Veterinary Integrative Sciences 2023; 21(3): 959 - 971 DOI; 10.12982/VIS.2023.069

Vet Integr Sci

Veterinary Integrative Sciences

ISSN; 2629-9968 (online)

Website; www.vet.cmu.ac.th/cmvj

Research article

Formulation and evaluation of saponin based alcohol - free polyherbal hand sanitizer

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Abstract

Sanitization is essential in limiting the spread of infectious diseases. Alcohol based sanitizer formulations consist of toxic compounds that are harmful to human health and not environmentally friendly. The purpose of the current research is to formulate a bio-derived alcohol-free hand sanitizer. Saponins are secondary metabolites that are stored in the roots of many plant species, where they may act as antimicrobial phytoprotectants. Saponins were extracted by Soxhlet extractor using methanol as solvent. Saponins were tested for their individual, synergetic antimicrobial (Agar plate diffusion method and Minimum inhibitory concentration) and anti-oxidant (Ferric Reducing Antioxidant Power (FRAP) assay) activity. The polyherbal sanitizer was formulated using saponins as the principal ingredient. It was evaluated for its physical parameters and antimicrobial properties. The synergistic effect of saponins was higher when compared to the individual saponins' antimicrobial activity. The formulated polyherbal sanitizer evinced acceptable organoleptic properties with slightly acidic pH and demonstrated antimicrobial properties against selected microorganisms with maximum activity against *Staphylococcus aureus* (concentration =100 µl, zone of inhibition diameter = 24±1 mm). The antimicrobial efficacy of the formulated sanitizer was comparable to that of a commercially available hand sanitizer

Keywords: Antimicrobial efficacy, Bio-derived, Polyherbal, Sanitizer, Saponins

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Article history; *received manuscript: 7 May 2023,*
revised manuscript: 6 June 2023,
accepted manuscript: 19 June 2023,
published online: 3 August 2023
Academic editor; *Korakot Nganvongpanit*

INTRODUCTION

Adequate hygiene and sanitization play an inevitable role in restraining the spread of infection in public places and healthcare institutions. WHO advises us to sanitize or wash our hands frequently with alcoholic hand sanitizer or soap as they disrupt the lipid membrane, expose the intercellular content and inactivate the virus. Overuse of soaps and surfactants damage the epidermis as they negatively affect the proteins, lipids and keratin composition (Khan and Yadav, 2020). The formulation of the available hand sanitizers mainly consists of isopropyl alcohol, ethanol and hydrogen peroxides in different combinations, which are toxic to human health and are not environmentally friendly. The skin seems to be deprived of oil and water and its permeability increases due to the excessive use of alcoholic sanitizer, which also causes irritation and allergic condition of eye. Prolonged exposure of alcohol hand sanitizer can result in cracking of skin with itching, peeling, dryness or redness. Damaged skin increases the risk of viral entry into skin (Mahmood et al., 2020). *Food and Drug Administration* (2023) warns that application of alcohol-based sanitizer can have adverse effects such as nausea, headache and dizziness, which are likely to have occurred due to vapours from hand sanitizer especially in enclosed spaces or poor air circulation places. The American Association of Poison Control Centers reported 9504 alcoholic hand sanitizer exposure cases in children under the age of 12 in the first five months of 2020, recognizing that even a small amount of alcohol can result in alcohol poisoning in children, leading to vomiting, drowsiness, and confusion, and, in severe cases, respiratory arrest and death (Mahmood et al., 2020). Natural herbal hand sanitizers can serve as an alternative to alcohol based hand sanitizers but commercially available ones also claim to possess alcohol.

Saponins, flavonoids, alkaloids, polyphenolic compounds are enriched in medicinal plants and exhibit antimicrobial, disinfectant and antiseptic activities (Alghamdi, 2021). Saponins are secondary metabolites widely spread in plant kingdom. They act as a shield to counter pathogens and thus play a major role in plant defense system (Cheok et al., 2014). Many important biological activities of saponins have been reported such as antifungal, antibacterial, antiviral, anti-ulcer, anti-inflammatory, hepatoprotective and haemolytic properties. The amphiphilic nature of saponins is responsible for rearrangement and disruption of membrane lipids, formation of pores and lysis of cell as well as inhibits the virus-host cell attachment process (Kregiel et al., 2017). Studies have revealed that saponins from different plant species like *Glycyrrhiza glabra*, *Asparagus racemosus, Withania somnifera* etc. exert inhibitory effects against several viruses (Huan et al., 2021; Patel C N et al., 2021; Kanchibhotla et al., 2022). Hence the objectives of the present study include extraction of saponins from species like *Glycyrrhiza glabra*, *Asparagus racemosus, Withania somnifera, Hemidesmus indicus* and formulation, testing and comparison of the antimicrobial efficacy of the formulated hand sanitizer with commercially available one.

MATERIALS AND METHODS

Sample preparation

Samples of *Glycyrrhiza glabra, Asparagus racemosus, Withania somnifera* and *Hemidesmus indicus* roots were collected and dried thoroughly under sunlight. The dried roots were then crushed using a mixer to a coarse powder and stored at room temperature for further analysis.

Extraction of saponins

100 g of root powder of each sample was subjected to defatting using 300 ml n-hexane using Soxhlet extractor to eliminate undesirable lipids. Residual solvent in the marc was removed completely by air-drying. The dried powder was subjected to Soxhlet extraction using methanol as solvent for 10 h. The extract was evaporated to dryness and dissolved in 100 ml distilled water. Equal volume of butanol was added, mixed well, transferred to a separating funnel and incubated overnight. This resulted in the formation of two layers – upper organic and lower aqueous layer. The upper organic layer was recovered and evaporated. The residue was dissolved in methanol and excess diethyl ether was added for precipitation. The precipitate was filtered, dried and weighed (Obasi et al., 2017).

Identification of saponins

Foam test:

0.5 g of the extract was added to 5 ml of distilled water in a test tube and warmed in a water bath. In accordance with the modified method of El et al. (2019), stable persistent foam at the top of the solution reveals the existence of saponins.

Lead acetate test:

1 ml of saponin extract was thoroughly agitated with 1 % lead acetate solution. The presence of saponins is indicated by the formation of white precipitate (Devmurari, 2010).

Quantification of saponins

The saponins were quantified using the formula (El et al., 2019):

Equation 1:

$$\text{Total Saponin Content (\%)} = \frac{\text{Weight of the saponin residue}}{\text{Weight of the original plant material}} \times 100$$

Estimation of antioxidant property by FRAP assay

FRAP assay was applied to determine the antioxidant property of the individual saponins (Mokrani et al., 2016). In brief, 1 ml of different concentrations of the samples (2-6 mg) were dissolved in 2.5 ml of phosphate buffer having pH-6 and 2.5 ml of Potassium ferricyanide (1 %) and incubated at 50 ºC for 20 minutes. Later, 2.5 ml of Trichloroacetic acid (10 %) was added. The solutions were subjected to centrifugation at 3000 rpm. 0.5 ml of Ferric chloride solution (0.1 %) and 2.5 ml of distilled water was added to 2.5 ml of

the supernatant of the centrifuged solution. A blank was prepared in similar manner without adding the extracts. Various concentrations of ascorbic acid were used as standard. The absorbance was read at 700 nm. A graph was plotted with concentration of sample versus absorbance and the antioxidant activity was expressed in terms of mg Fe/g saponin extract.

Antimicrobial activity of saponins

The microorganisms selected for evaluation of antimicrobial activity are as follows:

Gram positive Bacteria – 24 h cultured *Enterococcus faecalis, Staphylococcus aureus*

Gram negative Bacteria- 24 h cultured *Salmonella typhi, Escherichia coli.* Fungal strain – 24 h cultured *Candida albicans.*

The antibacterial and antifungal activity of individual saponin extract was determined using the agar plate diffusion method. Same method was used to test the synergistic effect of the saponins by taking 2.5 % of each saponin. 20 ml of media poured into sterilized petri plates. 100 µl of the selected test organisms were swabbed on top of the solid media. On agar plates, four wells with 6 mm diameter were made using the well borer in respective plates. 25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml of saponins were added to the respective wells. Chloramphenicol (positive control for bacteria) and Fluconazole (positive control for fungi) of concentration 25 µg/disc were placed at the centre of the plate. The antibacterial activity was measured after 24 hours of incubation at 37 °C by measuring the diameter of the zone of inhibition produced. The investigation was carried out in triplicate. Minimum inhibitory concentration (MIC) were performed according to Sen and Batra (2012).

Formulation of polyherbal sanitizer

The Polyherbal Sanitizer's composition is shown in Table 1. The formulation was prepared by gently mixing each saponin (2.5 % w/v) and Aloe Vera gel (2 % w/v) in 250 ml beaker. 0.5 % preservative and 0.2 % perfume was added to the above solution. The remainder formula was completed by adding deionised water (DI).

Table 1 Composition of Polyherbal Sanitizer.

Ingredients	Quantity (%)
Saponins each	2.5
Aloe vera gel	2.0
Preservative	0.5
Perfume	0.2
DI Water	QS

Evaluation of the formulated sanitizer

Organoleptic properties

Tests like odour, colour and clarity were carried out (Shaikh et al., 2020).

pH evaluation

A digital pH meter was used to determine the pH of the prepared polyherbal sanitizer (Shaikh et al., 2020).

Irritancy test

The formulated sanitizer was applied on the palm of 15 healthy volunteers and checked for dryness, redness, itching and irritation (Shaikh et al., 2020).

Comparison of formulated polyherbal sanitizer with commercially available sanitizer

The formulated polyherbal sanitizer was compared with the commercially available sanitizer using the zone of inhibition method as described above. Two wells were filled with 50 µl and 100 µl of formulated sanitizer and other two wells were filled with 50 µl and 100µl of commercially available sanitizer. After incubation for 24 h at 37 ºC, the zone of inhibition was measured.

Surface swab test

Two agar containing media plates were prepared, one containing the sanitizer and other without the sanitizer. The sterile swabs were wiped across the pre-marked surface (reception, wall corner and lab door) using an even pressure and holding the swabs flat against the surface. The swabs were then streaked onto the agar containing petri plates and incubated for 48 h. The plates were observed for the growth of microorganisms.

RESULTS

Identification of saponins

The precipitate obtained after extraction was identified as saponins by foam test and lead acetate test.

Foam test: As seen in Figure 1, the extract from each root shows the presence of stable persistent foam indicating the presence of saponins.

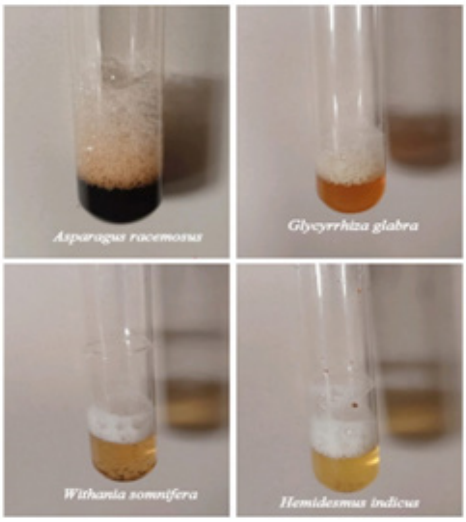

Figure 1 Foam formation as an identification test for saponins.

Lead acetate test: As indicated in Figure 2, the treatment of lead acetate with all the extracts resulted in the formation of lead-saponin precipitate which in turn proves the presence of saponins.

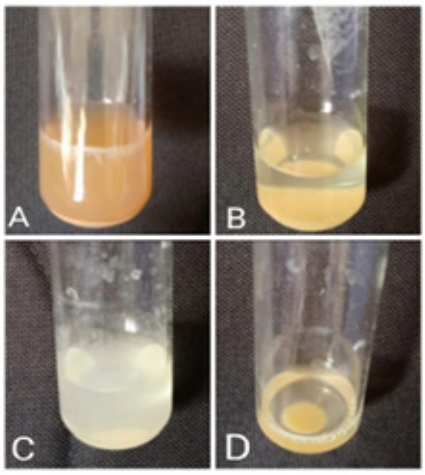

Figure 2 Lead-acetate test-White precipitate formed indicates saponin presence. A-*Asparagus racemosus,* B- *Glycyrrhiza glabra,* C- *Withania somnifera,* D- *Hemidesmus indicus*

Quantification of saponins

Table 2 shows the amount of saponin obtained from each plant. From Table 2 we can conclude that maximum amount of saponin was obtained from *Asparagus racemosus.*

Table 2 The saponin content from each plant expressed as percentage.

Plant	Total Saponin Content (%)
Glycyrrhiza glabra	06
Asparagus racemosus	10
Withania somnifera	07
Hemidesmus indicus	05

Reducing power assay (antioxidant assay)

Antioxidant assay of individual saponins are shown in Figure 3. All the saponins were found to exhibit good antioxidant activity. There was not much difference between the antioxidant activity of the Standard Ascorbic acid and *Glycyrrhiza glabra*. The highest antioxidant activity was shown by *Glycyrrhiza glabra* saponin extract (2831.573 mg Fe/g) followed by the saponin extract of *Hemidesmus indicus* (2338.921 mg Fe/g), *Asparagus racemosus (*1976.90 mg Fe/g*)* and *Withania somnifera (*1884.04 mg Fe/g*)*. The antioxidant activities exhibited by the other extracts were significantly lower than the standard ascorbic acid.

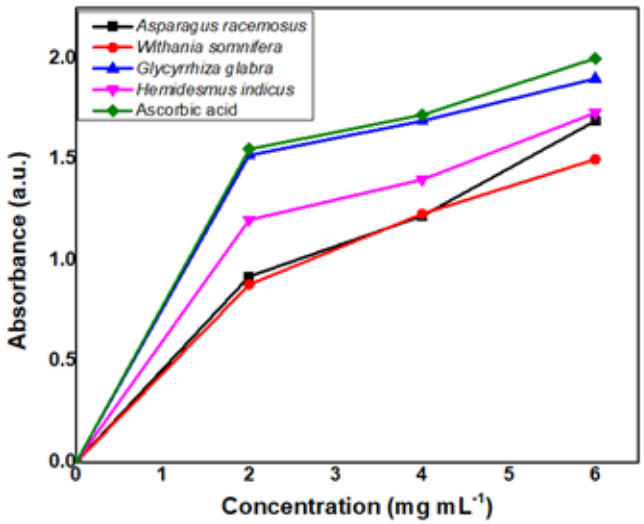

Figure 3 Antioxidant assay of individual saponins.

In vitro antimicrobial activity by agar plate diffusion method

Table 3 shows the antimicrobial activity of individual saponins and their synergistic effect against various microorganisms. The antimicrobial activity examined by agar plate diffusion method showed that the saponins extracted were active against both bacterial and fungal species. *Glycyrrhiza glabra, Asparagus racemosus* as well as *Withania somnifera* showed highest antimicrobial activity against *E. coli*. *Hemidesmus indicus* showed highest antimicrobial activity against *E. faecalis*. All the tested microorganisms were found to be sensitive against the saponin extracts.

Synergistic effect: The zone of inhibition formed by the cumulative effect of all the saponins (polyherbal) was larger than that formed by the individual saponins, which suggests that the activity of saponins increases when combined. The synergistic effect was maximum against *S.aureus.*

The results for MIC are shown in Table 4. The MIC of the extracts against the selected microorganisms ranged from 2.95 µg/ml to 3.35 µg/ml. The synergistic effect of the saponins showed the lowest MIC value with maximum activity against *S.aureus.*

Table 3 Antimicrobial activity of individual saponins and their synergistic effect against various microorganisms by agar plate diffusion method.

Plants/ Organisms	*G. Glabra*				*A. racemosus*				*W. somnifera*				*H. indicus*				Polyherbal				Positive Control
Concentration µg/ml	25	50	75	100	25	50	75	100	25	50	75	100	25	50	75	100	25	50	75	100	25
	Zone of Inhibition (mm)																				
S. aureus	11 ±2	14 ±1	17 ±1	20 ±2	14 ±2	17 ±1.5	19 ±2	22 ±1	11 ±2.2	15 ±2	189 ±1	20 ±1	13 ±2	14 ±2.1	16 ±1	19 ±1.6	15 ±2	19 ±1	21 ±0.9	23 ±2	26 ±2.5
E. faecalis	12 ±2	13 ±1.5	15 ±2	17 ±1.6	13 ±1.8	15 ±1	17 ±2	21 ±1.5	10 ±1	14 ±2	16 ±2	19 ±1	12 ±1.6	14 ±2.1	17 ±1.5	20 ±1.3	13 ±0.9	16 ±2	18 ±1.4	21 ±1.5	25 ±2
S. typhi	11 ±1.4	13 ±1.5	16 ±1.3	18 ±2	12 ±2.2	14 ±2.1	18 ±1.6	20 ±2.3	11 ±1.2	12 ±1.9	16 ±2	20 ±2	11 ±1.6	12 ±2.1	14 ±2.2	17 ±1.9	14 ±1.6	16 ±2.2	19 ±1	20 ±2	25 ±2
E. coli	14 ±1.5	16 ±1.3	18 ±1.6	20 ±2.2	15 ±1.2	18 ±2	20 ±2	23 ±1.9	13 ±2.3	15 ±1.7	18 ±2.1	21 ±2	10 ±2.1	13 ±1.6	16 ±1.5	18 ±2.3	16 ±2.0	18 ±2	20 ±1.6	22 ±1.6	27 ±3
C. albicans	10 ±1.5	12 ±1.9	15 ±2	17 ±1.5	11 ±2.1	13 ±2	16 ±2	19 ±1.5	12 ±2.3	14 ±2	15 ±1.6	18 ±2	10 v1	11 ±1	13 ±1.2	15 ±1.5	11 ±2	13 ±1	15 ±2	17 ±2	24 ±2

* Data are means of three replicates (n = 3) ± standard error.

Table 4 MIC (µg/ml) performance of different saponin extracts

Plants/ Organisms	*G. Glabra*	*A. racemosus*	*W. somnifera*	*H. indicus*	Polyherbal
S. aureus	3.15	3.10	3.15	3.20	2.95
E. faecalis	3.2	3.15	3.15	3.12	3.10
S. typhi	3.2	3.15	3.15	3.3	3.10
E. coli	3.12	3.05	3.12	3.2	3.00
C. albicans	3.30	3.25	3.25	3.35	3.20

Evaluation parameters

Organoleptic properties

The formulated hand sanitizer was organoleptically tested to assess the physical appearance of the prepared formulation, and it demonstrated acceptable organoleptic qualities. The colour of the sanitizer was found to be brown. It had a characteristic odour and was translucent.

Irritancy test and pH measurement

No individuals exposed to the formulation showed any side effects like redness, itching, dryness or any other allergic reactions. The optimal pH value for topical dose should be between 4.0 and 7.0 in order to avoid irritation or skin inflammation. The pH of the polyherbal sanitizer formulation was determined to be 5.15. It is slightly acidic due to Aloe vera which in turn hinders the growth of harmful microorganisms (Booq et al., 2021).

Comparison of formulated polyherbal sanitizer with commercially available sanitizer

Figure 4 shows the comparison between the formulated polyherbal sanitizer and commercially available sanitizer against microorganisms. The results tabulated in Table 5 showed that the formulated sanitizer was effective against *Enterococcus faecalis, Staphylococcus aureus, Salmonella typhi, Escherichia coli* and *Candida albicans*. Thus, it possesses both antibacterial and antifungal activity. It showed maximum activity against *Staphylococcus aureus*. The efficacy of the formulated polyherbal sanitizer was comparable to that of a commercially available hand sanitizer in terms of antimicrobial potency.

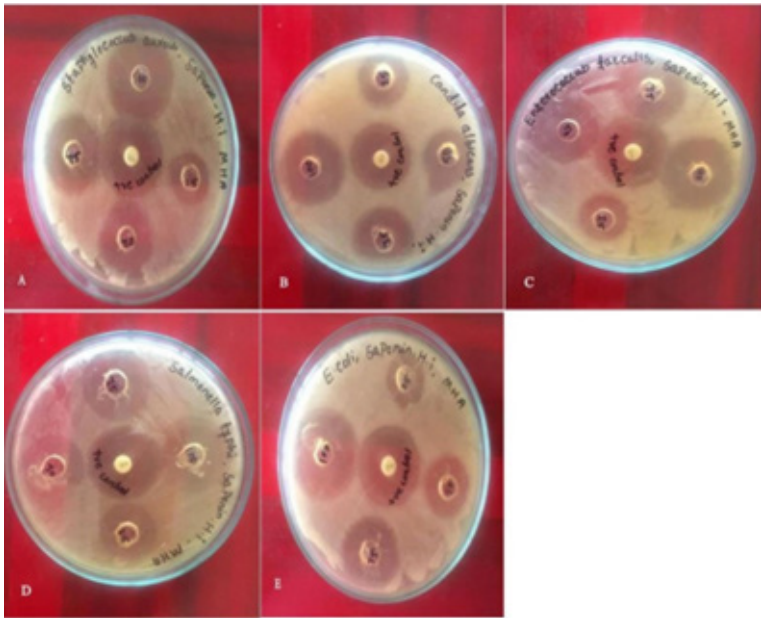

Figure 4 Zone of inhibition formed by polyherbal sanitizer against microorganisms: A- *Staphylococcus aureus*, B- *Candida albicans*, C– *Enterococcus faecalis*, D- *Salmonella typhi* and E- *Escherichia coli*.

Table 5 Comparison between the formulated polyherbal sanitizer and commercially available sanitizer

MICROORGANISMS	ZONE OF INHIBITION (mm)				
	Polyherbal		Commercial		Positive Control
	100µl	50µl	100µl	50µl	
Staphylococcus aureus	24±1	19±1.6	24±1.1	18±1.2	27±1.3
Enterococcus faecalis	22±1.4	17±1.4	23±1.1	17±1.5	26±2.0
Escherichia coli	23±1.2	18±1.4	23±1.6	19±1.1	28±1.7
Salmonella typhi	21±1.3	16±1.4	22±1.1	18±1.0	25±1.1
Candida albicans	20±1.7	16±1.4`	19±2.0	15±1.8	24±1.3

Surface swab test

Table 6 shows the swab test results before and after the use of sanitization. No growth was observed in the plates containing the formulated sanitizer. This shows the efficacy and antimicrobial potential of the formulated sanitizer.

Table 6 Swab test before and after the use of sanitizer

Sl No	Specimen	Before sanitization	After sanitization
1	Specimen Type - Reception Culture	Aerobic Spore forming grown in culture	No growth after 48 hours of incubation
2	Specimen Type - Wall Corner Culture	No growth after 48 hours of incubation	No growth after 48 hours of incubation
3	Specimen Type - Lab Door	Aerobic Spore forming grown in culture	No growth after 48 hours of incubation

DISCUSSION

Studies have been carried out to suppress the adverse effects of alcohol in sanitizers such as using herbal moisturizers for example coconut oil (Kuraeiad et al., 2022), but still there is a need to formulate a sanitizer completely devoid of alcohol to eliminate the detrimental effects of alcohol based sanitizers. In this study, the sanitizer is formulated using saponins as the major component. The presence of saponins was confirmed by foam test where the foam formed is due to the reduced surface tension of water owing to the amphiphilic nature of saponins when placed in aqueous solution (Kregiel et al., 2017). It was further confirmed by lead acetate test where the saponins present were precipitated as lead-saponin complex which was further decomposed by treatment with H_2S as a purification method suggested by Jurzysta (1973). Chen et al. (2014) has already reported that the reducing ability of saponins increases with the increase in concentration of saponins. The *Glycyrrhiza glabra* plant extracts showed the potency in antioxidant activity (Bhusal and

Sharma, 2020). *Asparagus racemosus* root methanol extracts can help reduce oxidative damage caused by free radicals, demonstrating its antioxidant activity (Tripathi et al., 2015). It is evident from Paul et al. (2016) study, that the root extract of *Withania somnifera* possesses good antioxidant activity. In vitro study by Nagat et al. (2016) indicated that *Hemidesmus indicus* extract is an effective source of natural antioxidant, which plays a role in preventing the various oxidative stresses. In addition, the saponins showed good antimicrobial activity with enhanced effect when used in combination. Secondary metabolites such as alkaloids, saponins and flavonoids contribute to the antibacterial activity of *Glycyrrhiza glabra* (Pastorino et al., 2018). The results from Shevale et al., (2015) study indicates that root extracts from *Asparagus racemosus* exhibited antimicrobial properties. The results of Pathak and Srivastav (2020) indicate that the anti-microbial activity of the methanolic extract of *Withania somnifera* was comparable with standard antibiotic. This shows that *Withania somnifera* has an anti-bacterial activity and this may be due to the extracted phytochemicals in methanolic extract. Antibacterial activity of saponins and tannins isolated from *Hemidesmus indicus* are well documented (Gayathri and Kannabiran, 2009). Thus, the results of this research are in accordance with the previous studies.

The formulated sanitizer proved to be effective against all tested strains and showed a comparable efficacy with the commercially available ones along with acceptable physical parameters. Further to test its applicability at commonly encountered areas in laboratories swab were collected from areas like reception, wall corners and lab door. These swabs when streaked upon sanitizer containing media plates showed no growth of microorganisms. These results suggest that the formulation might possess broad spectrum of antimicrobial activity.

CONCLUSIONS

Pandemic diseases like COVID-19 have highlighted the importance of sanitization, public health and hygiene, which has caused a surge in the use of sanitizers and disinfectants. Likewise, WHO has advised to use sanitizer containing 60% alcohol. Studies show that frequent and overuse of alcohol-based hand sanitizer is detrimental and has toxic impact on environment. Use of an alcohol-free herbal sanitizer serves as an alternative to alcohol-based sanitizer. Saponins are secondary metabolites which have an important role in plant defense mechanism. The amphiphilic nature contributes to the antimicrobial activity and prevents the virus from attaching to the host. The alcohol-free sanitizer was prepared using saponins as main active antimicrobial ingredient and using Aloe vera as an antimicrobial emollient. The formulated sanitizer showed significant antimicrobial activity and was comparable with the commercially available one in terms of antimicrobial potency. It can be concluded from the results that the prepared formulation has acceptable organoleptic and pH value, which is compatible with skin. As it is purely alcohol free, it is not flammable and safe to be used by children. Thus, the formulated saponin based polyherbal alcohol free hand sanitizer is effective, environment friendly, biodegradable with no toxic impact on humans. Furthermore, the antiviral effectiveness has to be carried out along with the evaluation of the shelf life.

AUTHOR CONTRIBUTIONS

Tanisha Rathore involved conception, technique, methodology visualization, inquiry, software, data curation, and first draft writing. **Vidya M** involved on article writing, formal analysis, resources, project management, data curation, and inquiry**. Sanjana C Shekar** participated in text editing suggestions, project monitoring, and project management. **Rubalakshmi Govindraj** involved in manuscript review and editing.

CONFLICT OF INTEREST

The authors state that they do not have any conflicts of interest.

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How to cite this article;

Tanisha Rathore, Vidya M, Sanjana C Shekar and Rubalakshmi Govindraj. Formulation and evaluation of saponin based alcohol - free polyherbal hand sanitizer. Veterinary Integrative Sciences. 2023; 21(3): 959 - 971