

Design, preparation and evaluation of germicidal *Toddalia asiatica* herbal antiseptic detergent

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ARTICLE INFO

Article history:

Received on: 23/06/2016

Revised on: 14/07/2016

Accepted on: 04/09/2016

Available online: 29/11/2016

Key words:

Toddalia asiatica; herbal;
antiseptic; detergent;
formulation.

ABSTRACT

To avoid skin dryness and irritation encountered with alcohol-based sanitizers, *Toddalia asiatica* stem bark extract was explored in formulation of a germicidal herbal antiseptic detergent. A L₉ (3⁴) Taguchi Orthogonal Array was used to optimize set factors for design and preparation. Antimicrobial activities were screened by disc diffusion method for formulated herbal detergent; blank detergent; commercial handwash and tap water. A model handwash efficacy experiment was carried out using volunteers, which involved enumeration of bacteria from water used for palm rinsing after a hand-wash with the test agents. Freeze-drying attained 11% yield from 500g plant material. The set factors for herbal antiseptic detergent preparation were optimized to 10% extract in 5g of sodium lauryl salt stirring for 15 minutes at 30°C. The herbal detergent displayed highest antimicrobial activity indicated by zone of inhibition diameters of 24mm (*M.R.S. aureus*) and 22mm (*M. gypseum*) compared to 22mm and 14mm respectively by the commercial hand wash. In the model hand wash efficacy experiment, the formulated herbal detergent attained 78.8% reduction of pathogenic load as compared to 67.9% reduction with the commercial handwash. The remarkable potency illustrated presents *T. asiatica* phytochemicals as promising germicidal non-synthetic active ingredients for formulation of antiseptic detergents.

INTRODUCTION

In the last decade, there has been an unprecedented investment in health care to accelerate the reduction of mortality. However, one of the main barriers to proven and affordable health care has been limited interventions for microbial infections (UNICEF, 2014). The skin as a protective covering of the body, averagely for an adult human being it covers a surface area of two square meters. In its intact state, the skin is a barrier, exposed to life threatening microorganisms. Meanwhile dermatology is an essential part of general medicine since diseases of the skin are a common occurrence accounting for a great deal of misery, suffering, incapacity and even economic loss (Roose-Amsaleg and Laverman, 2016). Hands have been noted to be the primary routes of transmission of multidrug resistant pathogens and other infectious germs.

This therefore necessitates use of antiseptic agents for washing hands and the skin (Pittet and Boyce, 2001). Currently on the market, there are alcohol-based sanitizers and chlorohexidine products as chemical antiseptics however; they have shortcomings like skin dryness and irritation as common adverse effects on frequent use (Pittet, 2005). Previously, studies have reported that resistance to the chemical antiseptics has led to outbreaks (Maury *et. al.*, 2000; Pittet *et. al.*, 2000).

Toddalia asiatica (L) Lam. (Rutaceae) is a medicinal plant commonly known as Orange climber (Eng.). Traditionally it is used as a browse for goats and as a hedge among the Maasai and Kipsigis communities of Kenya (Orwa *et. al.*, 2008). The most important use of *Toddalia asiatica* however is medicinal. It is noted that traditional medicine treatments that exploit *Toddalia asiatica* are increasingly becoming common. It is used to treat skin infections, nasal and bronchial pains, stomachache, snake bites, and in rituals (Gidwani *et. al.*, 2010). The use of *T. asiatica* in folk medicine to treat various ailments has been substantiated scientifically in studies focusing on phytochemical isolation, structure elucidation and antimicrobial testing.

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In one study, the ethyl acetate extract which was found to possess highest antimicrobial activity was subjected to activity guided fractionation by column chromatography over silica gel. This resulted in the isolation of the coumarin, Ulopetrol, an active principle besides Flindersine which had been reported earlier. Ulopterol showed activity against the bacteria *Staphylococcus epidermidis*, *Enterobacter aerogenes*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Escherichia coli* and fungi *Aspergillus flavus*, *Candida krusei* and *Botrytis cinerea* (Karunai *et. al.*, 2012). Much earlier, antibacterial and antifungal activities were observed in ethyl acetate extract. The active principle Flindersine (2,6-dihydro-2,2-dimethyl-5H-pyrano [3,2-c] quinoline-5-one-9cl) was isolated from the ethyl acetate extract. In the study, significant inhibition was realized at quite low concentrations against bacteria *Bacillus subtilis* (31.25 µg/ml), *Staphylococcus aureus* (62.5 µg/ml), *Staphylococcus epidermidis* (62.5 µg/ml), *Enterococcus faecalis* (31.25 µg/ml), *Pseudomonas aeruginosa* (250 µg/ml), *Acinetobacter baumannii* (125 µg/ml) and fungi *Trichophyton rubrum* 57 (62.5 µg/ml), *Trichophyton mentagrophytes* (62.5 µg/ml), *Trichophyton simii* (62.5 µg/ml), *Epidermophyton floccosum* (62.5 µg/ml), *Magnaporthe grisea* (250 µg/ml) and *Candida albicans* (250 µg/ml) were observed (Duraipandian *et. al.*, 2009). The Indian subcontinent is being considered as a vast repository of polyherbal formulations used in traditional medical treatments. The formulations are prepared by plant extracts either of the bark, leaves, stem or roots with suitable excipients standardized by various methods. A combination of chloroform extracts of leaves of *Tevetia peruviana*, *Waltheria indica* and *Toddalia asiatica* were used to prepare polyherbal formulations that were assessed for their cytotoxic activity by using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Results concluded that the formulation demonstrated significant protective activity that might be due to combined effect of all these extracts (Hemalatha *et. al.*, 2006).

Despite of its wide spread use in traditional health care systems and the variously reported *In-vitro* antibacterial antifungal activities, there is no report for use of *T. asiatica* extracts as the active ingredient in liquid detergents formulations. This study therefore reports for the first time design, and preparation of detergent with herbal extracts as the antiseptic agent together with a model for its evaluation as hand wash in palm rinse experiments.

MATERIALS AND METHODS

Reagents

The chemicals and solvents used were all Analar grade purchased from Kobian Kenya Ltd. Sodium hydroxide, Potassium hydroxide and sodium lauryl salt were obtained from Sigma Aldrich Chemical Co. St. Louis U.S.A. The solvents were of above 99% purity and hence used without further purification.

Plant samples collection and processing

The plant material (*Toddalia asiatica* stem) was collected during the short rain season in the month of July from its natural

habitat at the slopes of Kajulu hills (0°03'S 34°41'E) in the Lake Victoria basin, Kenya. Upon successfully authentication by plant taxonomist at the Department of Biological Sciences of Moi University Mr. Taabu Tepeny, specimen was deposited and voucher specimen accession number assigned as MU/BL/0038/2016. The mature plant stems were cut into pieces and carried in polythene bags to the laboratory for processing. Stem bark was scraped off, washed then dried at room temperature in open air for three weeks before grinding into fine powder using a hammer mill.

Stem bark powder (500g) was extracted by mercuration in 1 litre distilled water for 48 hours. The extract was filtered through Whatman No. 1 filter paper and freeze-dried using a laboratory lyophilizer (FD-1A-50). The extracts were then stored in sterile air-tight containers.

Pathogenic test organisms

Microbial organisms selected for screening efficacy of the prepared herbal antiseptic detergent was based on the recommendation of the National committee for clinical laboratory standards (NCCLS, 1992) as the important pathogens. Bacterial test strains were chosen in terms of their gram-staining properties. Gram positive included Methicillin Resistant *Staphylococcus aureus* (clinical isolate) while the gram negative were *Pseudomonas aeruginosa* (ATCC 27863), *Escherichia coli* (ATCC 2692) and *Salmonella typhi* (clinical isolate). Similarly the fungal test organisms were chosen categorically as yeasts entailing *Candida albicans* (ATCC 90038) and *Candida tropicalis* (ATCC 1750), while a dermatophyte as *Microsporum gypseum* (Clinical isolate). The clinical isolates were all identified with similar methods previously used by Nagy (2009).

Design and Preparation of the herbal antiseptic detergent

Taguchi experimental design (Taguchi and Konishi, 1987) implemented on the Design-Expert version 7 was used for designing and optimization of the variables in preparation to attain the herbal antiseptic detergent with highest germicidal activity. From preliminary screening experiments the factors that would affect the response (Zones of Inhibition) were found to entail Sodium lauryl sulphates (SLS) concentration, percentage extract incorporated, temperature and the stirring time. These were all evaluated in single factor tests. A L₉ (3⁴) Taguchi Orthogonal Array was then used to define the optimal conditions per the selected factors to produce the herbal antiseptic detergent formulae and the optimum temperature and the stirring time for preparation. Each of the nine experiments were performed in triplicate and means reported.

According to results of optimization the herbal antiseptic detergent was then prepared by the conventional incorporation method for semi solid preparation heating with a thermostated oil bath magnetic stirrer with heating. Blank detergent was prepared similarly but without addition of the extract in same amount of plain distilled water. The formulated detergent was compared to commercial antiseptic handwash marketed in Kenya.

***In-vitro* antimicrobial evaluation**

In the assays, the negative control was the blank detergent prepared without extract; positive control was the commercial antiseptic hand wash. Freshly growing microorganisms were obtained by sub-culturing stocked isolates of bacterial strains onto Muller Hinton agar No. CM0047 (Oxoid Ltd.) and incubating at 37 °C for 24 hours. Meanwhile fungal strains on Sabouraud Dextrose Agar No. CM 0441 (Oxoid LTD) incubating at 30 °C for 72 hours. A 0.5 McFarland turbidity standard was prepared in sterile distilled water and inoculated uniformly onto the growth media. A 6mm disc impregnated with the test solution was then aseptically placed onto the pates before incubating at respective temperatures. The zones of inhibition were then measured after 24 hours and 72 hours for antibacterial and antifungal assays respectfully.

Model hand wash efficacy study

Four volunteers groups (each three participants) were involved in the study categorized as those for the formulated herbal antiseptic detergent; for blank detergent; for commercial handwash and for tap water. The protocol was reviewed by the University Ethical Committee and all procedures approved as being within required ethical considerations. All the participants understood the protocol and informed consent was confirmed through duly signed acceptance to participate forms.

A rinse - wash method (Saad *et. al.*, 2011) was adopted. This involved rinsing palm for 15 seconds with tap water; palm is then lathered with the desired test agent before rinsing again with water for 30 seconds. The drains of the palm rinse were collected separately into a sterile beaker. Enumeration of bacterial flora was then carried out by serially diluting and plating of the palm rinse drains on media plates and incubation at 37°C for 24 hours. Point inoculation method was adopted for viable microbial count.

RESULTS AND DISCUSSION

Plant sample extraction percentage yield

For the extraction of the phytochemicals from the stem bark, percentage yield was calculated as the ratio of the obtained

freeze dried material to the total plant powder macerated in distilled water. Out of the 500g of plant material extracted, a yield of upto 11% was afforded on freeze-drying. This was considered a satisfactorily amount for utilization in further tests.

Design and Preparation of the herbal antiseptic detergent

In the investigation of the optimum factors for the preparation of the antiseptic herbal detergent, two responses were considered as the bacterial inhibition against *M.R.S. aureus* and the fungal inhibition against *M. gypsiium*. These were typical pathogenic organisms prevalent in the environment. The Taguchi Orthogonal Arrays of nine experiments gave the respective inhibitions for particular variables as indicated in table 1

On analysis of the responses together using the Design expert software, optimality was obtained as 10% water extract suspension to 5g of SLS and solution made homogeneous by stirring for 15 minutes at 30°C.

The optimized formula for the antiseptic herbal detergent was thus the one comprising of 5 g SLS, which gave a bacterial inhibition of 24mm diameter. The other factors optimized were the percentage extract as 10% water suspension; and stirring time of 15 minutes at 30°C, which also displayed a fungal inhibition of a zone reaching 22 mm in diameter this was therefore selected as the design for preparing the antiseptic herbal detergent.

ANOVA analysis and Factors Percent contribution of the Taguchi orthogonal design

ANOVA analysis was performed and results tabulated in table 2 accordingly for sum of squares, degree of freedom, mean squares and the F-value. The various factors contribution was also calculated by the formulae in equation (i.)

$$\text{Percent Contribution (PC)} = \frac{\text{Sum of Squares (SS}_F)}{\text{Sum of Squares Total(SS}_{Total})} \times 100(i.)$$

(Waddad *et. al.*, 2013)

Table 1: Optimization array.

Run	Factor 1: SLS amount (g)	Factor 2: % Extract (%)	Factor 3: Temperature (°C)	Factor 4: Stirring time (min)	Response 1: Bact-Inhibition (mm)	Response 2: Fungi-Inhibition (mm)
1	2.5	20	30	45	18	14
2	7.5	5	30	30	16	12
3	7.5	20	25	15	20	16
4	5	5	25	45	12	26
5	2.5	10	25	30	8	14
6	5	20	20	30	8	8
7	2.5	5	20	15	6	8
8	7.5	10	20	45	20	18
9	5	10	30	15	24	22

Table 2: ANOVA analysis and Factor's Percent contribution of the TOA.

FACTOR	SS ^a	DF ^b	MS ^c	F ^d	PC % ^e
Bacterial inhibition					
A – SLS concentration	210.89	2	105.44	70.95	20.3
B - % Extract	156.22	2	78.11	52.56	15.0
C – Temperature	402.00	2	201.00	135.25	38.7
D – Stiring time	270.22	2	135.11	90.92	26.0
Residual (error)	23.78	2			
Totals	1039.33	10			
Fungal inhibition					
A – SLS concentration	238.89	2	119.44	126.47	24.6
B - % Extract	150.22	2	75.11	79.53	15.5
C – Temperature	288.89	2	144.44	152.94	29.7
D – Stiring time	294.00	2	147.00	155.65	30.2
Residual (error)	15.11	2			
Totals	972	10			

^a Sum of Squares^b Degree of Freedom^c Mean Sum of Squares^d F – value^e Percent Contribution**Table 3:** Zones of inhibition for antimicrobial assays

Test agent (50 mg/ml)	Zone of Inhibition (mm)						
	<i>M.R.S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>S. typhi</i>	<i>C. albicans</i>	<i>C. tropicalis</i>	<i>M. gypseum</i>
Formulated antiseptic herbal detergent	24.30±0.67 ^d	18.00±0.58 ^d	16.00±0.58 ^d	19.67±0.67 ^d	22.00±1.00 ^d	18.33±0.67 ^d	21.67±0.67 ^d
Blank detergent	14.00±0.58 ^{ce}	12.33±1.20 ^c	9.67±0.33 ^c	8.00±0.00 ^c	8.33±0.33 ^c	12.00±0.58 ^c	14.33±0.33 ^{ce}
Commercial hand wash	21.67±0.33 ^{be}	19.67±0.67 ^b	13.67±0.33 ^b	18.33±0.33 ^b	20.33±0.88 ^b	14.33±0.33 ^b	14.00±0.00 ^{be}
Tap water	6.00±0.00 ^a	8.00±1.15 ^a	8.00±1.15 ^a	10.00±0.58 ^a	9.67±0.67 ^a	8.00±0.00 ^a	8.33±1.20 ^a

Values represent means ± standard error.

For each organism, means with same letters within a column are not significantly different (p<0.05, Tukey's standardised range test). N=3

Table 4: percent reduction in bacterial growth for palm rinse

Test agent	Control (cfu/ml)	Test (cfu/ml)	% reduction
Formulated Herbal detergent	26.9 X 10 ³	5.7 X 10 ³	78.8%
Blank detergent	23.6 X 10 ³	15.3 X 10 ³	35.2%
Commercial hand wash	25.2 X 10 ³	8.1 X 10 ³	67.9%

In-vitro antimicrobial evaluation

In-vitro antimicrobial assays revealed that the formulated herbal detergent had significant activity against all the bacterial and fungal strains tested (table 3). The highest activity was against *M.R.S. aureus* (24mm), this was significantly higher than the commercial hand wash (22mm) while tap water registered no inhibition and the blank detergent only elicited an inhibition reaching 14mm in diameter.

The formulated herbal detergent exhibited highest activity across all the strains of pathogenic microorganisms tested followed by the commercial handwash. Thus denoting the action of the active ingredient for which in the formulated herbal detergent was the extract of *T. asiatica* stem bark. Antibacterial antifungal activity of *T. asiatica* stem bark reported previously happens to be in the same range as that reported in this study. The findings of this study therefore confidently attributes the witnessed antiseptic activity to the phytochemicals in the plant stem bark based on the zones of inhibition that compares well to those obtained by Munyendo and group (2011) from the ethyl acetate extract of *T. asiatica* stem bark.

Meanwhile tap water registered minimal zone of inhibition diameter against all the pathogenic organisms screened, this is logically accepted since the water had no active ingredients

compared to blank detergent that showed smaller diameter of inhibition whose activity could be charged to the cleaning capabilities as dirty removers.

Statistical analysis of the antimicrobial activity data to compare significance of means variation by t-tests revealed that there was significant variations at p<0.05 level of confidence for most of the strains. *M. R. S. aureus* and *M. gypseum* inhibition did not significantly differ in particular with the blank detergent and commercial antiseptic detergent (table 3). This could be attributed to the fact that the bacteria *M. R. S. aureus* depicts resistance to common antiseptic agent while *M. gypseum* is a persistent dermatophyte that manifests as being chronic. This making susceptibility not differ significantly with this two particular test agents. However it is interesting to note that there was significant variation for the formulated antiseptic herbal detergent in comparison to tap water thus a confirmation statistically of the efficacy of the formulated herbal antiseptic product.

Model hand wash efficacy study

The skin carries a large number of bacterial and fungal organisms. Mainly the gram-positive bacteria are usually picked up from the various objects with which the skin comes in contact with. The pathogenic microorganisms growth reduction (table 4)

was obtained by use of equation (ii.), with data obtained from media plates bacteria enumeration after incubation of collected palm rinse up on washing with formulated herbal detergent, blank detergent and commercial hand wash

$$\% \text{ Reduction} = \frac{\text{control} - \text{Test agent}}{\text{control}} \times 100 \dots \dots \text{(ii)}$$

(Saad *et. al.*, 2011)

The formulated herbal detergent displayed greatest pathogenic microorganism reduction potential with *M. R. S. aureus* bacterial enumeration as the indicator. This was even higher than the commercial handwash and noted to be more than two times the blank detergent hence a confirmation of its efficacy. The activity against *M.R.S. aureus* is of significant interest, apart from the multi-drug resistance, *S aureus* is one of the natural flora commonly found on human hands and face and may not be removed easily by routine washing. The potency therefore illustrated from the significant % reduction by the formulated herbal detergent (78.8%) is remarkable presenting *T. asiatica* phytocompounds as promising active ingredients for formulation of antiseptic detergents. These results compares well with findings of Mann and Staba (2002) with formulations of herbal organic extracts against isolates from skin infections including boils and abscess.

CONCLUSION

The formulation containing the aqueous extracts of *T. asiatica* stem bark exhibited pronounced antimicrobial activity. This therefore suggest the use of formulated herbal antiseptic detergent as a superior and promising germicidal agent that comprises of non-synthetic active ingredients.

ACKNOWLEDGEMENTS

Author express their sincere gratitude to Maaida Abubaker Salim and Lilian Chepngetich for their tenacious workmanship during the laboratory experiments of this study. Authors also thankful to the entire School of Biological and Physical Sciences staff for accepting to participate as volunteers. We also acknowledge the Department of Chemistry and Biochemistry, Moi University for allowing access to necessary equipment for the study.

Financial support and sponsorship: Nil.

Conflict of Interests: There are no conflicts of interest.

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How to cite this article:

Were L. L. Munyendo, Ambrose K. Kiprop., Design, preparation and evaluation of germicidal *Toddalia asiatica* herbal antiseptic detergent. *J App Pharm Sci*, 2016; 6 (11): 100-104.