Original Research Article

Antifungal Activity of *Echinops echinatus* and *Fagonia cretica* from Cholistan Desert-Pakistan

ABSTRACT

Background: Fungal infections are getting worse due to their resistance against available antibiotics and have always remain a problem. Homoeopathic medicines made of mother tinctures of plants are one of natural ways of treatment with no side effects and have been proved effective against several infections.

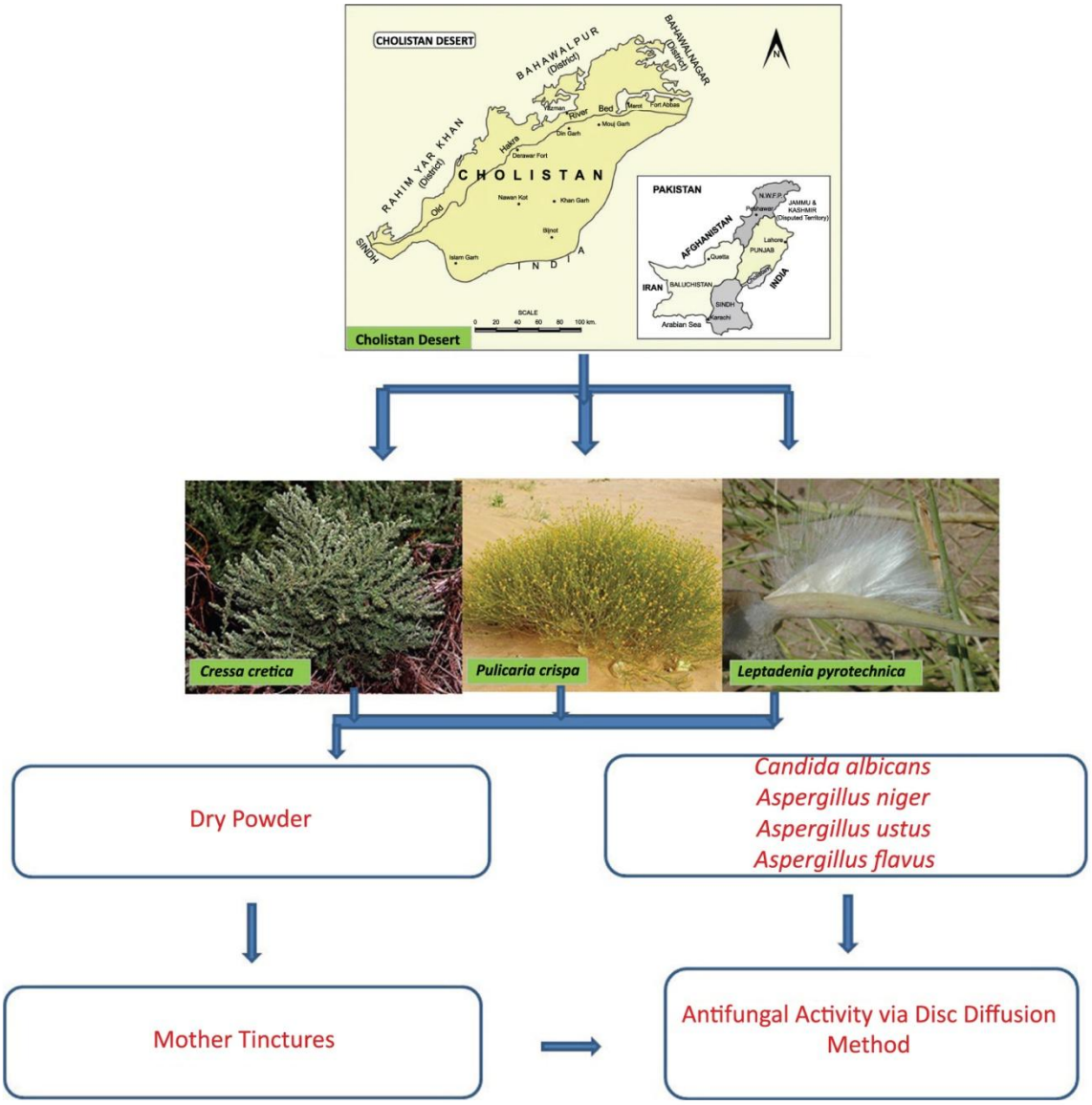
Methodology: Preparation of mother tinctures of plants *Echinops echinatus* and *Fagonia cretica* was done and used to evaluate the anti-fungal potential of these plants against potentially pathogenic fungal species like *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus ustus* and *Candida albicans* by agar disc diffusion method. Each tincture was evaluated at 0.25 ml, 0.5 ml and 1 ml volume per disc and zone of inhibition was measured in millimetres and compared with commercial drug Fluconazole (2 mg/ml) which was used as standard.

Results: The results indicated that both mother tinctures showed remakarble activity against pathogens. The mother tinctures showed activity ranging from zone of inhibition 17.33 – 30.33 mm.

Conclusion: Our study proved that mother tincture of these plants can be a potential new therapy to treat fungal infections and has the potential to rule out fungal problems. Further research using mother tincture of these plants against other fungal species has the potential to prove them a safer and widespread anti-fungal homoeopathic medicine. Due to these promising results, further *in vivo* studies over these plants can be conducted.

Keywords: Antifungal activity, Cholistan desert, homeopath mother tincture, *Echinops echinatus, Fagonia cretica*

GRAPHICAL ABSTRACT



Anti-Fungal Activity of *Cressa cretica*, *Leptadenia pyrotechnica* and *Pulicaria crispa*.

INTRODUCTION

Cholistan is called as Rohi desert in local area, that covers 26,000 km^2, having length of 480 km and width of 32-192 km near Bahawalpur city, Pakistan. This desert is rich in various untested medicinal plants and contains 128 species of desert plants, which belong to 32 families. Local herbalists are using these plants to treat many ailments. Many plants contains tannins,

sesquiterpenes, terpenoids, polyphenols, phenolics and flavonoids. They displayed antimicrobial properties against microrganisms (1).

Two medicinal plants obtained from Cholistan desert named as *Echinops echinatus* and *Fagonia cretica* were selected for antifunal activity. *Echinops echinatus* is related to the largest flowering plant families (Asteraceae) with about 3000 species, commonly known as "Brahmadandi" or "Utakatira". It is an xerophytic herbaceous plant, widely distributed in desert regions. Triterpenoids, alkaloids (2), acylflavone glucoside (3), isoflavone glycoside, echinosie, 7-hydroxyisoflavone, kaempferol, kaempferol-3-O-alpha-L-rhamnoside, kaempferol-4'-methylether, kaempferol-7-methylether and myrecetin-3-O-alpha-L-rhamnoside (4) have been isolated from *Echinops echinatus*. This plant diplayed some activities such as antifertility activity (5) and anti-inflammatory activity in rats (6). It is also used in treatment of sexual problems, leukorrhea, diabetes, eczema, skin papules, diarrhea, malarial fever, asthma, whooping cough, renal colic, heat stroke, jaundice, hysteria, dyspepsia, hoarseness of throat, polyurea, scrofula, migraine, heart diseases, joint pains, urinary infections and hemorrhoids. Its roots contains aphrodisiac properties as well as abortifacient. It is taken with honey to expel worms.

Antimicrobial activity of plant was also reported against *Alternaria tenuissima, Escherichia coli, Salmonella typhi* and *Pseudomonas aeruginosa*. Its antioxidant property was also reported in various *in-vitro* models. It also showed protective effect in prostate and liver. Its chloroform extract showed anti-irritant activity in *in-vivo* study (2). *Fagonia cretica* is related to family Zygophyllaceae (7). It has been used in eastern system of medicine for many ailments. It has been reported to contain various antioxidants (8). Plant contains natural compouds with potent DPP-4 inhibitory activity (9). This plant also showed antihaemorrhagic property against effect of black cobra venom (10). Plant also has antioxidant potential (11). Aqueous extract of plant showed antidiabetic activity (12). These plants were tested for their antifungal activity against notorious pathogens like *Candida albicans, Aspergillus niger, Aspergillus ustus* and *Aspergillus flavus.* In this study, their antifungal activity has been evaluated as they contain those compounds which have antimicrobial activity in other plants too.

MATERIALS & METHODS

Collection of Plant Material: All plant samples were collected from Shikarwala toba, near Fort Moj Garh, Cholistan, Desert Bahawalpur. The plants sample were identified and authenticated by Dr. Shazia Anjum Director Cholistan Institute of Desert Studies, The Islamia University,

Bahawalpur. The voucher specimens of *Echinops echinatus* (3513/CIDS/IUB) and *Fagonia cretica* (3515/CIDS/IUB) were deposited in the herbarium of CIDS, The Islamia University of Bahawalpur.

Preparation of Plant Material: Collected plant samples were crushed and washed to remove all the external dirt and unwanted material, and dried in open air under shade for 15 days and milled to fine powder in an electric grinder and stored in a well closed container (13).

Formation of Mother Tinctures: Powdered material of selected plants was used for preparation of Homeopathic mother tinctures. Mother tinctures were prepared by maceration process mentioned in Homoeopathic pharmacopeia. Plant material one part and solvent nine parts were used for preparation of mother tinctures. Solvents used were ethanol and distilled water (70% ethanol and 30% purified distilled water) (13).

Maceration: It is process in which medicinal part of plant is softened in solvent for a specific period of time under standered temperature and pressure. 100g of each powdered plant material was placed in a 2 liter glass flask. Powder was soaked for 15 days in 900 ml of 70% ethanol. Flask was sealed and kept in cool dark place. Flask was shaken daily for 10 minutes. After 15 days, soaked material of each plant was filtered through several layers of muslin cloth by coarse filtration. Coarse filtrate was then filtered through a Whatmann filter paper. Mother tincture so obtained was collected in glass containers (13).

Fungal Strains: Three species of *Aspergillus* i.e., *niger, flavus* and *ustus,* were obtained from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences (IAGS), University of Punjab Lahore. Whereas, *Candida albicans* strains were obtained from Microbiology department, Quaid-e-Azam Medical College (QMC) Bahawalpur, as well as from clinical isolates.

Preparation of the Medium: 65g of Sabouraud's Dextrose Agar (SDA) medium was dissolved in 1000 ml of distilled water. Medium was sterilized by autoclaving at 121^{o}C for 15 minutes at 15 psi pressure (13).

Antifungal Assay: Experiments were performed three times under strict aseptic conditions. Antifungal tests were done by using disc diffusion method. Mother tinctures were applied on these discs drop by drop. Discs with Fluconazole were used as positive standards. These impregnated discs were then aseptically transferred into Sabouraud's Dextrose Agar (SDA) plates freshly inoculated with test organisms *Candida albicans, Aspergillus niger, Aspergillus*

flavus and *Aspergillus ustus*. After autoclaving, media was poured into different petri plates and allowed to solidify. Fungi were then inoculated on agar plates by using sterilized culture swabs. Then, filter paper discs were placed in centre of petri plates by using sterilized forcep. Then, dropping of different concentrations of mother tinctures was done on discs. Three additional plates, one containing solvent, second containing positive control and third containing only fungi, were also maintained. Plates were incubated at 28^{o}C in incubator and observed after 48-72 hours. All experiments were done in triplicate. Antimicrobial activity was determined by measurement of inhibition of zone around each paper disc. Antifungal activity of mother tinctures was evaluated by disc diffusion method (13).

Statistical analysis: Statistical analysis was performed with Graph Pad software, all data of experimental groups were expressed as mean ± SEM in triplicate experiments. For statistical analysis, group means were compared by one-way ANOVA and Bonferroni's test was used to identify differences between groups. The threshold for statistical significance was set at $P<0.05$. Endnote was used to insert references.

RESULTS

Anti-fungal activity of two different plants, *Echinops echinatus* and *Fagonia cretica*, was evaluated by disc diffusion method against four common pathogenic fungal species *Aspergillus niger, Aspergillus flavus, Aspergillus ustus* and *Candida albicans.* Figure 1 shows the mean zone of inhibition data for three different dilutions of mother tincture of *Cressa cretica* against each of the fungal species *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus ustus* and *Candida albicans*. Images in figure 2 correspond to the same experiment and are illustrative plates showing the zone of inhibition created by mother tinctures. According to the data, the mother tincture showed good antifungal activity against all dilutions but the activity in 1ml volume was maximum for all.

In the case of mother tincture of *Echinops echinatus*, the best anti-fungal activity was obtained against *Aspergillus flavus*, considering the larger diameter of zone of inhibition, as compared to all other pathogens and this activity was higher for 1.0 ml dilution in comparison to the standard drug Fluconazole, as shown in figure.

In case of *Fagonia cretica* mother tincture, the best antifungal activity was observed against *Candida albicans* among all four pathogenic fungal species and this activity was slightly higher than our standard drug (figure 5).

DISCUSSION

The antifungal activity of the mother tincture was evaluated by the disc diffusion method against four fungal strains using *Fluconazole* as standards. In the screening, the mother tinctures showed strong antifungal activity with zone of inhibition of 17.33-30.33 mm. Antimycotic activity of mother tincture of *Echinops echinatus* was determined against *Aspergillus niger, Aspergillus flavus, Aspergillus ustus* and *Candida albicans,* in concentrations i.e., 0.25 ml, 0.50 ml and 1 ml concentrations per disc. Effects of drug of 0.25 ml and 0.50 ml on *Aspergillus niger* were same with mild difference and less significant. P value of both doses was less than 0.03, while concentration of 1 ml of tincture showed maximum zone of inhibition but level of antifungal activity was not significant with compared to positive control drug *Fluconazole*. Mother tincture of *Echinops echinatus* fungitoxic effect on *Aspergillus flavus* was considerable in concentrations of 0.25 ml and 0.50 ml and hydro alcoholic tincture of *Echinops echinatus* confirmed significant results. Antifungal activity of mother tincture against *Aspergillus ustus* was varied whereas lowest dose showed minimum inhibitory zone and high dose was maximum effects and all doses were significance and level of significant ranged from 0.03 to 0.05. Level of sensitivity of evaluated mother tincture of *Echinops echinatus* against *Candida albicans* was dose dependent and inhibitory of fungi in concentration of 0.25 ml and 0.50 ml were close to positive control. These results revealed with comparison of *Fluconazole* standard antifungal drug. Generally antifungal effects of *Echinops echinatus* mother tincture on *Aspergillus flavus* (28.91%) was maximum and *Candida albicans* (26%) was moderate while sensitivity of *Aspergillus ustus* (25.78%) and *Aspergillus niger* (23.34%) were minimum. These results are in line with those obtained by Mathur *et al*., which showed that hydro alcoholic extract of *Achyranthus asper* against *Candida albicans, Aspergillus niger* sensitivity was high while in present study, hydro alcoholic extract of *Echinops echinatus* revealed very close antimycotic effect against *Aspergillus Spp.* and *Candida albicans* (14). The antifungal activities of the mother tinctures were evaluated by the disc diffusion method against four fungal strains using *Fluconazole* as standards. In the screening, the mother tinctures showed strong antifungal activity with zone of inhibition of 19.00-37.00 mm while the highest antifungal activity was seen against *Candida albicans* and *Aspergillus ustus.* Fungitoxic activity of hydro alcoholic mother tincture of *Fagonia cretica* was tested against *Aspergillus niger, Aspergillus flavus, Aspergillus ustus* and *Candida albicans.* In concentrations of 0.25 ml, 0.50 ml and 1 ml, significant results were observed on fungi strains of *Aspergillus niger, Aspergillus flavus* and *Candida albicans*.

Level of antifungal activity in dose of 1 ml was significant in *Candida albicans* with compared to *Fluconazole*. Fungitoxic effects of mother tincture of *Fagonia cretica* on *Aspergillus niger*, *Aspergillus flavus, Aspergillus ustus* and *Candida albicans* were considerable and less significant however, hydro alcoholic extract of plant showed improved activity on *Aspergillus niger*, *Aspergillus ustus* and *Candida albicans* in dose of 1 ml as compared to activity of *Fluconazole* standard antifungal drug. On the whole, antimycotic effects of *Fagonia cretica* tincture on *Aspergillus ustus* (27.66%) and *Candida albicans* (26%) were maximum and on *Aspergillus niger* (23.83%) and *Aspergillus flavus* (22.46%) were minimum. Similar study of screening natural plant extracts against different fungal pathogens was well recorded in literature by Avasti *et al*. (2010) whereas aqueous extracts of *Trachyspermum ammi* showed 27.76 % inhibitory effects on *Aspergillus niger* in concentration of 10 %, while in present study, mother tincture of *Fagonia cretica* proved 23.83% antimycotic results which is very close to reported experiment.

CONCLUSION

The results show that the mother tinctures of all plants possessed antifungal activity against all the tested fungal strains. The isolation of active constituents showing antifungal activity can be more useful and work is to be done in this regard. In future, these plants may be used as in vivo in animal or man.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES

1. Ahmad M, Ghafoor N, Aamir M. Antibacterial activity of mother tinctures of cholistan desert plants in Pakistan. Indian journal of pharmaceutical sciences. 2012;74(5):465.

2. Maurya SK, Kushwaha AK, Seth A. Ethnomedicinal review of Usnakantaka (Echinops echinatus Roxb.). Pharmacognosy reviews. 2015;9(18):149-54.
3. Ram SN, Roy R, Singh B, Singh RP, Pandey VB. An acylflavone glucoside of Echinops echinatus flowers. Planta medica. 1996;62(2):187.
4. Singh S, Upadhyay RK, Pandey MB, Singh JP, Pandey VB. Flavonoids from Echinops echinatus. Journal of Asian natural products research. 2006;8(3):197-200.
5. Sharma KS, Mishra S, Mehta BK. Antifertility activity of Echinops echinatus in albino rats. Indian journal of medical sciences. 1988;42(2):23-6.
6. Singh B, Gambhir SS, Pandey VB, Joshi VK. Anti-inflammatory activity of Echinops echinatus. Journal of ethnopharmacology. 1989;25(2):189-99.
7. Qureshi H, Asif S, Ahmed H, Al-Kahtani HA, Hayat K. Chemical composition and medicinal significance of Fagonia cretica: a review. Natural product research. 2016;30(6):625-39.
8. Rawal A, Muddeshwar M, Biswas S. Effect of Rubia cordifolia, Fagonia cretica linn, and Tinospora cordifolia on free radical generation and lipid peroxidation during oxygen-glucose deprivation in rat hippocampal slices. Biochemical and biophysical research communications. 2004;324(2):588-96.
9. Saleem S, Jafri L, ul Haq I, Chang LC, Calderwood D, Green BD, et al. Plants Fagonia cretica L. and Hedera nepalensis K. Koch contain natural compounds with potent dipeptidyl peptidase-4 (DPP-4) inhibitory activity. Journal of ethnopharmacology. 2014;156:26-32.
10. Razi MT, Asad MH, Khan T, Chaudhary MZ, Ansari MT, Arshad MA, et al. Antihaemorrhagic potentials of Fagonia cretica against Naja naja karachiensis (black Pakistan cobra) venom. Natural product research. 2011;25(20):1902-7.
11. Iqbal P, Ahmed D, Asghar MN. A comparative in vitro antioxidant potential profile of extracts from different parts of Fagonia cretica. Asian Pacific journal of tropical medicine. 2014;7S1:S473-80.
12. Nazir I, Ur Rahman N, Alvi Z, Hafizur Rahman M, Sendker J, Zhang T, et al. Antidiabetic Activities of an LC/MS Fingerprinted Aqueous Extract of Fagonia cretica L. in Preclinical Models. Planta medica. 2017;83(14-15):1141-8.
13. Hadi F, Maqbool T, Khurshid S, Nawaz A, Aftab S, Tahir S, et al. Anti-Fungal Activity of Cressa cretica, Leptadenia pyrotechnica and Pulicaria crispa, Indigenous Plants of Cholistan Desert, Pakistan. Anti-Infective Agents. 2021;19(3):325-32.
14. Mathur A, Singh R, Yousuf S, Bhardwaj A, Verma SK, Babu P, et al. Antifungal activity of some plant extracts against clinical pathogens. Advances in Applied Science Research. 2011;2(2):260-4.

Table- 1: Composition of the Sabouraud's Dextrose Agar (SDA) medium

Sabouraud's Dextrose Agar (SDA) medium (1000 ml)	
Ingredients	**Amount**
Dextrose	40.0 gm
Peptone	10.0 gm
Agar	20.0 gm
Distilled water	q.s to 1000 ml
Ph	5.6

Table 2 Antifungal activity of *Echinops echinatus* mother tincture

Concentrations	***Aspergillus niger*** **Zone of Inhibition (mm)**	***Aspergillus flavus*** **Zone of Inhibition (mm)**	***Aspergillus ustus*** **Zone of Inhibition (mm)**	***Candida albicans*** **Zone of Inhibition (mm)**
0.25 ml	18.33 ± 0.88**	17.33 ± 0.88**	17.66 ± 0.88**	21.00 ± 1.15**
0.50 ml	20.33±1.76 **	20.33 ± 1.20 **	20.66 ± 0.88**	23.66 ± 2.02*
1.00 ml	22.33 ± 1.76*	27.66 ± 1.33	24.66 ± 1.76*	25.00 ± 2.08
Fluconazole (2 mg in 1 ml)	27.33 ± 0.881	28.66 ± 0.881	30.83 ± 1.364	30.33 ± 0.333

*≤ 0.05, **≤ 0.01

Table 3 Antifungal activity of *Fagonia cretica* mother tincture

Concentrations	***Aspergillus niger*** **Zone of inhibition (mm)**	***Aspergillus flavus*** **Zone of inhibition (mm)**	***Aspergillus ustus*** **Zone of inhibition (mm)**	***Candida albicans*** **Zone of inhibition (mm)**
0.25 ml	21.33 ± 1.452**	19.00 ± 0.57**	27.66 ± 0.66	29.00 ± 1.45
0.50 ml	24.66 ± 0.88	22.33 ± 1.33 *	30.66 ± 0.88	31.66 ± 0.88

1.00 ml	29.00 ± 1.154	27.33 ± 1.45	33.66 ± 0.333	37.00 ± 1.154**
Fluconazole (2 mg in 1 ml)	27.33 ± 0.881	28.66 ± 0.881	30.83 ± 1.364	30.33 ± 0.333

*≤ 0.05, **≤ 0.01

Figure Legends:

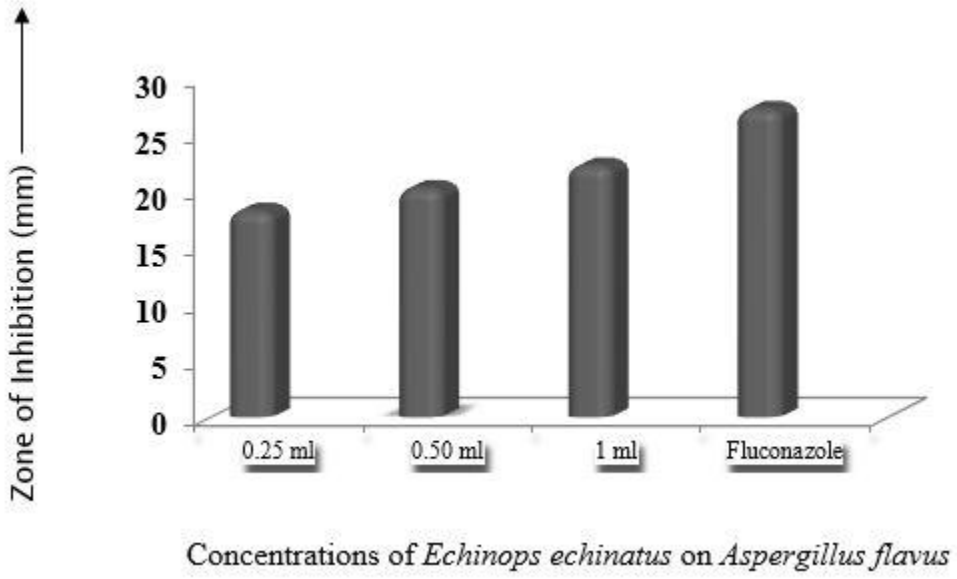


Figure 1: Effect of different concentrations of *Echinops echinatus* on *Aspergillus niger*.

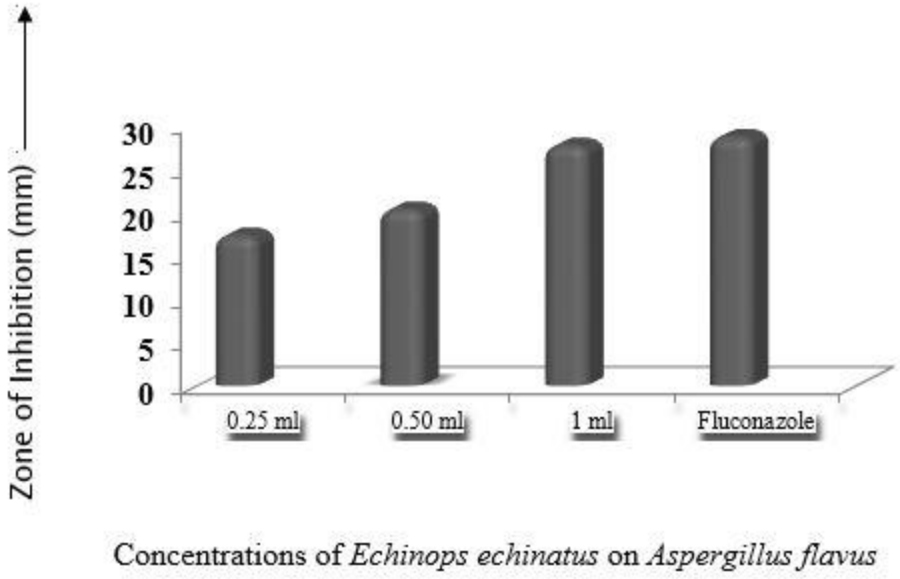


Figure 2: Effect of different concentrations of *Echinops echinatus* on *Aspergillus flavus.*

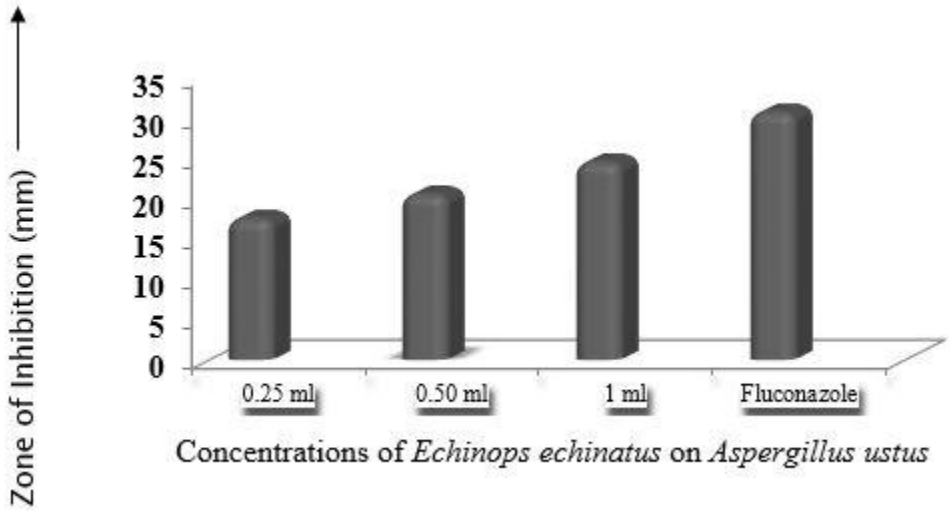


Figure 3: Effect of different concentrations of *Echinops echinatus* on *Aspergillus ustus.*

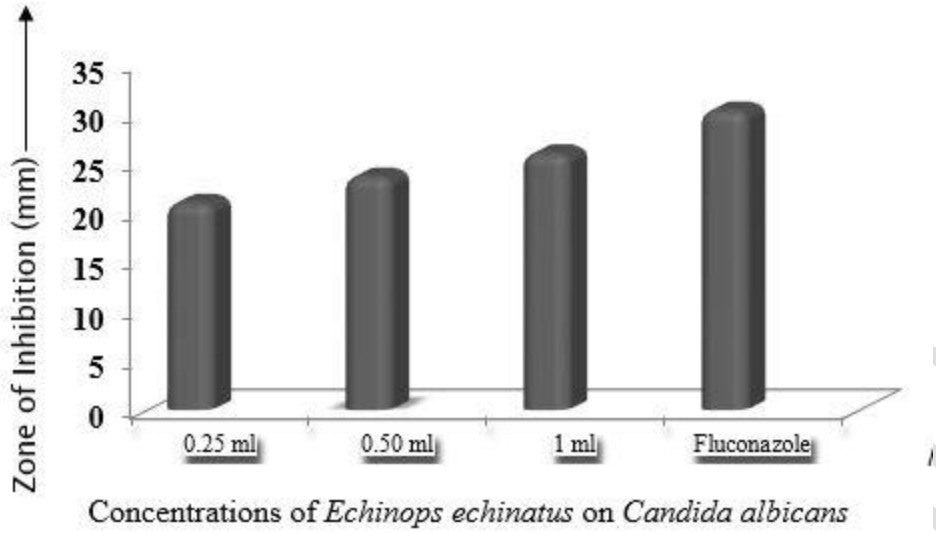


Figure 4: Effect of different concentrations of *Echinops echinatus* on *Candida albicans.*

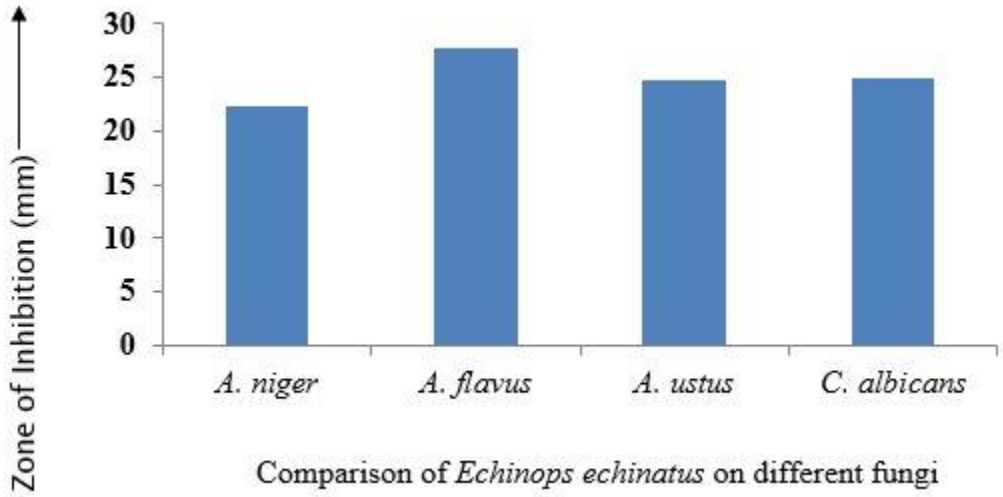


Figure 5: Antifungal activity of mother tincture of *Echinops echinatus* on different strains of fungi.

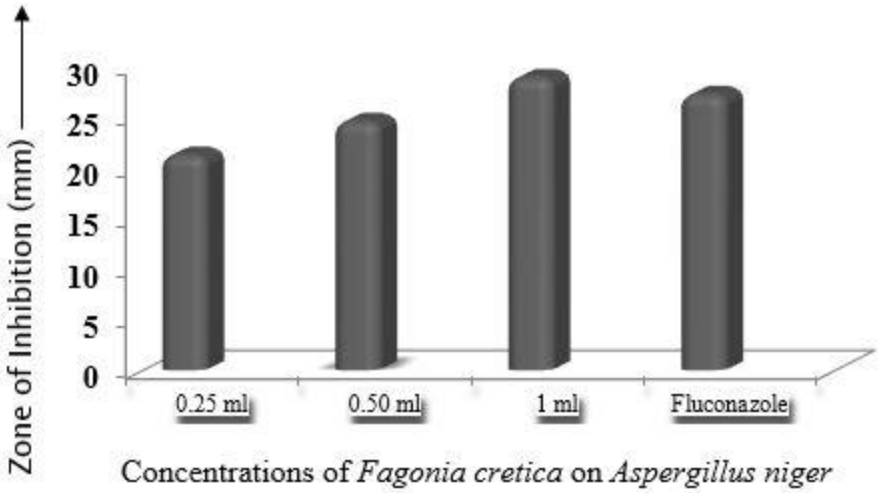


Figure 6: Effect of different concentrations of *Fagonia cretica* on *Aspergillus niger.*

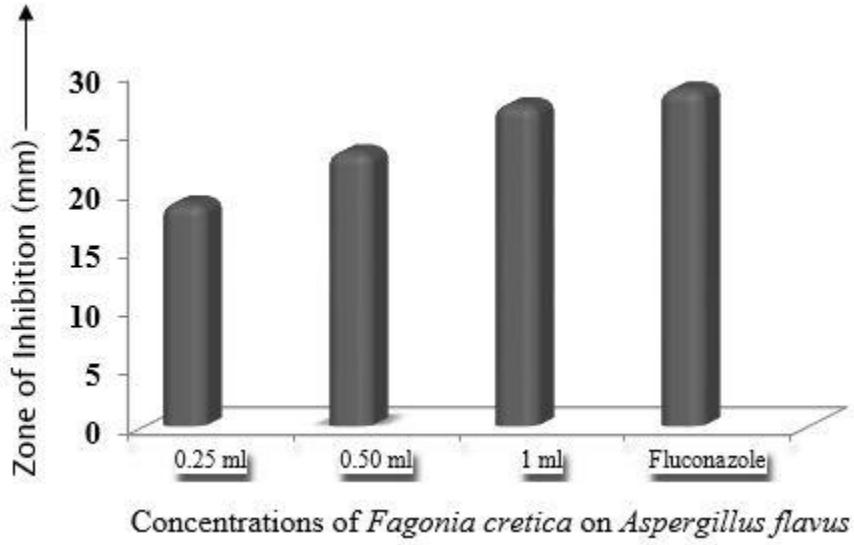


Figure 7: Effect of different concentrations of *Fagonia cretica* on *Aspergillus flavus*

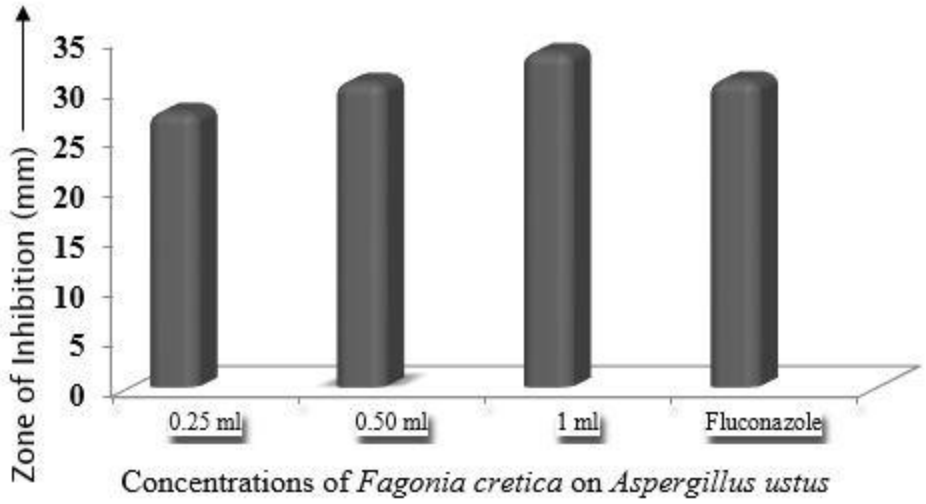


Figure 8: Effect of different concentrations of *Fagonia cretica* on *Aspergillus ustus*

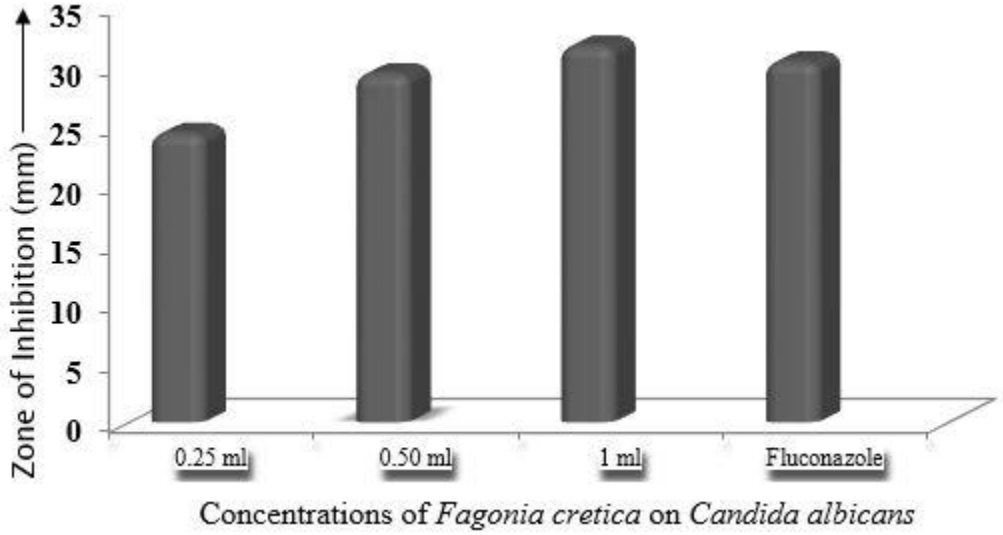


Figure 9: Effect of different concentrations of *Fagonia cretica* on *Candida albicans*

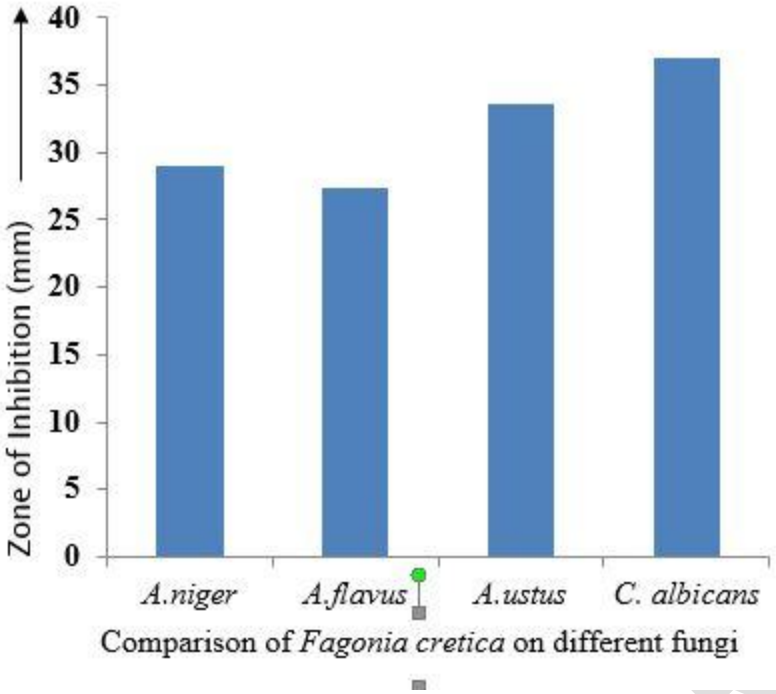


Figure 10: Antifungal activity of mother tincture of *Fagonia cretica* on different strains of fungi.