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**Maya Alshieka**Department of Pharmacognosy,  
Faculty of Pharmacy, Damascus  
University, Syria**Mohammad Isam Hasan Agha**Department of Pharmacognosy,  
Faculty of Pharmacy, Damascus  
University, Syria

## A comparative study of *in vitro* lipoxygenase inhibition and DPPH (1, 1-Diphenyl-2-Picrylhydrazyl) free radical scavenging activity of *Silybum marianum* and [*Notobasis syriaca* (L.) Cass.]Fruits

**Maya Alshieka and Mohammad Isam Hasan Agha**

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### Abstract

*Silybum marianum* and *Notobasis Syriaca* belong to the most widespread thorny plants in the Middle East, the Mediterranean, and large areas of Asia. Both plants are derived from the Asteraceae family.

The aim of this study was to compare the phytochemical content of the two plants, the scavenging activity of free radicals, and to evaluate the anti-inflammatory activity of their fruit extracts *in vitro*.

Methanol extracts of the fruits were prepared from both plants. The activity of scavenging free radicals was studied using DPPH, and the anti-inflammatory activity was investigated *in-vitro* using soybean lipoxygenase, in comparison with quercetin as standard.

The qualitative phytochemical screening of the fruits showed that both plants contained flavonoids, and coumarins. Both plants showed negative results with alkaloids, saponins, and anthraquinone. The fruit extract from *Notobasis Syriaca* has the highest capacity to scavenge free radical DPPH (IC<sub>50</sub>=22.1 mcg/mL), and it has higher ability to inhibit soybean lipoxygenase *in-vitro* (IC<sub>50</sub>= 2.7 mcg/mL) than fruit extract from *Silybum marianum* (IC<sub>50</sub>= 9.8 mcg/mL).

This study showed that *Silybum marianum* and *Notobasis Syriaca* have a high ability to scavenge free radicals; and that both plants possess anti-inflammatory properties.

**Keywords:** *Silybum marianum*, *Notobasis Syriaca*, chemical content, 1,1-Diphenyl-2- Picrylhydrazyl (DPPH), Soybean Lipoxygenase

### Introduction

Most diseases are caused by an oxidative imbalance which may lead to a cascade of biochemical pathways primarily related to inflammation and immune response [1].

Although the mechanisms and sequences of events by which free radicals interfere with cellular functions are not fully understood, the process of lipid peroxidation is one of the most prominent of these mechanisms; which it produces lipid peroxides as oxidation products, which leads to cell membrane damage [2].

The presence of lipoxygenase enzyme activity in plants, which stimulate the oxidation of fatty acids, was demonstrated in the last 60 years [3]. The lipoxygenase enzyme from soybean (*Glycine max*) was among the first enzymes to be isolated in the early 1970s by Axelord and Coworkers [3].

Some evidence indicates that lipoxygenase contribute to vascular changes associated with inflammatory process; while the lipoxygenase enzyme (LOX) is also associated with many diseases such as arthritis, allergic asthma, psoriasis, enteritis, cardiovascular disease, skin diseases, and neurological diseases such as Alzheimer's. In addition to its role in some types of cancers such as prostate, colon and lung cancer; among others [4].

There are many lipoxygenase inhibitors (LOX) such as "zileuton and minocycline", which have many side effects, including stomach pain, muscle pain, throat pain, photosensitivity, dizziness, and headaches. Consequently, their use has been limited, and it is necessary to develop new drugs that inhibit the lipoxygenase enzyme LOX with minimal side effects [5].

There are a number of natural compounds isolated from some plants, which reduce the inflammatory processes, by inhibiting the synthesis pathways of the enzymes cyclooxygenase (COX) and lipoxygenase (LOX), and perhaps through some other mechanisms as well. Flavonoids are among the most important of these compounds [6, 2].

One of the most important biological effects of flavonoids is the inhibitory effect of free radicals derived from oxygen, and the results of *in vitro* experiments have shown that flavonoids have anti-inflammatory, anti-allergic, anti-viral, and anti-cancer properties [2].

**Corresponding Author:****Maya Alshieka**Department of Pharmacognosy,  
Faculty of Pharmacy, Damascus  
University, Syria

*Notobasis Syriaca* and *Silybum marianum* are widely spread in the Mediterranean and large areas of Asia, and studies indicate that extracts of both plants have antioxidant and anti-inflammatory effects due to their content of polyphenols, mainly represented by flavonoids [7, 8]. The aim of this study was to evaluate the inhibitory activity of soybean lipoxygenase of extracts prepared from *Silybum marianum* and *Notobasis Syriaca* fruits.

### Materials and Methods Plant material

Both plants were collected from Zabadani Plain by Damascus, Syria; in May and June 2019.

The ripe fruits were separated from the aerial parts; and dried and grinded.

### Preparation of the extracts

30 g of the fruits powder of both plants were extracted with petroleum ether using Soxhlet Ext. method, to eliminate fatty substances. The extracts were then treated with methanol using Soxhlet Ext. method; and the process was repeated twice. The extracts obtained from both plants were condensed using a rotary evaporator at a temperature of 50 °C [9].

### Qualitative phytochemical detection

The fruit powder of two plants were tested for the presence of the main phytochemical groups such as flavonoids, coumarins, saponins, alkaloids, tannins; and anthraquinone [10, 11].

### Alkaloids

Concentrated extract (2 mL) was taken into a test tube and 1 mL HCl was added.

The mixture was heated gently for 20 minutes, cooled and filtered, then treated with following reagents:

- Wagner's reagent: formation of brown-reddish precipitate indicates presence of alkaloids.
- Mayer's reagent: formation of cream-white colored precipitation indicates the presence of alkaloids.

### Saponin

0,5 g fruit powder was mixed with 10 mL of distilled water then agitated For 10 minutes. Formation of foam indicates Saponin.

### Tannins

- Lead acetate test: 3 mL fruit extract was treated with 1% of lead acetate. Formation of cream gelatinous precipitation indicates tannins.
- Ferric chloride test: 3 mL extract was treated with 3 mL FeCl<sub>3</sub>. Formation of greenish to black color indicates a positive test.

### Coumarin

Aqueous fruit extract was diluted with a solution of NaOH, boiled for 3 minutes then was exposed to UV light to observe the yellow-greenish color that indicates coumarins.

### Flavonoids

Shinoda test: ethanolic extract of fruits were treated with Mg powder and concentrated HCl. The formation of crimson red color indicates Flavonoid.

**Alkaline reagent test:** extract was treated with 10% NaOH solution. Formation of intense yellow color indicates presence of Flavonoid.

### Anthraquinones

The fruit powder was boiled with dilute sulfuric acid. The filtrate chloroform was added and shaken well. The organic layer is separated to which ammonia was added slowly. The ammonia layer shows pink to red color due the presences of anthraquinone.

### DPPH free radical scavenging activity

The principle of the test is based on measuring the ability of the plant extract to scavenge the free radical DPPH by giving a hydrogen atom to the free radical. The change in color is measured by spectrophotometer at wavelength of  $\lambda = 517$  nm. The standard curve of quercetin was used to determine the inhibition ratio as shown in Figure (1). The reaction was carried out by mixing 1 mL of each plant fruit extract with 2 mL of DPPH solution (2.5 mg / 100 mL), then the tubes were incubated in the dark for 30 minutes. [12]. The experiment was repeated in triplicate, and the mean value was determined. Scavenging ability (%) was calculated by using the formula: DPPH radical scavenging activity (%) =  $[(A_0 - A_1)/A_0] \times 100$ , where A<sub>0</sub> is the absorbance of reaction control and A<sub>1</sub> is the absorbance of extracts or standards.

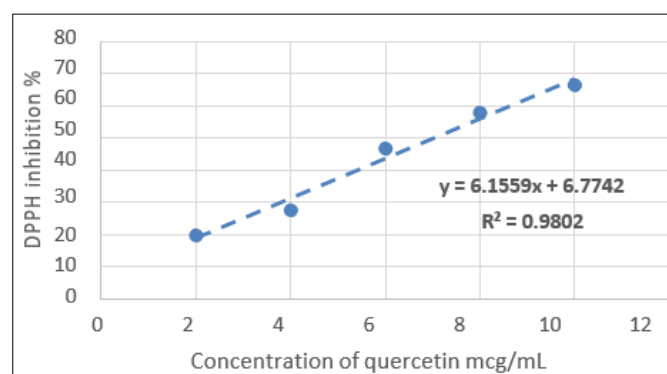
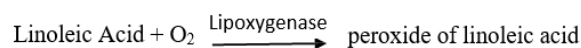


Fig 1: Standard curve of DPPH inhibition by quercetin

### In-vitro Soybean Lipoxygenase Inhibition

The inhibitory activity of the lipoxygenase enzyme is measured using spectrophotometer (Yawer *et al.* 2007) [13]. The lipoxygenase enzyme oxidizes linoleic acid according to the following reaction:



The peroxide of linoleic acid is expressed in the following formula: (9Z, 11E) - (13S) -13-Hydroperoxyoctadeca-9, 11-dienoate A series of diluted solutions of the methanol extract of the fruits of both plants were applied to the enzyme according to the following concentrations (10, 25, 50, 100, 250, 500 mcg / mL), and the absorbance of the enzymatic reaction was measured at wavelength of  $\lambda = 234$  nm.

The enzyme solution (10,000 U / mL) was mixed with borate buffer (0.2 mol PH = 9), then the solution of the plant sample was added; and the mixture was incubated for 10 minutes at 25 °C. Linoleic acid substrate solution (0.8  $\mu$ mol) was added; and the absorbance was determined.

Each trial was repeated in triplicate, and the mean was determined.

The percentage of inhibition activity of lipoxygenase was calculated by using the formula:

$$\% \text{ Inhibition} = \left[ \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \right] \times 100$$

Where Abs Control value is the value of the enzyme activity without the inhibitor, and the Abs Sample is the value of the enzyme activity in the presence of the inhibitor.

## Results and Discussion

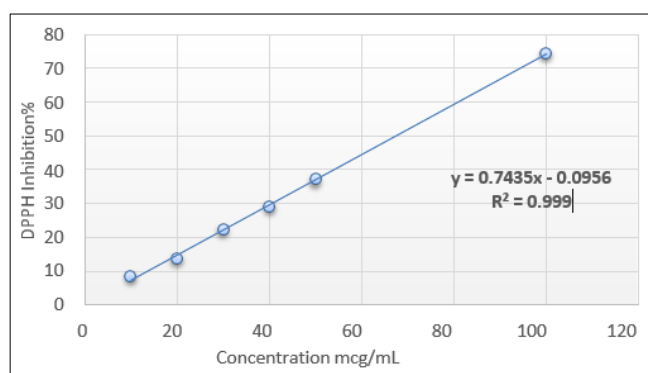
### Chemical constituents

The results of the qualitative phytochemical detection of fruits of *Silybum marianum* showed that it contains flavonoids, coumarins, and tannins. These results agree with the results shown by (Ibrahim J. Abed *et al.* 2015) [11].

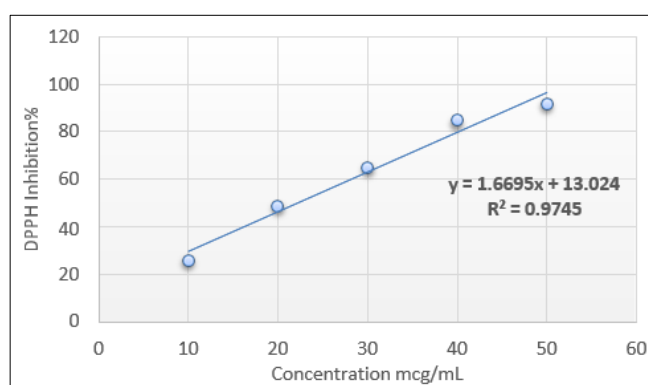
The fruits of *Notobasis Syriaca* also contain flavonoids; and coumarins, however; *Notobasis Syriaca* fruits did not produce a precipitate with lead acetate; but a green color appeared with iron chloride. This can indicate the presence of pseudo tannins. None of the plants contained alkaloids, saponins; or anthraquinones.

### DPPH free radical scavenging activity test

The results showed that the fruit extracts of both plants have a high activity as a scavenger for free radicals. The extract with the lowest IC<sub>50</sub> values have got a higher effectiveness in scavenging free radicals, and it was found that the fruit extract of *Notobasis Syriaca* has the highest free radical scavenging activity (IC<sub>50</sub> = 22.1 µg/mL), followed by the fruit extract from *Silybum marianum* (IC<sub>50</sub> = 67.3 µg/mL), Figure (2, 3).



**Fig 2:** Standard curve of DPPH inhibition by *Silybum marianum* fruit extract



**Fig 3:** Standard curve of DPPH inhibition by *Notobasis Syriaca* fruit extract

It was found that *Silybum marianum* has antioxidant capacity due to its high content of phenols and flavonoids [14, 15].

It is difficult to compare these findings in terms of IC<sub>50</sub> values, however, as these results differ according to the method used; and the phenotype of *Silybum marianum* [12]. The IC<sub>50</sub> value also varies according to the genotype of *Silybum marianum*, which can be attributed to the region from which it was collected [16].

Furthermore, the antioxidant capacity is influenced by environmental factors and surrounding climatic conditions. In the study by (Adnan Zahir *et al.* 2014), it was shown that the ability to scavenge free radical DPPH is affected by induced drought tests [17].

This ability is also affected by the structure and chemical nature of the flavonoids [18], as studies

showed that flavolignan compounds, like silybin, have less free radical scavenging activity than that of flavonols (quercetin, kaempferol) and flavones (apigenin, luteolin) [18].

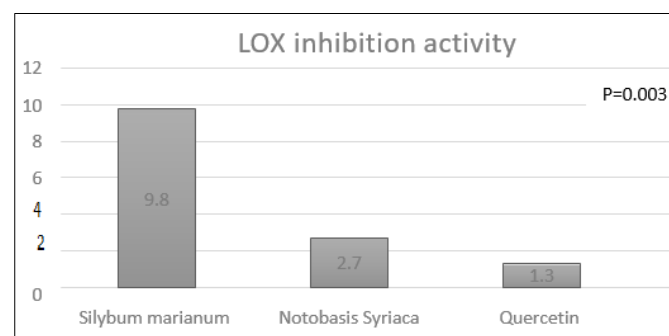
Fruits of *Silybum marianum* contain a mixture of flavonoids (quercetin, taxifolin, chrysoeriol, and luteolin), but it mainly contains flavolignans represented primarily by silybin [16, 19].

The aerial parts of *Notobasis Syriaca* contain (luteolin, chrysoeriol, apigenin, kaempferol, isorhamnetin) [20]. This supports the observation that *Silybum marianum* fruit extract have less free radical scavenging activity than those of *Notobasis Syriaca*.

### Inhibitory activity of lipoxygenase

By determining the IC<sub>50</sub> values it was found that the fruit extract from the *Notobasis Syriaca* (IC<sub>50</sub> = µg/mL) showed a statistically significant difference ( $P < 0.05$ ) than the fruits' extract of *Silybum marianum* (IC<sub>50</sub> = 9.8 µg/mL), and thus possessed higher inhibitory activity of the lipoxygenase enzyme, as shown in figure (4).

A dose-response curve of the plant extract was obtained.



**Fig 4:** IC<sub>50</sub> values for each of the two methanol fruit extracts compared to the quercetin standard

Many natural and synthetic free radical scavengers and antioxidant compounds are known as inhibitors of lipoxygenase [21]. Phenolic compounds can block the cascade of arachidonic acid by inhibiting the activity of the lipoxygenase enzyme, and can also scavenge the reactive free radicals which are produced during the metabolism of arachidonic acid [22].

In general, it is difficult to determine a common pattern of inhibition among polyphenols; and some studies assume that quercetin inhibits lipoxygenase enzyme by binding in the hydrophobic cavity of the enzyme, preventing the substrate from reaching the catalytic site [23].

Flavonoids act to chelate heavy metals; when the flavonoids bind to the active site of the lipoxygenase enzyme, it can result in a direct reduction of iron ions Fe<sup>3+</sup> to its inactive form Fe<sup>2+</sup> [23]. Another suggested mechanism in which flavonoids exhibit their anti-inflammatory properties; is through the effective reduction of hydroperoxide compounds, which are strong activators of lipoxygenase by converting the silent iron ions Fe<sup>2+</sup> into their active form Fe<sup>3+</sup> [23].

The lipoxygenase inhibitory activity of flavonoids cannot be attributed to its free radical scavenging properties. Despite the formation of free radicals during the lipoxygenase reaction,

these radicals are strongly bound to the active site of the enzyme and it cannot be reached by free radical scavengers. In some cases, however, these free radicals may escape from the active site of lipoxygenase and cause secondary lipid peroxidation, which are suppressed by free radical scavengers [24].

In order to explain our results for the two extracts, it was necessary to clarify the structure-activity relationship between the inhibiting compound and the lipoxygenase inhibitory effect:

The presence of a catechol group on the A or B ring, figure (5, 6), increases the inhibition of the enzyme, but their presence is not essential for the expression of inhibitory activity [24].

The catechol group is present in luteolin which has been isolated from aerial parts of *Notobasis Syriaca* [20].

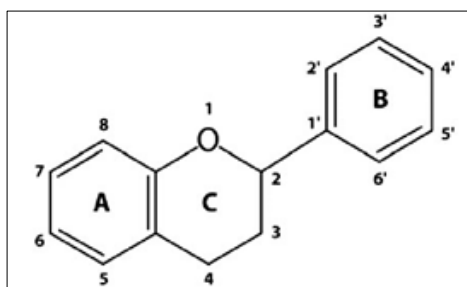


Fig 5: Basic structure of flavonoid

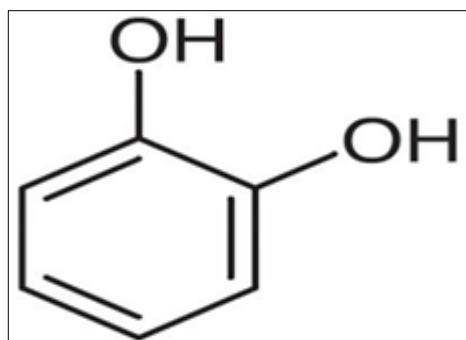


Fig 6: Catechol group

The catechol group is also found in quercetin, despite *Silybum marianum* fruits containing luteolin and quercetin, but silybin, which is the main component of *Silybum marianum* fruit, does not contain the catechol group; and that could reduce the ability of lipoxygenase inhibition by *Silybum marianum* fruit extracts [16, 19].

As for the compounds containing the catechol group, there is an inverse relationship between the total of hydroxyl groups and the necessary concentration of the inhibitory compound. This explains the low IC50 value shown by the fruits' extract of *Notobasis Syriaca* [24].

The presence of the methoxy group (O-CH<sub>3</sub>) increases the lipophilic property, which would increase the ability to bind to the hydrophobic cavity of lipoxygenase; and increase the inhibitory activity of the enzyme [24]. This group is found in kaempferol and isorhamnetin isolated from *Notobasis Syriaca*, and it is also found in chrysoeriol, which is present in both plants [20].

The presence of the C2-C3 double bond is necessary for the inhibitory activity of the enzyme, and its absence would significantly reduce the activity [24]. This bond is found in Luteolin, Quercetin, Chrysoeriol, and Kaempferol, while it is

not present in Silybin, the main component of *Silybum marianum* fruits.

The results of this study agreed with the study of Akula and colleagues 2008, in which methanol extracts of 18 different plants were applied to lipoxygenase enzyme, and the extracts from the plants of the Asteraceae family have specifically shown high lipoxygenase inhibitory activity. This indicates that the plants of Asteraceae family have high anti-inflammatory activity [22].

## Conclusion

This study showed that *Silybum marianum* and *Notobasis Syriaca* have a high ability to scavenge free radicals related to their content of phenols and flavonoids. Both plants also showed anti-inflammatory properties. These results justify further research and studies in the future into *Notobasis Syriaca*, to determine its beneficial pharmacological effects, as well as to determine the chemical composition of *Notobasis Syriaca* extract and its most effective ingredients.

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