PharmacologyOnLine

Archives • 2021 • vol.3 • 602-614

PHYTOCHEMICAL SCREENING, AND *IN VITRO* ANTIRADICAL AND IMMUNOSTIMULANT POTENTIAL OF *LINUM USITATISSIMUM* L.

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Abstract

The The present study investigates and evaluates the phytochemical composition, antiradical and immunostimulant potential of *Linum usitatissimum* seeds, a medicinal plant widely used in Moroccan traditional medicine for its wealth of bioactive compounds. The extraction and the phytochemical screening revealed a richness of flaxseed total sugars (73.48 ± 0.93 mg GE/g), mucilages (44.60 ± 1.32 mg/g) and fixed oils (32.50 %). As for secondary metabolites, these oilseeds contain 7.24 ± 0.14 mg GAE/g of total polyphenols, 1.74 ± 0.57 mg QE/g of flavonoids and 1.35 ± 0.03 mg/g of condensed tannins. The *in vitro* antiradical activity of flaxseed extract expressed as IC_{50} of DPPH free radical scavenging was 287.40 ± 9.93 µg/ml, against 39.70 ± 2.42 mg/ml noted for fixed oils. The hemagglutination test, performed on rat red blood cells, showed an excellent immunostimulant potential of flaxseed through its richness in lectins. In summary, our findings suggest that *Linum usitatissimum* is a plant with many virtues that can find applications in phytotherapy and food industry.

Keywords: *Antiradical, Flaxseed, Immunostimulant, Lectin, Phytochemistry, Polyphenols.*

Introduction

Aromatic and medicinal plants have always been an enormous reservoir of active substances due to their metabolites and bioactive molecules (coumarins, terpenes, polyphenols, tannins, alkaloids, mucilages...), which have the advantage of having a wide variety of chemical structures and a wide spectrum of biological activities such as antioxidant, antimicrobial, immunostimulant... [1].

Morocco, in particular, has a wide floristic variety (over 4200 species have been discovered) and a population that has a long history of using traditional medical methods based on medicinal plants owing to its bio-geographical location [2]. Flaxseed is a well-known medicinal plant in Morocco, and it is extensively utilized in traditional medicine due to its health advantages and abundance of important biomolecules [3].

Despite this floristic richness of aromatic and medicinal plants in Morocco, as well as the increased interest in their use, in the face of promising and increasingly demands nationally and internationally and also the growing concern for clean green chemistry and an awareness of the concept of sustainable development, little effort has been devoted to the development of natural therapeutic agents from these plants, the evaluation of which remains a very interesting task and is the subject of many more detailed studies and innovative research on plant resources [4].

In this light, and in order to contribute to the valorization and optimization of Moroccan medicinal plants, we were interested in investigating *Linum usitatissimum,* one of the most commonly used and effective plants in traditional medicine in Morocco, according to recent ethnobotanical studies [5], We were interested in studying its phytochemical composition and also its antiradical and immunostimulant activities. The choice of this oleaginous plant was guided by the vast expanses occupied by this Moroccan terroir product rich in bioactive molecules and fixed oils, as well as the lack of information on the biological virtues of Moroccan varieties.

Methods

1. Materials and reagent

All chemicals and reagents involved in this study were analytical grades. The Phosphate Buffered Saline (PBS) used in the hemagglutination activity is prepared by mixing (8g/l of NaCl, 0.2g/l of KCl, 1.44g/l of Na_2HPO_4 and 0.24g/l of KH_2PO_4) in 1L of distilled water. The absorbance at different wavelengths was measured by a VWR UV/Vis Spectrophotometer, UV-3100PC.

2. Plant material

The plant material utilized in our research is *Linum usitatissimum* seeds, which were collected in the region of Fez, Morocco (figure 1). Those were purchased from a herbalist in the Moroccan city of Fez towards the end of May 2020, then cleaned and air-dried for 15 days. The seeds were chosen since they are the most often utilized part of this plant in traditional medicine in the area. The samples were identified in the laboratory by botanists using books and plant catalogues. A specimen of the plant was deposited in the herbarium of the National Institute of Medicinal and Aromatic Plants in Taounate.

3. Phytochemical screening

1.1. Mucilage extraction

Extraction was carried out according to the protocol described previously [6]. First, 5g of flaxseeds/linseeds are macerated in 50mL of distilled water for 6 hours. The mixture is then boiled for 30 min and left to stand for one hour for the complete release of mucilage and then filtered through a muslin tissue. Acetone (3 times the filtrate volume) is added to the filtrate to precipitate the mucilages. After centrifugation, the mucilage separated from the supernatant is passed through an oven to be dried at a temperature of 45°C. They are then collected in powder form and weighed to calculate the yield before being stored in the refrigerator. The following formula calculates the yield of the mucilage:

Mucilage yield (%) = (Mm/Mp) x 100

Mm: mass (g) of mucilage obtained.

Mp: mass (g) of plant matter.

1.2. Extraction and dosage of total sugars

The extraction and the dosage of sugars were realized according to the adapted protocol of

Oomah et *al.* [7]. First, one gram of flaxseed powder is macerated in 50mL of distilled water at 85°C for 2 hours. The mixture is then centrifuged at 4000 rpm for 10min, and the supernatant containing the mixture of total sugars to be dosed is recovered.

The dosage is carried out using 0,5mL of supernatant to which 0.5mL of 5% aqueous phenol solution and 2ml of 98% concentrated sulphuric acid is added. After vortex homogenisation, the tubes are placed in a water bath at 90°C for 5 min, then cooled in the dark for 30 min, and their absorbance is read with a spectrophotometer at 492 nm against a blank consisting of 0.5ml of distilled water to which all the reagents of the assay have been added.

The sugar content is expressed in mg of glucose equivalent per gram of dry matter (mg GE/g DM), using an external calibration range prepared from a glucose solution under the same conditions as the samples. The sugar yield is also calculated according to the formula:

Total sugar yield (%) = (Ms/Mp) x 100

Ms: mass (g) of the total sugars obtained.

Mp: mass (g) of plant material.

1.3. Extraction of fixed oils

Due to their low polarity, lipids in general are insoluble in polar solvents (such as water) but highly soluble in non-polar or weakly polar solvents such as ether, chloroform, acetone, hexane, etc. Therefore, in this test 20g of flaxseed powder is macerated in 100mL of chloroform under agitation for one hour at room temperature. The mixture is then filtered and evaporated at 40°C in the oven to recover the fixed oils, the yield of which is expressed in ml/100g according to the following formula:

Fixed oil yield (%) = [(V/Mp) x 100]

Mp: mass (g) of the dry plant.

V: volume of fixed oil recovered (ml)

1.4. Extraction and dosage of phenolic compounds

The extraction using one gram of flaxseed powder macerated in 10 ml of 50% ethanol under agitation for one night. The extract is then centrifuged at 5000 rpm for 5min. The supernatant containing the phenolic compounds is recovered and is used for the determination of polyphenols and tannins.

The determination of polyphenols is carried out according to the protocol of Marigo [8], 0.5ml of the diluted extract is added to 3ml of distilled water and 0.5ml of Na_2CO_3 20%. The whole is mixed in the vortex and 0.5ml of Folin-Ciocalteu reagent is added after 3min. The tubes are placed in an oven at 40°C for 30min, and the absorbance is then read at 760nm. The total polyphenol content is expressed in mg equivalent of gallic acid per gram of dry matter (mg GAE/g DM) using a standard range of gallic acid made under the same conditions.

The tannins are dosed according to the protocol described by Ribéreau-Gayon and Stonestreet [9]. A tube containing 3 ml of diluted ethanolic extract and 3 ml of HCl is placed in a water bath at 100°C for 30 min followed by rapid cooling. Then, 0.5 ml of concentrated ethanol is added and the absorbance is read with a spectrophotometer at 550 nm. The content of condensed tannins is expressed in mg/g of dry matter in regard to a control containing the same reagents and carried out under the same conditions but at room temperature, and calculated according to the following relation:

Condensed tannin content (g/l) = (DO1 – DO2) × 19.33

DO1: optical density of the extract.

DO2: optical density of the control.

1.5. Extraction and dosage of flavonoids

The extraction was made from 1g of *Linum usitatissimum* seed powder which is mixed with 90ml of methanol. After agitation, the mixture is heated at reflux for 50 min. Then the solution is filtered and made up to 100ml with methanol (used to determine flavonoids). The rest of this methanolic extract is concentrated under a vacuum at 40°C, and the residue obtained is recovered with 10ml of methanol and kept in the freezer until its use for antioxidant activity.

The determination of flavonoids is carried out using the aluminium trichloride ($AlCl_3$) method [10]. First, to 2ml of methanolic extract, 2 ml of a methanolic solution of $AlCl_3$ (20g/l) are added. After 15 minutes of incubation, the absorbance is

measured at 425 nm against a blank which contains methanol instead of the extract.

The total flavonoid content is expressed in milligrams of quercetin equivalent (as standard solution) per gram of dry matter (mg QE/g DM).

4. Antiradical activity test

The free radical scavenging activity of flaxseed extracts and fixed oils was determined by spectrophotometry, following the procedure described by Chen [11]. First, 2.5ml of different concentrations of the extracts and oils (concentration range 0-400 µg/ml) of flax are added to 2.5ml of a solution of DPPH (100 µM) prepared in methanol. The reaction mixture was agitated immediately and then kept in the dark for 30min at room temperature. The absorbance of the reaction mixture was measured at 517 nm against a blank containing only methanol. The control contain 2.5 ml DPPH and 2.5 ml methanol. The same test was carried out with ascorbic acid as reference antioxidant. The results are expressed as the percentage inhibition of DPPH calculated as a result of the decrease in colour intensity of the mixture according to the equation:

Inhibition of DPPH (%) = (1- (Abs test / Abs control)) x 100

Abs control: Control absorbance at 517nm.

Abs test: Absorbance of the extract at 517nm.

Carrying out the kinetics of this activity makes it possible to determine the concentration corresponding to 50% inhibition of the DPPH radical (IC_{50}).

5. Hemagglutination test

The hemagglutination activity of flax was tested on rat red blood cells. This test, which often involves mammalian erythrocytes, is considered the simplest and most widely used test for the detection and characterization of lectins [12,13].

5.1. Preparation of the total extract

20g of *Linum usitatissimum* plant powder is suspended in a 10% PBS solution (pH 7.4). The mixture is stirred for 2 hours and then centrifuged at 3000 rpm for 15min. The supernatant obtained is then filtered, and the resulting filtrate is the total plant extract, which is finally stored at 4°C until it is used.

1.6. Preparation of red blood cells

Rat blood is collected in a heparinised test tube to prevent coagulation. Three washes of the red blood cells (erythrocytes) by successive centrifugations (15min at 3000rpm) are carried out using physiological water (NaCl 0.9%), the supernatant is discarded, the remaining pellet represents the red blood cells, which are then diluted at a rate of 3% with NaCl 0.9%.

1.7. Phytohemagglutination technique

The phytohemagglutination test is performed on 96-well round-bottomed titration microplates. Thus, 25µl of the total *Linum usitatissimum* extract is deposited in each of the three wells, with 50µl of 0.9% NaCl and 25µl of the 3% erythrocyte suspension. Concanavalin A (con A), a red bean lectin is known for its agglutinating effect, is used as a positive control in this test. The blank (negative control) contains physiological water instead of the plant extract. The plate is then covered and incubated in an oven at 37°C for 30min before the results are read with the naked eye and under the microscope.

A hemagglutination limit test is carried out to determine the minimum concentration of the flax extract that causes the agglutination of the red blood cells. The same protocol mentioned above is used but this time with a range of extract dilutions from 1/5 to 1/2000 times.

6. Statistical analysis

The different dosages were carried out in three repetitions. They were subject to statistical analysis (calculation of means, standard deviation, standard error) to examine the variability between the different extracts and dosages. The data were processed using GraphPad Prism 8 software (Microsoft software). A comparison of the means was made each time there was a significant effect of a factor studied by the ANOVA (Tukey test was performed at the 5% probability level).

Results

1. Phytochemical screening

The quantitative evaluation of the principal primary metabolites of *Linum usitatissimum* seeds is summarised in Table 1. On the basis of the results obtained, it appears that fixed oils, with a density of 0.798 g/ml, represent almost one third (32.5%) of the weight of flaxseed. These oilseeds also contain 73.48 ± 0.93 mg GE/g of total sugars and 44.6 ± 1.32 mg/g of mucilages, marking yields of 7.34% and 4.46% respectively.

The phytochemical screening of *Linum usitatissimum* seeds also focused on quantifying the most sought-after secondary metabolites in medicinal plants. The results are shown in Table 2. Examination of these results reveals that flavonoids represent 1.74 ± 0.57 mg QE/g of plant material of the flax seeds studied. The same flax seeds also contain 1.35 ± 0.03 mg/g of condensed tannins. As for total polyphenols, *Linum usitatissimum* contains an amount of 7.24 ± 0.14 mg GAE/g.

2. Antiradical activity

Tests expressing antioxidant potential can be classified into two groups: tests that focus on the ability to scavenge free radicals, and those testing the ability to inhibit the oxidation of lipids under accelerated conditions. However, the stable free-radical scavenging model is widely used to estimate antioxidant properties in a relatively short time and with high reliability compared to other methods [14]. In our case, the antiradical activity of the methanolic extract and fixed oils of *Linum usitatissimum* was evaluated by the scavenging capacity of the free radical 2,2-diphenyl-1 picrylhydrazyl (DPPH), a stable free radical that can receive hydrogen or an electron from an antioxidant to become a stable molecule.

As shown in figure 2, the flaxseed extract showed a strong anti-free radical effect depending on the concentration compared to fixed oils. To better discuss the results obtained, the concentrations of the test samples required to inhibit 50% of the DPPH (IC_{50}) are calculated and illustrated in Table 3. Taking into account that the IC_{50} is inversely proportional to the antiradical potential, the results thus obtained reveal that the methanolic extract of *Linum usitatissimum* has a higher antiradical activity than fixed oils, with IC_{50} values of 287.40 ± 9.93 µg/ml and 39.70 ± 2.42 mg/ml, respectively. However, these were significantly ($p < 0.001$) lower than that of the reference antioxidant ascorbic acid (IC_{50} = 3.10 ± 0.11 µg/ml).

3. Hemagglutination activity

The evaluation of the immunostimulant potential of *Linum usitatissimum* involved hemagglutination activity. The test used is based on the observation of agglutination or precipitation of the red blood cells due to phytolectins (hemagglutinnins) in order to assert their presence.

3.1. Phytohemagglutination test

In view of the results shown in Figures 3 and 4, the total extract of *Linum usitatissimum* shows a strong agglutination towards the rat red blood cells compared to the negative control. This agglutination was observed both with the naked eye and under the microscope, which proves that this plant contains lectins. The interaction between the lectins and the red blood cells generally occurs when the lectins are deposited in the well containing the red blood cells. The red blood cells will sediment at the bottom of the well as soon as they are deposited, while the lectins will interact with them, forming a homogeneous mass in the form of a gelatinous phase: this is the phenomenon of hemagglutination.

The lectin in the plant attaches itself to the receptor (glycoconjugates) on the surface of the red blood cells, and because it is polyvalent, it forms a network of red blood cells that are deposited in a thin, pinkish layer. This result is observed under the microscope and is called positive hemagglutination (Figure 5). In the absence of lectins, the cells roll to the bottom of the well where they accumulate in a dense red knob: hemagglutination is said to be negative in this case (Figure 6).

3.2. Hemagglutination limit

The determination of the equivalence point, which is the minimum concentration of lectins showing agglutination, is also studied for *Linum usitatissimum*. The hemagglutination limit is expressed as a function of the dilution ratio for which there is obvious hemagglutination, the results of which are shown in Table 4 and Figure 7.

Linum usitatissimum extract showed a strong agglutination towards red blood cells during its dilution at the first four wells, while it decreased at the next two wells and disappeared completely at the remaining wells. The hemagglutination unit (HU) is defined as the dilution factor at which hemagglutination is no longer observed. In our case the agglutination limit was 100 $HU.ml^{-1}$ which corresponds to a concentration of 2 mg/ml of *Linum usitatissimum* extract. In comparison the absence of agglutination at the other wells is due to the dilution performed.

Discussion

1. Phytochemical characterisation

The phytochemical characterisation of *Linum usitatissimum* seeds targeted the main primary and secondary metabolites of this plant. According to the results obtained, fixed oils represent 32.5% of these oilseeds, a result close to that of Diederichsen and Raney [15], in which they found a content of 38.3% for brown seeded flax considering the variability of extraction conditions. Flaxseed studied contains also 7.34% of total sugars. The work of Wanasundara showed a slightly higher sugar content of 7.69 % in flax. Most of the sugars of this plant constitute dietary fibre with a content varying between 20 and 25% [16]. Mucilages, which are multifunctional water-soluble polysaccharides with several pharmacological activities [17], depend during their extraction on several conditions such as temperature and extraction time [18]. This explains the variability in mucilage content between our results (4.46%) and those of Fedeniuk and Biliaderis in which they reported a lower yield of around 3.6 % [19].

As to secondary metabolites, flaxseed contains 1.74 ± 0.57 mg QE/g of flavonoids. This result is better than that found by Oomah ranging from 0.35 to 0.71 mg/g in Canadian flaxseed [20]. Anwar and Przybylski found flavonoid contents in ethanolic and methanolic extracts of flax between 1.9 and 4.8mg CE/g using catechin as standard [21]. We also recorded a condensed tannin content of 1.35 ± 0.03 mg/g in the flax seeds studied, a result close to that found by Wanasundara which was 1.36 mg/g of plant material. The same flax seeds contain 7.24 ± 0.14 mg GAE/g of total polyphenols. This result is higher than that found by Velioglu (5 mg GAE/g) in the hydro-methanolic extract of flaxseed [22], whereas 3 mg GAE/g of polyphenols are found by Anwar and Przybylski when 80% aqueous ethanol is used [21]. These different variations observed for the total phenolic content could be attributed to the different flaxseed varieties or the extraction technique used [23]. It is now widely accepted that phenolic compounds in general attract a lot of public and scientific interest due to their multiple biological activities. However, their content in a plant depends on a number of intrinsic and extrinsic factors such as geographical origin, climatic conditions, cultural practices, maturity at harvest and storage conditions [24].

2. Antiradical activity

In our study, the DPPH inhibition test is used to evaluate the antioxidant activity of *Linum usitatissimum* extract and fixed oils. By marking an IC_{50} of 287.40 ± 9.93 µg/ml, flaxseed extract generates a significant antiradical effect compared to that of fixed oils with a higher IC_{50} (39.70 ± 2.42 mg/ml). Some works on *Linum usitatissimum* seeds showed a significant antiradical activity of the methanolic extract of flax with up to 83.6 % inhibition of DPPH [21]. In the same sense, studies conducted by Bensaci on the fixed oil of *Pistacia lentiscus* revealed a less important antiradical activity, with an IC_{50} of up to 304.37 mg/ml.

In fact, the marked antioxidant potential in this plant is probably explained by its richness in free radical scavenging compounds, mainly polyphenols. In this regard, a major lignan called secoisolariciresinol diglycoside (SDG) present in flaxseed seems to be responsible for the high antioxidant activity of the plant [25]. In general and according to the bibliography, the anti-free radical effect becomes more important as the concentration of polyphenols in the extract increases. Anwar and Przybylski have shown that there is a significant correlation between the anti-radical activity against DPPH radicals and the content of polyphenols (R^2 = 0.7078) and flavonoids (R^2 = 0.8633) in methanolic and ethanolic extracts of *Linum usitatissimum* [21]. Several works have reported, in part, that antiradical activity is dependent on the number, position and nature of

substituents on the B and C rings (hydroxyl, metaxyl, glycosylated groups) as well as the degree of polymerisation of polyphenols [26]. According to recent study, the hydrolysis of alimentary proteins using enzymes generates certain peptides with antioxidant properties. This has been demonstrated in particular on proteins contained in oilseeds such as flaxseed [27], which should be taken into account in the biological activity. On the other hand, and due to their low antioxidant activity, the fixed oils oxidise easily after extraction from the flaxseed because they lose their protective antioxidants, which are mainly hydrophilic and do not accompany the extracted oils [28].

3. Hemagglutination activity

The hemagglutination potential of native rat red blood cells by *Linum usitatissimum* lectins being positive through this test, it thus demonstrates a good immunostimulant effect of this plant. Our results are in agreement with those of lectins extracted from the roots of plants such as *Moringa G* and *Moringa M*, which showed very strong agglutination when adding the rabbit red blood cell suspension [29]. The hemagglutination limit, which characterises the lowest concentration of the extract causing agglutination, was in our case in the order of 100 $HU.ml^{-1}$ which corresponds to a concentration of 2 mg/ml of *Linum usitatissimum* extract. *Terfezia bouderei* has shown strong agglutination up to a dilution of 128 $HU.ml^{-1}$ [30].

Lectins, as proteins of non-immune origin that bind to carbohydrates, are involved in various biological processes, including cell recognition, cell proliferation, cell migration, cell adhesion to the extracellular matrix and host-parasite interactions [31–33], and since the 1960s, plant lectins have been widely used as valuable tools in biomedical research. Most lectins of higher plants are located in the seed: they are formed during maturation and disappear during germination. They are especially common in Fabaceae such as peanuts, soybeans, lentils, canavalia, beans... [34].

Conclusion

From an ecological and pharmacological perspective, the present study was carried out in the sense of the evaluation and valorisation of an aromatic and medicinal plant belonging to the Moroccan terroir products, and which is widely used in traditional medicine in this country, it is *Linum usitatissimum*. This through the phytochemical characterization of this plant and the evaluation of its antioxidant and immunostimulant potential. The results obtained testify to flax's richness in active principles, particularly mucilages with laxative and emollient effects and fixed oils rich in omega-3, as well as phenolic compounds that constitute strong antioxidant agents that give this plant an antiradical potential against free radicals. Furthermore, the hemagglutination test highlighted the richness of *Linum usitatissimum* in lectins, which attribute to this plant an immunostimulant effect in favour of the immune system. All these results lead us to confirm that *Linum usitatissimum* is a plant with many virtues which can find application in phytotherapy and the food industry. It therefore deserves to be valued and protected in green chemistry and sustainable development approach. In perspective, this work must be completed by other studies concerning the identification of bioactive molecules, as well as other biological activities that enhance the value of this plant further.

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Table 1. Primary metabolite content (mg/g plant material) of Linum usitatissimum seeds (mean values ± SE).

	Primary metabolite content (mg/g)	Yield (%)
Mucilages	44.6 ± 1.32	4.46 %
Total sugars (mg GE/g)	73.48 ± 0.93	7.34 %
Fixed oils	259.50 ± 8.82	32.5 %

Table 2. Secondary metabolite content (mg/g plant material) of Linum usitatissimum seeds (mean values ± SE).

	Secondary metabolite content (mg/g)
Flavonoids (mg QE/g)	1.74 ± 0.57
Condensed tannins	1.35 ± 0.03
Total polyphenols (mg GAE/g)	7.24 ± 0.14

Table 3. IC50 values of ascorbic acid, extract and fixed oils of Linum usitatissimum (mean values ± SE).

	IC_{50} value
Ascorbic acid	3.10^{a} ± 0.11 µg/ml
Methanolic extract of *L. usitatissimum*	287.40^{b} ± 9.93 µg/ml
Fixed oils of *L. usitatissimum*	39.70^{c} ± 2.42 mg/ml

Values with different letters are significantly different (P<0.05).

Table 4. Hemagglutination limit of rat red blood cells by Linum usitatissimum extract.

	Dilutions of *Linum usitatissimum* extract									
	1/1	**1/5**	**1/10**	**1/20**	**1/50**	**1/100**	**1/200**	**1/400**	**1/1000**	**1/2000**
Hemagglutination	++	++	++	++	+	+	-	-	-	-

(++ : Strong agglutination; + : Weak agglutination; - : Absence of agglutination).

Figure 1. *Linum usitatissimum* seeds

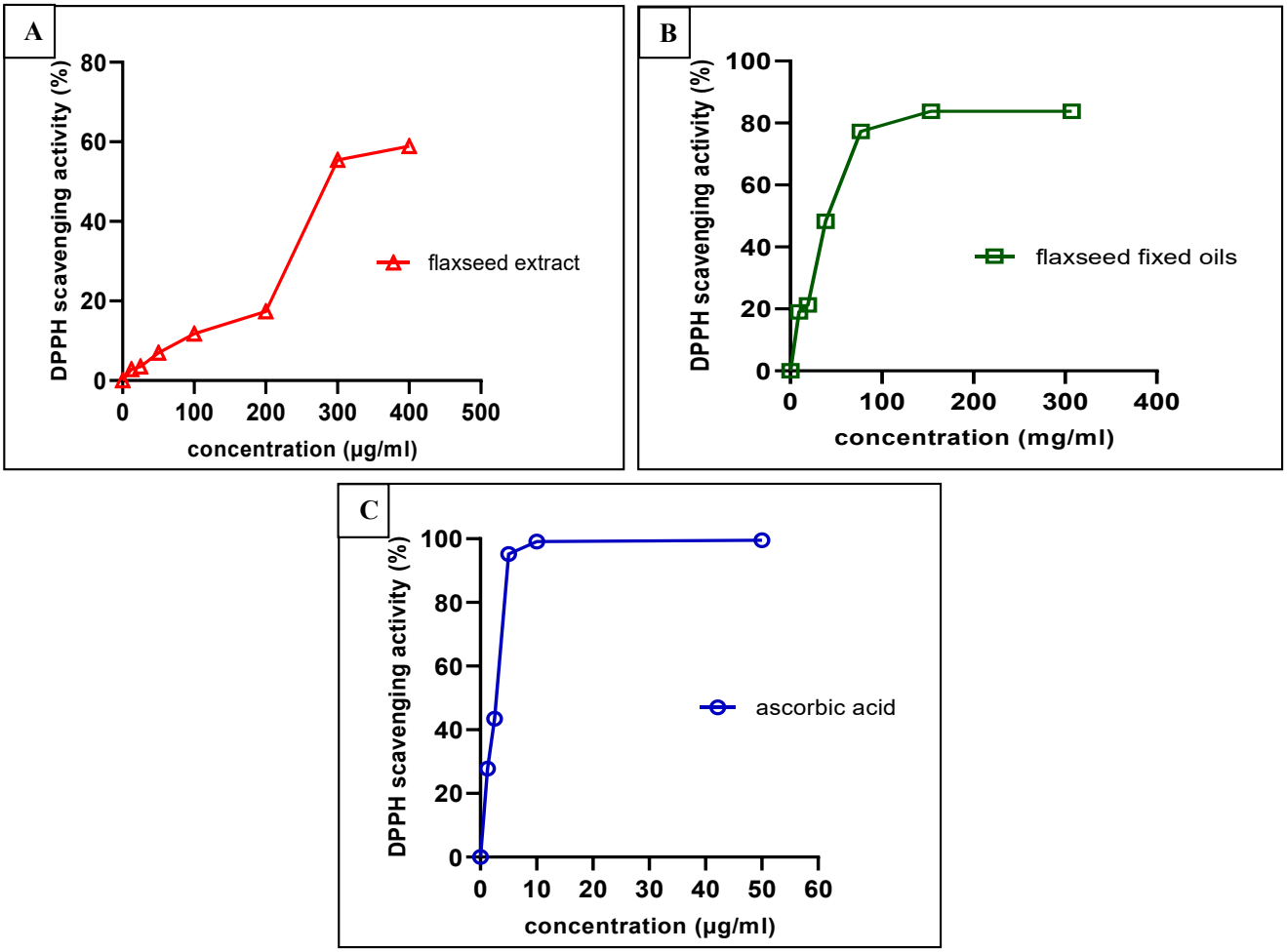

Figure 2. Scavenging activity of the free radical DPPH by ascorbic (C) acid and by the extract (A) and fixed oils (B) of *Linum usitatissimum*.

Figure 3. Hemagglutination test of rat red blood cells using *Linum usitatissimum* extract (Observation with the naked eye).

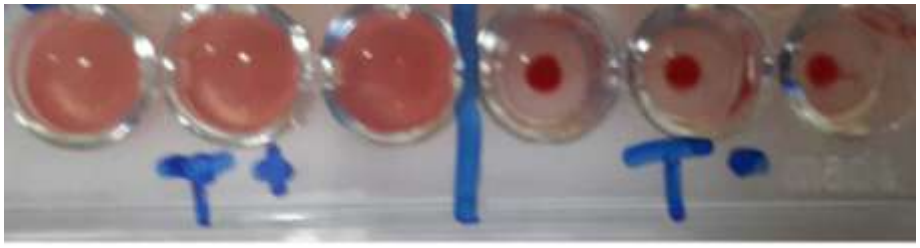

Figure 4. Positive control by Concanavalin A (T+) and negative control (T-) of the rat red blood cell hemagglutination test (Observation with the naked eye).

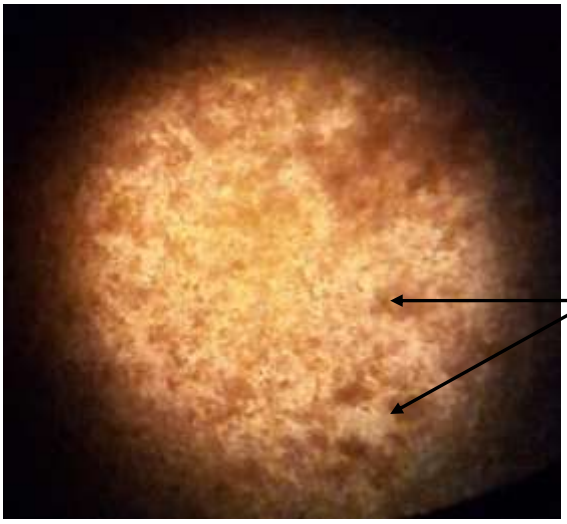

Figure 5. Microscopic observation of hemagglutination of rat red blood cells by *Linum usitatissimum* extract (magnification x 40).

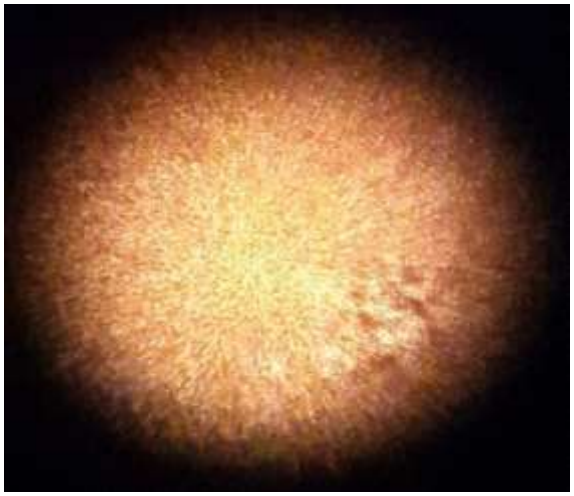

Figure 6. Microscopic observation of the negative control (T^-) of the rat red blood cell hemagglutination test (magnification x 40).

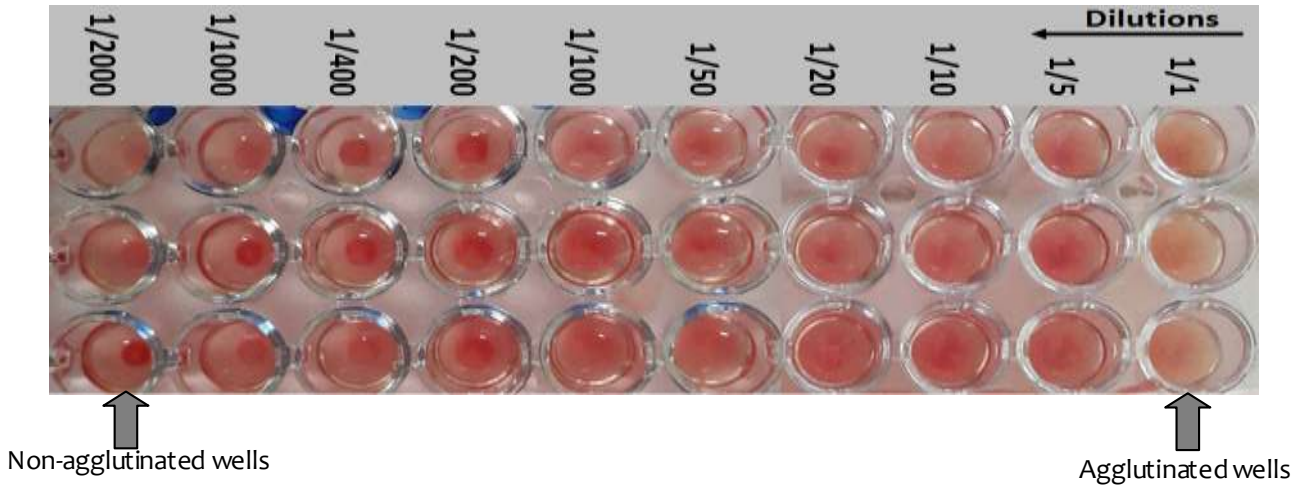

Figure 7. Limit of agglutination test of rat red blood cells by *Linum usitatissimum* extract (Observation with the naked eye).