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PREPARATION OF MEDICINAL SOAP PRODUCTS USING THE LEAF EXTRACTS OF *PUNICA GRANATUM* (POMEGRANATE)

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ABSTRACT

The present study was carried out to prepare medicinal soaps with antibacterial and/or antioxidant activities using leaf extracts of pomegranate. Leaf extracts of pomegranate were obtained by maceration, soxhlet extraction and sonication using a series of solvents. The extracts were screened for antibacterial activity using the disk diffusion assay carried out against B.cereus, S.typhimurium, S.aureus and E.coli. The Folin Ciocîteau (FC) and DPPH radical scavenging assays were used to determine the total antioxidant capacity (AOC) and the DPPH radical scavenging activity (RSA) respectively. Presence of phytochemical constituents of bioactive extracts was investigated. Bioactive leaf extracts were used to prepare a liquid and a solid soap and their effectiveness was determined. Methanol soxhlet extract was the best bioactive extract exhibiting considerable antibacterial activity against S.aureus and B.cereus and the highest total AOC of 603 µg PGE/mg. It displayed a 94% RSA as well. Therefore, this extract was used in the liquid and solid soap preparations. The liquid soap inhibited the growth of S.aureus and B.cereus at 25 mg/mL and 10 mg/mL concentrations respectively. A thumb impression test indicated a reduction of the microbial growth upon the usage of the medicinal soap displaying its antibacterial effectiveness. The liquid soap displayed 6.7 times better AOC whereas the solid soap indicated 2.5 times better AOC compared to their control soaps. The solid soap did not display any significant antibacterial activity even at a 100 mg/mL concentration. Leaf extracts of pomegranate can be successfully utilized to obtain medicinal soaps with improved antibacterial and antioxidant activities.

KEY WORDS

Pomegranate, antibacterial activity, antioxidant activity, medicinal soap.

INTRODUCTION

The soaps that are being used in our day to day life have a history going back for about six thousand years. The ancient Babylonians discovered that mixing animal fats with wood ash and water created a cleansing substance which was latterly known as "soap".[1] The basic method of soap making is known as saponification.[2] Irrespective of the physical status of the soap, a combination of oil and a base is used for soap preparation. In solid soaps NaOH is used as the base whereas KOH is used to obtain liquid soaps.[2]

Medicinal soaps are a simple variation of the normal soaps where synthetic or natural bioactive ingredients are added into the basic soap medium to give a vast variety of biological activities to the final product.[3,4,5,6,7] However, due to the undesirable side effects of synthetic substances, it is preferential to avoid the use harmful synthetic chemicals from medicinal soap products.[3] In recent years, the plant based natural products have become an attractive alternative to enhance the important biological characteristics of medicinal soaps.[3,4,5,6,7] The replacement of synthetic foaming agents such as SLS by saponins,[8] synthetic antibacterial agents such as Triclosan[9] by natural antibacterial agents and synthetic antioxidants such as BHT[3] by natural phenolic compounds have served to overcome many of the side effects associated with the medicinal soaps based on synthetic ingredients. Coconut oil, olive oil, neem oil, turmeric, sandalwood, venivel, jasmine and lemon essence are few of the most commonly found ingredients in skin care products including medicinal soaps.[10]

The fruit of *Punica granatum*, also known as pomegranate is extensively used for the value addition of cosmetic products[11] including medicinal soaps. Pomegranate is distributed throughout the Mediterranean region of Asia, Africa and Europe[12] and is known to possess a number of medicinal properties from ancient times. Various parts of the pomegranate plant including leaves, fruits, flowers and bark have been used to treat a number of diseases like dysentery, diarrhea, respiratory diseases, skin disorders, hemorrhage, arthritis etc. Furthermore, the investigations suggest that the fruit juice increases the high density lipoproteins, reduce blood pressure and therefore prevent strokes and heart attacks.[12] Pomegranate is known to possess antibacterial and antioxidant activities[13] and is reported to be rich in phytochemicals including flavonoids, condensed tannins and hydrolysable tannins.[14] The pomegranate fruit is highly expensive, seasonal and is a common food source, and thus replacing the pomegranate fruit with a different plant part could be more economically effective. However, there is only very limited publications are available for the use of other plant parts of pomegranate except the fruit. Pomegranate leaves are readily available, inexpensive and there have been various medicinal uses of it from ancient times.[12] Therefore, during this research, the effectiveness of using *Punica granatum* leaves in medicinal soap preparations was investigated as an alternative to its fruit towards value addition of consumer products. A wide range of extraction conditions were investigated towards identifying the extraction technique and solvent system that yields the best bioactive leaf extracts of pomegranate. The extract with the best antibacterial and antioxidant activities was incorporated into a liquid and a solid soap preparation.

MATERIALS AND METHODS

Materials

Mueller Hington Agar (MHA) was purchased from Royal Surgical, Colombo, Sri Lanka. Gentamycin, from Union Chemist Pvt. Ltd., Sri Lanka. Detergent grade soft soap was purchased from Glorchem Enterprises, Sri Lanka. Methylated spirit, Coconut oil and Palm oil were purchased from local market. All the other chemical reagents including pyrogallol and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were Sigma Aldrich and the bacterial culture media were bacteriological grade Agar and LB Broth all obtained from the Department of Chemistry, University of Colombo. Analytical grade solvents; methanol, ethyl acetate, hexane which were double distilled prior to use were also obtained from Department of Chemistry, University of Colombo.

Instruments

An electronic balance (OHAUS Pioneer PA313), Stirrer hot plate (IKEA RH B1 S22), Sonicator (Grant XUB12), Orbital incubator (Stuart S1500), Oven (Memmert Loading modell 100-800), Rotary evaporator (Type N-N ser no-10823000), Laminar flow (BIOBASE), Autoclave (KT 30-SD No-105370), Jenway 6300 spectrophotometer were used during this research study.

Plant identification and preparation of crude plant extracts

All plant parts were authenticated by the Department of Plant Sciences, University of Colombo, Sri Lanka. Extractions were carried out with well dried, coarsely ground *Punica granatum* leaves from maceration, soxhlet extraction and sonication using methanol, ethyl acetate, hexane as the solvents according to previously published protocols.[12,14] The macerated extract was obtained by adding 100 mL of the solvent to 5 g of coarsely ground leaves and keeping in an orbital incubator at 37 ˚C for 24 hours. The soxhlet extract was obtained by mixing of 10 g of coarsely ground leaves with 200 mL of the solvent for 3 hours using the soxhlet apparatus. Sonication was carried out with 5 g of coarsely ground leaves and 100 mL of the solvent at room temperature for 2 hours to obtain the sonicated extract. All the extracts were dried with anhydrous Na_2SO_4, filtered and concentrated to a minimum volume. The extracts were stored at 4 ˚C until further use.

Investigation of bioactivities of plant extracts

Antibacterial activity (Disk diffusion assay)

The 6 mm diameter sterilized filter paper disks were impregnated with plant extracts and the controls of known concentrations. Solvent was used as the negative control while 1 mg/mL Gentamycin was used as the positive control. The disk diffusion assays were carried out according to previously published protocols against *Bacillus cereus* (ATCC 11778), *Salmonella typhimurium* (ATCC 700720), *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 35218).[7,15] The plates containing the impregnated disks were incubated overnight at 37 ^{o}C and the diameters of the inhibition zones were measured. The plant extracts which indicated the highest antibacterial activity were tested for their MICs using the same disk diffusion assay protocol. All the experiments were carried out in triplicate.

Total antioxidant activity (Folin Ciocalteu assay)

To 0.1 mL of each test sample prepared using methanol, 2 mL of 2 % (w/v) $NaHCO_3$ solution was added and the content was kept for incubation in dark for 2 minutes. Next, a volume of 0.1 mL of the Folin Ciocalteu reagent was added to each tube and further incubated for 30 minutes at room temperature. The absorbance of the resulting solution was measured at 750 nm. The AOCs of each sample was determined using a pyrogallol standard curve.[16] All the experiments were carried out in triplicate.

Radical scavenging activity (DPPH radical scavenging assay)

To 50 µL of the plant extract of known concentration, 1.95 mL of a 24 mg/L DPPH solution prepared in methanol was added and mixed well. After 30 minutes of incubation at room temperature under dark conditions, absorbance of all the samples was measured at 517 nm against the blank. Percentage RSA (RSA%) of the extracts was calculated using the following equation.[17] All the experiments were carried out in triplicate.

$$= \frac{\text{(Absorbance of control-Absorbance of sample)} \times 100\ \%}{\text{Absorbance of control}}$$

$$= \frac{(A_c - A_t) \times 100\ \%}{A_c}$$

Absorbance of the test sample – A_t

Absorbance of the control – A_c

Preparation of medicinal soap products

Liquid soap

A 100 mg portion of the methanol soxhlet extract of pomegranate leaves was added to a 10 mL liquid soap solution (1 g of commercial soft soap dissolved in 10 mL of distilled water). [14,16,18,19]. Control soap was prepared using the same procedure without the addition of the plant extract.

A weight of 12 g of coconut oil and 54 g of palm oil was mixed and stirred well at room temperature for 15 minutes. Thereafter, 51 mL of 20 % NaOH solution was added and the soap mixture was well stirred approximately for 30 minutes until formation of soap was visible. An amount of 0.9 g the active pomegranate extract was added to 30 g of the above soap mixture and mixed well to prepare a solid soap containing 3% (w/w) methanol soxhlet extract. The mixture was poured into moulds and allowed to solidify for 12 hours. The resultant solid soaps were taken out of the moulds and kept in open air for 7 days for drying.[18] Control soap was prepared using the same procedure without the addition of the plant extract.

Determination of antibacterial and antioxidant activities of prepared medicinal soaps

Samples of the test and control liquid and solid soaps of known concentration were used to carry out the disk diffusion and FC assays using the protocols reported in the previous section (Investigation of bioactivities of plant extracts). The increment of the antibacterial or antioxidant activity of the test medicinal soap was monitored in comparison to their control soaps. The experiments were carried out in triplicates.

Thumb impression test

With proper distance, thumb impressions of hands exposed to the environment were placed on a sterile MHA plate. Then, the impressions of the two separate thumbs, one, washed with the medicinal soap and the other, with the control soap were placed on the same MHA plate without any overlaps of thumbprints. The pattern of microbial growth on the plates was observed after an incubation period of 24 hours at 37 $^{\circ}$C. [7, 19]

Phytochemical screening of pomegranate leaf extracts

The pomegranate leaf extracts with significant bioactivities were screened for the presence of various phytochemical constituents according to the previously published protocols. [12, 19]

RESULTS AND DISCUSSION

This research was carried out to prepare medicinal soap products using leaf extracts of *Punica granatum*. According to the available published data, incorporation of *Punica granatum* leaf extracts into any soap preparations have not been reported up to date.

Percentage yields of crude leaf extracts

Percentage yields for the crude leaf extracts of pomegranate obtained under different extraction technique and solvent combinations are indicated in Table 1.

Table 1: Percentage yields of the crude leaf extracts.

Solvent	Percentage yield (%)		
	Maceration	Soxhlet extraction	Sonication
Methanol	18	19	11
Ethyl acetate	4	5	2
Hexane	1	12	2

In general, irrespective of the extraction method, the polar methanolic extracts exhibited relatively high percentages of crude yields compared to the other solvents. The important bioactivities of these plant extracts were further analyzed through suitable bioassays.

Investigation of bioactivities of plant extracts

Antibacterial activity

The antibacterial activity results obtained for the different leaf extracts are shown in Table 2.

Only the methanoilc extracts of pomegranate leaves exhibited antibacterial activity whereas no significant antibacterial activity was observed for the ethyl acetate and hexane extracts. The methanolic soxhlet leaf extract exhibited the highest antibacterial activity with respect to the other methanolic extracts. However, the antibacterial activity of the methanoilc soxhlet extract was best against *S.aureus* and *B.cereus* and no growth inhibition was observed with *E.coli* and *S.typhimurium* at the tested concentration. The Minimum Inhibitory Concentrations (MICs) of the methanolic soxhlet extract against *S.aureus* and *B.cereus* were found to be 25 mg/mL and 10 mg/mL respectively. (Table 3)

Therefore, according to the findings of this research, methanolic soxhlet extract was selected to be used to attain antibacterial activity in medicinal soap preparation.

Table 2: Disk diffusion assay results of different leaf extracts of pomegranate.

Extraction method	Solvent	Diameter of inhibition zones (cm)[c]			
		B.cereus	***S.typhimurium***	***S.aureus***	***E.coli***
Maceration	Methanol[a]	NI	NI	0.8±0.1	NI
	Ethyl acetate[b]	NI	NI	NI	NI
	Hexane[b]	NI	NI	NI	NI
Soxhlet	Methanol	1.1±0.0	NI	1.1±0.1	NI
	Ethyl acetate	NI	NI	NI	NI
	Hexane	NI	NI	NI	NI
Sonication	Methanol	NI	NI	0.9±0.1	NI
	Ethyl acetate	NI	NI	NI	NI
	Hexane	NI	NI	NI	NI
Negative control		NI	NI	NI	NI
Positive control (gentamycin)		2.0±0.1	2.0±0.0	1.9±0.0	1.4±0.1

[a]100 mg/mL, [b]25 mg/mL, [c]mean±SEM, n=3

Table 3: MIC of the methanolic soxhlet extract of pomegranate leaves against *S. aureus* and *B.cereus*.

Concentration (mg/mL)	Diameter of inhibition zones (cm)[a]	
	S. aureus	*B.cereus*
5	NI	NI
10	NI	0.7±0.0
25	0.7±0.0	0.8±0.0
50	1.0±0.1	0.9±0.1
75	1.0±0.0	0.9±0.1
100	1.1±0.0	1.1±0.1
Positive control (gentamycin)	1.9±0.0	2.0±0.1
Negative control (methanol)	NI	NI

[a]mean±SEM, n=3

Antioxidant activity and DPPH radical scavenging activity

All the pomegranate plant extracts were tested with the FC assay to determine their total AOC and the results are shown in Table 4.

Table 4: AOCs of different leaf extracts of pomegranate from FC assay.

Solvent	AOC in µg (PGE)/mg[a]		
	Maceration	Soxhlet extraction	Sonication
Methanol	546±14	603±35	329±14
Ethyl acetate	31±1	17±2	7±1
Hexane	17±1	15±1	19±3

[a]mean±SEM, n=3

The yellow colored molybdotungstate complex [Mo(VI)] in the FC reagent is reduced to a [Mo(V)] complex which is blue in color in the presence of phenolic and other reducing substances in the sample.[20] This assay is usually utilized to measure the Total Phenolic Content (TPC) of a sample due to its high reactivity with various phenolic compounds.[20] However, since it reacts with many other reducing agents that can act as antioxidants, it is has been shown to be used as a method to determine the total AOC of a sample more accurately.[20,21] Total AOC could be somewhat greater for a natural plant extract than the TPC which is the net contribution from phenolic compounds in the sample.[21] According to the results of the FC assay, the total AOC of the methanolic soxhlet extract was significantly higher than that of the other pomegranate leaf extracts and it was further analyzed

for its radical scavenging activity, which was found to be 94±0% from the DPPH radical scavenging assay. Therefore, the methanolic pomegranate leaf extract obtained by soxhlet extraction was selected to be used to attain the required antioxidant activity of the medicinal soaps prepared during this study.

Phytochemical studies

Pomegranate leaves are well known for their antibacterial and antioxidant activities.[7-8] It has been reported that the pomegranate leaves contain punicalin, punicafolin as tannins and luteolin, apigenin as flavone glycosides.[14] Accordingly, when the methanolic soxhlet extract which displayed the best antibacterial and antioxidant activities during this study was subjected to phytochemical analyses, it revealed the presence of a number of secondary metabolites and reducing sugars (Table 5).

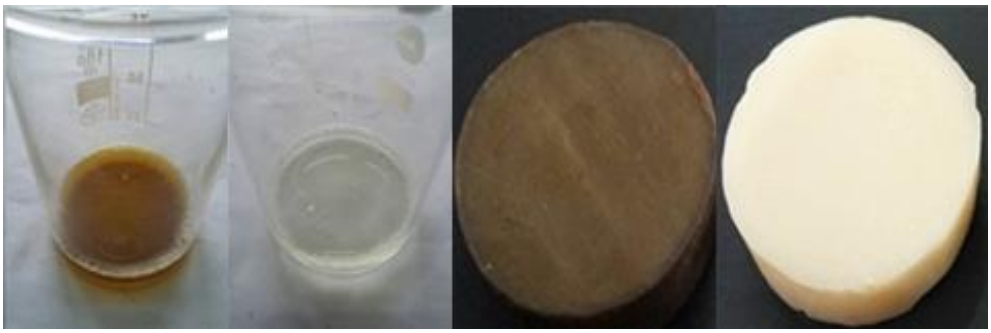
Table 5: Results of phytochemical analysis of the antibacterial and antioxidant active methanol soxhlet extract.

Alkaloiods	Phenols and tannins	Reducing sugars	Steroids and terpenoids	Saponins
+	+	+	+	+

The presence of phenols and tannins which are well known natural antioxidants reflect the high antioxidant capacity of this extract.[3,22] Additionally, the presence of phenols which are involved in plant defense mechanisms[3] also support the antibacterial properties displayed by this extract. The presence of compounds like saponins in pomegranate extract makes it ideal to be used in medicinal soap preparations since the saponins can act as natural foaming agents. This will also help to minimize the use of synthetic foaming substances in medicinal soap products.

Preparation of soap products based on pomegranate leaf extracts

Two medicinal soaps were prepared using the methanolic soxhlet leaf extract of pomegranate andtheir effectiveness was tested against the control soaps which did not contain the plant extract. The prepared soaps are shown in Figure 1.



a) b) c) d)

Figure 1: Various soaps prepared during the study a) liquid soap prepared with the methanolic soxhlet extract, b) control liquid soap c) solid soap prepared with the methanolic soxhlet extract d) control solid soap.

Assessing the biological activities of the prepared medicinal soaps

Antibacterial activity

The antibacterial activities of the prepared medicinal soaps are shown in Table 6.

Table 6: Disk diffusion assay results for the prepared soap products.

Sample type	Diameter of inhibition zones (cm)[c]	
	B. cereus	*S. aureus*
Test liquid soap[a]	1.6±0.1	1.0±0.1
Control liquid soap	1.0±0.1	0.7±0.0
Test solid soap[b]	0.7±0.0	NI
Control solid soap	0.7±0.0	NI
Positive control (gentamycin)	2.1±0.1	1.9±0.0

[a]100 mg/mL, [b]3% w/w, [c]mean±SEM, n=3

According to the results shown in Table 6, the methanolic soxhlet extract which showed antibacterial activity as the pure extract at a 100 mg/mL concentration (Table 2) was found to be active against the same bacterial species when the pomegranate liquid medicinal soap was prepared with the same final concentration of the extract. The activity of the soap was not tested against *S. typhimurium* and *E. coli* since they were not inhibited by the 100 mg/mL pure extract. The smaller sized inhibition zones observed for the control soaps could be due to the natural antibacterial activity of coconut oil which is used to prepare the soap base.[23] The enhanced growth inhibition displayed by the test liquid soap indicates the ability of the selected plant extract to provide the antibacterial activity to a liquid medicinal soap. However, the solid medicinal soap containing 3% of the pomegranate methanol soxhlet extract did not show a considerable antibacterial activity with respect to its control soap. This could be due to the degradation of antibacterial agents resent in the plant extract during the solid soap preparation process.

Further antibacterial tests were carried out to investigate the minimum amount of plant extract that need to be added to the liquid soap in order to attain a net antibacterial activity compared to the control soap. The results of this study are shown in Table 7. The liquid soap indicated antibacterial activity even at 10-25 mg/mL concentrations indicating the antibacterial effectiveness of the selected pomegranate leaf extract in liquid soap.

Table 7: Dose dependent antibacterial effect of the pomegranate methanolic soxhlet extract in the liquid medicinal soap against *S. aureus* and *B.cereus*.

Concentration (mg/mL)	Diameter of inhibition zones (cm)[a]	
	S. aureus	*B.cereus*
10	NI	1.2±0.1
25	0.8±0.0	1.4±0.1
50	0.9±0.1	1.4±0.2
75	1.0±0.1	1.4±0.1
100	1.1±0.1	1.5±0.1
150	1.1±0.1	1.6±0.1
200	1.1±0.1	1.6±0.1
Control liquid soap	NI	1.1±0.1
Positive control (gentamycin)	1.9±0.1	2.0±0.0

[a]mean±SEM, n=3

Furthermore, a thumb impression test was carried out to investigate the effectiveness of the liquid medicinal soap and the results are shown in Figure 2.

As expected, the number of bacterial colonies grown on the thumbprints kept with washed thumbs are lower than those grown on the thumbprints made with the unwashed thumbs [b and d vs. a and c in Figure 2]. The pomegranate liquid soap indicated a better efficiency in removing the microbes from the thumb washed with it which is indicated by the reduced number of bacterial colonies grown on the thumbprint made with the thumb washed with the pomegranate liquid soap (Figure 2 d), compared to the control thumbprint (Figure 2 b). When consider the overall results of the thumb impression test, it can be concluded that the liquid medicinal soap prepared

using the methanolic soxhlet extraction of pomegranate leaves can display considerable antibacterial activity.

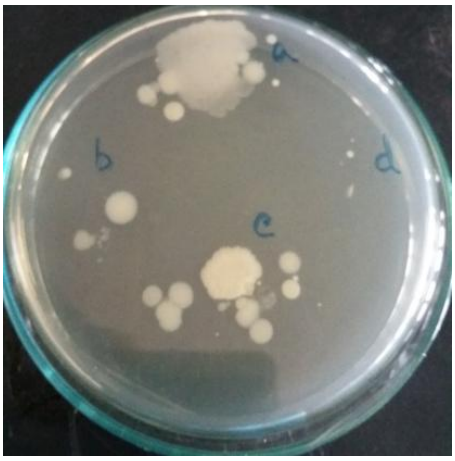


Fig. 2: Thumbprints of differently treated thumbs. a) and c) unwashed thumb, b) thumb washed with the control liquid soap, d) thumb washed with 100 mg/mL pomegranate liquid soap.

Antioxidant activity

The antioxidant activities of the prepared medicinal soaps were investigated using the FC assay and the results are indicated in Table 8. The AOC of the pomegranate liquid soap was about 6.7 times better than the control liquid soap. A similar observation was made with the solid pomegranate soap which indicated 2.5 times better AOC compared to the solid control soap.

Table 8: AOCs of the prepared soap products.

Soap type	AOC in µg (PGE)/mg[c]
Test liquid soap[a]	20±0
Control liquid soap	3±0
Test solid soap[b]	20±1
Control solid soap	8±1

[a]0.03% (w/v)[b], 3% (w/w), [c]mean±SEM, n=3

The liquid soap prepared during the study using the selected pomegranate leaf extract displayed both antibacterial and antioxidant activities while the solid soap displayed only antioxidant activity with respect to the respective control soaps. Therefore, it could be possible that two different compounds or classes of compounds might have been responsible for the antibacterial and antioxidant activities in these soap products and their biological activities could be different in the two different soap media. Processing of the solid soap after the addition of the extract to obtain the final solid product might have led the antibacterial active compounds to degradation, whereas in liquid form these compounds must have been able to retain their stability to produce the desired activity of the final soap product. However, the bioactive ingredients in the methanolic soxhlet extract that were responsible for its antioxidant activity seemed to be stable regardless of the nature of the soap preparation process and thus improved the antioxidant activity of both the solid and liquid soaps with respect to their control soaps.

CONCLUSION

Applicability of the leaves of pomegranate (*Punica granatum*) in preparation of medicinal soap products have not been extensively investigated so far whereas its fruit is widely used in such applications as

previuosly reported. According to this research, it was found that the methanolic soxhlet extract of pomegranate leaves could be an effective alternative to the expensive and seasonal pomegranate fruit towards preparing medicinal soap products. The methanolic soxhlet extract indicated the highest percentage yield and the best antibacterial and antioxidant activities among all the tested leaf extracts. The liquid medicinal soap prepared using this extract retained both the original antibacterial and antioxidant activities displayed by the extract whereas the solid medicinal soap only displayed the antioxidant activity. It can be concluded that the methanolic soxhlet extract of pomegranate is more suitable towards preparing liquid medicinal soap products to obtain both tested bioactivities.

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