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ANTIFUNGAL ACTIVITY OF ETHANOLIC *OCIMUM SANCTUM* EXTRACTS AGAINST FUNGAL PATHOGEN ISOLATED FROM SINUSITIS SUBJECTS

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ABSTRACT

Ocimum sanctum ethanol leaf extract was evaluated for its *in vitro* antifungal activity for isolates against Allergic fungal rhino sinusitis (AFS) positive subjects. Out of 25 clinical samples collected from different hospitals in Allahabad, seventeen subjects were diagnosed positive for noninvasive rhino sinusitis in the year 2003-2005. The fungal pathogens isolated were identified as *Aspergillus niger* (64%), *Penicillium sp*. (44%), *Alternaria sp*. (64%), *Fusarium sp*. (36%), *Exophiala sp*. (40%). Ninety percent of *A. niger* infections were reported among 30-39 year age group old victims, male being infected the most was reported. Antimycotic activity of the extract was screened using agar well diffusion method with *O. sanctum* ethanol leaf extract by taking concentration of 500mg/ml DMSO which had maximum zone of inhibition for *Fusarium* sp. (19.33mm) and minimum for *Alternaria* sp. (8mm). These results support inhibition of AFS pathogens and thus can be used for treatment of AFS, being economical, with no side effects and no pain.

Key words: Allergic/Eosinophilic fungal rhinosinusitis, *Ocimum sanctum, Aspergillus, Penicillium, Alternaria, Fusarium, Exophiala*.

INTRODUCTION

Fungi are ubiquitous, and our exposure to these organisms occurs on a daily basis with their common route of entry inside the body through sinonasal cavity. Four distinct histological categories of fungal sinusitis (FS) have been recognized: allergic, noninvasive fungal colonization (mycetoma or "fungus ball"); chronic invasive; and acute fulminant. Histological features, which are highly sensitive and specific for the diagnosis of allergic FS, includes: eosinophilic mucin with Charcot-Leyden crystals and collections of degenerating eosinophils. Allergic mucin is very characteristic but not pathognomonic for allergic fungal sinusitis (Mohammad, 2005). Allergic/Eosinophilic fungal rhinosinusitis (AFS) was first described in 1981 by Millar and for the past 25 years has been the subject of intense study and debate (Millar, 1981) AFS is a benign non invasive sinus disease, believed to be an allergic reaction to aerosolized environmental fungi. It is a form of chronic sinusitis characterized by nasal obstruction, sinus pain, rhinorrhoea, and frequent orbital symptoms (Mallick, 2008). AFS causing fungal pathogens includes *Aspergillus flavus, A. fumigatus, Fusarium sp. Rhizopus sp., Bipolaris sp.* and *Candida albicans* (Tilak, 2012). Fungi commonly identified in electron microscopy were from the dematiaceous family and *Aspergillus* sp. Dematiaceous fungi include the genera *Alternaria, Bipolaris, Cladosporium, Culvularia, Drechslera* and *Helminthosporium* (Gupta, 2012). The Ayurvedic Pharmacopoeia of India recommends the use of the leaves and seeds in rhinitis and influenza. Ursolic acid, isolated from leaves, exhibited significant protection of mast cell membrane by preventing granulation and decreased histamine release.

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Ocimum sanctum as sacred armoury used to cure upper respiratory tract infections and bronchitis since long the mankind evolution. *Ocimum sanctum* leaf is carminative, stomachic, antispasmodic, antiasthmatic, antirheumatic, expectorant, stimulant, hepatoprotective, antiperiodic, antipyretic and diaphoretic (Khare, 2007). *In vitro* antifungal activity of its leaves also reported against *Aspergillus niger, A. fumigates* (Bansod and Rai, 2008). *Ocimum sanctum* against some well known fungal etiological agents as *Candida albicans* (Ahmad and Beg, 2001; Geeta *et al*., 2001; Kaya *et al*., 2008; Sharma, 2010), *Fusarium solani* sp. Melongenae (Joseph *et al*., 2008), *Aspergillus flavus* and aflatoxin B1 (AFB1) production (Reddy *et al*., 2009), *Aspergillus niger* (Tewari and Robert, 2009; Mohan *et al*., 2011), *Aspergillus repens*, *Curvularia lunata* and *Fusarium moniliforme* were also reported (Amadi *et al*., 2010).
Being easily accessible, economical with diverse medicinal magic this plant was taken under to study its antifungal prospects to cure AFS. Our study is focused on identification of human fungal isolates from sinusitis patients and *in vivo* antifungal activity of *O. sanctum* ethanol leaf extract.

METHODS AND MATERIALS

Sample Collection and Processing

Nasal samples were collected from AFS positive subjects from Swaroop Rani Nehru Medical Hospital and Jeevan Jyoti Hospital, Allahabad, U.P. Awareness that fungi are colonizing the mucus prompted development of a simple noninvasive procedure to obtain maximum mucus for testing. Two puffs of phenylephrine hydrochloride (1%) were sprayed into each nostril to produce vasoconstriction which increased nasal lumen and consequently the yield from nasal lavage. After the injection of saline, the patients were asked to exhale forcefully through the nose and the return was collected in a sterile pan. The collected fluid was placed into centrifuge tubes and processed maintaining the sterile condition.

The specimen processed following the protocol of Ponikau *et al*., (1999). Briefly the collected specimen was suspended into an equal volume of diluted dithiothreitol and vortexed for 30 seconds. The mixture was allowed to stand at room temperature for 15 minutes followed by centrifugation at 3000*g* for 10 minutes. Retained sediment is vortexed for 30 seconds.

Isolation and Identification of Pathogenic Fungal Sinusitis from Clinical Samples

One-half ml of the prepared sediment was inoculated on the Sabouraud's dextrose agar (SDA) media and incubated at 25^{o}C for 15 days. Obtained fungal cultures were examined for their colony morphology considering color and texture. Microscopic characteristics observed using lacto phenol cotton blue staining technique for their mycelia and spore arrangement. Sugar fermentation test was performed using glucose and sucrose followed by acid and gas production.

Extract Preparation

Ocimum sanctum ethanol leaf extract (OSELE) was prepared using 10g of dried leaves soaked in 100 ml ethanol at 200 rpm for 24 hrs. at ambient temperature concentrated using vacuum rotary evaporator. Stock solutions of crude OSELE was prepared by diluting 0.2 g dried extracts with 1 ml of 10% dimethyl sulphoxide (DMSO) solution (Rathod *et al*., 2012).

Antimicrobial Susceptibility Test by Using Well Diffusion Method

Antifungal activity of OSELE was performed using agar well diffusion assay on pre-prepared SDA plates (Perez *et al*., 1990). Prepared wells (10 mm diameter) were filled with OSELE (0.1 ml, of 500mg/ml DMSO). Incubation period of 24-48 hours at 28^{0}C was maintained for observation of antifungal activity of plant extract. The antifungal activity was evaluated by measuring zones of inhibition (in mm) of fungal growth surrounding the plant extracts. The experiment was carried out in triplicates.

RESULT AND DISCUSSION

Twenty five clinical samples were examined for the incidence of the pathogenic fungal isolates collected from different hospitals of Allahabad city. Out of these, five positive fungal pathogens were isolated and identified as *Aspergillus niger, Penicillium sp., Alternaria sp., Fusarium sp., Exophiala sp.* The maximum incidences of AFS was found in male patients with 20-29 years of age. Similarly seventeen patients were identified between 2003 and 2005 that were diagnosed with noninvasive fungal rhino sinusitis. Fourteen patients (82%) had allergic fungal mucin with polyps, while two patients had positive fungal cultures and chronic rhino sinusitis without polyps. One patient (5.8%) had a positive fungal stain, negative culture and evidence of chronic rhino sinusitis. Eleven patients (65%) had a positive fungal culture, and 10 patients (59%) had a positive fungal stain. Eight out of the ten (80%) patients with a positive fungal stain subsequently had positive fungal growth on culture. Three of 11 (27%) patients with a positive fungal culture had a negative fungal stain. Identified organisms included *Aspergillus, Alternaria, Bipolaris, Curvularia* and *Cladosporium*. Fourteen patients were immuno-competent, while 3 had underlying immune suppression (HIV/AIDS, metastatic breast cancer, and post organ transplant). Pathological findings included edematous respiratory mucosa with numerous mononuclear inflammatory cells, eosinophils and

neutrophils. The most characteristic feature was the presence of allergic fungal mucin, which was grossly thick and dark with a consistency of peanut butter or wet clay. This fungal mucin consisted of inspissated basophilic, purple-colored mucous with large numbers of degenerated eosinophils. Fungal stains (GMS and PAS) demonstrated that the fungal hyphae were sparse and not always found. There were never fungal hyphae within the mucosal tissue (Mowry *et al.*, 2008).

A total of 55 sputum samples from 41 patients were analyzed. Each sample was obtained on a separate visit and was independently analyzed. A total of 10 culture plates were inoculated with each sample. Yeasts were isolated from 1 plate from all but one of the samples (98%) that were inoculated on 1 plate, and 29% of samples (16 of 55) yielded *A. fumigates* . There were no differences between recovery rates of yeasts from homogenized sputum compared to sputum plug detection of *A. fumigatus* was highly dependent on quantity of sputum inoculated onto the culture plate. Of the 16 samples that were *A. fumigatus* positive on 1 plate, growth of the fungus was not detected with diluted-homogenized sputum samples. Not considering differences due to the media, 19% and 44% of *A. fumigatus* positive samples were detected using 10µl and 100µl of homogenized sputum, respectively, and 94% when a neat sputum plug was inoculated. One sample was culture negative with a sputum plug, but culture positive with 100µl homogenized sputum. In the majority of cases (11 of 16) filamentous fungal growth was observed after two days incubation at 37°C, with fungi detected in three samples after 4 – 6 days of incubation and growth was not detected with two until the 7th day (Pashley *et al*., 2012).

In vitro antifungal activity was performed using agar well diffusion method which gave excellent result in form of maximum zone of inhibition for *Fusarium* sp. followed by *Penicillium sp., Aspergillus niger, Exophilala and Alternaria.* Sharma *et al.* (2012), reported that the *Ocimum tenuiflorum* was found to be effective against pathogenic fungal isolates such as *Aspergillus niger* (7mm) and *Candida albicans* (0.19mm) respectively. Kumar *et al.* 2013, reported that the *Ocimum sanctum* extract was effective against *Fusarium sp.* (8mm) showed maximum inhibition which conformed to the present results.

Figure 1. Incidence of fungal sinusitis subjects with respect to sex

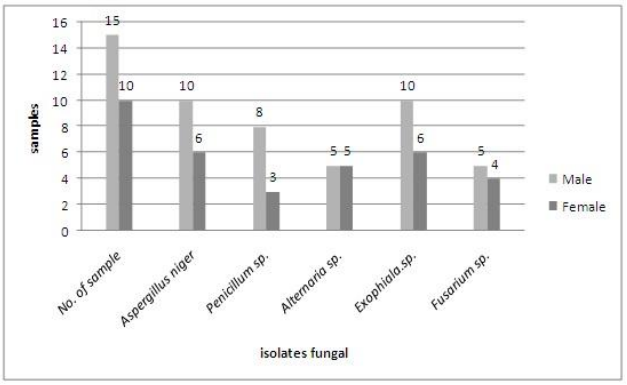

Figure 2. Incidence of fungal sinusitis subjects with respect to age

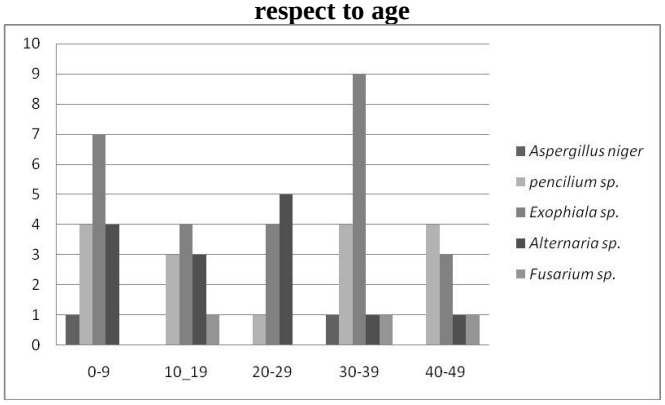

Figure 3. Fungal isolates from clinical samples

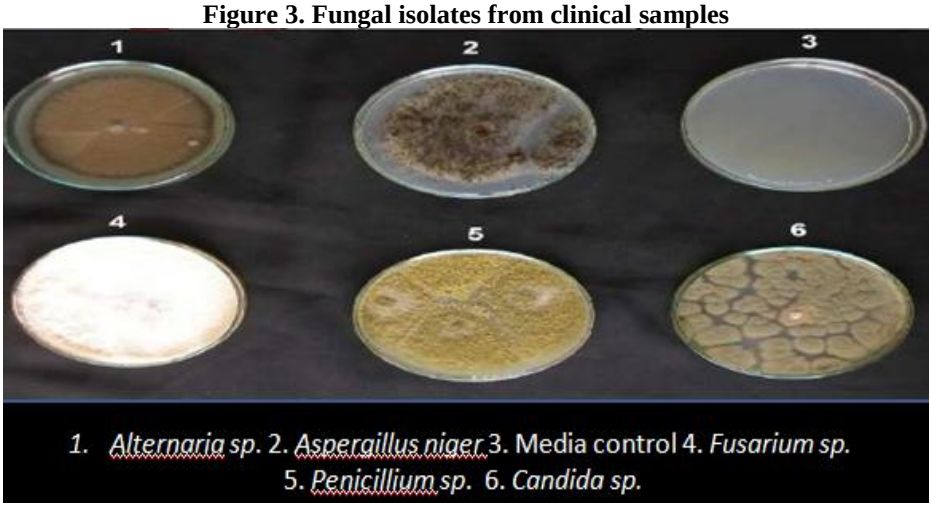

Figure 4. A. *Alternaria sp.*, B. *Aspergillus niger,* C. *Penicillium sp.* ,D. *Fusarium sp.*, E. *Exophiala sp.*

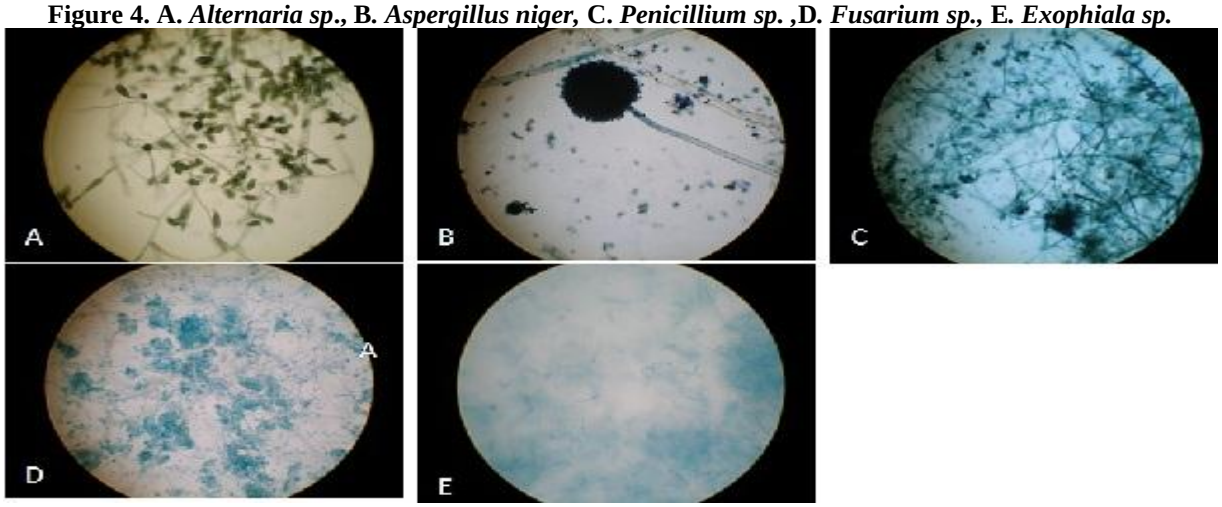

Figure 5. Antimicrobial activity of *Ocimum sanctum* against Human fungal isolates

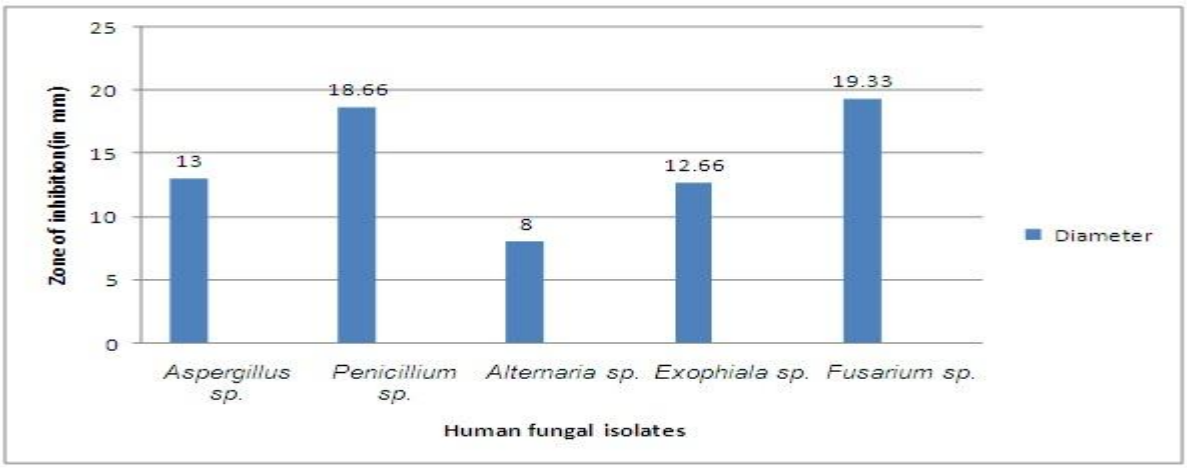

Table 1. Human fungal isolates from samples collected from sinusitis subjects from different hospitals

S. No	Subjects	Sex	Age	*Aspergillus niger*	*Penicillium sp.*	*Exophiala Sp.*	*AlternariaSp.*	*Fusarium Sp.*
1	1	M	12	Y	N	Y	N	Y
2	2	M	9	Y	N	N	Y	N
3	3	M	15	N	Y	N	Y	Y
4	4	M	20	N	Y	Y	Y	N
5	5	M	22	Y	N	N	Y	N
6	6	F	35	N	Y	Y	N	N
7	7	F	22	N	N	N	Y	Y
8	8	M	32	Y	Y	Y	N	N
9	9	M	20	Y	N	N	Y	N
10	10	F	11	Y	N	N	Y	Y
11	11	F	23	N	Y	N	N	N
12	12	F	21	Y	N	Y	Y	Y
13	13	F	15	Y	Y	N	Y	N
14	14	M	19	N	Y	N	Y	N
15	15	M	15	Y	N	N	N	Y
16	16	M	35	Y	N	Y	N	N
17	17	F	35	N	N	Y	Y	N

18	18	M	24	Y	Y	N	Y	N
19	19	F	21	Y	N	Y	Y	N
20	20	F	22	Y	N	N	N	Y
21	21	M	20	Y	Y	N	Y	N
22	22	M	21	N	Y	Y	Y	N
23	23	M	36	Y	N	N	N	Y
24	24	F	34	Y	N	Y	N	N
25	25	M	45	N	Y	N	Y	Y
	Total no. of fungal isolates			16	11	10	16	9

Table 2. Incidence of fungal sinusitis subjects with respect to sex

Sex	No. of sample	*Aspergillus niger*	*Penicillium sp.*	*Exophiala sp.*	*Alternaria sp.*	*Fusarium sp.*
Male	15	10(66.66%)	8 (53.33%)	5 (33.33%)	10(66.66%)	5(33.33%)
Female	10	6(60%)	3(30%)	5(50%)	6(60%)	4(40%)
Total	25	16(64%)	11(44%)	10(40%)	16(64%)	9(36%)

Due to organism F_{cal}= 7.31<F_{tab}=9.60,NS= Non Significant
Due to sex F_{cal}=29.004>F_{tab}=12.2; CD=6.1198; S=Significant

Table 3. Incidence of fungal sinusitis subjects with respect to age

Sex	No. of sample	*Aspergillus niger*	*Penicillium sp.*	*Exophiala sp.*	*Alternaria sp.*	*Fusarium sp.*
0-9	1	1(50%)	N.A.	N.A.	1(50%)	N.A.
10-19	6	4(66.6%)	3(50%)	1(16.66%)	4(66.66%)	4(66.66%)
20-29	11	7(63.63%)	4(36.36%)	4(36.36%)	9(81.81%)	3(27.27%)
30-39	6	4(66.6%)	3(50%)	5(83.33%)	1(16.66%)	1(16.66%)
40-49	1	N.A.	1(33.33%)	N.A.	1(33.33%)	1(33.33%)
Total	25	16(64%)	11(44%)	10(40%)	16(64%)	9(36%)

Due to organism F_{cal}=0.947<F_{tab}=3.01; NS=Non Significant
Due to age F_{cal}= 8.9140>F_{tab}=3.01, C.D= 2.0699; S= Significant
N.A.= Not available

Table 4. Identification of fungal isolates from clinical samples

Fungal Culture	**Morphological Identification**		**Biochemical identification w.r.t. Sugar fermentation**				**Culture Identified**
			Glucose		Sucrose		
	Colony Morphology	Microscopic Characteristics	Acid production	Gas production	Acid production	Gas production	
A	Surface is black with white border; thallus deep, Hypha erect white to cream, cluster of black conidia. Reverse white to cream.	Septate hyphae. Conidiophores long, smooth, may be brownish near top. Phialides radiate around entire vesicle, biseriate, with the metulae twice as long as the phialides. Conidia are round and dark`	+	–	+	–	*Aspergillus niger*
B	Thermally dimorphic at 25-30^{o}C on SDA, colony flat, powdery to velvety, tan, later becoming reddish yellow or white edge; bluish gray-green in the centre. A deep-reddish soluble pigment diffuses into	Smooth conidiophores with four or five terminal metulae, each metula bearing four to six phialides. Conidia are smooth or slightly rough and round to oval and form chains ; short, narrow extensions connect the conidia	–	–	–	–	*Penicillium sp.*

	the medium after 3-7 days; Reverse is brownish red.						
C	Surface is at first grayish white and woolly and later becomes greenish black or brown with a light boarder. May eventually become covered by short, grayish, aerial hyphae. Reverse is black.	Hyphae are septate and dark. Conidiophores are septate, of variable length, and sometimes have a zigzag appearance. Conidia are large and brown, have both transverse and longitudinal septations, sometimes produce germ tubes, and are found singly or in chains; they are usually rather round at the end nearest the conidiophore while narrowing at the apex, producing a clublike shape.	+	–	+	–	*Alternaria sp.*
D	At first white and cottony, but often quickly develops at pink or violet center with a lighter periphery. Some species remain white or become tan or orangey . *F. solani* is unique in becoming blue-green or bluish brown where clusters of conidiogenous cells develop. Reverse is usually light, but may be deeply coloured.	Septate hyphae. There are two types of conidiation: (i) unbranched or branched conidiophore with phialides that produce large, sickle or canoe-shaped macroconidia (with three to five septa.); (ii) long or short simple conidiophores bearing small oval, one or two celledнconidiasingly or in clusters.	+	–	–	+	*Fusarium sp.*
E	Initial colony is moist and black, becoming gray to black or brown and velvety. Good growth is observed at 25^{o}C on SDA.	Conidiogenous cells occur as either (1) annellides, or (2) thick-walled budding cells of varied sizes and shapes. Annelloconidia are formed outside the annellide. As these are formed and freed, they will often slip down the side of the conidiophores, giving the appearance, superficially, of being attached.	–	–	–	–	*Exophiala sp.*

Table 5. Antimicrobial activity of *Ocimum sanctum* against fungal pathogen

Culture	Diameter (mm)	Diameter (mm)	Diameter (mm)	Average(mm)
Aspergillus sp.	12	15	12	13
Penicillium sp.	20	18	18	18.66
Alternaria Sp.	8	8	8	8
Exophiala Sp.	13	12	13	12.66
Fusarium Sp.	20	18	20	19.33

*included well size of 5mm diameter, due to oraganisium: $F_{(cal)}$ 29.351>$F_{(tab)}$4.5; CD=6.605; S=Significant.

*included well size of 5mm diameter, due to zone: $F_{(cal)}$ 3.80< $F_{(tab)}$ 5.14. NS=Non Significant.

CONCLUSION

Present study revealed that the potent bioactive component from *O. sancutum* can be used to cure AFS thus providing relief to subjects without any surgical pain. It is economical and easily available and could be potent pharmaceutical drug.

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