ISSN 0975-6299 Vol 3/Issue 2/April – June 2012

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE MICROBIOLOGY

ANTI-MYCOTIC EFFECT OF 'KUSUM OIL' EXTRACT ON CANDIDA ALBICANS CLINICAL ISOLATES FROM ENDOPHTHALMITIS CASES

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ABSTRACT

Oil extracted from seeds of *Schleichera trijuga* which is an unexplored plant from a local natural reserve forest in Orissa (India) was tested in order to explore a possible indigenous source of anti-fungal agent. Ripen fruits of *S. trijuga* ("Kusum") were collected in its natural fruiting season and the oil fraction from sun dried seeds was extracted. This indigenous oil preparation (Kusum Oil) was tested for its anti-fungal activity against a total of 64 *Candida albicans* isolates collected from infectious vitreous fluid of endophthalmitis patients. Its minimum inhibitory concentration (MIC) was estimated to be 333.33 μl/ml when tested against *C. albicans* clinical isolates as well as the standard strain. Minimum detectable action time was found to be varying between 8 to 12 hours. The 'Kusum Oil' at its MIC was effective against 81.25% clinical isolates of *C. albicans* which was as efficient as Amphotericine-B and higher than Itraconazole (73.44%), Clotrimazole (71.875%), Nystatin (68.75%) and Fluconazole (59.375%). In our study 'Kusum Oil' was found to be an effective anti-mycotic agent against *C. albicans* clinical isolates that expanded its medicinal utility possibly as an alternative source of anti-fungal agent.

KEY WORDS

Kusum Oil, *Schleichera, Candida albicans,* Endophthalmitis

INTRODUCTION

Empiric knowledge about plants with medicinal properties has been accumulated through centuries, always following human evolution through the time (Ferro ., 2006). *Schleichera oleosa* (Synonyms: *Cussambium oleosum*; *Pistacia oleosa*; *Schleichera trijuga* Wild), is a tropical plant prevalent in Orissa and other parts of India (foliage: deciduous, and semi evergreen). That is locally called as "Kusum" (Hindi) and "Kusuma" (Oriya). It belongs to Family "*Sapindaceae*". Fruiting season is usually in the monsoon. Fruits are simple, fleshy and of 'Berry' category which is either one seeded or two seeded. Other plant characteristics of *Schleichera trijuga* include a deep and tap root system; erect cylindrical, hard wooded stem; and alternate, elliptical, oblong, pinnately compound, green leaves (Iwasa, 1997). The fruit of *Schleichera trijuga* is edible in Similipal and in other hilly regions of Orissa. The oil obtained from its seeds is called 'Kusum Oil'. The estimated availability of *Schlichera oleosa* seed is about 25, 000 oil potential per tones per annum. Seeds contain 40.3% of yellowish brown colored oil (Mallela et al, 2011; Chanida et al, 2008). Oleic acid was previously well-known as major fatty acid in 'Kusum Oil' (CSIR, 1972). Though *Schleichera trijuga* is well known before as the source of Indian macassar oil, very limited information is available on its medicinal properties. The oil which is traditionally used for the cure of itch, acne, burns, other skin troubles, rheumatism (external massage), hair dressing and promoting hair growth (Mallela et al, 2011; CSIR, 1972; Maharashtra State Gazetteers, 1953). Powdered seeds of *Schleichera trijuga* are applied to wounds and ulcers of cattle to remove maggots (Iwasa, 1997). The bark is astringent and used against skin imflammations and ulcers, while an infusion is taken against malaria. However there is a lack of published reports on identification of any specific compound from this plant and its role in specific microorganism.

It has been reported that *Candida albicans* is the most common cause of endogenous endophthalmitis resulting from hematogenous disseminations following trauma or eye surgery (Schiedler et al, 2004). An earlier study from our hospital also authenticates *C. albicans* to be the most common pathogen isolated in endogenous fungal endophthalmitis cases (Patra et al, 2012). Endogenous fungal endophthalmitis, including both chorioretinitis and vitritis needs an early and appropriate therapy. Because the eye is a protected compartment, penetration of systemically administered anti-fungal agents is highly variable. Advances in the development of new anti-fungals, might offer some new hope of improving the future of anti-fungal therapy. In the present study oil extracted from seeds of *S. trijuga* which is an unexplored plant from a local natural reserve forest is tested in order to explore a possible indigenous source of anti-fungal material.

MATERIALS AND METHODS

The Kusum Plant and Preparation of the Plant Extract (Kusum Oil)*:*

The *Schleichera trijuga* plant and its fruits were identified scientifically as per the morphological descriptions mentioned in various reference material (Maharashtra State Gazetteers 1953; CSIR, 1972; Iwasa, 1997). 'Kusum Oil' was extracted by conventional method (Manjula *et al*, 2001). Briefly, ripen fruits of 'Kusum' were collected from 'Similipal Natural Reserve' forest (in Orissa) in its normal fruiting season (i.e., June and July). Natural appearance of Kusum fruit and seeds are shown in Figure-1. The outer covering sheath and fleshy layer were removed

by crushing manually for harvesting the seeds which were then dried under natural sun light. Hard coat of the seeds were removed & ground. The grounded seeds were steamed for about 30-45 minutes until the oil fraction separated out and the oil was extracted by pressing with the help of 'Wooden Ghana' (locally used tool that is used for extracting oil from seeds).

Figure-1
Kusum Fruits and seeds

***Media and Chemicals*:**
Fungal culture media, chemicals and anti-fungal discs were procured from Hi-Media Laboratories Privet limited, Mumbai (India).

The test organism:
The test organism used was *C. albicans* clinical isolate. A total of 64 *C. albicans* isolates were collected from infectious vitreous fluid of endophthalmitis patients who attended a local tertiary care eye hospital between June 2008 to December 2010.

a) Isolation of C. albicans from patient
Vitreous fluid samples were collected from the patients by Ophthalmologist and inoculated on Sabouraud Dextrose Agar (SDA) medium plates with 0.05 mg/ml of Chloramphenicol. Plates were incubated at 35 ± 2^0 C temperature and observed on everyday for growth. When the growth was not appeared after 3-4 weeks than the samples were consider as negative (-ve) and then disposed as per the standard protocol (Ramakrishnan *et al*, 2009).

b) Identification of C. albicans
A portion of isolated colony was inoculated to approximately 0.5 ml of fresh human serum and incubated for 2 hours at 37^0 C. The positive germ tube test indicated *C. albicans*, while negative test required further testing. In case of negative germ tube test, a portion of colony was inoculated to Cornmeal Tween-80 agar and incubated for 24 hours. The production of chlamydospore reported as *C. albicans*. The *C. albicans* were identified by their characteristic morphological features and biochemical reactions as described in standard book (Gary S. Moore & Douglas M. Jaciow, 1979).

Determination of minimum inhibitory concentration (MIC):
The MIC of the 'Kusum Oil' was determined by tube dilution technique following the protocol described earlier **(**Janssen *et al*., 1986). Briefly, Sabouraud Dextrose Broth Sodium Taurocholate (SDBST) medium was prepared by supplementing 0.75% (w/v) Sodium taurocholate in to the Sabouraud dextrose broth (SDB). A

series of ten sterile test tubes were taken and 2.0 ml of kusum oil was added in to the first one. Rest were contained each 1.0 ml of sterile SDBST broth. Taking 1.0 ml of oil from 1^{st} tube, two-fold serial dilution was made up to 9^{th} tube &1.0 ml of suspension was discarded from the

9th tube. The tenth tube, which served as a negative control having no oil where as 1st one is positive control. Inoculated 1.0 ml of the diluted culture suspension (0.5-2.5 x 10^3 cfu/ml) of *C. albicans* into each of the tubes. The result was observed after 24 h at 35±2^0 C. The minimum inhibitory concentration of the oil was determined as the least concentration inhibiting the growth of the *C. albicans* in the test tube.

Anti-fungal Assay by Disc Diffusion Technique:

Oil was screened for their anti-fungal activity against isolated *C albicans* by disc diffusion method of Baur et al (1966) on Sabouraud Dextrose Agar Sodium Taurocholate (SDAST) medium plate (Rath et al, 2001). The sterile paper disc (5mm diameter, Whatmans filter paper No.42) containing MIC level of Kusum oil was soaked and test was performed in triplicate. Plates were incubated at 35±2^0 C for 48 h. within 15 minutes after applied the discs. Zones of inhibition in mm were determined and comparison of zones of sensitivity with standard anti-fungals (Amphotericin-B, Clotrimazole, Fluconazole, Itraconazole, Nystatin). Fluconazole was taken for standard positive control in this present study as per CLSI guidelines for interpretation of the sensitive or resistance of 'Kusum Oil' and a filter paper disc soaked with sterile distilled water was used as negative control. Zones of the oil were measured. The data of the parameters were statistically analyzed. Sensitive or resistant result was interpreted based on the diameter of the zone of inhibition after comparing with the standard anti-fungal disc as per CLSI guidelines (2004).

Determination of action time of the oil:

An *in-vitro* experiment was previously described (Patra et al, 2010) with slightly modification to determine the time required to kill the *C. albicans* by the 'Kusum Oil'. A set of ten test tubes with each one ml of SDBST at MIC level of 'Kusum Oil' were inoculated with 50µl of freshly grown broth culture of *C. albicans* (0.5-2.5 x 10^3 cfu/ml) in to each tube and incubated at 35±2^0 C. The incubated tubes were sub cultured on SDA plates at 0 minute, 30 minutes, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr, 8hr, 10hr and after overnight incubation. The activity was observed by incubating the plates at 35±2^0 C for 24 hours. No growth on the streaking line was considered to be the time required by the oil to kill the *C. albicans.*

Statistical Analysis:

Statistical significance was analyzed by estimating the P values on the basis of unpaired t test results calculated using GraphPad QuickCals.

RESULTS

Out of 312 vitreous samples, 64(20.51%) *C. albicans* strains were isolated from culture during the 24 months study. Figure-2 shows representative cases of infectious endophthalmitis that were later diagnosed to be due to *C. albicans* infection. A total 52 strains were found to be positive by the germ tube test as well as characteristic morphology under microscopic observation.

Figure-2
Endophthalmitis case later diagnosed to be due to *Candida albicans* infection

The minimum inhibitory concentration of 'Kusum Oil' was determined by tube dilution technique. The Concentration of 'Kusum Oil' varied for each isolated strain of *C. albicans.* However, the MIC of this oil at a concentration of 333.33 μl/ml was observed to be inhibitory against the standard strain of *C. albicans*. The minimum detectable action time was found to be varying between 8 to 12 hours as observed in the present study.

This preliminary study revealed 'Kusum Oil' to have inhibitory effect against *C. albicans* clinical isolates. The 'Kusum Oil' was 81.25% sensitive to *C. albicans* at its MIC level where as Fluconazole showed 59.375% sensitivity against *C. albicans*. The oil susceptibility result was varied according to the different standard disc used in this study. Amphotericine-B was showed 81.25% sensitive against *C. albicans*, followed by Itraconazole (73.44%), Clotrimazole (71.875%), Nystatin (68.75%) and Fluconazole (59.375%) (Table-1).

Table-1
Susceptibility of Different Antifungals and 'Kusum Oil' against C. albicans

Antifungals	Abbreviation	Concentration	Susceptibility test result (n=64)	
			Sensitive	*Resistance*
Amphotericin-B	Ap	100 units/disc	52 (81.25%)	12 (18.75%)
Clotrimazole	Cc	10 mcg/disc	46 (71.875%)	18 (28.125%)
Fluconazole	Fu	25 mcg/disc	38 (59.375%)	26 (40.625%)
Itraconazole	It	10 mcg/disc	47 (73.44%)	17 (26.56%)
Nystatin	Ns	100 units/disc	44 (68.75%)	20 (31.25%)
Kusum Oil **	KO	10 μl/disc	52 (81.25%)	12 (18.75%)

N: total number of isolates. Sensitive or resistant result was interpreted based on the diameter of the zone of inhibition after comparing with the standard table as per CLSI guidelines 2004. **The basis of interpretation of the result as 'sensitive' or 'resistant' of oil was taken according to the zone size of standard disc Fluconazole (Fu).

Sensitivity or resistance was interpreted based on the diameter of the zone of inhibition following the CLSI guidelines. The inhibited zone sizes were varied from 2 mm to 32 mm in diameter. The average zone of inhibition diameters in the susceptibility test to different antifungal agents as well as the 'Kusum Oil' are mentioned in Table-2. Antifungal sensitivity test on SDA culture plates are shown in Figure-3.

Table-2

Average zone of inhibition diameters in the susceptibility test to different antifungal agents

Anti-Fungal agent	Number of isolates (N)		Mean average diameter of ZOI	Standard Deviation (SD)	Significance (P value)
Ap	Sensitive	52	21.32692	4.566378	
	Resistant	12	1.333333	1.825742	0.0001
Cc	Sensitive	46	19.56522	3.733644	
	Resistant	18	1.888889	2.541164	0.0001
Fu	Sensitive	38	21.36842	3.744698	
	Resistant	26	2.08	3.340659	0.0001
It	Sensitive	47	21.25532	4.093835	
	Resistant	17	1.411765	2.123053	0.0001
Ns	Sensitive	44	20.95455	4.176092	
	Resistant	20	2.75	2.989015	0.0001
KO	Sensitive	52	20.57692	4.645665	
	Resistant	12	3.416667	4.144182	0.0001

ZOI: Zone of Inhibition; Ap: Amphotericin-B; Cc: Clotrimazole; Fu: Fluconazole; It: Itraconazole; Ns: Nystatin; KO: Kusum Oil. P values are based on the Unpaired t test results calculated using GraphPad QuickCals.

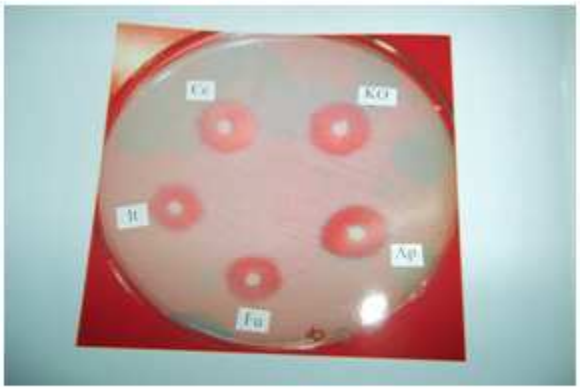

Figure-3
Antifungal sensitivity test

DISCUSSION

Either exogenous or endogenous spread of infectious agents affecting vitreous and anterior chamber of eye (endophthalmitis) pose as a major challenge both in diagnosis and successful clinical management across the globe (Mamalis et al, 2002). Ocular involvement in systemic candidiasis continues to be an important cause of visual morbidity with prevalence 0 to 78% (Rodrigues-Adrian et al, 2003). *C. albicans* is the main cause of mycotic endophthalmitis in intravenous drug abusers, patients on intravenous therapy, immunosuppressed individuals, and patients subjected to surgical treatment (Elliott et al, 1979; Essman et al, 1997; Schmid et al 1991). Although the current anti-fungal therapies have been significantly improved, the outcome is still far from satisfactory, partly due the limited number of

classes of clinically available anti-fungals, the development of resistance to current anti-fungals, and the challenges of proper and early diagnosis (Zhai and Lin, 2011). *C. albicans* resistance to azoles is reported (Niimi et al., 2010). Because of the increasing of antimicrobial resistance of *Candida*, many attempts have been made to find out a new antimicrobial agent to prevent candidal endophthalmtis. Medicinal plants could be a source of alternative antimicrobial agents to treat infectious diseases.

Fungal endophthalmitis is noted to be contracted more commonly during trauma or eye surgery either by direct inoculation or that can pose a serious condition secondary to hematogenous dissemination of the underlying pathogen. In an earlier study analyzing 1,662 vitreous fluid samples from fungal endophthalmitis cases, *C. albicans* was found to be the most common pathogen isolated in endogenous fungal endophthalmitis cases constituting 52.99%, followed by *Aspergillus sp*. (26.16%), *Fusarium sp* (15.08%), *Penicillium sp*. (1.77%), *Cephalosporium sp*. (0.44%) and *Curvularial sp*. (0.44%) (Patra et al., 2012). So the present study is justified to focus on the candidal endophthalmitis cases only where the effect of 'Kusum Oil' is experimented *in vitro* to find its possible anti-*Candida* effect where this plant is known to be prevalent locally in this geographic region.

All patients who clinically diagnosed as endophthalmitis were taken for the microbiological investigation, where 87.50% patients were from rural area and 12.50% patients were from urban area. The percentage of male was 71.88%. Majority of these patients belong to farmer, daily worker, or labor community. With respect to various predisposing systemic conditions, *Candida* endophthalmitis was noted in patients with a history of gastrointestinal surgery, catahyperalimentation, urinary tract infection, blood septicemia or diabetes mellitus. About 30% of patients had history of alcohol intake. History of other activities or condition viz., immunosuppression, intravenous drug abuse, bacterial sepsis, systemic antibiotics, corticosteroid therapy, and recent abdominal surgery, were found in limited cases.

C. albicans is a commensal organism that resides in the human body and is found normally in the female genital tract, the gastrointestinal tract, and the respiratory tract. These fungi are kept in check by the host's normal immune response. When a breakdown in the host's immune system occurs, fungi may spread throughout the body. Many organs, including the eye, can be affected by this spread of fungi through the bloodstream (Litheh *et al*, 2004). Patients with risk factors, such as chronic pulmonary diseases, orthotropic liver transplants, renal transplants, and bone marrow transplants, disseminated infection may inhibit for the propagation of infection (Litheh *et al*, 2004).

Globally about 85% of the traditional medicines used for primary health care are derived from plants. Herbal drugs obtained from plants are believed to be much safer; this has been proved in the treatment of various ailments (Mitalaya et al, 2003). Approximately 50% of new chemical molecules from natural products found between 2000 and 2006 have shown their importance in the development of drugs for the treatment of infectious diseases (Newman et al., 2007). The traditional use of plants as medicines provide the basis for indicating which plant extracts may be useful for specific medical conditions. Historically, many plant oils and extracts have been reported to have antimicrobial properties (Hoffman, 1987; Lawless *et al*, 1995). It is important to investigate scientifically those plants which have been used in traditional medicines as potential sources of novel antimicrobial compounds (Mitscher *et al*, 1987). Also, the resurgence of interest in natural therapies and increasing consumer demand for effective, safe, natural products means that quantitative data on plant oils and extracts are required.

Due to limited no of anti-fungal drugs, *Candida* endophthalmitis are commonly treated empirically; hence, drugs are typically selected without regard to susceptibility (Lalita *et al,*

2007). The present *in-vitro* study has shown that 'Kusum Oil' is highly effective against *C. albicans* when compared with the results from other medicinal plants such as *B. discolor*, *C. transvaalensis, P. capense*, *W. salutaris* (Samie *et al*, 2010). In our study 'Kusum Oil' was effective against 81.25% of the total isolates of *C albicans* clinical isolates. However, the effectiveness was found to be of different extent in case of different isolates of *C. albicans* when we consider diameter of the zone of inhibition as a parameter. Otherwise we can augment this finding when the concentration of the extract is considered. Therefore, more number of isolates were killed at 333.33 μl/ml of oil extract keeping the protocol same for each of the batch of experiment.

'Kusum oil' is a novel substance that has been used by tribal people for their disease like, cold, itch, ulcer for both human and cattle. It was confirmed after the discussion with native inhabitants in the area where the "Kusum' fruits were collected. Mallela et al (2011) have also described the medicinal use of the 'Kusum Oil' which is traditionally used for the cure of itch, acne, burns, other skin troubles, rheumatism (external massage), hair dressing and promoting hair growth. Similar result has been published in Maharashtra State Gazetteers (1953). Powdered seeds of *Schleichera trijuga* are used for ulcers and wounds of cattle to remove maggots and bark is used for skin imflammations and ulcers. The anti-mycotic effect of the oil expanded to its utility possibly in treating endophthalmitis cases and also it needs to be further evaluated against other infectious agents.

Though specific methods of purification of *Schlichera trijuga* seeds oil and other edible vegetable oils is documented (viz., alkaline-catalyzed esterification process) their use is limited to commercial production of biodiesel only (Gandhi et al., 2011). No scientific study is done before to verify its medicinal effect. The present study is the first of its kind to authenticate the scientific basis of its use against a fungal pathogen. Hence crude extract of the seed (Kusum Oil) is employed not to miss any important component in the extract having anti-mycotic function. However, identifying specific fraction from the crude extract and further characterization is warranted which was beyond the scope of the present study.

CONCLUSION AND RECOMMENDATION

In our study 'Kusum Oil' was found to be an effective anti-mycotic agent against a *C albicans* clinical isolates that expanded its medicinal utility. However, for development of 'Kusum Oil' as an alternative source of anti-fungal agent, further studies are required to evaluate its effect on other fungal pathogens. At the same time safety aspects as well as active compound identification in purified form should be explored.

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