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EVALUATION OF ANTIOXIDANT ACTIVITY AND CYTOTOXICITY OF *LINUM USITATISSIMUM* SEEDS

Enas Ali Hassan Alkangar[*1], Elaf Badr Aldeen Osman[2], Maab Yosif Osman[2], Eman Adil khalifa[2], Shaima Amin Mohamed Ahmed[2]

Omdurman Islamic University, Faculty of Pharmacy, Pharmacognosy Department.

***Corresponding Author: Dr. Enas Ali Hassan Alkangar**

Omdurman Islamic University, Faculty of Pharmacy, Pharmacognosy Department.

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ABSTRACT

Linum usitatissimum (Flax) is a member of the genus Linum in the family Linaceae. The main objectives of this study is to evaluate the antioxidant activity and cytotoxicity of *Linum usitatissimum* seeds and to identify the solvent of the highest capacity to extract it s antioxidant and cytotoxic components among solvent used. In this study *Linum usitatissimum* seeds was extracted by maceration with petroleum ether, chloroform and ethanol 70%, those yielded 8.8, 2.3 and 11.1% respectively, all extracts were analyzed by thin layer chromatograhy. The antioxidant activity and Cytotoxicity of all extracts were evaluated using DPPH method and brine shrimp lethality bioassay respectively. Phytochemical screening and thin chromatographic analysis of *Linum usitatissimum* were exhibited the presence of reducing sugars, essential oils, anthraquinones, terpenoids, coumarins, alkaloids, flavonoids but saponins and cardiac glycosides exhibited with TLC only. Aquatic ethanol extract accompanied with high tannin content (1.35 mg tannic acid equivalent/mg of dry extract). Evaluation of antioxidant activity and cytotoxicity revealed that no extract was induced antioxidant activity while chloroform extract was shown to have high cytotoxicity (LC50=36.4 µg/ml) among tested extracts.

KEYWORDS: *Linum usitatissimum*, Antioxidant, Cytotoxicity, Thin layer chromatography.

INTRODUCTION

Herbal medicines are being used about 80% of the world population primarily in the developing countries for primary health care. They have stood the test of time for their safety, efficacy, cultural acceptability and lesser side effects. The chemical constituents present in them are a part of the physiological functions of living flora and hence they are believed to have better compatibility with the human body.[1]

Cancer is the major cause of morbidity and mortality throughout the world. It is the second most frequent cause of death in Europe and is becoming the leading cause of death in old age.[2] Chemotherapy used to reduce the ability of cancer cell to grow and divide but severe side effects and high cost of chemotherapeutic agents intensify researches in this field to find out alternative or complementary cancer treatment. [3]

Flax is an erect annual plant that grows up to 120 cm tall, with slender stems. the leaves are alternate, greyish green, slender lanceolate, 2 to 4 cm long and 3 mm broad. the flowers are bright blue or white, 1,5 to 2,0 cm in diameter, with five petals. the fruit is a round, dry capsule, 5 to 9 cm in diameter, containing several glossy brown or yellow seeds shaped like an apple pip. the seeds are 4 to 7 mm long and become sticky when wet. the colour of the seeds depends on the variety. Flax is one of the few plant species capable of producing truly blue flowers, although not all flax varieties produce blue flowers .It was already cultivated in ancient Egypt and samaria 10,000 years ago to provide both fiber and oil . The center of origin of cultivated flax is believed to be the Middle East ,although secondary diversity centers were identified in the Mediterranean basin, Ethiopia, Central Asia and India.[4] Flaxseed is a multicomponent system with bio-active plant substances such as oil, protein, dietary fiber, soluble polysaccharides, lignans, phenolic compounds, vitamins (A, C, F and E) and mineral (P, Mg, K, Na, Fe, Cu, Mn and Zn.[5] Flax seed product are used widely for numerous indication . seeds from the plant have historically been used for upper respiratory infections , constipation, abdominal pain , urinary infection ,and skin inflammation . oil derived from the seeds of the plant has been used topically as a skin salve/demulcent and orally for constipation,arthritis,cancer,vaginitis,weight loss, heart disease and benign prostatic hypertrophy. The oil may share the lipid-lowering properties of flaxseed but not the laxative or anticancer abilities.[6]

MATERILAS AND METHODS

Collection and Identification of the plant: *Linum usitatissimum* (*L. usitatissimum*) seeds was obtained from herbalist in Omdurman market during March and authenticated by medicinal and aromatic plant research institute, khartoum, sudan on 3/2017.

Preparation of plant material and extraction: The flax seeds were cleaned and air dried then crushed. Three weights of the sample was taken, each weight (100 g) of the plant material was soaked in selected organic solvent; petroleum ether, chloroform or ethanol 70% for 48 hours, each extract was filtered, air dried and yield percentage of different extracts was calculated using equation: yield percentage=(weight of extract/total weight of sample)*100. The different extracts were preserved in refrigerator for further studies.

Phytochemical analysis of *Linum usitatissimum*

Preliminary phytochemical screening for the major constituents of the extracts: General phytochemical screening for the presence of Reducing sugar, Anthraquinone, Terpenoids, Flavonoids, Saponins, Tannins, Alkaloids and Cardiac glycosides was done for all extracts using method described by [7] with some modification.

Test for Reducing sugars (Fehling's test): 200 mg of each extract was added to boiling Fehling's solution (A and B) in a test tube. The solution was observed for a colour reaction.

Test for Anthraquinones: 200 mg of each extract was boiled with 10 ml of sulphuric acid and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes.

Test for Terpenoids (Salkowski test): 200 mg of each extract was dissolved in 2 ml of chloroform. 3 ml of Conc. H_2S0_4 was carefully added to form a layer. A reddish brown colouration at the interface indicates the presence of terpenoids.

Test for flavonoids: Two methods were used to test for flavonoids. First, dilute ammonia (5 ml) was added to a portion of an 200 mg of each extract. Concentrated sulphuric acid (1 ml) was added. A yellow colouration that disappear on standing indicates the presence of flavonoids. Second, a few drops of 1% aluminium solution were added to a portion of the filtrate. A yellow colouration indicates the presence of flavonoids.

Test for Saponins: 200 mg of each extract was dissolved in 5 ml of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

Test for Tannins: 200 mg of each extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.

Test for Alkaloids: 200 mg of each extract was diluted to 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate was added 2 ml of dilute ammonia. 5 ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into three portions. Mayer's reagent was added to one portion and Hager's and wagner's reagents to the other portions. The formation of a cream (with Mayer's reagent) was regarded as positive for the presence of alkaloids.

Test for Cardiac glycosides (Keller-Killiani test): To 200 mg of extract diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Thin layer chromatographic analysis: From each extract, about 20-100 micro were applied using capillary tubes to a precoated silica gel plates, 7 plates were prepared to cover each of the main classes of constituents. Each having different solvents system and detection method (Table 1).[8]

Table 1: Solvent systems and detection methods used in TLC of *L. usitatissimum* seeds

Plant constituent	Solvent system	Detection method
Essential oils	Toluene-ethyl acetate(93:7)	Vanillin/H_2SO_4
Coumarins	Toluene-ethyl acetate(93:7)	10% ethanolic KOH and U.V
Anthraquinones	Ethyl acetate – methanol-water (100-13.5-10)	Borntrager and U.V
Cardiac glycosides	Ethyl acetate – methanol-water (100-13.5-10)	Conc. H_2SO_4 and U.V
Saponins	Ethyl acetate –methanol-water (100-13.5-10)	Vanillin/H_2SO_4
Alkaloids	Ethyl acetate- methanol- water(100-13.5-10)	Drangendroff reagent and U.V
Flavenoids	Ethyl acetate- methanol-water(100-13.5-10)	U.V

Total flavonoid content: Aliquots of each extracts were pipette out in series of test tubes and volume was made up to 2 ml with distilled water, 0.3 ml of sodium nitrite (5%) was added to each tube and incubated for 5 min. at room temperature, 0.3 ml of aluminium chloride solution (10%) was added and incubated for 5 min, 2 ml of sodium hydroxide (1M) were added. Absorbance was measured at 415 nm against a reagent blank. A calibration curve was constructed, using quercetin (in conc. 0.1-0.9 mg/ml) as standard. Total flavonoids content was expressed as quercetin (mg/ml) using the following equation based on the calibration curve: $y = 0.8065x + 0.0349$ $R^2 = 0.9855$, where y was the Absorbance and x was the concentration of quercetin in (mg/ml) corresponding to absorbance. total flavonoids content of the extracts (mg/ml) expressed as mg quercetin equivalents.[9]

Total Tannin content: The tannins content was determined by using FeCl3 and gelatin test method as described by Shivakumar et al., (2012) with some modification. About 1 ml of extract (mg/ml) was transferred to vials, 1ml of 1% K3Fe (CN) 6 and 1 ml of 1% FeCl3 were added, and the volume was made up to 10 ml with distilled water. After 5 min absorbance was measured at 510 nm against a reagent blank. A calibration curve was constructed, using tannic acid (0.1-0.8 mg/ml) as standard. The total tannins content of the extracts (mg/ml) was calculated using the following equation: $y = 2.8072 x + 0.0824$ $R^2 = 0.9936$, where y= Absorbance, x= concentration of tannic acid (mg/ml) corresponding to Absorbance and expressed as mg tannic acid equivalent.[9]

Biological Screening of *Linum usitatissimum*

Antioxidant activity using DPPH method: The DPPH (2,2-diphenyl-1-picryl hydrazyl) radical scavenging was determined according to the method of [10] with some modification. In 96-wells plate, the test samples were allowed to react with 2.2Di(4-tert-octylphenyl)-1-picryl-hydrazyl stable free radical (DPPH) for half an hour at 37° c. The concentration of DPPH was kept as (300 µM). the test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 512 nm using multiplate reader spectrophotometer. Percentage of radical scavenging activity (RSA%) by samples was determined in compassion with DMSO treated control group. All tests and analysis were run in triplicate.

Evaluation of cytotoxicity by Brine Shrimp Lethality Bioassy: The brine shrimp assay was developed by.[11] It is considered a useful tool for preliminary assessment of toxicity and it has been used for the detection of fungal toxins, plant extract toxicity, heavy metals, pesticides and cytotoxicity testing of dental materials.[12] It is widely used as a simple, reliable and cheaper prescreens method to determine the cytotoxicity of crude plant extract and pure natural compounds, especially antitumor compounds from the natural source. The test was carried out according to the method prescribed by [13]. Eggs of *Artemia salina* (Family, Artemiidae) were stored at low temp. (4ºC), they will remain viable for many years. Brine shrimp eggs, *Artemia salina* were hatched in artificial seawater prepared by dissolving 38g of sea salt in one liter of distilled water. After 24-72 h incubation at room temperature (37°C), the larvae were attracted to one side of the vessel with a light source and then collected with pipette. Larvae were separated from eggs by aliquoting them three times in small beakers containing artificial seawater. Bioactivity of the extract was monitored by the brine shrimp lethality test.. 50 mg of (Leach) eggs were added to a hatching chamber containing artificial Sea water (75ml). The hatching chamber was kept under an inflorescent bulb for 48h for the eggs to hatch into shrimp larvae. 20 mg of test extracts of the various plant species were separately dissolved in 2 ml of methanol, then 500, 50, and 5µl of each solution was transferred into vials corresponding to 1000, 100, and 10 µg/ml, respectively. Each dosage was tested in triplicate. 10 larvae of *A. salina* Leach (taken 48–72h after the initiation of hatching) were added to each vial. The final volume of solution in each vial was adjusted to 5ml with Sea water immediately after adding the shrimps. One drop of dimethyl sulphoxide (DMSO) was added to the test and control vials before adding the shrimps to enhance the solubility of test materials. The numbers of survivals were counted after 24 hour, the experiment was performed in triplicate, the % of mortality was then determined and the main of mortality values was calculated. LC50 value was obtained from the best fit line by plotting concentration versus % of mortality.

Statistical analysis: Data analyzed using excel sheet 2010. Data were reported as means ± SD.

RESULTS

Phytochemical analysis of *Linum usitatissimum* Seeds: Yield percentage and phytochemical screening of *L. usitatissimum* seeds: Different yield % was obtained with different menstruum, Phytochemical screening revealed the presence of various phytochemical (table 2).

Table 2: Phytochemical screening of all extract of *linum usitatissimum* seeds

Extract	Yield		Phytochemical tested							
	%	Description	Anthraquinones	flavonoids	Saponins	Tannins	Alkaloids	Cardiac Glycosides	Reducing Sugers	Terpenoids
PET	8.8	Oily, yellow brown	+	-	-	+	+	-	-	+
$CHCL_3$	2.3	Oily, yellow brown	+	+	-	+	+	-	-	+
EtOH	11.1	Sticky, brown	-	+	-	+	+	-	+	-

Key: *+: present , -: abscent, PET:Petroleum ether, $CHCL_3$:Chloroform, EtOH: Ethanol 70%.*

Thin layer chromatographic analysis of all extract of *Linum usitstissimum* L.

TLC anlaysis of *L. usitstissimum* seeds exhibited various compounds (table 3).

Table 3: Thin layer chromatographic analysis of *L. usitstissimum* seeds

Extract	Compound		Detection Method
	Code/R_f	Colour	
Chromatographic Analysis of Essential oils in all extracts			
PET	C1/0.270	Blue (uv)	Vanillin/H_2SO_4
$CHCL_3$	C1/0.270	Blue (uv)	
EtOH 70%	-	-	
Chromatographic Analysis of Coumarins in all extracts			
PET	C1/0.293	Blue (uv)	10% ethanolic KOH and U.V
$CHCL_3$	C1/0.267	Blue(uv)	
EtOH 70%	-	-	
Chromatographic Analysis of Anthraquinones in all extracts			
PET	C1/0.958	Yellow (vis)	Borntrager and U.V
$CHCL_3$	C1/0.845 C2/0.761 C3/0.169	Blue (uv) Yellow (vis) Blue (uv)	
EtOH 70%	C1/0.972 C2/0.789	Yellow (vis) Yellow (vis)	
Chromatographic Analysis of Cardiac glycosides in all extracts			
PET	C1/0.878 C2/0.878	Violet (vis) Brown(vis)	Vanillin/H_2SO_4
$CHCL_3$	C1/0.311 C2/0.959	Violet (vis) Brown(vis)	
EtOH 70%	-	-	
Chromatographic Analysis of Saponins in all extracts			
PET	C1/0.919 C2/0.946	Yellow(vis) Yellow (vis)	Vanillin/H_2SO_4
$CHCL_3$	C1/0.959	Yellow (vis)	
EtOH 70%	-	-	
Chromatographic Analysis of Alkaloids in all extracts			
PET	C10.859 C1/0.944	Blue (uv) Blue (uv)	Drangendroff reagent and U.V
$CHCL_3$	C1/0.944	Blue (uv)	
EtOH 70%	C1/0.830	Blue (uv)	
Chromatographic Analysis of Flavenoids in all extracts			
PET	C1/0.057 C2/0.486 C3/0.957	Blue (uv) Brown (vis) Blue (uv)	U.V
$CHCL_3$	C1/0.971	Brown (vis)	
EtOH 70%	C1/0.871	Brown (vis)	

Key: *PET: Petroleum ether, $CHCL_3$: Chloroform, EtOH: Ethanol 70%, C: compound, vis.: on visible light, U.V: under ultraviolet.*

Total flavonoid and tannin contents of aquatic ethanol extract: The measured Absorbance of different conc. of standard quercetin and tannic acid (table 4) were used to set the calibration curve equations (Figure 1 and 2). Calculated flavonoid and tannins content was expressed as mg Quercetin and Tannic acid equivalent respectively (table 5).

Table 4: Absorbance of different conc. of Quercetin and Tannic acid

Quercetin		Tannic acid	
Conc. (mg/ml)	**A**	**Conc. (mg/ml)**	**A**
0.1	0.1253	0.1	0.3947
0.2	0.1937	0.2	0.5984
0.3	0.2872	0.3	0.9048
0.4	0.3337	0.4	1.1799
0.5	0.4048	0.5	1.6069
0.6	0.5382	0.6	1.7208
0.7	0.6137	0.7	2.0285
0.8	0.7234	0.8	2.3311
0.9	0.7234	-	-

Key: *A: absorbance of the standards*

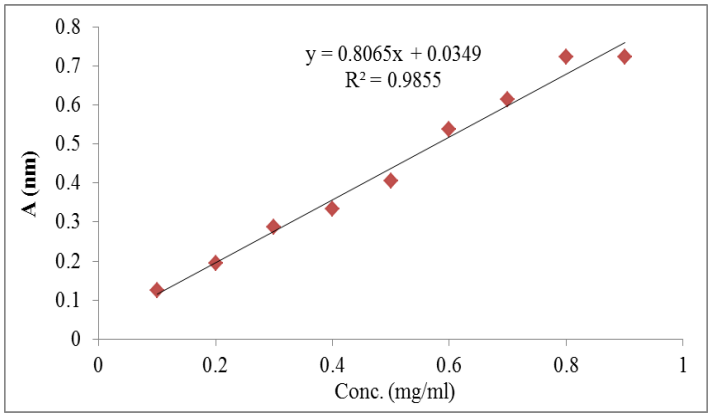

Figure 1: Absorbance of different conc. of standard Quercetin

Key: A and Y: absorbance of the quercetin at ʎ 415 nm, x=conc. of quercetin (mg/ml)

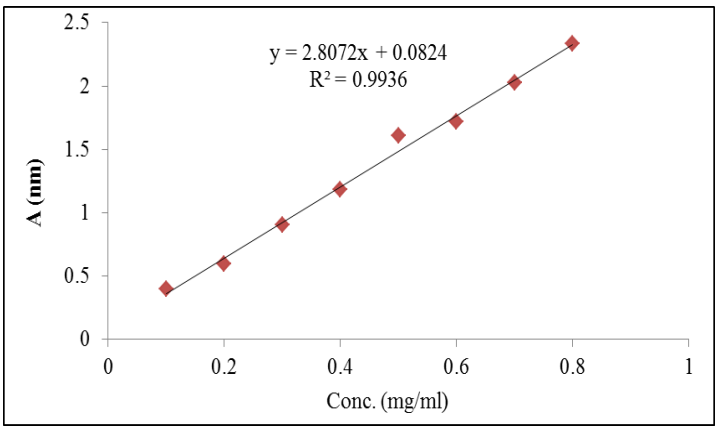

Figure 2: Absorbance of different conc. of standard Tannic acid

Key: A and y: absorbance of tannic acid at ʎ 510 nm, x=concentration of tannic acid (mg/ml).

Table 5: Total flavonoid and tannin content of aquatic ethanol extract

Extract	Flavenoids		Tannins	
	A	**Conc. (mg QE/mg of extract)**	**A**	**Conc. (mg TAE/mg of extract)**
Ethanol 70%	-0.4146	0	1.980033333	1.35

Key: *QE: Quercetin equivalent, TAE: Tannic acid equivalent.*

Biological screening of *Linum usitatissimum* seeds

Antioxidant activity

All tested extracts of *L.usitatissimum* were found to be in active using DPPH assay (table 6).

Table 6: RSA% of all extracts of *Linum usitatissimum* seeds

Extracting solvent	RSA % ±SD
PET	Inactive
$CHCL_3$	Inactive
EtOH70%	2± 0.09
Propyl galate	92±0.01

Key: *PET : Petroleum ether, $CHCL_3$: Chloroform, EtOH: Ethanol 70%, RSA%: Percentage of radioactive scavenging activity.*

Cytotoxicity Assay: *L.usitatissimum* was revealed high toxicity against brine shrimp (table 7). Extracts with LC50 above 1000 µg/ml are non-toxic, LC50 of 500 - 1000 µg/ml are low toxic, extracts with LC50 of 100-500 µg/ml are medium toxic, extracts with LC50 of 0 - 100 µg/ml are highly toxic.[14]

Table 7: LC50 of tested extracts of *L. usitatissimum* seeds

Extract	Conc.(µg/ml) / % Mortality ± SD				LC50 (µg/ml)	Toxicity profile	Calibration curve Equation
	0	10	100	1000			
PET	0	16.67±4.7	20 ±8.2	20±8.2	4171.6	Non toxic	y = 0.0092x 11.621 R^2 = 0.2149
$CHCL_3$	0	70±8.1	83.33±4.7	83.33±4.7	36.4	Highly toxic	y = 0.038x+48.617 R^2 = 0.212
EtOH 70%	0	16.67±9.4	16.67±4.7	20±8.2	3944.7	Non toxic	y = 0.01x + 10.553 R^2 = 0.2882

Key : *PET: Petroleum ether, $CHCL_3$: Chloroform, EtOH: Ethanol 70%, LC50: concentration which induce 50% mortality, Extracts with LC50 above 1000 µg/ml are non-toxic, 500 - 1000 µg/ml are low toxic, 100 - 500 µg/ml are medium toxic, 0 – 100 µg/ml are highly toxic, y: Mortality %, x: conc. of extract, SD: standard deviation, R: regression coefficient.*

DISCUSSION

The medicinal value of plants refer to its phytochemical constituents which produce specific physiological action on the animal and human body.

The phytochemical screening show the presence of alkaloids, terpenoids, flavonoids, tannins, and anthraquinones and exhibited absence of saponins and cardiac glycosides, TLC support these results except for saponins and cardiac glycosides, some of these metabolites are known to have antioxidant activity and cytotoxicity effect on the human body. No antioxidant activity was seen with all tested extracts which disagreed with many studies,[15] concluded that 80% aqueous ethanol and pure methanol are the effective solvents for recovering antioxidant components from flaxseed while the pure ethanol has shown the lower efficacy. Low cytotoxicity with high tannin content of aquatic ethanol extract exclude the significant role of tannin as cytotoxic component of flax but may roles out the safety of flax seeds aquatic extracts. Chloroform extract revealed the highest cytotoxicity, so low polar components may responsible about cytotoxicity of flax seeds.

CONCLUSION

This study showed that *Linum usitatissumum L.* seeds contain an important phytochemicals; essential oils, alkaloids, terpenoids, coumarins ,flavonoids, cardiac glycosides tannins, saponins and anthraquinones which responsible about various therapeutic uses of the plant, high tannin content was observed with aquatic ethanol extract, no antioxidant activity was seen with all extracts while chloroform extract revealed high cytotoxicity that may highlight the possible antitumor activity of the flax seeds.

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