Effect of planting date and plant density in the bio-activity for the leaves and seeds of Flax plant (*Linum usitatissimum* L.)

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ABSTRACT

Two field experiments were conducted in the field of medicinal plants belonging to College of Agriculture, the University of Basra during the agricultural seasons (2015/2016 and 2016/2017) with clay loam soil. The study included six treatments which are the combination between two planting dates (1/11 and 1/12), the cultivating with three plant densities (20, 30, 40 kg.ha^{-1}) and their interactions in the activity of methanolic extract for the leaves and seeds of the flax plant (*Linum usitatissimum* L.) as Anti-inflammatory and antioxidant. The highest anti-inflammatory effect in vitro for the methanolic extract for the leaves and cultivated seeds of the plant at 1/11 with a plant density of (20 kg.ha^{-1}), which amounted of (97.80, 94.78, 98.0, 96.3), respectively, and anti-oxidant activity amounted to (98.04, 95.67, 66.58, 55.11%), respectively. Treatment with Flavonoids for the leaves or seeds of flax plant had an anti-inflammatory effect in vivo that was close to the activity of Diclofenac drug.

تأثير موعد الزراعة و الكثافة النباتية في الفعالية الحيوية لأوراق و بذور نبات الكتان *Linum usitatissimum* L.

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الخلاصة

نفذت تجربتين حقليتين في حقل النباتات الطبية التابع لكلية الزراعة/جامعة البصرة أثناء الموسمين الزراعيين 2015/ 2016 و 2016/ 2017 بتربة مزيجية طينية، إذ تضمنتا ست معاملات عامليه وهي عبارة عن التوافيق بين موعدي زراعة 11/1 و 12/1 والزراعة بثلاث كثافات نباتية 20 و 30 و 40 كغم. هكتار$^{-1}$ وتداخلاهما في فعالية المستخلص الميثانولي لأوراق وبذور نبات الكتان *Linum usitatissimum* L. المضادة للالتهابات والمضادة للأكسدة. إن أعلى فعالية مضادة للالتهابات خارج الجسم الحي للمستخلص الميثانولي لأوراق وبذور النباتات المزروعة في 11/1 بالكثافة النباتية 20 كغم. هكتار$^{-1}$ بلغت 97.80 و 94.78 و 98.0 و 96.3, على التوالي و الفعالية المضادة للاكسدة بلغت 98.04 و 95.67 و 66.58 و 55.11%, على التوالي. وان المعاملة بفلافونيدات أوراق او بذور نبات الكتان كان لها فعالية مضادة للالتهابات داخل الجسم الحي أقتربت من فعالية عقار الدايكلوفيناك.

1. INTRODUCTION

Flax plant (*Linum usitatissimum* L.) is an annual Herbaceous plant has a total root at little depth, belonging to the *Lineaceae* family. Its native country in Western Asia and the Mediterranean region, it was cultivated 5000 years ago, It is considered an important plant that has been cultivated for its high nutrient content of protein, Soluble fiber in water, lignin, mucilage and Linamarin (a cyanogenic glycoside) and enzymes. It is currently cultivated mainly to obtain the oil of its seeds containing about 30-40% fatty acids, including linoleic acid (omega-6), stearic acid, linoleic acid (omega-3), oleic acid and α-linolenic acid. The percentage of Omega-6: Omega-3 is about

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1: 3, This combination does not exist in any other vegetable oil, which is the essential fatty acids for the human being because it cannot be synthesized within the body of the organism and must be obtained from food. It has many benefits for human health. It is important in the treating Neurological Disorders, visual Disorders, and hemorrhagic dermatitis. It is possible to change the percentage of different fatty acids by physiological and/or field processes (8). Flaxseed is considered a rich source for Lignins, which is a plant nutrient that is 100 times more valuable than other plants seeds such as wheat, oats, and soybeans, which have recently gained considerable attention because of its anti-cancer properties, specifically breast and colon cancers, where Lignin of Flaxseed reduces the risk of breast and prostate cancer (4, 22). Moreover, it has an anti-bacterial, fungal and viral effect (32). Flaxseeds are a rich source for flavonoids, the majority of which are found in cotyledon seeds (14). Control of planting date has an important role to play in improving the quality traits for the used seeds (31). Several factors influence the accumulation of flavonoids in plants, including genetic factors as well as ultraviolet radiation 27 and seasonal variations (16). It is well known that plant spacing plays an important role in crop production. Spacing between plant depends on the expected growth of a given crop type in a particular agricultural climate. Therefore, the optimal spacing of plants is one of the most important factors in increasing the yield per hectare. Optimal spacing ensures better growth and improves yield and quality. The compounds that have antioxidant effectiveness work to prevent the formation of free radicals (ROS) and reduce the concentration of active oxygen compounds and the most important antioxidant compounds are flavonoids and alkaloids, Which are known for their antioxidant capacity and plants that containing high concentrations of these compounds have great therapeutic value and have the potential to treat many diseases (3). Flavonoids are a wide range of plant secondary metabolites that have attracted attention as natural oxidation inhibitors to prevent nutrient degradation and to provide benefits for the metabolic effects of animals (10). The difference in flaxseed content from active substances is due to climate effects (23). Oomah et al., (24) explained that the flaxseed content of flavonoid was affected according to the cultivar of plant and climatic factors. Flaxseeds contain different types of phenols, which have an antioxidant effect that significantly reduced the effects of free radicals (5). Pant et al., (25) found that the methanolic extract for flaxseed had an antioxidant effect and this attributed to the free radicals are unstable molecules have a pair electron that appeared in oxidative stress. In India, Kamtekar et al., (20) Found that the total Flavonoids content was affected by the extraction method, where amounted to (96.1556 mg of quercetin equivalent/ 100 g (aqueous extract), 85.1881 mg of quercetin equivalent / 100 g (alcoholic extract), and 96.0122 mg of quercetin equivalent / 100 g (Ethanolic extract). In India, Kamtekar et al., (20) Found that the effectiveness of antioxidant flaxseed in vitro for vitamin C amounted to 6.436 mg of ascorbic acid equivalent / 100 g (aqueous extract), 6.7242 mg of ascorbic acid equivalent / 100 g (alcoholic extract), and 5.4616 mg of ascorbic acid equivalent / 100 g (ethanolic extract). Slavova-Kazakova et al., (30) found that flaxseed extract had antioxidant effect. There are many anti-inflammatory drugs to treat the causes of these infections, which are due to anti-inflammatory drugs (steroidal and non-steroidal). These studies suggest that these drugs are not free of gastrointestinal side effects that cause Bleeding or damage (29). It can cause acute renal failure (13). For these reasons, many researchers have focused on finding medical plants have the properties of anti-inflammatory drugs and their future use as safe therapeutic drugs (17). Flavonoids are thought to be effective anti-inflammatory and Cancer diseases (18). Several studies have been conducted on plants to investigate the possibility of their use as anti-inflammatory as well as to know their active ingredients and

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their association with anti-inflammatory properties. The study of the effectiveness of anti-inflammatory in vitro is done by the stability of the erythrocytes membranes for human and in vivo is done by injecting the palm of the upper left side for the mice. Many studies have found, where Joseph et al., (19) found When using the extract of petroleum ether and chloroform for the fresh leaves of the Clerodendron inerme plant, with a concentration of (200, 400 mg.Kg^{-1}), so the best anti-inflammatory effect resulted from both concentrations. Saleem et al., (28) noted when using the aqueous and alcoholic extract for leaves of Gendarussa vulgaris plant in both in vivo and in vitro, the concentration (300 mg.mL^{-1}) from the alcoholic extract gave an anti-inflammatory effect similar to the activity of the diclofenac sodium compound in vitro. In a study of Chippada et al., (7) in vitro to know the effect of the Centella asiatica plant note that the concentration of (2 mg.mL^{-1}) for this plant worked on the survival of 94.97% from red blood cell membranes. This has been attributed to the fact that plant extracts contain active ingredients such as triple turbines and flavonoids, which have been observed by its bio-effective. Hossain et al., (17) noted when using the extract of ethanol, hexane and ethyl estrogen at a concentration of (1 mg.mL^{-1}) for leaves of Spilanthes paniculata plant have an anti-inflammatory effect, and hexane extract was the most effective. Chamlagai and Singh, (6) showed when using the extracts of Sikkim viscum articulatum plant and Acorus calamus plant each separately, with six concentrations of (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mg.mL^{-1}), so all of them have an anti-inflammatory effect and that the extract of Acorus calamus plant is the most effective. For the importance of flax plant in terms of economic and medical and to know the percentages for antioxidant compounds in leaves and seeds, which give significance in the evaluation of the plant medically, and the absence of previous studies, this study was conducted.

2. MATERIALS AND METHODS

Two field experiments were conducted in the field of medicinal plants belonging to the College of Agriculture, the University of Basra during the agricultural seasons (2015/2016 and 2016/2017) in clay loam soil with pH (7.14, 7. 93), electrical conductivity (E.C) (4.14, 5.03 $ds.m^{-1}$), organic matter (0.7, 1.3%), for both seasons respectively. The study included the effect of two planting dates (1/11 and 1/12), the cultivating with three plant densities (20, 30, 40 $kg.ha^{-1}$) and their interactions in the growth and yield of seeds for flax plant (*Linum usitatissimum* L.) and its content of Fixed oil by using the Randomized Complete Block Design (RCBD), with three replicates. So the number of experimental units is 18 experimental units. GenStat program was used to compare the averages according to the least significant difference (L.S.D) test at the probability level of 0.05 (2). The soil of the experiment was plowed twice perpendicularly and it was smoothed, settled fertilized with animal fertilizer at a rate of (32 $tons.ha^{-1}$), Phosphate Fertilizer in the form of Super Phosphate (45% P2O5) at level of (80 kg.ha-1) (12). It was divided into plots with an area of 2 m2 (2 x 1) for one plot, the plots were covered with a light layer of reverine mixture and the seeds were then spread with a density of (20, 30, 40 $kg.ha^{-1}$) for both cultivars (1/11, 1/12) and then covered with a light layer of soil, The cultivated plots were irrigated. Seeds germinated after 5 days of cultivating. The plants were fertilized with nitrogen fertilizer in the form of urea (46% N) at (200 $kg.ha^{-1}$) (15) on the two batches, the first one after a month of cultivation and the second one after a month from the first batch. For protection from insect, plants were sprayed three times with Morisban4 (4) at a concentration of (1 $ml.L^{-1}$) after 35 days of cultivating with a 14-day interval between spraying and another. To prevent fungal infections, Plants were sprayed with Topsin-M at a concentration of (0.5 $g.L^{-1}$), three times starting after 40 days of cultivating with a 14-day interval between a one spraying and another. All agricultural operations were conducted from irrigation, weeding, and

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removing thicket whenever needed, as recommended for the cultivation of this crop. Plants were harvested on 1 and 30/4 for both seasons, respectively.

Table 1: Maximum and minimum temperatures and relative humidity during growth seasons *

Date	Maximum temperatures (°C)		Minimum temperatures (°C)		Relative humidity (%)	
	2015-2016	2016-2017	2015-2016	2016-2017	2015-2016	2016-2017
11/10 -1	26.68	25.21	17.11	4.79	83.99	75.34
11/21-11	23.96	26.51	11.72	5.26	80.76	62.58
11/30 -21	24.42	19.9	10.26	1.48	75.71	39.55
12/10-1	18.63	19.73	6.77	9.33	83.51	76.35
12/20 -11	18.49	17.28	7.63	3.97	88.52	76.66
12/31-21	15.96	20.25	7.25	6.62	94.67	76.66
1/10 -1	15.29	20.25	7.23	6.62	94.67	76.66
1/20 -11	19.14	18.84	6.57	5.95	89.07	73.09
1/31-21	16.51	18.38	5.54	5.30	74.30	78.72
2/10 -1	20.04	17.51	5.95	3.23	79.34	56.52
2/20 -11	21.60	19.02	8.86	6.30	73.14	69.77
2/30 -21	25.16	21.7	12.67	6.69	85.14	65.20
3/10 -1	27.51	25.76	13.85	11.14	73.09	72.04
3/21-11	26.87	27.26	13.65	14.45	67.73	71.20
3/31-21	29.87	27.26	13.85	11.14	73.09	72.04
4/10 -1	35.86	31.06	15.76	17.10	80.53	66.93
/4/20 -11	32.84	34.49	17.89	19.32	67.90	54.34
4/31-21	37.45	33.44	20.34	22.02	51.67	38.22

* Iraqi Meteorological Organization and Seismology at Al-Bargesia Station for Agricultural Sciences.

The preparing method for the plant extract to estimate flavonoids

A 20 g from the plant powder for the milled and dried leaves. A 200 mL of organic solvent (Hexane) was added and put it in the Soxhlet extractor at 100 °C for 24 hours to remove the oil in it. The sample was then extracted from the device and left for 24 hours to dry. The sample was then re-added to the Soxhlet extractor device and 200 mL methanol alcohol was added to it for 24 hours at a temperature of 120 °C to extract the largest amount of flavonoids, The evaporation of alcohol from the extract was conducted using a rotary evaporator at 65 °C to complete the evaporation of alcohol and then left the samples at room temperature to dry completely.

Measuring the effectiveness of anti-inflammatory in vitro

Adebayo et al., (1) was followed, Blood samples were collected for healthy volunteers and mixed with equal sizes from the sterilized Alsever's Solution, which prepared by dissolving (2.05% glucose, 0.42% sodium chloride, 0.8% trisodium citrate and 0.55 citric acid) in distilled water. This solution was used to store red blood cells, placing the blood mixture in the centrifuge at (3000 cycles.min^{-1}) for 10 min, the red blood cells were then collected, the red blood cells were washed with Isosaline solution (0.9% sodium chloride), 1 ml of the above suspension was taken and add to it 9 ml of Isosaline solution. After that, take 0.2 g from the extract of leaves or seeds flax and dissolving it in 1 ml of ethanol. A 1 ml of Phosphate buffer (pH7), 2 ml of Hyposaline (0.7% sodium chloride) and 0.5 mL of hemoglobin suspension H R B C were then added to the extract solution of leaves or seeds. The solutions were incubated at a temperature

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of 37 °C for half an hour and then subjected to centrifugation at (3000 cycles.min^{-1}) for 10 min, the Leachate was taken and the absorption was measured to it with the spectrophotometer device along the 560 nm wavelength. The following equation was applied:

The percentage for the prevention = 100 - $\frac{\text{Absorption of the sample}}{\text{absorption of the control}} \times 100\ \%$

Measuring the effectiveness of anti-inflammatory in vivo

The effectiveness of anti-inflammatory was measured on the males of the white albino Swiss mice, their weight was 25 ± 5 g, which was placed under standard conditions within the animal house and providing equal amounts of food and water after dividing into four groups, each group consisting of six mice. All experiment mice were dosed before an hour from the induction of infections by oral :

1- The first group (negative control group) with distilled water.
2- The second group (positive control group) with Diclofenac at a concentration of (10 mg.kg^{-1}).
3- The third group was treated with a Flavonoids extract for leaves at a concentration of (100 mg.kg^{-1}) dissolved in 0.2 ml distilled water.
4- The fourth group was treated with a Flavonoids extract for seeds at a concentration of (100 mg.kg^{-1}) dissolved in 0.2 ml distilled water.

Inflammation was induced in all experiment mice by injecting the left upper limb of each mouse with 20 µL from the albumin of fresh chicken eggs. The results of inhibition in the size of the mouse palm in (mm) were compared with the control group that dosed distilled water only and diclofenac drug (10 mg.kg^{-1}) as a standard which dosed by oral (28).

Measuring the effectiveness of antioxidant for the leaves and seeds of the flax plant

It was estimated according to (Gorinstein et al. 12), where 2 mg of plant extract (leaves and seeds) was dissolved separately in 10 mL methanol, 13 mg of (1,1-diphenyl-2-picrylhydrazyl (DPPH)) was dissolved in 500 ml of methanol, then take 2 ml of the solution prepared in the first step and add to it 2 mL of the solution in the second step. The mixture was then shaken well and kept in the dark for 30 min and at room temperature, The absorbance was measured at a wavelength of 517 nm. The effectiveness of antioxidant was estimated using a standard curve in which vitamin E (produced by T and D Pharma GmbH. KleineKnopheide 4.32657 Lemgo, Germany) was used. The effectiveness of antioxidant was calculated by applying the following equation:

Percentage of inhibition = $\frac{\text{Absorption for the control } (A_0) - \text{Absorption with sample presence } (A_1}{\text{Absorption for the control } (A_0)}$ 100

$A_0 = 0.184$

A_1 = sample Absorption

Calculation of the Lethal Dose for half of the experiment animals LD_{50}

Thirty-six male mice were selected from the white albino Swiss mice with a weight of 25 ± 5 g. It was divided into six groups. Each group containing six mice which placed inside the animal house belonging to the College of Pharmacy, University of Basra at 25 C. It was given equal amounts of diet and water and left for a week to acclimatization. In order to determine the lethal dose for half of the experiment animals, the first group was dosed with distilled water by Stomach tube. This group was considered as the control group. Other groups were given in the same method graduated doses of flaxseed extracts at concentrations (1, 2, 3, 4 and 5 g.kg^{-1}) from body weight. The animals were monitored for 72 hours to record data on the apparent changes in behavior and calculate the number of deaths (21).

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3. RESULTS AND DISCUSSION

Table (2) show that the study factors and their interactions have a significant effect on the effectiveness of methanolic extract for anti-inflammatory leaves and seeds for both seasons. where the cultivated plants in 1/11 were significantly excelled in the percentage of inflammatory inhibition compared with those cultivated in 1/12. The plant density had a significant effect on this trait, where the percentage of anti-inflammatory activity increased as the plant density decreased. Bi-interaction had a significant effect on this trait, where the cultivated plants in 1/11 with a plant density (20 kg.ha^{-1}) recorded the highest percentage of inhibition amounted to (97.80, 94.78, 98.0, 96.3%), compared to the lowest percentage amounted to (79.23, 73.27, 59.1, 48.1%), from plant cultivated in 1/12 with plant density of (40 kg.ha^{-1}), for both seasons, respectively

Table 2: Effect of the planting date, plant density and their interactions in the percentage of anti-inflammatory activity (%) in leaves and seeds of the flax.

Planting date	Plant density	The percentage of anti-inflammatory activity for the leaves of the flax plant		The percentage of anti-inflammatory activity for flaxseeds	
		2016/2015	**2017/2016**	**2016/2015**	**2017/2016**
11/1	20	97.80	94.78	98.0	96.3
	30	95.09	88.77	91.9	86.7
	40	93.14	82.33	63.2	81.7
12/1	20	93.88	89.85	89.9	89.9
	30	86.69	81.99	76.0	63.2
	40	79.23	73.27	59.1	48.1
LSD0.05 for interaction		**5.58**	**5.70**	**11.30**	**11.45**
Average effect of planting date	11/1	95.34	88.63	84.4	88.2
	12/1	86.60	81.70	75.0	67.1
LSD0.05 for planting date		**3.22**	**3.29**	**6.53**	**6.61**
Average effect of plant density	20	95.84	92.32	94.0	93.1
	30	90.89	85.38	84.0	74.9
	40	86.19	77.80	61.2	64.9
LSD0.05 for plant density		**3.95**	**4.03**	**7.99**	**8.10**

Table (3) shows that the average size of the mouse's palm increased when inflammation was induced and it was most intense during the first hour of inducing for all treatments, The average size of mice treated with flavonoids was significantly decreased compared to those treated with distilled water and the effect was increased a long time with the treatment. The two treatments of Flavonoids were approached in their effect from the standard anti-inflammatory treatment (diclofenac).

Table 3: Effect of treating with flavonoids of leaves and flaxseed in the average size of the mouse's palm (mm).

Group	The average size of the mouse's palm (mm)			
	0 hr	**1 hr**	**2 hr**	**3 hr**
Control	3.25 ± 0.22	4.40 ± 0.38	4.21 ± 0.17	3.97 ± 0.21
Diclofenac	3.13 ± 0.20	3.91 ± 0.33*	3.71 ± 0.18*	3.51 ± 0.26*
Leaves	3.24 ± 0.19	3.77 ± 0.22*	3.73 ± 0.19*	3.56 ± 0.19*
Seeds	3.31 ± 0.17	3.95 ± 0.41*	3.74 ± 0.33*	3.58 ± 0.28

* Statistical analysis according to ANOVA.

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The effect of the study factors on the developing plant growth and the efficiency of its bio-processes, which led to the increase of active substances, such as Triterpenoids and flavonoids, vitamin C and phenolic acid and Lignins have been observed to be effective bio-activity for anti-inflammatory. These results agree with Hossain et al., (17) in their study on the Spilanthes paniculata plant and Chamlagai and Singh, (6) on Sikkim Viscum articulatum plant and Acorus calamus plant. Table (4) show that the study factors and their interactions have a significant effect on the effectiveness of methanolic extract for antioxidant leaves and seeds for both seasons. where the cultivated plants in 1/11 were significantly excelled compared with those cultivated in 1/12. The plant density had a significant effect on this trait, where the percentage of antioxidant activity increased as the plant density decreased. Bi-interaction had a significant effect on this trait, where the cultivated plants in 1/11 with a plant density (20 kg.ha^{-1}) recorded the highest percentage of antioxidant activity in leaves and seeds amounted to (98.04, 95.67, 66.58, 55.11%), compared to the lowest percentage amounted to (72.83, 80.27, 31.92, 34.33%), from plant cultivated in 1/12 with plant density of (40 kg.ha^{-1}), for both seasons, respectively. The activity of antioxidant leaves and flaxseed is due to its effect on flavonoids compounds and active phenolic acids in the holding of free radicals.

Table 4: Effect of the planting date, plant density and their interactions in the percentage of antioxidant activity (%) in leaves and seeds of the flax.

Planting date	Plant density	The percentage of antioxidant activity for the leaves of the flax plant		The percentage of antioxidant activity for flaxseeds	
		2016/2015	**2017/2016**	**2016/2015**	**2017/2016**
11/1	20	98.04	95.67	66.58	55.11
	30	94.51	93.25	53.35	48.46
	40	89.76	86.08	51.06	44.76
12/1	20	92.56	92.81	51.13	52.30
	30	84.85	89.20	33.09	38.34
	40	72.83	80.27	31.92	34.33
LSD0.05 for interaction		**5.55**	**2.26**	**4.81**	**4.93**
Average effect of planting date	11/1	94.10	91.67	57.00	49.45
	12/1	83.41	87.43	38.71	41.66
LSD0.05 for planting date		**3.20**	**1.30**	**2.77**	**2.85**
Average effect of plant density	20	95.30	94.24	58.85	53.71
	30	89.68	91.22	43.22	43.40
	40	81.30	83.18	41.49	39.54
LSD0.05 for plant density		**3.92**	**1.59**	**3.40**	**3.49**

Calculation of the Lethal Dose for half of the experiment animals LD_{50}

The results of this test showed that the concentrations of the extracts used in this study did not have any toxic effect on the laboratory mice. No mortality or behavioral changes were observed during the observation period, but the opposite was observed, which led to increasing the activity and vitality. This means that LD_{50} for flaxseed extract is more than (5 g.kg^{-1}) of body weight. So, in principle, it can be said that this extract is safe within the dosage limits used in this experiment.

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