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The Anti-Inflammatory and Antioxidant Effects of Methanol Seed Extracts of Sunflower (Helianthus annuus) and Level of Uric Acid in Gout Induced Albino Rats

Almustapha, Lawal[1*], Mukhtar Jangebe Ladan[1], Muhammad Sani Isma'ila[2] and Jamila Husaini, Sifawa[1]
1. Department of Biochemistry and Molecular Biology, Faculty of Science, Usmanu Danfodiyo University, Sokoto-Nigeria
2. Department of Basic Veterinary Sciences, School of Veterinary Medicine, Faculty of Medical Science, University of the West Indies Trinidad and Tobago
*Email of the Corresponding Author: lawal.almustapha@udusok.edu.ng

Abstract
The seeds of *Helianthus annuus* (*H. annuus*) are known for their economic importance, especially in vegetable oil production. The work aimed to study the anti-inflammatory and antioxidant effects of methanol seed extracts of *H. annuus* and the level of uric acid in gout-induced Albino rats. In an experiment, using monosodium urate (3mg/kg), gouty arthritis was induced on the left-leg ankle of the albino rats subcutaneously and three different concentrations of the seed extracts; 250, 350, and 450 mg/body-weight used to fed the induced rats; D, E and F groups, and C group were given allopurinol (positive control), while B and A groups were negative and normal controls. In a dose-dependent pattern, methanol seed extracts increased the activity of antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPX). Furthermore, it significantly decrease the level of uric acid, Interleukin 6 (Ile 6), and xanthine oxidase activity ($p<0.05$) in the treated groups. These makers are involved in oxidative and inflammatory processes that cause pains in gouty arthritis. The study suggests that the methanol seed extracts have antioxidants and anti-inflammatory potential against gouty arthritis in albino rats.
Keywords: gouty arthritis, anti-inflammatory, antioxidants, ROS, *Helianthus annuus* seed.
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1.0 Introduction

The sunflower plant (*Helianthus Annuus*) is a tall annual or perennial plant that has one or more wide, terminal capitula (flower heads). It is a plant that is comprised of about 70 species. It has been described as a relatively young crop, which has been cultivated since the middle of the 19th century, and undergoes intensive crop breeding (Schilling *et al.,* 2006; Jovan et al., 2006). It has been the most important oil plant in many countries around the world and serves as an excellent source of high-quality oil for human consumption (Jovan et al., 2006). Sunflower (*Helianthus annuus*) seeds serve as a good source of antioxidants, such as flavonoids, phenolic acids, trace elements such as minerals, specifically calcium, iron, magnesium, phosphorous, potassium, selenium, zinc, and vitamins (Blicharska, *et al.,* 2014). Some of the flavonoids that were identified are heliannone, quercetin, kaempferol, luteolin, apigenin, and those phenolics are caffeic acid, chlorogenic acid, caffeoylquinic acid, gallic acid, protocatechuic, coumaric, ferulic acid, sinapic acids, etc., which characterised the sunflower seeds as a good source of antioxidant and could have antioxidant properties (Kamal, 2011; Pająk, *et al*., 2014).

A condition characterised by excess uric acid disposition especially at the supersaturated body fluids, tissues, and around the joints leading to pain around joints, is referred to as gouty arthritis (Kim *et al*., 2003; Falasca, 2006; Sharma, *et al.,* 2009). Its most common manifestation could be excruciatingly painful arthritic joint, and sudden onset of inflammation most often in the big toe (Schlesinger, 2011). It has also been characterised as a condition that can be repeatedly re-occurrent that could lead to a red, tender, hot, and swollen joint (Dalbeth *et al.,* 2021; Hui *et al.,* 2019). Different studies have shown that gouty arthritis has a frequency of 3 out of every 1000 reported pain. It predominantly affects older males and studies have shown the risk factors are in those who regularly eat red meat, seafood, drink beers, or are overweight can be predisposed to gouty arthritis (Richet and Bardin 2010; Belyl *et al.,* 2016). Gout which is synonymous with gouty arthritis is associated with several metabolic insufficiencies, genetics, and lifestyle changes (Richette *et al.,* January 2010; Hui *et al*., 2019). Some food's content has been reported to contain higher purines compounds that are broken down into uric acid metabolically by xanthine oxidase enzyme. In humans, purines are converted into uric acid by oxidation of hypoxanthine to xanthine and xanthine to uric acid by xanthine oxidase (XO), which can be removed in the urine (Mamat *et al.,* 2014). Consequently, the excessive formation and accumulation of uric acid resulted in hyperuricemia and gout (Richette & Bardin, 2010). Other compounds reported to be formed as a result of the conversion of hypoxanthine to uric acid are superoxide anion (O_2-), and nitric oxide (NO) (Pouliot, *et al.,* 2012). In addition, the interaction of these molecules promotes the generation of peroxy-nitrite (ONOO-) and hydrogen peroxide, which in turn increases apoptosis, degradation of connective tissues, and joint damage (Pouliot, *et al.,* 2012, So and Thorens, 2010).

Complex interactions within joints and various cell types, including neutrophils, macrophages, mast cells, endothelial cells, and synovial fibroblasts, have been suggested to play an important role in the modulation of an inflammatory response to uric acid crystals (MSU) formed in patients suffering from gout arthritis (Zheng *et al*., 2015; Scanu *et al.,* 2010). The Reactive Oxygen Species (ROS) generated upon activation of inflammasome that leads to the oxidation of mitochondrial DNA (mtDNA) and released into the cytosol may induce the activation of the (NLRP3) inflammasome (Zhong *et al.,* 2018). These mechanisms are yet to be fully unraveled have suggested that NLRP3 protein can sense and activate a response under the concept of the inflammasome, which includes K^+ efflux and maturation of the proinflammatory cytokines IL-Iβ and IL-18 follows by others like IL6, TNF-α, etc (Martinon *et al.,* 2009; Stamp, *et al.,* 2014; Kim *et al.,* 2016). In this research, a work effort was made to study the therapeutic effect of crude methanol seeds extract of sunflower (*Helianthus annuus)* in gout-induced albino rats by considering antioxidant activity and anti-inflammatory makers.

2. Experimental

2.1 Material and Methods

2.1.1 Material

2.1.1.1 Sunflower and Seeds Sowing

The sunflower (*Helianthus annus*) seeds were obtained from Bosso Local Government Area of Niger State, Nigeria. The soil pH of 7.03 was used to plant the seeds, at the Department of Biological Science garden in Usman Danfodiyo University Sokoto, Sokoto State, Nigeria. The germinated plants were watered twice a day until grown to a matured flower (45 days).

2.1.1.2 Seeds Collection and Preparation from Matured Sunflower Plants

The flower containing the seeds from matured sunflower plants were collected by hand pulling method. The seeds were carefully removed and washed vividly with freshly prepared sterilised water to remove all sorts of dirt. The seeds were dried at room temperature and grounded using mortar and pestle into a fine powder. The powdered samples were weighed using a digital laboratory weighing balance and stored in a desiccator at room temperature before use.

2.1.1.3 Methanol Crude Seeds Extract Preparation

Twenty grams (20 g) of the dried sunflower seeds powder was used for the extraction in 200ml of 80% Methanol at 200 °C for 1 hour.

2.2 Chemicals and Reagents

All chemicals and reagents used are of analytical grade.

2.3 Methods

2.3.1 Preparation of monosodium urate crystals (MSU crystals).

The MSU crystals were prepared in the department of Biochemistry Usmanu Danfodiyo University Sokoto**,** Nigeria, by using a procedure reported by Ortiz-Bravo *et al*. (1993). One gram of uric acid was dissolved in 6 ml of 1 M NaOH and 194 ml of distilled water to use to make the volume to 200ml. The mixture was heated at 180ºC for 30minutes. 1N HCl was then added into the mixture dropwise to maintain pH at 7.2. The mixture was then stirred slowly at room temperature and then stored at 4 C for 24hr to form the crystals. At last, the MSU crystals in the mixture were then removed, dried, after washing, and suspended in sterile saline (100 mg/ml).

2.3.2 Experimental Design for the MSU and Allopurinol Administration

Thirty-five (35) male albino rats with an average weight of 160-180g were used for the study. They were purchased from the Department of Biological Science, Usmanu Danfodiyo University Sokoto, and the rats were acclimatised for one week before the induction of gouty arthritis using MSU. In a randomised design, the rats were grouped into six groups and each group has 5 rats. Group A, the normal control group (Normal Control) was fed orally equal volume of saline, group B, the model group (Negative Control) was injected with 3 mg/body-weight of MSU crystal at the left ankle synovial space but did not receive any treatment, also group C, (positive control group) received an equal volume of MSU but were treated with 10mg/body-w of Allopurinol. Groups D, E, and F (methanol crude extract-treated groups), were injected like other groups with MSU, these groups received orally 250, 350, and 450 mg/body-w of sunflower methanol seeds extract for 9 days. At the end of the experimental day (day 9), 1 h after the final extract administration, blood was collected from the caudal vein of the rats following approved guidelines from the Department of Veterinary Medicine Usman Danfodiyo University Sokoto (UDUS). Serum was collected after centrifugation and kept at 20 ^{0}C until required for biochemical analysis.

2.3.3 Measurement of Swelling Ratio

The left ankle circumference of all the rats after MSU injection at different hours: 0.00 hr, 12 hr, 24 hr, and 48 hr were measured using Vernier caliper. The percentage swelling ratio was calculated as the change in circumference by using the following formula:

$$(\%)swelling\ ratio = \frac{(Ct-C0)}{C0} * 100 \quad ----------1$$

Where Ct represented the circumference at different times and Co represented the circumference at 0.00 hours

2.3.4 Determination of *In-vitro* Antioxidants Capacity.

2.3.4.1 DPPH Activity (Methods of Matano, *et al.* 1998; Gow-Chin and Hui-Yin, 1995)

Several serial dilutions were carried out on the methanol crude seeds extract by taking out 0.2, 0.4, 0.6, 0.8, and 1.0 ml of the extract in triplicates, and the volume was brought up to 4ml with distilled water, 1ml of DPPH (1.0 mmol) was added to each test tube to make the final volume of 5 ml, the mixture was shaken vigorously and left standing at room temperature for 30 min. The absorbance of the resulting solution was then measured at 517 nm after 30 min.

2.3.4.2 Ferric reducing antioxidant power (Benzie and Strain, 1996)

Principle: The antioxidant capacity of the medicinal plants was estimated spectrophotometrically following the procedure of Benzie and Strain (Benzie and Strain. 1996). The method is based on the reduction of Fe^{3+} tripyridyl triazine (TPTZ) complex (colourless complex) to Fe^{2+}-tripyridyl triazine (blue coloured complex) formed by the action of electron-donating antioxidants at low pH. This reaction is monitored by measuring the change in absorbance at 593 nm.

2.3.5 Determination of Total Phenolic Content

The total phenolic content in the methanol crude seeds extract was determined spectrophotometrically as according to the Folin-Ciocalteu method (Singleton, *et al.*, 1999). Gallic acid was used to set up the standard curve. The content of phenolic compounds of the triplicates samples was expressed as garlic acid equivalents (GAE) in mg per gram dry weight.

2.3.6 Determination of Total Flavonoid Content

Exactly, 20µl of the sample extract was added unto a solution of 2% $AlCl_3.6H_2O$. The mixture was vigorously vortex. The total volume was brought up to 10 ml using double distilled water. The mixture was measured and incubated at 25 ^{0}C for 10 min. The absorbance of the reaction mixture was measured at 440nm Flavonoid content was expressed as quercetin equivalents in mg per gram dry material.

2.4 Biochemical Assay

2.4.1 Liver Sample Preparation

The liver of the rats was excised immediately after blood collection, washed in 0.9% cold saline, and rapidly stored at 80^0 C until further handling. Enzyme extraction was performed as described elsewhere (Haidari *et al*., 2009). Briefly, Rats livers were homogenized in 5mL of 80mM sodium phosphate buffer (pH 7.4) and, then, the homogenate was centrifuged at 3000 g for 10 min at 4^0C. Lipid layer was carefully removed, and the supernatant was further centrifuged at 10,000g for 60 min at 4^0C. The final supernatant was used for enzyme assays.

2.4.2 Superoxide Dismutase

The activity of the superoxide dismutase enzyme was determined using Caymans SOD Assay Kits (USA) and strictly followed the guidelines provided by the manufacturers.

2.4.3 Glutathione Peroxidase

The activity of the glutathione peroxidase enzyme was determined using Caymans GPx Assay Kits (USA) and strictly followed the guidelines provided by the manufacturers.

2.4.4 Determination of Malonaldehyde (MDA), Interleukin 6, and Xanthine oxidase were carried out according to the manufacturer's guidelines (Nangin Pars Biochemical ELISA Kits, China).

2.4.5 Uric acid was determined using the enzymatic method **(Uricase Tops method)**

Principle:

Uricase transforms uric acid into allantoin, with the formation of hydrogen peroxide. In the presence of peroxidase (POD) it reacts with ethyl- sulphopropyl toluidine (ESTP) and 4- amino phenazone, to produce a colored complex whose color intensity is directly proportional to the uric acid concentration of uric acid in the sample.

Enzymatic determination of uric acid according to the following reactions.

$$\text{Uric acid} + 2H_2O \xrightarrow{Uricase} \text{Allantoin} + CO_2 + H_2O_2 \quad ---------- 1$$

$$\text{Amino antipyrin} + \text{Tops} \xrightarrow{Peroxidase} \text{Voilet coloured complex} + 2H_2O_2 \quad ---------- 2$$

(Tops = Ethylene – N (Sulphopropyl) -3-Methyl Aniline).

2.5 Data Analysis

The data generated were analysed using Instat Graph Pad software 3 San Diego USA. One-way analysis of variance (ANOVA) and Bonferroni multiple comparisons were carried out and values considered statistically significant difference at $p < 0.05$.

3. Results and Discussion

3.1 Monitoring the Progress of the Induced Gouty Arthritis

3.1.1 Dimension of the Ankle Joints of the Induced Rats

The administration of MSU into the sub-peritoneal around the ankle region induced arthritis (Fig. 2) and increased the swelling volume observed from 12 hours to 48 hours after the induction (Table 2). The result showed a non-significant increase (P>0.05) in the swelling ratio between different hours after induction as presented in **Table 1**. **Figure 1A** shows the inflammations and swelling of the ankle joint of the left leg of the rats. The swelling and inflammatory response were a result of deposition of MSU which causes pain around the ankle joint. Treatment was followed using methanol extract of *Helianthus annuus* at different doses as presented in **Figure 1B.** From the result, inflammation and swelling have subsided significantly when a physical comparison was made between the induction and after receiving the methanol seed extracts at different dosages.

(A) Before Treatment Started (B) After Treated with methanol seeds extract

Figure 1: Monitoring gouty arthritis induced using monosodium urate crystal (MSU) in the left ankle of the rats which causes a swelling and inflammatory response in the rats. In **(A)**, 24 hr after rats were injected with MSU and rats no treatment started, and **(B)** as the days pass by and the rats received treatment of methanol seeds extract of sunflower at different dosages, the swelling and inflammation subsided.

Table 2: - The swelling ratio of Rat's ankle after gouty arthritis induction.

GROUPS	12 hrs	24 hrs	48 hrs
A	-	-	-
B	0.424±0.16[a]	0.545±0.340[a]	0.404±0.20[a]
C	0.382±0.02[a]	0.412±0.127[a]	0.338±0.12[a]
D	0.434±0.20[a]	0.590±0.226[a]	0.473±0.08[a]
E	0.428±0.12[a]	0.320±0.121[a]	0.396±0.12[a]
F	0.585±0.25[a]	0.517±0.172[a]	0.563±0.19[a]

Group A was the normal control group (Normal saline), Group B was the modal control group, **Group C** are positive control, and **Groups D, E, & F** were the experimental groups, respectively. Values are Mean ± SD of triplicates measurements with the same superscript across the columns indicates no significant difference at p>0.05.

3.2 Qualitative and Quantitative Analyses and Antioxidant Capacity of Methanol Seed Extracts of *Helianthus annus*

Plants are generally shown to be effective in the treatment of many diseases and different countries use herbs as a source of ingredients for the synthesis of drugs (Strivastara *et al.,* 1996; Mahesh and Satish, 2008; Masaudi *et al.,* 2016). A wide variety of plant parts are used as drugs because they possess varied medicinal properties which can serve as a source of a new therapeutic agent. The result showed that the methanol seed extracts of *Helianthus annus* possess antioxidant properties, which was further corroborated by Kamal (2011). The antioxidants activity of the methanol crude seed extracts showed total phenolic content (34.12 ± 0.01732), Ferric reducing antioxidant power (FRAP) (153.56 ± 5.82), and total flavonoid (23.8 ± 0.0400). These compounds have been reported to contribute directly to reducing oxidative stress (Kamal, 2011). The presence of functional hydroxyl groups in these compounds gives the capacity to suppress the generation of reactive oxygen species (ROS) by inhibiting the activity of some enzymes/chelating trace elements and metal ions which are crucial in preventing the free radical generation (Leopoldini *et al.,* 2006 and Kumar, 2017).

The qualitative and quantitative phytochemicals screening of methanol seed extracts of *Helianthus annuus* (**Table 2**), revealed the presence of some essential secondary metabolites such as; flavonoids, glycosides, saponins,

alkaloids, volatile oils, cardiac glycosides, steroids, tannins, saponin glycoside, and balsams. Similarly, the quantitative analysis of the seed extracts indicated higher content of alkaloids 15.01 g%, followed by saponins 1.05 g% and flavonoids 0.39 g% whereas glycosides and tannins were quantitative as 0.17 g% and 0.08 g% respectively.

Table 2: - Qualitative and quantitative analyses of some phytochemicals in Methanol Crude Seeds Extract of *Helianthus annuus*

Secondary metabolite	Detection	g%
Flavonoids	+	0.39
Glycosides	+	0.17
Saponins	+	1.05
Alkaloids	+	15.01
Volatile oils	+	-
Cardiac glycosides	+	-
Steroids	+	-
Tannins	+	0.08
Saponin glycosides	+	-
Balsams	+	-
Anthraquinones	ND	-

Key: + = detected, ND = Not detected, and – not determine

Phenolic compounds constitute mainly secondary metabolites are widely distributed in the plant kingdom. These compounds play a critical role in both the growth and development of plants and serve as important antioxidants because of their ability to donate a hydrogen atom or an electron (H^+) to form stable radical intermediates. Therefore, they prevent the oxidation of various biological molecules (Cuvelier et al. 1992).

3.4 DPPH Scavenging Activity

The scavenging activity (%) on the DPPH radical of the methanol seed extracts of sunflower seeds samples on the DPPH radical are presented in **Figure 2**. The results showed that the methanol extract of sunflower seeds possesses marked scavenging ability from 11.5% to reach a maximum of 90.07% at increasing extracts concentration from 20 to 100µg/ml on DPPH radical. Meanwhile, the results indicated that the methanol seed extracts of sunflower seeds at levels between 60 to 100 µg/ml had higher marked scavenging activity of DPPH radical. This result corroborates with submission by Yen and Duh (1995) that found the radical-scavenging activity of 89.3% inhibition in 1500 mg/ml methanol extract from peanut hulls on the DPPH radical and Abdeldaiem and Hoda (2014) that showed the ethanolic extract of non-irradiated sunflower seeds hull possess marked scavenging ability from 79.85% to 94.18% with increasing extract concentration from 50 to 200µg/ml on DPPH radical in comparison with non-irradiated and irradiated sunflower hull samples. The DPPH radicals are widely used as antiradical to investigate the scavenging activities of several natural compounds and plant materials (Uddin *et al.,* 2012). Synthesis of uric acid causes the release of several ROS as a result of xanthine oxidase activity that converts xanthine to uric acid (Zhuang *et al*., 2014).

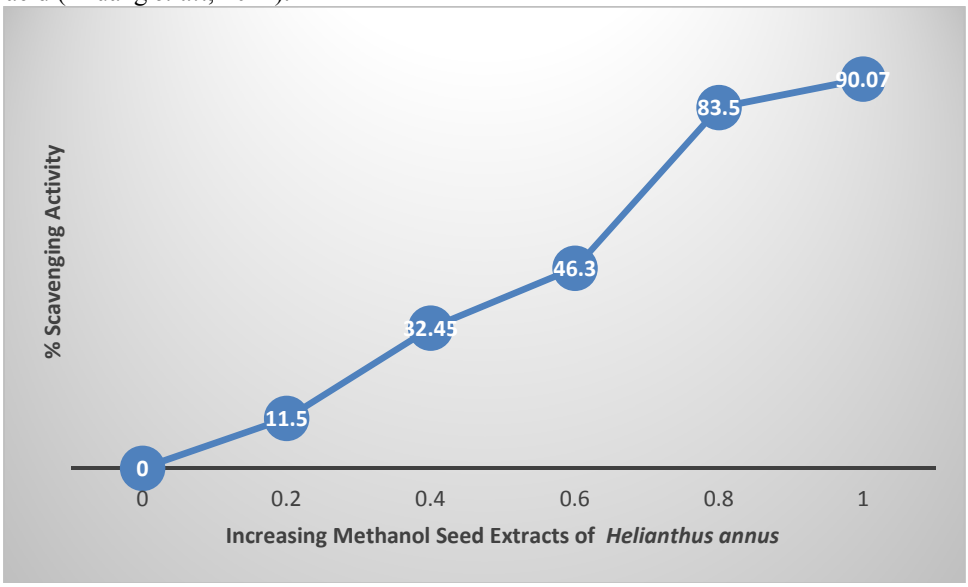

Figure 2: The Antiradical activity of methanol seed extracts on 2, 2 – diphenyl – 1 – picrylhydrazyl (DPPH) radical using an increasing concentration of the extracts against a constant volume of known DPPH concentration.

3.5 Antioxidants Enzymes, Oxidative Stress Marker and Inhibitory Activity on Xanthine Oxidase, the Levels of Uric Acid and Interleukin 6 (IL6) in Gouty Arthritis Induced Rats.

Figure 3 presents a study on the effect of methanol seed extracts of *Helianthus annuus* on the activity of antioxidants enzymes; glutathione peroxidase (GPX), superoxide dismutase (SOD), and oxidative stress marker malonaldehyde (MDA) showed a significant increase ($p<0.05$) in the activity of both SOD and GPX as compared to the model control group (group B). However, the level of oxidative stress marker MDA was significant dropped in group C, treated with Allopurinol (10mg/bw) while Groups D, E, and F were administered with the crude extract at different concentrations 250, 350, and 450 mg/bw as compared against the model control group (group B), which were administered only MSU.

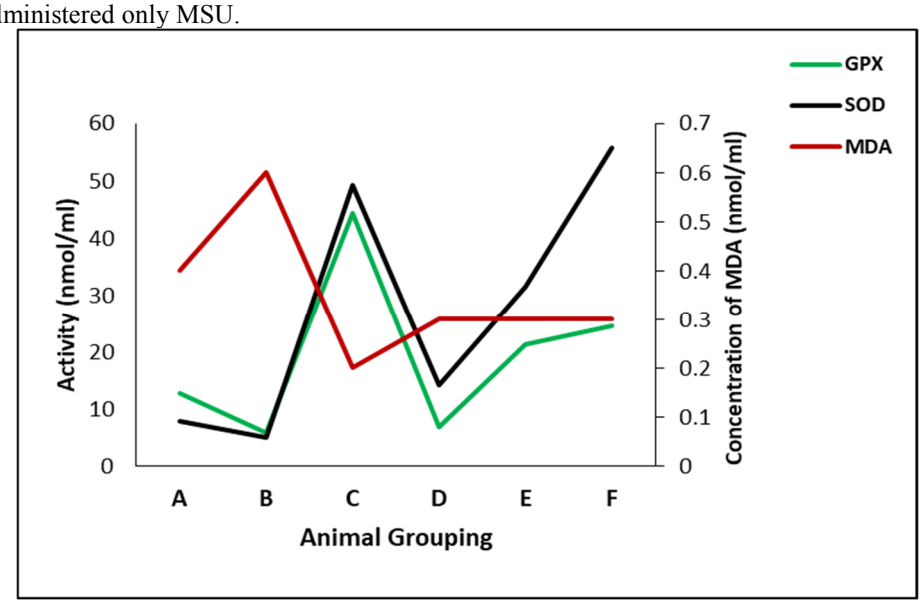

Figure 3: The effect of methanol crude extract of *Helianthus annuus* seed on the activity of antioxidants enzymes; glutathione peroxidase (GPX), superoxide dismutase (SOD), and oxidative stress marker malonaldehyde (MDA) in rat induced gouty arthritis. Group A was the normal control, Group B was the modal control given MSU only., Group C is positive control treated with Allopurinol (10 mg/bw) and Groups D, E, F, each administered 250, 350 and 450 mg/bw of methanol crude seeds extract, respectively.

Likewise, **Figure 4** shows the inhibitory effects of methanol seed extracts on the activity of xanthine oxidase showed a significant decrease ($p<0.05$). Similarly, the levels of uric acid and Interleukin 6 (Il6) in the induced rats indicated a significant decrease ($p<0.05$) as compared to the non-treated induced group B (model group).

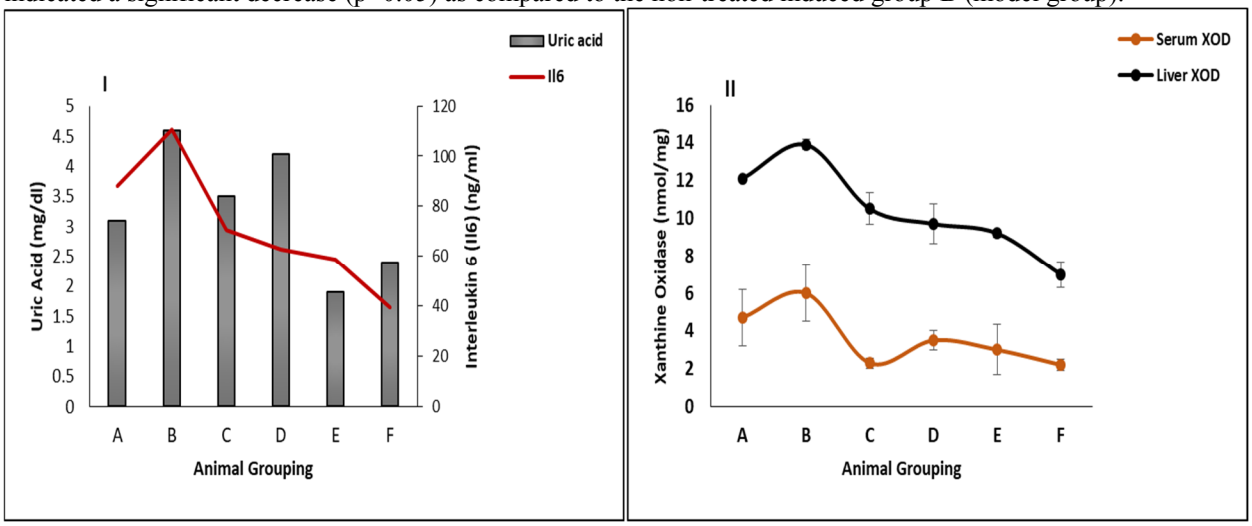

Figure 4: I) The Serum Levels of Uric acid, Interleukin 6 (Il6), **II**) Inhibitory effects of Methanol Seed Extracts of *Helianthus annuus* on the activity of Xanthine Oxidase (XOD) in rat induced gouty arthritis. **A** is the normal control group, **B**- is the modal control group given MSU only, **C** is the positive control group treated with Allopurinol (10 mg/bw), and **D**, **E**, and **F** groups are administered with 250, 350, and 450 mg/bw of methanol seed extracts, respectively.

Additionally, high serum levels of SOD, GSH-Px, and low levels of MDA were observed in gout-induced rats treated with crude seed extract suggesting its potential as a scavenger to the ROS. The levels of uric acid and

activity of xanthine oxidase have been lower in both allopurinol treated (positive control) and methanol crude seeds extract treated groups (**Figures 3 & 4**). This showed that the plant extracts have inhibitory properties against xanthine oxidase and anti-inflammatory activity, which caused a decreased level of IL6. The *in vitro* studies of some researchers have shown that the presence of antioxidant compounds such as flavonoids, alkaloids, and essential oils possessed a dual mechanism as antioxidant and anti-gout by inhibition of xanthine oxidase (XO) (Ahmad *et al*., 2018). Also, studies have linked the release of Ile 6 and overproduction of ROS by the enzyme xanthine oxidase that causes cellular oxidative stress, which ultimately leads to inflammation through the destruction of tissues and release of danger signals by necrotic cells (Mittal *et al.,* 2014).

4. Conclusion Remarks

In conclusion, the methanol seed extracts of *Helianthus annuus* have shown the potential of antioxidant and anti-inflammatory properties by both reducing the swelling and increasing the activity of antioxidant enzymes GPX, SOD also reduces the level of MDA. The seed extracts have shown inhibitory activity against xanthine oxidase thereby reducing gouty arthritis by decreasing the inflammation in the induced albino rats. This indicates its scavenging potential toward ROS generation and inflammatory processes during gouty arthritis attacks which are important.

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