

In vitro Antibacterial activity of the Aqueous and Ethanol extracts of *Allium sativum* L. (Garlic) and *Sapindus laurifolia* Vahl (Soap nut) against clinical isolates of *Gardnerella vaginalis*

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ABSTRACT

Introduction: *Gardnerella vaginalis* is a significant pathogen in bacterial vaginosis and other vaginal tract infections in women. Antibiotics have always been a challenge for treatment of *G.vaginalis* infection and there is a requirement for newer drugs. The aim of this study was to investigate the antibacterial potential of *Allium sativum* L. (garlic) and *Sapindus laurifolia* Vahl (soap nut) against clinical isolates of *G. vaginalis*.

Material and Methods: The aqueous extracts of garlic bulb and soap nut were prepared by maceration and ethanol extraction by Soxhlet extractor method. The antibacterial activity of these extracts were analysed against 72 isolates of *G. vaginalis* by agar punch well and disc diffusion methods. Among these 40 isolates were tested for minimum inhibitory concentration (MIC) of aqueous and ethanol extracts of soap nut by agar dilution method.

Results: Of the 72 *G. vaginalis* isolates, 54.1% showed zones of inhibition by garlic and 79.1% by soap nut aqueous extracts, whereas 59.7% were inhibited by garlic and 81.9% by soap nut ethanol extracts by punch well technique. By the disc diffusion method, 38.8% were inhibited by garlic and 81.9% showed sensitivity to soap nut aqueous extracts, whereas 44.4% were inhibited by garlic and 83.3% by soap nut ethanol extracts. The MIC ranges of soap nut aqueous and ethanolic extract was 78 to 2500 µg/ml.

Conclusion: The aqueous and ethanol extracts of soap nut exhibited good antibacterial activity by both methods compared to garlic extracts. Result of the present study suggests purified phytochemicals obtained from soap nut could be considered as new treatment option towards *G.vaginalis*.

Keywords: *Gardnerella vaginalis*, Agar dilution method, *Allium sativum* L Aqueous and Ethanol extracts, *Sapindus laurifolia* Vahl, Minimum inhibitory concentration.

INTRODUCTION

Gardnerella vaginalis (*G.vaginalis*) a gram variable microaerophilic coccobacillus is the commonest pathogen present in the female vaginal microbiota. Bacterial vaginosis (BV) is a polymicrobial infection which is associated with a significant increase in the number of microorganisms, especially obligate anaerobes such as, *Bacteroides fragilis* group, *Prevotella*, *Porphyromonas* and *Mobiluncus species*.^{1,2} Regarding treatment option metronidazole and clindamycin are recognized as effective drugs in case of BV. At present standard drugs like metronidazole and clindamycin are unable to fully inhibit the growth of *G.vaginalis*, hence it requires novel treatment strategies.³ In this context, few herbal antimicrobial agents are reported to inhibit the growth of *G.vaginalis* like, garlic (*Allium sativum* L) and soap nut (*Sapindus laurifolia* Vahl) which are routinely used as natural remedies.^{4,5} The importance of garlic and soap nut extracts had been

documented in literature. It was observed that a variety of phytochemical molecules presents in these antimicrobial agents like, alkaloids, flavonoids, glycosides, saponins, flavonoids, protein, tannins, oils and fats in aqueous and ethanolic extracts of garlic and soap nut, which could be the needed for their fungicidal, virucidal and bactericidal inhibition.^{6,7}

It has been noted that herbal treatment options for *G.vaginalis* is lacking in studies, our finding demonstrates the *in vitro* antibacterial action of garlic and soap nut by agar punch well (ditch) and disc diffusion methods. The minimum inhibitory concentration (MIC) of aqueous and ethanol soap nut extracts by agar dilution method which could be helpful in future against *G.vaginalis* as a treatment option for female genital tract infections.

MATERIAL AND METHODS

A. Collection, isolation and identification of *G.vaginalis* isolates.

The study was conducted in a tertiary care hospital in Coastal Karnataka South India. Females between the ages of 15-45 years with complaints of vaginal discharge were included in the study. Women who were menstruating at the time of the specimen collection and women, who were on medication for any bacterial, fungal, parasitic or viral infections for up to one month prior to the specimen collection, were excluded. The study has been approved by the Institutional Ethics Committee (Ref. No FMCC/ FMIEC/ 1298/ 2013) and written informed consent was collected from the patients or attenders, clinical history of each woman. Vaginal discharge was collected into sterile tube containing 0.5 ml normal saline and immediately transported to the department of microbiology for further processing. *G.vaginalis* isolates were identified according to standard methods.^{8,9}

B. Preparation of garlic and soap nut aqueous and ethanol extracts

Garlic bulbs and soap nut fruits purchased from reputed retail store in Mangalore and were submitted for authentication to National Ayurvedic Dietetics Research Institute (Central Council for research in Ayurveda and Siddha, Department of

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AYUSH, Ministry of Health and Family Welfare, Government of India), Bangalore. Table 1 shows authentication numbers of herbs. Extracts were prepared at the Shree Devi College of Pharmaceutical Sciences Mangalore. Garlic bulbs and soap nuts were dried and reduced to coarse powder.

Preparation of aqueous extracts of garlic and soap nut by Maceration Method

100 g of the coarse powder of garlic bulbs and soap nut were extracted separately and set to 100 ml distilled water by simple maceration method. In the process of extraction, coarse drugs were kept in contact with the solvents for few hours to several days with several shaking /stirring at room temperature. Finally the extracts were coarse filtered after the concentration and later sterilized with the help of membrane filters of 0.2µl pore size. The extracts were stored at 10°C for further use.¹⁰

Preparation of ethanol extracts of garlic and soap nut by Soxhlet Extractor Method

100g of each coarse powder was extracted with 150 ml of ethanol and materials to be extracted were filled in a thimble made of cellulose or cloth and placed in the extractor part of the soxhlet extraction unit. The solvent (ethanol) was placed

in the lower part of the apparatus (round bottomed flask of different capacity) and connected to the extractor part. The other end of the extractor was connected to the condenser. The solvent in the lower container was heated slowly to boil. The vapours of the solvent were passed through the side arm into the reflux condenser, liquefied and then dropped into the thimble containing material to be extracted. The warm solution was percolated through the wall of the thimble and extracted gradually and collected in the extractor part. Once the extract height reached the siphon, the entire liquid flowed back into the lower part of the soxhlet extraction unit containing the solvent. The process was repeated several times and filtered after the concentration and later sterilized with the help of membrane filters of 0.2µl pore size. The extracts were stored at 10°C for further use.¹⁰

C. Screening of antimicrobial action of garlic and soap nut aqueous and ethanol extracts

Preparation of dilutions of garlic and soap nut aqueous and ethanol extracts

In the present study one gram of garlic and soap nut each extract was dissolved in 10 ml DMSO (Dimethyl sulphoxide)

Common name	Scientific name	Family	Part of the plant used	Authentication number
Garlic	<i>Allium sativum L.</i>	Liliaceae	Bulbs	RRCBI-MUS-149
Soap nut	<i>Sapindus laurifolia Vahl</i>	Sapindaceae	Fruits	RRCBI-14082
Herbs used in extraction of Antimicrobial Action				

Table-1: Authentication details of Natural Herbs

No	Extracts	Punch well (Ditch) method						Disc diffusion method					
		Grading				Number of Sensitive isolates	Number of Resistant isolates	Grading				No of Sensitive isolates	Number of Resistant isolates
		1+	2+	3+	4+			1+	2+	3+	4+		
1.	Garlic aqueous extract	15	10	10	4	39 (54.1%)	33 (45.8%)	8	9	6	5	28 (38.8%)	44 (61.1%)
2.	Garlic ethanol extract	14	11	11	7	43 (59.7%)	29 (40.2%)	5	11	10	6	32 (44.4%)	40 (55.5%)
3.	Soap nut aqueous extract	16	13	16	12	57 (79.1%)	15 (20.8%)	12	21	15	11	59 (81.9%)	13 (18.0%)
4.	Soap nut ethanol extract	18	15	15	11	59 (81.9%)	13 (18.0%)	14	16	17	13	60 (83.3%)	12 (16.6%)

Tests variables were analysed by chi square test, all of them showed highly significant difference $p \leq 0.001$.

Key: 9mm-12mm → 1+, 13mm-16mm → 2+, 17mm-20mm3+, >20mm → 4+.

In Vitro Antimicrobial Action of Garlic and Soap nut aqueous and ethanol extracts

Table-2: Results of susceptibility of 72 Isolates of *G.vaginalis* by Punch well (Ditch) method and Disc diffusion technique

No.	Nature of the extracts with isolates number	Various concentration of soap nut, aqueous and ethanol extracts in 1:10 dilution (µg/ml)								
		10000 µg/ml	5000 µg/ml	2500 µg/ml	1250 µg/ml	625 µg/ml	312 µg/ml	156 µg/ml	78 µg/ml	39 µg/ml
1.	No. and % of isolates in MIC of soap nut aqueous extract	-	-	(5) 12.5%	(12) 30.0%	-	(4) 10.0%	(5) 12.5%	(14) 35.0%	-
3.	No. and % of isolates in MIC of soap nut ethanol extract	-	-	(5) 12.5%	(15) 37.5%	-	(6) 15.0%	(9) 22.5%	(10) 25.0%	-
4.	MICs ranges of soap nut aqueous extract	78 - 2500 µg/ml								
5.	MICs ranges of soap nut ethanol extract	78 - 2500 µg/ml								

Details of isolates inhibited at various concentrations of soap nut extracts, (-) = Absent of isolates.

Table-3: Results of Minimum inhibitory concentration (MIC) in Soap nut aqueous and alcohol extracts on 40 numbers of *G. vaginalis* isolates.

to solubilise the agent and brought to volume with water. A total 72 isolates of *G.vaginalis* were employed in the study. The antimicrobial efficacy of the extracts were tested by agar punch (Ditch) well and disc diffusion methods.

Preparation of inoculums

A standard bacterial suspension (1.5×10^8 colony forming unit/ml) was prepared from 48 hours culture of *G.vaginalis* in citrate phosphate buffered saline of pH 5.5 and the bacterial suspension turbidity was matched with 0.5 Mc Farland.

Agar punch (Ditch) well method

Bacterial suspension adjusted to opacity Mc Farland 0.5 was swabbed onto Brucella blood agar plates which were already punched using sterile metallic template of 6mm diameter. The 50µl of each extract was put into the separate ditch well and DMSO served as negative control. Extracts were transferred to the wells with sterile micropipette tips. The plates were incubated in the candle jar (5-10% CO₂) at 37°C for 48 hours. After incubation period, the zone of inhibition was measured in millimetres.¹¹

Disc diffusion method

One millilitre each of various extracts were added to separate sets of 100 sterile discs of 6 mm diameter (whatmann No.1 filter paper). Standard bacterial suspension was swabbed onto Brucella blood agar plates and extracts impregnated discs were placed onto inoculated plates. DMSO incorporated disc was served as negative control. The plates were incubated in the candle jar (5-10% CO₂) at 37°C for 48 hours. The antimicrobial effects of extracts were measured in millimetres.¹¹

The agar punch well and disc diffusion methods were performed in triplicate and *G.vaginalis*, ATCC 14018 was tested as parallel with each isolate. The results were calculated as diameter of zone of inhibition like, 9mm-12mm → 1+, 13mm-16mm → 2+, 17mm-20mm → 3+, >20mm → 4+.¹²

Detection of MIC of the soap nut aqueous and ethanol extracts by agar dilution method.

A total 40 isolates of *G.vaginalis* were employed to detect MIC of soap nut aqueous and ethanol extracts by agar dilution.^{13,14}

a. Preparation of dilutions of soap nut aqueous and ethanol extracts and media

The each concentrate extract was dissolved in DMSO and made up to the required concentration using sterile distilled water and incorporated into the agar plate to obtain concentration such as, 10000 µg/ml, 5000 µg/ml, 2500 µg/ml, 1250 µg/ml, 625 µg/ml, 312 µg/ml, 156 µg/ml, 78µg/ml and 39 µg/ml. Two millilitre of each diluted extract, 1 ml of sterile lysed sheep blood were added to 17 ml Brucella blood agar base supplemented with vitamin K1 and hemin, after autoclaving at 121 °C for 15 min, poured into Petri plates and was refrigerated at 4°C (Each plate contained 17 ml of agar + 1 ml of laked sheep blood + 2 ml of respective dilution of extract).

b. Agar dilution method

Enriched plates were warmed to room temperature before the test run. Spot inoculations were done using inoculum of *G.vaginalis* isolates (diluted to 0.5 Mc Farland density) on to the marked area in the supplemented Brucella blood agar plate those had varying concentrations of the extracts. Two inoculated Brucella blood agar plates served as control without extracts.

After inoculation plates were kept at room temperature for 10 minutes so that the inoculum was absorbed. All the test plates were incubated in the candle jar (5-10% CO₂) and another plate incubated aerobically (aerobic contaminant control) at 37° C for 48 hours. MIC was determined and results were recorded as the lowest concentration of drug yielded no growth, a haze, one discrete colony or multiple tiny colonies. MIC of these extracts was interpreted with *G.vaginalis*, ATCC 14018.

STATISTICAL ANALYSIS

SPSS version 16 was used. Chi square test was used to compare between different variables. P<0.05 was considered as significant.

RESULTS

A total 72 *G.vaginalis* clinical isolates were investigated for their antimicrobial activity against garlic and soap nut aqueous and ethanol extracts by punch well technique and disc diffusion method. A comparison of tests revealed most of the *G.vaginalis* isolates were sensitive to soap nut rather than garlic extract and ethanolic extracts were more effective than aqueous by both the methods. The antimicrobial assay results of isolates against the extracts by both methods were shown in Table 2. Garlic extracts showed better results with the punch well than with disc diffusion method. Soap nut extracts showed slightly better results by disc diffusion rather than punch well technique. When the variables were analysed by chi square test, all of them showed highly significant difference $p \leq 0.001$. Results of sensitivity pattern were graded on the diameter of Zone of inhibition 1+ to 4+ presented in Table 2. Table 3 demonstrated, MIC results of 40 isolates of *G.vaginalis* against soap nut aqueous and ethanol extract by agar dilution method.

DISCUSSION

G.vaginalis, the principle pathogen in BV is unable to eradicate completely by standard antibiotics like metronidazole which is the selective drug for anaerobes associated BV. According to previous studies many antibiotics such as Metronidazole, Erythromycin, Clindamycin, Trimethoprim, Vancomycin, Ampicillin, Amikacin, Imipenem, Ciprofloxacin, Nitrofurantoin and Sulfonamide were employed as treatment options for *G.vaginalis* associated infections.^{8,15} One recent study showed that 76% *G.vaginalis* was sensitive to clindamycin.¹⁶ However antibiotic treatment still remains a challenge against *G.vaginalis* infections.

Garlic and soap nut are the medicinal plants which have large contribution to human health and wellbeing. There are many reports available regarding the antimicrobial effects of garlic and soap nut against various organisms such as aerobic bacteria and fungi. Ethanolic extract of garlic inhibited species of *Aspergillus* and *Penicillium*.¹⁷ Aqueous and methanol extracts of garlic was found to be active against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* by agar well diffusion method.⁶ In another study, Garlic methanol extract inhibited *Salmonella paratyphi*, *Bacillus subtilis*, *Klebsiella pneumoniae* and species of *Candida* by agar diffusion method.¹⁸ Ameh and colleagues demonstrated antimicrobial action of methanol, acetone and 1,4 Dioxan *Sapindus saponaria* leaf extracts against *Staphylococcus aureus*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Micrococcus albus*.¹⁹

The extracts of the Seaweed *Ulva pertusa* was found to be natural agents for *G.vaginalis* activity by using the agar disk-diffusion method by Ha YM and colleagues.²⁰

However no reports are available on the antimicrobial action of these extracts on microaerophilic bacteria. The present study demonstrates the antimicrobial action of aqueous and ethanol extracts of garlic and soap nut on *G.vaginalis* by punch (Ditch) well technique and disc diffusion method. Turvovskiy and chikindas reported strong antibacterial potential of soap nut extract and zinc lactate with lactocin 60 against *G.vaginalis*.⁵ Few researches highlighted various activities of soap nut such as surfactant, anti inflammatory, contraceptive, mild detergent hepatoprotective, antipruritic, antihyperlipidemic, antimicrobial.^{5,7,19} In present study antibacterial effect of aqueous extract of soap nut was found to be 79.1% by punch well method and 81.9% by disc diffusion method. Ethanol extracts of soap nut exhibited 81.9% activity by punch well and 83.3% by disc diffusion technique. MICs of both extracts were done by agar dilution method and the results ranged from 78 to 2500 µg/ml. The results of both the techniques were comparable.

The screening of antibacterial efficacy of the soap nut extracts in our study has provided justification for developing a potential of soap nut to control vaginal tract infections caused by *G.vaginalis*.

Mohammadzadeh and colleagues conducted a randomized controlled clinical trial study on therapeutic effects of garlic tablet and metronidazole on BV with similar results.²¹ A recent study demonstrated the mycocin vaginal cream, made from garlic and thyme was found to be an appropriate medicine in BV cases.²² In the present study aqueous garlic extract inhibited 54.1% of *G.vaginalis* by punch well technique and 38.8% by disc diffusion method, whereas, ethanolic garlic extract inhibited 59.7% by punch well technique and 44.4% by disc diffusion method. Results of this study indicate that the antibacterial efficacy of aqueous and ethanol garlic extracts and better results were obtained by punch well technique compared to the disc diffusion method. Earlier studies investigated that garlic has been used to cure various infections like gastrointestinal infections, urinary tract infections, guinea worm sores, open wounds, toothaches, cold, cough and viral infections.^{6,17,18} In the current study the antibacterial efficacy of soap nut extracts which have remarkable potency compare to garlic extracts may be helpful as a therapeutic potential.

CONCLUSION

The present study concludes that the aqueous and ethanol extracts of soap nut have therapeutic effects against *G.vaginalis* as an alternative medicine especially in developing countries which will be reduce the cost of treatment.

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