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Research Article

PRELIMINARY PHYTOCHEMICAL SCREENING AND IN-VITRO EVALUATION OF ANTIOXIDANT ACTIVITY OF IRAQI SPECIES OF *SILYBUM MARIANUM* SEEDS

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ABSTRACT
Silybum marianum is medicinally important plant species from Asteraceae family. It has been used for centuries as a herbal medicine for the treatment of many diseases particularly liver diseases. In the present work the seeds of the plant were extracted by two methods: maceration using 80 % ethanol and soxhlet extraction using petroleum ether and methanol, respectively. Fractional separation of ethanolic extract was carried out using solvents with various polarities like: petroleum ether, chloroform, ethyl acetate and n-butanol. Ethanolic extract was subjected to phytochemical screening to detect the presence of bioactive compounds. The study revealed the presence of flavonoids, phenols, tannins, terpenoids, carbohydrates and proteins. All fractions and extracts were subjected to TLC analysis to determine the phytochemical compounds. Results of the retention factor (R_f) obtained were compared with that of the silymarin standard TLC system. The TLC finger print of the methanolic, ethanolic extracts and ethyl acetate fraction using the solvent system chloroform : acetone : formic Acid (75: 16.5:8.5), showed the spots having R_f = 0.63, 0.4, 0.35 which was equivalent to silybin, taxifolin and silichristin, respectively. Evaluation of antioxidant activity was carried out by two methods, the free radical scavenging activity of the 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), and total antioxidant capacity (TAC) by the phosphomolybdenum method. Estimation of total phenolic contents was performed using Folin-Ciocâlteu method. Antioxidant assays revealed that ethyl acetate fraction exhibited highest percent inhibition of DPPH followed by methanolic and ethanolic extracts. It also showed the highest value of total antioxidant capacity as well as highest amount of total phenolic compounds. Our findings revealed that Iraqi species of *Silybum marianum* seeds can be considered as a valuable source of natural antioxidants which may be attributed to the presence of silymarin a powerful antioxidant herbal drug which can protect biological systems against the oxidative stress.
Keywords: *Silybum marianum*; phytochemicals; antioxidant; DPPH; total antioxidant capacity; total phenolic contents.

INTRODUCTION

Plants are potent biochemical factories and have been components of phytomedicine since times immemorial. Medicinal plants play an important role in human health care. About 80 % of the world population relies on the use of traditional medicine, which is mainly based on plant material[1]. Recently, various medicinal plants and their phytoextracts have shown numerous medicinal properties like antioxidant, anti-inflammation, anti-cancer, anti-microbial, anti-diabetes, anti-nociceptive action, etc.[2] The Reactive Oxygen Species (ROS), such as free radical superoxide anion (O_2•–), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH•), have a great effect on humans both from within the body and the environment. They are continuously produced during cell metabolism, or sometimes the immune cells produced them to neutralize the foreign bodies. Moreover, environmental factors such as radiation, pollution, herbicides, cigarette smoke and certain foods can also create free radicals[3]. ROS play an important role in the physiological process; however, because of their toxicity, their levels must be controlled by the endogenous antioxidant system. But when ROS production is increased, an imbalance is promoted between these and the antioxidant molecules; phenomenon known as Oxidative Stress (OS) which can adversely affect various cellular bio molecules like proteins, RNA and DNA, causing serious damage to tissues and organs resulting in chronic disease such as: cancer, heart diseases, autoimmune diseases, diabetes mellitus, arthritis, and neurodegenerative disease[4]. Antioxidants act as free radical scavengers, reducing agents, quenchers of singlet oxygen molecule, and activators for anti oxidative enzyme to suppress the damage induced by free radicals in biological system[5]. Synthetic antioxidants like butylated hydroxyl toluene (BHT), propylgallate (PG) are known to prevent oxidative damages but they have been restricted due to their carcinogenic and toxic effects. Therefore, investigations of antioxidants are focused on naturally occurring substances, especially plant phytochemicals, like flavonoids and phenolic compounds[6]. *Silybum marianum*, commonly known as (milk thistle), belonging to Asteraceae family, is an annual or biennial herb, native to the Mediterranean and North African regions, but now widespread throughout the world.[8] The plant has an erect, tall stem, each stem bears a single, large, purple flower ending in sharp spines. The seeds are black to glossy brown in color and bear white silky pappus[9], the leaves are alternate, large and glabrous, contain milky white veins. The common name, milk thistle, is derived from these "milky white" veins, which, when broken open, yield a milky sap.

Silymarin, the principal active constituents of milk thistle is an isomeric mixture of flavonolignans, the main components of silymarin are silybin, isosilybin, silychristin and silydianin (Figure 1). Silymarin localized mainly in the external cover of the seeds (1.5-3 %). Other constituents are essential fatty acids, flavonoids e.g. taxifolin (Figure 1), kaempferol and quercetin.[10] The major pharmacological activities of milk thistle are hepatoprotective, antioxidant, anticancer, antibacterial, antidepressant, anti-inflammatory, cardio protective, demulcent, digestive tonic, immune stimulatory and a neuroprotective[9]. Because of these activities the plant has been used to treat a wide variety of diseases like; liver cirrhosis, acute and chronic hepatitis, toxin-induced liver damage (prevention of liver damage from Amanita phalloides ('death cap' mushroom poisoning), gallbladder disorders. lung ailments, migraine, motion sickness, diabetes mellitus, psoriasis, spleen disorders, skin cancer, hypercholesterolemia, Alzheimer's disease, menstrual

disorders and infections.[8,10] The present work was aimed to investigate the phytoconstituents, estimate the total phenolic contents and evaluate the antioxidant potency of Iraqi species of *Silybum marianum* plant, therefore, justifying the use of plant in ethno-medicine for treatment of various ailments.

MATERIALS AND METHODS

Collection and Identification of Plant materials

The top flowers of plant *Silybum marianum* were collected from different regions of Mosul, botanically identified by Dr. Yonis kasim Ahmad. Agriculture College/Mosul University, the seeds were separated from the plant and washed with sufficient water, dried under shade for several days, then powdered in an electrical blender.

Chemicals and reagents

All the chemicals and reagents used in the research were of analytical grade.

Preparation of Extract

Soxhlet extraction

A (100 g) of powdered seeds of *Silybum marianum* were subjected to soxhlet extraction using petroleum ether for (4 h) in order to deffating the seeds. The solvent was discarded; the residue was dried in air then extracted with methanol for (5 h). The methanolic extract was concentrated under reduced pressure using rotary evaporator at 45°c. Then freeze dried using lyophiliezer to achieve complete dryness.[10]

Extraction by maceration and fractionation of the plant Constitutes

The air dried powdered seeds of *Silybum marianum* (500 g) were macerated with 80 % ethanol by 3 days soaking with stirring using magnetic stirrer. After maceration the extract was filtered, the ethanolic extract was concentrated under reduced pressure using rotary evaporator at 45°c. The resultant gummy solution was freeze dried using lyophiliezer until complete dryness. For further separation of the polar and non-polar components of the ethanolic extract, Liquid–liquid partitioning was applied using petroleum ether, chloroform, ethyl acetate and *n*-butanol (3 × 100 ml) in a separatory funnel and each fraction was concentrated and dried using rotary evaporator at 45°c. Then the fractions were labeled for further investigations.

Phytochemical Screening

Phytochemical screening were carried out using standard procedures.[11-14]

Test for Alkaloids

Dragendroff's Test

In a test tube containing 1 ml of extract, few drops of dragendroff's reagent was added and the color developed was noticed. Appearance of orange color indicates the presence of alkaloids.

Mayer's Test

To 1 ml of the extract, 2 ml of mayer's reagent was added, a dull white precipitate indicates the presence of alkaloids.

Wagner's Test

To 1 ml of the extract, 2 ml of wagner's reagent was added. The appearance of a reddish brown precipitate indicates the presence of alkaloids.

Test for Flavonoids

A few drops of 1 % aluminium solution were added to 1 ml of the extract. A yellow color indicates the presence of flavonoids.

NaOH test

1 ml of extract was treated with aqueous NaOH and HCl, observed for the formation of yellow orange colour which disappears on addition of HCL.

H_2SO_4 test

1 ml of extract was treated with concentrated H_2SO_4 and observed for the formation of orange color.

Test for Phenols

Ferric chloride test

1 ml of the extract was treated with 10 % aqueous ferric chloride and observed for formation of deep blue or black color.

Test for Saponins

Distilled water 2 ml was added to 2 ml of extract and shaken vigorously in a graduated cylinder for about 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Test for Tannins

To 1 ml of the extract, 2 ml of 5 % ferric chloride was added. Formation of a dark blue or greenish black color showed the presence of tannins.

Test for Terpenoids (Salkowski Test)

2 ml of chloroform was added to 0.5 g of the extract. Concentrated H_2SO_4 (3 ml) was added carefully to form a layer. A reddish –brown coloration at the interface indicates the presence of terpenoids.

Test for Quinones

1 ml of extract was treated with concentrated HCl and observed for the formation of yellow color precipitate.

Test for Anthocyanin

NaOH test

1 ml of extract was treated with 2 M NaOH and observed for the formation of blue green color.

Test for Proteins

Ninhydrin test (aqueous)

1 ml of extract was treated with aqueous ninhydrin. Purple color indicates the presence of protein.

Test for Carbohydrates

Benedict's test

To 1 ml of the extract, 5 ml of Benedict's reagent were added. The mixture was heated; appearance of red precipitate indicated the presence of reducing sugars.

Iodine test

To 1 ml of the extract, 2 ml of iodine solution were added; appearance of dark blue or purple color indicates the presence of polysaccharides.

Test for Anthraquinones

Few drops of 2 % HCL were added to 1 ml of the extract. Appearance of red precipitate indicates the presence of anthraquinones.

Thin Layer Chromatography

Thin layer chromatography was performed on ethanolic, methanolic crude extracts, chloroform, ethyl acetate and n-butanol fractions using TLC analytical plates over silica gel GF 250. These plates were developed in various solvent systems like: S_1: chloroform - acetone -formic Acid having a ratio of (75: 16.5:8.5) and S_2: ethyl acetate- glacial acetic acid-formic acid -water (100:11:11:26), to select the mobile phase that achieves the best separation[15]. Detection of the spot was done using: vanillin sulfuric acid reagent, aluminum chloride spray reagent, iodine vapor, UV light 365 nm. The retention factors values (R_f) of the samples spots were then determined and compared with silymarin standard.

Determination of Total phenolic content by Folin-Ciocalteu method

Total phenolic content of ethanolic, methanolic crude extract and ethyl acetate fraction was determined by Folin-Ciocalteu method[16]. An aliquot of 0.5 ml of the sample (1 mg/ml) was mixed with 1.25 ml FC reagent and kept for 5 minutes and then 2.5 ml of 20 % sodium carbonate solution was added and made up to10 ml with distilled water. The mixture was kept in the dark for 30 minutes and absorbance was measured at 765 nm by UV-visible spectrophotometer. Total phenolics were expressed as milligrams of tannic acid equivalents (TAE) per gram of the sample using standard calibration curve constructed for various concentrations of tannic acid. Results were expressed as (mg TAE/g of dry extract).

Evaluation of *In-vitro* Antioxidant activity

The following antioxidant assays were performed on ethanolic, methanolic extracts and ethyl acetate fraction.

Evaluation of free radical scavenging activity by DPPH method

The determination of the radical scavenging activity of the extract was carried out using the stable DPPH (1, 1-diphenyl-2 picryl hydrazyl) assay as described by Brand-Williams *et al*[17]. Briefly 250 µl of the extract (1 mg/ml) and standard ascorbic acid was diluted with distilled water to 10 ml. Aliquots of 200 µl of sample was mixed with 2 ml of 100 µM DPPH methanolic solution. The mixture was placed in the dark at room temperature for 120 minutes. The absorbance of the resulting solution was then read at 520 nm against methanol as a blank in the spectrophotometer, after (30, 60, 120) minutes. The antiradical activity was expressed in terms of the percentage reduction of the DPPH. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0-A_1)/A_0]*100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample

Evaluation of total antioxidant capacity (TAC) by Phosphomolybdenum method

The total antioxidant capacity of the extract was evaluated by the phosphomolybdenum method according to the procedure described by Prieto et al. (1999)[18]. An aliquot of 100 µL of sample solution (100 mg/mL) was mixed with 1 mL of reagent (600 mM sulphuric acid, 28 mM sodium phosphate and 4 mM. ammonium molybdate). Then the tubes were capped and incubated in a water bath at 95°C for 90 minutes. After cooling to room temperature, the absorbance of the samples was measured at 695 nm against a blank (1 mL of reagent and 100 µL of the methanol). Ascorbic acid used as a standard. The total antioxidant activity was expressed as the absorbance value at 695 nm.

RESULTS AND DISCUSSION

Phytochemical screening

The various chemical tests were performed for establishing profile of the extract for its chemical composition. These qualitative phytochemical tests provides an essential information that are necessary for the discovery of any new drug having pharmacological importance, since they provides information about the chemical constituents and the presence of any particular primary or secondary metabolites having a clinical significance[19]. The ethanolic seed extract of Silybum marianum was screened for the presence of various bioactive phytochemical compounds. Phytochemical analysis revealed the presence of flavonoids, phenols, Terpenoids, tannins, carbohydrates and protein and the absence of alkaloids, quinines, saponin, anthocyanins and anthraquinons. (Table 1) The findings are also in line of previous findings and reported literature[20]. The presence of wide range of phytochemical constituents particularly the flavonoids and phenols indicate that this plant exhibits multiple biological activities and can be used in the treatment of various disorders.

TLC Analysis

Thin layer chromatography was used to determine the phytochemical compounds of flavonoids present in different solvents extract of the plant. TLC analysis showed methanolic, ethanolic extract and ethyl acetate fraction gave Rf values (0.35-0.4- 0.63) using the best mobile phase chloroform: acetone: Formic Acid (75:16.5:8.5) corresponding to silychristin, taxifolin and silybin, respectively. These Rf values are the same as those obtained from TLC of silymarin standard[15].

Total Phenolic Content

Phenolic compounds, a large group of plant secondary metabolites, are potent free radical scavengers and also exhibit multiple medicinal and physiological functions in human and plant. Phenolic compounds play an important preventive role in the development of cancer, heart diseases and ageing related diseases. Table 3 shows the total phenol content (TPC) expressed as tannic acid equivalents (TAE) achieved by FC method. The ethyl acetate fraction showed the highest amount of total phenolic compounds 425 TAE mg/g while that of methanolic extract was found to be 215 TAEmg/g, the total phenolic contents of ethanolic extract was 60 TAE mg/g. It is clear from these results that the total phenolic contents in Silybum marianum extract depends on the type of extract, i.e. the polarity of solvent used in extraction. The Folin-Ciocalteu method is a colorimetric assay commonly used to measure the total concentration of phenolic hydroxyl groups in the plant extract. Phenolic compounds undergo a complex redox reaction with the phosphotungstic and phosphomolybdic acids present in the Folin Ciocalteu (Fc) reagent to form a blue complex that can be quantified by visible-light spectrophotometry[21].

DPPH Radical Scavenging Activity

The DPPH assay is a quick, sensitive and reproducible parameter. It has been commonly used to evaluate the antioxidant activity of pure compounds as well as plant extract[22]. Methanolic, ethanolic extract and ethyl acetate fraction were evaluated for their DPPH scavenging activity and the antioxidant activity was determined in time dependent manner. All extract showed a significant scavenging activity compared to the standard antioxidant ascorbic acid. This activity was increased in time dependent manner. The maximum scavenging activity was achieved after 120 minutes. The ethyl acetate fraction showed a highest scavenging activity 74.6 %, followed by methanolic extract 72.5 %, then ethanolic extract 54 %. The DPPH test is based on the exchange of hydrogen atoms between the antioxidant and the stable DPPH free radical. Practically, the reaction brings about the reduction of DPPH radicals to the corresponding hydrazine; that is manifested by a color change from violet to yellow, which is monitored spectrophotometrically[23]. The prominent scavenging activity of *S. marianum* may be due to synergistic antioxidant activity of various phytochemicals present in the plant extract like: flavonoids and phenols[24]. Flavonoids are class of secondary plant metabolites, with significant antioxidant and chelating activities. Biological and pharmacological properties of flavonoids depend on their antioxidant activity. Chemically the flavonoids are polyphenolic compounds, antioxidant activity of flavonoids depends on the number, structure and substitution pattern of hydroxyl groups, the more hydroxyl moieties the higher antioxidant activity[25]. The presence of flavonoids in the extract might contribute to the free radical scavenging activity because their hydrogen donating ability helps in terminating the chain reaction[26].

Total Antioxidant Activity by Phosphomolybdenum Method

The total antioxidant activity of methanolic, ethanolic extract and ethyl acetate fraction were measured spectrophotometrically by the phosphomolybdenum method, which is based on the reduction of Mo (VI) to Mo (V) by the sample and the subsequent formation of green phosphate/Mo (V) compounds at acidic pH, with a maximum absorption 695 nm[27]. A high absorbance value of the sample indicates its strong antioxidant activity. The total antioxidant activities of these extracts were compared with the standard antioxidant ascorbic acid, the results shown in Table 2. All the extracts showed a good total antioxidant capacity, ethyl acetate fraction showed the highest TAC 0.93, followed by methanolic extract 0.83 and ethanolic extract was 0.64. TAC of both ethyl acetate fraction and methanolic extract were higher than that of standard ascorbic acid 0.79. TAC of the phosphomolybdenum model measures both water-soluble and fat-soluble antioxidant capacity (total antioxidant capacity)[28]. The total antioxidant capacity of S. marianum may be attributed to total phenolic and flavonoid contents.

Table 1: Phytochemical screening of ethanolic seed extract of *Silybum marianum*

phytochemicals	Ethanol extract
Flavonoids	++
phenols	++
Terpenoiods	+
Tannins	+
Anthocyanin	--
Saponins	--
Alkaloids	--
Proteins	+
Carbohydrates reducing sugar polysaccarides	 + --
Quinns	--
Anthraquinons	--

Present; ++ Strongly Present; - Absent +

Table 2: Free radical scavenging activity

Sample	%DPPH scavenging activity			Total phenolic contents (mg TAE/g dry extract)	Total antioxidant activity
	After30 minutes	After 60 minutes	After 120 minutes		
Ethyl acetate fraction	57	66.8	74.6	425	0.93
Methanolic extract	52	65	72.5	215	0.83
Ethanolic extract	30	45	54	60	0.64
Ascorbic acid	92	96	96.3	--	0.79

a:Total phenolic contents expressed as tannic acid equivalents (TAE) /g of dry extract.
b : The total antioxidant activity was expressed as the absorbance value at 695 nm.

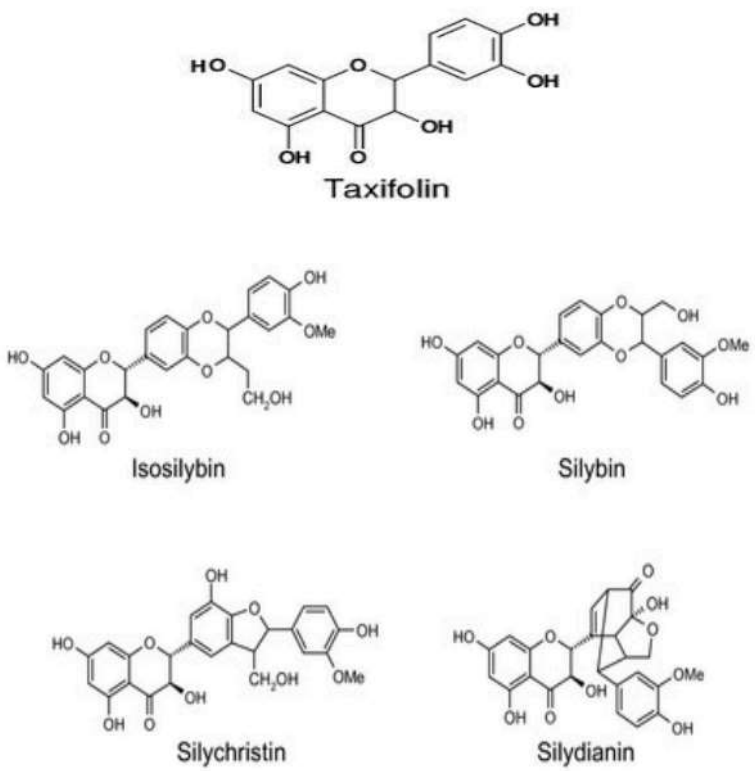

Figure 1: Chemical structures of silymarin components and flavonoid taxifolin

CONCLUSION

In our present work we conclude that extracts of Silybum marianum are rich in phytochemicals particularly phenols and flavonoids, The results of antioxidant evaluation based on two models (DPPH and TAC) used in this study revealed that different extracts and especially ethyl acetate extract of this plant have a powerful antioxidant activity. Our findings reinforce the potentials of Silybum marianum as a valuable source of natural antioxidants and support its medical uses in the treatment of many diseases.

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