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Original Research Article

Preliminary evaluation of in-Vivo and in-Vitro antifungal activity of Piper longum, Origanum majorana, Embelia ribes and Butea monosperma with Gas chromatography-mass spectrometry analysis of phytochemical property of plant extracts against Candida species causing dermatological *Candidiasis*

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ABSTRACT

Background: Globally, fungi infections cause more than 1.5 million fatalities annually, mostly in those with weakened immune systems with Candida albicans found to be the chief pathogen. Approximately one to two billion people are affected by dermatological fungal infections involving skin and skin appendages, perhaps very difficult to estimate its exact prevalence due to under-reporting, this augments the requirements for more attention towards new molecules to combat resistance.
Objective: Evaluation of antifungal activity of Piper longum, Origanum majorana, Embelia ribes, Butea monosperma both in-vivo and in-vitro analysis against Candida species.
Materials and Methods: This experimental study conducted for evaluating both in-vitro as well as in-vivo anti-fungal activity of Piper longum, Butea monosperma, Embelia ribes and Origanum majorana, and evaluation of phytoconstituent through Gas Chromatography and Mass Spectrometry analysis by GC-MS-QP-2010 plus system to identify the active bio-component. Fluconazole used as the standard control in-vivo and Itraconazole used as a standard drug for in-vitro infection and analysis. Ethical Committee approval was taken (IMS, BHU Dean/2018/CAEC/818). The antifungal activity of the extracts in-vitro was evaluated by Resazurin microtiter assay against Candida sp. Micro broth dilution methods. Rats were given an injection of 0.2 mL (intravenously/i.v) of a 10° UFC/mL inoculum made from a fresh 48-hour Candida albicans culture in sterile saline to cause *candidiasis* infection. Induction of candidemia in rats was confirmed using qualitative Gram-stained smear.
Results: The extracts of Butea monosperma (Alc.), Piper longum (Alc.), Origanum majorana (Aq.), and Piper longum (Aq.) showed effective antifungal activity against Candida sp. in-vitro research with a minimum inhibitory concentration (MIC) of less than 0.25 mg/mL.
Conclusion: This study is contributing towards the search for a natural drug candidate to combat resistance caused by the Candida genus, as it is one of the highest contributors to fungal dermatological/skin infections.

1. Introduction

Fungal infections share a significant mortality and morbidity burden with the world's population with more than 1.5

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million fatalities annually, mostly in those with weakened immune systems.[1] Its prevalence is far greater than might be thought. As these infections are infrequent, some are common in particular regions, and even not categorized under notifiable diseases making global estimation regarding prevalence of these subcutaneous infections difficult. Due to under reporting, WHO(world health organization) considered these infections to be Neglected Tropical Diseases.[2]

Human skin is often colonized with fungal and bacterial communities, among fungi species of Canidida, Malassezia, Cryptococcus and Rhodotorula have been detected as skin commensals however variety species are also disease causing i.e. pathogenic. Pathogenic species of fungi can be categorized into two groups: dermatophytes and yeast, with Candida species belonging to the latter. Candida has approximately 200 known species, among them C. albicans is responsible for majority symptomatic dermatological infections.[3]

The contributive and causative components can be designated as endogenous and exogenous factors. Endogenous factors, like Chronic disease, diabetes mellitus, obesity, endocrinopathy, age related, immunological and physiological changes in pregnancy whereas exogenous factors include skin cleanliness, climatic variations, foot bath habits and contact with patients of same disease.[4]

A systematic review found that 10.34% of the C. albicans isolates in India were resistant to medication.[5] Some fungi produce mycotoxins, which are responsible for multiple health hazards. Pneumocystis, Aspergillus, and Candida are the fungi responsible for more than 80 % of all fungal infections seen in hospitalized patients. Candida is more common in immunosuppressed patients, like organ transplants and HIV, overall mortality rates vary from 6% to 12%.[6] Alterations to the ERG11 gene in fungi, changes to sterol production, decrease in intracellular concentration of target enzymes, and overexpression of drug efflux are some of the reasons for fungus resistance.[7] The annual incidence of Invasive *Candidiasis* in India is 34/100000 population, with *C. albicans* accounting for ~21% of cases.[8]

Growing resistance against conventional therapies and the emergence of newer symptoms only amplifies the requirement for more attention towards reverse pharmacology and the study of phytochemicals in the field of modern medicine and pharmacology. The aim of our experimental study is the assessment of the antifungal activity of Origanum majorana, Embelia ribes, Piper longum, and Butea monosperma. The primary goal of this study is evaluation of in-vivo and in-vitro antifungal activity of methanolic as well as aqueous extract of the fruit of Embelia ribes, the fruit of Piper longum, the seed of, the seed of Butea monosperma. The secondary objective is the evaluation of the Phytochemical constituents of Embelia ribes, Butea monosperma, Piper longum, and Origanum majorana through GC-MS analysis and to identify antifungal phytoconstituent of these extracts. The null hypothesize is, the antifungal activity of Embelia ribes, Butea monosperma, Piper longum, and Origanum majorana, is not as effective as Standard Control Drugs (Itraconazole and Fluconazole).

2. Material and Methods

2.1. Ethical approval

Ethical committee approval was taken, letter number: Dean/2018/CAEC/818.

2.2. Extract preparation

The seeds and fruits have been verified by the faculty of Ayurveda, Dravyaguna department, IMS-BHU, Varanasi. The plant's name was validated using http://www.theplantlist.org.[9] The methanolic and aqueous extracts were prepared using the Maceration method.

2.3. Fungal isolate

A clinical isolate of Candida albicans from the infection control section's repository, was employed. The isolate was revived from storage in the stock medium at -20^{0}C by culture on a plate of blood agar.

2.4. Determination of in-vitro antifungal activity

The Resazurin microtiter test was used to measure the extracts' antifungal efficacy against Candida sp. [Figure 4] The micro broth dilution technique was used to calculate MICs with a concentration range of 32 to 0.25 g/ml. In 96-well microplates, yeast suspensions in the log growth phase were serially diluted with extracts and cultured overnight at 37°C with a concentration of 0.5 MacFarlane. Standard Drug (Itraconazole), at the same concentration range, was used as a control. To verify the experiment, wells with only sterile media were used as the media control. Every experiment was carried out twice.

2.5. Determination of In-vivo Antifungal activity

Experimental animals: Animals: Charles Foster rats of either sex, 40 in number, weighing 200-250 grams were used in the study. The investigation excluded pregnant female rats, aged rodents, and sick and ill rats. The animals were acclimatized in the laboratory for 8 days. Rats were kept in groups of 12 in a conditioned room at 22+20 degrees celsius with a schedule of 12 hours each of lighting and darkness. The animals were given unrestricted access to the usual pellet meal as well as tap water. Throughout the trial, CPCSEA criteria were followed.

2.6. Drug administration

The extract was suspended in CMC and delivered orally through an orogastric tube based on dosage and rat body weight. There were 6 groups, 4 Methanolic extracts of Embelia ribes (Group 1).[10] Butea monosperma (Group 2),[11,12] Origanum majorana (Group 3),[13] Piper longum (Group 4).[14] The dose given of all the extracts was 200 mg/kg, p.o, Oral fluconazole (Group 5) was10 mg/kg per day[15] and Group 6 was the Control group.

2.7. Induction of systemic infection in rats

All the animals were weighed before the experiment. Blood slides were prepared, and complete blood counts were analyzed before the initiation of the experiment. Methylprednisolone: 10 mg/Kg 12 hourly i.v., was continued for 3 days. On the third day, rats were infected with Candida albicans using 0.2mL of i.v. injection of 10° CFU/mL inoculum generated from a fresh 48 h Candida albicans culture in sterile saline.

2.8. Determination of viable yeasts in blood and organs

Induction of candidemia in rats was confirmed by a qualitative Gram-stained smear of peripheral blood samples (PBS) after three days of injection of fungal culture. Following treatment with extracts, semiquantitative Gram stain scoring of PBS and quantitative blood culture were done at 48 hrs, and at 120 hrs and quantitative blood, as well as organ culture, were done post dissection. Briefly, blood samples were serially diluted up to a concentration of 10^{-6} and 0.1 ml of these dilutions were plated on Sabouraud dextrose agar (SDA). Fungal colonies were counted, and the mean colony count was calculated. [Figures 1 and 2]

2.9. Quantification of candida in organs

Ether inhalation was used to sacrifice overnight-fasted rats after 15 days of therapy. To quantify the Candida organism in organs, both kidneys and the intestine, liver, as well as spleen were removed. Organ punch has been taken on slides for quantitative investigation.

2.10. Gas chromatography-mass spectrometry (GC-MS) investigation

Utilizing a split injection mode, samples with a split ratio of 10: 1 injected. The GC-MS-QP-2010 plus equipment was used for the gas chromatography-mass analysis. (Shimadzu Co., Kyoto, Japan). In accordance with the manufacturer's instructions the mass spectrometer was calibrated. Rxi-5 SIL MS column was utilized (30M 0.25 mm id 0.25 u, film thickness). Helium used as carrier gas with 1.21 ml min-1 maintained flow rate. Temperature for injection set to 260°C, interface to 270°C, and ion source at 220°C. Temperature program includes isothermal heating at 60°C, at 250^{o}C for 2minutes each and 21 minutes at 280^o C. On comparing average peak area for different components to the overall area, the relative percentage quantity of all scomponent was calculated. The mass lab software was used for analyzing the chromatograms and mass spectra. (Thermo Quest, Manchester, UK). Within the mass lab technique format, a retention time along with mass spectrum library for automated peak measurement of metabolite derivatives was developed.

2.11. Statistical analysis

Using Microsoft Excel (Microsoft Corporation), ANOVA: Two-factor was used to examine the mean growth inhibition across various extracts and fluconazole at different time intervals. (2018). Furthermore, the impact of the Piper longum extract was compared to the standard drug fluconazole using ANOVA single factor.

3. Results

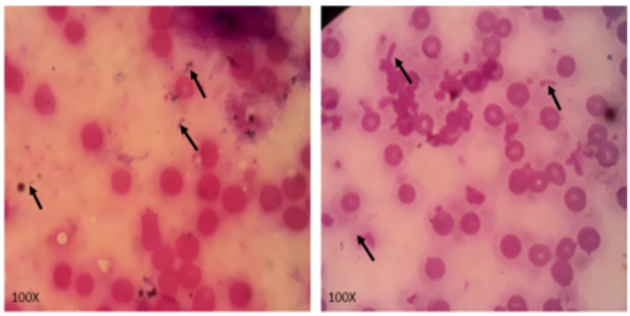

Figure 1: Gram-stained smears of peripheral blood showing the presence of yeast cells (marked with arrows)

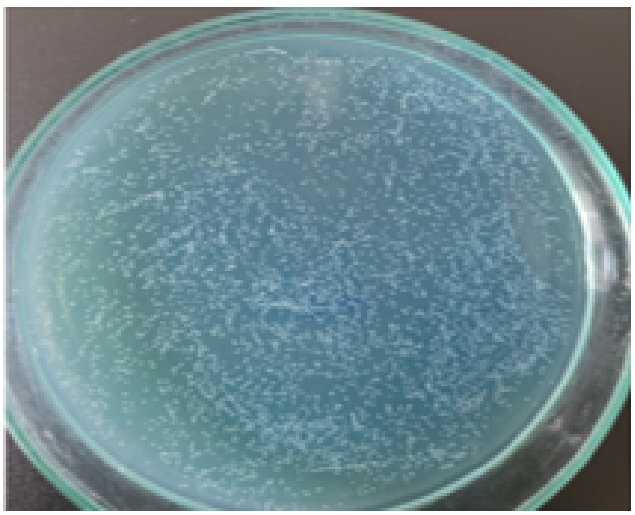

Figure 2: Growth of Candida albicans by quantitative blood culture.

3.1. *In-vitro study*

The extracts of Butea monosperma(Alc.), Piper longum(Alc.), Origanum majorana (Aq.) and Piper longum (Aq.) showed effective antifungal activity with ≤ 0.25 mg/ml i.e. a minimum inhibitory concentration (MIC), against Candida sp.. The MIC of extracts of Embeliaribes (Aq.), Butea monosperma (Aq.) and Origanum majorana (Alc.) was detected to be 0.5 mg/ml, 8 mg/ml and ≥ 32 mg/ml respectively. The standard drug, Itraconazole exhibited a MIC value of ≤ 0.25 mg/ml against test microorganism.

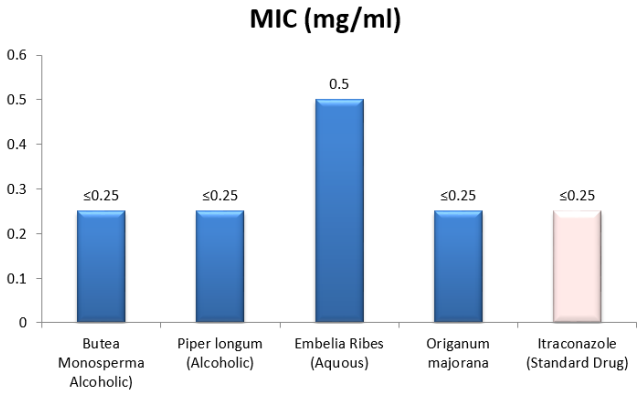

Figure 3: Graph showing theminimum inhibitory concentration of extracts against the test microorganism.

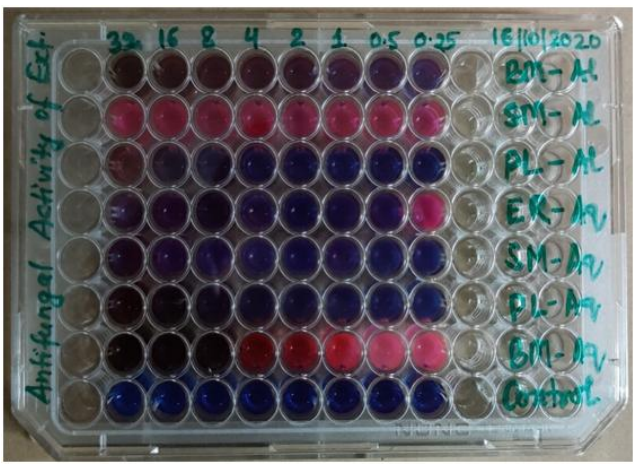

Figure 4: Showing 96 well plate micro broth dilution method using Resazurin dye for the determination of the MIC of the plant extracts against the Candida sp.

3.2. *In-vivo activity*

Semi-quantitative Gram stain scoring: This showed that fungal load in blood was at its maximum on Day 1 in rats. However, on the day of dissection, semiquantitative Gram stain from blood could not detect fungi from Piper longum, Emelia and fluconazole.

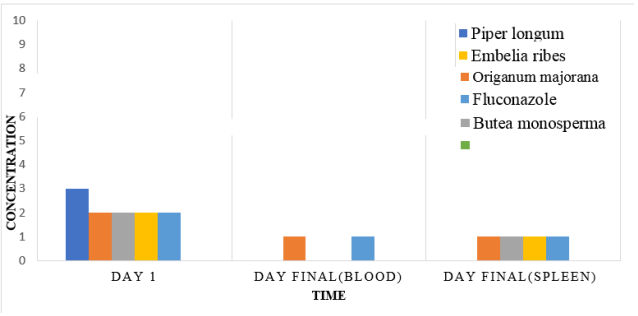

Figure 5: Semi-quantitativegram stain scoring

3.3. *Quantitative culture*

Quantitative blood culture showed the maximum effect of Piper longum followed by Embelia ribes and Butea monosperma.

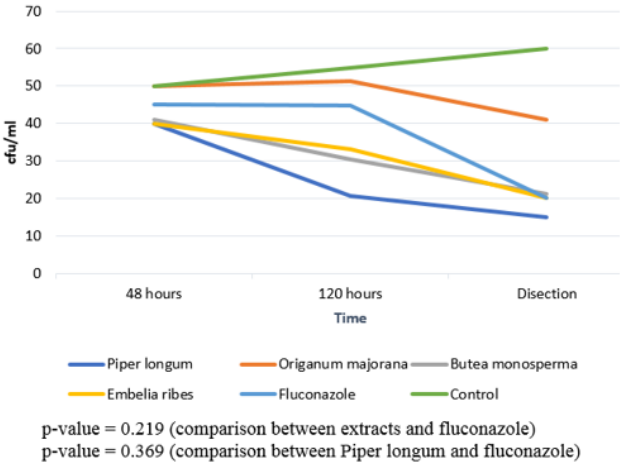

Figure 6: Graphical representation of quantitative blood culture

Gas chromatography-mass spectrometry (GC-MS) investigation.

4. Discussion

The null hypothesis is rejected as all four extracts show comparable antifungal activity in both in-vivo as well as in-vitro analysis. In-vitro study, the extracts of Butea monosperma (Alc.), Piper longum (Alc.), Origanum majorana(Aq.) and Piper longum (Aq.) demonstrated effective antifungal property against Candida sp. at a minimum inhibitory concentration (MIC) of ≤ 0.25 mg/ml. The MIC of extracts of Embelia ribes (Aq.), Butea monosperma (Aq.) and Origanum majorana (Alc.) was found to be 0.5mg/ml, 8mg/ml and ≥32 mg/ml respectively.

Table 1: Phytoconstituents of Butea monosperma with references which indicate Antifungal Activiy of Butea monosperma

Peak#	R.Time	Area	Area%	Name	
3	16.377	90451	0.03	1,2-Benzoldicarbonsaeure	13
6	21.432	1539268	0.49	N-Hexadecanoic Acid	14
13	23.764	459456	0.15	Octadecanoic Acid	14
15	27.268	833787	0.27	1,2-Benzenedicarboxylic Acid	15

Table 2: Phytoconstituents of Origanum majorana with references which indicate Antifungal Activity of Origanum majorana

Peak#	R.Time	Area	Area%	Name	
3	16.351	468514	0.06	Caryophyllene Oxide	17
8	20.880	27979476	3.47	Hexadecanoic Acid	18
11	22.906	13958368	1.73	9,12-Octadecadienoic Acid	18
33	39.006	8771918	1.09	Beta-Sitosterol	19

Table 3: Phytoconstituent of Piper longum (Phytoconstituent of Piper longum with references which indicate antifungal activity of Piper longum)

Peak#	R.Time *	Area	Area%	Name	
53	32.808	448770925	18.57	Piperine	20
6	14.350	1322680	0.05	Humulene	21

The standard drug, Itraconazole also showed an MIC value of ≤ 0.25 mg/ml towards the test microorganism. [Figure 3]

Quantitative blood culture results from the day of the dissection indicated that Piper longum had the greatest impact, followed by Embelia ribes and Butea monosperma. Semi-quantitative Gram stain scoring showed that fungal load in blood was maximum on Day 1 in rats with the Piper group and till the day of dissection, it showed the disappearance of fungi from Piper, Embelia and Fluconazole. [Figures 5 and 6] Phytoconstituent analysis done by GC-MS analysis showed the presence of 63 compounds in Piper longum, 35 compounds in Origanum majorana, and 20 components in Butea monosperma extract out of which antifungal activity was seen in compounds 1,2-Benzoldicarbonsaeure, Octadecanoic Acid, 1,2-Benzenedicarboxylic Acid, N-Hexadecanoic Acid, Nonadecane2,5-Cyclohexadiene-1,4-Dione,2,6-Bis (1,1-Dimethyl Propyl) concluded in many reference studies. [Table 1]

In research by Senthamilselvi S. and Kumar P., the latex obtained from Artocarpusheterophyllus Lam (jackfruit) was tested for antimicrobial and antioxidant properties. 1,2-benzoldicarbonsaeure was found in latex using GC-MS analysis as a significant peak which is responsible for its antimicrobial activity.[16] In the current GC-MS study, an identical component was discovered at RT-16.377 in the methanolic extract of Butea monosperma. Octadecanoic acid, Kojic acid, and n-Hexadecanoic acid were identified from an endophyte, Aspergillus flavus, in research by Premjanu N et al., these chemicals are recognized for their antibacterial properties. Therefore, the synergistic effects of these chemicals may be the cause of their effectiveness against fungi like Candida albicans and Malasseziapachydermis. When these substances docked with the target protein, they were able to bind at an active site like that of the well-known inhibitor fluconazole. A similar molecule was found in Butea monosperma methanolic extract at RT: 21.432 (n-Hexadecanoic acid), RT: 23.764 (Octadecanoic acid), which may be contributing to its antifungal action of Butea monosperma as found in this research.[17]

According to Shuwu Zhang and Bingliang Xu's work showing the antifungal activity of Trichoderma longibrachiatum T6, 1,2-benzenedicarboxylic acid is one of the compounds that Trichoderma longibrachiatum T6 produces with the greatest antifungal activity .[18] A similar substance was also discovered in the Butea monosperma methanolic extract at RT 27.268. These bioactive compounds establish Butea monosperma for antifungal properties, and our findings are consistent with those of previous research on these bio-constituents.

In a study by Rani et al., eight different types of fungi were used to test Embelia ribes antifungal properties, and different seed extract concentrations reduced the development of fungi, and the highest-level action was at a concentration of 2.0 mg.[19] The results of the current investigation u nequivocally proved that Embelia ribes has antifungal capabilities. The antifungal impact of Embelia ribes shown in current research is concomitant with the findings of the prior study.The Origanum majorana (Aq.) extract showed effective antifungal activity against Candida sp. With active bioconstituent as Caryophyllene Oxide, Hexadecanoic Acid, 9,12-Octadecadienoic Acid, Beta-Sitosterol. [Table 2]

According to research by Jassal K. and Kaushal S., the main components of beta-caryophyllene and caryophyllene

oxide in Psidiumguajava Linn leaves' essential oil have antifungal properties. At 1250 g/ml, caryophyllene and caryophyllene oxide, respectively, showed 79.43 and 84.25% mycelial growth inhibition.[20] The bioactive component responsible for the antifungal action of Origanum majorana, caryophyllene oxide, is found at RT: 16.351 in the extract. Antimicrobial properties have been found for muricata plant extract, which contains possible bioactive compounds methyl esters of hexadecanoic acid (C17H34O2) and 9-octadecenoic acid (Z) (C19H36O2). Octadecadienoic acid causes DNA to break down and produce antifungal secondary metabolites.[21] According to the GC-MS analysis hexadecanoic acid and 9-octadecenoic acid (Z) are bioactive compounds in Origanum majorana.

The antifungal activity of B. Papyrifera was the greatest (MIC50, 1 ug/ml) against the fungus Madurellamycetomatis. Beta-amyrin, beta-Sitosterol, beta-amyrone and stigmatise were detected by GC-MS of B. papyrifera. The extract of Origanum majorana contains a bioactive constituent that is similar to beta-sitosterol.[22] Gram-stained smears of peripheral blood and blood cultures were used to confirm the presence of Candida. Many biologically active chemicals were present in the extract of piperine by GC-MS analysis, and the Piper longum group had an outstanding activity with a minimum inhibitory concentration (MIC) of less than 0.25 mg/ml. A semiquantitative Gram stain from blood on the day of dissection was unable to identify fungus from Piper longum, Embelia ribes, or fluconazole.

According to Priya A. and Pandian SK's study, the key virulence factors in the *C. albicans* biofilm are believed to be hyphal development and yeast-to-hyphal morphological transitions. Piperine further shows its distinct antivirulence capability by drastically reducing in-vivo colonisation and increasing the longevity of Caenorhabditis worms infected with *C. albicans*. Transcriptomic research revealed that piperine dramatically reduced the expression of numerous hyphal and biofilm-specific genes. (ALS3, HWP1, EFG1, CPH1, etc.). Piperine may be a possibility for the treatment of *C. albicans* infection linked to biofilms, particularly in cases of oral *candidiasis*.[23] Piperine is one of the primary bioactive substances included in the methanolic extract of Piper longum at RT: 32.808. We had positive findings in the Piper longum-treated group after semiquantitative gram staining since no fungal hyphes were seen. [Table 3]

The antifungal effects of the essential oil from C. sylvestris leave, and their fractions, have been researched for the first time by Pereira FG and Marquete R in their study against Candida albicans, C. glabrata, C. krusei and Saccharomyces cerevisiae. These sesquiterpenes included -copaene (8.5%) and -humulene (17.8%), as well as the oxygenated sesquiterpene pathulenol (11.8%). Humulene was one of the constituents found in Piper Longum extract at Rt 14.350.[24] [Table 3]

In a study Sardorbek A et al on P.longum concluded that its ethanolic extract, exhibited potent activity to esclate the melanin content and has weak stimulatory effect on the tyrosinase activity in a concentration-dependent patern,[25] similarly a study by Ahmad T et al also showed that both chloroform and ethanolic extract of P.longum exhibits maximum zone of inhibition against Staphylococcus saparophyticus, Staphylococcus aureus and Escherichia coli.[26][26]indicating that P. longum is a efficient natural source of leading derivatives, helpful for various dermatological/skin disorders.

5. Conclusion

The presence of different phytoconstituents and their antifungal activity clearly indicate the antifungal potential of these plant extracts. In the present study, Piper longum, Butea monosperma and Embelia ribes have shown high antifungal potential against Candida. On the day of dissection, semiquantitative Gram stain from blood could not detect fungi from Piper longum, Emelia and fluconazole group. The extracts of Butea monosperma (Alc.), Piper longum (Alc.), Origanum majorana (Aq.) and Piper longum (Aq.) showed effective antifungal property against Candida sp. with a minimum inhibitory concentration (MIC) of $\leq$ 0.25 mg/ml. We propose that active constituents Butea monosperma, Piper longum, and Origanum majorana will be a potential option to combat resistance as these extracts show confirmed antifungal activity with potentially bioactive compounds, indicating that these plants derivatives could be an efficient source of antimicrobial agents to battle multi drug resistance (MDR) and lead to discovery of natural bioactive products in the form of antimicrobial phytochemicals that perhaps be help for the emergence of new medicinal drugs, combatting fungal infection especially *Candidiasis*, which is one the most prevalent dermatological /skin pathology leading to increased well-being of patients, with minimization of side effects too.

6. Limitations of the study

This is a preliminary evaluation and confirmation of the antifungal activity of extracts of Embelia ribes, Butea monosperma, Origanum majorana, Piper longum and, an extensive study on each phytoconstituent of these plants is required.

7. Strength of the Study

In-vivo, antifungal confirmative studies of these extracts were not found in an extensive literature search. In present studies, Antifungal activity is confirmed through In-vivo and in-vitro analysis. Detail GC-MS analysis was done, selection of phytoconstituent showing antifungal activity and linking it with mechanism was done in this study.

8. Institutional Review Board Statement

The above project is approved (ethical approval number: Dean/2018/CAEC/818) by the Institutional Animal Ethical Committee, IMS-BHU.

9. List of Abbreviation

Methanolic extract of Embelia ribes: MER, Methanolic extract Butea monosperma: MBM, Methanolic extract Origanum majorana: MOM, Methanolic extract Piper longum: MPL, Oral fluconazole: OF, Methanolic: Alc, Aqueous: Aq, p.o: per oral, i.v.: Intravenous, *C. albican*s: Candida albicans, GCMS: Gas-Liquid-Chromatography Mass Spectrometry, PBS: Peripheral Blood Smear, MIC: Minimum inhibitory concentration, L. decurrens: Laggeradecurrens, VOCs: Volatile organic chemicals, SDA: Sabouraud dextrose agar

10. Source of Funding

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11. Conflict of Interest

None.

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