ORIGINAL ARTICLE

Antimicrobial activity of a novel polyherbal combination for the treatment of vaginal infection

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ABSTRACT

The present study evaluated the antimicrobial activity of *Azadirachta indica* (AI), *Cichorium intybus* (CI), and *Trigonella foenum-graecum* (TFG) against bacterial and fungal pathogens responsible for the vaginal infections. The AI, CI, and TFG were selected to include antimicrobial and antifungal action against wide range of microbes. The different extracts of the herbs were evaluated for antibacterial and antifungal activity by well diffusion assays. Based on the results, the combination was selected and evaluated, "polyherbal antimicrobial (PHA)." The developed PHA extract demonstrated synergistic broad-spectrum antimicrobial activities including antibacterial and antifungal activity (minimum inhibition concentration: 5–7 mg/ml).

Key words: Antimicrobial, Azadirachta indica, Cichorium intybus, polyherbal combination, Trigonella foenum-graecum

INTRODUCTION

Vaginal infection is a common problem among women, and about 75% of women will have at least one episode of vaginitis during their lifetime. Vaginal tract can be infected by diverse pathogens, resulting in diverse diseases such as urinary tract infections, bacterial vaginosis, vulvovaginal candidiasis, vaginitis, trichomoniasis, and sexually transmitted diseases.[1] Various antimicrobial compounds have been studied to treat these vaginal infections including antibacterial, antifungal, antiparasitic, and antiviral agents. Most of these agents used to date for treating vaginal infections have myriads of side effects and emergence of problem of drug resistance. Since ancient times, herbal medicines have served as a platform for the prevention and cure of diseases, and to date, many more constituents of these natural sources are yet to be explored.[2-4] There is a pressing need to develop a natural formulation, which can act against the microorganisms causing vaginal infections.[5] Over the past decade, interest in herbal medicine has increased tremendously. According to the World Health Organization, 60%–80% of population in developing countries depends essentially on plants for primary health-care needs.[6] The resurgence of herbal medicines has increased the international trade enormously, and the global herbal medicine market is expected to reach about USD 117 billion by 2024, driven by the rising popularity of herbal medicine compared to conventional drugs. The literature revealed *Azadirachta indica* (AI),[7] *Cichorium intybus* (CI),[8] and *Trigonella foenum-graecum* (TFG)[9] as herbs that possess antibacterial and antifungal activity. Pharmaceutical companies have established renewed concern in exploring plants as a major source for new lead structures and for the development of standardized phytotherapeutics with promising quality, safety, and efficacy.[10] The aim of the present study is to screen out a novel polyherbal combination for antimicrobial activity with the objective to treat infections of the vagina.

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MATERIALS AND METHODS

Plant raw materials

Three herbs, namely AI, CI, and TFG, were selected and procured from Moonlight Traders, Delhi; Asian Traders, Khari Baoli, Delhi; and Green Earth Products, International Exporters, New Delhi, respectively.

Preparation of extracts

The leaves of AI and CI and the seeds of TFG were powdered and extracted with aqueous, alcoholic, and hydroalcoholic solutions prepared using hot extraction method. Soxhlet apparatus was used for carrying out hot extraction process, where temperature was controlled at 75°–80°C for all the three herbs. Various extracts were separately collected and evaporated to dryness under reduced pressure at 40°–45°C using a rotary evaporator. The dried extracts were weighed, and the extraction efficiency (%) was calculated with reference to the air-dried substance. The dried extracts were dissolved in various solvents at varying concentrations, and their comparative antimicrobial activity was evaluated by comparing the zones of inhibition. Chloramphenicol (20, 30, and 40 µg/mL) was used as the standard for *Staphylococcus aureus* and *Streptococcus agalactiae*; ciprofloxacin (5, 10, and 15 µg/mL) was used as the standard for *Escherichia coli*; and miconazole (15, 20, and 25 µg/mL) was used as the standard for *Candida albicans* and *Aspergillus fumigatus*.

Inoculum and culture media

The primary subcultures for each microorganism were procured from authentic sources. The bacterial strains employed were *E. coli* (Strain No. 8739-8/03/15), *S. aureus* (Strain No. B-17-DF-EB-IITD), and *S. agalactiae* (Strain No. T-15-DF-TK-IITD). The fungal strains employed were *C. albicans* (Strain No. B778-8/03/15) and *A. fumigatus* (Strain No. F-2/21-DBE3-IITD).

Antimicrobial screening using agar well diffusion method

The medium (25 mL) was poured into presterilized Petri dishes and set aside for solidification for 5 h. Cotton swabs charged with 0.1 mL of diluted inoculum (10^5 cfu/mL) of test microorganisms were inoculated and spread evenly over the surface of agar plates. Uniform sized wells of 8 mm diameter were aseptically punched into the seeded agar with a flamed cork borer to make three holes at equidistant positions on the Petri dishes. In each Petri dish, two out of three holes were filled with 0.2 mL of sample solution with varying concentrations, and the third hole was filled with the standard solution. The solvent blanks were also taken similarly as negative controls.

Incubation

All the plates were incubated at 37°C for 24 h for bacteria cultures and at 28°C for 48 h and 120 h for fungal cultures of *C. albicans* and *A. fumigatus*, respectively. The zone of inhibition produced by sample and standard solutions was recorded using sliding calipers and compared for their antimicrobial activity evaluation. The antimicrobial screening study of each sample was carried out in triplicate ($n = 3$).

Statistical analysis

Statistical analysis of the results for polyherbal formulation was performed using Prism 7 software (GraphPad, USA). Differences were considered to be statistically significant when $P < 0.05$. The data were presented as mean ± standard deviation and were analyzed by one-way ANOVA.

RESULTS AND DISCUSSION

The extraction process for each of the herb was optimized in terms of maximum yield based on solvent system and time duration required for extraction. The percent yields observed for dried extracts of AI, CI, and TFG were found to be 31.19%–37.43%, 21.94%–27.65%, and 18.72%–27.16%, respectively [Table 1]. The data [Tables 2-4] showed that hydroalcoholic and water extracts of AI (30 mg/mL) had comparable antibacterial activity at similar doses against *S. aureus*, *S. agalactiae*, and *E. coli*. The activity was highest for the former extract against *S. agalactiae*. Hydroalcoholic and ethanol extracts of CI (15 mg/mL) showed the greatest activity against *S. aureus*, but poor antifungal activity and no activity against *E. coli*. Water extract of CI showed higher antifungal activity, but no antibacterial activity. Ethyl acetate extract of CI did not show any antifungal activity. Hydroalcoholic and ethanol extracts of TGF showed the highest activity against *A. fumigatus* and good activity against *C. albicans* at 6 mg/mL concentration; the hydroalcoholic extract was found to inhibit bacterial growth to a greater extent than the ethanol extract. Based on the results, the hydroalcoholic extracts of AI, CI,

Table 1: Percentage yield of various extracts

Herb	Sample Qt (g)	Solvent system	Duration (min)	Yield (%)
AI (leaves)	120	n-Hexane	720	34.71
	120	95% ethanol (v/v)	360	31.19
	120	Water-ethanol (1:1, v/v)	360	37.43
	120	Water	360	37.11
TFG (seeds)	135	n-Hexane	720	18.72
	135	95% ethanol (v/v)	360	24.31
	135	Water-ethanol (1:1, v/v)	360	27.16
	135	Water	360	27.16
CI (leaves)	90	Ethyl acetate	720	23.57
	90	95% ethanol (v/v)	360	27.65
	90	Water-ethanol (1:1, v/v)	360	26.83
	90	Water	360	21.94

AI: *Azadirachta indica*, CI: *Cichorium intybus*, TFG: *Trigonella foenum-graecum*

Table 2: Results for antimicrobial activity of various extracts of the leaves of *Azadirachta indica*

Test sample (mg/ml)	Antimicrobial Activity[a,b]			Antifungal Activity[a,b]	
	Staphylococcus aureus	*Streptococcus agalactiae*	*Escherichia coli*	*Candida albicans*	*Aspergillus fumigatus*
W1	16.3±0.6	16.0±1.0	17.3±0.6	-	-
W2	20.7±1.15	17.0±1.0	20.3±0.6	10.3±0.6	10.3±0.6
W3	20.7±1.15	17.7±1.15	20.7±1.15	11.0±1.0	10.0±1.0
E1	-	-	-	11.0±0.0	-
E2	14.3±0.6	15.3±0.6	-	11.0±0.0	12.0±0.0
E3	20.3±0.6	22.0±1.0	17.0±1.0	12.0±0.0	12.0±1.0
H1	-	-	10.3±0.6	-	-
H2	13.0±1.0	-	11.3±0.6	-	-
H3	13.0±1.0	12.3±0.6	11.0±0.0	-	-
HA1	22.0±0.0	19.0±1.0	17.3±0.6	-	-
HA2	22.0±0.0	20.0±0.0	17.0±0.0	11.3±0.6	10.0±0.0
HA3	22.0±0.0	24.0±0.0	18.3±0.6	12.0±0.0	12.3±0.6
HA4	-	-	-	-	-
HA5	-	-	-	-	-
R1[c]	40.0±0.0	38.0±0.0	-	-	-
R2[c]	-	-	37.0±0.0	-	-
R3[c]	-	-	-	35.0±0.0	32.0±0.0

[a]Values are an average of three replicates (n=3). W1, W2, and W3: Water extracts at concentrations of 10, 20, and 30 mg/mL, respectively; E1, E2, and E3: Ethanol extracts at concentrations of 20, 30, and 40 mg/mL, respectively; H1, H2, and H3: n-Hexane extracts at concentrations of 30, 60, and 80 mg/mL, respectively; HA1, HA2, HA3: Hydroalcoholic extracts at concentrations of 10, 20, and 30 mg/mL, respectively, [b]Zone diameter value is given in mm, [c]Reference: R1: Chloramphenicol (30 μg/mL); R2: Ciprofloxacin (10 μg/mL); R3: Miconazole (20 μg/mL)

Table 3: Results for antimicrobial activity of various extracts of the leaves of *Cichorium intybus*

Test sample (mg/ml)	Antimicrobial Activity[a,b]			Antifungal Activity[a,b]	
	Staphylococcus aureus	*Streptococcus agalactiae*	*Escherichia coli*	*Candida albicans*	*Aspergillus fumigatus*
W1	-	-	-	12.3±0.6	10.0±1.0
W2	-	-	-	13.0±1.0	12.0±1.0
W3	-	-	-	15.0±1.0	16.0±1.0
E1	20.3±0.6	14.0±1.0	-	-	10.0±1.0
E2	22.3±0.6	14.3±0.6	-	-	11.3±0.6
E3	25.0±1.0	15.0±1.0	-	-	12.0±1.0
EA1	16.0±1.0	15.0±1.0	-	-	-
EA2	16.0±1.0	15.0±1.0	-	-	-
EA3	19.3±0.6	15.3±0.6	-	-	-
HA1	12.0±1.0	-	-	10.0±0.0	10.3±0.6
HA2	16.0±1.0	11.0±1.0	-	10.0±0.0	11.7±1.15
HA3	19.0±1.0	11.3±0.6	-	10.0±0.0	11.0±1.0
HA4	24.0±1.0	15.0±1.0	-	12.0±1.0	11.3±0.6
HA5	10.3±0.6	11.3±0.6	-	11.3±0.6	12.3±0.6
R1[c]	40.0±0.0	38.0±0.0	-	-	-
R2[c]	-	-	37.0±0.0	-	-
R3[c]	-	-	-	35.0±0.0	32.0±0.0

[a]Values are an average of three replicates (n=3). W1, W2, and W3: Water extracts at concentrations of 5, 10, and 15 mg/mL, respectively; E1, E2, and E3: Ethanol extracts at concentrations of 5, 10, and 15 mg/mL, respectively; EA1, EA2, and EA3: Ethyl acetate extracts at concentrations of 10, 15, and 20 mg/mL, respectively; HA1, HA2, HA3, HA4, and HA5: Hydroalcoholic extracts in the ratio of 1:1, 1:2, 1:3, 1:4, and 2:1, respectively, at a concentration of 15 mg/mL, [b]Zone diameter value is given in mm, [c]Reference: R1: Chloramphenicol (30 μg/mL[1]); R2: Ciprofloxacin (10 μg/mL); R3: Miconazole (20 μg/mL)

and TFG at a concentration of 20 mg/ml, 15 mg/ml, and 5 mg/ml, respectively, were selected to prepare polyherbal antimicrobial (PHA) extract. The PHA extract formed by mixing hydroalcoholic extracts of TFG, CI, and AI in the ratio of 1:3:5 contains 12.5 mg of TFG, 37.5 mg of CI, and 50 mg of AI per 100 mg of PHA extract. The PHA extract was then evaluated for the synergistic antimicrobial activity at four different concentration levels:

a. Dose 1: 1% of the actual dose
b. Dose 2: 10% of the actual dose

c. Dose 3: 25% of the actual dose
d. Dose 4: 50% of the actual dose.

PHA extract exhibited synergistic antimicrobial activity, and the extract at 25% of the actual dose (1.25 mg TFG, 3.75 mg CI, and 5.0 mg AI per mL) demonstrated enhanced and broad-spectrum antibacterial and antifungal activity [Table 5]. The microbiological activity of D3 against all the tested microorganisms, except *E. coli*, was significantly higher ($P < 0.05$) than D2. However, the microbiological activity of D3 is not significantly different ($P > 0.05$) from that of D4. Representative photographs for comparative zones of inhibition, as observed for PHA extract, are shown in Figure 1. This PHA extract could further be developed into the formulation for effective application and treatment of local vaginal infections.

CONCLUSION

The developed PHA extract using a novel combination of antimicrobial agents from polyherbal sources demonstrated enhanced antibacterial and antifungal activity and could be an effective formulation for the treatment of vaginal infections.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

Table 4: Results for antimicrobial activity of various extracts of the seeds of *Trigonella foenum-graecum*

Test sample (mg/ml)	Antimicrobial Activity[a,b]			Antifungal Activity[a,b]	
	Staphylococcus aureus	*Streptococcus agalactiae*	*Escherichia coli*	*Candida albicans*	*Aspergillus fumigatus*
W1	16.0±0.0	-	14.3±0.6	15.0±1.0	17.0±1.0
W2	16.0±0.0	12.7±1.15	14.0±0.0	15.3±0.6	17.3±0.6
W3	16.7±1.15	16.0±0.0	16.0±1.0	16.0±0.0	18.0±0.0
E1	12.0±0.0	10.0±0.0	10.3±0.6	18.3±0.6	15.0±1.0
E2	15.0±0.0	13.0±1.0	16.3±0.6	18.3±0.6	22.0±1.0
E3	17.3±0.6	15.0±0.0	17.0±0.0	19.0±1.0	22.0±1.0
H1	10.3±0.6	-	12.0±0.0	-	-
H2	12.0±1.0	-	12.0±0.0	-	-
H3	16.0±1.0	14.3±0.6	12.0±0.0	-	-
HA1	14.0±1.0	-	12.0±1.0	14.0±0.0	15.0±1.0
HA2	16.0±0.0	12.0±1.0	12.0±0.0	14.3±0.6	15.3±0.6
HA3	17.0±0.0	12.0±1.0	13.0±1.0	17.3±0.6	19.0±0.0
HA4	19.0±0.0	14.0±0.0	18.0±1.0	18.3±0.6	21.0±1.0
HA5	19.0±1.0	13.0±0.0	14.0±0.0	17.3±0.6	17.0±0.0
R1[c]	40.0±0.0	38.0±0.0	-	-	-
R2[c]	-	-	37.0±0.0	-	-
R3[c]	-	-	-	35.0±0.0	32.0±0.0

[a]Values are an average of three replicates (n=3). W1, W2, and W3: Water extracts at concentrations of 4, 6, and 8 mg/mL, respectively; E1, E2, and E3: Ethanol extracts at concentrations of 4, 6, and 8 mg/mL, respectively; H1, H2, and H3: n-Hexane extracts at concentrations of 4, 6, and 8 mg/mL, respectively[1]; HA1, HA2, HA3, HA4, and HA5: Hydroalcoholic extracts in the ratio of 1:1, 1:2, 1:3, 2:3, and 2:1, respectively, at a concentration of 6 mg/mL, [b]Zone diameter value is given in mm, [c]Reference: R1: Chloramphenicol (30 μg/mL[1]); R2: Ciprofloxacin (10 μg/mL); R3: Miconazole (20 μg/mL)

Table 5: Results for antimicrobial activity of polyherbal antimicrobial extract

Test Sample (mg/ml)	Antimicrobial Activity[a]			Antifungal Activity[a]	
	Staphylococcus aureus	*Streptococcus agalactiae*	*Escherichia coli*	*Candida albicans*	*Aspergillus fumigatus*
D1	20.0±0.0 (S)	19.3±0.6 (M)	15.0±1.0 (M)	16.7±1.15 (M)	16.3±0.6 (M)
D2	20.7±1.15 (S)	19.3±0.6 (M)	15.7±1.15 (M)	18.7±1.15 (M)	16.3±0.6 (M)
D3	23.7±1.15 (S)	21.0±1.0 (S)	16.7±1.15 (M)	21.7±1.15 (S)	19.0±1.0 (M)
D4	23.7±1.15 (S)	22.0±1.0 (S)	17.7±1.15 (M)	22.0±1.0 (S)	18.7±1.15 (M)
R1	40.0±0.0 (S)	38.0±0.0 (S)	-	-	-
R2	-	-	37.0±0.0 (S)	-	-
R3	-	-	-	35.0±0.0 (S)	32.0±0.0 (S)

[a]Values are an average of three replicates (n=3). Dose 1: 4 mg/mL; Dose 2: 4.5 mg/mL; Dose 3: 5 mg/mL; Dose 4: 5.5 mg/mL. Zone diameter value is given in mm. R1: Chloramphenicol (30 μg/mL); R2: Ciprofloxacin (10 μg/mL); R3: Miconazole (20 μg/mL). S: Susceptible, M: Medium

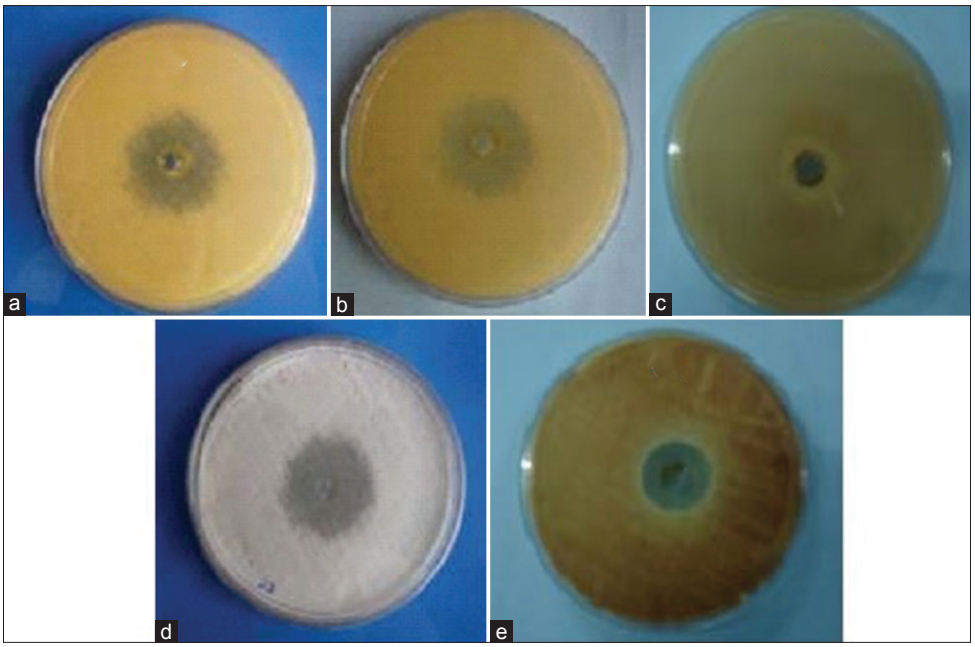

Figure 1: Antimicrobial activity of polyherbal antimicrobial extract against (a) *Staphylococcus aureus*, (b) *Streptococcus agalactiae*, (c) *Escherichia coli*, (d) *Candida albicans*, and (e) *Aspergillus fumigatus*

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