Differential inhibitory effects of medicinal plant extracts on proline uptake in clinically isolated three *Candida spp.*

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Abstract: Estimation of proline, a stress amino acid with help of antifungal plant extracts. Proline was estimated with Bate's acid ninhydrin method. The selected ten test plants like *Allium cepa, Aegle marmelos, Allium sativm, Aloe vera, Cinnamomum zeylanicum, Eugenia caryophyllus, Moringa oleifera, Mentha viridis, Piper nigrum, Zingiber officinale,* were collected for the experiment. *Candida* species are reported to germinate in high proline medium and the *Candida spp*. change from yeast phase to mycelial phase, the virulence phase. With the addition of plant extracts to the medium along with maximum utilizable concentration of proline, growth of all the three *Candida spp.* was inhibited .After 48hrs incubation at 37°C , the findings presented that *Allium sativum* strongly inhibited the growth as *Candida albicans* with biomass 0.232 ± 0.01 g / 50 mL , *Candida parapsilosis* with biomass 0.265 ± 0.01 g / 50 mL and *Candida tropicalis* with biomass 0.206 ± 0.01 g / 50 mL in comparision to control (2.077 ± 0.08 g / 50 mL in *C.albicans*, 0.943 ± 0.01 g / 50 mL in *C.parapsilosis* and 1.192 ± 0.08 g / 50 mL in *C.tropicalis*). On extraction of proline from respective broth cultures of three *Candida spp.* it was found that the enzyme proline-permease which helps in the uptake of proline into the *Candidal* cell may be inhibited. So proline cannot be utilized by the *Candida spp.* Bioactive compounds from plants in purified form, can replace synthetic drugs and used efficiently against dreadful diseases. [Nature and Science 2010;8(9):132-139]. (ISSN: 1545-0740).

Key words: *Candida spp.*; proline uptake; selected plant extracts; inhibitory effects.

1. Introduction

Nature always stands as a golden mark to exemplify the outstanding phenomenon of symbiosis. The biotic and abiotic elements of nature are all interdependent. The plants are indispensable to man for his life. The three important necessities of life food, clothing and shelter and a host of other useful products are supplied to him by the plant kingdom. Nature has provided a complete store-house of remedies to cure all ailments of mankind. The knowledge of drugs has accumulated over thousands of years as a result of man's inquisitive nature so that today we possess many effective means of ensuring health care(Juglal *et al*.,2002).

In the presence of certain body fluids *in vitro*, or in tissue *in vivo*, the yeast form of *Candida albicans* will convert to a filamentous form. This was first demonstrated in blood and serum acquired from debilitated individuals by Reynolds and Braude (1956). Filamentation in combination with certain host factors may be an important factor in pathogenesis consequently a better understanding of the mechanism controlling the yeast-mycelial balance might lead to a means of controlling systematic disease. In the majority of *in vitro* experiments, either serum or various complex artificial media were used to induce filamentation (Alexander and Pfaller, 2006; Gordana *et al.,* 2008).

Among several factors which affects yeast to mycelia transition of the pathogenic pleiomorphic yeast *Candida albicans*, various nutrients play an important role. Amino acids which enter metabolism by their conversion to 2-oxoglutarate are generally more active in including germ tube formation in *Candida albicans*. L-proline, one such amino acid is known to be an efficient germ tube stimulator. Proline, a non-essential amino acid, is of interest clinically because of hyperprolinemia resulting from a deficiency of proline oxidase in association with congental renal defects and hematuria(Al-Fattani and Douglas,2006; Holmes and Shepherd,1987; Jethwaney *et al*.,1997) .

Living organisms are exposed to diverse forms of environmental stress including changes in temperature, water content, osmolarity , pH , oxidation , nutritional starvation and chemical compounds. Under severe stress conditions, cellular macromolecules such as

proteins, nucleic acid and membranes are seriously damaged and lead to growth inhibition or cell death. To survive these stresses and to avoid potentialy lethal damage the cells adapt a variety of stress proteins, accumulation of compactible solutes etc. Proline is an important amino acid which is also known as a stress substrate. It is believed to have multiple functions as it stabilizes proteins and membranes and scavenge reactive oxygen species. It has been reported that proline level increases in the blood serum when the body has an infection. *Candida* species are reported to germinate in high proline medium and the *Candida spp.* change from yeast phase to mycelial phase, the virulence phase(Shepherd, 1991). So work is carried out to find out

i. The effect of increasing concentration of proline on *Candidal* growth.
ii. Effect of some medicinal plants on uptake of proline from the medium.

2. Materials and methods

2.1 Test organisms

Candida spp. from urine, sputum, pus, blood, throat swab, stool and high vaginal swab of patients attending to S.C.B. Medical College, Cuttack were isolated by the conventional methods using bacteriological media (MacConkey agar and blood agar) as *Candida* is able to grow in such media while carrying out the bacteriological analysis (Chander, 2002; Ellen *et al*., 1990). Then identification upto species level was made using Chromagar *Candida* tube test. All the species identified by this method were reconfirmed by using other conventional methods (Hospenthal *et al*., 2008; Odd and Bernaerts, 1994).

2.2 Extraction and Estimation of proline

Proline was estimated with acid ninhydrin method of Bates (1973). A proline grade ranging from 0 - 100 µg/mL was prepared and final volume was adjusted to 2mL with distilled water 2 mL of acid ninhydrin reagent and 2 mL of glacial acetic acid were added and boiled for 1hr and cooled in ice bath immediately, 4mL toluene was added and shaken thoroughly to extract the colour. OD was read at 520 nm. The *Candidal* cultures were allowed grown in Sabouraud dextrose broth for 2 to 3 days. Once sufficient growth has been achieved, pellets were extracted from the cultures through centrifugation at 7000 rpm. The pellets were washed with sterile distilled water and centrifuged. The supernatant were discarded and pellets were allowed to be dried for proline extraction and estimation.

Homogeneate dry pellet in a mortar and pestle and 2mL of sulphosalicylic acid (3% aqueous solution) was added then centrifuged and the supernatant was used for estimation of proline (mentioned above). Blank was prepared with sulfosalicylic acid.

2.3 Estimation of proline in presence of selected plant extracts

The selected each test plants like *Allium cepa, Aegle marmelos, Allium sativm, Aloe vera, Cinnamomum zeylanicum, Eugenia caryophyllus, Moringa oleifera, Mentha viridis, Piper nigrum, Zingiber officinale,* were collected and washed first in tap water and then in distilled water. These were macerated in mortar with a pestle. Selected cocentration as 10g/5mL were prepared with distilled water and were filtered off with sterile Whattman No. 1 filter paper into clean sterilized tubes.

Antifungal activity was performed by taking Sabouraud dextrose broth in which 1ml of each respective plant extracts along with 40µL proline and 0.1ml of three stock cultures (200x 10^5 CFU/mL) of *C.albicans, C. parapsilosis, C.tropicalis* were allowed to incubate for 48hrs at room temperature. Controls for three Candida spp. were prepared without adding extracts. One sufficient growth has been achieved; the incubated cultures were filtered off with sterile Whattman No. 1 filter paper into clean sterile tubes. The biomasses were allowed to homogeneated in a mortar and pestle and 2mL of sulphosalicylic acid (3% aqueous solution) was added, centrifuged and the supernatant was used for estimation of proline. To the 2mL supernatant, 2mL acid ninhydrin and 2mL glacial acetic acid was added and incubated for 1hr at 100˚C covering the tubes. The tubes were transferred immediately to ice bath; 4mL of toluene was added and shaken thoroughly for 20mins. The toluene extract was taken and OD was measured at 520 nm. Blank was prepared with sulfosalicylic acid.

3. Statistical analysis

The experiment for the antifungal activity was conducted and analyzed as a factorial experiment with three replications in a completely randomized design. The results were presented as the means with SEM (standard error of the mean).

3.1 Results

Shepherd (1991) reported that the transition to mycelial morphology requires the presence of amino acid proline in the medium. The first protuberances were observed 60-90 minutes after exposure to the

proline at 37°C. Growth increased up to a concentration of 40µg in *C.albicans*, 60µg in case of *C.parapsilosis* and *C.tropicalis. C.albicans* showed maximum growth at 40µg (0.902±0.01) where as *C.parapsilosis* and *C.tropicalis* showed maximum growth at concentration 60µg, the OD value being 0.784±0.01 and 0.82±0.01 respectively. The growth remained constant with increase of proline concentration in medium (Table-1).

When both proline and antifungal plant extracts were added to the broth medium , it has been seen that growth of three *Candida spp.* were inhibited by antifungal plant extracts even in the presence of proline.

Table 1. Growth rate of three *Candida spp.* in presence of proline (absorbance at 520nm)

Proline in microgram	***Candida albicans***	***Candida parapsilosis***	***Candida tropicalis***
Control	0.632±0.01	0.527±0.01	0.54±0.01
10	0.868 ± 0.01	0.758 ±0.01	0.776 ±0.01
20	0.879 ±0.01	0.766 ±0.01	0.797 ±0.01
30	0.893 ±0.01	0.772 ±0.01	0.81 ±0.01
40	0.902 ±0.01	0.779 ±0.01	0.812 ±0.01
50	0.902 ±0.01	0.782 ± 0.01	0.812 ±0.01
60	0.902 ±0.01	0.784±0.01	0.82 ±0.01
70	0.902 ±0.01	0.784 ±0.01	0.82 ±0.01
80	0.902 ±0.01	0.784±0.01	0.82 ±0.01
90	0.902 ±0.01	0.784±0.01	0.82 ±0.01
100	0.902 ±0.03	0.784±0.01	0.82 ±0.01

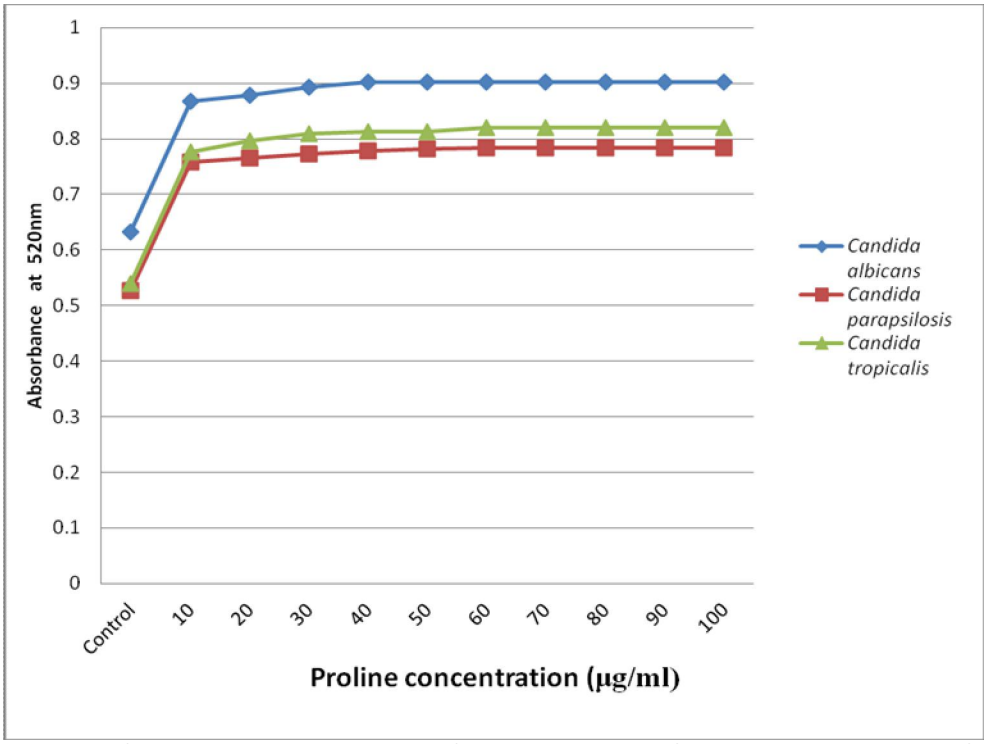

Figure 1. Growth rate of three *Candida spp.* in presence of proline. (Proline was found to increase the growth of all the three *Candida spp.* to a concentration of 40µg in *C.albicans* and 60µg in other two species *C.parapsilosis* and *C.tropicalis.).*

Table 2. Effect of selected ten plant extracts on biomass (gms/50mL) of three *Candida spp.* in presence of proline (40µg/50mL).

Names of selected plants	***Candida albicans***	***Candida parapsilosis***	***Candida tropicalis***
Control	2.077±0.08	0.934±0.01	1.192±0.08
Allium cepa	0.652±0.01	0.619±0.01	0.714±0.01
Aegle marmelos	0.906±0.02	0.714±0.02	0.856±0.01
Allium sativum	0.232±0.01	0.265±0.01	0.206±0.01
Aloe vera	0.803±0.01	0.723±0.01	0.84±0.01
Cinnamomum zeylanicum	0.65±0.01	0.457±0.01	0.629±0.01
Eugenia caryophyllus	0.247±0.01	0.33±0.01	0.332±0.01
Moringa oleifera	0.809±0.01	0.65±0.01	0.74±0.01
Mentha viridis	0.321±0.01	0.332±0.01	0.368±0.01
Piper nigrum	0.841±0.01	0.58±0.01	0.829±0.01
Zingiber officinale	0.835±0.01	0.546±0.01	0.849±0.01

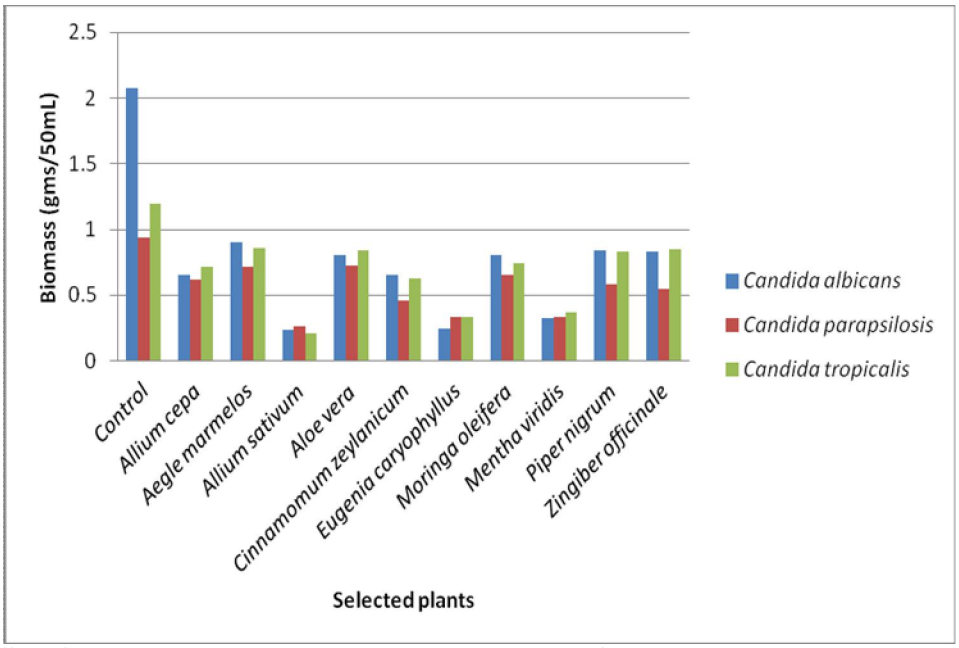

Figure 2. Effect of selected ten plant extracts on biomass (gms/50mL) of three *Candida spp.* in presence of proline (40µg/50mL)(With the addition of plant extracts to the medium along with maximum utilizable concentration of proline, growth of all the three *Candida spp.* was inhibited).

Table 3. Effect of selected ten plant extracts on three *Candida spp.* in presence of proline (40µg/50mL).

Names of selected plants	***Candida albicans***	***Candida parapsilosis***	***Candida tropicalis***
Control	32.67 ± 2.13	22.33 ± 1.66	25.67± 1.96
Allium cepa	8± 0.47	8.67± 0.72	9.67 ± 0.54
Aegle marmelos	15 ± 0.94	14.33± 0.72	15.33± 0.72
Allium sativum	4.33± 0.54	5 ± 0.47	3.67 ± 0.47
Aloe vera	11.67 ± 0.72	11 ± 0.47	13 ± 0.47
Cinnamomum zeylanicum	8± 0.82	7.67 ± 0.54	8.33 ± 0.27
Eugenia caryophyllus	4.67 ± 0.72	5.33 ± 0.54	6.67 ±0.72
Moringa oleifera	11.33 ± 0.72	13.67 ± 0.72	10.67 ± 0.72
Mentha viridis	6.33 ± 0.27	8.83± 0.49	6.67± 0.54
Piper nigrum	13.67 ± 0.54	14.67± 0.98	12 ± 0.47
Zingiber officinale	12.67 ± 0.54	13± 0.94	13.67 ± 0.98

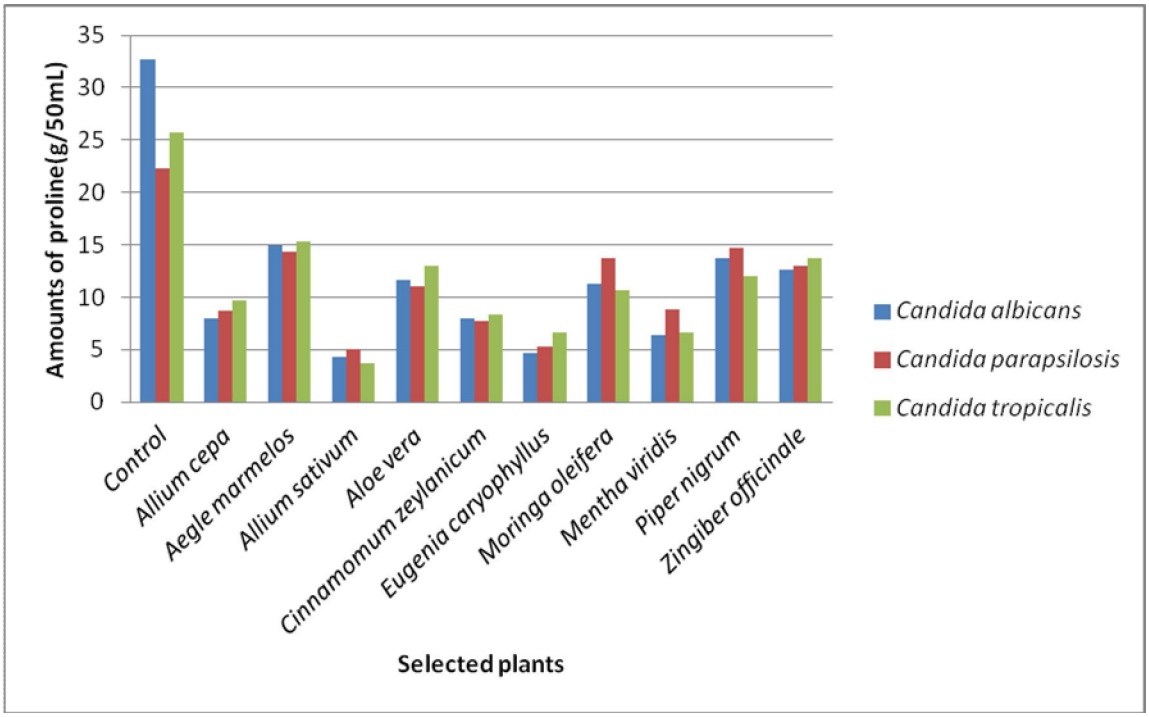

Figure 3. Effect of selected ten plant extracts on three *Candida spp.* in presence of proline (40µg/50mL) (With the addition of plant extracts to the medium along with maximum utilizable concentration of proline, of all the three *Candida spp.* was inhibited).

Figure 4. Proline concentration of three *Candida spp.* (L à R: Blank, *C.albicans, C.parapsilosis, C.tropicalis*)

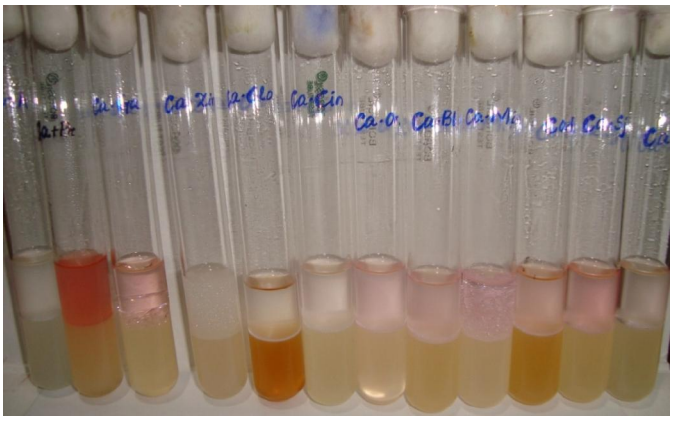

Figure 5. Effect of selected ten plant extracts on *Candida albicans* in presence of proline (left to right- blank, Ca=Control *C.albicans*, Ca.As= *C.albicans* treated with *Allium sativum*, Ca.Zi= *C.albicans* treated with *Zingiber officinale* , Ca.Ec=*C.albicans* treated with *Eugenia caryophyllus*, Ca.Cz= *C.albicans* treated with *Cinnamomum zeylanicum*, Ca.Ac=*C.albicans* treated with *Allium cepa*, Ca.Am=*C.albicans* treated with *Aegle marmelos* , Ca.Mv= *C.albicans* treated with *Mentha viridis*, Ca.Av=*C.albicans* treated with *Aloe vera*, Ca.Mo=*C.albicans* treated with *Moringa oleifera*, Ca.Pn=*C.albicans* treated with *Piper nigrum*)

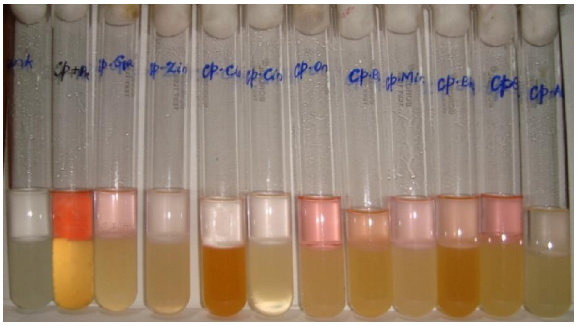

Figure 6. Effect of selected ten plant extracts on *Candida parapsilosis* in presence of proline.
(left to right- blank,
Cp=Control *C.parapsilosis*,
Cp.As= *C. parapsilosis* treated with *Allium sativum*,
Cp.Zi= *C. parapsilosis* treated with *Zingiber officinale*,
Cp.Ec=*C. parapsilosis* treated with *Eugenia caryophyllus*,
Cp.Cz= *C. parapsilosis* treated with *Cinnamomum zeylanicum*,
Cp.Ac=*C. parapsilosis* treated with *Allium cepa*,
Cp.Am=*C. parapsilosis* treated with *Aegle marmelos*,
Cp.Mv= *C. parapsilosis* treated with *Mentha viridis*,
Cp.Pn=*C. parapsilosis* treated with *Piper nigrum*,
Cp.Mo=*C. parapsilosis* treated with *Moringa oleifera*,
Cp.Av=*C. parapsilosis* treated with *Aloe vera*).

Figure 7. Effect of selected ten plant extracts on *Candida tropicalis* in presence of proline.
(left to right- blank,
Ct=Control *C. tropicalis*,
Ct.As= *C. tropicalis* treated with *Allium sativum*,
Ct.Zi= *C. tropicalis* treated with *Zingiber officinale*,
Ct.Ec=*C. tropicalis* treated with *Eugenia caryophyllus*,
Ct.Cz= *C. tropicalis* treated with *Cinnamomum zeylanicum*,
Ct.Ac=*C. tropicalis* treated with *Allium cepa*,
Ct.Am=*C. tropicalis* treated with *Aegle marmelos*,
Ct.Mv= *C. tropicalis* treated with *Mentha viridis*,
Ct.Pn=*C. tropicalis* treated with *Piper nigrum*,
Ct.Mo=*C. tropicalis* treated with *Moringa oleifera*,
Ct.Av=*C. tropicalis* treated with *Aloe vera).*

After 48hrs incubation at 37°C , the findings presented that *Allium sativum* strongly inhibited the growth as *C.albicans* with biomass 0.232 ± 0.01 g / 50 mL , *C.parapsilosis* with biomass 0.265 ± 0.01 g / 50 mL and *C.tropicalis* with biomass 0.206 ± 0.01 g / 50 mL in comparision to control (2.077 ± 0.08 g / 50 mL in *C.albicans*, 0.943 ± 0.01 g / 50 mL in *C.parapsilosis* and 1.192 ± 0.08 g / 50 mL in *C.tropicalis*) (Table – 2).Similarly *Allium cepa* , *Cinnamomum zeylanicum* , *Eugenia caryophyllus* and *Mentha viridis* showed good inhibition effect against three *Candida spp.* in comparision to *Aegel marmelos* , *Aloe vera* , *Moringa oleifera* , *Piper nigrum* , *Zingiber officinale* .It has also seen that the concentration of proline remained as such in the medium after the incubation period .On extraction of proline from respective broth cultures of three *Candida spp.* it was found that after treatment with *A.sativum*, 4.33 ± 0.54 µg / 50 mL amount of proline from *C.albicans* , 5 ± 0.47 µg / 50 mL from *C.parapsilosis* and 3.67 ± 0.47 µg / 50 mL from *C.tropicalis* were extracted in comparision to control 32.67 ± 2.13 µg / 50 mL from *C.albicans*, 22.33 ± 1.66 µg / 50 mL from *C.parapsilosis* and 25.67 ± 1.96 µg / 50 mL from *C.tropicalis* followed by *A.cepa* (8 ± 0.47 µg / 50 mL from *C.albicans* , 8.67 ± 0.72 µg / 50 mL from *C.parapsilosis* and 9.67 ± 0.54 µg / 50 mL from *C.tropicalis*.) , *C. zeylanicum* (8 ± 0.82 µg / 50 mL from *C.albicans* , 7.67 ± 0.54 µg / 50 mL from *C.parapsilosis* and 8.33 ± 0.27 µg / 50 mL from *C.tropicalis*) , *E. caryophyllus* (4.67 ± 0.72 µg / 50 mL from *C.albicans* , 5.33 ± 0.54 µg / 50 mL from *C.parapsilosis* and 6.67 ± 0.27 µg / 50 mL from *C.tropicalis*) , *M. viridis* (6.33 ± 0.27 µg / 50 mL from *C.albicans* , 8.83 ± 0.49 µg / 50 mL from *C.parapsilosis* and 6.67 ± 0.54 µg / 50 mL from *C.tropicalis*) (Table – 3). *A. marmelos* , *A. vera* , *M. oleifera* , *P. nigrum* and *Z. officinale* showed moderate inhibitory effect. This may be due to the fact that the enzyme proline-permease which helps in the uptake of proline into the *Candidal* cell may be inhibited. So proline cannot be utilized by the *Candida spp.*

4. Conclusions

Proline was found to increase growth of all the three *Candida spp.* It was showed that among amino acids that induced germination, those that enter the metabolism by conversion to α-ketoglutarate are generally the more active in enhancing growth. This was also confirmed by Dabrowa and Howard (1981). In this experiment proline was found to increase the growth of all the three *Candida spp.* to a concentration of 40µg in *C.albicans* and 60µg in other two species *C.parapsilosis* and *C.tropicalis.* This may be due to uptake of proline and its metabolism inside the cells. Beyond that concentration proline was found to have

no positive effect in enhancing the biomass growth. So, after 40µg and 60µg growth remained constant (Figures 1and 4).

Proline gets converted to α-ketoglutarate an intermediate of TCA cycle. α-ketoglutarate gets converted to other metabolism and in this process reducing power $NADH_2$ and energy are produced which accelerate the growth of *Candida spp.* (Jethwaney *et al*.,1997). Thus concentration beyond 60 did not further enhance the growth but proline concentration increases in the biomass. This may be due to the fact that at higher proline concentration, the active transport system of proline was accompanied by diffusion. Though more amount of proline was taken in this cannot be metabolized further due to reduction in TCA cycle activity because it has been seen that accumulation of more organic acids in the cellular environment inhibits the functioning of the TCA cycle. So higher concentration of proline was found to have no increasing effect on the biomass growth of three *Candida spp.*

With the addition of plant extracts to the medium along with maximum utilizable concentration of proline, growth of all the three *Candida spp.* was inhibited (Figure -2 and 3). This may be due to the inhibition of proline-permease, the enzyme essential for the transport of proline into the *Candidial* cell. Proline cannot be metabolized the species, cannot get energy and reducing power. Thus growth was inhibited (Figures 5, 6 and 7).

Scientists from divergent fields are investigating different plant extracts with an eye to their antimicrobial usefulness. Laboratories of the world have found literally thousands of physiochemical that have inhibitory effects on all types of micro-organisms *in vitro*. Evaluation of a crude drug means its identification and determination to its purity and quality. Quality control of a crude drug and its pharmaceuticals can be attempted by different methods evaluated depending upon the morphological and microscopical studies of the crude drugs or their physical, chemical and biological behavior. Bioactive compounds from plants in purified form, can replace synthetic drugs and used efficiently against dreadful diseases. Health foundations have to increase their funding of these studies and research to help saving the lives of many peoples. This will also offer a great help in facing the emergence spread of antimicrobial resistance.

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References

[1] Alexander,B. D. , Pfaller,M.A. Contemporary tools for the diagnosis and management of invasive mycoses. Clin Infect Dis. 2006:43:15-27.

[2] Al-Fattani,M.A. , Douglas,L.J. Biofilm matrix of *Candida albicans* and *Candida tropicalis* : chemical composition and role in drug resistance. Journal of Medical Microbiology. 2006:55:999-1008.

[3] Bates, L.S. Rapid determination of the proline for water-stress studies. Plant soil. 1973:39:205-207.

[4] Chander,J. A textbook of Medical Mycology. 2nd ed. 2002:212-227.

[5] Dabrowa, N. and Howard, D.H. Proline uptake in *Candida albicans.* Jour. of General Microbiology. 1981:127:391-397.

[6] Ellen,J.B., Lance,R.P., Syoney,M.F. Bailey & Scott's Diagnostic Microbiology. Mosby.9th ed.1990:219-233.

[7] Gordana,M.B.M., Momir,M.M., Svetlana,M.G.K. The importance of genus *Candida* in human samples. Proc Nat Sci,Matica Srpska Novi Sad. 2008:114: 79-95.

[8] Hospenthal,D.R., Murray,C.K., Beckius,M.L., Green,J.A., Dooley,D.P. Persistence of pigment production by yeasts isolates grown on CHROMagar Candida medium. J Clin Microbiol. 2002:40:4768-4770.

[9] Holmes, A. R., Shepherd, M. G. Proline-induced germtube formation in *Candida albicans*: role of proline uptake and nitrogen metabolism. J Gen Microbiot.1987:133: 3219-3228.

[10] Jethwaney,D., Hofer, M., Khaware, R.K., Prasad,

R. Functional reconstitution of a purified proline permease from *Candida albicans* : interaction with the antifungal cispentacin. Microbiology.1997:143:397-404.

[11] Juglal,S.,Govinden,R., Odhav,B. Spices oils for the control of co-occuring mycotoxin producing fungi. Journal of Food Protection.2002:65:638-687.

[12] Odd,F.C., Bernaerts,R. CHROMagar Candida, a new differential isolation medium for presumptive identification of clinically important *Candida* species. J Clin Microbiol. 1994:32:1923-1929.

[13] Reynolds, R., Braude, A. The filament inducing property of blood for *Candida albicans* : its nature and significance. Clin. Res. Proc. 1956:4: 40 –47 1956.

[14] Shepherd, M.G. Morphogenesis in *Candida albicans.* In *Candida albicans*: Cellular and Molecular Biology, Edited by R. Prasad. Heideberg : Springer –Verlag. 1991: 5-19.

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