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Original article

Pharmacopeial constants, secondary metabolites and antibacterial activity of calli induced *in vitro* from flaxseed sprouts explants

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ABSTRACT

In the present study, some important pharmaceutical compounds in flaxseed plant have been done *via* biotechnology techniques. The seeds of *Linum usitatissimum* were surface sterilized germinated for one week on hormone-free MS culture media for 10 days. Cotyledonary leaves, hypocotyls segments (1 cm) and rootlets segments (1 cm) of the obtained sprouts were used as explants for callus induction on MS supplemented with BA (benzyladenine) and NAA (Naphthalene acetic acid). Maximum callus fresh weight from rootlets explants was recorded and reached up to 3.72 gram in response to the treatment with 0.5 mg/l BA plus 0.25 mg/l NAA after 4 weeks of treatment. The highest callus fresh weight from cotyledonary leaves and hypocotyls segments were recorded in response to the treatment with 0.75 mg/l BA plus 0. 5 mg/l NAA and 0. 5 mg/l BA and NAA, respectively. The results obtained have revealed many differences between the resulted calli with respect to pharmacopeial constants and secondary metabolites determined (Phenolics, glycosides, flavonoids and saponins). Methanolic extracts of calli obtained from different explants exhibited antibacterial activity against both the Gram-positive and Gram-negative tested pathogenic bacterial strains. The maximum inhibition zone (26 mm) was obtained in response to the treatment of *staphylococcus aureus* with extract of callus obtained from cotyledonary leaf explants. This study may indicate that the callus of *L. usitatissimum* can be adopted in the future as a source of valuable metabolites.

Graphical abstract

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1. Introduction

Flaxseed, also known as flaxseed, is a rare plant of the flax family. Linum usitatissimum is the Latin name for flaxseed meaning "useful" and its main types are yellow, yellow and brown [1]. The flaxseed plant used in this study is thought to be of Egyptian origin and cultivated worldwide for its oil and fiber [2]. In addition, flaxseed contains many nutritionaland medicinal properties that are beneficial for health [3]. It is also believed that the nutritional and health benefits of flaxseed are beneficial in terms of healthy omega3 fatty acids required for human consumption, as well as phenolic compounds that are expected to have many health benefits [4]. Flaxseed activities include antibacterial and antifungal [5]. Flaxseed contains high amounts of phenolic compounds and many lignans [6].

Flax lignans, flaxseed oil and fiber have many health benefits as they help treat many diseases such as atherosclerosis, heart disease, cancer, diabetes, arthritis, osteoporosis, autoimmune and neurological diseases [7]. Degreased flaxseed meal (residue of linseed oil extraction) is rich in lignans, dietary fiber and protein that are beneficial to human health [8]. Also, in another study, the therapeutic effect of L. usitatissimum oil as an antibiotic was discussed in bovine mastitis, which is considered a microbial disease that causes many diseases of inflammation [2].In the search for alternative production of pharmaceutical compounds from plants, biotechnological approaches, specifically tissue cultures technique are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites **[9]**.

The main objective of the present study is to induce callus formation from different flaxseed sprout explants and determine the pharmacopeial constants, some important secondary metabolites and antibacterial activity of callus extracts.

2. Materials and Methods

This work was carried out in the Plant Tissue Culture and Biotechnology lab., Faculty of Science, Botany and Microbiology Department, Al-Azhar University, Cairo, Egypt during 2021-2022 academic years.

1- Preparation of Plant Material for *In Vitro* Callogenesis

Li-num usitatisemum seeds were dipped in 70% ethanol solution with continuous gentle agitation for one minute before being transferred to 100 ml Erlenmeyer flasks containing 20% commercial sodium hypochlorite solution (1% active chlorine) for permanent surface sterilization. Gentle stirring for 7 minutes [10]. Seeds were cleaned by pouring disinfect-ant and aseptically rinsed three times in conjunction with sterile water. The seeds were then dried in Petri dishes on two layers of sterile filter paper. Using a sterile scalpel, transfer the seeds to pots without MS hormone and let them germinate for 7 days at room temperature. The cotyledons, hypocotyls and well roots of the shoots were cut into the same explants under aseptic conditions for callus initiation.

2- Culture Media and Hormonal Treatments

Hormone–free Murashige and Skoog's basal medium (MS) **[11]** augmented with sucrose (2%) gelled with phytagel (7%) was used for germination of the seeds of *linum usitstisemum*. For callus formation from different explants benzyladenine and naphthalene acetic acid at different factorial combinations were added to MS.

3- Determination of Pharmacopeial Constants

I. Total crude fibers

Total crude fibers were determined according to **[12].** Two grams of defatted plant powder were boiled with 200 ml of 1.25% sulphuric acid under reflux for 30 minutes and then filtered. The residue was washed with distilled water. To the residue, 200 ml of boiled sodium hydroxide 1.25% was added and the mixture was allowed to boil again for 30 minutes with reflux. The mixture was rapidly filtered and washed again with distilled water and the residue was then dried at 100 ^{0}C. The difference between ash dry weight and residue represents the total crude fibers.

II. Organic and Inorganic matter

Two grams of dry plant powder in a clean crucible of known weight were placed in a muffle furnace and ignited at 800 ^{0}C for 8 to 12 hours. The crucible was then left to cool in a desiccator at room temperature and then reweighed and the amount of total ash per gram dry powder was calculated. Organic matter was calculated by subtracting the total ash content from the dry plant powder used for determination of total ash **[13]**.

III. Water and acid soluble ash fractions

The acid insoluble fraction of the of the total ash was determined by boiling a known weight of the total ash obtained with 25 ml of 10% HCl for 5 minutes, separating them from the acid-soluble ash of the ash by filtration using ashless filter paper. After washing the residue with several rinses of hot distilled water, the ashless filter paper together with the acid-insoluble fraction collected on its surface was ignited on a muffle furnace and weighed after ignition to calculate the acid insoluble fraction. The acid soluble fraction of the ash was calculated by subtracting the acid insoluble fraction from the total ash. Water soluble and insoluble fractions of the total ash content were determined using 25 ml of boiling distilled water instead of HCL for initial extraction **[14]**.

2- Determination of Secondary Metabolites

I. Total phenolics

The amounts of phenolics calli originating from different explants were determined by using Folin-Ciocalteu as a reagent. Macerated known weight callus in 80% ethanol then extracted to exhaustion. The crude extract was dried, and a known weight of the dried extract was dissolved in 10.0 ml methanol. Add 0.25 ml of 10% diluted Folin-Ciocalteu reagent to 5.0 ml of each sample and incubate the resulting mixture at 450 °C for 15 min. Resulted color absorbance was measured spectrophotometrically at 765.0 nm. Then, a standard curve was made using different concentrations of gallic

acid (mg) equivalent per g dry mass of callus material (mg GAE/g dw) **[15]**.

II. Total flavonoids

In a test tube (in triplicate), mix: 0.1 ml of methanol callus extract + 1.5 ml of methanol + 0.1 ml of 10% $AlCl_3$ + 0.1 mL of 1 M CH_3COOK + 2.8 mL of distilled water. The previous mixture was then left at room temperature for half an hour until a yellow color appeared, then measured at 415.0 nm. Concentrations of all flavonoids in callus extracts were expressed as quercetin equivalents and standard curves were prepared using quercetin at concentrations ranging from 20 to 100 mg/l in methanol. [16].

III. Total saponins

Total saponins estimated by using, 0.5 ml of methanol extract for each callus extract and 0.5 ml of o.5 % reagent (p-anisaldehyde) was mingled and kept for 10.0 minutes. Then add 2 ml of 50% sulfuric acid and vortex. It was then placed in tempura water continuously for 10 minutes at 600°C, then cooled. The absorbance of the yellow color was measured at 435 nm. The amount of saponin is calculated as saponin equivalents according to the standard saponin 100 - 1000 µ/ml measurement curve. [17].

IV. Total glycosides

Total glycosides were determined using Baljet reagent (95.0 ml of picric acid (1%) and 5.0 ml of sodium hydroxide (10.0%). Transfer 8 mL of callus extract to a 100 mL bottle; 60 mL HO and 8 mL 12.

Add 5% aluminum acetate, mix and filter. Transfer 50 ml of H2O and 8 ml of filter to another 100 ml bottle, add 8 ml of 47% Na_2HPO_4, mix well, add distilled water to volume and filter twice. Transfer 10.0 ml of purified filtrate to a sterile bottle and treat with 10.0 ml of Baljet reagent. Let stand at room temperature for 1.0 hour until fully colored. The color used was then measured colorimetrically at 495.0 nm with 10.0 mL of distilled water and 10.0 mL of distilled water as a blind. 10.0 ml of Baljet reagent and incubate for 1.0 hour under the same conditions [18].

V. PREPARATION OF CALLUS EXTRACT FOR ASSAY OF ANTIBACTRIAL ACTIVITY

Calli were harvested and their fresh weights were recorded. Then, they were dried in oven at 50 º C. After weights were constant, calli grinded into a fine powder**.** Then 1.0 g of calli dry powder was steeped in 70.0% ethanol (150 ml) at room temperature for 7 days with shaking. Then after filtration, the residue was washed with three successive rinses (100.0 ml) of ethanol 80.0%. The filtrate and washings were mixed and evaporated to dryness, weighed and then dissolved in 1 ml of 80% ethanol**.**

VI. Test microorganisms and an assay of antibacterial activity:

The strains used in this study were Escherichia coli [ATTCC 11229], Pseudomonas auruginosa [CS.25], Klebsiella pneumonia [ATCC 13883], and Staphylococcus aureus [NCTC7447]. Antibiotics were made the discplate diffusion method, and extracts were made with a callusdry extract amount of 25 mg per disc.

For the determination of total flavonoids, aluminum chloride colorimetric method used in milligram quercetin equivalents.

by After the organic solvent has completely evaporated, place the disc in a 9 cm diameter petri dish on nutrient agar inoculated with the test bacteria. Then place the plate at 37.0 °C for 24.0 hours. The test was carried out 3 times under sterile conditions. Then, the antibiotic was determined by measuring the area of the inhibition zone and the mean value was calculated.

3. Result and Discussion

1- Callogenesis

Trials of callus induction from different explants (cotyledonous leaves, hypocotyls and rootlets) of the 10 days germinated sprouts of *Linum usitatisemum* using MS culture media supplemented with benzyladenine and naphthalene acetic acid were fruitful.

Concerning the cotyledonous leaf explants, callus induction was possible using the different plant growth regulators combinations. The highest callus fresh weight was obtained using the combination of 0.75 mg BA plus 0.50 mg/l NAA. All the deviations from this hormonal combination resulted in a decrease in callus fresh weight.

Concerning hypocotyls segments explants, the results illustrated in table (1) may show that the highest fresh weight obtained per callus (which reached up 0.627 g.) could be obtained using 0.5 mg/l of each of BA and NAA while on the other hand, the increase of both plant growth regulators to 0.75 mg/l resulted in the lowest callus fresh weight (0.225 g.). This may show the high sensitivity and specificity of hormone explant interaction.

The results illustrated in table (1) have revealed that callus formation from rootlets explants was possible using various plant growth regulators used but with various degrees depending on their concentrations. The highest callus fresh weight was obtained using 0.5 mg/l BA plus 0.25 mg/l NAA and reached up to 3.720 g. The increase of either BA or NAA exhibited negative effects on the average callus fresh weight. The smallest callus fresh weight was obtained using 1 mg/l BA plus 0.75 mg/l of NAA (0.653 g/callus).

From **table (1)** and results it may be deduced that various explants could be used for callus production successfully but with different degrees; maximum callus growth depended on the type of explant (may be due to the nature, biochemical composition, anatomical features or physiological activity of cells and tissues of the explant) and that the response of the explant depended on the plant growth substances formulation or composition in the culture media. The results obtained may indicate also that the most suitable explant for callus formation is the rootlets segments of the 10 days old sprouts of *Linum usitatissimum* and the recommended hormonal combination is 0.5 mg/l BA plus 0.25 mg/l NAA.

The results obtained in this study may run in parallel with the results obtained in earlier and recent studies carried out on other plants like *Bixa orellana* [19],

Mimosa harmata [20] *Morus alba* [21], *Rauvolfia tetraphylla* and *Physalis minima* **[22]**, *Flaveria trinervea* **[23]** and *Passiflora edulis* **[24-27]** declared that plant tissue culture as a perpetual source for the production of industrially important pharmaceutical bioactive compounds.

Table (1): Effect of different hormonal treatments on fresh weight (g.) of callus obtained from various explants of 10 days old flax seed sprouts.

Explant	NAA mg/l	BA concentration mg/l		
		0.5	0.75	1.0
Cotyledons leaves	0.25	0.807	0.862	0.947
	0.50	0.875	1.421	0.593
	0.75	0.995	1.141	1.016
Hypocotyl segments	0.25	0.420	0.576	0.503
	0.50	0.627	0.405	0.376
	0.75	0.461	0.225	0.333
Rootlets segments	0.25	3.720	3.490	0.972
	0.50	3.193	1.486	1.070
	0.75	1.025	1.172	0.653

Each value is a mean of 10.0 determinations.

Pharmacopeial constants of calli

Concerning pharmacopeial constants, the results obtained in this study which is illustrated in table (2) have shown that the amounts of crude fibers in callus cultures reached up to 0.173, 0.159 and 0.264 mg/g dry callus matter for calli obtained from cotyledonary leaves, hypocotyls and rootlets explants. The increase in fiber content in root calli over the others may come back to the histological nature and physiological activities of the cells from which explants were taken. The organic matter contents in calli generally ranged between 700 and 760 mg/g while the inorganic matter ranged between 240 to 302 mg/g. The amounts of the water-soluble and insoluble fractions, as well as the acid soluble and insoluble fractions of the total ash content, are illustrated in the same table where the maximum amount of water-soluble fraction (240 mg) was obtained in calli produced from root callus while the minimum amount of water-soluble ash (133 mg) was recorded in calli derived from hypocotyls explants. The increase in water-soluble fraction was accompanied by a decrease in the amounts of water-insoluble fractions and vice versa. This is probably due to the nature and degree of differentiation of the tissues from which calli were obtained. The results presented in the same table show that the amount of the acid-soluble fraction of the total ash content of calli ranged between 162 -273 mg while the acid-insoluble fractions ranged between 61 -89 mg. The results obtained generally showed some variations in the contents of the measured pharmacopeial constants. These variations or more specifically, shift water or acid-soluble to insoluble may represent adaptation strategies of the cultured cells to the in vitro cultural and/or nutritional conditions.

Plate (1): Callus produced from rootlets segments of *L. usitatisemum* after 4 weeks on MS augmented with 0.5 mg/l BA plus 0.25 g/l NAA.

Table (2): Pharmacopeial constants of callus cultures mg/g dry callus biomass

Type of explant	Crude fiber	Organic matter	Inorganic matter	Water soluble ash	Water insoluble ash	Acid soluble ash	Acid insoluble ash
Cotyledons leaves	0.173	740	260	159	102	162	89
Hypocotyl segments	0.159	760	240	133	101	179	61
Rootlets segments	0.264	700	302	240	60	273	29

2- Secondary metabolites

Results showed that calli of *L. usitatisemum* which were obtained from different parts of germinated seeds (cotyledonous leaves, hypocotyls and rootlets) were able to synthesize important plant secondary metabolites like saponins, flavonoids, glycosides and phenolics but with some significant capacities in the biosynthesis or accumulation of these metabolites. It seems from the results obtained in general that calli which were produced from hypocotyl explants were less active than the cotyledonous leaves and rootlets in the biosynthesis of the estimated plant secondary metabolites. Table (3) has shown that amounts of dry methanol extract callus cultures obtained were different

according to various explants taken cotyledonary leaves, hypocotyls segments and rootlets segments were (370, 500 and 900 mg/g dry biomass) respectively.

Concerning saponins, the results obtained and illustrated in table (4) have shown that calli obtained from leaf explants contained 52.191±0.043 mg/g dry weight followed by calli obtained from rootlet explants and reached up 44.956±0.008 mg/g dry callus matter. On the other hand, calli obtained from hypocotyl explants synthesized almost half the amount of saponins that were produced by the other types of explants. The differences in amounts of saponins that were synthesized by calli of different explant type origins were statistically significant.

Table (3): Amounts of dry methanol extracts of different callus cultures obtained from various explants taken from 10 days old *Linum usitatisemum* sprouts.

	Callus from explants of		
	Cotyledonary leaves	Hypocotyls segments	Rootlets segments
Methanol extract mg/g dry biomass	370	500	390

Flavonoid contents obtained as illustrated in table (4) were 8.790±0.028, 4.725±0.010 and 8.363±0.019 mg/g dry weight of calli C1, C2 and C3 obtained from cotyledons leaf explants, produced from hypocotyls and rootlets explants respectively. There were no statistically significant differences between C1 and C3. Calli of C2 (obtained from hypocotyl segments) synthesized almost half the amounts of flavonoids that were synthesized by the other types of calli and the difference was statistically significant.

Table (4): Contents of some secondary metabolites in calli obtained from different explants of L. usitatisemum obtained in vitro as mg/g dry callus biomass.

Type of explant	Secondary metabolite measured as mg/g callus dry biomass			
	Saponins	Flavonoids	Glycosides	Phenolics
Cotyledons leaves	52.19±0.04 A	8.79±0.03 A	2.40±0.13 B	11.85±0.59 A
Hypocotyl segments	24.30±0.04 C	4.73±0.01 C	1.11±0.05 C	8.04±2.20 C
Rootlets segments	44.96±0.01 B	8.36±0.02 A	3.64±0.15 A	10.88±0.22 A

Each value is a mean of three determinations ± standard error. Dissimilar letters means that the difference is significant.

The amounts of glycosides for the three types of calli (as mg/g dry biomass) were 2.395±0.126, 1.109±0.048 and 3.636±0.154 respectively and the differences between them were statistically significant. The contents of phenolic in calli produced by cotyledonous explants reached up 11.848±0.590 mg/g dry callus biomass and 8.036±2.204. For calli produced from hypocotyls explants and 10.879±0.216 for calli obtained from rootlets explants

Biotechnological methods, especially callus cultures, are attractive alternative sources to the whole plants to produce specific secondary metabolites like saponins, flavonoids, glycosides and phenolics with significant amounts **[28]**. Many reports have been conducted about the *in vitro* production of flavonoids and others using different biotechnological methods, including callus cultures **[29]**. Also, Callus cultures have been carried out in several plants for the production of secondary metabolites ex. Flavonoids **[30]**. Also, Agarwal and Kamal **[31]** studied the total flavonoid in *Momordica charantia* and observed that the highest amount of total flavonoid (1.83 mg/l dry wt.) accumulated in 6-wk-old callus.

3- Antibacterial activity

Results which are illustrated in Table (5) and Figure (2) have shown that the methanol extracts of calli obtained from various explants were active against all the tested pathogenic bacterial strains but with different zones of inhibition. The most pronounced effect is probably with *Staphyllococcus aureus* (which reached up to 26 mm of inhibition zone diameter) and the least pronounced effect is probably with *Psudomonas aeroginosa* (inhibition zone diameter = 12).

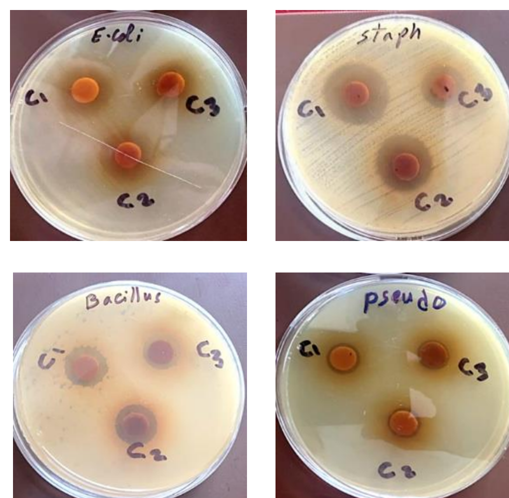

Plate (2): Inhibition of bacterial growth (as measured by the disc-plate diffusion method) in mm by extracts of different *L. usitatissimum* callus cultures. C_1 is callus from cotyledonary leaves, C_2 is callus from hypocotyl segments and C_3 is callus from rootlets segments.

Phenolic compound in *L. usitatissimum* can enhance degradation of bacterial DNA **[32]**. Also, the second cause for antibacterial activity of *L. usitatissimum* is the lignans with bacterial cell walls thus, combat bacterial growth **[33]**. Also. Hussien and Aziz **[34]** conclude that *L. usitatissimum* L. extract has antibacterial activity mainly against three types of Gram-negative bacteria Shigella flexneri, Salmonella typhimurium and E. coli.

Table (5): Inhibition zones (in millimeters) of methanolic extracts of calli by the disc-plate diffusion method against some pathogenic bacterial strains.

Sources of callai	Gram-+Ve		Gram -Ve	
	E. coli	*S. aureus*	*B. subtilis*	*P.aeroginosa*
Cotyledonous leaves	**18**	**26**	**15**	**12**
Hypocotyls segments	**19**	**22**	**16**	**12**
Rootlets segments	**19**	**15**	**15**	**13**

Note: diameter of discs used is 9 mm

With respect to *E. coli*, *S. aureus*, *B. subtilis* and *P. aeroginosa*, the callus from cotyledonary leaves resulted in inhibition zones of 18, 26, 15 and 12 mm, the callus originated from hypocotyls resulted in inhibition zones of 19, 22, 16 and 12 mm and the extract of calli developed from rootlets could inhibit bacterial growth with inhibition zones of 19, 15, 15 and 13 mm respectively.

However, it may be strongly suggested that the antibacterial activity of the calli tested may refer to the potential of callus and tissues to synthesize the previously detected secondary metabolites which are known generally to have powerful antioxidant, antimicrobial and anti-cancer effect.

4. Conclusion

In the current study, the seeds of *L. usitatissimum* were surface sterilized and germinated under aseptic conditions on Murashige and Skoog's culture media. Different explants were cultured on MS culture media containing different auxin /cytokinin concentrations to induce callus formation. Calli was dried and extracted with 80 % methanol. Methanolic extracts were subjected to phytochemical analysis and antibacterial activity. The results indicated the self-capacity of callus cultures to synthesize active important secondary metabolites and inhibit bacterial growth. In future perspectives we are ambitious to apply this research in the field of medicines and the formation of medicinal drugs.

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