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Journal of Advanced Scientific Research

Available online through https://sciensage.info

ISSN: 0976-9595
Research Article
DOI: 10.55218/JASR.202213115

EVALUATION OF ANTIOXIDANT, ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL SCREENING OF AQUEOUS METHANOLIC EXTRACT OF *LINUM USITATISSIMUM*

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ABSTRACT

The present study was carried out to evaluate the antioxidant, antimicrobial study and phytochemical assessment of 80% v/v aqueous methanol extract of *Linum usitatisimum*. Flax seeds (*Linum usitatisimum*) are an ample source of lignans, which have antioxidant properties and promisingly reduce the effects of free radicals. The antioxidant activity of the extract was determined by free radical scavenging activity using DPPH radical and the activity was in the range of 26.7% to 98.6%. The average inhibitory concentration (IC_{50}) was calculated. According to DPPH method, the IC_{50}value of 80% v/v aqueous methanol extract of flax seeds was 5.56mg/ml. An *in vitro* antimicrobial assay by disc diffusion method was carried out. The results showed that 80% v/v aqueous methanol extract of seeds were effective against various test microorganisms. This paper also presents total phenolic assessment of 80% v/v aqueous methanol extract of flax seeds; the content was 20.91%. Furthermore, 80% v/v aqueous methanol extract was examined for qualitative phytochemical analysis and the results illustrate the presence of alkaloids, flavonoids, phenols, glycosides, steroids, terpenoids and proteins.

Keywords: Flax, Antioxidant activity, Polyphenol, DPPH, Antimicrobial activity, Phytochemicals.

1. INTRODUCTION

Linum usitatissimum, commonly called as Flax, is a member of the *Linaceae* family [1], can be grown in every country with moderate climate. Flax becomes up to 1.2 meters tall. From June to August, flax plants carry flowers consisting of 5 petals, colored red or blue. Flax is grown for linseed oil and in European countries flax is generally used as a source of fiber [2, 3]. Flax seed contains around 30%-40% oil, 20%-25% protein, 20%-28% total dietary fiber and the oil contains vitamins A, B, D and E, minerals and amino acids. Flax seed has often fallen into several categories: ''functional food'', ''bioactive food'' and an ''endocrine active food'' for the incidence of physiologically active food components that may provide health benefits beyond the basic nutrition [4].

Plants are used as a source of treatment for diseases since ancient times. The usage of herbal medicines grew due to their lesser side effects and natural origin. Numerous plants, parts of plants have been exploited for this purpose. The medicinal values of these plants could produce certain physiological action on the human body; this may be due to the presence of some chemical substances [5].

flax seed contains numerous bioactive compounds. Among plant foods, flax seeds have the highest content of lignans [6], largely in the form of secoisolariciresinol diglucoside; flax seed oil has a very high concentration of the essential omega-3 fatty acid alpha-linolenic acid [7]. Scientific evidences backing the consumption of flax seed enhance the prevention of some chronic diseases such as many types of cancer, diabetes, cardiovascular diseases and cerebrovascular stroke as it has a high content of omega-3, omega-6 rich oil, α-linolenic acid, lignans, high quality proteins and fibres that are biologically active [8].Other bioactive compounds of flax seed are from the class of phenolic compounds, including lignans, flavonoids and phenolic acids [9, 10]. Lignans, phenolic acids and flavonoids are preventative in the decreasing rate of tumour growth and the decreasing incidence of breast, prostate, and colon cancers [1, 11]. Also the natural antioxidants play an important role in slowing down oxidation processes by quenching free radicals, chelating catalytic metals and

scavenging oxygen or oxidizing chain reaction [1,12-14]. The present study was carried out to study the antioxidant, antimicrobial activities and phytochemical assessment of *Linum usitatisimum* (flax seeds) by using 80% v/v aqueous methanol. Furthermore, due to its excellent nutritional profile and potential health benefits, currently it became an attractive ingredient in the diets specially designed for specific health benefits [15].

2. MATERIAL AND METHODS

Samples of flax seeds (whole grains) were obtained from a local store in Chennai, India. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) was procured from Sigma Aldrich, Folin-Ciocalteu's reagent was purchased from LobaChem, India, as the 2N reagent. All the other chemicals /reagents and solvents used in this study were purchased from Merck, India Pvt. Ltd as analytical reagent grade materials and applied without subsequent purification.

2.1. Extraction of flax seeds

Flax seeds were ground using a grinder; ground material was extracted with 5 ml of 80% aqueous methanol for 0.5g seed material. Extracts were obtained at room temperature using a separator set at 5000 RPM for 20 minutes. The supernatants were decanted and transferred into conical flasks. Freshly prepared extracts were tested.

2.2. Evaluation of antioxidant activity using DPPH method

Free radical-scavenging capacity of flax seed extracts was determined spectrophotometrically. The modified method described by Oyedemi et al (2011) was used to determine the DPPH scavenging activity of the flax extract [16]. A solution of 0.135mM DPPH was prepared in methanol. Different concentrations of extracts 1.5, 3, 6, 12, 24 and 36 etc. (0.5ml) were mixed with 2.5 ml of DPPH solution. The reaction mixture was vortexed thorough and left in the dark at room temperature for 30 min. The absorbance was measured at 517 nm. Ascorbic acid was used as the reference drug. The ability of the flax extract to scavenge DPPH radical was calculated from the following formula:

% DPPH inhibition = {(OD of control-OD of test)/ (OD of control)} x100

2.3. Evaluation of antimicrobial activity

2.3.1. Anti-bacterial activity of test Sample against the E.coli and Staphylococcus aureus

The samples were screened for their antibacterial activity by disc diffusion technique. Compounds were screened *in vitro* for their anti-microbial activity against *E.coli* (MTCC 443) and *Staphylococcus aureus* (MTCC 96) and were compared with standard drug Streptomycin (10µg). The zones of inhibition formed for the compounds against organisms were calculated. The antibacterial activities of test samples were carried out by disc diffusion method. The concentrations of 1000µg and 2000µg test samples were taken in aqueous methanol. The targeted microorganisms were cultured in Mueller-Hinton broth (MHB). The suspensions were adjusted to standard subculture dilution after 24 h. The petri dishes that contain Muller Hinton Agar (MHA) medium were cultured with diluted bacterial strain.

Discs made of Whatman No.1, diameter 6 mm were presterilized and were maintained in aseptic chamber. Each concentration was injected to the sterile disc papers. The prepared discs were then placed on the culture medium. Standard drug Streptomycin (10µg) was used as a positive reference standard to determine the sensitivity of each microbial species tested. Then the inoculated plates were incubated at 37°C for 24 h. The diameter of the clear zone or zone of inhibition around the disc was measured and expressed in millimeters as its anti-microbial activity.

2.3.2. Anti-fungal activity of test Sample against the Candida albicans

The samples were screened for antifungal activities by disc diffusion technique. Compounds were screened *in vitro* for their antifungal activity against *Candida albicans*a nd are compared with standard drug Clotrimazole. The zone of inhibition formed for the compounds against organisms was calculated.

Potato dextrose agar (PDA) was sourced for fungal cultures. The culture medium was inoculated with the fungal strains distinctly suspended in Potato dextrose broth. The samples of 1000µg and 2000µg were then applied on sterile disc. Standard antibiotic (Clotrimazole 10µg) was used as positive control. The prepared fungal plates were incubated at 37°C for 72h. The zone of inhibition observed was measured.

2.4. Determination of total phenolic content

Total phenolic content of the sample was determined

using the Folin-Ciocalteu's method with Gallic acid as standard [17].

2.4.1. Acid Stock Solution

Transferred 0.010g of dry gallic acid into a 100-mL volumetric flask, added 10 ml of ethanol and diluted to volume with water.

2.4.2. Sodium Carbonate Solution

Tewnty g of anhydrous sodium carbonate was dissolved in 80 ml of water and boiled. Cooled the solution to room temperature, after cooling, added a few crystals of sodium carbonate and after 24 hours filtered and added water to make up to the volume 100ml (pH ~14).

2.4.3. Preparation of calibration curve

To prepare a calibration curve, different concentrations (0, 2, 3, 4, and 10 ml) of the stock solution were added into 100 ml volumetric flasks, diluted to the volume with water. These solutions will have phenol concentrations of 0, 2, 4, 8, 16, 32, 64 mg/L Gallic acid. 20 µL of the sample, or blank from each calibration solution were pipetted out into separate cuvettes, and added 1.58 ml water to each cuvette, added 100 µL of the Folin-Ciocalteu reagent and mixed well. After 5 min, 300 µL of the sodium carbonate solution was added and mixed well. The solutions were kept at room temperature for 2 hrs. Absorbance was measured at 765 nm for each solution. Based on the absorbance, the concentrations of phenolics were interpreted (mg/ml) from the calibration line; then the content of phenolics in the extracts was expressed in terms of Gallic acid equivalent (mg of GA/g of extract).

2.5. Phytochemical screening

Phytochemical evaluation for various phytoconstituents of the extracts were undertaken using standard qualitative methods [18, 19]. The extracts were screened with the presence of biologically active compounds like alkaloids, carbohydrates, glycosides, amino acid, proteins, steroids, diterpenes, flavonoids, phenolic, tannin, etc.

To the 1mL of extract, 2ml of Mayer's reagent was added, a dull white precipitate revealed the presence of alkaloids (Mayer's Test). 5 ml of water was added to 1 ml of the extract and the tube was shaken vigorously. Copious lather formation indicates the presence of Saponins. To the 1 ml extract, ferric chloride was added, formation of a dark blue or greenish black colour showed the presence of tannins. To 1ml of the extracts, 1ml of concentrated H_2SO_4 was added and allowed to stand for 2 min. a reddish colour precipitation indicates the presence of glycosides.1mL of the extract was treated with 10 % NaOH solution, formation of intense yellow colour indicates presence of Flavonoid. To the 1mL of extract, 3 ml of 10% lead acetate solution was added. A bulky white precipitate was formed indicating the presence of phenolic compounds.1mL of the extract was dissolved in 10 ml of chloroform and equal volume of concentrated H_2SO_4 acid was added from the side of the test tube. The superior layer turns red and H_2SO_4 layer showed yellow with green fluorescence. This indicates the presence of steroid. 5ml of extract was mixed with 2ml of chloroform, and concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration of the interface indicates the occurrence of terpenoids.1mL of the extract was treated separately with alc. KOH solution. Manifestation of colours ranging from red to blue indicates the presence of Quinones. To 2 ml of the extract, 0.1 ml of Conc. HCl and 1ml of Ninhydrin reagent was added and heated for 5 min at 100°C. Formation of the bluecoloursignifies the presence of protein.

3. RESULTS AND DISCUSSION

3.1. Evaluation of antioxidant activity using DPPH method

The study showed the ability of the flax seed,its free radical scavenging activity, also expressed by the antioxidant activity was from 26.7% to 98.6 %. The lowest concentration 1.5 mg/ml corresponded to 26.69±1.46812 %, while for the highest concentration 60 mg/ml it was 98.52±0.22782 %. However, the graph was linear from 1.5mg/ml to 24 mg/ml, presented in Table 1. The experiment was continued further in 36, 48 mg/ml, the activity was 96.53± 0.22782 %, 97.65±0.27158 %, correspondingly.

Table 1: Percentage scavenging of 1,1-Diphenyl-2-Picrylhydrazyl radical

Concentration of Extract mg/mL	Percentage scavenging activity
1.5	26.69±1.46812
3	36.77±1.06078
6	43.65±1.38889
12	61.38±0.77058
24	84.79±0.78918
36	96.53 ± 0.22782

The concentration required for 50% DPPH reduction (IC_{50}) was 5.56 mg/mL, which was calculated from the

linear regression equation: y=mx+c, where y = 2.160x +26.69, R^2 = 0.943, are presented in Fig.1. The IC_{50} value is defined as the concentration of the extract required for 50% scavenging of radicals. Lesser IC_{50} value corresponds to a higher antioxidant activity [20].

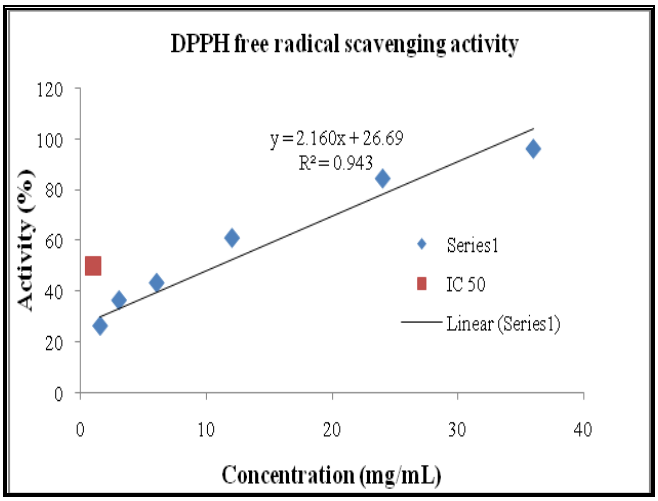

Fig. 1: Antioxidant activity of flax seeds based on DPPH method

According to Katarzyna et al, 2014, IC_{50}values for defatted, non-defatted flax seed extracts were 35.5 mg/ml and 29.9 mg/ml respectively [21]. However, as per the studies of Amin and Thakur, the percentage scavenging of DPPH radical of non-defatted flax seeds were at the lowest concentration; 100µg/ml corresponded to 32.33%, while for the highest concentration; 500 µg/ml was 82.53 %. The IC_{50} value of ethanolic extract of flax seeds was 256.313µg/ml [22]. Gaafar et al., also have reported that different defatted flax seeds cultivars possessed antioxidant, scavenging activity in the field from 5.57±0.28% to 9.05±0.76 %, which corresponded to the lowest concentration of flax seeds 5µg/ml. The highest concentration of defatted flax seeds 50 µg/ml was characterized by percentage scavenging of DPPH radical ranging from 55.17±0.56 % to 88.56±0.28 %. These differences between flax seed cultivars may be caused by country of origin of testing seeds of flax [23].

3.2. Evaluation of antimicrobial activity

The zones of inhibition shown by 80% v/v of an aqueous methanol extract of flax seeds are effective against *Staphylococcus aureus, Escherichia coli*, the zone of inhibition observed for 2000µg concentration was 8mm for both the organisms, whereas 1000µg concentration did not show any activity (Table 2). However, both the concentrations did not show any activity against *Candida albicans* (Table 3). Plant-derived phytochemicals are a wide group of chemical compounds synthesized in the secondary metabolism. Alkaloids, phenolic compounds, and flavonoids originate naturally in plants, have been established *in vitro* to have antimicrobial activities [24, 25]. Our results are in concurrence with the fact that the phyochemicals are known to exhibit antibacterial activity, also with the results reported by Andreea I. PAG et al. (2014) [26].

Table 2: Antibacterial activity

Sample	Zone of Inhibition (mm)	
	S. aureus	*E. Coli*
Extract (1000 µg)	-	-
Extract (2000 µg)	8	8
Streptomycin (10 µg)	21	24

Table 3: Antifungal activity

Sample	Zone of Inhibition (mm)
	Candida albicans
Extract (1000 µg)	-
Extract (2000 µg)	-
Clotrimazole (10 µg)	16

3.3. Determination of total phenolic content

Total phenolic content of the samples was calculated using standard calibration curve of Gallic acid (standard curve equation: y =0.0247x+0.2124 and R2 = 0.9972) and is presented in Fig. 2. The phenolic content in the 80% aqueous methanol extract of Flax seeds was found to be 21.54 mg/g. The values obtained are expressed as GAE/g of extract (GAE = Gallic Acid Equivalent). Total polyphenol contents were estimated by Folin-Ciocalteu colorimetric method. The absorption of which is the maximum in the wavelength range of 700 nm to 750 nm, proportional to the amount of polyphenols present in the extracts [27].

The results obtained are higher than the values reported for 70% acetone (12 mg/g), 70% ethanol (15.5 mg/g), 70% methanol (13.5 mg/g) and water (11.5 mg/g) [28]. Whereas same as the value reported (21.52mg/g) by Amin and Thakur (2014) [22].

Flax holds three types of phenolic compounds: phenolic acids, flavonoids and lignans. The majority of phenolics are believed to exhibit antioxidant and anticancer effects in humans [29, 30]. Flax contains about 8-10 g of total phenolic acids per kilogram of flax. Flax is a significant

source of a lignan called secoisolariciresinol diglucoside (SDG), which can be found in amounts ranging from 1 mg/g of seed to nearly 26 mg/g of seed [31].

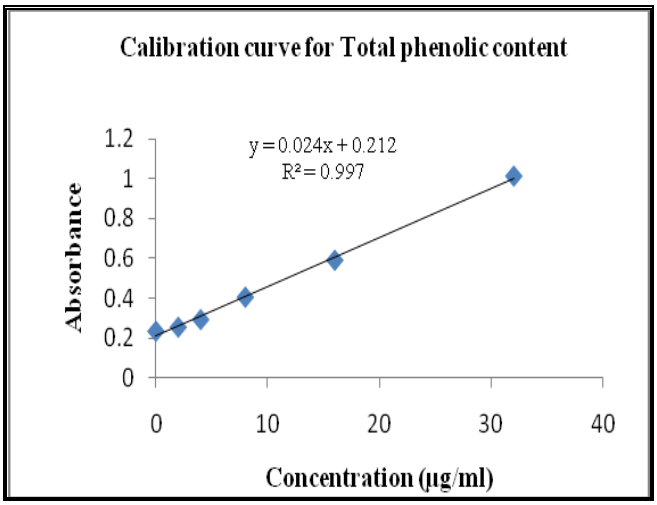

Fig. 2: Calibration curve of Gallic acid

3.4. Phytochemical screening

The results of preliminary qualitative phytochemical study are depicted in Table 4. The study carried out on 80% aqueous extract of flax seed, shows the presence of phytochemicals such as alkaloids, flavonoids, phenols, glycosides, steroids, terpenoids and proteins while saponnins, tannins and quinones were absent.

Hanaa*et al*. (2017) carried out the phytochemical screening in four different extracts of flax seed. 70% acetone extract of flax seed showed the absence of tannins, 70% ethanol and water extracts showed the absence of saponnins, tannins, alkaloids and glycosides, 70% methanol showed the absence of only saponnins [28]. Amin and Thakur (2014) reported that phyto-chemical analysis of ethanol extract of flax seeds showed the absence of saponins, sterols, and glycosides [22]. Whereas Nazir *et al*. (2012) reported the absence of tannins, glycosides, reducing sugars and steroids in the methanol extract of flax seeds [32].

Table 4: **Phytochemical analysis of 80% v/v aqueous methanol**

Name of the analyte	Name of the reagent /tests	80% Aq Methanol Extract
Alkaloids	Mayer's Test	+
Saponins	Distilled water	-
Tannins	5% Ferric chloride	-
Flavonoids	Alkaline reagent test	+
Phenols	Lead acetate test	+
Glycosides	Conc. H2SO4 test	+
Steroids	Chloroform, Acetic acid, H_2SO_4	+
Terpenoids	Salkowski test	+
Quinones	Alc. KOH	-
protein	Ninhydrine test	+

(+) indicates presence; (-) indicates absence

4. CONCLUSION

This study evaluates that the antioxidant, antimicrobial study and phytochemical assessment of 80% aqueous methanol extracts. 80% aqueous extracts showed better antioxidant activity compared to methanol, ethanol and chloroform extracts. Whereas the extract did not show any activity against C*andida Albicans*. Further exploration may be essential to investigate the activities of flax seed thoroughly to understand and utilize the novel benefits for human health.

Conflict of interst

None declared

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