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ORIGINAL RESEARCH ARTICLE

In vitro screening for Antimycotic Activity of Various Extracts of *Centratherum anthelminticum* Seeds by the Microtiter Plate Based Assay

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ABSTRACT

Plants are the natural and traditional source of the drug. Investigations were carried out to evaluate the anti mycotic properties of the various extract of the seeds of *Kalijiri (Centratherum Anthelminticum)*. The dried fruits were successively extracted with ethanol, petroleum ether, hexane, chloroform, diethyl ether using Soxhlet Apparatus. All the extracts and powdered drug were screened for its antimycotic activity using Microtiter plate based assay method by the CLSI (Previously NCCLS) protocol M27 – A2. Amphotericin B was used as a standard drug as a positive control. IC_{50} value was calculated using the software Graphpad Prism *ver.* 5.0. During study we found that Ethanolic extract showed most potent activity against *Candida tropicalis* and *Candida parapsilosis* while chloroform extract, N-Hexane extract, Diethyl ether and petroleum ether extract. Ethanolic extract have also shown significant activity against Aspergillus fumigatus, Issatchenkia orientalis, and Candida albicans. Chloroform extract shown potent activity against Aspergillus fumigates and less potent activity against Candida Albicans, Candida parapsilosis, Candida tropicalis and Issatchenkia orientalis. Diethyl ether, N-Hexane extract and petroleum ether extract have not shown significant activity against any fungal strains. Further study should be done with separated and purified phytochemical constituent which is responsible for antimycotic activity of the extract.

Key Words: *Centratherum Anthelminticum,* Antimycotic Acitivity, Microtiter Plate based assay.

INTRODUCTION

Kaliziri is an Indian medicinal plant though it is vastly used in culinary purposes. The seeds of Kaliziri are generally popular for its usage in culinary practices and the entire plant is considered to be useful for treating diseases. Kaliziri is scientifically named as *Centratherum anthelminticum* Kuntze [1].

Fig 1: Seeds of *Kalijiri*

Centratherum anthelminticum (Kalajiri, Somraj) of the Asteraceae family is an erect, annual herb. It has been used for a number of medicinal purposes since ancient times as its seeds are anthelmintic and antiseptic. They are used to cure kidney troubles, asthma, hiccups, sores and white leprosy. Powdered seeds are also used externally to treat paralysis of legs. Roots are useful in curing diarrhea, stomachache, ulcers and cough and the flowers are purgative [2].

Centratherum anthelminticum seeds contain 18% fixed oil and .02% volatile oil and also reported to contain flavonoids. The major constituents are, 7(Z)(28)-Stigmastadienol, 5-stigmasten-3β-ol, 7,22-stigmastadienol, 8,14(Z)-24(28)-stigmastatrienol acetate (Frost *et al.*, 1970), verovan, p-hydroxybenzoyl vernovan, vernodalin, butein, 3,4,2‘,4‘,5‘-pentahydroxy-6‘-methoxy-2-methylchalcone, daucosterol, vernolic acid, acacetin-7-0-β-D-glucopyranosyl(1—4)-α-D-xylopyranoside.and others like stigmasterol, brassicasterol, 4α-methylvernosterol, vernosterol, avenosterol, (24αS)-stigmasta-5,22-dien-3β-ol, (24αS)-stigmasta-7,22-dien-3β-ol, (24αR)-

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stigmasta-7-en-3-one, (24αR)-stigmasta-7,9(11)-dien-3one: βamyrin; linoleic acid, oleic acid and sugars [3].

Ethanolic extract of seeds showed anthelmintic, hypotensive and laxative effects in rats. Ethanolic and ethanol: water (l: 1) extracts of seeds showed smooth muscle stimulant activity in guineapigs. Ethanol extract of seeds showed spermicidal activity in rats. Acetone, ethyl acetate and methanol extracts of seeds showed inhibition of spontaneous activity of whole worm as well as nerve muscle preparation of *Setaria cervi*. The alcoholic, ethereal and aqueous extract of seeds showed *In Vitro* anthelmintic activity against *Hymenolepis nana, Fasciolopsis buski* and *Ascaris lumhricoides* and *In Vivo* activity against *H. nana*. Further, the alcoholic extract also showed *In Vivo* anthelmintic activity against *F. buski* [4].

The search for biologically active natural products complements the traditional chemically oriented isolation of phytochemicals. A large number of bioassays are available for the testing of plant extracts and isolated compounds like bioautography, agar well diffusion method, Microtiter plate based assay [5].

The National Committee for Clinical Laboratory Standards (NCCLS) has set benchmark methodology by providing laboratory tested reproducible, consensus peer-reviewed standards. NCCLS is now referred as CLSI (Clinical and Laboratory Standards Institute). The end-point for present investigation was determined by visual determination using turbidity levels as stated in the NCCLS M27-A2 protocol[5], the spectrophotometric determination using a plate reader, and the colorimetric determination using an oxidationreduction indicator - Resazurin.

The objective of the present work is to perform the *in vitro* screening for Antimycotic activity of various extracts of *Centratherum anthelminticum* seeds using Microtiter plate based assay method by NCCLS protocol agaist tested microorganisms.

MATERIAL AND METHOD

Preparation of Extracts

Extraction of the *Centratherum anthelminticum* seeds were carried out by the percolation method using the Soxhlet apparatus. Kalijiri seeds were obtained from the local market and identified by botanist in Krushi Vighyan Kendra at Ganpat University. The dried fruits were then extracted using solvents ethanol, petroleum ether, hexane, chloroform, diethyl ether.

The soxhlet solvents extracts were obtained by soxhlet extraction of 25g of dried seeds in 100ml-200ml of solvents at 65°C using soxhlet apparatus. The extract was then concentrated to 10ml on a water bath and dried at room temperature. Percentage yield of various extracts were noted in each solvent.

The assay was performed using the CLSI (formerly NCCLS) Protocol M27 – A2 [6].

Preparation of broth medium: RPMI-1640 medium supplemented with glutamine and phenol red, without bicarbonate and 3-(N-morpholino) propanesulfonic acid (MOPS) was dissolved in distilled water. pH was adjusted to 7.0 at 25°C with sodium hydroxide. The volume was made up with water and was filtered, sterilized and was stored at 4°C until required.

Preparation of Inocula: Pure culture of *Aspergillus fumigatus (MTCC 2550), Candida albicans (MTCC 183), Candida tropicalis (MTCC 184), Candida parapsilosis (MTCC 1744), Issatchenkia orientalis (MTCC 3020), Cryptococcus albidus (MTCC 2661)* and *Cryptococcus layrentii (MTCC 2898)*. were obtain from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. Fungal strains were sub-cultured on to their respective growth medium and incubated for 48 hrs at 25-30°C. From these plates, several colonies were transferred to 5 ml of sterile distilled water. The suspensions were mixed for 15 second to ensure homogeneity and were subsequently diluted to match the turbidity of a 0.5 McFarland standard (i.e. OD = 0.12 – 0.15 at λ = 530 nm, corresponding to 1 – 5 X 10^6 CFU/ml). Then the working suspensions were prepared by a 1 in 30 further dilution of the stock suspension in sterile distilled water to yield 1–5 X 10^3 CFU/ml. 0.1ml sterilized solution of resazurin (20 mg/ml in water) was supplemented to the working suspension.

Preparation of samples: Stock solutions of the plant extracts were prepared in 1% Dimethyl sulphoxide (DMSO) at the concentrations of 1000 mg/ml. Further it was diluted to 1:100 in broth.

Preparation of plates: Microdilution susceptibility testing was performed in flat-bottom 96-well clear plates containing broth medium (50µl) in each well. Sample solutions (100µl) were subsequently serially diluted two-fold in the plates with the broth, starting with the final concentration of 1 mg/ml. The working inoculum

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suspension (50µl) was added to give a final inoculum concentration of 0.5–2.5 X 10^3CFU/ml. Amphotericin B was used as the standard antifungal drug. Sterility and growth controls in the presence of DMSO were also included. The plates were then incubated at 37°C for 48 h. The amount of growth was measured using plate reader at λ = 450nm.

RESULTS AND DISCUSSION

Fungal Infection is a condition in which fungi pass the resistance barriers of the human or animal body and establish infections. The human body is covered with a vast amount and diverse range of germs. These germs live harmlessly within the body and on the skin. However, certain types of fungus can build up on the skin and cause infections. A fungal infection is usually not serious and cannot normally be spread easily from person to person. An infection deeper within the body is more serious.

In the present study each fungal strain was inoculated and pre incubated for 24 hrs in 96 well micro-titer plates along with resazurin. Then test sample were added in 2 fold dilution manner, to make test compound's concentration ranges from 0.020 µg/ml to 100 µg/ml. Plates were further incubated for 24 hrs. After the 24 hour the absorbance was measured at the 450 nm by the plate reader. Results for each test samples were reported as the percent of growth inhibition (IC_{50}).

All the extracts were evaluated *in-vitro* against *Aspergillus fumigatus, Candida albicans, Candida tropicalis, Candida parapsilosis, Issatchenkia orientalis, Cryptococcus albidus* and *Cryptococcus layrentii*. Reason for choosing these fungal strains are that each belongs to most common mycosis producing strain in the human.

Reactions were performed with reaction mixture containing serially diluted various Extract concentration (0.2, 0.39, 0.78, 1.56, 3.12, 6.25, 12.50, 25, 50, 100 µg/ml), Resazurin dye (20mg/ml), Content was incubated for 48 hours. Data were analyzed with GraphPad Prism, non linear regression; competitive inhibition equation was used with statistical confidence limit of 95% results mentioned in (**Table 1**) indicate that out of 4 samples only 3 of them shows IC_{50} value below 100 µg/ml and that is against various fungal strains. The standard drug, Amphotericin B showed activity against all fungal strain with the IC_{50} value ranging from 6.338 µM to 8.871 µM.

For each tested compound, Dose Response Curve (DRC) against all fungal strain was plotted with 10 analysis points i.e. with 10 different drug concentrations. The concentration causing 50% growth inhibition (IC_{50}) was determined from DRC using *GraphPad Prism* software (v*er*. 5.0) and MS-Excel application.

Table 1: IC_{50} value (in µg/ml) of *Centratherum Anthelminticum* with reference to Amphotericin B against various fungal strains by Microtiter plate based assay method.

Fungal strains	Ethanolic Extract		Chloroform Extract		N - Hexane Extract		Diethyl Ether Extract		Petroleum Ether		Amphotericin B*	
	IC_{50}	R^2	IC_{50}	R^2	IC_{50}	R^2	IC_{50}	R^2	IC_{50}	R^2	IC_{50}	R^2
Aspargillus fumigatus	20.12	0.9834	21.33	0.9643	>100	0.9865	> 100	0.9754	>100	0.9452	19.02	0.9771
Candida albicans	36.30	0.9459	58.06	0.9503	>100	0.9210	> 100	0.9369	>100	0.9882	28.43	0.9544
Candida parapsilosis	18.46	0.9732	35.67	0.9120	>100	0.8182	> 100	0.8182 0.8473	>100	0.9352	15.96	0.9703
Candida tropicalis	15.05	0.9870	29.88	0.9436	>100	0.9529	> 100	0.9542	>100	0.9320	8.21	0.9703
Cryptococcus albidus	>100	0.9924	>100	0.9948	>100	0.9839	> 100	0.9543	>100	0.9912	22.32	0.97851

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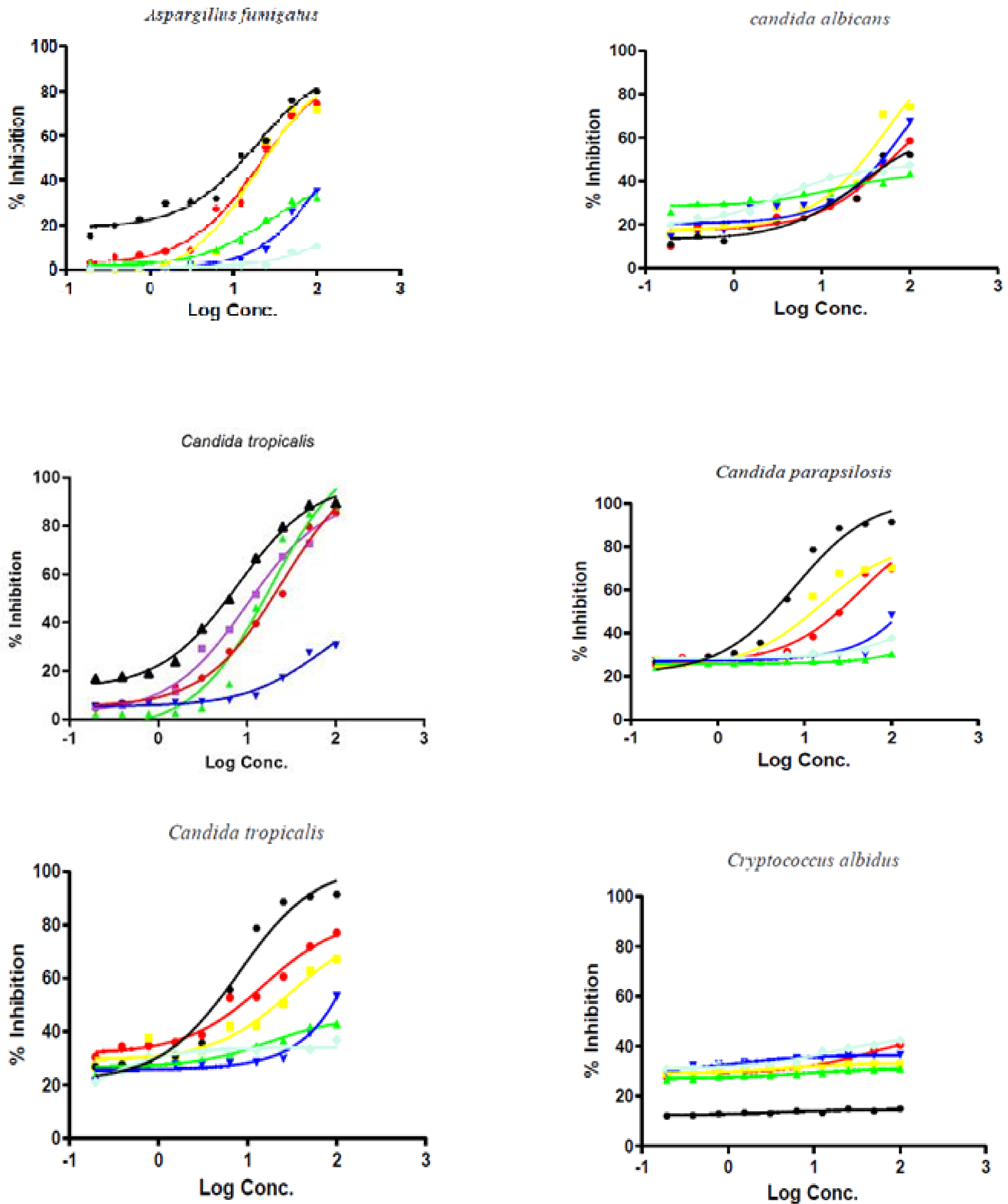
Cryptococcus laurentii	>100	0.9708	>100	0.9928	>100	0.9862	> 100	0.9825	>100	0.9661	20.53	0.9823
Issatchenkia orientalis	63.31	0.9459	62.88	0.8654	> 100	0.8334	> 100	0.8276	7.936	0.9703	14.22	0.9677

* IC_{50} value in µM

Fig. 1: Drug Response Curves of ethanolic extract, Chloroform, ethyl acetate and N – Hexane Extract with Compare to Amphotericin B against the various Fungus Species by Microtiter plate based assay method.



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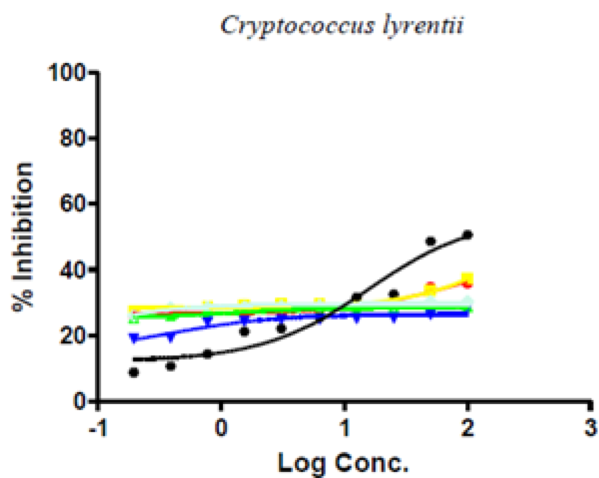

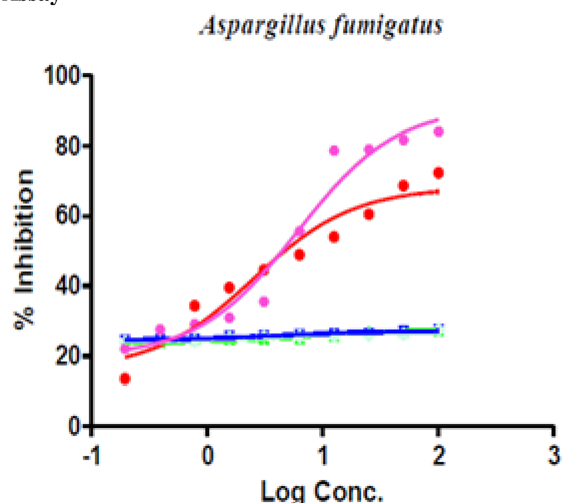

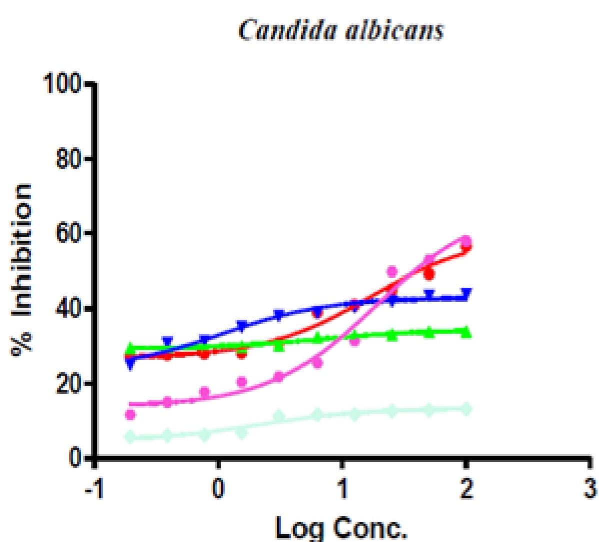

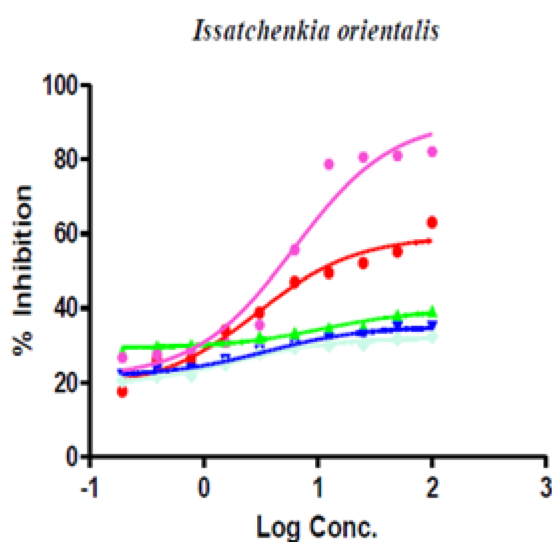

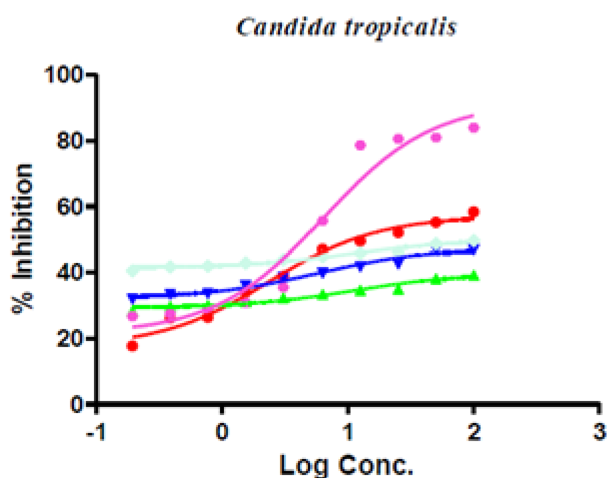

Ethanolic
Chloroform Extract
Ethyle Acetate
N - Hexane
Amphotericin B

CONCLUSION:

From the above study, it can be concluded that the ethanolic extract showed most potent activity against *Candida tropicalis* and *Candida parapsilosis* among all rest extract of *Centratherum anthelminticum* seed which are chloroform extract, N-Hexane extract, Diethyl ether and petroleum ether extract. Ethanolic extract have also shown significant activity against Aspergillus fumigatus, Issatchenkia orientalis, and Candida albicans. Ethanolic extract showed relatively close potency as that of the Amphotericin B. Chloroform extract shown potent activity against

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Aspergillus fumigates and less potent activity against Candida Albicans, Candida parapsilosis, Candida tropicalis and Issatchenkia orientalis. Chloroform extract has relative less potency than that of the Amphotericin B. Diethyl ether, N-Hexane extract and petroleum ether extract have not shown significant activity against any fungal strains which could have been due to the absence of prominent components in *Centratherum anthelminticum* i.e. flavanoids & tannins. Among all successive extract of ethanolic residue, ethanol extract produced effective % inhibition on all fungal strain except *Cryptococcus albidus,* and *Cryptococcus laurentii.*

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