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# *In vitro* antioxidant activity of a flavonoid compound isolated from methanolic extract of *Helianthus annuus* leaves (Asteraceae)

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**ABSTRACT**

*Natural products have been considered for many years ago to become a rich source of novel chemical structures. The present study was aimed to isolate a pure compound with significant antioxidant activity from Helianthus annuus leaves. The plant material was extracted successively by Soxhlet apparatus by using petroleum ether, chloroform, and methanol. These extracts were tested in vitro by using 2, 2- diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The antioxidant activity of methanol extract was (55± 0.05 RSA %) at 500 µg/ ml. Column chromatography was used to separate the methanol extract into eight fractions. The fractions were again tested by using DPPH radical scavenging assay, fraction seven (F7) has shown the highest ability to reduce DPPH with percentage 65% at 500 µg/ ml, it was even higher than the radical scavenging activity of the whole crude methanol extract. The $IC_{50}$ of fraction seven has shown 329 µg/ ml. TLC technique was applied to confirm the purity of fraction seven. Cyanidin reaction and $AlCl_3$ colorimetric assay method were applied to confirm that the isolated compound was a flavonoid most probably a flavone type. The isolated flavonoid compound is now under the process of determination of its chemical structure.*

***Key words:*** *Antioxidant activity, DPPH, Flavonoids, Helianthus annuus leaves.*

## INTRODUCTION

Free radicals are chemical compounds which contain unpaired electrons, they are formed either endogenously (from nutrient metabolism) or exogenously (from tobacco smoking, ionizing radiation, air pollution, organic solvents, pesticides, etc.), they are cytotoxic compounds which can cause tissue injuries, excessive amount of these reactive oxygen species (ROS) is harmful because they initiate intracellular molecular oxidation which leads to cell death [1]. The damages caused by free radicals may lead to many chronic diseases like cancer, cardiovascular diseases, neural disorders, Alzheimer's disease, mild cognitive impairments, Parkinson's disease, ulcerative colitis, aging and atherosclerosis [2].

Medicinal plants are important source of antioxidant compounds; these natural antioxidants reduce the risk of many chronic diseases [3]. The secondary metabolites- like phenolic compounds and flavonoids- from plants have been reported to be the potent free radical scavengers, they are found in all parts of plants such as leaves, fruits, seeds, roots and barks [4]. Therefore, screening for highly potent, less toxic, and cost effectiveness antioxidant molecules from medicinal plants is highly required.

*Helianthus annuus* L. is mainly cultivated to produce edible oil from its seeds; it also has many traditional uses like treatment of inflammatory disorders, respiratory diseases, and renal disorders [5]. The antioxidant activity of *H. annuus* has been reported; its methanol seeds extract has shown recognizable antioxidant and antimicrobial activities [6]. The antitumor and antioxidant activities of some fixed oils from Sudanese medicinal plants were evaluated, the antioxidant activity result of the fixed oil of *H. annuus* showed moderate antioxidant activity [7]. The methanol extract and its fractions of *H. annuus* leaves have shown promising anti-diabetic and antioxidant activities, the effects were attributed to the presence of saponins, tannins, alkaloids, flavonoids, and terpenes in which further purification and characterization is needed [8]. The antioxidant activity of the chloroform extract of *H. annuus* leaves was also tested and has shown significant activity [9].

The aim of this study was to apply bio-activity guided fractionation to the most active antioxidant extract of *H. annuus* leaves, which latter was led to isolation of a pure flavonoid compound which was determined qualitatively (by TLC technique and cyanidin reaction) and quantitatively (by $AlCl_3$ colorimetric assay).

## MATERIALS AND METHODS

### *Collection of the plant material*

The *H. annuus* leaves were collected (immediately after the harvesting of their seeds) from Eldamazeen (Blue Nile state) in southeast Sudan in November 2012. They were identified and authenticated by the taxonomist in Medicinal and Aromatic plants Research Institute, National Center for Research in Khartoum.

### *Extraction process*

The collected plant material was dried under shade, then it was pulverized by a pestle and mortar, then 50 grams was weighted and was extracted successively with petroleum ether, chloroform, and methanol by Soxhlet apparatus. The extracts were dried under reduced pressure and the yield percentages were calculated with the reference to the dry powder. The extracts were kept in well closed containers in the refrigerator at 4ºC.

### *DPPH radical scavenging assay*

The DPPH radical scavenging was determined according to the method by (Shimada *et al.*, 1992) [10] with some modification. In 96-wells plate, the test samples were allowed to react with 2, 2-di (4-tert-octylphenyl)-1-picrylhydrazyl stable free radical (DPPH) for half an hour at 37 °C. The concentration of DPPH was kept as 300 µm. The extract was dissolved in DMSO (500µg/ml. concentration), while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517 nm using multiple plate reader spectrophotometer. Percentage of radical scavenging activity by samples was determined in comparison with a DMSO treated control group and propyl gallate (PG). All tests and analysis were run in triplicate.

Antioxidant activity% = 100 - (As/Ac) × 100%

Where,
At = Absorbance value of sample;
Ac =Absorbance value of control.
All data were presented as means ± S.D. Statistical analysis for all the assay results were done using Microsoft Excel program (2007).

### *Antioxidant activity by using qualitative assay*

A spot from the active methanol extract of *H. annuus* leaves was put on TLC plate; another spot from a known antioxidant flavonoid compound (quercetin) was put on another TLC plate; both spots were sprayed by DPPH (4 mg of DPPH was dissolved in 10 ml of methanol), the change from the violet color of DPPH to yellow indicates the presence of antioxidant activity.

### *Determination of $IC_{50}$*

The $IC_{50}$ of the most active antioxidant fraction was determined by monitoring the effect of different concentrations of that active fraction ranging from (500- 31.25 µg/ ml). The $IC_{50}$ was calculated by using EZ- fit Enzyme Kinetic Program (Perrella Scientific Inc, USA).

### *Isolation and identification techniques*

Column chromatography and TLC techniques were used for isolation and identification, the methanol extract of *H. annuus* leaves was loaded on silica gel-60 mesh size packed in a column (3 x 50 cm) by using wet method, then it was gradient eluted by petroleum ether, chloroform, ethyl acetate, methanol and finally water. The fractions were monitored by TLC by using toluene: ethyl acetate: formic acid (5:4:1) and also petroleum ether: ethyl acetate: methanol (1:1:1); then similar fractions were combined, and finally the fractions were dried and stored in well closed containers inside the refrigerator at 4 $^0$C.

### *Detection of flavonoids in the active antioxidant fraction*

Small amount from the most active antioxidant fraction was taken and was dissolved with 1 ml methanol. Then small piece of Mg metal followed by drop wise addition of conc. HCl were added, appearance of reddish pink color indicates the presence of flavonoids while appearance of orange color indicates the presence of flavones.

### *Confirmation of the purity of the isolated flavonoid active fraction*

TLC technique was used to confirm the purity of the active antioxidant fraction, several mobile phases with different $R_f$ values were tried, and development of single separated spot was the criteria used to confirm the purity of the isolated flavonoid compound.

### *Aluminum chloride colorimetric assay method*

Total flavonoid content was measured according to Patel *et al.* (2010) method [11]. Quercetin was used to make the calibration curve in which 10 mg of quercetin was dissolved in 80% ethanol and then different dilutions (10- 100 µg /ml) were prepared. Then the most active fraction of methanol extract and different dilutions of standard solution of quercetin were added in 10 ml volumetric flask containing 4 ml of distilled water. To the above mixture, 0.3 ml of 1 M $NaNO_2$ was added. After 5 minutes, 0.3 ml of 10% of $AlCl_3$ was added. After 6 minutes, 2 ml of 1 M NaOH was added and the volume was completed to 10 ml by distilled water. Then the solution was mixed well and the absorbance was measured against freshly prepared reagent blank at 510 nm. Total flavonoid content of the most active antioxidant fraction was expressed as percentage of quercetin equivalent/ 100 g dry weight of sample.

### *Preliminary phytochemical screening of H. annuus*

The powdered plant material of *H. annuus* leaves was screened for the presence of some biologically active compounds like flavonoids, anthraquinones, cyanogenic glycosides, alkaloids, saponins, cardiac glycosides, steroids, tannins and coumarins.

## RESULTS AND DISCUSSION

The *H. annuus* leaves powder was extracted successively by Soxhlet. Petroleum ether, chloroform and methanol solvents were used, the methanol extract has shown the highest yield percentage with moderate antioxidant activity (Table 1), it was only the active extract among others with about 55% inhibition percentage of radical scavenging activity.

**Table-1: The yield percentage and percentage of radical scavenging activity (% RSA) of petroleum ether, chloroform, and methanol extracts of *H. annuus* leaves. The test was done as triplicate.**

| ***H. annuus* extract** | **Yield %** | **% RSA* ± SEM (DPPH)** |
|---|---|---|
| **Petroleum ether extract** | 3.48% | 3% ± 0.02 |
| **Chloroform extract** | 4.60% | 11% ± 0.06 |
| **Methanol extract** | 21.86% | 55% ± 0.05 |
| **Control (PG)**** | - | 90% ± 0.02 |
| **Key:** RSA*= Radicals scavenging activity. Control (PG) ** = Propyl Gallate. | | |

Then a spot from the methanol extract was put on a plate, while another spot of quercetin was put on another plate **(Figure 1)**, they were sprayed together by DPPH, immediately after spraying, the color of both spots was changed to yellow color (the color of DPPH reagent was changed from violet to yellow); this antioxidant qualitative assay encouraged us to continue for further fractionation of the methanol extract of *H. annuus* by column chromatography.

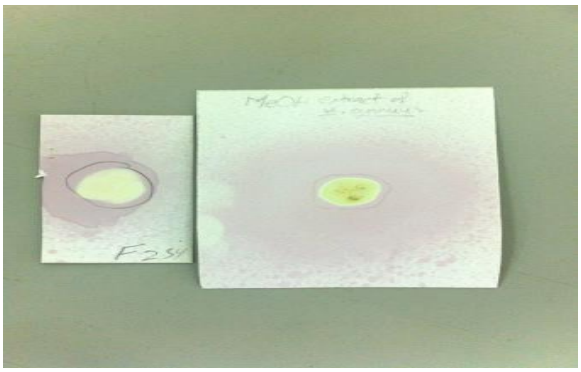

**Figure-1: A spot of methanol extract of *H. annuus* (right) and a spot of quercetin (left) after spraying with DPPH, the change from violet to yellow color indicates the presence of antioxidant activity.**

After that, 2.5 grams of methanol extract was packed on a column, eight fractions have been obtained and they were collected, dried and weighted.

Next, the inhibition percentages of RSA for the eight fractions were also determined at dose 0.5 mg/ml (Table 2). Fraction seven (F7) was the most active one which has shown about 65% of radical scavenging activity by DPPH; it was greater than the inhibition percentage RSA obtained by the crude methanol extract (55%); our explanation for this result is that the purification of the whole methanol extract by column chromatography allowed the most active fraction (F7) to act freely in reducing DPPH, so the fractionation of the methanol extract has caused separation of the most active antioxidant fraction (F7) from other less active fractions. Although some fractions were also active as antioxidant (like fraction 5 which showed 41% RSA), but still fraction 7 (F7) was the most active fraction with activity even greater than the whole methanol crude extract.

Fraction seven (F7) and propyl gallate (PG) were diluted to 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/ml and were used to determine $IC_{50}$, the obtained result has shown the decrease in the antioxidant activity of both F7 and PG with the decrease in their concentrations (Table 3); the $IC_{50}$ for F7 was calculated by using EZ- fit Enzyme Kinetic Program and was found to be 329 µg/ml (Table 4).

**Table-2: Percentage of radical scavenging activity for the eight fractions from *H. annuus* methanol extract. Propyl gallate (PG) was used as positive control.**

| Fractions of methanol extract of *H. annuus* | % RSA ± SEM (DPPH) |
|---|---|
| **1** | 10% ± 0.01 |
| **2** | 13% ± 0.02 |
| **3** | 32% ± 0.07 |
| **4** | 21% ± 0.08 |
| **5** | 41% ± 0.04 |
| **6** | 14% ± 0.02 |
| **7*** | 65% ± 0.01 |
| **8** | 11% ± 0.01 |
| **PG** | 91% ± 0.02 |
| *: Most active fraction (Mean ± SEM, n=3). | |

**Table 3: Determination of $IC_{50}$ of the most active fraction (F7) and of the positive control (PG).**

| Concentration (mg/ml) | % RSA ± SD (F7) | % RSA ± SD (PG) |
|---|---|---|
| **0.5** | 67% ± 0.72 | 91% ± 0.2 |
| **0.25** | 42% ± 0.28 | 90% ± 0.34 |
| **0.125** | 29% ± 5.36 | 66% ± 2.8 |
| **0.0625** | 21% ± 1.96 | 46% ± 0.6 |
| **0.03125** | 15% ± 0.54 | 26% ± 0.6 |

**Table-4: $IC_{50}$ value of fraction 7 (F7). The $IC_{50}$ was calculated by using EZ- fit Enzyme Kinetic Program (Perrella Scientific Inc, USA).**

| | **$IC_{50}$ ±SEM (µg/ml) (DPPH)** |
|---|---|
| F7 | 329 ±0.04 |
| PG | 77 ±0.01 |

The next step was to attempt structure elucidation to F7; more than twenty mobile phases were tried in which all of them have shown development of only one single spot with $R_f$- values ranging from 0.1 to 0.9. Some of these mobile phases were sprayed by vanillin/ $H_2SO_4$ and 1% $AlCl_3$, then all were seen under UV light in which all plates have shown the appearance of just single spot and hence we decided to name fraction seven as compound F7. After spraying by $AlCl_3$, all spots had stained with yellow color, also the spot was dark color under long UV, this observation allowed us to detect for the presence of flavonoids by cyanidin reaction (Table 5), orange color was developed which indicates the presence of flavonoids most probably of flavone type. Moreover, determination of total flavonoid content by using $AlCl_3$ colorimetric method had also proved the presence of flavonoids (Table 6). There was a study conducted for separation and isolation of flavonoids from different subspecies of *H. annuus* leaves collected from different areas in USA [12], this study has shown the presence of two types of flavonoid aglycones, they were flavones and chalcones aglycones, the isolated flavones were luteolin, nepetin, hispidulin, jaceocidine and nevadensin, while the chalcone aglycones were isoliquiritigenin, 2`,4-dihydroxy-4`chalcone and unknown third chalcone, they occurred mainly in the external structures on the leaves surfaces, also flavonol glycosides are present in both leaves and floral tissues [12]. Although nevadensin represents the highest frequency among flavonoid compounds in *H. annuus* leaves [12], but its free radical scavenging activity was not significant [13]. Therefore, it is early in this stage to conclude which type of flavonoids had caused the antioxidant activity, so further application of other spectroscopic methods like IR and NMR methods are required to detect the chemical structure of F7 and to use it as a lead for drug development.

**Table-5: Detection of flavonoids in fraction 7 (F7).**

| **Method** | **Observation** |
|---|---|
| **Cyanidin reaction** | Appearance of orange color |
| **TLC sprayed by 1% $AlCl_3$** | The separated spot get yellow color which was dark under long UV |

**Table-6: Determination of total flavonoid content (TFC) by $AlCl_3$ colorimetric assay for fraction 7 (F7).**

| | **%w/w of total Flavonoid** |
|---|---|
| **F7** | 1.509% of quercetin equivalent/ 100g dry matter |

The results of preliminary phytochemical screening of *H. annuus* leaves powder are shown in (**Table 7)**; the results were identical when compared to previous studies [5]. Application of HCl/ amyl alcohol test has revealed the presence of flavonoids as aglycone form but not as glycoside form, this observation was identical with Rieseberg *et al.*, (1987) observations [12].

**Table-7: Preliminary phytochemical screening of *H. annuus* leaves powder**

| **Phyto constituent** | **Result** |
|---|---|
| Flavonoids | + |

| | |
|---|---|
| Coumarins | + |
| Tannins | + |
| Cardiac glycosides | - |
| Saponins | + |
| Sterols | + |
| Anthraquinones | - |
| Alkaloids | + |
| Cyanogenic glycosides | - |

## CONCLUSION

This study has showed the importance of plant extracts and plant- isolated compounds as source for new molecules and leads for drug discovery and development in pharmaceutical field. The methanol leaves extract of *H. annuus* has showed moderate antioxidant activity. After fractionation of the methanol extract, the antioxidant activity of the isolated flavonoid compound from fraction 7 was further active than the crude methanol extract, but still less active than propyl gallate. Hence, structure elucidation for the isolated flavonoid compound is highly recommended in order to determine its chemical structure and then to use as lead compound for improving its antioxidant capacity with less toxic effects.

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