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*Research Article*

# Essential phytochemicals determination and their antioxidant activities and oil properties of coat variety Linseed extract oils

Adem Ali Muhabie[1,*], Asnakew Amare Tadesse[1]

[1]*Woldia University, Faculty of Natural and Computational Sciences, Department of Chemistry, Woldia, Ethiopia*

*Corresponding author: alimohabe2003@gmail.com

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**Abstract**

Linseed (*Linumusitatissimum* L.) oils consumption exhibits potential health benefits that include reducing coronary heart disease, cancer, neurological disease, diabetes, and hormonal disorders. Phytochemical screening revealed the presence or absences of secondary metabolites such as phenolics, flavonoids, lignin, fatty acids, saponins, Coumarins, tannis and alkaloids in the extract of linseeds. The quality of extracted oils of white and brown linseed were evaluated by analyzing the physico-chemical properties (acid, peroxide, free fatty acids, and saponification values) using titration methods. The oil extracted from brown revealed high quality than white linseed extracts. The total phenolic contents (TPC) and total flavonoids content (TFC) of linseed extract oils were analyzed by UV-visible spectrophotometer. Total content of flavonoids found in white and brown linseed extract were 33.407±3.63 and 34.889 ± 23.782 mg QEQ/100 g of samples, respectively. The total phenolics content for the brown and white linseed extracts were 53.681±3.362 and 52.984 ±8.685 mg TAEQ/100 g of sample, respectively. The brown linseed extract oil revealed high content of phenolic and flavonoid compounds as compared relative to the white linseed extracts, indicating brown linseed oil extract was responsible for the higher antioxidant activity. The total antioxidant capabilities of linseed extract oils were studied by using Phosphomolybdenum assay and ferric reducing assay power (FRAP). From Phosphomolybdenum method, total antioxidant capacity of Brown and white linseed extract oils were 196.138±0.656 and 188.825±2.031 %, respectively. From ferric reducing assays, power (FRAP), the total antioxidant capacity or reducing power of brown and white linseed extract oils exhibited were 69.104 and 63.57 % respectively. The commercial development of plants as sources of antioxidants to enhance health and food preservation is of current interest.

**Keywords:** antioxidant, phytochemicals, linseed extract, oils, reducing power

## Introduction

Antioxidants are compounds that help to prevent various oxidation reactions caused by free radicals such as superoxide, singlet oxygen, hydroxyl radicals, peroxyl radicals and peroxynitrite and thereby promoting destruction to the cells and tissues.[1-4] Antioxidants limit the free radical damage from Oxidizing cholesterol, which can proliferation the risk of atherosclerosis, damaging the cell's DNA, which may lead to cancer, initiating inflammation and Impairing immune function, promoting platelet adhesion, which can increasing the risk of heart disease or stroke, blocking the normal endothelial cell function and vasodilatation.[5] Antioxidants are the organic compounds that prevent the very reactive intermediate, in which free radicals from oxidizing bioorganic molecules in the body. The possible action of the mechanism of antioxidants are chelators of pro-oxidant metals, such as quenchers of singlet oxygen, free radical scavengers, and reducing agents, thereby reducing the oxidative damage of cellular biomolecules such as proteins, lipids, and nucleic acids.[6-8] Antioxidants could be categorized as synthetic which are most efficient antioxidants, approved by Food and Drug Administration for addition to foods whereas natural antioxidants are present in foods, plants, seeds and fruits and has been reported as being protective against propagation of free radical that cause for certain types of cancer, risk of cardiovascular and cerebrovascular.[9-11] Synthetic antioxidants such as butylated hydroxytoluene (BHT), tertbutylhydroquinone (TBHQ) and butylated hydroxyanisole (BHA) have been used as an antioxidant in foods for years.[12-14] However, physical properties of synthetic antioxidants such as their high instability and volatility at elevated temperatures, side effects and strict legislation on the use of synthetic food

additives.[15] Consumers are gradually evading foods prepared by adding preservatives of chemical origin, and natural alternatives are therefore needed to achieve sufficiently a long shelf life of foods and a high degree of safety.[16] Therefore, plants have been coming as sources of antioxidants to care health and food preservation is of present interest compared to synthetic antioxidants.[17] Various seed oils contain potent antioxidant activity and as retard lipid oxidative rancidity in oils.

Linseeds are among the seed oils and their oils are known to possess many bioactive and health-keeping phytochemicals. However, little information is currently available on the physico-chemical properties and antioxidant activity of the bioactive phytochemicals present in linseed oils.[18] Epidemiological information shows that the consumption of linseed may exert several cardioprotective effects, which are speculated to arise from their phytochemicals that includes phenolic, unsaturated fatty acids, flavonoids, phytosterols and tocophenols.[19, 20] These antioxidants constituents can act as pro-oxidants such as free fatty acids and hydroperoxides, tocopherols, tocopherols and carotenoids, phenols, and possibly phospholipids are important antioxidants and improve oil stability and thus oils naturally rich in these constituents are preferred.[21] However, during refining the oil the crucial phytochemicals are partially removed during the process, bleaching and deodorization that most commercial vegetable oils undergo.[22]

In this study, we have developed new extraction method that appropriate solvents, equal ratio of non-polar (petroleum ether) and polar solvent (ethanol) prepared to extract almost all types of phytochemicals. Much of the existing literature attributes the beneficial health effects of linseed and linseed oils to their nutritional value. However, very little research has been conducted on the qualitative and quantity compositions of phytochemicals and the activities of their components. As far as we know, no research has been conducted on the effect of color change of seeds on phytochemicals, physic-chemical properties, and antioxidant activities of brown and white oil extracts. A further objective of this work was to compare the brown and white colored linseed extract oils with an equal ratio of petroleum ether and ethanol components present. this work aimed to screen and determine phytochemicals of extracts of linseed oils, including analysis of phenolics, flavonoids, lignin, fatty acids, saponins, coumarins, tannis, alkaloids, tocopherols, sterols and stanols, as well as to examine the antioxidant activity of oils using a number of in vitro assays. Eventually, the quality of the extracted oils was evaluated by analyzing acid, peroxide, free fatty acids, and saponification values using titration methods.

## Materials and Methods

### *Chemicals*

Analytical grade chemicals and reagents were used in the analysis of the linseeds. potassium hexacyanoferrate$K_3[Fe(CN)_6]$, trichloroacetic acid ($CCl_3COOH$), ferric chloride ($FeCl_3$), ascorbic acid, hydrochloric acid, sodium hydroxide, sodium phosphate dibasic($Na_2HPO_4$), and sodium phosphate monobasic dihydrate ($NaH_2PO_4.2H_2O$), sulfuric acid, ammonium molybdate. sodiumthioshulphate, sulfuric acid, sodium phosphate, $AlCl_3$, ascorbic acid, trichloroacetic acid, $NaNO_2$, NaOH, $NaCO_3$, KOH and HCl were collected from chemical store.

### *Samples preparation*

### *Oil Extraction – Solvent Stripping Techniques*

A 200 mg sample was crushed in a mixer grinder to obtain a fine powder. The 100 mg of powder was blended with an equal mixture of organic solvents such as petroleum ether/ethanol. The oily extracted layer was filtered using Buchner funnel and the filtrate was transferred to flasks, the oils were heated over a water bath for 12 h until the solvents and impurities completely evaporated to obtain pure extract. Extracted seed oil was stored in freezer at 4°C for subsequent experimental analysis.

### *Characterization methods*

Ultraviolet-visible spectrophotometry (SANYO SP65UV/Vis. U.K) was used to analyze the total content of phenols/flavonoids and antioxidant activities of the oil extracts. The titration method was applied to determine the physicochemical properties of *Linumusitatissimum L*. Digital analytical balance (Ohaus, Switzerland) was used to weight the fruit samples and solid chemicals. Electronic blending device (Mixer

grinder, india) and centrifuge were used for grinding the seed and clarifying the supernatant, respectively. The pH meter of the solution was measured with a digital pH meter (Hanna model 3310, Italy). Refrigerator and oven was used throughout the experiment.

***Experimental procedures***

***Physicochemical properties of Linum usitatissimum L.***

The quality of extracted oils of white and brown linseed were evaluated by analyzing the physico-chemical properties (acid, peroxide, free fatty acids, and saponification values) using titration methods.[23]

**Acid value:** Each oil sample (1.0 g) was weighed and dissolved with 50 ml of petroleumether/ethanol in a conical flask. Two drops of phenolphthalein indicator were added and titrated to the pink end point (which persisted for 15 minutes) with 0.1 N potassium hydroxide solution (KOH). Acid value was calculated using ***Equation 1***.

$$\text{acid value} = \frac{56.1 \text{x VxC}}{\text{m}} \quad \text{.......................................................} 1$$

Where 56.1 is the equivalent weight of KOH, V is the volume in ml of the standard volumetric KOH solution, C is the exact concentration of KOH solution (0.1 N); m is the mass in grams of the test portion (1 g). Percentage free fatty acid (% FFA) (oleic) was determined by multiplying the acid value with the factor 0.503. Thus: % FFA = 0.503 x acid value.

**Saponification value:-**Saponification values of the oil samples were determined as described below: 0.5 g of each oil was dissolved in 12.5ml of 0.5% ethanolic KOH and the mixture refluxed for 30 minutes. 1ml of phenolphthalein indicator was added and the hot soap solution titrated with 0.5 N HCl to the end point until the pink colour of the indicator just disappeared. A blank determination was also carried out under the same condition and saponification value determined using ***equation 2.***

$$\text{Saponification value} = \frac{56.1 C (V_1 - V_2)}{m} \quad \text{.......................................} 2$$

Where 56.1 is the equivalent weight of KOH, $V_1$ is the volume in ml of standard HCl solution used for the blank test, $V_2$ is the volume in ml of the standard HCl solution used for the sample, C is the exact concentration of the standard HCl (0.5 N) solution and m is the mass in gram of the test portion

**Peroxide value:-**Oil sample (1 g) was added into a boiling tube containing 1 g powered potassium iodide. Glacial acetic acid/chloroform mixture (20 ml; 2:1) was added, the boiling tube was placed in boiling water for 1 min after which its content was poured into a conical flasks containing potassium iodide solution (20 ml; 5%). The boiling tube was rinsed twice with distilled water (25 ml) and the content added into the conical flask. The whole content was titrated with sodium thiosulphate (0.002N ) solution to a colorless end point using starch as indicator. Results are expressed as mMol/kg. Peroxide value of the oil sample was calculated by using ***equation 3***:

$$\text{peroxide value} = \frac{10 \text{x} (\text{V}_1 - \text{V}_2)}{\text{m}} \quad \text{................................................} 3$$

***Determination of total contents, flavonoids and phenols in linseed oil extracts***

**Total flavonoid content**:-Total flavonoid content of the extracts was also measured according to the method described below. A diluted crude extract (2 ml) was added to 4 ml distilled water. 0.3 ml of (5% w/v) $NaNO_2$ was added. Then 0.3 ml of (10% w/v) $AlCl_3$ was added. Finally, 2 ml of 1 M solution of NaOH was added. The mixture was shaken vigorously and the absorbance of the mixture was read at 510 nm. A calibration curve was prepared using a standard solution of quercetin. Results were expressed on a fresh weight basis as mg quercetin equivalents (QEQ)/100 g sample.

**Total phenolic content:-** The total phenolic content was determined using Folin-denis method with some modifications. 1 ml of linseed oil extract was added to 1 ml Folin-denis reagent, followed by the addition of 1 ml of 7% sodium carbonate solution. The absorbance was measured at 760 nm using a UV-Vis Spectrophotometer (UV-1650 PC Spectrophotometer, Shimadzu, Japan). The amount of TPC was expressed as miligram of tannic acid equivalents (TAE) per 100g of fresh weight of sample.

***Anti-oxidant reducing power of linseeds extract oils***

**Ferric reducing power: -** a spectrophotometric method was used for the measurement of reducing power. Different concentrations of samples, 20, 30, 40, & 50 % v/v solutions were prepared from stock solution. 2ml of from each of the extracts were measured and mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1 % potassium ferric cyanide (10 mg/ml). The mixture was incubated at 50 ºC for 10 min, then rapidly cooled, mixed with 2.5 ml of 10 % trichloroacetic acid, and centrifuged at 3000 rpm for 10 min. An aliquot (2.5 ml) of the supernatant was separated and diluted with distilled water (2.5 ml) and then ferric chloride (0.5 ml, 0.1 %) was added and allowed to place for 10 min. The absorbance of each sample was recorded spectrophotometrically at 700 nm. The antioxidant capacity of each sample was expressed as ascorbic acid (A.A) equivalent using the linear equation established using ascorbic acid as standard.

**Phosphomolybdenum reducing power: -** The total antioxidant activity of the crude extract was evaluated by the phosphomolybdenum method according to the procedure described by Prieto et al with little modification. a 1ml samples were added to 4 ml of reagent solution (28 mM sodium phosphate, 0.6 M sulfuric acid, and 4 mM ammonium molybdate). The tubes containing the reaction solution were plugged and incubated in a boiling water bath at 95°C for 90 min at acidic condition. The assay is based on the reduction of molybdenum, Mo (VI) reduced to Mo (V) by the samples and the consequent formation of a green phosphate/Mo (V) complex at acidic pH. After cooling to room temperature, the absorbances of the solutions were measured at 695 nm using a UV-visible spectrophotometry. Those equivalent antioxidant capacities, TFC and TPC were expressed as mg of the standard per 100 g of sample.[30] the antioxidant capacity, total flavonoids/phenolic contents were calculated using the ***equation 4***

$$\text{mg standard equivalent per 100 g sample} = \frac{(\text{activity})(\text{dilution factor})(V_{\text{extract(ml)}})100}{g_{\text{sample}}} \quad \ldots\ldots\ldots\ldots 4$$

Where mg standard equivalent per 100 g sample = Values of FRAP, total antioxidant assay, TFC and TPC

$g_s$= initial weight of sample in grams.

Dilution factor = final volume/aliquot volume

$\text{Activity} = \frac{y\text{-}c}{a}$, y = absorbance of the sample, c = y-intercept, a = slope

The percentage reduced of the sample was calculated using **equation 5**.

$$\text{percentage of reducing power} = [1\text{-}(1\text{-}\frac{A_E}{A_S})]\,x\,100 \quad \ldots\ldots\ldots\ldots 5$$

Where: As = absorbance of the standard at maximum concentration tested.

$A_E$ = absorbance of seed extract at maximum concentration tested

***Statistical analysis***

All research experiments were conducted in triplicate; origin statistical software were used to evaluate the significance of differences among different mean values and sketch the graph, respectively.

**Results and Discussion**

***Qualitative determination of phytochemical chemicals of linseed oils extract***

The secondary metabolites in brown and white linseed extract oils were qualitatively determined by their corresponding phytochemical tests as shown in Table 1. The Secondary metabolites are biological important compounds such as phenols, flavonoids, fatty acids, alkaloids, tannins, and lignans, tocopherols, sterols, stanols, carotinoids were confirmed present in brown linseed oil by phytochemical test but saponisns and coumarins were not existed.[22]

***Table 1.*** *Phytochemicals confirmed tested from linseed extract oils.*

| No. | Pytochemicals | Brown Linseed Oil | White Linseed Oil |
|---|---|---|---|
| 1 | Phenol | + | + |
| 2 | Flavonoids | + | + |
| 3 | Fatty Acid | + | + |
| 4 | Alkaloids | + | + |
| 5 | Tannins | + | + |
| 6 | Saponins | - | - |
| 7 | Coumarins | - | - |
| 8. | Lignins | + | + |
| 9. | Tocopherols | + | - |
| 11. | Sterols | + | + |
| 12. | Stanols, | + | - |
| 13. | Carotinoids | + | - |

- = Negative (absent); + = Positive (present)

In the white linseed oil, phenols, flavonoids, fatty acids, alkaloids, lignins, tannins, lignans, and Sterols are present in the sample. Others phytochemicals such as tocopherols, stanols, carotinoids, saponins, and coumarins are not exist in all samples. This difference comes due to the difference of composition components within the two samples. The brown linseed is very rich with more phytochemicals and leads to high quality oils and the best antioxidant activity performance than the white colored linseed. The phytochemical evaluation of the samples in this study is in line with previous work. [24, 25]

***Physico-Chemical Behavior of Oils Extracted From Linseeds***

Linseed oils were extracted from samples with solvent extraction techniques by a mixture of organic solvents, which are the appropriate solvents to absorb the non-polar and polar compounds. The produced oil displayed a yellow color, good viscosity, and mosturity behavior.[26] The quality of extracted oils depends on the accuracy of the measured acid, peroxide, free fatty acids, and saponification values compared to standard values. Figure 1 shows the physico chemical properties of both linseed oil extracts. The peroxide, free fatty acids and saponification and acid values of white linseed oil are 11.00 ± 0.07, 1.846 ± 0.016, 367.83 ± 0.06 and 3.36 ± 0.06 respectively, whereas the peroxide, free fatty acids and saponification and acids values of brown linseed oil are 8.17± 0.08, 1.32 ± 0.02, 293.59 ± 0.08 and 2.62 ± 0.03 respectively. The physio-chemical properties, which are observed in this research, are almost similar to the previous works reported.[27-29]

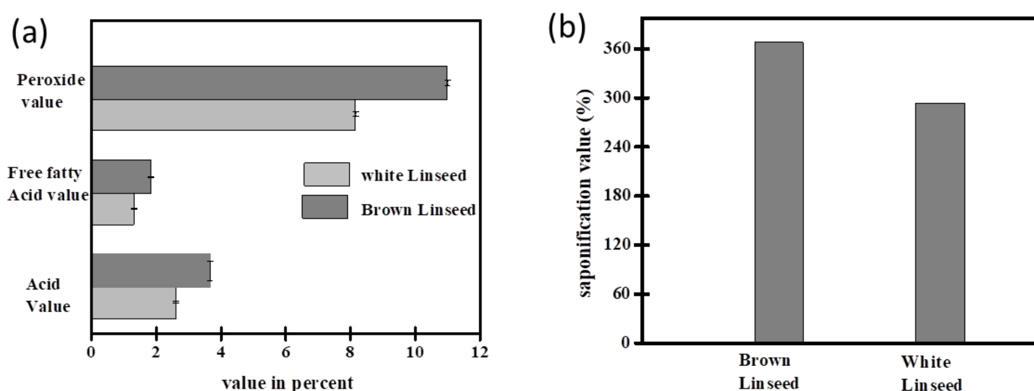


***Figure 1.*** *(a) peroxide, free fatty acids, and acid value, (b) saponification values of value oils*

***Quantitative Determination of Selected Phytochemicals***

UV- visible spectrometry was applied to assess the total content of some selected secondary such as flavonoids and phenol in linseed extracted oils. From figure 2, it can be seen that the Total flavonoid content found in white and brown linseed are 33.407±3.63mg and 34.889 ± 23.782 mg(QEQ)/ 100 g of samples respectively. Brown linseed extract shows slightly high amount of flavonoid compounds than the white linseed and the same as previously reported by Oomah et A.[37]. The total phenolics content in the brown and white linseed extracts are 53.681±3.362mg and 52.984 ±8.685 mg TEQ/ 100 g of sample, respectively. The brown linseed extracts has high content of phenolic compounds relative to the white linseed extracts. The higher the total phenolic and flavonoid content of brown samples, the higher the antioxidant activity. The phytochemical of linseed oil has been studied by a number of research groups who have reported values more likely similar to the present work. [24], [30] The relatively strong antioxidant activities of linseed oil extracts may be due to their high content of the possible phytochemical.[31, 32]

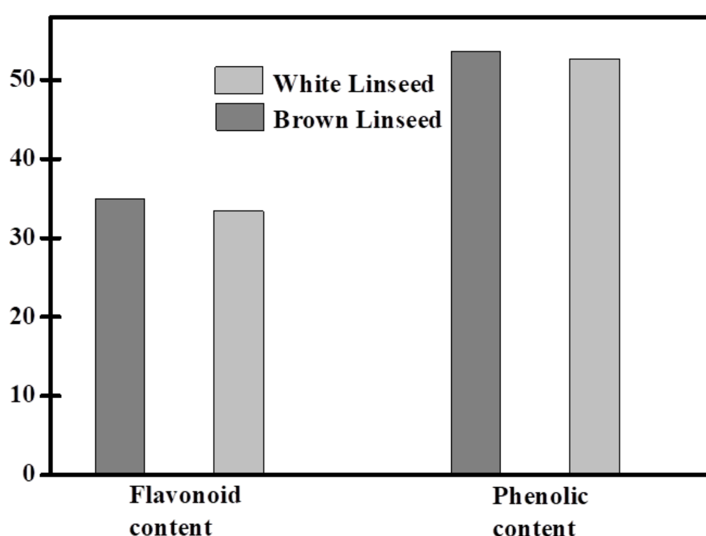


***Figure 2****. The Total flavonoid and phenolic Content of linseeds oils*

***Antioxidant activity of linseed extract oils***

The antioxidant activity of secondary metabolite compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, decomposing peroxides, quenching singlet and triplet oxygen.[36] these methods involve two components in the reaction mixture, antioxidants and oxidant. They are based on the following electron transfer reaction: oxidant + e (from antioxidant) → reduced + oxidized antioxidant. [33]

**Ferric reducing assay: -** The reducing power was expressed as a percentage value using ascorbic acid as standard. From the measurement of the reductive ability, it has been found that the $Fe^{3+}$ to $Fe^{2+}$ transformation occurred in the presence of antioxidant capacity of linseed extract oils. The total antioxidant capacities or reducing powers of the brown and white linseed aqueous extract are 69.104 % and 63.57 %, respectively. These results are in line with previous work.[34, 35] Therefore, the brown seed extract displayed a higher reducing power compared to the white linseed extract oil. The reducing ability of the extract served as a significant indicator of its potential antioxidant activity. [36, 37]

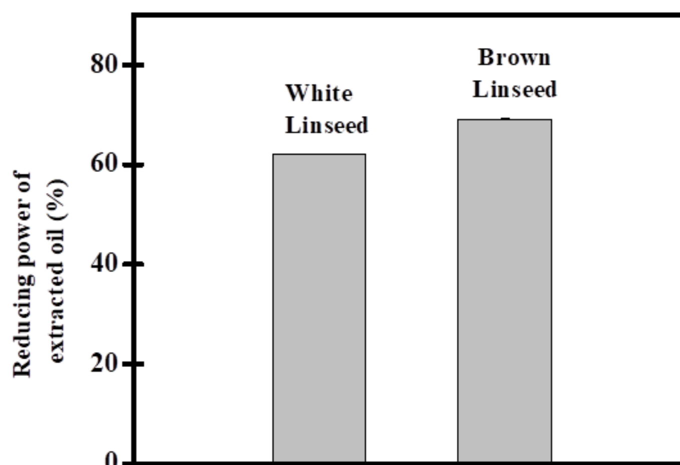


***Figure 3.** Anti-oxidant activity of linseed oils by Ferric reducing assay*

**Phosphomolybdenum method:-** figure 3 shows the antioxidant activities of extracted oils against the reduction of molybdenum. The total antioxidant capacities of the brown and white linseed aqueous extract were 196.138±0.065 and 188.825±0.031 %, respectively. These values are inconsistent with the previously reported works.[38, 39] the antioxidant capacity of white linseed extract was found to be lower than brown linseed. While incubation of the solution of extracted oils and phoshomolbdnum reagents, the formation of green phosphate Mo (V) complex was observed.[40] This indicates the reduction capacity of oils. In the present study, the Phosphomolybdenum antioxidant method was consistent with the ferric reducing antioxidant power.[41] The results indicate that under linseed extract were found to be a powerful antioxidant. This is due to the presence in the content of secondary metabolites such as phenols, flavonoids, tannins, and vitamin E compound estimation etc. The secondary metabolite compounds were associated with antioxidant activity and play an important role in stabilizing lipid peroxidation and radical scavenging ability due to their hydroxyl groups. [42] [43, 44]

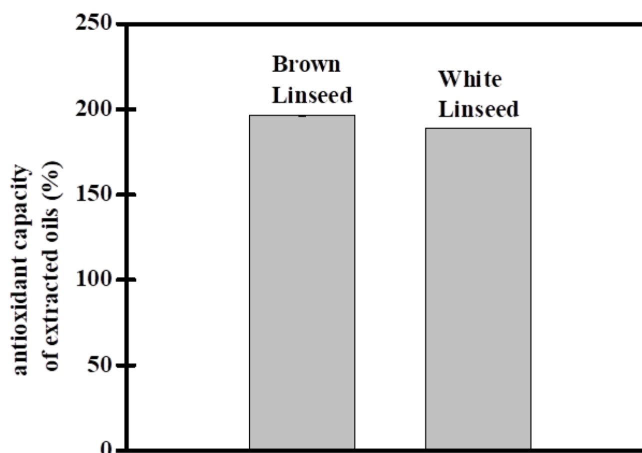


***Figure 4**: Antioxidant activity of linseed oils by Phosphomolybdenum method.*

***Conclusions***

The antioxidative components of linseed oils were extracted using a solvent stripping extraction technique. Linseed oils extract contained phenolics, flavonoids, lignins, fatty acids, saponins, coumarins, tannins, and alkaloids. The petroleum ether/ethanol extracted oils had higher amounts of phenolic and flavonoid compounds than their hexane extracted counterparts. The antioxidant activities of linseed oils phytochemical extracts were assessed using the Ferric reducing assay and Phosphomolybdenum method. Results of these studies demonstrated that the extracts of petroleum ether/ethanol extracted oils of brown linseed oil possessed higher antioxidant activities than ex-extracted counterparts. The present's results confirmed that linseed extract oils are rich in phytochemicals and highly valuable source of natural antioxidants and free radical scavengers.

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***Supplementary Materials***

0.025 g of ascorbic acid was dissolved in 100 ml of volumetric flask, 2, 8, 14, and 20 ppm were prepared from stock solution by taking 0.4, 1.6, 2.8 and 4 ml of stock solution to 50 ml of volumetric flask respectively, the same procedure was done for quercetin and tannic acid standards. The absorbances were measured by UV/vis spectrophotometer for three standards of different concentrations. The calibration curves for all standards were plotted as absorbance versus concentration. The equations with linear regression coefficient of curves for ascorbic acid (figure S1), quercetin (figure S2), and tannic acid (figure S3) were $Y = 0.0231X + 0.0108$ with ($R=0.998$), $Y = 0.0179X - 0.0088$ with $R= 0.997$ and $Y= 0.058X + 0.0242$ with $R=0.999$, respectively.

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