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Phytochemical effectiveness of some ethanomedicinal plants of Balochistan, Pakistan against urogenital infections

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Abstract *Neisseria gonorrhea* and *Escherichia coli* are urogenital infection causing bacteria which leads to gynaecological problems like vaginitis and cervicitis and impaired sperm motility leading to infertility in male and female. Crude ethanolic extracts of *Acorus calamus, Cichorium intybus* and *Fumaria indica*, the ethnomedicinal plants of Balochistan (Pakistan), were screened for their phytochemical composition i.e., alkaloids, anthraquinones, flavonoids, saponins, steroids, tannins, and terpenoids. Moreover, these selected plants were also tested for their antibacterial activity against ATCC strains of *E.coli* and *Neisseria gonorrhoeae*, by agar well diffusion assay with the tested concentrations of 10, 15, and 20 µL, and Kirby-Bauer disk diffusion method with the tested concentrations of 04, 06 and 08 µL. Streptomycin and dimethyl Sulphoxide (DMSO) were used as a positive and negative control, respectively against the bacterial strains. It was observed that *C.intybus* produced comparatively more effective results in disk diffusion method against *E.coli* (15.67±0.58, 17.34±0.58, 19±0, respectively) but *A.calamus* was found effective against *N.gonorrhoeae* (12.41±0.60 and 13.41±0.61 at 10 and 15 µL, respectively). According to agar well diffusion assay, *A.calamus* expressed most antimicrobial activity against *N.gonorrhoeae* (14±11±0.43, 16.67±0.58, 19.34±0.58, respectively) whereas, *C.intybus* was found to be most toxic for *E.coli*. These findings favored the traditional medicinal usages of tested plants against urogenital infections and a cure for infertility.

Keywords: Antimicrobial activity, Agar well diffusion, Disk diffusion, *N.gonorrhoeae*, Urogenital infections, Zone of Inhibition (ZOI)

Introduction

Infertility diagnosed in humans, is a medical and social issue globally. Physical health may not be influenced by infertility, but it can exert some impact on the social and mental health of infertile couple. There are 60-80 million infertile couples reported world widely, of which 10-15% married

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couples are affected (Dwivedi and Mishra, 2017). Sexual dysfunction is a serious medical issue damaging social and biological relationships, occurs 63% in women and approximately 52% in men. There are plenty of synthetic remedies in drug market world widely; however, these drugs may exert some side effects. Thus, common people tend towards the usage of herbal/medicinal plants which may safely cure the issues related with the reproductive organs (Dutta and Sengupta, 2018). The most common reproductive tract infections i.e., cervicitis and vaginitis cause severe reproductive disorders in women. Gram-positive and Gram-negative bacteria and yeast are responsible for this microflora alteration. *Escherichia coli* (*E. coli*) and *Neisseria gonorrhoeae* are the most responsible microbes associated with cervicitis and vaginitis (Moro and Ali, 2018). *N. gonorrhoeae* is responsible for oxidative stress (OS) which is associated with male infertility (Agarwal *et al*., 2018). *E. coli* is the most common etiologic agent of infertility (Mu'azu *et al*., 2021).

The gyanecological problems such as vaginitis are caused by the presence of *E. coli* and lack of lactobacilli. Owing to atrophic vaginitis in postmenopausal period, estrogen level falls which resultantly reduces mucosal epithelial barrier, shortens lactobacilli in the vaginal flora and lessens vaginal pH that leads to, predominant colonization of *E. coli* in vagina (vaginitis) (Kim *et al*., 2018). Cervicitis is least common than vaginitis which is the most marked gynaecological problem occurs owing to change in the microfloral ecosystem of vagina.

E. coli is considered as the most frequent isolated microorganism in male patients with genital tract infection, and its effect on sperm motility. Gonorrhea is a common and well known sexually transmitted disease of both male and female reproductive system. This disease is initiated by *Neisseria Gonorrhea*, often called Gonococcus, which contains fimbriae that allow bacteria to attach the epithelial cells. In addition, it also contains a kind of lipopolysaccharide endotoxin present in the outer membrane structure that improves its pathogenicity (Zuhair *et al*., 2019).

Natural products were the only source of treating diseases up to the end of 19^{th} century. Even, half of the current drugs are developed from the natural products including herbs (Christensen, 2021). In Pakistan, there are approximately 6000 species of medicinal plants out of which 600 are considered to have a significant effect in medicinal point of view (Malik *et al*., 2019). Balochistan, a largest province of Pakistan, has the decent habitats for the growth of medicinal plants due to several ecologic zones (Alamgeer *et al*., 2018).

Phytochemicals are compounds derived from plants through diverse forms of release, mostly by extraction. They are attractive and strategic products of bioprospecting with application in medicine (Oyewole *et al*., 2021).

The current study aimed to use the crude extracts of three ethnomedicinal plants, collected from various parts of Balochistan, which were analyzed for their phytochemical investigation and their extracts were tested as an alternative therapeutic agent against *N. gonorrhea* and *E. coli* (potential urogenital infection causing agents).

Materials and methods

The test plants were collected from various areas of Balochistan province i.e. Harboi, Quetta, Kalat, Pishin and Ziarat, conditional to the time of growth and accessibility of these plants. These plants were shade-dried for 9 days at 25–28 ℃, in order to avoid decomposition and were ground in a grinding machine. About 50g of these whole plant sample powders were soaked in 300 mL of ethanol and their extracts were obtained after 48 hours through rotary evaporation. The crude extracts were preserved at 4 ℃ for further use.

Preliminary phytochemical analysis

A 20mg of each plant was subjected to extraction through rotary evaporator and dissolved in 1 mL of DMSO. Dimethyl sulfoxide (DMSO) ranks high in terms of choice of solvent for plant extracts/ drug delivery. It is an organic amphiphilic molecule that is widely used in cell biology; it exhibits a number of capabilities such as vasodilatory, diuretic, anti-inflammatory and bacteriostatic functions (Nguyen *et al*., 2020). These crude extracts were further utilized for preliminary phytochemical tests to analyze them for their chemical composition.

Test for alkaloids (Meyer's Test)

Each plant's residue was collected by evaporating its extract till it dried up. The mixture of residue and 2% hydrochloric acid was heated on a boiling water bath. Few drops of Meyer's reagent were added to the filtrate of mixture after cooling. The yellow precipitation/ turbidity indicated the presence of alkaloids (Sharma *et al*., 2020).

Test for anthraquinones

Borntrager's test was used for the presence of anthraquinones. A 3 mL of plant extract was mixed with a 3 mL of chloroform. The chloroform layer was separated after shaking on vertex and 5% potassium hydroxide solution was

added to it. The presence of quinones was confirmed by indication of red color in alkaline phase. The test samples displaying yellow coloration with green fluorescence were mixed with one drop of 6% hydrogen peroxide. The enthrones derivatives were confirmed by appearance of red coloration (Mar á *et al*., 2018).

Test for flavonoids

Flavonoids test was done by the lead acetate protocol. The concentrated plant extracts and few drops of lead acetate solution were mixed together. The flavonoid class of compounds was indicated by the exhibition of yellow colour precipitates (Ushie *et al*., 2016).

Test for saponins

Phytoextract of 0.5 mg was diluted with 20 mL double distilled water and properly shaken on a vertex for 15 min. Saponins were confirmed by formation of foam up to length of 1 cm (Deshmukh and Theng, 2018).

Test for steroids

For determination of steroids, a 0.30 g measured weight of plant was soaked in 20 mL of ethanol. The extraction was carried out after 2 hrs' time period. In an ethanolic extract of 5 mL, 2 mL of acetic anhydride was added, followed by addition of 2 mL concentrated tetraoxosulphate (VI) acid. Appearance of violet to blue or green colouration pointed to steroids in the samples (Ezeonu and Ejikeme, 2016).

Test for tannins

A plant extract of 5 mL was boiled with 5 mL of 45% solution of ethanol for 5 min, followed by cooling and filtration. The filtrate was utilized in ferric chloride analysis. 2 mL of distilled water was used to dilute 1 mL plant filtrate followed by addition of two drops of ferric chloride to it. Tannins were pointed out owing to greenish to black color occurrence (Okoro *et al*., 2016).

Test for terpenoids (Salkowski test)

Five mL phytoextract sample, 2 mL chloroform and 3 mL concentrated sulphuric acid (3 mL) were cautiously added near the edge of the test tube. Terpenoids were indicated by exhibition of reddish brown colouration at the interface (Edeoga *et al*., 2005).

Biological assay

Antimicrobial susceptibility analysis

The antibiotic activity investigations were performed by Paper Kirby-Bauer disc diffusion method (Drago *et al*., 1999) and agar well diffusion (Muller Hinton) method.

Strains, media and inoculums

In this study, ATCC strains of bacteria i.e. *Escherichia coli* (ATCC-8739) and *Neisseria gonorrhoeae* (ATCC-31149) were used. Nutrient broth solution and nutrient agar media for bacterial strains culturing were prepared on the guidelines of manufacturer i.e. 13 g/L and 23 g/L respectively. The pH was maintained at minimum 6.5 (using 0.1M sodium acetate buffer). For investigation of antibacterial activity of ethnomedicinal plants, Muller Hinton agar (MHA) media was prepared i.e. 38 g of Muller Hinton Agar was mixed with distilled water. All prepared media were subjected to sterilization by autoclaving (121℃, 15 Psi, 15 min). One-two colonies of pure culture of different organisms from stock culture were transferred through sterile loop to the respective sterile test tubes containing nutrient broth and later incubated for 4 hours. After incubation, the test tubes were matched with McFarland (turbidity) standard. The bacterial cell density was maintained at 1.5×10^8 CFU/mL.

For inoculation on MHA and Sabouraud dextrose agar (SDA) plates (Okla *et al*., 2021), sterile cotton swab was dipped into the standard working inoculums to spread it all over the MHA plates in the angle of 60° by rotating the plates. Each organism was streaked in a triplicate and left to dry at room temperature for about 10 min with closed lid (Antika *et al*., 2020).

Antimicrobial and antifungal activity

For agar well diffusion method, the cork borer having 6 mm diameter was sterilized and used to prepare wells in the MHA plates. On each plate, five wells were prepared. The disk diffusion assay was performed according to the standard protocol of Kirby Bauer (Loo *et al*., 2018).

The test quantity of 20 mg/mL of each extract was dissolved in DMSO (Dimethyl Sulfoxide). In order to detect potential antimicrobial activity in the plant extracts, 4 µL and 6 µL and 8 µL volume of each plant extract stock solution were impregnated on Whatman No.1 filter paper discs (diameter 12 mm). The same quantity was used for agar well diffusion assay too. The surface of agar plates were inoculated with the culture of bacteria, discs soaked with each of the test solutions containing different extract solutions at varying volumes. The discs were placed separately in each quarter of the plate under aseptic conditions. The standard drug solution of streptomycin and the control-blank of 2 µL DMSO were used for both assay techniques. Three replications

were prepared for each of the plant crude extract and later incubated at 37 ℃ for 24 h. The zones of inhibition were subsequently measured in mm by following the method stated by (Eloff, 2019).

Results

The study was conducted on the bioactivity screening of some selected ethnomedicinal plants of Balochistan. The antimicrobial assay was carried out through Kirbey Bauer's Paper disk diffusion and agar well diffusion techniques against three ATCC bacterial strains i.e. *Escherichia coli* (ATCC-8739) and *Neisseria gonorrhoeae* (ATCC-31149). The diameters of zones of inhibition (ZOI) were measured for each medicinal plant. The zone of inhibition is an antibiotic/ drug sensitivity test of micro-organisms. The clear zone around an antibiotic-paper disk/ well is a visual measure of the inhibitory potency of an antibiotic/ plant extract/ or drug. The phytochemical screening was carried out for identification of alkaloids, anthraquinones, flavonoids, saponins, steroids, tannins and terpenoids (Table 1).

Table 1. The phytochemical components of *A. calamus*, *C. intybus*, *F. indica* based on the preliminary crude extract screening

Name of Plants	Alkaloids	Anthraqui-nones	Flavonoids	Saponins	Steroids	Tannins	Terpenoids
A. calamus	+	-	+	+	+	+	+
C.intybus	+	+	+	-	+	+	+
F.indica	+	+	+	-	-	+	-

Phytochemical investigation

The results of phytochemical investigation revealed the presence of alkaloids, flavonoids and tannins in all three test strains. Nonetheless, steroids and *terpenoids* were found to be missing in *F.indica*, while *anthraquinones* activity was exhibited by *C. intybus* and *F.indica*. Saponin was the exclusive property of *A. calamus*.

Biological assay

Disk diffusion method

The treatment of crude ethanolic extracts of selected ethnomedicinal plants i.e. *F.indica*, *A. calamus* and *C. intybus* against *E.coli* is shown in Figure 1. The

positive control, streptomycin, could only produce 10.33 mm average ZOI. Whereas, *F. indica* represented the highest ZOI of 15.66 mm and 17.33 mm and 19 mm at 4 µL, 6 µL and 8 µL plant extract concentrations, respectively.

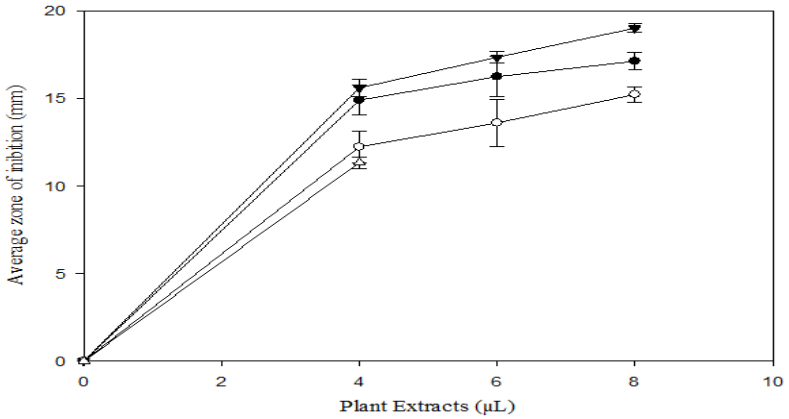

Figure 1. Average antibacterial activity (mm) of selected ethanomedicinal plants against *E. coli at* 4 µL, 6 µL and 8 µL through disk diffusion method, *A. calamous* (●), *C. intybus* (○), *F. indica* (▼), *Streptomycin* (4 µL) (△)

The average antimicrobial activity of test plants against *N.gonorrhea* at increasing concentrations of 4 µL, 6 µL and 8 µL is shown in Figure 2. *A.calamous* experimentally proved to be the most effective as it produced the maximum zone of inhibition of 12.4 mm at 4 µL and 13.4 mm at 6 µL, whereas *C. intybus* produced ZOI of 17.34 mm at 8µL concentrations of ethanolic extracts. Streptomycin produced 7.67 mm ZOI.

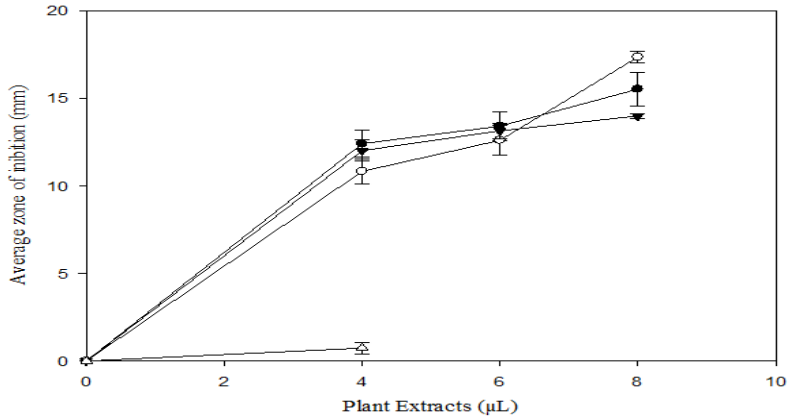

Figure 2. Average antibacterial activity (mm) of selected ethanomedicinal plants against *N. gonorrhea at* 4 µL, 6 µL and 8 µL through disk diffusion method, *A. calamous* (●), *C. intybus* (○), *F. indica* (▼), *Streptomycin* (4 µL) (△)

Agar well diffusion method

A. calamus showed no biological activity against *E.coli* as seen in Figure 3. *C.intybus* exhibited the highest ZOI at 10 µL, 15 µL and 20 µL which were 13.67 mm, 15.34 mm and 19 mm, respectively. Streptomycin (10 µL), the positive control drug, could only produce 9 mm ZOI.

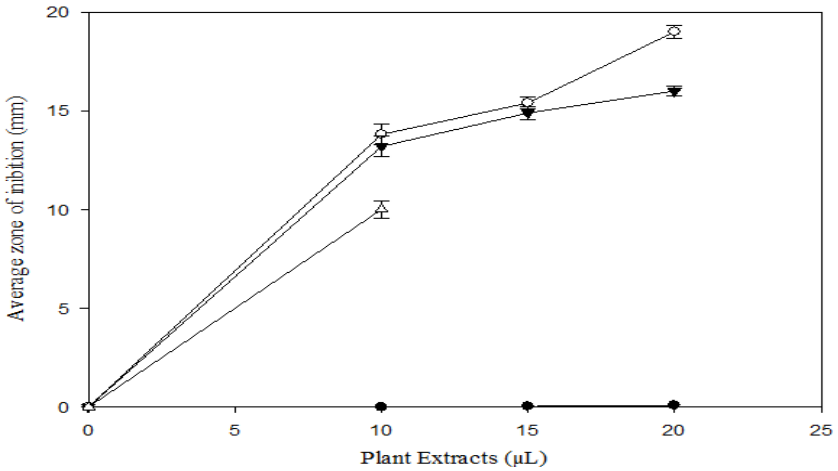

Figure 3. Average antibacterial activity (mm) of selected ethanomedicinal plants against *E. coli at* 10 µL, 15 µL and 20 µL through agar well diffusion assay technique, *A. calamous* (●), *C. intybus* (○), *F. indica* (▼), *Streptomycin* (4 µL) (△)

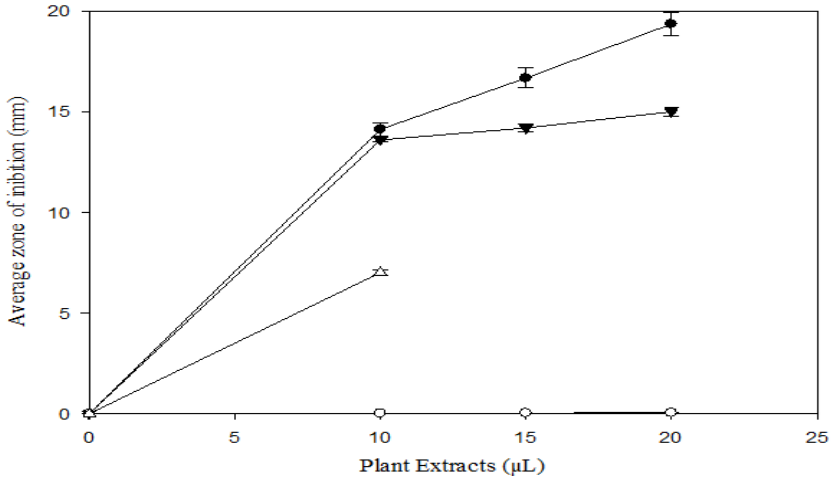

Figure 4. Average antibacterial activity (mm) of selected ethanomedicinal plants against *N. gonorrhea at* 10 µL, 15 µL and 20 µL through agar well diffusion method, *A. calamous* (●), *C. intybus* (○), *F. indica* (▼), *Streptomycin* (4 µL) (△)

The selected medicinal plants except *C. intybus* displayed antibacterial activity against *N. gonorrhoeae* at 10 µL, 15 µL and 20 µL concentration of their crude ethanolic extracts (Figure 4). *A.calamous* produced highest ZOI i.e. 14 mm, 16.67 mm and 19.34 mm at 10 µL, 15 µL and 20 µL, respectively. Streptomycin could only inhibit the bacterial activity on 7 mm ZOI at 10 µL concentration.

Discussion

The phytochemical screening of *A. calamus* revealed the presence of alkaloids (Hao *et al*., 2021), flavonoids, phenolic compounds, tannins, steroids, saponins, and terpenoids (Elshikh *et al*., 2022). The absence of anthraquinones in *A. calamus* was confirmed by the similar finding of Porwal *et al*. (2021). The results of this study for the presence of secondary metabolites in *Cichorium intybus* were similar to that of Choudhary *et al*. (2021). The absence of steroids, terpenoids and saponins in *F. indica* are in contrast with the observations of Ali *et al*. (2020), and Riaz *et al*. (2018). The phytochemical investigations revealed the differences in results as compared to previous studies which may be attributable to distribution of herbs in different geographical conditions; particularly, effect of climate on phytochemical composition (Dinchev *et al*., 2008).

Various previous studies have also supported the potential effect of the crude extract i.e. methanol, chloroform (Ali *et al*., 2020) and EtOAc (Riaz *et al*., 2019) of *F. indica* fractions against *E. coli*. Chicory plant is active against gram negative bacteria (Janda *et al*., 2021) but it produced least ZOI against *E. coli* as compared to the rest of the tested plant extracts. *A. calamus* ethanolic extract inhibited *E. coli*. *A. calamus* extract of 8 µL concentration was slightly higher than the activity of *F. indica* at 4 µL concentration which were similar to the findings of Maharjan *et al*. (2012) and Rahamoz-Haghighi *et al*. (2014). *F. indica* extract at a concentration of 100 mg/mL after 24 hours inoculation was fond to be the most effective against *N. gonorrhoeae* with zones of inhibition of 18.333 ± 2.081 at 8 µL concentration (Toor *et al*., 2015).

It is concluded that ethnomedicinal plants are still used in many parts of the world as safe and traditional drugs. The experimental design against the three selected plants that were believed to be medicinally effective, exhibited the antimicrobial activity and effectiveness of them against test microbes named as *N. gonorrhea* and *E. coli* which are among the most common microorganisms involved in urogenital infections. In disk diffusion assay *F.indica* was found to be the most effective whereas in agar well diffusion assay *A.calamous* was observed to be more active against test microorganisms

that may be depending upon the experimental techniques or environmental conditions. The phytochemical screening was carried out for identification of alkaloids, anthraquinones, flavonoids, saponins, steroids, tannins and terpenoids. It was found that *A.calamous* only lacked anthraquinones and showed positive results for the rest of phytochemicals. *C. Intybus* depicted positive results for all tests. Whereas, *F.indica* displayed negative results for saponins, steroids and terpenoids.

In the nutshell, A. calamus was found to be the best as compared to the rest of the tested plants on the bases of phytochemicals screening. However, the results of antimicrobial susceptibility through various techniques indicated that all tested plants were having good antimicrobial activity against urogenital infection causing agents i.e., *E. coli* and *N. gonorrhoeae.*

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