Research Article

INACTIVATION OF *CANDIDIA ALBICANS* IN CULTURE MEDIA BY EIGHT SPICES NATIVE TO INDIAN SUBCONTINENT

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ABSTRACT

Plant parts of eight spices namely *Allium sativum, Brassica nigra, Cinnamomum cassia, Cinnamomum zeylanicum, Cuminum cyminum, Curcuma longa, Trigonella foenum-graecum* and *Zingiber officinale* were screened for their anticandidal activities towards *Candida albicans (NCIM 227),* in culture media. Aqueous extracts, essential oils and powdered forms of reference spices constituted the test materials for present study. Spice agar method was followed for investigating anticandidal activities of powdered spice samples, while impregnated paper disc method and broth dilution technique were opted for screening inhibitory potentials of aqueous extracts and essential oils. Results revealed that essential oils most effectively inhibited the test microbe followed by powdered forms and aqueous extracts. Among all the powdered spice samples tested, *C. zeylanicum* inhibited *C. albicans* most effectively, and among essential oils, *B. nigra* produced widest growth inhibitory zone against test yeast strain. Minimum inhibitory concentrations of different spice forms were also determined.

Keywords: Anticandidal, antimicrobial, essential oils, medicinal plants, spices.

INTRODUCTION

Spices need fewer introductions and for people throughout the world, they stimulate the appetite, add flavor and texture to otherwise monotonous and insipid foods and create visual appeals in meals. Called as *behart* (Arabic), *besamin* (Hebrew), *epices* (French), *especerias* (Spanish), *kimem* (Ethiopian), *krooder* (Norwegian), *masala* (Hindi), *rempah* (Malaysian and Indonesian), *sheng liu* (Mandarin) and *specerjein* (Dutch), these vital culinary addendums have been savored and sought for their preservative, aphrodisiac and medicinal faculties, since the dawn of civilization. There is at present growing interest, both in the food as well as pharmaceutical industry for spices because of their antimicrobial properties and from the point of view of safety[1]. Moreover, current economic and biological assessment upon withdrawal of most of the conventional synthetic preservatives have elicited widespread interest in providing new perspectives for the development and commercialization of future antimicrobials based on natural substances particularly of plant origin, those are socially more acceptable[2].

Thus, present study (*in vitro)* was undertaken to assess the inhibitory activities of eight spices, widely used in domestic culinary practices and as traditional medicines in Indian subcontinent, towards *Candida albicans. C. albicans* is an opportunistic yeast responsible for oral and genital infections in humans and for spoilage of soft drinks, canned or frozen fruit juices, fruit jams, pickles, mushrooms, cheeses and meats etc.

MATERIALS AND METHODS

Procurement of spice samples

The dried plant parts of the spices i.e *Brassica nigra*, *Cinnamomum cassia*, *Cinnamomum zeylanicum*, *Cuminum cyminum*, *Curcuma longa* and *Trigonella foenum-graecum* were procured in a single lot, in the amounts of 500 g each, from a wholesaler spice-seller, local market, Hisar, India. These spice samples were cleaned manually for extraneous material, ground to powdered form and were kept in airtight containers. Fresh plant parts of *Allium sativum*, and *Zingiber officinale* were purchased in the amounts of 1 kg each from grocery shop, local market, Hisar, India. The outer coverings of *A. sativum* bulbs and *Z. officinale* rhizomes were peeled off manually with the help of knife. Fresh forms of these spices were washed with distilled water to remove the foreign particles and were dried in shade for 5 days (temperature 24-27°C; Relative Humidity 55±5%) followed by their grinding in the laboratory grinder. These ground forms of *A. sativum* and *Z. officinale* were kept in airtight containers till further use. Plant parts of different spices used in current study and botanical information of test spices are given in Table 1.

Essential oils of *A. sativum, C. cassia (Blume), C. zeylanicum, C. cyminum, C. longa, T. foenum-graecum* and *Z. officinale* were procured from Aroma Chemicals Pvt. Limited, India. Quality of the spice essential oils was assured by the company to be more than 99.0 %. Essential oils were stored in the dark amber colored, screw capped glass bottles and were kept away from light to avoid physicochemical changes in their compositions. These bottles were closed tightly to check the loss of volatiles and were opened only for a short while, whenever required.

Table 1: Different plant parts of spices investigated and botanical information of test spices

Botanical names	Family	Indian names	English names	Plant parts used
Allium sativum	*Liliaceae*	Lehasun	Garlic	Bulbs
Brassica nigra	*Crucifereae*	Sarson	Mustard	Seeds
Cinnamomum cassia	*Lauraceae*	Dalchini	Cassia	Bark
Cinnamomum zeylanicum	*Lauraceae*	Dalchini	Cinnamon	Bark
Cuminum cyminum	*Apiaceae*	Jeera	Cumin	Seeds / Fruits
Curcuma longa	*Zingiberaceae*	Haldi	Turmeric	Rhizomes
Trigonella foenum-graecum	*Leguminosae*	Methi	Fenugreek	Seeds
Zingiber officinale	*Zingiberaceae*	Adarak	Ginger	Rhizomes

Chemicals and culture media

Yeast extract potato dextrose agar (YEPDA) and yeast extract potato dextrose broth (YEPDB) were obtained from Hi-Media Pvt. Ltd, India. Dimethylsulphoxide (DMSO) and Tween 80 were procured from Central drug house Pvt. Limited, India.

Yeast strain

Pure cultures of *Candida albicans* (NCIM 227) were obtained from National Collection of Industrial Microorganisms, Pune, India. The reference yeast strain was maintained on YEPDA (yeast extract potato dextrose agar (Hi-Media)) slants, subcultured bimonthly to maintain their viability and were stored at 4±1° C.

Inoculum preparation

A flamed sterile wire loop was used to dislodge the lawn of test yeast strain from its respective pure culture slant (24-48 h. old), using 10 ml of sterile Tween 80 (0.05%) under aseptic conditions. Dislodged dense suspension of yeast strain was adjusted with Tween 80 (0.05%) to contain approximately 1×10^7 cfu /ml based on total counts obtained by surface spreading on YEPDA.

Preparation of aqueous extracts

Aqueous extracts of powdered forms of all the eight reference spice samples were prepared[3]. Powdered spice samples were steeped overnight (temperature: 24-27°C) in sterilized distilled water in a ratio of 1:1 (w: v), followed by their homogenization in a blender at high speed for 2 min. The homogenized spice mixtures were filtered through Whatman No. 1 filter paper. Filtrates thus obtained, were sterilized by passing through syringe filters containing 0.45 um pore size membrane filters under aseptic conditions, collected in sterilized glass vials and were stored at 4±1°C. These stored aqueous extracts were further used within the 2 h. of their preparation.

Screening anticandidal activities of powdered spices

Anticandidal activities of powdered forms of spice samples were examined using spice agar method[4], with a slight modification. Erelenmeyer flasks (100 ml capacity) containing 20 ml of YEPDA and powdered spices at different concentration levels (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 (%,w/v)), were autoclaved at 121° C for 20 minutes. After autoclaving, spice agar mixtures (cooled but still molten) were poured into sterilized petriplates under aseptic conditions and these plates were kept undisturbed for 30 min. for proper setting of agar. Freshly prepared inoculum of test yeast strain at 100 ul level was evenly spread over the entire surface of solidified YEPDA in petriplate using a sterile bent glass rod. Seeded petriplates were incubated in B.O.D. incubator at 25±2°C and were examined for candidal growth at 12h. intervals, throughout the incubation period of 30 days. A similar experiment was carried out without any spice sample that served as control. The time for initiation of yeast growth on control (without spice samples) and media supplemented with different concentration of spices were recorded.

Determination of minimum inhibitory concentration (MIC) values of powdered spices

MIC values of the powdered spice samples towards microbe under observation were determined from the observations of spice agar method[5]. For determining MIC values, concentrations of spices (%, w/v) were plotted on x-axis and days of inhibition on y-axis of the graph. The days elapsed prior to initiation of yeast growth were subtracted from the days taken by the test yeast strain to grow in the control samples (without spice). The 80% level of incubation period was calculated first i.e. 24 days, and then a horizontal straight line was drawn from this level to intersect the curve. From the point of intersection a perpendicular line was drawn indicating the MICs of the reference spice samples.

Screening anticandidal activities of aqueous extracts and essential oils

Impregnated paper disc method[6] was followed. Fresh inoculum (100 µl) of *C. albicans* was spread evenly with sterile bent glass rod over the plates containing solidified YEPDA. Under aseptic conditions, empty sterilized filter paper discs (Whatman No.1, diameter 6 mm) impregnated with different concentrations of the test substances were placed on the surface of the seeded plates. Sterilized filter paper discs moistened with DMSO served as negative control. All petridishes were sealed with laboratory parafilm to avoid eventual loss of volatile test samples. These plates were left at room temperature for at least 1 h. to allow the even diffusion of

impregnated components and were then incubated without inversion at 25±2°C in B.O.D. incubator for 5-8 days depending on the yeast strain implicated in the study. After the incubation period, zones of inhibition formed around the discs were measured in mm and the results were expressed as the net zone of inhibition (mm) which represented the subtraction of the diameter of the paper disc (6 mm) from the measured zone.

Determination of Minimum inhibitory concentration (MIC) values aqueous extracts and essential oils

MIC values of aqueous extracts and essential oils were determined by broth dilution method[6], with a slight modification. The media (yeast extract potato dextrose broth) containing 2000 µl/ml of test substances were serially diluted twofold each with the media (yeast extract potato dextrose broth) to give concentrations of 1000, 500, 250, 125, 62.50, 31.25, 15.62, 7.81, 3.90, 1.95, 0.97, 0.48, 0.24, 0.12, 0.06 µl/ml. Sterilized dimethylsulphoxide (DMSO), instead of test samples of spices served as negative control. To the diluted solution, 100 µl of freshly prepared inoculum of yeast strain was added. These mixtures were incubated in the B.O.D. incubator at 25±3°C, for appropriate incubation periods. After the completion of incubation, 100 µl of the above mixture was evenly spread on the surface of solidified YEPDA petriplates with the help of sterile bent glass rod. These petriplates were incubated in an inverted position to observe the minimum concentration of test substances, at which visible growth of *C. albicans* were fully inhibited for 48 h.

Statistical analysis

All the experiments were performed in triplicates with two independent trials and the results obtained were highly reproducible. Values of growth inhibitory zones are mean ±SD (standard deviation) of three replicates.

RESULTS AND DISCUSSION

Anticandidal activities of powdered forms

Data pertaining to anticandidal activities of powdered forms of test spice samples, at various concentration levels (%, w/v) are presented in table 2. Results revealed that YEPDA petriplates supplemented with different concentrations of *B. nigra*, *C. cassia*, *C. zeylanicum* and *C. cyminum* inhibited *C. albicans* in culture media and gave different levels of inhibition. However, *A. sativum*, *C. longa*, *T. foenum-graecum* and *Z. officinale*, up to 6.0% level, remained ineffective in arresting the test yeast strain, and visible growth of microbe under observation was noticed on 2nd day of incubation, as in control sets of YEPDA petriplates, containing no spice sample. *C. zeylanicum* at concentration as low as 0.4%, delayed the growth of *C. albicans* by 4 days, while higher concentrations of *B. nigra*, *C. cassia* and *C. cyminum* were required to produce growth inhibitory effect. However, a direct and positive relationship was noticed between the concentration of spices and different levels of inhibition produced (table 3), and at a concentration level of 3.0%, *B. nigra*, *C. cassia*, *C. zeylanicum* and *C. cyminum* arrested the growth of *C. albicans* up to 7, 19, 28 and 4 days respectively. Moreover, *C. zeylanicum* at 3.5% level, exhibited desired growth inhibitory activity i.e. arrested reference microbe for more than 30 days. On the other hand, *B. nigra* produced same growth inhibitory effect at a concentration of 6.0%, and *C. cassia* gave at 4.5% level, while *C. cyminum* up to its highest level of 6.0% in current experiment, inhibited *C. albicans* up to 25 days of the total incubation period of 30 days.

Table 2: Anticandidal activities of powdered spice samples

Spice Concentrations (%, w/v)	Spices							
	As	*Bn*	*Cc*	*Cz*	*Ccy*	*Cl*	*Tf-g*	*Zo*
0.0	2	2	2	2	2	2	2	2
0.1	2	2	2	2	2	2	2	2
0.2	2	2	2	2	2	2	2	2
0.4	2	2	2	4	2	2	2	2
0.6	2	2	2	5	2	2	2	2
0.8	2	2	3	7	2	2	2	2
1.0	2	2	4	8	2	2	2	2
1.5	2	2	8	12	2	2	2	2
2.0	2	2	11	18	2	2	2	2
2.5	2	4	15	24	2	2	2	2
3.0	2	7	19	28	4	2	2	2
3.5	2	11	22	>30	7	2	2	2
4.0	2	14	26	>30	10	2	2	2
4.5	2	18	>30	>30	14	2	2	2
5.0	2	21	>30	>30	17	2	2	2
5.5	2	27	>30	>30	21	2	2	2
6.0	2	>30	>30	>30	25	2	2	2

As:A.sativum, Bn: Brassica nigra, Cc: Cinnamomum cassia, Cz: Cinnamomum zeylanicum, Ccy: Cuminum cyminum, Cl: Curcuma longa, Tf-g: Trigonella foenum-graecum, Zo: Zingiber officinale.

Table 3: Different levels of inhibition and MIC values produced by powdered spice samples towards *C. albicans*

Levels of inhibition	Species							
	As	*Bn*	*Cc*	*Cz*	*Ccy*	*Cl*	*Tf-g*	*Zo*
40%	ND	3.75	2.12	1.50	4.26	ND	ND	ND
60%	ND	4.50	2.93	2.00	5.19	ND	ND	ND
80% (MIC)	ND	5.25	3.78	2.50	5.84	ND	ND	ND

As: sativum, Bn: Brassica nigra, Cc: Cinnamomum cassia, Cz: Cinnamomum zeylanicum, Ccy: Cuminum cyminum, Cl: Curcuma longa, Tf-g: Trigonella foenum-graecum, Zo: Zingiber officinale.
MIC: Minimum inhibitory concentration

Test microbe responded differently towards all the powdered spice samples. The exact reason for this differential behaviour of *C. albicans* towards powdered spice samples is not clear, but it may be attributed to different concentrations and compositions of essential oils of test spices and their differential relative permeability to the target site of microbial cell to cause cell lysis or cell death. It is well documented that antimicrobial properties of spices reside in their volatile aromatic secretions commonly known as essential oils[7]. These essential oils in turn are composed of a number of compounds such as, aldehydes (cinnamic aldehyde in *C.*

cassia and *C. zeylanicum;* cuminic aldehyde in *C. cyminum*), sulphur compounds (allicin in *A.sativum* and allylisothiocyanate in *B. nigra*), phenols (curcumin in *C. longa*) and terpene compounds (zingiberene in *Z. officinale*)[8]. These compounds show inhibitory activity against microorganisms either by binding to biochemicals (enzymes, proteins, nucleic acids etc.), those are essential for microbial metabolism and thereby rendering them unavailable for their growth[9] or by microbial membrane/ cell wall disruption[10] causing the leakage of vital ions and other cell contents[11-13]. Furthermore, the inert response of *C. albicans* towards powdered forms of *A. sativum, C. longa, T. foenum-graecum* and *Z. officinale* may be either due to lower essential oil concentrations of aforementioned spices, or may be attributed to the loss of highly volatile essential oil components from spice samples during the process of drying and grinding.

Minimum inhibitory concentration (MIC) values of reference spices towards *C. albicans* ranged from 2.50-5.84 % (w/v). On the basis of MIC values, anticandidal effectiveness of spices in descending order may be put in the following order : *C. zeylanicum> C. cassia> B. nigra> C. cyminum> A. sativum= C. longa= T.foenum-graecum> Z. officinale.*

Anticandidal activities of aqueous extracts and essential oils

Results of disc diffusion assay and broth dilution technique revealed that aqueous extracts of all the spices under observation were found ineffective in inhibiting the test microorganism (table 4 and table 5). The inertness of aqueous extracts towards microbes may be due to the non extraction of the lipophilic constituents of essential oils of reference spice in aqueous phase or their subsequent losses during the milling operation and extraction procedure. Moreover, filtration of extracts in the present study was done through Whatmann filter paper no.1, which might have led to the removal of components, responsible for any antibacterial activity

Impregnated paper disc method results showed that essential oils of all the test spices at 5 ul disc^{-1} , displayed distinct zones of inhibition towards *C. albicans,* except *C. longa, T. foenum-graecum* and *Z. officinale* (Table 4). Diameter of inhibitory zones varied with the type of spice essential oil implicated in the study. Essential oil of *B. nigra* exhibited widest growth inhibitory zone (diameter : 35.10 mm) towards test yeast strain, followed by *C.zeylanicum* (diameter: 33.50 mm). Reasons for anticandidal activities of essential oils of spices are same as mentioned in previous section of powdered spice samples. On the basis of zones of inhibition produced towards *C. albicans,* effectiveness of spice essential oils in descending order may be presented as: *B.nigra> C. zeylanicum> C. cassia> A. sativum> C. cyminum> C. longa= T.foenum-graecum= Z. officinale.*

Table 4: Anticandidal activities of aqueous extracts and essential oils

Spices	Test Substances	Concentration of Test Substances (µl)	Zones of Inhibition (mm)
As	AE	80	0.00
		100	0.00
	EO	5	22.50±0.45
Bn	AE	80	0.00
		100	0.00
	EO	5	35.10±0.11
Cc	AE	80	0.00
		100	0.00
	EO	5	33.50±0.18
Cz	AE	80	0.00
		100	0.00
	EO	5	33.95±0.16
Ccy	AE	80	0.00
		100	0.00
	EO	5	21.00±0.48
Cl	AE	80	0.00
		100	0.00
	EO	30	0.00
Tf-g	AE	80	0.00
		100	0.00
	EO	30	0.00
Zo	AE	80	0.00
		100	0.00
	EO	50	0.00
	DMSO	100	0.00

As: sativum, Bn: Brassica nigra, Cc: Cinnamomum cassia, Cz: Cinnamomum zeylanicum, Ccy: Cuminum cyminum, Cl: Curcuma longa, Tf-g: Trigonella foenum-graecum, Zo: Zingiber officinale.

AE: Aqueous extract, EO : Essential oil, DMSO: Dimethylsulphoxide.

During broth dilution assay, it was observed that except the essential oil of *T. foenum-graecum,* all the other spice essential oils under investigation inhibited the growth of test microbe in YEPD broth. Minimum inhibitory concentrations of substrate components ranged from 7.81-1000 µl/ml. It is also worth mentioning here, that essential oils of spices namely *C. longa* and *Z. officinale* behaved differently towards *C.albicans* during impregnated disc diffusion and broth dilution assays. Growth inhibitory activities of these two essential oils during broth dilution technique may be attributed to liquid phase of media (broth), which might have allowed the easy and quick diffusion of spice essential oil components to reach the target site of *C.albicans.*

Table 5: MIC values of aqueous extracts and essential oils of spices towards *C. albicans*

Test Substances	MIC (µl/ml)							
	As	***Bn***	***Cc***	***Cz***	***Ccy***	***Cl***	***Tf-g***	***Zo***
AE	ND	ND	ND	ND	ND	ND	ND	ND
EO	7.81	7.81	15.62	7.81	31.25	1000	ND	125
DMSO	ND	ND	ND	ND	ND	ND	ND	ND

As: sativum, Bn: Brassica nigra, Cc: Cinnamomum cassia, Cz: Cinnamomum zeylanicum, Ccy: Cuminum cyminum, Cl: Curcuma longa, Tf-g: Trigonella foenum-graecum, Zo: Zingiber officinale.

MIC : Minimum inhibitory concentration, AE: Aqueous extract, EO : Essential oil, DMSO: Dimethylsulphoxide.

CONCLUSION

Concluding, results of present *in vitro* study were quite encouraging as powdered forms of four spices and essential oils seven spices effectively inhibited *C. albicans.* Hence, these spice forms may be investigated further for their antimicrobial potentials against other microorganisms of spoilage and health significance. Moreover, exact mechanisms of mode of action of spice essential oils towards microbes are not well understood, so far. Thus, it would be the next line of research.

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