absorbance of sample in the presence of the DPPH; A_2 is the absorbance of sample without DPPH radical (blank absorbance). The scavenging ability of the samples was expressed as EC_{50} value, which is the effective concentration at which 50% of DPPH radicals were scavenged; the EC_{50} value was calculated from the curve of percentage scavenging activity (%) versus concentration of the respective sample.

RESULTS

Percentage yields of aqueous and organic extracts of Panchawalkala

Direct aqueous extracts: The hot extraction of Panchawalkala by the reflux method followed by freeze-drying furnished a reddish-brown powder with 1.8% yield. The corresponding

Table 1. Percentage yields of Panchawalkala extracts

Extraction method	Percentage yields %				
	Direct aqueous extracts	Hexane	Sequential extracts DCM	EtOAc	MeOH
Reflux method	3.8	ND*	ND	ND	ND
Bottle-shaker method	1.12	0.23	0.08	0.2	4
Soxhlet method	ND	0.26	0.06	0.24	4.8

* ND – Not done

Table 2. Phytochemical screening results of the constituent plants of Panchawalkala

Plant extracts	Phytochemical screening tests						
	Alkaloids	Saponins	Phenols/tannins	Terpenoids	Flavonoids	Triterpenoids	Reducing sugars
<i>F. benghalensis</i>	-*	-	+	+	+	-	+
<i>F. racemosa</i>	+	-	+	+	+	-	-
<i>F. religiosa</i>	-	-	+	+	+	-	-
<i>F. tsiela</i>	-	-	+	+	+	+	-
<i>G. cambogia</i>	-	+	+	+	+	-	-

*+ presence of the class of compounds

*- absence of the class of compounds

Table 3. Minimum inhibitory concentration values (MIC) of Panchawalkala extracts obtained by different extraction methods, against 6 wild MRSA strains, *S. aureus* ATCC 25923 strain and *S. aureus* NCTC 6571 susceptible strains

Bacteria	Minimum inhibitory concentration (mg/mL)			
	Direct aqueous extracts		Sequential MeOH extracts	
	Reflux method	Bottle-shaker method	Soxhlet method	Bottle-shaker method
MRSA 1	0.625	2.5	5	5
MRSA 2	0.312	2.5	1.25	2.5
MRSA 3	0.312	1.25	2.5	2.5
MRSA 4	0.625	2.5	1.25	2.5
MRSA 5	0.312	1.25	5	5
MRSA 6	0.625	2.5	2.5	5
<i>S. aureus</i> ATCC	1.25	1.25	5	5
<i>S. aureus</i> NCTC	0.625	1.25	1.25	2.5

yield from the bottle-shaker method at ambient temperature was 1.1 % (Table 1).

Sequential extracts: Panchawalkala was extracted sequentially using hexane, DCM, EtOAc and MeOH by two methods (Soxhlet method and bottle-shaker method) and the yield of each extraction procedure is given in Table 1.

Preliminary phytochemical screening of the constituent plants of Panchawalkala

Hot aqueous extract of each constituent plant of Panchawalkala was tested for phytochemical constituents and its results are given in Table 2. Preliminary Phytochemical analysis showed the presence of phenols, tannins, terpenoids, flavonoids and reducing sugars in the hot aqueous extracts of *F. benghalensis* (Table 2). In *F. racemosa* extract contains alkaloids, phenols, tannins, terpenoids, flavonoids and triterpenoids. The corresponding phytochemical screening results of *F. religiosa*, *F. tsiela* and *G. cambogia* is given in Table 2.

Antibacterial activity of direct aqueous and sequential organic extracts of Panchawalkala

Screening for antibacterial activity (Cut-well diffusion method)

Direct aqueous extracts: The mean diameters of the zones of inhibition (ZOIs) for direct aqueous extracts (10 mg/mL) against the tested panel of bacteria (6 MRSA wild strains and *S. aureus* ATCC 25923 and NCTC 6571 susceptible strains) are given in Figure 1. The extract obtained from the reflux method displayed relatively larger ZOIs (12-19 mm) against 6 MRSA strains than those for the bottle-shaker method.

Mean diameter of ZOI for each direct aqueous extract of Panchawalkala

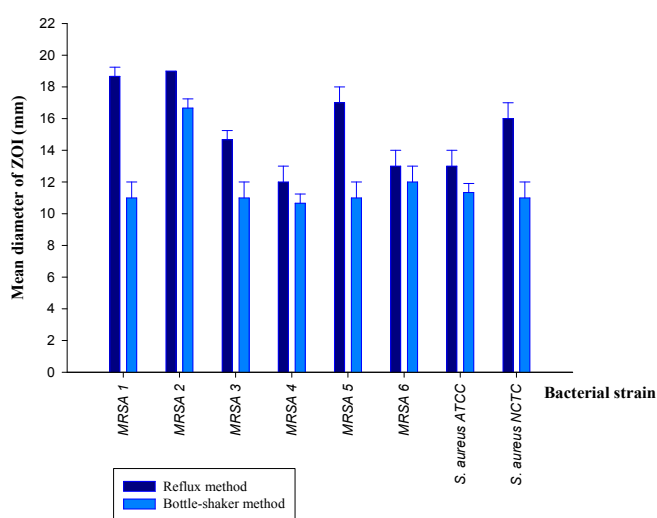


Figure 1. Mean diameter of ZOI for each direct aqueous extract of Panchawalkala (well diameter = 8 mm)

Sequential extracts

The sequential hexane, DCM and EtOAc extracts did not exhibit ZOI in preliminary screening (in Cut-well diffusion method). Therefore, these extracts were excluded from further antibacterial studies. The screening results (mean diameters of ZOIs) for sequential MeOH extracts (10 mg/mL) obtained

from the two extraction methods are given in Figure 2. The MeOH extract obtained from the Soxhlet method consistently displayed higher ZOI (10-15 mm) compared to the MeOH extract obtained from the bottle-shaker method.

Mean diameter of ZOI for each sequential methanol extracts of Panchawalkala

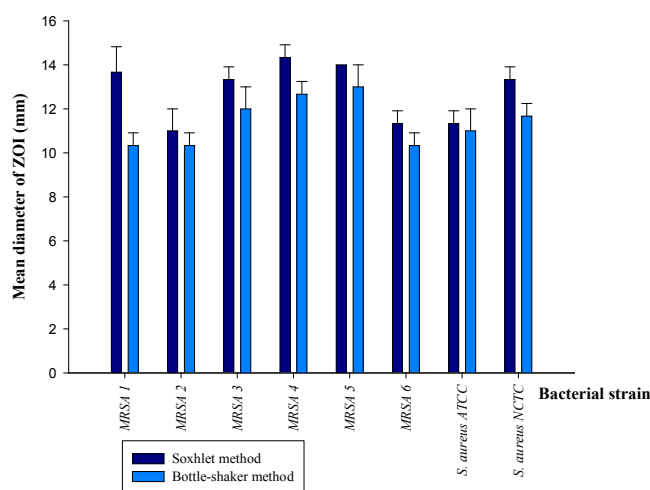


Figure 2. Mean diameter of ZOI for each sequential methanol extracts of Panchawalkala (well diameter = 8 mm)

Determining MIC of aqueous and organic solvent extracts of Panchawalkala

Table 3 demonstrates that the hot aqueous extract obtained by reflux method exhibited antibacterial activity with MIC values ranging from 0.312 to 0.625 mg/mL against 6 MRSA strains. The cold aqueous extract (bottle-shaker method) exhibited comparatively high MIC values than it for hot aqueous extract (Reflux method) (Table 3). The sequential MeOH extracts obtained from both hot and cold conditions exhibited comparable MIC values ranging from 1.25 to 5 mg/mL against tested panel of bacteria.

DPPH radical scavenging activity of direct aqueous extracts and sequential organic extracts of Panchawalkala

Direct aqueous extracts and sequential organic extracts of Panchawalkala was tested for DPPH radical scavenging activity and its results are given in Figure 3.

DPPH radical scavenging activity of aqueous and organic extracts of Panchawalkala

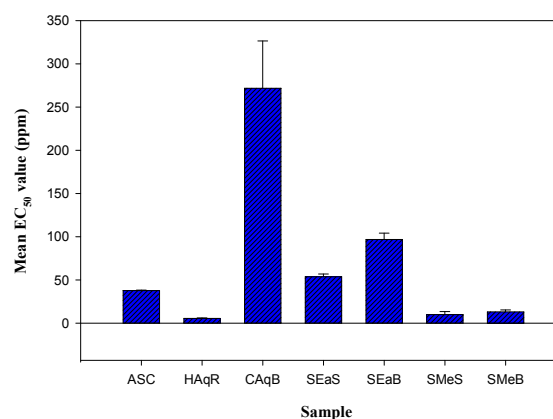


Figure 3. DPPH radical scavenging activity of aqueous and organic extracts of Panchawalkala

ASC- L-ascorbic acid, HAQR- Hot aqueous extract from reflux method, CAQB- Cold aqueous extract from bottle shaker method, SEaS- Sequential ethyl acetate extract from Soxhlet method, SEaB- Sequential ethyl acetate extract from bottle shaker method, SMeS- Sequential methanol extract from Soxhlet method, SMeB- Sequential methanol extract from bottle shaker method.

DISCUSSION

The discussion of the results begins with the percentage yields of different solvent extracts of Panchawalkala. The extracts obtained under hot conditions (direct aqueous extract obtained by reflux method and sequential MeOH extract obtained by Soxhlet method) demonstrated high percentage yields compared to extracts obtained under cold conditions (Table 1). Moreover for sequential extraction process, as the polarity increases from hexane to MeOH, the percentage yield also increases significantly. This implies the presence of high polar secondary metabolites in Panchawalkala. All the constituent plants of Panchawalkala displays positive results for phenols, tannins, terpenoids and flavonoids. In addition, only the *F. racemosa*, *G. cambogia*, *F. tsiela* and *F. benghalensis* shows positive results for alkaloids, saponins, triterpenoids and reducing sugars respectively (Table 2). The antibacterial potency of Panchawalkala may be attributed to single or combined effect of the mentioned chemical groups. Several reports are available in support of antibacterial activity of several phytochemicals present in plant extracts. Antibacterial activity of tannins and saponins isolated from plant species are well documented (Proestos *et al.*, 2005). However, further studies are needed to evaluate the antibacterial activity of isolated phytochemicals such as tannins and saponins from these constituent plants against pathogenic bacterial strains. During the preliminary antibacterial screening, hexane, DCM and EtOAc extracts did not show ZOI; hence those extracts were excluded from further antibacterial studies. This finding suggests that, the active principles which responsible for the antibacterial activity are high polar secondary metabolites. In Ayurvedic medicine, the decoction of Panchawalkala is usually prepared by heating the dried barks of constituent plants in boiling water until initial volume reduced to 1/10. Under the hot conditions (> 100 °C) there is a possibility of degrading the thermolabile active principles, if present. To test this scenario, in the current investigation, the extraction was performed under both hot and cold conditions. To represent the cold condition, the bottle-shaker method was introduced in the view of extracting thermo-labile active principle, if present (Dharmaratne *et al.*, 2013). The hot aqueous extract obtained from the reflux method consistently displayed higher ZOIs compared to the cold aqueous extract obtained from the bottle-shaker method (Figure 1). From the data in Figure 2, It is apparent that the sequential MeOH extract obtained by Soxhlet method showed larger ZOIs compared to sequential MeOH extract obtained by bottle-shaker method.

MIC was determined using micro-broth dilution assay against 6 MRSA and 2 *S. aureus* susceptible strains. For all tested bacterial strains (8 strains), the hot aqueous extract obtained from reflux method showed lower MIC values (0.312-0.625 mg/mL) compared to the cold aqueous extract obtained from bottle-shaker method (Table 3). The fact that the aqueous extraction under reflux condition provided the most potent extract implies that the antibacterial compounds present in Panchawalkala are thermally stable. It is noteworthy that the

MIC of the extracts for MRSA ranged from 0.312 to 1.25 mg/mL whereas the MIC for *S. aureus* NCTC 6571 and ATCC 25923 were 0.625 to 1.25 mg/mL. Since the mechanism of resistance in MRSA is due to change in the penicillin binding proteins (PBP) in the cell wall of *S. aureus*, these results suggest that the extracts being investigated in Panchawalkala have compounds which are operating in a mode of action different to that of the β -lactam antibiotics which bind to the PBP proteins. There is a significantly low ($p < 0.05$) EC_{50} value for the hot aqueous extract obtained from reflux method (5.47 ± 0.55 ppm) than it for cold aqueous extract (271 ± 54.73 ppm) and L-ascorbic acid. The sequential MeOH extracts obtained by both Soxhlet (9.86 ± 3.60 ppm) and bottle-shaker (13.03 ± 2.35 ppm) method have demonstrated significantly high DPPH radical scavenging activity than it for sequential EtOAc extracts and L-ascorbic acid (Figure 3).

Conclusion

All the constituent plants of Panchawalkala displayed positive results for phenols, tannins, terpenoids and flavonoids while only *F. racemosa*, *G. cambogia*, *F. tsiela* and *F. benghalensis* showed positive results for alkaloids, saponins, triterpenoids and reducing sugars, respectively. The hot aqueous extract of Panchawalkala exhibited the highest antibacterial activity against MRSA strains when compared to the other extracts. The hot aqueous extract obtained from reflux method and the sequential MeOH extracts obtained from Soxhlet and bottle-shaker method exhibited significantly high DPPH radical scavenging activity compared to the other extracts tested for Panchawalkala. Results of the present study suggest that the high polar solvent extracts of *Panchawalkala* possess antibacterial activity against MRSA strains and significant antioxidant activity. Therefore, highly active solvent extracts of Panchawalkala and its purified fractions can be used to develop antibacterial chemotherapeutic agent to combat multi-drug resistant bacterial infections.

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Conflicts of Interests

The authors declare that they have no conflict of interests.

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