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Preparation of Liquid Medicinal Soap Products Using *Adhatoda Vasica* (Adhatoda) Leaf Extracts

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ABSTRACT

Main objective of this research was to prepare medicinal soaps based on Adhatoda vasica. Optimum extraction conditions to obtain antibacterial and antioxidant active extracts from adhatoda were selected from a range of extraction conditions carried out using maceration, soxhlet extraction and sonication. The antibacterial activity of the extracts was determined by the disk diffusion assay against B.cereus, S.typhimurium, S.aureus and E.coli. The Folin Ciocltеau (FC) assay and the DPPH radical scavenging assay were used to obtain the antioxidant capacity (AOC) and the percentage radical scavenging activity respectively. An antibacterial liquid soap and an antioxidant liquid soap were prepared using the adhatoda leaf extracts and these bioactivities of the prepared medicinal soaps were determined with respect to their control soaps. Furthermore, phytochemical analyses of the bioactive extracts were carried out to investigate the presence of different secondary metabolites.

Methanolic soxhlet extract of adhatoda exhibited significant antibacterial activity against all the tested bacterial strains. The ethyl acetate soxhlet extract displayed the highest AOC of 109±2 µg PGE/mg by the FC assay and a 22±1% radical scavenging activity in the DPPH assay. These extracts were used to prepare the antibacterial and antioxidant adhatoda soaps respectively.

Antibacterial adhatoda soap indicated significant antibacterial activity at a 50 mg/mL concentration against S. aureus compared to the control. According to the results of a thumb impression test, a reduction in the number of bacterial colonies was observed in the thumb impression of the hand washed with the new adhatoda antibacterial soap compared to the control. The antioxidant adhatoda soap displayed approximately three times better AOC (10±0 µg PGE/mg) relative to the control soap (3±0 µg PGE/mg).

Incorporation of adhatoda extracts into soap products to enhance their medicinal properties has not been reported so far and the promising results of this study indicate the possibility of this approach.

KEYWORDS: *Potassium application, capsicum anthracnose, fruit quality*

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1. INTRODUCTION

Soaps; the products of basic saponification reactions (Chukwulozie, 2014) have been used among people since its discovery by the ancient Babylonians as a cleansing substance (Warra, 2010). At present, there are a number of commercially available medicinal soap products which mostly come as solid bar soaps or liquid soaps. The difference of solid and liquid soap is that NaOH is used as the base in solid soap preparation, whereas KOH is used in the preparation of liquid soaps (Chukwulozie, 2014).

Soaps have been enriched with various natural ingredients in order to provide different medicinal properties such as antibacterial, antifungal, antioxidant, anti-inflammatory etc. to the final product (Kole, 2005). Additionally, natural ingredients also have an impact on the color, texture and odor of the soap products. When preparing medicinal soap products it is important and more attractive to substitute natural ingredients for harmful synthetic substances, to give the desired bioactivities. Triclosan and BHT (2,6-di tert-butyl-4-methylphenol) are examples of synthetic substances which have been most commonly used in such consumer products to give antibacterial and antioxidant effects respectively. However, the natural ingredients attained from common medicinal plants could be used as substituents for these synthetic substances. Neem, turmeric, sandalwood, venivel, jasmine and lemon essence are few of the most commonly found ingredients in skin care products including medicinal soaps (Kole, 2005). During this study, efforts were made to prepare medicinal soap products using bioactive leaf extracts of adhatoda.

2. BACKGROUND

Adhatoda vasica is an evergreen shrub, which usually grows well in low moisture areas and dry soils to a height of 2-3 meters. Its leaves are simple, opposite, large and lance shaped. The plant is native to Asia and can be seen in countries like Sri Lanka, India, Nepal and Pakistan (Sampath, 2010). *A. vasica* contains a variety of alkaloids including vasicine, quinazoline etc. It also contains compounds such as lycopenes, ascorbic acid etc. (Wankhede, 2015).

It has multiple traditional applications in folk medicine. *A. vasica* is commonly prescribed as an expectorant, mainly to treat asthma, bronchitis and cough due to its bronchodilatory activity (Sampath, 2010). Leaf juice is used to cure diarrhea and dysentery as well. *A. vasica* is also known to possess antibacterial, antioxidant, anti-diabetic, anti-hemorrhagic, anthelmintic and anti-rheumatic properties (Sampath, 2010). However, applicability of *A. vasica* in the preparation of medicinal soaps has not been reported up to date according to the available data. Adhatoda is an easily cultivated and readily available plant and therefore can be suitable even for an industrial scale application. Therefore, during this study, optimized conditions to obtain bioactive extracts from *A. vasica* were investigated and such extracts obtained were incorporated into soap products to enhance their medicinal values.

3. MATERIALS AND METHODS

3.1. Plant identification

The preserved plant parts were authenticated by the Department of Plant Sciences, University of Colombo.

3.2. Preparation of crude plant extracts

Mature *Adhatoda vasica* leaves obtained from Colombo and Kurunegala areas were well dried, coarsely ground and the extractions were carried out by maceration, soxhlet extraction and sonication using methanol, ethyl acetate and hexane as the solvents.

i. Maceration

A mass of 5 g of coarsely ground leaves was added into 100 mL volume of the solvent in a conical flask. Then it was kept in an orbital

incubator for 24 hours at 37 ˚C. Thereafter, it was filtered using a no. 01 whatmaan filter paper followed by drying with anhydrous Na_2SO_4. The filtrate was collected and it was concentrated using a rotary evapotaor, followed by solvent evaporation using a water bath maintained at 50 ˚C.

ii. Soxhlet extraction

A mass of 10 g of coarsely ground leaves were added to the thimble of the soxhlet apparatus. A volume of 200 mL of the solvent and few boiling stones were added to the round bottom flask and attached to the extractor apparatus. Extraction was carried out for 3 hours. The resulting solution was filtered using a no. 01 whatmaan filter paper followed by drying with anhydrous Na_2SO_4. The filtrate was concentrated using a rotary evaporator, followed by evaporation of solvent using a water bath maintained at 50 ˚C.

iii. Sonication

A mass of 5 g of coarsely ground leaves were added into 100 mL volume of the solvent in a conical flask. The extraction was carried out in a sonicator for 2 hours at room temperature. The resulting solution was filtered using a no. 01 whatmaan filter paper followed by drying with anhydrous Na_2SO_4. The filtrate was concentrated using a rotary evaporator followed by evaporation of solvent using a water bath maintained at 50 ˚C.

3.3 Investigation of the bioactivities of adhatoda leaf extracts

i. Antibacterial activity by the disk diffusion assay

The sterilized filter paper disks with 6 mm diameter were impregnated with known concentrations of the plant extracts and the controls. Gentamycin (1 mg/mL) was used as the positive control and methanol was used as the negative control. The disk diffusion assays were carried out against *Bacillus cereus* (ATCC 11778), *Salmonella typhimurium* (ATCC 700720), S*taphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 35218).

Few milliliters of a sterilized 0.9 % NaCl solution was inoculated with each bacterial strain separately to match with the turbidity of the 0.5 McFarland solution and the resulting inoculums were used to prepare bacterial spread plates. After about 5 minutes, the impregnated disks were placed on the spread plates and the plates were incubated overnight at 37 ^{0}C. At the end of the incubation period, the diameters of the inhibition zones were measured. All the experiments were carried out in triplicate (Driscoll, 2012).

ii. Antioxidant capacity by the Folin Ciocalteau assay

A standard series of pyrogallol was prepared in methanol (62.5-750 µg/mL). A volume of 2 mL of a 2% (w/v) $NaHCO_3$ solution was added to 0.1 mL of each standard separately. The mixture was kept in dark for 2 minutes for incubation. Then, 0.1 mL of the Folin Ciocalteau reagent was added to each tube and further incubated for 30 minutes at room temperature. The absorbance of the resulting series was measured at 750 nm. Test samples with known concentrations were prepared using methanol and the same procedure was carried out to determine their antioxidant capacities with respect to pyrogallol equivalents (PGE) (Namjooyan, 2010).

iii. Radical scavenging activity by the DPPH radical scavenging assay

A 24 mg/mL DPPH solution was prepared in methanol. An aliquot of 1.95 mL of the prepared DPPH solution was mixed with 50 µL of the plant extract. Blank was prepared by mixing 50 µL of the plant extract with 1.95 mL of methanol. Absorbance values of all the samples were measured at 517 nm after 30 minutes of incubation at room temperature under dark conditions. The percentage radical scavenging

activity of each extracts was calculated using the following equation (Padmanabhan & Jangle, 2012).

Percentage radical scavenging activity (RSA %)

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

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

Absorbance of the test sample – A_t
Absorbance of the control – A_c

3.4 Preparation of liquid medicinal soap products with bioactive adhatoda extracts

A liquid soap was prepared by dissolving 1 g of commercial soft soap in 10 mL of distilled water and adding a known amount of the relevant adhatoda extract. Antioxidant adhatoda soap was prepared to contain a 50 mg/mL concentration of the methanolic soxhlet extract whereas the antioxidant soap was prepared to contain a 10 mg/mL concentration of the ethyl acetate soxhlet extract (Wongthongdee & Inprakhon, 2013).

3.5 Determination of the bioactivities of the prepared medicinal soaps

i. Antibacterial activity

Sterile filter paper disks were impregnated with the liquid medicinal soap. Liquid soap without the plant extracts were used as the negative control. The disk diffusion assays were carried out in triplicate to check for the antibacterial activity of the prepared antibacterial medicinal soap compared to that of the control soap.

ii. Test for the effectiveness of liquid medicinal soap (thumb impression test)

Thumb impressions of bare hands (exposed to the environment) were made on a MHA (Muller Hinton Agar) plate with proper distance. Then, one thumb was washed with the prepared medicinal soap and the other with control soap. The thumb impressions of the washed hands were then placed on the same MHA plate at a suitable position without any overlaps. The plates were incubated at 37 ˚C for 24 hours and the pattern of microbial growth was observed (Kaur, 2014).

iii. Antioxidant activity

The AOC of the adhatoda antioxidant medicinal soap was measured using the Folin Ciocalteau assay. Experiments were carried in triplicate and the AOC of the medicinal soap compared to that of the control soap.

3.6 Phytochemical screening of plant extracts with significant bioactivities

i. Test for alkaloids

To a small amount of the prepared plant extract, few drops of the Hager's reagent (saturated solution of picric acid) was added. Yellow color precipitate can be observed in the presence of alkaloids (De, 2010).

ii. Test for tannins and phenolic compounds

To a small amount of the prepared plant extract, few drops of 5 % $FeCl_3$ was added. Blue green color was observed in the presence of tannins and phenolic compounds (De, 2010).

iii. Test for reducing sugars

Equal volume of Fehling's A and Fehling's B reagents were mixed with each other to prepare the Fehling's reagent. Few drops of the plant extract was mixed with a small amount of the Fehling's reagent and boiled. The formation of a brick red precipitate indicated the presence of reducing sugars (De, 2010).

iv. Test for steroids and terpenoids

Few drops of the plant extract was treated with a small volume of chloroform and few drops of conc. sulfuric was added. The mixture was shaken well and allowed to stand for some time.

Appearance of red or reddish brown color at lower layer (organic layer) indicated the presence of steroids and terpenoids (De, 2010).

v. Test for saponins

A small amount of the extract was added to 1 mL distilled water and well shaken. Appearance of stable froth indicated the presence of saponins (De, 2010).

4. RESULTS AND DISCUSSION

This research was mainly focused on extracting bioactive fractions from *Adhatoda vasica* leaves to prepare medicinal soap products. During the extraction of natural products from adhatoda, the following percentage yields were observed for the crude plant extracts obtained by different extraction technique and solvent combinations. (Table 1)

Table 1. Percentage yields of the crude leaf extracts

Solvent	Percentage yield (%)		
	Maceration	Soxhlet extraction	Sonication
Methanol	14	6	14
Ethyl acetate	2	7	3
Hexane	1	2	5

The solvent polarity decreases from methanol to ethyl acetate to hexane. When considering the percentage crude yields of all three solvents, in general, the polar methanolic extracts displayed relatively high percentages of crude yields compared to the other solvents.

However, as the percentage crude yield alone is not a good indicator for the presence of bioactive ingredients, the plant extracts were further analyzed for their bioactivities such as the antibacterial and antioxidant activities.

4.1 Investigation of the bioactivities of adhatoda extracts

i. Antibacterial activity

All the adhatoda extracts prepared in this study were screened for their antibacterial activity and the results are summarized in Table 2.

According to the results shown in Table 2, only the methanolic extract of adhatoda obtained by soxhlet extraction indicated considerable antibacterial activity at the tested 25 mg/mL concentration against all the four bacterial strains. The other leaf extracts of adhatoda did not indicate any significant antibacterial activity against any of the tested pathogens at 25 mg/mL concentration.

Therefore, it can be concluded that soxhlet extraction of leaves in methanol to be the best extraction condition to obtain antibacterial extracts from adhatoda, possibly indicting that the antibacterial compounds are polar in nature. Hence, the methanolic soxhlet extract of adhatoda was selected to be used during the preparation of antibacterial adhatoda soap.

Table 2. Antibacterial assay results for the 25 mg/mL adhatoda extracts.

Extraction method	Solvent	Diameter of zones of inhibition (cm) ± SEM			
		B.cereus	*S.typhimurium*	*S.aureus*	*E.coli*
Maceration	Methanol	NI	NI	NI	NI
	Ethyl acetate	NI	NI	NI	NI
	Hexane	NI	NI	NI	NI
Soxhlet	Methanol	1.8 ± 0.0	1.8 ± 0.1	1.5 ± 0.1	0.9 ± 0.0
	Ethyl acetate	NI	NI	NI	NI
	Hexane	NI	NI	NI	NI
Sonication	Methanol	NI	NI	NI	NI
	Ethyl acetate	NI	NI	NI	NI
	Hexane	NI	NI	NI	NI
Negative control		NI	NI	NI	NI
Positive control		3.0 ± 0.1	2.9 ± 0.0	2.6 ± 0.1	2.0 ± 0.1

*NI = No Inhibition

ii. Antioxidant capacity

All the adhatoda plant extracts were tested with the FC assay to determine their antioxidant capacity (AOC) with respect to a standard series of pyrogallol. The AOCs determined for different leaf extracts of adhatoda are shown in Table 3.

Table 3. AOCs of different leaf extracts of adhatoda determined by the FC assay.

Solvent	AOC in µg PGE/ mg		
	Maceration	Soxhlet extraction	Sonication
Methanol	9 ± 1	19 ± 3	5 ± 0
Ethyl acetate	79 ± 3	109 ± 2	107 ± 0
Hexane	29 ± 0	26 ± 2	22 ± 1

According to the findings of this research, ethyl acetate which is a solvent with an intermediate polarity gave the highest AOC over methanol and hexane extracts of adhatoda regardless of the extraction technique used. The highest AOC was observed for the ethyl acetate extract obtained by soxhlet extraction (109 ± 2 µg PGE/mg). However, as sonication is a more convenient extraction technique, it could be more practically useful in large scale trials, especially since it has resulted a similar AOC with the ethyl acetate solvent.

The [Mo(VI)] complex in the FC reagent which is in yellow color is reduced to form a blue color [Mo(V)] complex in the presence of phenolic and other reducing substances in the sample (Prior, 2005). This assay was previously considered as an assay to measure the Total Phenolic Content (TPC) of a sample. However, as the FC reagent can react with other reducing agents that can act as antioxidants, it can be more accurately considered as a method to determine the total AOC of a sample (Everette, 2010).

iii. DPPH radical scavenging activity

Percentage radical scavenging activity of the best antioxidant active ethyl acetate soxhlet extract of adhatoda was found to be 22 ± 1 % from the DPPH radical scavenging assay. Even though a high AOC was observed for the ethyl acetate soxhlet extract, it yielded a relatively low radical scavenging activity.

However, the ethyl acetate soxhlet extract of adhatoda was selected to be used to give the desired bioactivity to the antioxidant adhatoda soap prepared during this study.

4.2 Phytochemical studies

The methanol and ethyl acetate extracts of adhatoda obtained by soxhlet extraction were screened to investigate the phytochemicals present in each extract. The methanol soxhlet extract of adhatoda indicated the presence of a number of phytochemicals whereas the ethyl acetate soxhlet extract only indicated the presence of phenols and tannins. (Table 4)

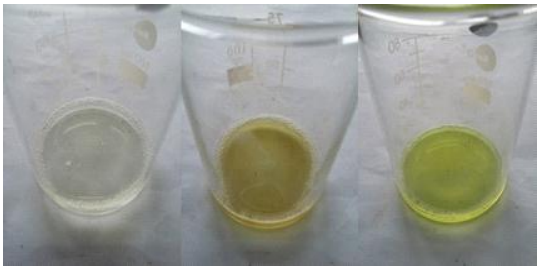
Table 4. Phytochemical analysis of methanol and ethyl acetate extracts of adhatoda obtained by soxhlet extraction.

Solvent	Alkaloiods	Phenols and tannins	Terpenoids	Steroids and terpenoids	Saponins
Methanol	+	+	+	+	-
Ethyl acetate	-	+	-	-	-

Adhatoda is known to possess alkaloids such as vasicine and vasicinone which are responsible for its antibacterial activity (Pa & Mathew, 2012). Therefore, the significant antibacterial activity observed in the methanol soxhlet extract of adhatoda (Table 2) could be attributed to the presence of alkaloids in the methanolic extract as indicated above. Even though both the extracts contained phenols and tannins according to the phytochemical results, the highest AOC was observed for the ethyl acetate extract. With the support of the phytochemical analysis which indicated the presence of important secondary metabolites that could be responsible for the observed bioactivities, these extracts reflected their ability to serve as biologically active extracts that could be used in the preparation of medicinal adhatoda soap products.

4.3 Preparation of adhatoda soap products

Two medicinal soaps were prepared with the aforementioned bioactive extracts (methanol soxhlet extract and ethyl acetate soxhlet extract). A control soap was made without the addition of extracts. The prepared soaps are shown in Figure 1.



a) b) c)

Figure 1. Adhatoda liquid soaps.

a) control soap
b) antibacterial soap made with the methanol soxhlet extract
c) antioxidant soap made with the ethyl acetate soxhlet extract.

4.4 Assessing the biological activities of the prepared adhatoda medicinal soaps

i. Antibacterial activity

The antibacterial activity of the prepared antibacterial adhatoda soap was investigated using the disk diffusion assay and the results obtained are shown in Table 5.

Table 5. Antibacterial activity of the soap products.

.Sample	Average diameter of inhibition zones (cm)			
	B. cereus	*S. aureus*	*S. typhimurium*	*E. coli*
Antibacterial soap	1.0 ± 0.1	1.0 ± 0.0	NI	0.7 ± 0.0
control soap	1.0 ± 0.1	0.7 ± 0.0	NI	0.7 ± 0.0
Gentamycin	2.0 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	1.4 ± 0.1

Even though a 25 mg/mL concentration of the methanolic soxhlet extract of adhatoda displayed an antibacterial activity as the pure extract (Table 2), the concentration of the extract used to prepare the medicinal liquid soap was doubled to ensure its activity in the final product. However, after the addition of the methanoilc extract into the soap product, it was active only against *S. aureus* with an inhibition zone of 1.0 ± 0.0 cm at the 50 mg/mL concentration of the extract (Table 5). As the antibacterial components in the extract might not have been stable in the liquid soap medium, the concentration used for the preparation of the soap product might not have been enough to inhibit the growth of the other three bacterial species. However, the growth of all these species was inhibited at a concentration of 25 mg/mL of the pure extract (Table 2). Compared to the test soap, a smaller inhibition zone was observed for the control soap; 0.7 ± 0.0 cm (Table 5) and this might be due to the natural antibacterial activity of coconut oil (Vermén, 2008) used to prepare the soap base.

Test for the effectiveness of the prepared antibacterial adhatoda soap (Thumb impression test)

Effectiveness of the antibacterial adhatoda soap was further tested using the thumb impression test explained in the methodology section. During this experiment, one hand was washed with the test soap and the other one was washed with the control soap and thumbprints of both hands were made on a sterile agar plate to monitor the microbial growth on the area of the thumbprints. The results of the experiment are shown in Figure 2.

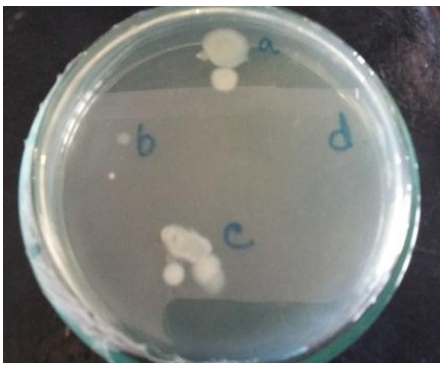


Figure 2. Thumbprints of differently treated thumbs.

a) and c) unwashed thumbs b) thumb washed with the control soap, d) thumb washed with the antibacterial adhatoda soap.

It can be observed that the number of bacterial colonies grown on the thumbprints kept with washed thumbs are lower in number and smaller in size than those grown on the thumbprints made with unwashed thumbs (b and d thumbprints *vs* a and c thumbprints in Figure 2). The thumbprint kept from the hand washed with the antibacterial adhatoda soap, indicated no bacterial growth in the relevant area (area d in Figure 2) whereas the thumbprint of the hand washed with the control soap indicated the presence of 2-3 bacterial colonies on it (area b in Figure 2). According to the overall results of these thumb impression test, it can be concluded that the antibacterial medicinal soap prepared during this study is effective against removing the bacteria which could be found on ones hands in addition to the specific bacterial strains that were tested in the laboratory. This also reveals the promising antibacterial nature of the liquid medicinal soap prepared during this study.

ii. Antioxidant activity

The antioxidant adhatoda medicinal soap was prepared using the ethyl acetate soxhlet extract. The AOC of the prepared medicinal soap was investigated using the FC assay and the results are shown in Table 6 with respect to its control soap.

Table 6. AOCs of the prepared soap products.

Soap type	Amount of plant extract contained in 100 mg of the final soap product (mg)	AOC µg PGE/mg
Control soap	0	3 ± 0
Antioxidant soap	10	10 ± 0

The adhatoda liquid soap was about 3.3 times better than the control soap in its AOC. A slight AOC for the control soap has also been observed and this might be due to the ingredients such as coconut oil which is a constituent in the liquid soap base which is known to be antioxidant active itself (Yeap, 2015). The antioxidant soap was able to retain a similar AOC to that indicated by the original plant extract used to prepare the medicinal soap product. These results clearly indicate the improvement of the AOC of the soap product upon addition of the respective adhatoda extract to it.

5. CONCLUSION

Amongst the different extracts of adhatoda, methanol soxhlet extract was found to be the best antibacterial active fraction and the best antioxidant active extract of adhatoda was found to be the ethyl acetate soxhlet extract (AOC = 109±2 µg PGE/mg and % RSA = 22±1%). When consider the medicinal adhatoda soaps prepared in this study, the successful antibacterial and antioxidant properties observed in them indicate the promising nature of using adhatoda extracts in the preparation of value added medicinal soaps. This approach is especially attractive due to the novelty of this application, as there has not been any published literature so far indicating the use of adhatoda in medicinal soap products according to our knowledge and available published data. Further investigations can be carried out to incorporate these bioactive extracts into other cosmetic products as well.

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