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Research article

Vaginitis phyto therapy against vaginal pathogen and molecular identification of isolated vaginal pathogen

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ABSTRACT

Female genital tract infections are major public health problems, with considerable economic consequences. Drugs like metronidazole and clindamycin are unable to control the growth of vaginal infection completely, which requires alternate novel treatment strategies. Some herbal antimicrobial agents are reported to inhibit the growth of vaginal infection. *Boerhaavia diffusa* and*Azadirachta indica* are such herbs with antimicrobial, antioxidant and anti-inflammatory properties. In the present study, vaginal fluid samples from infected patients were collected and cultured using different media. Isolated pathogens were identified using 16S rRNA sequence. Four bacterial pathogens were isolated and identified. The pathogens identified were *Aeromonas cavia, Lactobacillus, Staphylococcus aureus* and *Klebsiella pneumomiae. A*nti-vaginalis activity of the two herbs were analysed in an isolated pathogen. Maximum zone of inhibition was observed against *S. aureus* and *Aeromonas caviae*. The present investigation confirmed that, *Boerhaavia diffusa* and*Azadirachta indica* herbal extracts were able to control the vaginal pathogens without any side-effects

Keywords: Biochemical analysis, Bacterial pathogens, Phytochemicals, 16S rRNA sequence, Vaginitis.

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INTRODUCTION

Vaginal ecosystem contributes various synergistic communications between the host and different microflora colonizing the vaginal mucosa [1]. When the vaginal mucosa colonized more by *Lactobacillus* spp., then it seems to be a healthy vaginal environment, which acts as a defence against vaginal pathogens [2]. These bacteria produce antimicrobial compounds and acts by competition for adherence to the vaginal epithelium. The biosurfactants produced by *Lactobacilli* microbiota will provide a restoration that further displace the pathogenic species [3]. When this beneficial Lactobacillus is depleting due to any conditions, it could increase infection and modulate immune responses, favour the growth of pathogens and would ease the development of several diseases [4]. Vaginal inflammation, or vaginitis, is the most frequent gynaecological problem and may be caused by various vaginal microflora, allergies or due to chemicals exposure [5].

Bacterial Vaginosis (BV), the most common reason of vaginitis and Vulvovaginal candidosis (VVC) is the second most common reason of vaginitis. Anaerobic micro-organisms such as Gardnerellavaginalis, Prevotella, Peptostreptococcus and Bacteroid es spp. are responsible for bacterial Vaginosis with symptoms of malodorous vaginal discharge or local irritation [6] whereas symptoms of vulvovaginal candidiasis includes pruritus (itching), soreness, irritation, vulvar burning change in vaginal discharge, dyspareunia and cottage cheese-like vaginal discharge [7]. Tricomonas vaginitis is a sexually transmitted disease caused by Trichomonas vaginalis with symptoms of vaginal discharge green to brown colour, foul door, edema or erythema and colpitis macular is. Vaginitis has its own serious sequelae [8].

Antibiotics usage for genital infections are less effective with side effects and recurrences. In addition, antibiotics will disturb healthy non-pathogenic microbiota present in vagina [9]. Such condition is the cause for recurrent infection as elimination of the commensal microorganisms may increase the susceptibility to recolonize the resistant pathogens [10]. Alternatively, medicinal plants with unlimited bioactive compounds can be used as antimicrobial agents expecting that plant extracts recognizing target sites are other than those used by antibiotics and will be active against drug-resistant microbial pathogens [11].

Boerhaavia diffusa and*Azadirachta indica* are using as herbal medicine due to its antimicrobial, antioxidants and anti-inflammatory properties. In the present study, the antimicrobial activity of these two herbs were analysed against vaginal pathogens. The objectives of the study are to collect vaginal fluid samples from infected patients, identification of vaginal pathogens using 16S rRNA sequence and to analyse the antimicrobial property of the two herbal extract against isolated vaginal pathogens.

MATERIALS AND METHODS

Clinical sample collection

From 15 infected patients, vaginal swabs were collected from primary health centre, Puthur Uthamanur, Lalgudi, Tiruchirappalli, Tamil Nādu. The samples were properly labelled and transported to the Private laboratory BBRC located in No.1 Tollgate which took around 15 minutes of distance about 12kms. In the laboratory, samples were processed within 1-2 hour from the time of the collection.

Isolation of Microorganisms

Samples were inoculated on selective and differential media such as MacConkey agar, Nutrient agar, Chocolate agar, Cetrimate agar and incubated under aerobic or micro aerophilic environment at 37° C for 24 hours. Microscopic observations like size, shape and motility for different morphological characters among microorganisms was revealed using simple staining, gram staining and hanging drop methods [12]. Biochemical tests such as methyl red test, Voges Proskauer test, citrate utilization test and catalase test were done to identify the isolated microorganisms [13].

Sequencing of 16S rRNA gene

Genomic DNA was extracted from obtained pure colonies of each bacterial culture. The concentration of the extracted DNA was determined using UV-Vis spectrophotometer. For identification of specific bacteria, 16S rRNA PCR amplification was done and the finalized sequence of amplified 16S rRNA fragment from each bacterial culture was blasted against the collection of nonredundant nucleotide sequence database of NCBI. The species were identified based on hits analysis from mega blast (highly similar sequences) output [14].

Collection of herbs

Plants such as *Boerhaavia diffusa* and*Azadirachta indica*were collected from the local areas of Tiruchirappalli, Tamil Nādu. Fresh leaves chosen from each plant were washed with sterile distilled water thrice, and shade dried at room temperature for two weeks and made into a coarse powder.

Preparation of herbal aqueous extract and ethanolic extract

In order to prepare the aqueous extract, 30g of the powder was taken and added to 100 cc of distilled water at a temperature of 70-80°C. This extract was prepared using vacuum distillation method as described.[15]Using percolation method, ethanolic extract was prepared by adding 50g of herbal powder to decanter and by adding ethanol to the decanter and repeat this step till herbs were completely immersed. After an hour, the solvent was exited and the whole process was repeated thrice. The solvent was then separated by the method of vacuum distillation [16].

Microbial load assessment for water and alcohol extract

About 1g of water extract with 10ml of water was taken as a master dilution. To 1ml of the master dilution, 9ml of distilled water was added to mark it as 10^{-1}. The same procedure of dilution was done until 10^{-5}. Then pour plate technique was followed for each dilution. Same procedure was followed for alcohol extract.

Antimicrobial assay

Sterile Petri plates of nutrient agar medium were seeded with 0.01ml of 18 hours old test bacterial culture. Various concentration of herbal extract was impregnated into the sterile 6mm diameter discs, dispensed on the solidified medium with incubation at 37℃for 24 hours. The effect of antibacterial activity was based on the measurement of diameter of the inhibition zone formed. Streptomycin sulphate was used as a positive control and Dimethyl sulfoxide (DMSO) was used as a negative control [17]. Statistical comparisons was performed with Student's *t* tests at *P* value of 0.05 was considered to be significant and Mean values ± S.D. were calculated for the parameters.

Biochemical test for phytochemicals

Ethanolic herbal extract was analyzed for its total flavonoid content and was determined photo metrically using aluminum chloride ($AlCl_3$) assay. Total phenolic content was also determined using Folin-Ciocalteu reagent and using thin layer chromatography [18]. Other qualitative tests for alkaloids, saponins, tannins, steroids and coumarin were also analyzed.

RESULTS AND DISCUSSION

Isolation of microorganisms

Diagnosis and treatment of female genital tract infections are major public health problems, with considerable economic consequences. Some herbal antimicrobial agents are reported to inhibit the growth of vaginal infection and therefore in this study, we selected two herbs such as *Boerhaavia diffusa* and*Azadirachta indica* and their antimicrobial activity was analysed. Females between the ages of 15-45 years with complaints of vaginal discharge were included in the study. Vaginal samples were collected and allowed to culture on the separate nutrient agar medium as shown in Table 1. Four bacterial pathogens were isolated from patients, which were subjected for morphological, and biochemical identification as shown in Table 2.

Table 1: Identification of pathogens based on selective and differential media

media	isolate 1 *s.aureus*	isolate 2 *k.pneumoniae*	isolate 3 *aeromonas cavia*	isolate 4 *lactobacillus*
macconkey agar	lf colonies	red mucoid colonies	nlf colonies	lf colonies
blood agar	np	colorless colonies	non-haemolytic colony	no growth observed
baired parker agar	np	no growth observed	np	Np
nutrient agar	white color colonies	large muciod colonies	pigmented blue green colonies	swarmingwhite color colonies
cetrimide agar	np	no growth observed	green color colonies	Np
rajhans medium	greenish colonies	np	no growth observed	Np
xld agar	yellow color colonies	yellow color colonies	red color colonies	yellow color colonies with black center

**LF Colonies-Lactose Fermenting colonies, NFL-Non-Lactose Fermenting colonies and NP-Not performed*

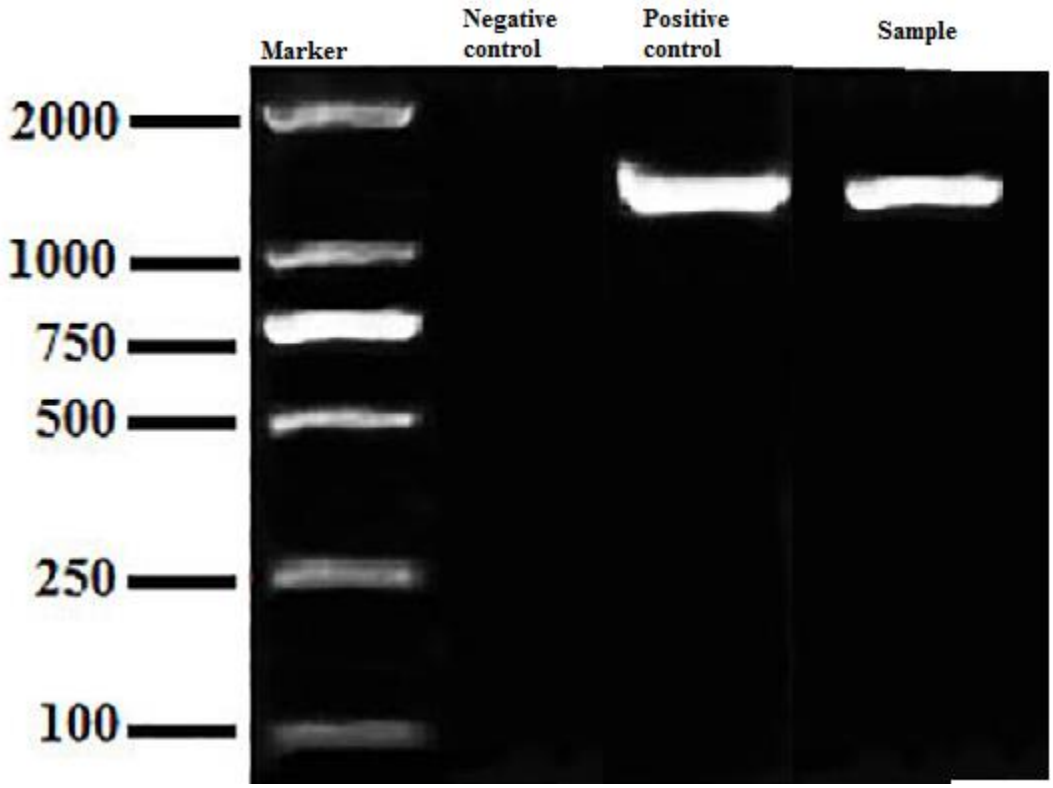
Table 2: Biochemical tests for identification of isolated microorganisms

Test	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Gram staining	+	-	-	+
Shape	**Cocci**	**Rod**	**Rod**	**Rod**
Motility	**M**	**NM**	**M**	**M**
Methyl red test	+	-	-	+
Vogesproskaur test	+	+	-	-
Citrate test	-	+	+	-/+
Catalase test	+	+	+	-

Sequencing of 16S rRNA gene

Sequencing of 16S rRNA gene is used for providing reference identifications of unknown strains, as this genetic marker is present in almost all bacteria [19].The four isolated pathogens were identified asIsolate 1*:* S*taphylococcus aureus*, Isolate 2*: Klebsiella pneumomia*, Isolate 3: *Aeromonas cavia* and Isolate 4: *Lactobacillus* based on 16S rRNA gene sequencing. The full length 1400 bp of 16S rRNA gene was performed on the DNA extracted from vaginal sample and confirmed by electrophoretic analysis as shown in Figure 2 (*Aeromonas cavia* as a representative). The results showed that this isolated *Aeromonas cavia* contains 16S rRNA in molecular weight of 1400 bp, which appeared as a single band that resulted from the successful binding between specific primers.

Figure 2: PCR amplification of the 16S rRNA gene (*Aeromonas cavia)*



Antimicrobial activity of aqueous and ethanolic herbal extract

Many higher plants accumulate extractable phytochemicals to be economically used as antibiotics. Herbal plant extracts such as aqueous and alcoholic extracts from*Boerhaavia diffusa* and*Azadirachta indica*are used in this study. Antibacterial effect of the herbal extracts in different concentration such as 10 and 20 and 30 mg/ml for aqueous extract and ethanolic extract was shown in Table 3. Both aqueous and ethanolic herbal extracts had comparable antibacterial activity at similar doses against S*taphylococcus aureus*, *Klebsiella pneumomia*, *Aeromonas cavia* and *Lactobacillus.* In general, the activity was highest for ethanolic extract which showed greatest activity against *S. aureus* and A*eromonas caviae.* Water extract also showed higher antibacterial activity against the same isolates. The minimum inhibition zone was observed against

Lactobacillus in both extracts. Moderate amount of inhibition zones was recorded in *Klebsilla pneumoniae.*

Table 3: Antimicrobial activity of aqueous and ethanolic extracts of *Boerhaavia diffusa* and*Azadirachta indica*

Herbal extract (mg/ml)	Anti-bacterial activity			
	Staphylococcus aureus	***K.pneumoniae***	***Aeromonas caviae***	***Lactobacillus***
Aqueous extract	12.8 ± 1.0	11.5 ± 0.6	12.5 ± 1.0	9.8 ± 0.0
Ethanolic extract	13 ± 0.6	12 ± 1.0	12.8± 1.0	10.5 ± 1.0

[a]Values are an average of three replicates (n=3).

Similar result was observed by Abdali et al. 2015 [20]. Our current findings are also consistent with other study reported by Vazini H et al. 2017[21]. In one study conducted in 2012, crude extracts of the plant showed remarkable antibacterial activity against tested microorganisms [22]. Drug resistance in human pathogenic microorganisms has forced to search for herbal antimicrobial substances and therefore i*n vitro* evaluation of plants for antimicrobial property is an important step for developing eco-friendly herbal management of infectious diseases. Based on the results, it was proved that herbal extracts of *Boerhaavia diffusa* and*Azadirachta indica* has significant acceptable efficacy on the elimination of bacterial vaginosis. Considering these anti-microbial action, the herbal extracts was screened for its phytochemicals through qualitative analysis and by thin layer chromatography. Such plant active components usually interfere with growth and metabolism of microorganisms [23]. It was reported that neem bark extracts were effective against both gram negative pathogenic bacteria and gram-positive bacteria [24]. In different study conducted by Kaviya et al. in 2022, *B. diffusa* root ethanol extract was effective against the growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus* with zones of inhibition of about 8 mm and 20 mm at maximum 200 µg concentration [25].

Biochemical test for phytochemicals

The phytochemical screening of herbal extracts showed the presence of alkaloids, flavonoids, tannins, phenol and coumarin. Various phytochemical compounds present in the leaves extracts suppressed the growth of bacteria in the study done by Khadikar*et al.*, (2001) support our result of phytochemical constituents present in the selected plants. Thin Layer Chromatography was performed and spots were identified. The spots indicted the presence of phenol and in general the phenol shown to have antimicrobial activity [26].

CONCLUSION

Present standard drugs like metronidazole and clindamycin are unable to inhibit the growth of vaginal infection completely and hence it requires novel treatment strategies. Some herbal antimicrobial agents are reported to inhibit the growth of vaginal infection. In the present study, four pathogens were isolated from vaginal fluid samples and molecular characterization based on 16S rRNA gene confirms the identity of the organisms. Herbal extracts of *Boerhaavia diffusa* and*Azadirachta indica* was analysed for its anti-vaginalis activity and was found that present herbal extracts were significantly effective against *Staphylococcus aureus* and *Aeromonas caviae*. Presence of secondary metabolites in the herbal extracts are capable of inhibiting or slowing the growth of bacteria, yeasts and moulds. It can be a suitable alternative for metronidazole after implementation of *in-vivo* investigations on laboratory animals.

Conflict of Interests:

The authors declare no conflict of interest.

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