INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com ISSN : 0976-4550

Volume-8, Issue-2, April-June-2017 Coden IJABFP-CAS-USA **Copyrights@2017**

Received: 10th Feb 2017 ***Revised: 11th Mar 2017*** ***Accepted: 12th Mar 2017***

DOI: 10.21276/Ijabpt, http://dx.doi.org/10.21276/ijabpt ***Research article***

ANTIBACTERIAL AND ANTIBIOFILM ACTIVITY OF TEA TREE OIL AND COCONUT VINEGAR AGAINST *GARDNERELLA VAGINALIS*.

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ABSTRACT

Introduction: *Gardnerella vaginalis* is a significant pathogen associated more frequently with genital and rarely extragenital infections in women of reproductive age. Few natural antimicrobial agents such as soap nut, garlic, calendula oil are reported to inhibit the growth of *G.vaginalis*. The present study was intended to investigate the antibacterial and antibiofilm actions of Tea Tree oil and Coconut Vinegar against clinical isolates of *G.vaginalis*.

Material and Methods: Antimicrobial activity of these agents were determined by Disc diffusion method. Minimum inhibitory concentration (MIC) was determined by Agar dilution technique. Biofilm and Antibiofilm activity of *G.vaginalis* was detected by tissue culture plate method.

Results: By the disc diffusion method, 73.6% were inhibited by Tea tree oil and 80.2% by Vinegar. The MIC range of Tea tree oil and Vinegar varied from 0.78 to 12.5ul/ml. The minimum biofilm inhibition concentration (BIC_{50}) was determined at 200ul/ml concentration.

Conclusion: Tea tree oil and Vinegar exhibited good antibacterial and antibiofilm activitiy against *G.vaginalis.*

Key Words: *Gardnerella vaginalis,* Tea tree oil, Vinegar, Biofilm formation, Biofilm inhibition concentration (BIC_{50}), Agar dilution method, Minimum inhibitory concentration

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INTRODUCTION:

Gardnerella vaginalis (G.vaginalis) is a microaerophilic bacterium associated with bacterial vaginosis (BV) which is the leading vaginal disorder in women of reproductive age. This organism can predispose to obstetrical infections, post abortal pelvic inflammatory disease, postpartum endometritis, preterm labour etc. (Winn.W.C et.al., 2006). Biofilm formation is one of the most important virulence factors which can initiate infectious process. A study reviewed the influence of biofilm formation by *G.vaginalis* and anaerobes in BV cases and the synergistic interaction between them. (Machado.A, et.al., 2015). Treatment option for *G.vaginalis* infections employing antibiotics still remain as a challenge. Studies are lacking in literature for herbal treatment options against *G.vaginalis*, therefore an attempt is done in this study to find some alternate drug options from herbal agents like Tea tree oil (TTO) as well as Vinegar which have antibacterial and antibiofilm potential against *G.vaginalis.* Various phytochemical volatile molecules were revealed in TTO such as terpene, 1,8-Cineole, Terpinolene, pinene, Aromadendrene, Cadinene, Limonene, Sabinene , Viridiflorol, Globulol (C.F.Carson, et.al., 2006). Carbohydrates, fat, saponins, flavanoids, tannins, quinones, terpenoids and coumarines were found in vinegar as compounds which play a role in antimicrobial activity. (Praveena.R.J, et.al., 2014). The present study investigates the biofilm formation of *G.vaginalis* and the in vitro antibacterial and antibiofilm activity of TTO and vinegar against *G.vaginalis.*

MATERIAL AND METHODS

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

The study was conducted in a tertiary care hospital in Coastal Karnataka South India. 91 strains of *G.vaginalis* isolated from females of reproductive age with abnormal vaginal discharge were included in the study. The collection of specimen, isolation and identification of *G.vaginalis* were done according to standard procedures. (Betty.A.F, et.al., 2007, Aroutcheva.A.A, et.al., 2001) as well as the our previous report. (Nisha.K, et.al., 2016). The study was approved by the Institutional Ethics Committee (Ref. No FMCC/ FMIEC/ 1298/ 2013).

B. Screening of antimicrobial action of Tea tree oil and Vinegar

In the present study a total 91 isolates were subjected to disc diffusion technique to determine the antibacterial efficacy of the TTO and Coconut vinegar which were purchased from reputed retail store in Mangalore. TTO is an essential oil with camphoraceous odour, appear pale yellow to colourless. It is prepared from the leaves of the *Melaleuca alternifolia*, which is native to Southeast Queensland, Australia. Vinegar was prepared from coconut water by a reputed manufacturer.

Antibacterial efficacy by Disc diffusion method:

Whatmann No.1 filter paper discs were saturated with various herbal agents used in the study. Bacterial suspension of *G.vaginalis* was prepared in citrate phosphate buffered saline adjusted to 0.5 Mc Farland opacity and streaked on to Brucella blood agar. The plates were incubated in the candle jar (5-10% CO_2) at 37^0C for 48 hours. The antimicrobial effects of these agents were measured in millimetres. Dimethyl sulphoxide (DMSO) incorporated disc was served as negative control. (Wilkinson J.M., 2006). The disc diffusion method was performed in triplicate and *G.vaginalis,* ATCC 14018 was tested in parallel with each isolate. The results were graded depending on the diameter of zone of inhibition such as 9mm-12mm ➔ 1+, 13mm-16mm ➔ 2+, 17mm-20mm3+, >20mm➔ 4+. (Alves.T.M.A, et.al., 2000).

C) Agar dilution method for the Detection of MIC of the Tea tree oil and Vinegar

The agar dilution method was employed to detect MIC of TTO and Vinegar according to Wadsworth-KTL anaerobic bacteriology manual sixth edition (Jousimies-Somer.H.R, et.al., 2002). A total 40 isolates of *G.vaginalis* which showed antimicrobial efficacy to both the herbal agents were subjected to detect MIC.

i. Preparation of plates for agar dilution technique:

The undiluted TTO (essential oil) and Vinegar were dissolved in DMSO and made up to the required concentration and incorporated into the agar plate to obtain concentration such as, 100 µl/ml, 50 µl/ml, 25 µl/ml, 12.5 µl/ml, 6.25 µl/ml, 3.12 µl/ml, 1.56 µl/ml, 0.78µl/ml and 0.39 µl/ml. Two millilitre of each diluted herbal extract and 1 ml of sterile lysed sheep blood were added to 17 ml sterile Brucella blood agar base supplemented with vitamin K1 and hemin, poured into petri plates and refrigerated at 4^0C.

ii. Determinatinon of MIC

Spot inoculations were done using standard inoculum of *G.vaginalis* isolates on to the marked areas in the supplemented Brucella blood agar plate with varying concentrations of the TTO and Vinegar. Two Brucella blood agar plates without herbal agents inoculated with strains served as control. All the plates were incubated in the candle jar (5-10% CO_2) and another plate incubated aerobically (aerobic contaminant control) at 37^0 C for 48 hours. The lowest concentration of drug yielded no growth, a haze, one discrete colony or multiple tiny colonies was recorded as MIC.

D) Detection of biofilm

The detection of biofilm production was done according to modified technique of Christensen et al. (Mathur.T, et.al, 2006). In brief, *G.vaginalis* were grown in BHI broth supplemented with 1% yeast extract, 2% gelatin and 0.1% starch, incubated in candle jar at 37^0c for 24 hours. (Patterson.J.L, et.al., 2010). A standard bacterial suspension was diluted in citrate phosphate buffered saline of pH 5.5. 200 µl of the suspension was dispensed in to micro titre plate wells. The plate was incubated in the candle jar at 37^0 C for 48 hours. The micro titre plate wells were washed with 0.85% sterile saline and 100 µl of Bouin's fixative was added which incubated at room temperature for ten minutes. The contents were aspirated and washed in 0.85% saline, 20 µl of 1% crystal violet was added for one minute, washed with water and air dried. The absorbance was read at 490 nm by using an ELISA reader. The test was done in triplicate and the mean value was taken as, OD of < 0.1 – weak or non-biofilm producers, OD of 0.1–0.2 – moderate and an OD of > 0.2 – high. 58 strains of *G.vaginalis* were subjected to the study. Sterile saline which was incorporated into one of the wells, served as a blank. *Pseudomonas aeruginosa* ATCC 27853 was used as a positive control.(Suman.E, et.al., 2008).

E) Detection of Antibiofilm Activity

A total 38 strong biofilm producing *G.vaginalis* clinical isolates were subjected to detect antibiofilm potential. These strains also showed good antibacterial action against *G.vaginalis* by disc diffusion as well as MIC detection assay.

TTO and Vinegar were applied to test the inhibition of biofilm activity on *G.vaginalis* isolates. Two fold serial dilutions of these reagents were made in sterile 96 polystyrene tissue culture plates. The test concentration range was from 200 µl/ml to 3.125 µl/ml. A 100µl of fresh standard bacterial suspension (0.5 McFarland) was made in citrate phosphate buffered saline (Citrate PBS) of pH 5.5 and added to each well.

The first row was served as the Growth control (bacterial suspension and medium without herbal extract). Media control (Only Citrate PBS) and blank control (Citrate PBS +herbal agents) were also included. The plate was incubated in candle jar (5-10% CO_2) at 37^0 C for 48 hours.After incubation the micro titre plate wells were washed with 0.85% sterile saline and 100 µl of Bouin's fixative was added incubated at room temperature for ten minutes and stained with crystal violet and proceeded as for biofilm formation. Antibiofilm activity was assessed by measuring the absorbance (OD-Optical density) at 490 nm by using microplate ELISA reader (BIORAD,USA). All tests were performed in triplicate and the % of biofilm inhibition was calculated using the formula. The biofilm iinhibition concentration (BIC_{50}) was defined as the lowest concentration of herbal agents showed 50% inhibition on the biofilm formation.(Chaieb.K, et.al., 2011, Nikolic.M, et.al., 2014)

Antibiofilm activity- (BIC_{50}) was calculated using the following formula

$$BIC_{50} = \frac{[OD\text{growth control} - OD\text{ sample}]}{[OD\text{ growth control}]} \times 100$$

*Growth control : Medium without herbal agents
** Sample : Medium with herbal agents

RESULTS

A total 91 *G.vaginalis* clinical isolates were analysed for their antimicrobial activity against TTO and vinegar by disc diffusion method. The sensitivity pattern of these agents were is given in the in Table 1. The zone of inhibition varied from 1+ to 4+ grades. MICs value of TTO and vinegar against 40 isolates of *G.vaginalis* by agar dilution method varied from 12.5 - 0.78µl/ml which shown in Table 2. The 58 clinical isolates of *G.vaginalis* were subjected to biofilm formation out of that 38 biofilm positive isolates were examined for anti biofilm activity (BIC_{50}) using TTO and Vinegar. The results of antibiofilm activity of these isolates were demonstrated in Table 3.

Table-1. Results of susceptibility of 91 Isolates of *G.vaginalis* by Disc diffusion technique

No	Herbal agents	Disc diffusion method					
		Grading				No. of Sensitive isolates and percentage	No. of Resistant isolates and percentage
		1+	2+	3+	4+		
1.	Tea tree oil	23	23	14	7	67 (73.7%)	24 (26.3%)
2.	Coconut Vinegar	19	22	20	12	73 (80.2%)	18 (19.7%)
Key : 9mm-12mm ➔ 1+, 13mm-16mm ➔ 2+, 17mm-20mm3+, >20mm➔ 4+.							
In Vitro Antimicrobial Action of Tea tree oil and Vinegar							

Table-2: Results of Minimum inhibitory concentration (MIC) in Tea tree oil and Coconut vinegar on 40 numbers of *G.vaginalis* isolates.

NO.	Nature of the herbal agents with isolates number	Various concentration of Tea tree oil and Vinegar in 1:10 dilution (µl/ml)								
		100 µl/ml	50 µl/ml	25 µl/ml	12.5 µl/ml	6.25 µl/ml	3.12 µl/ml	1.56 µl/ml	0.78 µl/ml	0.39 µl/ml
1.	No. & % of isolates in MIC of Tea tree oil	-	-	-	(5) 12.5%	(12) 30.0%	(4) 10.0%	(5) 12.5%	(14) 35.0%	-
3.	No. & % of isolates in MIC of Coconut Vinegar	-	-	-	(5) 12.5%	(16) 40.0%	(5) 12.5%	(4) 10.0%	(10) 25.0%	-
4.	**MICs ranges of Tea tree oil**	0.78 - 12.5 µg/ml								
5.	**MICs ranges of Coconut Vinegar**	0.78 - 12.5 µg/ml								
Details of isolates inhibited at various concentrations of Herbal agents, (-) = Absent of isolates.										

Table-3. Antibiofilm effect of Tea tree oil and Coconut vinegar against 38 biofilm positive *G.vaginalis* isolates.

No.	Herbal reagents	Number of isolates BIC50 (µl/ml)	Percentage (%)
1.	Tea tree oil	10	26.3
2.	Tea tree oil 1:10 dilution	10	26.3
3.	Coconut Vinegar	11	28.9
4.	Coconut Vinegar 1:10 dilution	11	28.9

DISCUSSION

G.vaginalis is a primary pathogen whose presence strongly correlates with BV which shows various virulence features such as biofilm formation. (Yeoman.C.J, et.al., 2010). It is reported that *G.vaginalis* is one of the main initiators of vaginal biofilm and this creates a favourable environment for anaerobic bacteria such as *Atopobium vaginae* and this finding was demonstrated in vitro by employing a multiplex FISH assay. (Hardy.L,et.al., 2015) . Regarding treatment options for *G.vaginalis*, antibiotics have always been a challenge. In one of the recent studies 26% of resistance was reported to clindamycin. (Nagaraja.P, 2008). There are no reports available in India about the antibacterial and antibiofilm activities of herbal agents against *G.vaginalis.* Horvath and colleague have reported the antibacterial activity of TTO against the Gram-negative luminescence tagged plant pathogenic bacterium *Pseudomonas syringae* pv. *maculicola* (Psmlux) and the Gram-negative, naturally luminescent marine bacterium *Vibrio fischeri*. (Horvath.G, et.al., 2013).

TTO is approved as an antibacterial and antifungal agent in the Western Countries. A diluted preparation of TTO or insertion of a non applicator tampon in olive or coconut oil with few drops of TTO were found to be effective as a home remedy for BV.(Dover.S.E, et al., 2008). TTO was used in various concentration for various aliments as reported in the literature such as bacterial vaginosis, onychomycosis, tinea pedis, skin lesions, wounds and ulcers.TTO is incorporated as the active ingredient in many topical formulation and used widely to treat cutaneous infections in the Western Countries. The antibacterial and anti inflammatory properties of TTO are documented in the literature. (Carson.C.F, et.al., 2006). However in vitro activity against *G.vaginalis* is not reported earlier and in the present study, TTO has found to have good antibacterial and antibiofilm activity against *G.vaginalis.*

Vinegar is reported as an antioxidant radical scavenger that can protect the human body from free radicals that may cause many diseases, including cancer and contribute to the aging process. (Praveena.R.J, et.al., 2014). Wash with a diluted vinegar solution to restore the naturally acidic vaginal pH was found to be effective as a home remedy for BV. (Dover. S.E et.al., 2008). Coconut vinegar was employed in the study to test its antibacterial efficacy against *G.vaginalis*. TTO exhibited 73.7% and vinegar has shown 80.2% susceptibility against *G.vaginalis* by disc diffusion methods. MICs of these agents were evaluated by agar dilution method which ranged from 0.78 to 12.5 µl/ml.

Of the 58 isolates which showed good activity to the herbal reagents tested, 38 strains produced biofilm with high positivity and these were subjected to antibiofilm activity of TTO and Coconut vinegar. The concentrated and 1:10 TTO showed biofilm inhibition in 10 (26.3%) isolates of *G.vaginalis* whereas concentrated and 1: 10 diluted vinegar inhibited 11 (28.94%) isolates. The screening of antibacterial activity and antibiofilm activity of the TTO and Vinegar in the present study has provided justification for developing a potential of herbal remedies to control female genital tract infections caused by *G.vaginalis*, especially in developing countries.

CONCLUSION

G.vaginalis is a significant pathogen from BV. The ability to produce biofilm might be helpful to initiate infection. This study shows bactericidal and anti biofilm activity of TTO and Coconut vinegar on *G.vaginalis.* The results of this study might be helpful to reduce biofilm formation and in turn vaginal colonisation.

ACKNOWLEDGEMENT

The authors would like to acknowledge Mr. Ramanath Karicheri, Research scholar, Department of Microbiology, Fr.Muller Medical College for the technical help rendered during the detection of antibiofilm activity of the herbal extracts.

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ISSN : 0976-4550

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