Gemmomodification: An emerging source of natural antioxidants from *Silybum marianum*

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Abstract: Gemmomodification is a form of herbal medicine in which young freshly growing buds of plants are used. At germinating stage, plants metabolic activities are maximum and various nutrients, hormones enzymes and bioactive phytoconstituents are released and available at this stage. Plants may be promising source of natural antioxidants at growing stage. Oxidative stress leads to many chronic and degenerative diseases. Antioxidants are very essential for human body; they can protect the body from damage caused by free radical induced oxidative stress. This research project had been designed to investigate the antioxidant potential of gemmo modified and native (dry leaves) extract of *Silybum marianum*. Total phenolic contents was calculated by using Folin-Ciocalteu reagent and antioxidant potential was evaluated by using four radical assays (DPPH, ABTS, Super oxide and nitric oxide), reducing power assay and lipid peroxidation assay spectrophotometrically. Gemmo modified extract showed significantly higher ($p<0.050$) TPC (830 mg GAE/g of plant extract) as compared to native extract (800 mg GAE/g.) Results of this study revealed that gemmo modified extract demonstrated better antioxidant potential than natively used parts of *Silybum marianum.*

Keywords: *DPPH, ABTS, gemmo modification, Silybum marianum.*

INTRODUCTION

In addition to the conventional use of medicinal plants, a newly emerging way of therapy, the gemmotherapy is getting more considerations of scientists. Gemmotherapy is a therapeutic system, where plant bud extracts and other young tissues freshly collected from living growing plants are used. Gemmotherapy have great function in many detoxification processes. Various diseases can occur when the dislodged bioenergetics toxin are not drained from the body, due to lack of an effective elimination process. Embryonic plant materials like buds and young shoots of the selected plants were collected in the spring. At germinating stage, plants activities are at peak and many nutrients, vitamins, hormones enzymes and antioxidant phytoconstituents are released and available at this stage.

Reactive oxygen species (ROS), comprising of free radicals like super oxide anion(O^{2-}), Hydroxyl radical (OH), and non radical species like hydrogen peroxide(H_2O_2)and singlet oxygen and reactive nitrogen species (RNS) including $NO^{\cdot}$, NO_2; as free radical and HNO_2, N_2O_2 as non-radical are generated in the body as consequence of many metabolic activities. Over production of reactive species under many situations such as environmental pollution, pesticides, aging and exposure to radiation leads to oxidative stress (Davies, 2000; Vadlapudi and Naidu, 2009). Which results in more than one hundred diseases in humans including arthritis, atherosclerosis, ischemia and reperfusion injury of many tissues, gastritis, cancer, AIDS and central nervous system injury (Cook and Samman, 1996; Kumpulainen and Salonen, 1999; Pourmorad *et al*., 2006; Vadlapudi and Naidu, 2009; Adedapo *et al.,* 2009).

Antioxidants are compounds that slow down or delay the oxidation process by obstructing the initiation or proliferation of series of oxidizing reactions. Main source of antioxidants are the medicinal plants. In plants a wide range of phenolic compounds are present that vary from simple phenolic acid to highly polymerized compound such as tannins and flavonoids and termed as antioxidants (Borchardt *et al*., 2008). Recently, medicinal plants as a rich source of antioxidant have emerged as substantial therapeutic agents for the treatment of different diseases such as diabetes, cardiovascular diseases, cancer, anemia and malaria (Fola, 1993; Biapa *et al*., 2007). The availability and relatively cheaper cost of medicinal plants, makes them more attractive as curative agents when compared to 'modern' medicines (Biapa *et al*., 2007).

Silybum marianum is an important medicinal plant of family Compositae. It is commonly known as milk thistle and has been widely used in traditional medicines. It has a long history of use as a medicine for liver problems and depression. Recent research has confirmed that it has an ability to protect the liver from damage caused by alcohol and other poisons. The whole plant is astringent, bitter, and diuretic, hepatic and tonic. It is used internally in the treatment of liver and gall bladder diseases, hepatitis, jaundice, cirrhosis (www.pfaf.org/database/plants).

Due to great importance of antioxidants from medicinal plant, this research project has been designed to investigate the antioxidant potential of gemmo modified and native extract of *Silybum marianum*.

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MATERIAL AND METHODS

Collection of plants

Fresh plant material ((200 g) of *Silybum marianum* was collected during spring seasons (March) from Botanical Garden of University of Agriculture, Faisalabad, identified by plant taxonomist, Dr. Muhammad Mansoor, and compared with specimen No 10286 in herbarium of Department of Botany University of Agriculture, Faisalabad. Plant material was equally divided into fresh growing young leaves and dried plant material. Fresh growing plant material immediately immersed in the solvents to prepare gemmo-modified plant extract and the dried samples were air dried at room temperature for two weeks and used for the preparation of native extract.

Extract preparation

Preparation of gemmomodified extract

Fresh plant material (100 g) in the form of paste was macerated in alcohol and glycerin. The mixture was left to stand for one month in a cool, shaded environment. During that time it was agitated at different intervals. The extract was decanted and filtered after completion of time. The solvent was evaporated under reduced pressure in rotary evaporator.

Preparation of native extract

Native extract of *Silybum marianum* was prepared by refluxing dry leaves powder (10g) in methanol (100mL) for 2 hours. Then extract was filtered and solvent was evaporated.

Determination of total polyphenolic contents

Folin-Ciocalteu was the method of choice to quantify the total polyphenolic compounds in gemmo modified and native extract of *Silybum marianum* (Folin and Ciocalteu, 1927; Pourmorad *et al*., 2006). The calibration curve was prepared with ten different concentrations of gallic acid. Plant extract (1mL, 0.01g/mL) was mixed with ten folds diluted Folin-Ciocalteu reagent (5mL), after three minutes sodium carbonate (4mL, 20%) was added into this mixture and incubated at ambient temperature for 60 minutes. Absorbance of blue color was measured at 765 nm with a UV visible spectrophotometer. The total polyphenolic contents were determined as gallic acid equivalents (GAE) from calibration curve of standard by using following formula:

$$T = C \times V / M$$

Where

T = total contents of phenolic compound in mg GAE/g plant extract.

C = the concentration of galic acid calculated from calibration curve in mg/mL.

V = the volume of extract in mL.

M= the weight of plant extract in grams.

Determination of antioxidant activity

DPPH scavenging activity

The assay of DPPH radical quenching activity was carried out by the method of Yen and Chen (1995). Freshly prepared solution of DPPH in methanol (1mL, 0.1mM) was mixed with five different concentration (20-100µg/mL) of *Silybum marianum* extracts and then solution were left for 30 minutes in dark . The absorbance was noted at 517 nm against a blank solution. Synthetic antioxidant (butylated hydroxy toluene, BHT) as well as natural antioxidants (Vit. C) were used as standards. The effect of plant extract on inhibition of DPPH radical was calculated with following formula:

$$\text{DPPH Inhibition (\%)} = [1 - A_1/A_0] \times 100$$

Where

A_1 = Absorbance of sample.

A_0 = Absorbance of control.

Antioxidant activity with Stable ABTS radical cation

2, 2 Azinosib-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation assay was conducted according to the method of Re *et al*., 1999. ABTS was generated as a result of a reaction between aqueous solution of ABTS (7mM, 25 mL) and potassium per sulphate (24 mM, 25 mL). This mixture was incubated in the dark at ambient temperature for 14 hours. ABTS solution was diluted with methanol to maintain an absorbance of 0.7 at 734 nm. The absorbance was noted after the addition of diluted ABTS solution into different concentrations (20-100µg/mL) of polyphenolic extract (2 mL). Percentage inhibition was calculated as follows:

$$\text{\% Inhibition of ABTS Radical} = [1 - A_1/A_0] \times 100$$

Where

A_1 = Absorbance of sample.

A_0 = Absorbance of control.

Super oxide radical scavenging assay

Super oxide radical scavenging activity of *Silybum marianum* extract was measured according to method described by Vaidya *et al*. (2008). The activity was carried out by using NBT (Nitro blue tetrazolium reagent). Various concentrations (25, 75, 100 µg/mL) of standards (BHT and ascorbic acid) and *Silybum marianum* extracts were prepared. In this method aliquots (1mL) of extracts were taken in a test tube, to which sodium carbonate (1mL, 5%), of NBT (0.4mL, 150 µM) and of EDTA (0.3mL, 0.5%) were added. The reaction was started by the addition of hydroxyl-amine hydrochloride (0.4mL, 1%) to the above solution. Reaction mixture was incubated at 25°C for 5 minutes. The reduction of NBT was measured at 560 nm. A parallel control was also treated in the similar manner. Percentage inhibition was calculated by following formula:

$$\text{\% Inhibition of Super Oxide Radical} = [1 - A_1/A_0] \times 100$$

Where

A_1 = Absorbance of sample.

A_0 = Absorbance of control.

Nitric oxide scavenging assay

Nitric oxide radical scavenging activity of plant extract was evaluated according to the method described by Govindarajan *et al*. (2003). Sodium Nitro-purside in saline buffer (1mL) was mixed with different concentration of *Silybum marianum* extracts (100- 500 µg/mL) this mixture was kept for 30 minutes at room temperature. After incubation time Griess reagent (1% Sulphonamide, 2% Phosphoric Acid, 0.1% coupling reagent) was added into this mixture. The absorbance of resulting purple color was noted at 500 nm. Natural and synthetic antioxidants used as standard to compare the antioxidant activity of extracts Percentage inhibition was calculated as follows:

$$\% \text{ Inhibition of Nitric oxide} = [1 - A_{1}/A_0] \times 100$$

Where

A_1 = Absorbance of sample.

A_0 = Absorbance of control.

Determination of reducing power

Reducing power was measured according to the method described by Yen and Chen 1995. Various concentrations (2- 10 mg/mL water) of plant extracts were prepared. Equal volumes (2.5 mL) of phosphate buffer (2.5 mL, 0.2 M , pH 6.6) and potassium ferricyanide (1%) was mixed with plant extract (2.5 mL)in a test tube. The control contained all the reaction reagents except plant extract. The mixture was kept for 20 minutes at 50°C in oven. The reaction was stopped by the addition of TCA (2.5mL 1% w/v) and centrifuged at 3000 rpm for 10 min. Upper layer (2.5 mL) of centrifuged mixture was diluted with water (2.5 mL). Absorbance of this solution was noted at 700 nm after the addition of 0.5 mL ferric chloride solution (0.1% w/v).

Lipid peroxidation assay

This assay was performed according to the method previously conducted by Duan *et al*., 2007). Equal weights (0.2804g) of linoleic acid and emulsifier (Tween 20) were homogenized in phosphate buffer (50mL). The extracts (500 µg) were added into linoleic acid emulsion (2.5mL) and phosphate buffer (2 mL, 0.04 M, pH 7) and incubated at 37° C for 72 hours to accelerate the oxidation process. After regular intervals (each 24 hour), the incubated sample (2mL) was removed and mixed with $FeCl_2$ (0.5mL, 0.02 M) and ammonium thiocynate (0.5mL, 30% w/v). The amount of peroxide was determined by measuring the absorbance at 500 nm. Butylated hydroxy toluene (BHT) and ascorbic acid were used as standards. The percent inhibition of lipid peroxidation of linoleic acid emulsion was calculated as follows:

$$\% \text{ Inhibition of Lipid Peroxidation} = 100 - [A_1/A_0 \times 100]$$

Where

A_1 = Absorbance of sample

A_0 = Absorbance of control

STATISTICAL ANALYSIS

The results were expressed in terms of Mean ± S.D. Data was analyzed using analysis of variance ANOVA in SPSS 15 software ($p<0.05$)

RESULTS

Total phenolic contents

Total phenolic contents of *Silybum marianum* was determined by using Folin-Ciocalteu reagent and results were expressed as mg gallic acid equivalent (GAE mg/g extract of *Silybum marianum*). The results showed total phenolic contents present in gemmo modified extract (830mg/g GAE) were higher than native (dry leaves) extracts (800mg/g GAE). This might be due to high amount of phenolics in young fresh plant leaves and buds.

Determination of Antioxidant Potential

DPPH scavenging activity

The DPPH scavenging activity of ascorbic acid, BHT and different extracts of *Silybum marianum* are shown in the fig. 1. The methanolic extract of *Silybum marianum* leaves inhibited 63.48 ± 2.15% radical at the concentration of 100 µg /mL. Gemmomodified extract of *Silybum marianum* showed less free radical quenching activity (63.48 ± 2.15%) in comparison to methanolic extract of leaves and standard BHT (68.34 ± 2.80%).

Antioxidant activity with stable ABTS radical cation The ABTS assay is used to evaluate total antioxidant power of single compound and complex mixture of various plants (Chang *et al*., 2008). In fig. 2 results of percentage inhibition of ABTS radical of *Silybum marianum* were presented. The results showed the trend of % inhibition in the following order. BHT > gemmo modified extract > methanol extract of leaves > water extract. The extracts of *Silybum marianum* showed lesser inhibition as compared to standard (BHT

Super oxide radicals scavenging assay

Fig. 4 represented the results of superoxide scavenging activity of *Silybum maranium*. The order of free radical scavenging potency for the tested extracts and standards was as follow; Ascorbic acid (71.61%) > BHT (67.67%) > Gemmo modified extract (63.61%) > Native extract (57.68%).

Nitric oxide scavenging activity

The spectrophotometeric analysis for the scavenging of nitric oxide was performed to determine antioxidant activity of compounds. Results of % inhibition of Nitric oxide by *Silybum marianum* are shown in fig. 4. Significant differences were observed between different extracts of *Silybum marianum*. Gemmo modified extracts showed the highest percentage inhibition for nitric oxide radical. Results showed that gemmo modified extract of

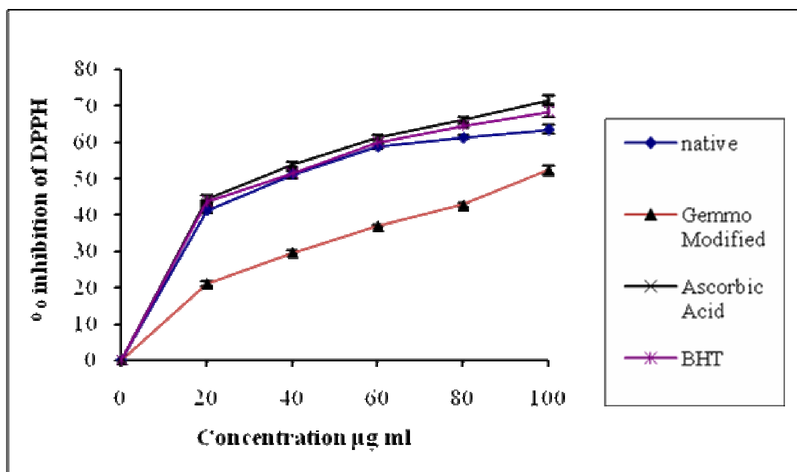

Fig. 1: Percentage (%) inhibition of DPPH radical by different extracts of *Silybum marianum.* All values are an average of triplicate experiment and represented as mean ± SD.

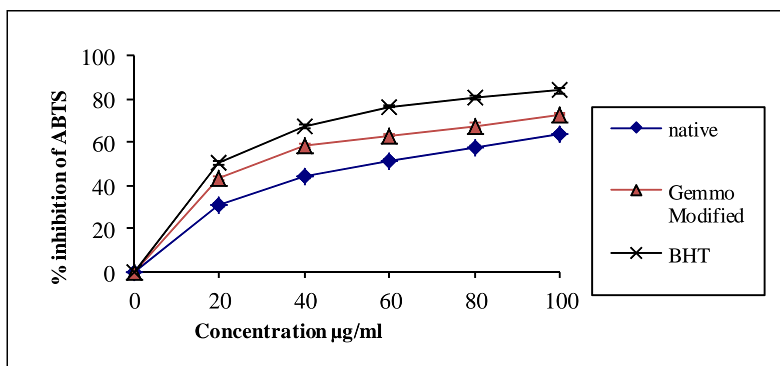

Fig. 2: Percentage (%) inhibitions of ABTS radical by different extracts of *Silybum marianum*. All values are an average of triplicate experiment and represented as mean ± SD.

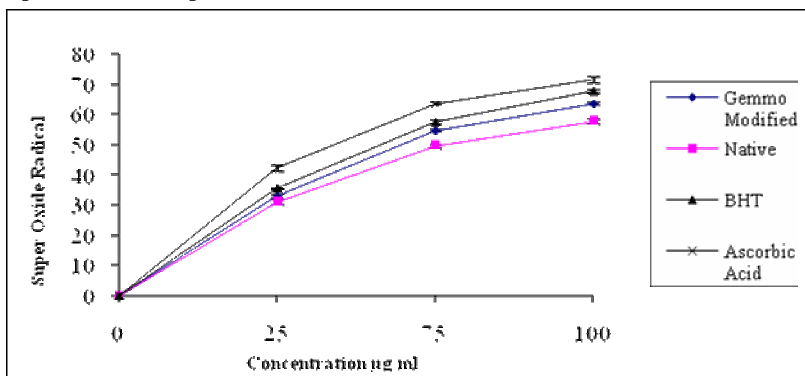

Fig. 3: Percentage (%) inhibition of Super Oxide Radical by different extracts of *Silybum marianum*. All values are an average of triplicate experiment and represented as mean ± SD

Silybum marianum have greater potential to inhibit NO radical than both standard BHT and ascorbic acid. Native extract showed lowest inhibition.

Reducing power assay

The results of reducing power are presented in fig. 5. In this study the reducing power of different extracts of *Silybum marianum* was found to increase in direct proportion to the increasing concentration of extracts from 2 to 10 mg/mL. It was observed that reducing power increased by increasing concentration, this might be due to increase in phenolic contents with the increase of concentration of extract.

Lipid peroxidation assay

Percentage inhibition of lipid peroxidation is presented in Fig.6. Following trend was observed in inhibition of lipid peroxidation; BHT > Grmmo > Native extract> ascorbic

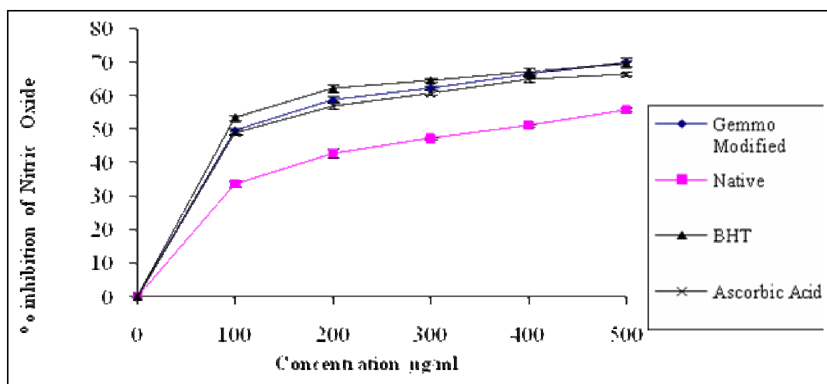

Fig. 4: Percentage (%) inhibition of Nitric Oxide by different extracts of *Silybum marianum*. All values are an average of triplicate experiment and represented as mean ± SD.

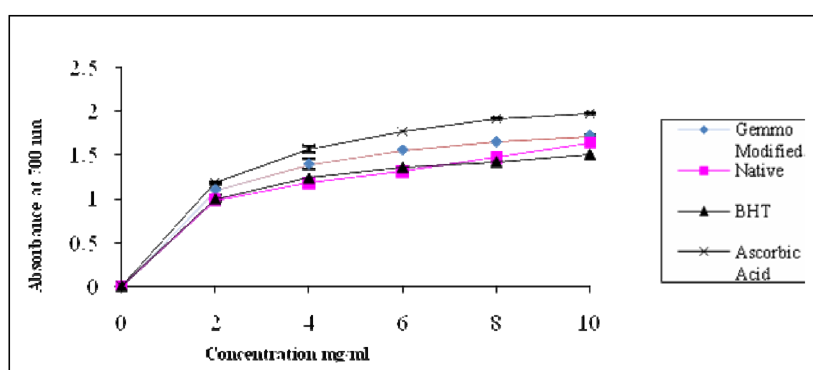

Fig. 5: Reducing power of different extracts of *Silybum marianum*. All values are an average of triplicate experiment and represented as mean ± SD.

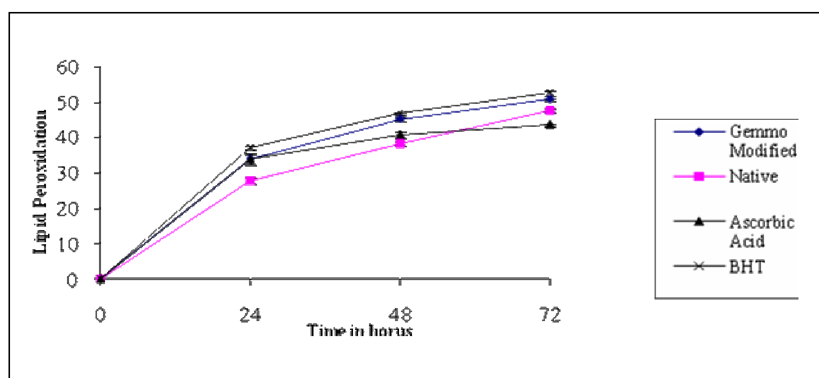

Fig. 6: Percentage (%) inhibition of Lipid Peroxidation by different extracts of *Silybum marianum*. All values are an average of triplicate experiment and represented as mean ± SD.

acid. Significant variation in antioxidant activity with respect to different timing was observed. By increasing incubation time percentage inhibition increased.

DISCUSSION

Gemmo modified extract (fresh leaves, young shoots, buds, mercerized in glycerin and methanol) demonstrated greater antioxidant potential toward all assays, than natively used extracts (leaves). Few studies have been reported about antioxidant activity of *Silybum marianum* (Asghar and Masood, 2008), but the antioxidant activity of gemmomodified is performed first time in Pakistan. Gemmotherapy; is an untapped research field, in which embryonic and freshly growing parts of plants are used. The fact is that at this stage of growth, activities of enzymes and hormones are high. Some other reports have been indicated that plant poly phenolics are natural defense compound for plants. During early stage of germination when biological activities are high excessive production of free radicals is initiated oxidation stress. A variety of phytoconstituents are synthesize during

photosynthesis, therefore phenolic contents are higher in young leaves and shoots (Shahidi and Nackz 2004).

Plant phenolics are multifunctional antioxidants. These compounds were reported to reduce reactive oxygen species by donating hydrogen atom or an electron (Huda-Faujan *et al*., 2007). Literature reports showed that phenolic compounds and antioxidant activity are highly correlated (Aliyu *et al*., 2009). The result of our findings for native extract was different from previous study of Asghar and Masood (2008). This disagreement between phenolic compounds can be explained by different variables such as the geographical area and seasonal time of collection of plant (Jastrzebski *et al*., 2007).

The DPPH assay is a quick, reliable and reproducible parameter and has been largely used to investigate the antioxidant power of specific pure compounds as well as plant extracts (Aliyu *et al*., 2009). Results of our study differ from the previously reported by Asghar and Masood (2008). They evaluated the antioxidant activity by using DPPH radical. The antioxidant activity was 131 ± 10.5, 622.7 ± 13.6, 242.3 ± 10.8, 535 ± 24.3 and 169.3 ± 21.9 µM/L in silymmarin, vitamin C, vitamin E, BHT and green tea respectively. This difference might be due to geographical area and seasonal time of collection of plant (Jastrzebski *et al*., 2007).

The ABTS assay is used to evaluate total antioxidant power of single compound and complex mixture of various plants (Chang *et al*., 2008). Results of this study showed that by increasing the concentration percentage inhibition increases that might be due to increase in phenolic contents by increasing concentration. The highest inhibition was observed at conc. 100 µg/mL. Same trend was reported in some previous studies (Katalinic *et al*., 2006; Adedapo *et al*., 2009).

Gemmo modified extract of *Silybum maranium* was very effective superoxide and nitric oxide radical scavenger. Percentage inhibition is related positively with TPC and increases with increase in concentration. The results are in agreements with earlier reports that phenolic compounds significantly contribute to antioxidant potential of plants (Jadhav *et al*. (2009) and Shyur *et al*. 2005).

It has been observed that reducing power is associated with antioxidant activity. The reducing power of a compound may serve as a significant indicator of its potential to scavenge free radical (Duan *et al*., 2007). These results are in agreement with findings reported by Asghar and Masood, 2008; Aliyu *et al*., 2009 and Sanja *et al*., 2009).

In Lipid peroxidation a significant correlation between antioxidant potential and phenolic contents was observed (Aliyu *et al*., 2009).

CONCLUSION

The results this study indicated that gemmomodified extract of *Silybum marianum* have greater antioxidant potential as compared to natively used part of plant.

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