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Antioxidant Activities, Total Flavonoids and Phenolics Content in Different Parts of Silybum Marianum L. Plants

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Silybum marianum L. is used for the production of silymarin, a flavonoid utilized for regenerating damaged hepatic tissues. Herein, the total flavonoid content (TFC) and total phenolic content (TPC) in the roots, main stems, leaves, fruit receptacles, and pappi of *Silybum marianum* were determined. The antioxidant activities of plant ethanol extracts were assessed to validate the medicinal potential of the various plant parts. The pappi exhibited the highest TFC (17.10 mg rutin/g of dry plant material), followed by the fruit receptacles (15.34 mg/g). The TPC varied from 9.80±0.13 to 48.97±0.41 mg gallic acid/g dry plant material; again, the highest TPC was obtained from the pappi. At 50 ug/mL, the pappi ethanol extract showed the highest 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity (69.68%), followed by the roots (66.02%). The IC_{50} values of the pappi (24.20 μg/mL) and roots (25.16 μg/mL) also indicated high scavenging activity. The same order was obtained for the ferric reducing antioxidant power (FRAP) of the ethanol extracts of different parts. Thus, the pappi and roots of *Silybum marianum L.* can be used as valuable sources of natural antioxtidants.

1. Introduction

Silybum marianum L. (milk thistle) is an important herb native to the region extending from Southern Europe to North Africa. The plant is cultivated throughout the world for the medicinal potential of its seeds. The main active compound in the seeds is a flavonoid known as silymarin, which is widely used for regenerating damaged hepatic tissues (Al-Anati et al, 2009; Jayaraj et al, 2007) .

Free radicals are produced by oxidation reactions in the human body, especially during stress. Chain reactions that sometimes damage cells and cause serious diseases such as various cancers, cardiovascular diseases, and neurodegenerative diseases can be induced by these toxic radicals (Ahmad et al, 2012; Astley, 2003). Antioxidants are compounds that delay or prevent oxidation reactions by undergoing oxidization themselves (Ahmad et al, 2011). Apart from their important role as health benefactors, antioxidants are also used to prevent the deterioration of food (Hotta et al, 2002). Most of the antioxidants utilized nowadays are manufactured synthetically, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), with side effects when taken in vivo. Because of the disadvantages of synthetic antioxidants, the search for natural antioxidants is becoming more prevalent. Secondary metabolites which are the main active compounds of medical plants are a potential source of natural antioxidants. Phenolic compounds are the main antioxidant agents of medicinal plants and several thousand phenolic structures have been identified (Dai and Mumper, 2010; Zuorro et al, 2014). Similarly, flavonoids, also exist in many edible plant sources and constitute important secondary metabolites with antioxidant, hepatoprotective, anti-inflammatory, and antiallergic activities (Tapas et al, 2008).

Hepatoprotective and antioxidative activities of Silybum marianum L. plants have been reported (Banna et al, 2011) . Nisar Ahmad evaluated the antioxidative activity of Silybum marianum L. and its association with plant development. The results suggested that the maximum antioxidant activity of leaves could be obtained from 80-day-old plants (Ahmad et al, 2013a). The roots were also reported to have high antioxidative activities (Ahmad et al, 2013b). The exact constituents of the plant extract responsible for the antioxidative properties have not been identified. Moreover, although the pappi and fruit receptacles, which are harvested along with

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their seeds, are also important plant parts, no research has been conducted on their active ingredients and antioxidant activities.

The main objective of the present study was to evaluate the antioxidant activities of ethanol extracts from Silybum marianum L. by DPPH radical scavenging activity and the ferric reducing antioxidant power (FRAP) assays. The total flavonoid content (TFC) and total phenolic content (TPC) in the roots, main stems, leaves, fruit receptacles, and pappi were determined in order to assess their roles as potential sources of natural antioxidants.

2. Materials and methods

2.1 Chemicals

DPPH, Folin–Ciocalteu phenol reagent, rutin and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma Chemicals Co. (St, Louis, MO, USA). BHT, sodium hydroxide, sodium nitrite, sodium carbonate, aluminum nitrate, ferric chloride, hydrogen chloride and other chemicals (analytical grade) were obtained from Merck (Germany).

2.2 Plant material and maintenance

Test plants were collected in harvest time (July, 2015) from Panjin City, Liaoning Province, China. Different parts including mature leaves, main stems, roots, fruit receptacles and pappi of Silybum marianum L. were collected and shade dried (28 ± 2°C) for 7 days. Plant materials were dried (45°C) in a cabinet drier (Shanghai Yiheng Scientific Instrument Co., Ltd, China) to constant weights prior to be used in the extraction. Vouchers specimens were deposited in Key Laboratory of deep processing of agricultural products, Liaoning province, China.

2.3 Extract preparation

Dried materials of different parts of Silybum marianum L. were ground and sieved through a 60-meth size screen to get fine powder from which the extracts were prepared. 2 gram of the powder was extracted continuously in an ultrasonic cleaner (Kun Shan Ultrasonic Instruments Co., Ltd, China) with ethanol (50 ml, 60% v/v) at 50°C for 2 h. This procedure was repeated twice with fresh solvent. After filtered (Whatman No. 1 filter paper), the final extracts were concentrated separately to dryness by rotary vacuum evaporator (Shanghai Yarong Instrument Co., Ltd, China) at 45°C and stored at 4°C.

2.4 Determination of TFC

A modified method(Sultana et al, 2009) was used for the determination of TFC: extract sample containing flavonoids (10ml) and 50g/L $NaNO_2$ (1.5 ml) were mixed for 6 min, then 10g/L $Al(NO_3)_3$(1.5 ml) was added and mixed together. 6 minutes later, 40g/LNaOH (20 ml) was added. After standing for 10 min, the absorbance of the solution was measured at 510 nm with a spectrophotometer (UV6300, Shanghai Mapada Instruments Co., Ltd. China). Rutin was used as a standard for the construction of a standard curve. 0.008, 0.016, 0.024, 0.032, 0.040, 0.048mg/ml rutin were prepared in ethanol and their absorbance was read at 510 nm using a spectrophotometer. The concentration of flavoniods in the samples was expressed as mg rutin /g of dry plant material, averaged from three measurements.

2.5 Determination of TPC

The TPC was determined by a modified method(Kim et al, 2003) as follows: 1mL of extract sample was added to deionized water (1.0 mL) and Folin–Ciocalteu phenol reagents (1.0mL), After 5 minutes, 10% Na_2CO_3 (1.0 mL) was added to the mixture. The mixture was kept at room temperature for 2 h, the absorbance was measured at 760 nm using a spectrophotometer (UV6300, Shanghai Mapada Instruments Co., Ltd. China). The same procedure was repeated for the solution of gallic acid which was used as a standard. The results were expressed as mg gallic acid /g of dry plant material, averaged from three measurements.

2.6 DPPH radical scavenging assay

The radical scavenging ability of the extracts were determined according to the procedure described earlier (Benzie and Strain, 1996). Briefly, 0.04g/L ethanol solution of DPPH (2 ml) was added to samples (2.0 ml) containing different concentrations of ethanol extracts in a dark place at room temperature. Their absorbance was read at 517 nm after 30 min. BHT was used as positive controls. IC_{50} values (μg/ml) (concentrations of the test samples that provided 50% inhibition of the DPPH radical) were calculated from the DPPH absorption curve of each test sample at 517 nm.

2.7 FRAP assay

A modified method reported by Benzie and Strainwas used for the determination of FRAP assay in plant extracts(Agarwal et al, 2006). The working reagent included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in distilled water, which were mixed together in the ratio of 10:1:1.

The solution was heated to 37°C in water bath before it was used. Plant extracts of various concentrations (50 µL) were allowed to react with 3.0ml of working FRAP reagent in darkness for 30 min, the absorbance of the mixture was measured at 593 nm. $FeSO_4$ (100-1,500 µM) was used as a standard for the construction of a standard curve. BHT and ascorbic acid were used as positive controls. Results were expressed as mmol Fe (II)/ g of dry extract.

2.8 Statistical analysis

All measurements were carried out in triplicate and expressed as mean (n=3) ± standard deviation (n=3) of three replicates. Construction of curves and graphical presentation were performed by MS Office Excel, 2007. SPSS Statistics (Version 19.0, IBM Inc., USA) was used for identifying the differences between values. A probability value of $p \leq 0.05$ was considered to be significant.

3. Results and discussion

3.1 Total Flavonoid and Phenolic Content

Table 1: Total flavonoid and phenolic content in different parts of Silybum marianum L.

Parts	Total flavonoids[1]	Total phenolics[2]
Leaf	9.82±0.32	11.84±0.21
Main stem	6.64±0.43	9.80±0.13
Root	10.88±0.45	13.32±0.25
Fruit receptacle	15.34±0.58	22.19±0.38
Pappus	17.10±0.56	48.97±0.41

1: Expressed as mg rutin/g of dry plant material; 2: Expressed as mg gallic acid/g of dry plant material

The quantities of phenolics and flavonoids in the different parts of examined plants are presented in Table 1. Among the five investigated parts of Silybum marianum L. (i.e., roots, main stems, leaves, fruit receptacles, and pappi), the pappi yielded the highest TFC (17.10 mg rutin/g of dry plant material), followed by the fruit receptacles (15.34 mg/g). The TFC in the main stems was the lowest (6.64 mg/g). Differences in the TFC among the five parts were significant ($p \leq 0.05$). The TFC in the pappi was higher than in other medicinal plants such as *Zingiber officinale Roscoe* (5.54 mg/g) (Ghasemzadeh et al, 2010) and *Salvia amplexicaulis Lam* (5.08 mg/g)(Alimpić et al, 2014), but was lower than that in the Silybum marianum L. fruits (30 mg/g) (Morazzoni and Bombardelli, 1995). Pappi and fruit receptacles were harvested with seeds together, which were discarded after seeds were observed. Our results indicated that flavonoids are important components of both the pappi and fruit receptacles, which could be used for flavonoid extraction.

In this study, the TPC was expressed in terms of gallic acid equivalents using the standard curve equation, $y = 105.58x + 0.0414$, $r^2 = 0.9997$. The TPC of the different parts ranged from 9.80±0.13 to 48.97±0.41 mg gallic acid/g of dry plant material and varied significantly among the different plant parts ($p \leq 0.05$). The pappi contained the greatest TPC (48.97 mg/g), while the main stems had the lowest TPC (9.80±0.13 mg/g). Flavonoids and phenolics could also be extracted by other solvents such as methanol and acetone. The TFC and TPC in Silybum marianum L. plants may be affected by different solvents and extraction techniques and should be investigated in future.

3.2 DPPH radical scavenging activity

The DPPH radical scavenging activity of ethanol extracts obtained from different parts of the plant is depicted in Figure 1. BHT was used as a positive control. The radical scavenging ability (RSA%) of the sample extracts was reported as the percent of DPPH scavenged. A greater RSA% correlates to a higher antioxidant activity. Increasing the concentrations (10–30 µg/mL) of ethanol extracts was found to be effective in improving the radical scavenging activity of the five plant parts; however, the RSA% increased slowly from 30 to 50 µg/mL in the roots, fruit receptacles, and pappi (Figure 1). At 50 µg/mL, the scavenging activity of the pappi ethanol extract was the highest (69.68%), followed by roots (66.02%), fruit receptacles (58.87%), leaves (56.82%), and main stems (42.11%). The scavenging activity of all the five parts was lower than that of BHT (82.5%) at 50 µg/mL.

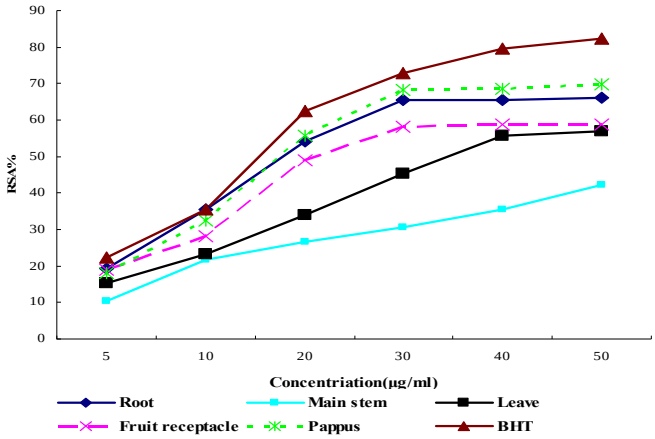

Figure 1: DPPH radical scavenging activity of the ethanol extracts from different parts of Silybum marianum L.

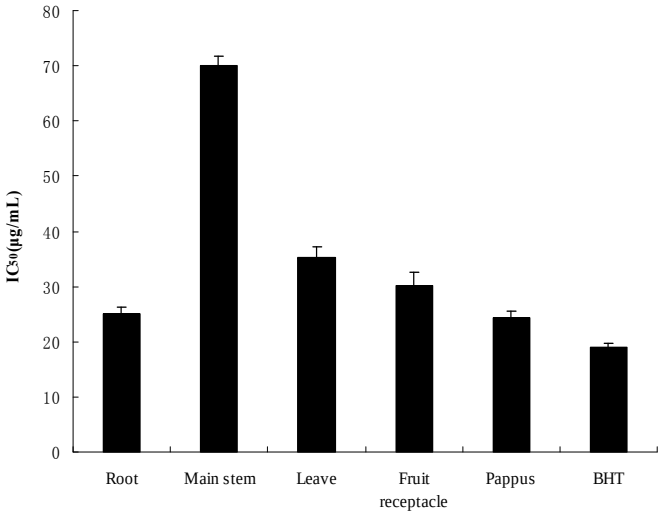

Figure 2: IC_{50} values of the ethanol extracts from different parts of Silybum marianum L.

The IC_{50} values among the different parts are shown in Figure 2. Lower IC_{50} values correlate to higher antioxidant activities. The pappi exhibited the highest scavenging activity with an IC_{50} value of 24.20 μg/mL, while the main stems demonstrated the lowest scavenging activity (IC_{50} 69.99 μg/mL). The descending order of radical scavenging activity among the different parts was as follows: pappi (IC_{50} 24.20 μg/mL) > roots (IC_{50} 25.16 μg/mL) > fruit receptacles (IC_{50} 30.17 μg/mL) > leaves (IC_{50} 35.18 μg/mL) > main stems (IC_{50} 69.99 μg/mL). Differences among the five parts were significant ($p \leq 0.05$). The extracts from all the five parts exhibited lower radical scavenging activities than that of BHT (IC_{50} 19.01 μg/mL). Nisar Aman studied the DPPH free radical scavenging activities and phenotypic differences in Silybum marianum L. Their research revealed that the DPPH radical scavenging activity of the roots was highest compared to that of the leaves and main stems (Ahmad et al, 2013b) , which was in accord with the results obtained in this study. Therefore, both the pappi and root can be used as a source of natural antioxidants.

Hydrogen donating ability is related to the effect of antioxidants on DPPH (Zin and Abdul, 2002). The results obtained in this study suggested that extracts from the pappi and roots contained a large amount of radical scavenging compounds with hydrogen donating abilities. However, the order of radical scavenging activity was not in accord with the TFC or TPC order shown by the different plant parts. Specifically, the TPC of the root was lower than that of the fruit receptacle, yet the radical scavenging activity of the ethanol extract from the root was higher than that from the fruit receptacle. Therefore, it is possible that the antioxidant properties of Silybum marianum L.plants were affected by many compounds besides flavonoids and phenolics,

especially in the roots. Other active compounds such as phenols, acids, sugars, and glycosides could be included in the ethanol extracts of medical plants (Hiroe and Nobuji, 1994). The synergistic effect of these compounds may be present in Silybum marianum L.plants. Further studies should be conducted to determine other active compounds in the plants.

3.3 FRAP Assay

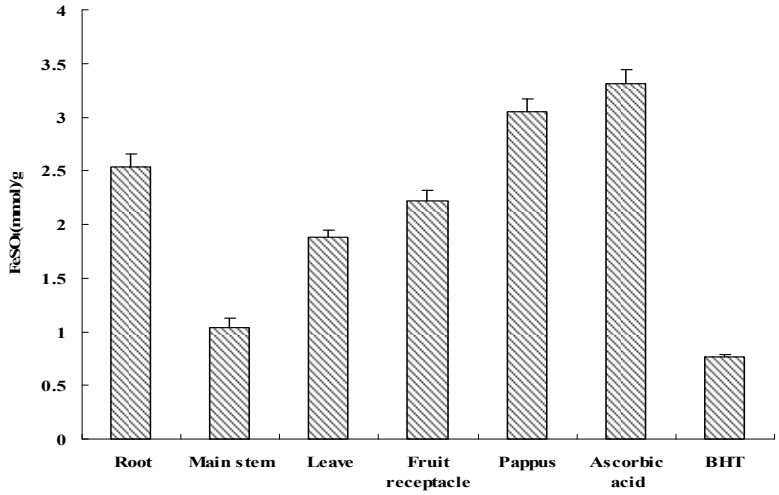

Figure 3: FRAP of the ethanol extracts from different parts of Silybum marianum L.

The FRAP assay is widely used to determine the reducing ability of antioxidants. Antioxidants can react with the ferric tripyridyltriazine complex and produce a colored ferrous tripyridyltriazine. A deep color of the reaction solution indicates a higher reducing ability. The FRAP values of the ethanol extracts from different parts of Silybum marianum L. ranged from 1.036±0.085 to 3.050±0.118 mmol/g of dry extract (Figure 3). The highest reducing activity was recorded for the pappus extract (3.050±0.118 mmol/g), followed by the root extract (2.534±0.121 mmol/g), whereas the main stem extract had the lowest reducing ability (1.036±0.085 mmol/g). The order was in accord with the DPPH radical scavenging activity shown by the different plant parts .The differences in the FRAP values among the various parts were significant ($p \leq 0.05$). The FRAP values of all the extracts were higher ($p \leq 0.001$) than that of BHT (0.765±0.024 mmol/g), but lower ($p \leq 0.001$) than that of ascorbic acid (3.308±0.132 mmol/g).

4. Conclusions

This study indicated that among the five parts of Silybum marianum L. (i.e., roots, main stems, leaves, fruit receptacles, and pappi) that were investigated, the highest TFC and TPC were obtained from the pappi, followed by the fruit receptacles. Thus, both these parts can be used for flavonoid extraction. The antioxidant activity of the ethanol extract from the pappi was the highest, followed by the roots. Further work is required to determine other active compounds in the plant, especially in the roots. The identity of the flavonoids in the pappi and fruit receptacles that may have contributed to the antioxidant activity should also be established.

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