

Original article

Phytochemical analysis and antioxidants activity of *Silybum marianum* (L.) Gaertn.

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Abstract

The present study was an attempt to evaluate the phytochemical constituents and antioxidants potential of the methanol extract of leaves of *Silybum marianum* (L.) Gaertn., a known hepatoprotectant. For the evaluation of antioxidant activities of silymarin, assayed of total antioxidant activity, total reducing power (FRAP), 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH), superoxide anion radical scavenging, hydrogen peroxide scavenging and ferrous ions (Fe²⁺) chelating activity. The phytochemicals of silymarin were determined qualitatively and quantitatively by using standard methods. Qualitative analysis revealed that *S. marianum* is rich in phytochemicals, viz., alkaloids, terpenoids, steroids, phenols, flavonoids and glycosides. Quantification of flavonoids (223 mg/g) and phenols (312 mg/g) were also evaluated. The DPPH and chelating activity of silymarin were significantly high, i.e., 95% and 85%, respectively at 21 µg/ml concentration, whereas FRAP and phosphomolybdenum reduction assay showed maximum activity, i.e., 0.88 and 0.089, respectively at 21 µg/ml concentration. Free radicals scavengers like hydrogen peroxide and superoxide anions showed maximum inhibition percentage, i.e., 83% and 96%, respectively at 21 µg/ml concentration. The research study concludes that the leaves of *S. marianum* showed the highest antioxidant activities at 21 µg/ml. It could be used in low concentrations in herbal medicine, pharmacological and food industry effectively.

Keywords: *Silybum marianum* (L.) Gaertn., silymarin, methanol extract, total antioxidant activity, FRAP, DPPH, radical scavenging

1. Introduction

Silybum marianum (L.) Gaertn. (milk thistle), family Asteraceae, is a hepatoprotective medicinal plant, used for the treatment of liver diseases like hepatitis. *Silybum* is a type of weed which usually grows in an empty land, roadside areas and field. It is native to Europe and Asia. But, due to the presence of medicinal properties, it is cultivated for seeds, throughout the world. Approximately, 40% of patients with chronic hepatitis C virus (HCV) infection have reported the use of silymarin herbal products (Strader *et al.*, 2002; Seeff *et al.*, 2008). Silymarin is the collective name of cards marianus, milk thistle, blessed milk thistle, marian thistle, mary thistle, Saint Mary's thistle, mediterranean milk thistle, variegated thistle and flavonolignans. Extraction of milk thistle, is one of the most frequently reported herbal remedies, representing 72% of all herbals used by patients with HCV (Strader *et al.*, 2002; Ghany *et al.*, 2009).

It has three major components, silibinin, silydianin and silychristin. Silibinin is the main active component of silymarin. It efficiently

reduces and potentially regenerates the damaged hepatic tissues (Al-Anati *et al.*, 2009). An antioxidant delay prevents and removes the oxidative damage to a target molecule (Sravanthi *et al.*, 2013; Gireesha and Raju, 2016). DNA, lipids and proteins are usually damaged due to the action of free radicals (Halliwell and Gutteridge, 2010), leading to prevention, protection of various diseases like cancer, diabetes, cataract, ageing, *etc.*, and as an oxygen scavengers (Bhatia *et al.*, 1999; Sharma *et al.*, 2003; Hogan *et al.*, 2007; Mokhtari, 2008; Halliwell, 2011). Recently, silymarin has shown its potential as an anticholesterolaemic agent by enhancing ribosomal protein synthesis. This helps in regenerating hepatocytes (Zongguo *et al.*, 2014).

An antioxidant is a highly stable molecule, they donate an electron to a destructively free radical and neutralize it, thus minimize its capacity to damage. Antioxidants delay cellular and molecular damage mainly through their free radical scavenging property (Lobo *et al.*, 2010; Ahmad *et al.*, 2010; Katerina *et al.*, 2014; Ansari, 2016; Mathe, 2018; Mates *et al.*, 2018). Antioxidants of plants origin are also used as anticancer, antimutagenic and antiageing agents (Cook and Samman, 1999). Secondary metabolites like phenols act as important antioxidant agents (Shaker *et al.*, 2010). They are released naturally during different plant growth phases, while some are produced against stress conditions (Ahmad *et al.*, 2010). As a result, antioxidants like thiols, ascorbic acid or polyphenols are often used as reducing agents. Natural antioxidants, especially present in

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medicinal plants, exhibits antioxidant, immunomodulatory, antifibrotic, antiproliferative, antiviral as well as vasodilatory activities (Pant *et al.*, 2017).

To protect against this oxidative stress, the human body has its own system including various enzymes, proteins and vitamins, which are known as antioxidants. Ageing declines the production of such antioxidants, hence it requires an external source of antioxidants to defend free radicals (Gucin *et al.*, 2003). Plants are good sources of antioxidants which provide protection against free radicals and have been strongly associated with reduced risk of chronic diseases (Tanizawa *et al.*, 1992; Devasagayam *et al.*, 2004). The antioxidant activity of these natural phytoconstituents products is due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Halliwell, 1997). Due to herbal and safer properties of silymarin and has no side effects is reported, from proper administration of designed curative prescribed amount (Nada *et al.*, 2015).

The main objective of the silymarin study was to evaluate the phenols and flavonoids contents in the leaves of *S. marianum*, using methanol as a solvent and to assess their antioxidant potential.



Figure 1: Habit of *Silybum marianum* (L.) Gaertn.

2. Materials and Methods

2.1 Sample collection

Samples of whole plant were collected in and around RPCAU, Pusa, Samastipur, Bihar, India. The plant material was identified by the corresponding author of this article. The voucher specimen (No: 246/PB/2017) was deposited in herbarium of this university. The samples were sundried and ground finely, sieved and stored in air tight containers for further analysis.

2.2 Chemicals and reagents

All chemicals used were of analytical grade, procured from HIMEDIA Laboratory Pvt. Ltd, India.

2.2.1 Extract preparation

50 ml of methanol was added to 5 gm powdered material with periodic shaking for a week (it was stirred every 18 h using a sterilized glass rod), filtered and the filtrate was collected. This

procedure was repeated three times with fresh methanol. The filtrates were pooled and concentrated at 40°C and evaporated to dryness and stored at 4°C in an airtight bottle. The extracts were dissolved in 10 ml of methanol independently to get stock solutions.

2.3 Qualitative phytochemical analysis

Secondary metabolites present in the methanol extract of leaves of *S. marianum* was analysed by standard methods (Harborne, 1973; Trease and Evans, 1993; Zafar Mujeeb, 2002).

2.4 Quantitative estimation

2.4.1 Estimation of total phenols by Folin-Ciocalteu reagent method

Total phenolic compound contents were determined by the Folin-Ciocalteu method (Singleton *et al.*, 1999) with slight modifications. The silymarin extract samples (1 ml of different dilutions) were mixed with Folin-Ciocalteu reagent (1 ml, 1:10 diluted with distilled water) for 5 min and aqueous Na₂CO₃ (1 ml, 1 M) was added. The mixture was allowed to stand for 15 min and the phenols were determined by UV-VIS double beam spectrophotometer at 765 nm. Gallic acid was used as the standard. The total phenol content was expressed in terms of gallic acid equivalent (mg/g).

2.4.2 Estimation of total flavonoids by AlCl₃ method

Aluminium chloride was used to determine the total flavonoids (Chang *et al.*, 2002) with slight modifications. 1 ml of methanol extract of leaves of silymarin was mixed with 0.5 ml of 10 % aluminium chloride, 0.5 ml of 1M potassium acetate and 1 ml of distilled water. It was incubated at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with UV-VIS double beam spectrophotometer. The total flavonoid contents were expressed as in terms of quercetin equivalent (mg/g) which served as the standard.

2.5 Antioxidant activities

2.5.1 DPPH radical scavenging assay

The antioxidant activity of methanol extract of leaves of silymarin was measured on the basis of the scavenging activity of the stable 2, 2- diphenyl-1-picrylhydrazyl (DPPH) free radical by Brand-Williams *et al.* (1995) with slight modifications. 1 ml of 0.1 mM DPPH solution in methanol was mixed with the extract. Quercetin was taken as the reference standard. Blank was 1 ml methanol and 1 ml DPPH solution. The decrease in absorbance was measured at 517 nm after 30 min in dark using UV-Vis spectrophotometer. The inhibition % was calculated using the following formula:

$$\text{DPPH scavenging effect \%} = (1 - \text{As}/\text{Ac}) \times 100$$

where Ac is the absorbance of control and As is the absorbance of the test sample.

2.5.2 Chelating activity on ferrous ion (Fe²⁺)

The chelation of Fe²⁺ by methanol extract of leaves of silymarin was estimated by the method of Dinis *et al.* (1996). Methanol extract was added in different concentrations (3 to 21 µg/ml) to a solution of 1 mM FeCl₂ (0.05 ml). The reaction was initiated by the addition of 1 mM ferrozine (0.1 ml), and the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was

measured spectrophotometrically at 562 nm. EDTA was used as the standard reference. The percentage of inhibition of ferrozine Fe^{+2} complex formation was calculated by using the following formula given below:

$$\text{Inhibition \% of Fe}^{+2} = (1 - \text{As}/\text{Ac}) \times 100$$

where Ac is the absorbance of control and As is the absorbance of the sample (Dorman *et al.*, 2003).

2.5.3 Ferric (Fe^{3+}) reducing power assay (FRAP)

The reducing power of methanol extract of leaves of silymarin was determined by a slightly modified method of Oyaizu (1986). 1 ml of each plant extract concentration (3-21 mg/m *S. marianum*) was mixed with phosphate buffer (1 ml, 0.2 M, pH 6.6) and potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) (1 ml, 1%). The mixtures were then incubated at 50°C for 20 min. 1 ml of trichloroacetic acid (10%) was added to each mixture, which was then centrifuged for 10 min at 5000 rpm. The upper layer of the solutions (1 ml) was mixed separately with distilled water (1 ml) and FeCl_3 (0.5 ml, 0.1%), and the absorbance was measured at 700 nm using a spectrophotometer. Ascorbic acid was used as the standard reference.

2.5.4 Total antioxidant capacity (Phosphomolybdenum reduction) assay

The total antioxidant capacity of the methanol extract of leaves of silymarin was evaluated by the phosphomolybdenum reduction assay method according to the procedure described by Prieto *et al.* (1999). The assay is based on the reduction of Mo (VI) to Mo (V) by the methanol extract of leaves of silymarin and subsequent formation of green phosphate/Mo (V) complex at acid pH. 1 ml of various concentrations (3-21 $\mu\text{g}/\text{ml}$) of the extract were combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer. Gallic acid was used as the standard reference.

2.5.5 Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging assay was carried out, following the procedure of Ruch *et al.* (1989). The principle of this method is there to decrease in absorbance of H_2O_2 upon oxidation of H_2O_2 . A solution of 40 mM H_2O_2 was prepared in 0.1M phosphate buffer (pH 7.4). Silymarin at the mg/ml concentration in 3.4 ml phosphate buffer was added to 0.6 ml of H_2O_2 solution (40 mM) and the absorbance of the reaction mixture was recorded at 230 nm. A blank solution contained the sodium phosphate buffer without H_2O_2 . The concentration of H_2O_2 ion of hydrogen peroxide (mM) in the assay medium was determined by using a standard (catechin) curve and was calculated using the following equation:

$$\text{H}_2\text{O}_2 \text{ scavenging effect \%} = (1 - \text{As}/\text{Ac}) \times 100$$

where Ac is the absorbance of control and As is the absorbance of the sample (Manach *et al.*, 2004).

2.5.6 Superoxide anion radical scavenging activity

Superoxide radical scavenging activity of silymarin was performed according to the method of Beauchamp and Fridovich (1971), described by Zhishen *et al.* (1999) with slight modification. Superoxide radicals are generated in riboflavin, methionine, illuminate and assayed by the reduction of NBT to form blue

formazan. All solutions were prepared in a 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed using fluorescent lamps (20 W). The concentration of silymarin in the reaction mixture was 30 mg/ml. The total volume of the reaction mixture was 3 ml and the concentrations of the riboflavin, methionine and NBT were $1.33 \cdot 10^{-5}$, $4.46 \cdot 10^{-5}$ and $8.15 \cdot 10^{-8}$ M, respectively. The reaction mixture was illuminated at 25°C for 40 min. The photochemically reduced riboflavin generated O_2^- which reduced NBT to form blue formazan. The unilluminated reaction mixture was used as a blank. The absorbance was measured at 560 nm. Silymarin was added to the reaction mixture, in which O_2^- was scavenged, thereby inhibiting the NBT reduction. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The percentage of superoxide anion scavenged was calculated by using the following formula:

$$\text{O}_2^- \text{ scavenging effect \%} = (1 - \text{As}/\text{Ac}) \times 100$$

where Ac is the absorbance of the control and As is the absorbance of silymarin and BHT was used as the standard.

2.6 Statistical analysis

The experimental results were performed in triplicate. The data were recorded as Mean \pm standard deviation and analysed by SPSS (operating system windows 2007). One-way analysis of variance (ANOVA) was performed by procedures. Significant differences between means were determined by Duncan's Multiple Range tests, and $p \leq 0.05$ was regarded as significant and $p \leq 0.01$ was very significant.

3. Results

The formulations of drugs are based on the extraction of natural and synthetic antioxidants. It is mainly used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer (Sofowora, 1999). The methanol extract of leaves of silymarin was subjected to various qualitative chemical tests (Ganapaty *et al.*, 2007). The methanol extract showed the presence of phytochemical constituents such as alkaloids, terpenoids, steroids, flavonoids, phenol compounds and glycosides as presented in Table 1. The qualitative analysis showed the major presence of phenols and flavonoids contents such as 312 mg/g and 223 mg/g, respectively as presented in Table 2.

Table 1: Qualitative estimation of phenols and flavonoids of methanol extract of leaves of *S. marianum*

Phytoconstituents	Chemical reagents	Results
Alkaloids	Dragendorff's reagent	++
	Wagner's reagent	++
Terpenoids	CHCl_3 + conc. H_2SO_4	++
Steroids	Liebermann-Burchard's reagent	++
Phenols	FeCl_3 solution	++
Flavonoids	Shinoda (NaOH solution) test	++
	Magnesium filaments + HCl test	++
Glycosides	Fehling's test	++
	Molisch's test	++

++:Strongly present, +:Mild present and - : complete absent

Table 2: Quantitative estimation of phenols and flavonoids of methanol extract of leaves of *S. marianum*

S.No.	Components	Concentration (mg/g)
1.	Phenols	312 ± 5.6
2.	Flavonoids	223 ± 3.1

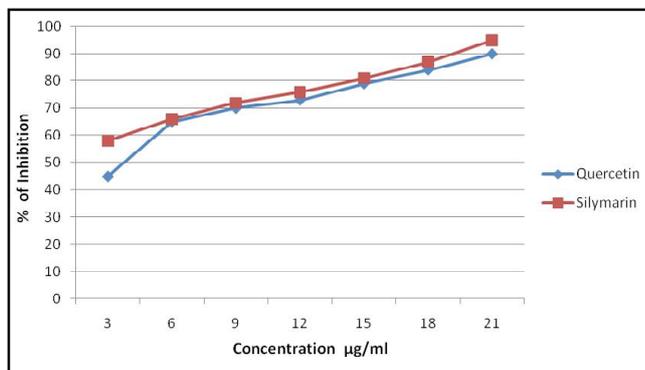


Figure 2: DPPH radical scavenging assay of *S. marianum*.

Table 3: Scavenging activities of methanol extract of leaves of *silymarin*

Scavenging radicals	Standard µg/ml	Concentration µ/ml							
		3.00	6.00	9.00	12.00	15.00	18.00	21.00	
DPPH	Quercetin	45.00	65.00	70.00	73.00	79.00	84.00	90.00	
	Silymarin	58.00	66.00	72.00	76.00	81.00	87.00	95.00	
Chelating Activity (Fe ²⁺)	EDTA	15.00	25.00	30.00	50.00	70.00	75.00	87.00	
	Silymarin	17.00	22.00	38.00	62.00	72.00	73.00	85.00	
FRAP (Fe ³⁺)	Ascorbic acid	0.35	0.48	0.60	0.65	0.69	0.75	0.84	
	Silymarin	0.36	0.45	0.55	0.63	0.65	0.71	0.88	

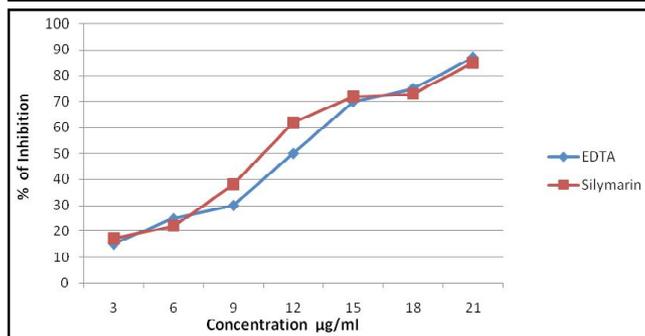


Figure 3: Fe²⁺ chelating assay of *S. marianum*.

The antioxidant activity of methanol extract of leaves of silymarin was evaluated by DPPH free radical scavenging assay, Fe²⁺ chelating assay, ferric (Fe³⁺) reducing power assay (FRAP) and total antioxidant capacity (phosphomolybdenum reduction assay) and the results were presented in the Tables 3 and 4. The increase in percentage of DPPH radicals scavenging activity as well as Fe²⁺ chelating activity increases with an increase in concentrations of silymarin. The maximum DPPH radical scavenging activity showed the % of inhibition was 95.0 and the standard quercetin was 90.0 at 21 µg/ml

concentration (Figure 1). The maximum chelating activity of silymarin was 85.0 and the standard EDTA was 87.0 at 21 µg/ml concentration (Figure 2).

The ferric reducing power (FRAP) assay showed maximum activity of 0.88 and also the phosphomolybdenum reduction assay showed maximum activity of 0.089 at 21 µg/ml concentration, which were compared with the standards showed the activity of 0.84 and 0.080, respectively. The ascorbic acid as standards was used for FRAP and gallic acid as standard used for phosphomolybdenum reduction assay (Figures 3 and 4).

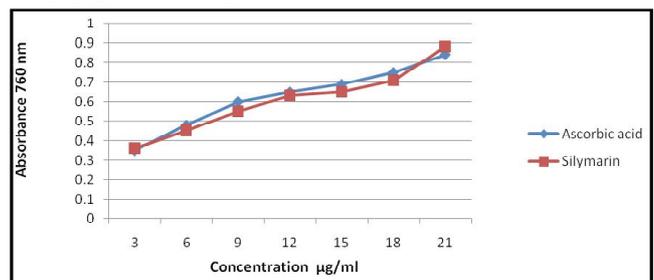


Figure 4: Reducing power assay (FRAP) of *S. marianum*.

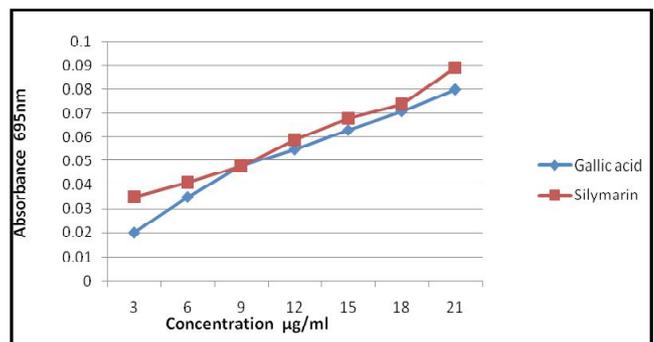


Figure 5: Phosphomolybdenum reduction assay of *S. marianum*.

Hydrogen peroxide scavenging activity of silymarin at 21 µg/ml was found to be 83 %, whereas standard catechin was found to be 81 % (Figure 5). The results showed that the silymarin has an effective hydrogen peroxide scavenging activity (Figure 6). At the above concentration, the hydrogen peroxide scavenging effect of silymarin was more than standard.

Table 4: Scavenging activities µg/ml of methanol extract of leaves of *S. marianum*

Scavenging radicals	Standard µg/ml	Concentration µ/ml							
		3.00	6.00	9.00	12.00	15.00	18.00	21.00	
Total antioxidant activity	Gallic acid	0.02	0.04	0.05	0.06	0.06	0.07	0.80	
	Silymarin	0.04	0.04	0.05	0.06	0.07	0.07	0.09	
Hydrogen peroxide scavenging activity	Catechin	31.00	42.00	58.00	64.00	73.00	78.00	81.00	
	Silymarin	28.00	48.00	52.00	73.00	78.00	80.00	83.00	
Superoxide anion radical activity	BHT	38.00	53.00	69.00	76.00	82.00	88.00	93.00	
	Silymarin	35.00	52.00	65.00	77.00	79.00	92.00	96.00	

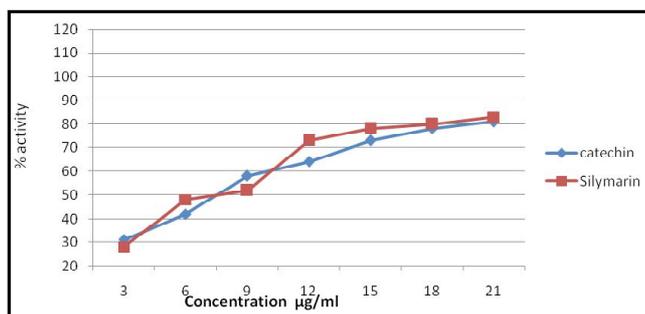


Figure 6: Hydrogen peroxide assay of *S. marianum*.

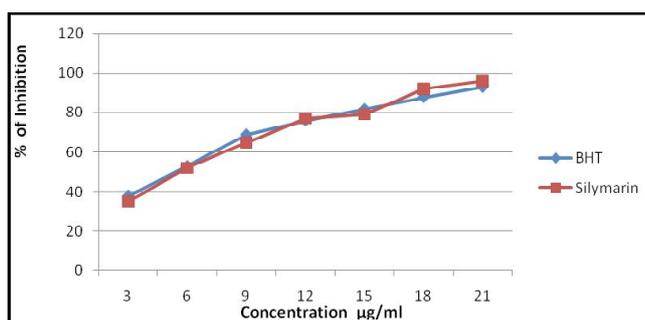


Figure 7: Superoxide anion radical assay of *S. marianum*.

The inhibition of superoxide anion radicals' generation by silymarin was somewhat higher than standard BHT (Figure 7). It was seen in the inhibition of superoxide radical of silymarin which was found to be 96.0 % as compared to the standard 93% to be at 21 µg/ml concentration.

4. Discussion

Flavonoids and plant phenolic compounds possess highly antioxidant properties and they have abilities to trapping free radicals and reactive oxygen species such as singlet oxygen, free radicals and hydroxyl radicals. They play an important role in stabilizing lipid peroxidation (Gulcin and Dastan, 2007; Gulcin, *et al.*, 2006) by scavenging and neutralizing free radicals, quenching singlet and triplet oxygen. They are continuously prevented from generating free radical by breaking of a chain reaction of the fatty acids during oxidative stress. The observation of the present study showed that silymarin plants have also high content of phytochemicals like phenols and flavonoids, *i.e.*, 312 and 223 mg/g, respectively (Table 2). The study revealed that the phenols and flavonoids of plants are correlated in metabolism and involved in different types of biosynthesis processes like shikimate, pentose phosphate, phenylpropanoid pathways, *etc.* (Singleton *et al.*, 1999).

Plants phenolic and flavonoids, both compounds are protecting elements of the cells means that they act as a defence mechanism. Other than this, they are also involved in the formation of structural polymers like lignin, attractants of plants pigments like carotenoids and flavonols, defence response chemicals like tannins and phytoalexins, UV screen like flavonoids, *etc.* In humans, phenolic compounds act as antioxidants, anti-ageing, anti-inflammatory and anti-proliferative activities, *etc.* Therefore, if the foods contain high antioxidants activities, indicate that reduced the chances of chronic diseases like diabetes, cardiovascular and cancer. So, the antioxidants activities of phytochemical constituents mainly phenolics,

flavonoids and flavonols are attributed to the medicinal effect of the plants (Miliauskas *et al.*, 2004). It is also known that phenolic compounds are strongly chain-breaking antioxidants (Shahidi and Wanasundara, 1992). The scavenging properties of phenolic compounds are due to the presence of hydroxyl groups (Hateno *et al.*, 1987). Other than the hydroxyl group, the o-dihydroxy group is also showing very strong antioxidants activities as well as scavenging activities.

The total antioxidant activities of plants are assessed by DPPH radical scavenging assay, Fe²⁺ chelating assay, FRAP assay and phosphomolybdenum reduction assay. The activities of DPPH are increased, showed the high contents of phenolic components such as flavonoids, phenolic acids and phenolic diterpenes (Oyaizu, 1986). The same studies were conducted by Chandra *et al.* (2014) and Sravanthi and Rao, (2014). In DPPH assay, the antioxidants present on silymarin reduce the violet coloured stable 2,2-diphenyl-1-picryl-hydrazyl radicals to the yellow coloured 2, 2-diphenyl-1-picryl hydrazine. The molecules of 2,2-diphenyl-1-picryl-hydrazine are identified as a stable free radical by the decolourisation of used electrons of the molecules. The different doses response of DPPH radical scavenging activity of silymarin was depicted in Figure 2.

The transition metal ion, Fe²⁺ have the capability to move single electron by virtue of which, it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals (Dinis *et al.*, 1996). The main strategy to avoid the generation of ROS that is associated with redox active metal catalysis involves chelating of the metal ions.

Metal-chelating property is measured of the antioxidant activity based on absorbance measurement of Fe²⁺ p-ferrozine complex after prior treatment of Fe²⁺ ion solution with the test material. Ferrozine complex compound formation with free Fe²⁺ ions but Fe²⁺ not bound to other chelators, hence decrease the amount of ferrozine Fe²⁺ complex formed after the treatment of the presence of antioxidant chelators.

The ferrozine Fe²⁺ complex develops the red colour compound with the absorbance can be measured of 562 nm wavelength of light (Koksai *et al.*, 2008). EDTA is a strong metal chelator. So, it is used as a standard metal agent in this study. Silymarin has a high capacity for iron binding, suggesting that its main action as a peroxidation inhibitor may be related to its iron binding capacity.

In this assay, silymarin interfered with the development of the ferrous-ferrozine complex. It suggests that silymarin has chelating property and is able to capture Fe²⁺ ion before ferrozine. Silymarin may chelate the Fe²⁺ with its OH⁻ group (Figure 3). Fiorucci and co-workers confirmed that quercetin chelated metal ion in the same way (Fiorucci *et al.*, 2007). The DPPH (2, 2-diphenyl-2-picryl hydrazyl) free radical scavenging activity and Fe²⁺ show the chelating activity. In non-antioxidant enzyme like FRAP is known for powerful free radicals scavengers and always they are used in the laboratory for measuring maximum trapping of oxidant radicals. The presence of antioxidant, the Fe³⁺ /ferricyanide complex reduce to Fe²⁺ ferrous form (Benzie *et al.*, 1996).

Reduction of Fe³⁺ solution is often used as an indicator of electron accepting activity, which is an important mechanism of phenolic. An antioxidant activity can be correlated with other antioxidant properties (Gulcin *et al.*, 2006). By estimating the formation of

pearl's prussian blue at 700 nm, it is conceivable to determine the concentration of ferrous (Fe^{2+}) ions. Increase in absorbance of the reaction solution signifies to reducing the power of the extract (Figure 4). The reducing power assay showed greater reduction capacity compared with the standard ascorbic acid curve. Hence, silymarin plant proved to be higher phytoconstituents with the potential antioxidant activity.

The phosphomolybdenum reduction assay was based on the decrease of the oxidation state of Mo (VI) to Mo (V), in presence of antioxidant and consequent formation of a green phosphate Mo (V) complex on higher temperature and acidic pH. The phosphomolybdenum reduction assay increases with increase in concentration (Figure 5). Hydrogen peroxide is generated by several oxidase enzymes such as catalase, peroxidase, superoxide dismutase, glutathione peroxidase and ascorbic oxidase.

There is increasing evidence that H_2O_2 , either directly or indirectly *via* its reduction product OH^- , act as a messenger molecule in the synthesis and activation of inflammatory mediators. Secondary messenger permeable to the membrane and oxidize slowly in various compounds. The hydrogen peroxide scavenging capacity of silymarin plant was depicted in Figure 6. Silymarin had effective hydrogen peroxide scavenging activity. H_2O_2 is toxic for cell and induces cell death because they degrade many cellular organelles like mitochondria and chloroplast or energy producing systems. Hydrogen peroxide inactivated glyceraldehydes-3-phosphate dehydrogenase, a glycolytic enzyme, the imitation of apoptosis process (Hyslop *et al.*, 1988).

The free radical reaction is most accepted as a common mechanism of lipid peroxidation. Free radical scavengers may directly react with and quench peroxide radicals to terminate the lipid peroxidation chain reactions and enhance the quality as well as the stability of food products. Superoxide anion has played an important role in the formation of other ROS such as H_2O_2 , OH radical and singlet oxygen which induces oxidative damage in the biomolecules. Superoxide free radicals are strongly oxidizing agents; they are commonly formed and their effects can be magnified because they are produced to other type of the free radicles in metabolic reactions in the cells. In this assay, the superoxide reduces NBT yellow dye to formazan blue colour, the activity measured on visible wavelength 560 nm through spectrophotometer. Antioxidants are capable to inhibit the blue NBT formation (Parejo *et al.*, 2002).

The decrease of absorbance at 560 nm with antioxidant indicates the consumption of superoxide in the reaction mixture. The unilluminated reaction mixture was used as a blank. It showed the maximum inhibition of superoxide radical generation by 21 $\mu\text{g}/\text{ml}$ concentration of silymarin with respect to standards (BHT) as depicted as in Figure 7.

5. Conclusion

The results of the present investigation clearly showed, that the silymarin plants have various phytochemical content and their respective antioxidant activities. Secondary metabolites of plant are a mostly phenolic compound which possesses on their structure a benzene ring with hydroxyl group. These metabolites are ubiquitously distributed in the plant and their parts; exhibit a wide range of pharmacological and curative properties, including anti-inflammatory, anticarcinogenic, vasodilatory actions. Plants are the

good resources for natural antioxidants. Antioxidant is one of the vital for preventing *in vivo* oxidative damages. The plants were used to evaluate the antioxidant potential using DPPH free radical scavenging assay, Fe^{2+} chelating assay, ferric (Fe^{3+}) reducing power assay, phosphomolybdenum reduction assay methods, chelating activities, hydrogen peroxide assay and superoxides anion radical scavenging activity. The methanol extract of silymarin showed higher phenols content as well as flavonoids content and it contributes to the higher antioxidant activity. Considering the results obtained, it could be concluded that the plant contains essential phytochemical constituents and possess active antioxidant property. The different studies are also carried out to find active component of the extract and to confirm the mechanism of action in animals. A number of beneficial health effects of silymarin have been reported. The above discussion revealed that silymarin can be used as pharmaceutical products, for decreasing the formation of toxic oxidation, keeping nutritional quality and increasing shelf life of pharmaceuticals.

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Conflict of interest

The authors declare that no conflict of interest exists in the course of conducting this research. All authors had final decision regarding the manuscript and the decision to submit the findings for publication.

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