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Isolated linseed oil from *Linum usitatissimum* exhibited pro-coagulant property

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Abstract

Current study describes isolation, characterization and the role of *Linum usitatissimum* oil on blood coagulation cascade. *Linum usitatissimum* also commonly known as flax seed. Flax seed withholds many macro and micro nutrients such as proteins, vitamins, carbohydrates and minerals. Although, flaxseed encompasses a fixed oil known as linseed oil or Flax Seed Oil (FSO) which contains unsaturated fatty acids like linoleic acid, oleic acid and linolenic acid. Linseed oil found to exhibit anti-inflammatory, anti-oxidant and anti-microbial property. Despite of its therapeutic potential, the role of linseed oil on plasma re-calcification time was not reported. Preliminary phytochemical screening was analyzed by simple test method and instrumentation method such as RP-HPLC, IR and GC-MS. Furthermore, plasma re-calcification time and direct hemolytic activity assays were performed for Flax Seed Oil (FSO) by freshly drawn human blood. FSO showed maximum amount of lipids and steroids. The obtained results from IR predicted the presence of different functional groups such as OH, CH_2, C=O, C-O and cyclic ring. While, the RP-HPLC and GC-MS profiles of FSO predicted the presence of several lipids and steroids in the extract. FSO induce the clot formation process of citrated human plasma in both PRP and PPP from the control 200sec to 100sec and 220sec to 140sec respectively. Moreover, FSO did not hydrolyze RBC cells suggesting its nontoxic property.

Keywords

Flax Seed Oil (FSO), GC-MS, RP-HPLC, IR and pro-coagulant.

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INTRODUCTION

Linum Usitatissimum L generally termed as flax belongs to family Linaceae, it is an annual plant cultivated mainly for industrial purpose due to its richest source of fibers and oils [1]. Flax seeds were major crops in Russia, Poland, France, Spain, Greece, Italy, Croatia, Egypt, Syria, Lebanon and it also common plant in Asian countries. Flaxseed was very

well known and largely consumed in India as food source and as well as for medical purpose. In India flaxseed was widely grown in Maharashtra, Madhya Pradesh, Bihar and Chhattisgarh. However, Canada is the major producer of flax seed in worldwide [2]. Flaxseed mainly contains 41% of fats/lipids, 28% of total dietary fibers, 20% of proteins, 7.7% of moisture, 3.5% of ash and only 1% of carbohydrates and also fiber content of flaxseed was used to treat cardiovascular diseases [3]. Whereas, flax seed oil is rich in omega-3 fatty acid which is beneficial for human health and it also found to exhibit anti-inflammatory [4], anti-arthritic [5], anti-ulcer [6] and anti-diabetic [7], properties. In recent days flax seed oil plays a major role in medical field due to its disease prevention properties [8]. Due to the therapeutic applications of biologically active substances such as ALA, SDG and dietary fiber content of flaxseed, it made to attain the main focus of cultivating interest from the group of medical researchers and nutritionists. ALA prevents hypercholesterolemia, thrombosis and also reduces platelet adhesiveness property. Moreover, few patents were also listed on flaxseed components, namely on lignans (four) gums (seven) as animal feed (four) and oil (three) respectively [9]. In our previous study we demonstrated that the presence of cysteine protease in the flax seed buffer extract and that was responsible for anticoagulation, antiplatelet and clot dissolving abilities [10]. Apart from all the above mentioned therapeutic applications of flaxseed oil, there was no report on its role on plasma coagulation cascade. Thus in the current study an effort was made to the identification of probable role of flaxseed oil on plasma coagulation cascade.

MATERIALS AND METHODS

All the chemicals used were of analytical grade. Fresh human blood was collected from healthy donors for Platelet Rich Plasma (PRP).

Extraction of Oil from flaxseed

Flaxseeds were purchased from local market of Tumakuru. From the flaxseeds, oil was extracted by the solvent hexane using Soxhlet extraction method. The finally obtained oil was termed as FSO (Flax Seed Oil) and it utilized for further assays.

Viscosity test

To know the viscosity of extracted oil, 10mL of FSO was added to pre-weighed glass vessel (Viscometer flask). Then weight was taken and calculates as follows

$$\textbf{Calculation: } \frac{W_2 - W_1}{\text{Sample volume}}$$

W_1- Weight of empty glass vessel (Viscometer flask)
W_2-Weight of oil + glass vessel (Viscometer flask)

Karl fisher titration method

Moisture content of FSO was identified by Karl fisher (Kf) titration method. Briefly, 10mL of FSO (in conical flask) was titrated against Kf reagent containing free iodine using Kf instrument (light brown end point) and note down the burette reading.

$$\textbf{Calculation: \% of moisture} = \frac{\text{Burette reading} \times 5.8(\text{Kf value}) \times 100}{\text{Weight of sample}}$$

Residue on ignition

To quantify the amount of inorganic metal ions in the extract, 2mL of FSO was added to pre-weighed crucible and weight was recorded. Then it was kept in muffle furnace at 350°C for 2hr. After the incubation period crucible was again weighed and record the weight.

$$\textbf{Calculation: } \frac{W_3 - W_1}{W_2 - W_1} \times 100$$

W_1- Weight of empty crucible
W_2-Weight of oil + crucible (before incubation time)
W_3-Weight of oil + crucible (after incubation time)

Test for Carbohydrates
FSO was mixed with few drops of Benedict's solution and boiled in water bath. Observed for reddish brown precipitation.
Test for Proteins
FSO was treated with 10% NaoH solution and add 2 drops of 0.1% $CuSO_4$ solution. Observed for violet pink color.
Test for Lipids
FSO was treated with 0.5N alcoholic KoH and add 1 drop of phenolphthalein as indicator. This solution was heated in water bath for 1 hr. Observed for white color foam.
Test for Alkaloids
FSO was treated with few drops of Hager's reagent saturated picric acid solution. Observed for yellow precipitation.
Test for tannins
FSO was treated with gelatin solution. Observed for white precipitation.
Test for steroids
FSO was mixed with few-drops of acidic anhydride boiled and cooled. Then concentrated sulphuric acid was added by sides of the test tubes. Observed the formation of brown ring at the junctions of two layers.
Test for Flavonoids
FSO was treated with sulphuric acid and observed for the formation of orange color.
Phenol test
FSO was treated with 5% ferric chloride and observed for the formation of deep blue or black color.
Glycosides Test
FSO was hydrolyzed with concentrated HCL for 2hr on a water bath and filtered. Few mL of above filtrate was shaken with chloroform and add 10% of ammonia. Formation of pink color indicated the presence of glycosides.
IR spectrum
Fourier Transform Infrared (FT-IR) spectrum was recorded on Agilent FT-IR-4100 spectrophotometer in the spectral range of 650-4000cm^{-1}taking the sample in the form of ATR powder discs [11].
Reverse Phase High Performance Liquid Chromatography analysis
FSO was subjected to RP-HPLC using C_{18} column (150mm×4.60mm, particle size 5µm) with PDA detector in shimadzu LC-20AD prominence. The column was pre-equilibrated with 0.1% Trifluoroacetic acid (TFA) in water and it was eluted at the flow rate of 1ml/min in linear gradient mode.
GC-MS
GC-MS analysis of samples was analyzed on quadrupole mass spectrometers in the electron-capture negative-ion chemical ionization (ECNICI) mode with capillary column (30X0.25mm IDX1EM df, composed of 100% Dimethyl poly siloxane). Helium (99.9%) gas was used as carrier gas at the flow rate of 1ml/min and the injection volume of 0.5 El (split ratio of 10:1). Temperature program was set as follows, injector temperature 250°C; ion-illuminator temperature 280°C, oven temperature 110°C (isothermal for 3min) with an increase in temperature of 20°C/min to 220°C, thereafter 5°C/min to 300°C. Mass spectrum was taken at 80ev; a scan interval of 0.5s [12].
Preparation of platelet-rich plasma and platelet-poor plasma
The PRP and PPP were prepared as described by Ardlie and Han [13]. The platelet concentration of PRP was adjusted to 3.1×10^8 platelets/ml with PPP. The PRP has to be used within 2hr from the time of blood drawn at 37°C. All the above preparations were carried out using plastic wares or siliconized glass wares.
Plasma re-calcification time
The plasma re-calcification time was determined according to the method of Quick [14]. Briefly, the FSO (1-10µL) was pre-incubated with 0.2ml of citrated human plasma in the presence of 10mM Tris HCl (20µl) buffer pH 7.4 for 1min at 37°C. Clotting time was recorded after the addition of 20µl $CaCl_2$ (0.25M) to the pre-incubated mixture.
Direct hemolytic activity
Direct hemolytic activity was determined by using washed human erythrocytes. Briefly, packed human erythrocytes and Phosphate Buffer Saline (PBS) (1:9v/v) were mixed; 1ml of this suspension was incubated independently with the various concentrations of FSO (0-100µL) for 1hr at 37°C. The reaction was terminated by adding 9ml of ice cold PBS and centrifuged at 1000g for 10min at 37°C. The amount of hemoglobin released in the supernatant was measured at 540nm. Activity was expressed as percent of hemolysis against 100% lysis of cells due to the addition of water (positive control), whereas PBS served as negative control.
Statistical analysis
The data are presented as mean ± SD. Statistical analyses were performed by Student's T-test. A significant difference between the groups were considered if $P < 0.01$.

RESULTS AND DISCUSSION

Physical and chemical Characterization of FSO
The extracted oil was look like fairly golden color, pungent odor with lower viscosity. To know the chemical composition of FSO, simple test tube

chemical analysis was done. Interestingly, FSO shows positive response to only for lipids and steroids tests, this clearly suggests that FSO withholds only lipids and steroids with trace amount of inorganic metal ions and 2% of moisture content (Table 01).

Fourier Transform Infrared (FT-IR) spectroscopy

The IR spectrum of FSO at (v, cm-1) 3011;89.535, 2854;69.711 and 2925;59.793 bands were stretching vibrational band for OH and aromatic C-H group. Although new vibrational bands appeared at 1748;50.062, 1659;95.584, 1465;80.644, 1380;86.941, 1242;79.505, 1164;63.007 and 1100;73.803 were characteristic of coordinated carbonyl (C=O) stretching of CHO group. Simultaneously bands appeared at 918;87.279, 810;88.484, 795;90.104 and 724;70.881 (Fig.01) indicates the bending form C-H aliphatic groups.

RP-HPLC

Further the presence of lipids and steroids in FSO was confirmed by RP-HPLC chromatogram. In RP-HPLC, assessment of compound was based on the retention time of obtained chromatogram. Due to isomeric form of compounds 11 peaks were resolved at the retention time of 1.6, 2.1, 2.7, 3.0, 3.8, 5.3, 7.9, 8.5, 10.6, 12.6 and 16.3min respectively (Fig.02).

Quantification by GC–MS

Moreover, the presence of isomeric form of compounds in FSO was also adjudged by GC-MS chromatography technique. Interestingly, as like RP-HPLC chromatogram in GC-MS also 11 major peaks were obtained at the retention time 8.6, 10.9, 11.4, 11.7, 12.5, 13.23, 13.29, 13.38, 13.89, 14,56 and 16.79min respectively (Fig.03).

Plasma Recalcification Time

To identify the probable role of FSO on coagulation cascade, plasma recalcification time was performed using both human platelet rich plasma (PRP) and platelet poor plasma (PPP). Remarkably, FSO exhibited Pro-Coagulant effect by decreasing the clotting time of both PRP and PPP from control 180s to 100s and 180s to 120s respectively. The maximum concentration consumed in both the cases was found to be 8µL and remain unchanged upon increased dose to 10µL (Fig.04). Hemostasis is a highly regulated physiological phenomenon which helps in the prevention of blood loss during a tissue injury [15]. Due to some genetic alteration and environmental factors it leads to uncontrolled blood loss at the site of injury known as hemophilia disorder a pathological phenomenon which ultimately leads to death [16]. Pro-coagulants play a major role in order to treat hemophilia disorder [17]. Due to the exhibition of pro-coagulant activity by FSO, it could be useful to treat hemophilia disorder. Although, several pro-coagulants were synthesized and adopted for medication, it has some side effects such as nausea, vomiting and miscarriage [18]. Therefore, identification of novel pro-coagulants from natural products without any side effects sounds to be good. Many, pro-coagulants and anti-coagulants [19] were isolated from plants [20] and animal [21] sources [22]. Facile capped nanoparticle [23] and Benz-imidazole compound [24] also exhibited anticoagulant property. Moreover, FSO did not hydrolyze RBC suggested its nontoxic property (Fig.05).

Table 01: Chemical composition of FSO

SL.No	Test	Result
01	Carbohydrates	-Ve
02	Proteins	-Ve
03	Lipids	+Ve
04	Alkaloids	-Ve
05	Tannins	-Ve
06	Flavonoids	-Ve
07	Steroids	+Ve
08	Phenol	-Ve
09	Glycosides	-Ve
10	Metal ions	0.8%
11	Moisture content	2%

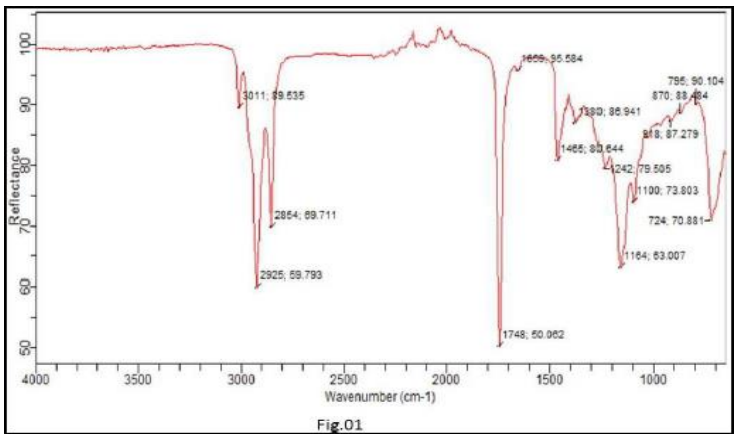

Fig.01

Fig.01 FT-IR Spectra of FSO

IR-spectrum of FSO was obtained in Agilent FT-IR-4100 spectrophotometer at the spectral range of 650-4000 cm-1 in ATR disc.

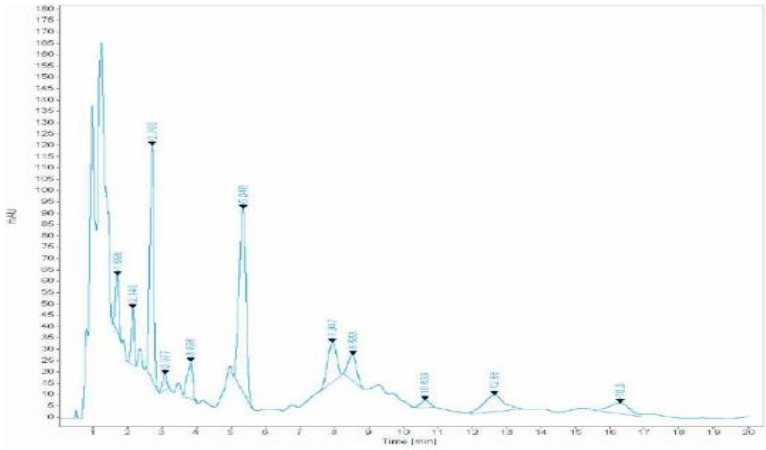

Fig.02

Fig.02 RP-HPLC chromatogram

FSO (5µL) was injected to C_{18} Column (5mm, 0.21X25cm) which was pre-equilibrated with 0.1% Trifluoro Acetic Acid (TFA) in water and sample was eluted in gradient mode by increasing the concentration (0-100%) of 0.1% TFA in acetonitrile for 20min at the flow rate of 1ml/min and monitored at 280nm.

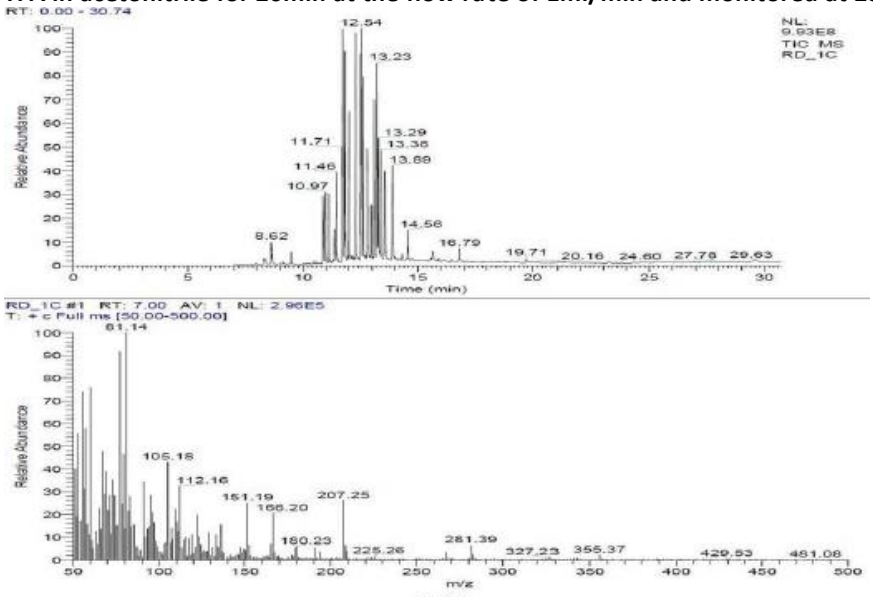

Fig.03

Fig.03 GC-MS Chromatogram of FSO

FSO (5µL) was analyzed in GC-MS with single quadrupole mass spectrometer in the electron-capture negative-ion chemical ionization (ECNICI) mode with capillary column (30X0.25mm). Helium was used as carrier gas at the flow rate of 1ml/min with gradient temperature system and the injection volume of 0.5 El (split ratio of 10:1).

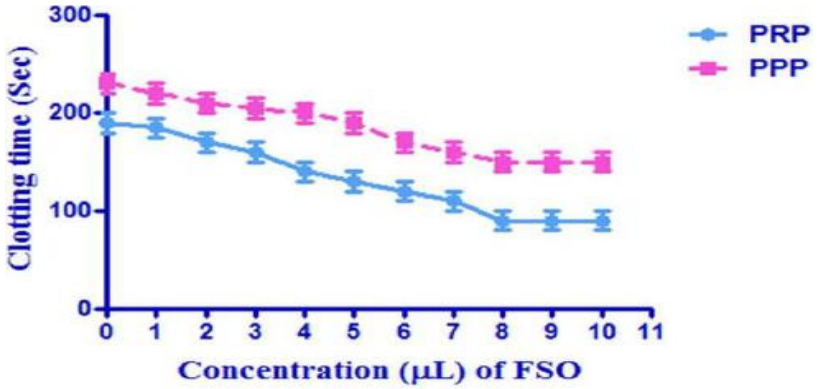

Fig.04

Fig.04 Plasma re-calcification time of FSO

FSO (0–10µL) was pre-incubated with 0.2ml of citrated human plasma PRP/PPP in the presence of 20µl 10mM Tris–HCl buffer (pH 7.4) for 1min at 37°C. 20µl of 0.25M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded.

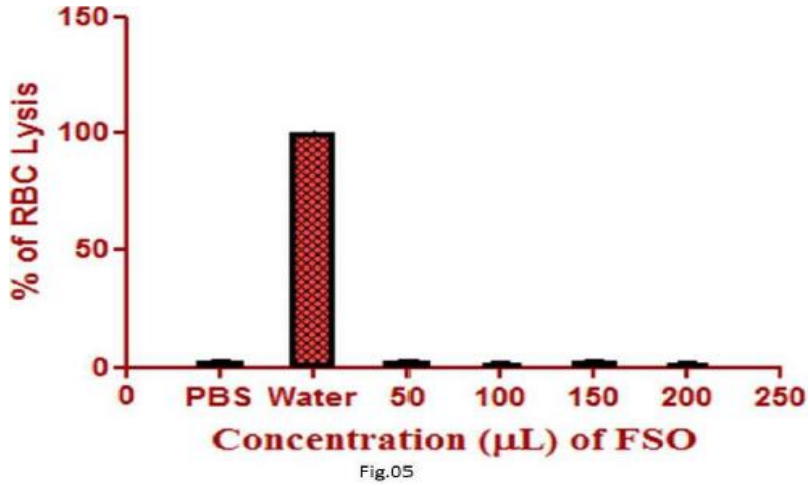

Fig.05

Fig.5 Hemolytic activity of FSO

FSO (0–200 µL) was pre-incubated for 30 min with PBS treated packed RBC. The lysis percentage of RBC was calculated based on the presence of free hemoglobin in the supernatant and it was measured at 540 nm.

CONCLUSION

In conclusion, this study demonstrates the characterization and pro-coagulant activity of FSO. Thus, isolation and purification of active compound sounds to be good.

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DECLARATION OF CONFLICT OF INTEREST

The authors declared no potential conflict of interest with respect to the authorship and publication.

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