

In vitro evaluation of antifungal activity of aerial parts of medicinal plants *Balanites aegyptiaca* Del. and *Spilanthes acmella* Murr.

Razia Khatoon^{1,2*}, Noor Jahan^{1,2}, Siraj Ahmad³, Anwar Shahzad⁴

¹Department of Microbiology, Era's Lucknow Medical College and Hospital, Lucknow-226003, India. ²Department of Microbiology, Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh-202002, India. ³Department of Community Medicine, Teerthanker Mahaveer Medical College and Research Centre, Teerthanker Mahaveer University, Moradabad-244001, India. ⁴Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh-202002, India.

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ABSTRACT

Aqueous and alcoholic extracts of endangered medicinal plants *Balanites aegyptiaca* Del. and *Spilanthes acmella* Murr. were evaluated for antifungal potential against various pathogenic and opportunistic fungi by in vitro agar well diffusion method. All the alcoholic extracts showed wide range of activity against the tested fungi as compared to aqueous extracts which showed limited antifungal activity. The alcoholic extract of fruit of *B. aegyptiaca* showed good antifungal activity against most of the tested *Candida* species and few opportunistic fungi, whereas, alcoholic extract of flower head of *S. acmella* showed good activity not only against *Candida* species but also against most of the tested *Aspergillus* species. Minimum inhibitory concentrations (MIC) of the alcoholic extracts were determined by broth microdilution method. The MIC of alcoholic extract of *B. aegyptiaca* and *S. acmella* against tested fungi ranged from 3.05 to 24.0 µg/ml and 1.53 to 49.0 µg/ml respectively. The present study leads to conclusion that extracts of *Balanites aegyptiaca* and *Spilanthes acmella* contain good antifungal activity and thus could be used as alternative medicine in the treatment of various opportunistic and life threatening fungal infections especially in immunocompromised patients which otherwise pose problem of resistance to the currently used antifungal agents.

INTRODUCTION

The fungi cause opportunistic infections in immunocompromised patients like those suffering from AIDS, cancer, diabetes, or undergoing treatment with immunosuppressive agents after transplant surgeries. Amongst these, candidiasis has become a major public health problem as it is one of the leading causes of fungal infection in immune-suppressed population, particularly in AIDS patients leading to oropharyngeal and oesophageal candidiasis (Al Ashaal *et al.*, 2010). A vast majority of synthetic antibiotics control the growth and development of microorganisms effectively, but they are highly toxic at their optimum dosage level (Reddy, 2009). Also, antibiotic usage for the prevention and treatment of infections in these high-risk patients leads to selection pressures resulting in.

the emergence and spread of resistant organisms (Panghal *et al.*, 2011). This situation of increasing failure of chemotherapeutics and antibiotic resistance as well as the undesirable effects of certain antifungal agents has forced the researchers to search for new antimicrobial substance from various sources including medicinal plants (Phongpaichit *et al.*, 2005; Parekh and Chanda, 2008). Antimicrobials of plant origin have proved to be effective in the treatment of several infectious diseases and also they produce fewer side effects as compared to the synthetic antimicrobials (Samy and Ignacimuthu, 2000).

Balanites aegyptiaca Del. an endangered medicinal plant belongs to family Zygophyllaceae. It is also known as 'desert date' in English and 'Hingoli' in Hindi. The plant is a small evergreen thorny tree found in drier parts of India and Africa (particularly in Sudan). It grows to 6-10 m in height, is highly resistant to stresses such as sandstorms and heat waves, and grows with minimal available moisture. The trees produce date-like fruits between March and October.

* Corresponding Author

*Dr. Razia Khatoon, Department of Microbiology, Era's Lucknow Medical College and Hospital, Lucknow-226003, India.

Email:- drrazia@rediffmail.com

All parts of this plant have medicinal properties and have been used as folk medicine in many regions of Africa and Asia. Literatures have revealed that bark, unripe fruits, and leaves of this plant have anthelmintic, antifertility, purgative, antifeedant, antidiabetic, molluscicide, contraceptive, anti-tumor and antidysenteric properties. Dried fruits of this plant are being used as abortifacient by local healers. This plant has also been used in treatment of various ailments such as stomach aches, syphilis, epilepsy, haemorrhoids, malaria, herpes zoster, and yellow fever (Mohamed *et al.*, 2000; Ojo *et al.*, 2006; Gaur *et al.*, 2008; Gnoula *et al.*, 2008).

Spilanthes acmella Murr. an endangered plant belongs to the family Asteraceae. It is a flowering herb native to Brazil, and is grown as an ornamental plant in various parts of the world. It is found throughout India, up to 5000 feet in the Himalayas and other mountains. Its growth peaks in November to March and requires frequent watering. It is commonly known as 'Akarkara' or 'toothache plant'.

The entire plant is medicinally active and non-toxic to humans. The Indian traditional healers use the flower heads of this plant in dental and gum care. It is one of the major ingredients in popular herbal tooth powders and paste. The flower heads and roots have been used for treatment of scabies, psoriasis, scurvy, toothache, infections of gums and throat, paralysis of tongue and as a remedy for stammering in children. Its extracts also possess properties which cause repair of functional wrinkles, hence it is used in anti-age creams. The leaves and flower heads contain analgesic, antifungal, anthelmintic, antimalarial, antibacterial, diuretic and immunostimulating activity (Ratnasooriya *et al.*, 2004; Rani and Murty, 2006; Barman *et al.*, 2009; Prachayasittikul *et al.*, 2013).

The present study was carried out to evaluate the antifungal potential of medicinal plants *Balanites aegyptiaca* and *Spilanthes acmella* by testing their activity against an exhaustive range of fungal isolates, including both standard as well as clinical strains.

MATERIALS AND METHODS

Collection of plant materials

Fruit pulp of a 15 years old plant of *Balanites aegyptiaca* was obtained from Tissue culture Laboratory, Department of Botany, Gujarat University, Ahmedabad, and young flower head was collected from 4 months old plant of *Spilanthes acmella* grown in the Medicinal Plant Nursery of Tamnaar, Raipur, Chhattisgarh.

Preparation of plant extracts

Both aqueous and alcoholic extracts of the plants were tested for antifungal activity. The extracts were prepared according to the method of Singh and Singh (2000) with some modifications as described below. To prepare aqueous extracts, fresh fruit (15 g) of *B. aegyptiaca* and flower head (15 g) of *S. acmella* were taken and surface sterilized in 70% ethyl alcohol for 1 min and then

washed 3 times with sterilized double distilled water (DDW). These were then grounded with sterilized pestle and mortar in 150 ml of DDW separately. The homogenized tissues were then centrifuged at 5000 rpm for 15 min and the supernatant was filtered and taken as aqueous extract. Similarly, alcoholic extracts were prepared using 150 ml of 95% ethanol in place of DDW. The extracts were immediately used for experimentation.

Fungi Tested

The clinical fungal strains tested were *Candida albicans*, *Candida parapsilosis*, *Candida krusei*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Alternaria* spp., *Penicillium* spp. and *Fusarium* spp. isolated from various clinical specimens in the Department of Microbiology, Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh, India. The fungal control strains included in our study were *C. parapsilosis* (ATCC 22019), *C. krusei* (ATCC 6258) and *A. fumigatus* (ATCC 204305), obtained from New Drug Discovery Research, Ranbaxy, Gurgaon, India. The fungi were grown at 25°C in biological oxygen demand incubator and maintained on Sabouraud's Dextrose agar slants.

Antifungal susceptibility testing

Antifungal activity of the extracts of these plants was determined on Sabouraud's Dextrose agar (SDA) plates by using agar well diffusion method (Akinpelu, 2001), with some modifications as described below. An inoculum size of 2×10^6 yeast cells or fungal spores was used for inoculating the susceptibility plates.

Two sets of SDA plates (one each for aqueous and alcoholic extracts) were lawn cultured with fungal suspensions with the help of sterile swabs. Wells of 5mm diameter were made in each plate using a sterile borer. Plant extracts (20µl) were poured in the wells using micropipette. Sterilized DDW and 95% ethanol (20µl each) were used as negative controls in the aqueous and alcoholic plates respectively. A broad spectrum antifungal agent voriconazole (500µg/20µl) was used as positive control. The plates were kept upright for 5-10 min until the solution diffused into the medium and then incubated aerobically at 25°C in a biological oxygen demand (BOD) incubator for 2-5 days. Later, the zone of inhibition was measured and recorded. All experiments were performed in triplicate.

Determination of minimum inhibitory concentrations (MIC)

MICs of the alcoholic extracts were determined by broth micro-dilution method performed according to Clinical and Laboratory Standards Institute (CLSI), formerly known as National Committee for Clinical Laboratory Standards, NCCLS (1997) for yeasts and NCCLS (2002) for filamentous fungi. Doubling dilutions of the extracts were prepared using RPMI-1640 (HiMedia, India) broth supplemented with 0.3g/L L-glutamine (HiMedia, India), 0.165mol/L of 3-[N-morpholino] propanesulfonic acid (MOPS) buffer (HiMedia, India) and 0.01% of Dimethyl sulphoxide (DMSO) (Qualigens Fine Chemicals,

India). Extracts were dissolved in DMSO, and further diluted 1:50 in RPMI-1640 medium, and each resulting solution was used for a doubling dilution series. Microtitre plates were prepared containing 100µl of undiluted extracts in the first well, followed by doubling dilutions of extracts. The standardized inoculum of each fungal species was added to the respective dilution wells, including the first well.

The final concentrations of the extracts ranged from $25 \times 10^3 \mu\text{g/ml}$ to $48 \times 10^3 \mu\text{g/ml}$. For each test there was a sterility control well containing alcoholic extract in RPMI-1640 broth plus DMSO and a growth control well containing fungal suspension without alcoholic extract. The microtitre plates were incubated at 35°C for 48 hours with their upper surface covered by sterile sealers. The lowest concentration of the extract that did not show any visible growth of the tested fungal species was considered the MIC of the extract for that fungus. All the MIC experimentations were performed in duplicate.

Statistical analysis

All the experiments of antifungal susceptibility testing were performed in triplicate. The results were expressed as the mean \pm standard error (SE). Data were analyzed statistically by one way analysis of variance (ANOVA) followed by Tukey's multiple analysis test (SPSS Software, Chicago, III, version 10). P values were calculated by one-sample T-test and $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Antifungal activities of aqueous as well as alcoholic extracts of fruit of *B. aegyptiaca* and flower head of *S. acmella* against the tested fungal species are shown in Table 1 and 2 respectively. Amongst the negative controls used, sterilized DDW did not show any zone of inhibition, whereas, absolute ethanol showed the zone of inhibition in the range of 0.00 to 8.67 ± 0.33 mm. Positive control (voriconazole) showed the zone of inhibition in the range of 9.33 ± 0.33 to 13.00 ± 0.58 mm. Alcoholic extracts of both the plants showed better antifungal activity as compared to their aqueous extracts. The alcoholic fruit extract of *B. aegyptiaca* showed significant antifungal activity ($P < 0.05$) against *Candida albicans* ($P=0.003$), *Candida parapsilosis* ($P=0.012$) and *Penicillium* spp. ($P=0.017$), and its MIC ranged from 3.05 to 24.0 µg/ml (Figure 2).

The aqueous fruit extract of *B. aegyptiaca* showed mild but significant activity against *Candida albicans* ($P=0.038$) and *Penicillium* spp. ($P=0.024$). It is interesting to note that extracts of *B. aegyptiaca* effectively controlled the growth of most of the organisms which are important fungal pathogens responsible for causing opportunistic fungal infections in immune compromised patients especially those suffering from AIDS.

Various studies have been done previously by different researchers to analyze the antifungal potential of *B. aegyptiaca* (Al Ashaal, 2010; Panghal *et al.*, 2011). They showed significant antifungal activity of this plant against *Candida albicans*, this

supports our present research findings. On the other hand a study done by Abdallah *et al.*, (2012) showed significant antifungal activity of this plant against *Aspergillus niger* and *Fusarium* species. These findings are in contrast with our study. This variation in findings could be due to different concentrations of extracts used in their study as well as variation in active metabolites present in plant extracts derived from different places.

The alcoholic extract of flower head of *S. acmella* showed significant ($P < 0.05$) activity against *Candida krusei* ($P=0.007$), *Candida parapsilosis* ($P=0.005$), *Aspergillus fumigatus* ($P=0.012$), *Aspergillus niger* ($P=0.027$) and *Fusarium* spp. ($P=0.038$), and its MIC ranged from 1.53 to 49.0 µg/ml (Figure 3). Aqueous extract of flower head of *S. acmella* showed significant antifungal activity against *Candida krusei* ($P=0.024$) and *Aspergillus fumigatus* ($P=0.038$). One may note that the organisms which are found to be susceptible to the extracts of *S. acmella* are important pathogens responsible for causing opportunistic fungal infections such as Aspergilloma (fungal ball) in patients suffering from pulmonary tuberculosis and carcinoma lung, thus increasing the problem of treatment of such high-risk patients.

Few studies have been conducted in the past to evaluate the antifungal potential of *Spilanthes acmella* (Phongpaichit *et al.*, 2005; Rani and Murty, 2006). They showed significant activity of extract of *Spilanthes acmella* against *Aspergillus niger* and *Fusarium* species but no activity against *Candida albicans*. This supports our present research findings.

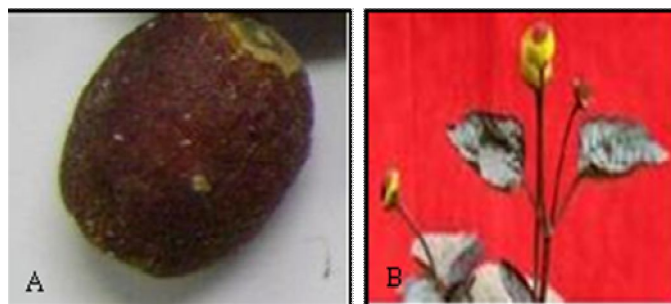


Fig. 1: shows various plant materials included in our study. A) Fruit of *Balanites aegyptiaca* and B) Flower of *Spilanthes acmella*.

CONCLUSION

In nutshell, alcoholic extracts of *Balanites aegyptiaca* and *Spilanthes acmella* contain remarkable antifungal potentials against most of the important fungal pathogens responsible for causing life threatening opportunistic fungal infections in immune-suppressed patients such as those suffering from immunodeficiency diseases, cancer, diabetes and AIDS. Though synthetic antibiotics have been developed for their treatment but at optimum dosage level these antifungal agents usually produce undesirable side effects. Also, resistance to even newly synthesized antifungal agents is on rise. Hence, in future extracts derived from both these plants may be used as novel antifungal agents. As these are herbal in nature, they may be more easily affordable and quite acceptable to such vulnerable patients.

Table 1: Antifungal activity of *Balanites aegyptiaca* against pathogenic and opportunistic Fungi.

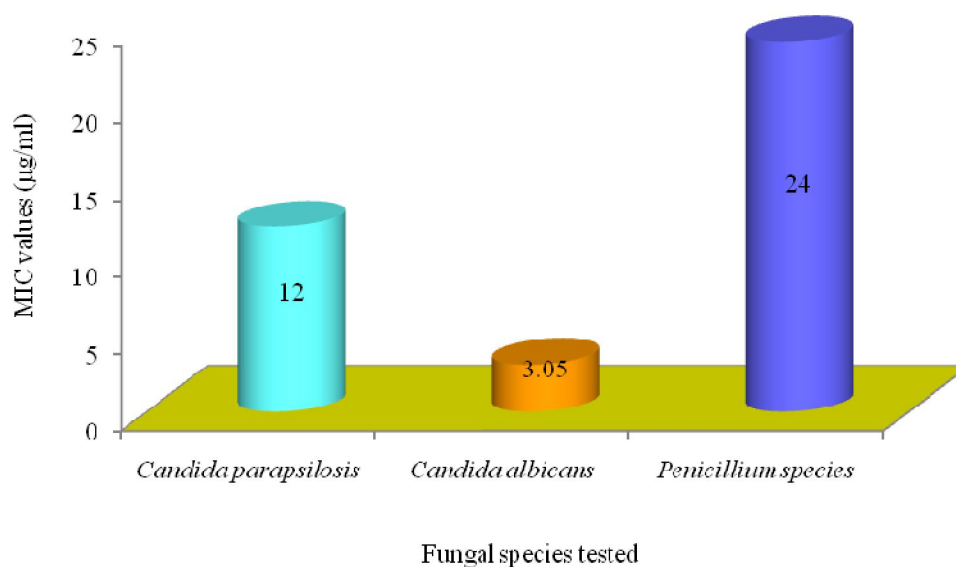
Fungi tested	Zone of inhibition (mm) ± SE				
	Alcoholic fruit extract ^Δ	Aqueous fruit extract ^Δ	DDW [†] (negative control)	Ethanol [†] (negative control)	Voriconazole [£] (positive control)
<i>Candida albicans</i>	15.33±0.67 ^a	14.67±0.33 ^a	0.00±0.00 ^a	8.33±0.33 ^b	12.33±0.33 ^b
<i>Candida krusei</i>	0.00±0.00 ^d	0.00±0.00 ^c	0.00±0.00 ^a	7.67±0.33 ^c	11.67±0.33 ^c
<i>Candida parapsilosis</i>	14.67±0.33 ^{ab}	0.00±0.00 ^c	0.00±0.00 ^a	8.67±0.33 ^a	12.67±0.33 ^{ab}
<i>Aspergillus fumigatus</i>	0.00±0.00 ^d	0.00±0.00 ^c	0.00±0.00 ^a	7.67±0.33 ^c	10.33±0.33 ^{de}
<i>Aspergillus flavus</i>	0.00±0.00 ^d	0.00±0.00 ^c	0.00±0.00 ^a	7.33±0.33 ^d	9.67±0.33 ^e
<i>Aspergillus niger</i>	0.00±0.00 ^d	0.00±0.00 ^c	0.00±0.00 ^a	0.00±0.00 ^e	9.33±0.33 ^f
<i>Alternaria</i> spp.	0.00±0.00 ^d	0.00±0.00 ^c	0.00±0.00 ^a	0.00±0.00 ^e	9.67±0.33 ^e
<i>Penicillium</i> spp.	13.00±0.58 ^c	12.67±0.33 ^b	0.00±0.00 ^a	8.33±0.33 ^b	11.67±0.33 ^c
<i>Fusarium</i> spp.	0.00±0.00 ^d	0.00±0.00 ^c	0.00±0.00 ^a	0.00±0.00 ^e	9.33±0.33 ^f
<i>C. parapsilosis</i> (ATCC 22019)	14.33±0.33 ^b	0.00±0.00 ^c	0.00±0.00 ^a	8.67±0.33 ^a	13.00±0.58 ^a
<i>C. krusei</i> (ATCC 6258)	0.00±0.00 ^d	0.00±0.00 ^c	0.00±0.00 ^a	8.33±0.33 ^b	12.33±0.33 ^b
<i>A. fumigatus</i> (ATCC 204305)	0.00±0.00 ^d	0.00±0.00 ^c	0.00±0.00 ^a	7.67±0.33 ^c	10.67±0.33 ^d

† = concentration of negative controls used in test i.e. 20 µl each of DDW and 95% ethanol. Δ = concentration of extracts used in the test i.e. 2 mg / 20 µl. £ = concentration of voriconazole used in test i.e. 500 µg / 20 µl. Diameter of zone of inhibition is a mean of triplicates ± SE (mm). Differences were assessed statistically using one way ANOVA followed by Tukey's test. P<0.05 was considered as significant. The mean represented by same letter is not significantly different within the column.

Table 2: Antifungal activity of *Spilanthes acmella* against pathogenic and opportunistic Fungi.

Fungi tested	Zone of inhibition (mm) ± SE				
	Alcoholic flower head extract ^Δ	Aqueous flower head extract ^Δ	DDW [†] (negative control)	Ethanol [†] (negative control)	Voriconazole [£] (positive control)
<i>Candida albicans</i>	0.00±0.00 ^f	0.00±0.00 ^e	0.00±0.00 ^a	8.33±0.33 ^b	12.33±0.33 ^b
<i>Candida krusei</i>	13.33±0.33 ^{bc}	12.33±0.33 ^b	0.00±0.00 ^a	7.67±0.33 ^c	11.67±0.33 ^c
<i>Candida parapsilosis</i>	15.00±0.58 ^{ab}	0.00±0.00 ^e	0.00±0.00 ^a	8.67±0.33 ^a	12.67±0.33 ^{ab}
<i>Aspergillus fumigatus</i>	12.33±0.33 ^{cd}	11.00±0.58 ^d	0.00±0.00 ^a	7.67±0.33 ^c	10.33±0.33 ^{de}
<i>Aspergillus flavus</i>	0.00±0.00 ^f	0.00±0.00 ^e	0.00±0.00 ^a	7.33±0.33 ^d	9.67±0.33 ^e
<i>Aspergillus niger</i>	11.33±0.33 ^d	0.00±0.00 ^e	0.00±0.00 ^a	0.00±0.00 ^e	9.33±0.33 ^f
<i>Alternaria</i> spp.	0.00±0.00 ^f	0.00±0.00 ^e	0.00±0.00 ^a	0.00±0.00 ^e	9.67±0.33 ^e
<i>Penicillium</i> spp.	0.00±0.00 ^f	0.00±0.00 ^e	0.00±0.00 ^a	8.33±0.33 ^b	11.67±0.33 ^c
<i>Fusarium</i> spp.	10.67±0.33 ^e	0.00±0.00 ^e	0.00±0.00 ^a	0.00±0.00 ^e	9.33±0.33 ^f
<i>C. parapsilosis</i> (ATCC 22019)	15.33±0.33 ^a	0.00±0.00 ^e	0.00±0.00 ^a	8.67±0.33 ^a	13.00±0.58 ^a
<i>C. krusei</i> (ATCC 6258)	13.67±0.33 ^b	12.67±0.33 ^a	0.00±0.00 ^a	8.33±0.33 ^b	12.33±0.33 ^b
<i>A. fumigatus</i> (ATCC 204305)	12.67±0.33 ^c	11.33±0.33 ^c	0.00±0.00 ^a	7.67±0.33 ^c	10.67±0.33 ^d

† = concentration of negative controls used in test i.e. 20 µl each of DDW and 95% ethanol. Δ = concentration of extracts used in the test i.e. 2 mg / 20 µl. £ = concentration of voriconazole used in test i.e. 500 µg / 20 µl. Diameter of zone of inhibition is a mean of triplicates ± SE (mm). Differences were assessed statistically using one way ANOVA followed by Tukey's test. P<0.05 was considered as significant. The mean represented by same letter is not significantly different within the column.

**Fig. 2:** MIC determination of alcoholic fruit extract of *Balanites aegyptiaca* against tested pathogenic and opportunistic fungi.

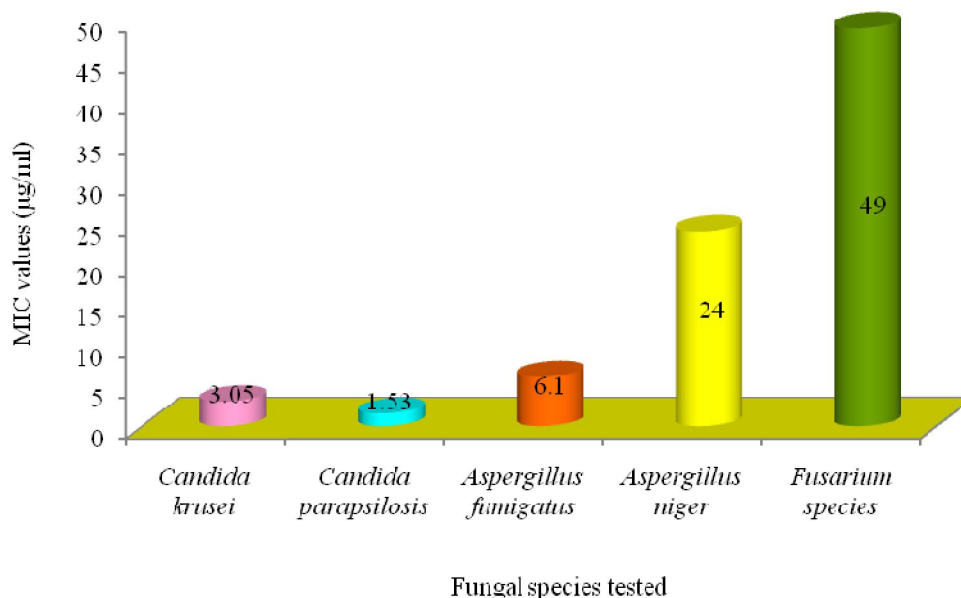


Fig. 3: MIC determination of alcoholic flower head extract of *Spilanthes acmella* against tested pathogenic and opportunistic fungi.

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