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ANTIMICROBIAL ACTIVITY OF THE SEED AND CALLUS BIOMASS EXTRACT OF *LINUM USITATISSIMUM* L. AGAINST SOME STRAINS OF CLINICALLY IMPORTANT PATHOGENS

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ABSTRACT

Antimicrobial activity of seed extract and callus biomass extract of *Linum usitatissimum* L. was studied using ethanol as solvent against some strains of clinically important pathogens that include bacterial strains *Bacillus cereus*, *Klebsiella pneumoniae,* and fungal strains *Candida albicans* and *Aspergillus niger.* The extraction was done by the Soxhlet method and its inhibitory effect was investigated using agar disc diffusion method. *Bacillus cereus* showed the highest zone of inhibition 8.3±0.57 mm for seed extract and 13.3±1.15mm for callus biomass extract, against *Klebsiella pneumoniae*. These results were compared with the reference drug Amikacin which was found extremely resistant at the concentration of 100 mcg/mL for both *Bacillus cereus* and *Klebsiella pneumoniae*. In case of the fungal strains, *Candida albicans* showed the highest zone of inhibition 9.3±0.57mm for seed extract and 14.6±0.57mm for callus biomass extract, against *Aspergillus niger*. The reference drug Fluconazole was resistant at the concentration of 150 mcg/mL against *Candida albicans* but non-resistant against *Aspergillus niger*. However, it is interesting to note that callus ethanolic extracts revealed a higher degree of antimicrobial activity than seed ethanolic extract.

KEYWORDS: ***Antifungal and Antibacterial activity. Callus extract Linum usitatissimum , Seed extract.***

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INTRODUCTION

The traditional use of medicinal plants for the production of commercial drugs and its substitutes has been in vogue from ancient times. These drugs help in the cure of ailments caused by bacterial and fungal pathogens and in other important health-related activities. [1-4] However, since past few decades, application of plant tissue culture and the commercial development of this technology as means of producing valuable phytochemicals has been the area of increasing scientific interest.[5-8] The plant *Linum usitatissimum* L. commonly known as Flax belongs to the family Linaceae. The plant is native to India and the eastern Mediterranean but distributed throughout the world including Canada, China, United States (Montana, Dakota, Minnesota, etc.), Ethiopia and all over Europe.[9] It is also cultivated for its flexible fibers. The plant has well known medicinal properties. Historically, linseed oil, derived from flaxseed, has been used as a topical demulcent and emollient, as a laxative, and as a treatment for coughs, colds, and urinary tract infections. It is the rich source of bioactive components Phenylopropanoids - the secondary metabolites exhibiting the strong antioxidant properties and thus possessed the inhibitory effect on bacteria, viruses and fungi.[10] Flax seed contains higher levels of lignans than most other foods. Lignans are the phenolic compounds which serve an antimicrobial, anti-inflammatory and antioxidant activity in basic research models of human diseases.[11,12] The principal lignan precursor found in flaxseed is secoisolariciresinol di-glucoside. The effective antibacterial activities of flaxseed proteins have also been reported.[13] The phytochemical estimation of aqueous flaxseed extract revealed the presence of glycerides, saponins, alkaloids and flavonoids.[14] Flaxseeds are the richest sources of a plant-based omega-3 fatty acids, called alpha-linolenic acid ALA.[15] These medicinal properties of *Linum usitatissimum* make it as the important genus to exploit its phytoconstituents and bioactive compounds for the formulation of pharmaceutical products to be used against diseases caused by several microbial infections. The test organisms used in this study are important as they are the strains of clinical pathogens, mostly opportunistic and nosocomial which target a group of immunocompromised as well as non-immunocompromised individuals. *Klebsiella Pneumoniae* is mainly responsible for pneumonia and urinary tract infections. *Bacillus cereus* is a type of bacteria that produce toxins. These toxins can cause two types of illness: one type characterized by diarrhea and the other called emetic toxin, by nausea and vomiting. *Candida albicans* causes bloodstream infections referred as 'candidemia' and have been associated with a high mortality rate. It is also the cause of most fungal infections in AIDS patients. *Aspergillus niger* causes black mold of onions, grapes, peanuts and ornamental plants. In humans, it is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss and in severe cases damage the ear canal and tympanic membrane.[16] Several investigations have been made on the antimicrobial activity of plant extract and their secondary metabolites. However, very few studies are presented on *in vitro* derived callus extract with this regard. Therefore, the main objective of this study was to emphasize on the ability of the *in vitro* derived callus to be used for the antimicrobial activities against these clinically significant microorganisms using agar disk diffusion method using the economically and medicinally important plant *Linum usitatissimum*..

MATERIALS AND METHODS

Preparation of Explant

Flaxseeds were purchased from the market in Mumbai. The seeds were soaked in water for 03 hours before use and surface sterilized with 0.1% $HgCl_2$ (Mercuric Chloride) solution for 5 minutes. The seeds were thoroughly washed for four to five times with sterile distilled water and used for inoculation onto the Murashige and Skoog MS medium.

Culture Conditions

The MS medium fortified with 8 % sucrose and growth regulators IAA+BAP was used with suitable hormonal concentrations viz., 0.5, 1.0 and 1.5 mg/L. since this media combination was most responsive. Agar 8 % (agar agar, J. M. Vaz Pereira, Lisboa Portugal) was added to the medium as a solidifying agent. The pH was adjusted to 5.8 before autoclave. Seeds with small incision were directly inoculated on MS medium aseptically. Around 10 seeds in each jar were placed. The culture bottles were transferred to an incubation room at 22°C temperature conditions for 16 hrs. photoperiod. Observations were recorded up to 40 days of incubation (Fig.1).

Extract preparation

For the seed extract preparation, flaxseeds were ground to form a fine powder. For callus extract, the freshly harvested callus biomass of *Linum usitatissimum* was homogenized with the minimum quantity of ethanol using mortar and pestle. In a separate set of experiment, 10 gm. each of flaxseed powder and homogenized callus biomass was extracted in 100 ml. Ethanol using Soxhlet apparatus (40 ºC, 21 hrs.). The extract samples were filtered through Whatman filter paper no.1, dried, weighed and again reconstituted in ethanol (1gm./mL) and stored at 4ºC for the antimicrobial assay.

W (Wt. of extract reconstituted) = W2-W1

where, W2 =Wt. of crucible containing dried extract and W1=Wt. of empty crucible
For seed ethanolic extract, 78.59 -77.56 =1.03 gm. and
For callus ethanolic extract, 67.59 - 66.58 = 1.01gm.

Isolates

The test organisms used were the strains of clinical isolates, obtained from the Department of Microbiology, Institute of Science, Mumbai that include gram positive bacteria *Bacillus cereus* (MTCC 121), gram negative bacteria *Klebsiella pneumoniae* (MTCC 7407), and two fungal forms namely, *Candida albicans* (MTCC 183) and *Aspergillus niger* (MTCC 1847).

Preparation of bacterial suspension

Nutrient Agar (NA) was used for the inoculation of the studied bacteria. Active cultures for bacteria were prepared by allowing the growth overnight in nutrient broth (NB) medium at 37º C under shaking conditions at 600 rpm. The bacterial inoculums having turbidity standard 0.5 McFarland standard equal to 1-2 x 10^8 cfu/ ml were used.

Preparation of fungal suspension

Sabouraud's dextrose agar (SDA) was used for the inoculation of the studied fungi. Active cultures for the experiment were prepared by seeding a loop full of fungi into Sabouraud's broth and incubated without agitation for 48 hrs. at 25^{O}C. The surface spores from the growth were harvested into 0.85% saline and a concentration of 2 x 10^5 cfu/ ml (OD-0.02 at 530 nm) achieved.

Agar disk diffusion method and impregnation of paper discs

The inhibitory effect of ethanolic extracts of seed and callus biomass of *Linum usitatissimum* was carried out using agar disk diffusion method. [17] The circular Whatman filter paper discs were prepared with the size of 10 mm in diameter. The discs were put into a petri dish and then sterilized in the oven at 160 ^{o}C for 2 hrs. About 200 µL of the specified bacterial and fungal inoculum of each isolate was inoculated on the NA and SDA plates respectively and spread uniformly with the spreader. The paper disc was impregnated with 10 µL concentration of each specified test solutions per disc i.e. ethanol as control, ethanolic callus extract and seed extract of *Linum usitatissimum,* with standard antibiotic Amikacin 100 mcg/mL for bacteria and Fluconazole 150 mcg/mL for fungi were placed on agar plates with a sterile forceps and incubated at 37^{o}C for 24 hrs. for bacteria and at 25^O C for 96 hrs. for fungi. After incubation, the diameter of the resulting zone of inhibition around each paper disc was measured to the nearest millimeter.

RESULTS

Table 1

Response of experimental plant system Linum usitatissimum in varying hormonal combinations for callus induction from seed explant

S.N	Hormonal Combination and Concentrations (in mg/L)	Callus induction
1	IAA: BAP 0.5: 0.5	--
2	IAA: BAP 1.0:1.0	+++
3	IAA: BAP 1.5:1.5	++++

(--) : No Response, (+++) :Very good, (++++) : Excellent

Table 2

Antimicrobial susceptibility testing of ethanolic seed extractand seed callus biomass extract of Linum usitatissimum against clinically important pathogens

S. N	Test solutions	Inhibition zone in (mm)			
		G+Ve Bacteria	G-Ve Bacteria	Fungi	
		Bacillus cereus	*Klebsiella pneumoniae*	*Candida albicans*	*Aspergillus niger*
1	Ethanol as control	NI	NI	NI	NI
2	Callus extract	13.3±1.15	12.6±0.57	14.6±0.57	12.3±0.57
3	Seed extract	8.3±0.57	7.3±0.57	9.3±0.57	7.3±0.57
4	Amikacin (Standard) 100 mcg/mL	24.6±0.57	29.3±1.15	__	__
5	Fluconazole (Standard) 150 mcg/mL	__	__	10.3±0.57	NI

Values are the means of two replicates. NI= No inhibition

Figure 1
Induced Callus in seeds of Linum usitatissimum (a) Seeds inoculation in MS medium on 1st day (b) Callus on 14th day (c) 40 days old callus in MS medium

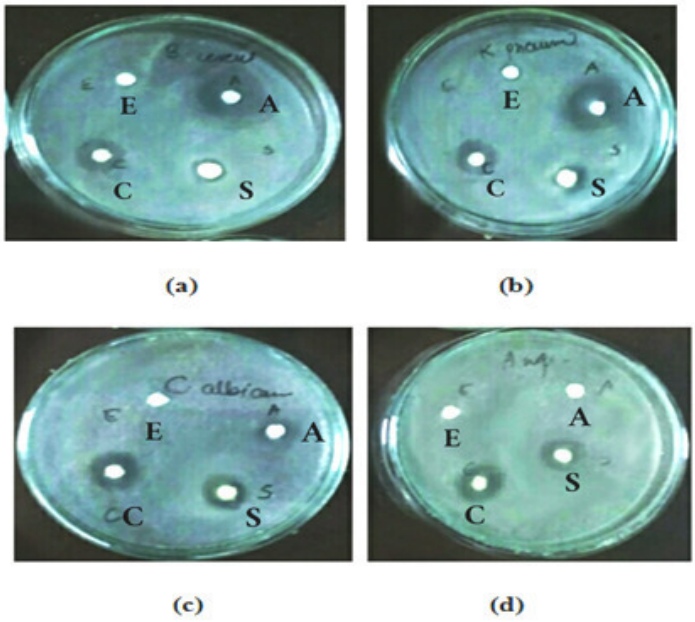
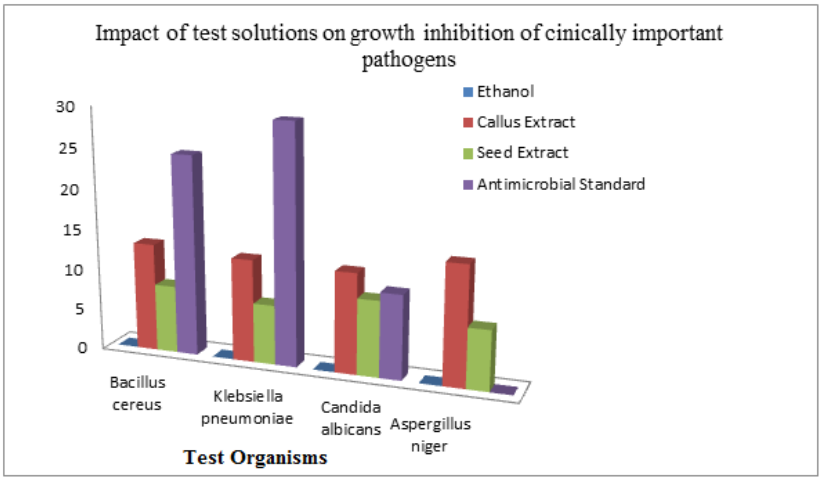


Figure 2
Antibacterial and antifungal susceptibility testing for ethanolic callus and seed extract of Linum usitatissimum against (a) Bacillus cereus, (b) Klebsiella pneumoniae, (c) Candida albicans and (d) Aspergillus niger.

Where, E= Ethanol as control, A = Standard Antibiotic, C= Callus extract and S= Seed extract.



Graph 1
Impact of test solutions on growth inhibition of clinically important pathogens

DISCUSSIONS

The findings in the present work revealed that the MS basal medium supplemented with 8% Sucrose and 1.5:1.5 IAA+BAP hormonal combination is favorably effective for the generation of profused callus from the seed explant in *Linum usitatissimum* (Table1). The callus was friable and light green in color (Fig1). The auxin IAA and cytokinin BAP alone and in combination has been reported by many workers as an effective protocol for callus regeneration in many important plants such as *Capsicum annum*, *Costus pictus, Solanum nigrum, Picrorhiza kurroa* and *Cajanus cajan* etc.[18-22] The highest antibacterial activity was recorded for *Bacillus cereus* with the inhibition zone of 8.3±0.57mm by ethanolic seed extract compared to *Klebsiella pneumoniae* and the highest antifungal activity for *Candida albicans* with the inhibition zone of 9.3±0.57mm against *Aspergillus niger.* The callus biomass extract exhibited highest antibacterial activity again for *Bacillus cereus* with the inhibition zone of, 13.3±1.15mm compared to *Klebsiella pneumoniae*. The highest antifungal activity of callus biomass extract was recorded against *Candida albicans* with the inhibition zone of, 14.6±0.57mm compared to *Aspergillus niger*. It is evident that the tested bacterial and fungal forms have responded well for anti proliferation activities mediated by ethanolic seed and callus extract of *Linum usitatissimum*. Similarly, it is to be noted that the impact range of antibacterial and antifungal activities was more prominent in ethanolic callus extract compared to seed extract. These studies are supported with reference to the studies performed on *in-vitro* callus induction and antimicrobial activities of callus and seeds extracts of *Nigella Sativa* L. where *Nigella sativa* callus extracts exhibited higher activity than seeds methanolic extract against *Escherichia coli.* Similar effects were observed in *Premna serratifolia* where increased inhibitory activities of callus extracts were found to be the best when compared to the natural plant material extracts against the tested microorganisms. The comparative antimicrobial activity of callus and natural plant extracts of *Solanum trilobatum* have revealed that the stem and leaf callus extracts indicate more significant activity against the tested microorganism, *Escherichia coli* and *Staphylococcus aureus* than the natural sample. These views are well reported by the workers cited earlier [under 5-8]. It is well known that *in vitro* cultures are able to produce secondary metabolites and these phytoconstituents may possess the good potential of applicable activities. This may be the reason that the callus extracts show significant activity than the natural plant extracts against the tested microorganisms. Thus, it can emphasize biotechnological method in the development of new antibiotic. The standard antibiotic Amikacin was resistant and showed the bactericidal effect at the concentration of 100 mcg/mL against both the bacterial strains, *Bacillus cereus and Klebsiella pneumoniae.* The Fluconazole standard was resistant at the concentration of 150 mcg/mL against *Candida albicans* but non-resistant against *Aspergillus niger*. (Table 2, Fig 2, Graph 1). These results may be attributed to the accumulation of freshly synthesized metabolites in the callus culture cells of *Linum usitatissimum* compared to ethanolic seed extract. Ethanol is known as a good solvent for the extraction of bioactive metabolites and therefore, its partial role in obtaining experimental output cannot be ruled out. To this context, there is greater hope to study other solvents such as methanol, petroleum ether and chloroform for the extraction of phytoconstituents. It is evident from earlier reports that the plant callus is effective in enhancing the production of secondary metabolites at a low concentration of growth hormones.[23] Cytokinins are known to enhance the production of secondary metabolites and play an important role incytodifferentiation[24] and subcellular differentiation *e.g*. anthocyanin production in *Camptotheca acuminata.*[25] Modulation of secondary metabolites production by plant growth regulators is very old,[26] but the marked effect of production of terpenoids and phenolics with auxins and cytokinin in callus and cell cultures of *Commiphora wightii* and isoflavonoids in cell cultures of *Pueraria tuberosa* is reported earlier.[27]

CONCLUSION

It is concluded that the callus ethanolic extract of *Linum usitatissimum* has the good potentiality of antibacterial activity against *Bacillus cereus* compared to the seed ethanolic extract. Similarly, the highest antifungal potentiality was recorded for callus biomass extract against *Candida albicans* compared to the relative impact of seed ethanolic extract. However, in both the cases, reference drug samples were extremely resistant compared to all the studied samples of seed and callus extract of *Linum usitatissimum* except for *Aspergillus niger* which did not show any inhibition against the drug used. But the present evaluation has explored the ability of *in vitro* grown callus biomass towards antimicrobial activities and has its own significance since it is a biological material. Thus, present work highlights the ability to utilize plant biotechnology technique and use of *in vitro* generated plant materials which are biocompatible, sustainable and harmless, towards the development of desirable metabolic constituents for minimizing pathogenic infections.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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