

# Unfolding the antioxidative and free radicals scavenging potential of stem bark of the wonder tree, *Prosopis cineraria* (L.) Druce: An *in vitro* approach

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## Abstract

**Objective:** The present experimental investigation was conducted to unravel and analyze the antioxidant potential of sequential extracts (petroleum ether, chloroform, ethyl acetate, ethanol, hydroethanol, and aqueous) of *Prosopis cineraria*. **Materials and Methods:** Quantitative assays for estimating content of phytochemicals (phenols, flavonoids, proanthocyanidins, tannins, and saponins), *in vitro* antioxidant and free radicals scavenging assays were performed. Reducing power capability of various extracts was done. The research investigated and calculated the half-inhibitory concentration (i.e.,  $IC_{50}$ ) values for various sequential extracts. **Results:** The research study revealed that hydroethanol extracts showed maximum antioxidant activity and free radical scavenging potential in contrast to other extracts. Hydroethanol extracts of the stem bark of the studied plant had comparatively lower  $IC_{50}$  values in comparison to other extracts. Quantitative measurements of phytoconstituents also revealed that the plant extracts are rich in bioactive constituents and antioxidants. **Conclusions:** This investigation would encourage researchers toward the extracts of stem bark of *P. cineraria*, which have significant antioxidative potential. The plant extracts can be further explored for understanding the mechanism underlying the free radicals scavenging, by conducting *in vivo* studies.

**Key words:** Antioxidant,  $IC_{50}$ , phytochemicals, *Prosopis cineraria*, reactive oxygen species

## INTRODUCTION

Medicinal plants are reservoirs of novel medicinal therapeutics due to the presence of various bioactive constituents or phytochemicals.<sup>[1]</sup> Most of these bioactive entities are antioxidant in nature. The antioxidants from plants usually are secondary metabolites that have the capacity to alleviate oxidative stress rendered by the activity of reactive oxygen species (ROS). These antioxidants are generated in the plant as a result of their own defense mechanisms. In the past and recent years, a number of medicinally important plants have been studied for their ability of neutralizing specific ROS, such as hydroxyl radicals, superoxide radicals, and nitric oxide radicals.<sup>[2]</sup>

India is rich of such pharmacologically important known medicinal plants. *Prosopis cineraria* (L.) Druce, commonly known as “Khejri,” is the state tree of Rajasthan.<sup>[3]</sup> The

genus *Prosopis* belongs to the family: leguminosae and subfamily: mimosaceae. This tree possesses ability to grow under harsh climatic conditions prevalent in dry, semi-arid, and arid regions of India, Afghanistan, Iraq, Pakistan, and Arabia. In India, it is commonly found in Rajasthan, Punjab, Haryana, Gujarat, Western Uttar Pradesh, and other drier regions of Deccan.<sup>[4]</sup>

It is a multipurpose tree due to the fact that all the parts of *P. cineraria* are useful for therapeutic purposes, and hence, it is referred as “Kalpavriksha” in the ancient literature of India.<sup>[5]</sup> *P. cineraria* has therapeutic importance and it is used for curing

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**Received:** 08-11-2019

**Revised:** 17-01-2020

**Accepted:** 28-01-2020

serious diseases and possesses pharmacological activities such as antifungal, anthelmintic, anticancer, antibacterial, antiviral, antihyperglycemic, antihyperlipidemic, and antioxidative.<sup>[6]</sup> Numerous phytoconstituents such as tannins, steroids, flavone derivatives (namely, prosogerin A, B, C, and E), rutin, patulitrin, luteolin, patuletin, and alkaloids have been reported and isolated from different parts of this medicinal plant.<sup>[7]</sup>

Considering the multiple uses of *P. cineraria* for various medicinal purposes, the present study is designed to validate these traditional folkloric medicinal uses.

## MATERIALS AND METHODS

The chemicals and all reagents mentioned in the present experimental research were of high purity (98–99%) and analytical grade. Chemicals were purchased from reliable firms and sources.

### Procurement of Plant

The stem bark of *P. cineraria* was collected from the local region of Banasthali Vidyapith, Rajasthan. The collected plant was identified by Farm Manager at Krishi Vigyan Kendra, Banasthali Vidyapith, Rajasthan, and authenticated at Herbarium unit of Banasthali Vidyapith, Rajasthan, India (Herbarium No.-BVR11359/2017).

### Preparation of Plant Extracts

The plant material, i.e., stem bark was shade dried and then powdered using mixer grinder. The powdered stem bark (30 g) was packed in a thimble and subjected to sequential Soxhlet extraction with petroleum ether, chloroform, ethyl acetate, and ethanol. After the sequential extraction, the marc obtained was extracted in water by maceration method.

### Preparation of hydroethanol extract

The powdered plant material was extracted with petroleum ether and then the obtained marc was subjected to Soxhlet extraction by 80% ethanol.

The extracts were evaporated to dryness using rotary evaporator and stored in airtight jars at 4°C for further experimental usage. For experimental use, mixed the plant extracts with their respective solvents to achieve a concentration of 1 mg/ml.

### Quantitative Assays

#### Total phenol content

To the sample of plant extract and standard (gallic acid), 1 ml of Folin–Ciocalteu’s reagent (diluted) was added. Five minutes later, 7% Na<sub>2</sub>CO<sub>3</sub> and distilled water were added to

the reaction mixture. The reaction test tubes were allowed to incubate for about 90 min (in dark) and further centrifuged at 10,000 rpm for 5 min. After centrifugation, the supernatant was taken and absorbance was recorded at 750 nm and total phenolic content was expressed as mg gallic acid equivalent (GAE) per g of plant sample.<sup>[8]</sup>

#### Total saponin content

To experimental plant extracts and standard plant saponin, 0.400 ml of 5% vanillin-acetic acid solution and perchloric acid (1.6 ml) were mixed and temperature was raised to 70–75°C for nearly 15 min. After heating, cooled the reaction mixture on ice for about 1–2 min and then, added 2.5 ml of glacial acetic acid (GAA). After mixing, the absorbance was recorded at 550 nm against blank.<sup>[9]</sup>

#### Total flavonoid content (TFC)

For determining the TFC, the samples of plant extracts and standard quercetin (1 mg/ml), distilled water (2 ml), and 15% NaNO<sub>2</sub> (0.15 ml) were added. After 6 min, 0.15 ml of AlCl<sub>3</sub> (10%) was added. After 6 min of incubation, 2 ml of NaOH (4%) was added to the reaction mixture and distilled water was also added to make up the total final volume of 5 ml. The reaction mixture was allowed to rest for about 15 min and then absorbance was recorded at 510 nm against blank.<sup>[10]</sup>

#### Total proanthocyanidin content

Total proanthocyanidin content in the plant extracts was estimated by mixing different concentrations and catechin (standard) with 1 ml of ethanol (70%), 25% hydrochloric acid, and distilled water (5%) and later on incubated at 85–90°C in the water bath for 80 min. After cooling, absorbance was measured at 545 nm after adding *n*-butanol (1.5 ml).<sup>[11]</sup>

#### Total tannin content (TCC)

For determining TTC, 1 ml of different concentrations of plant extracts and tannic acid (as a standard) were diluted with double-distilled water (8 ml), diluted Folin–Ciocalteu’s reagent and 20% Na<sub>2</sub>CO<sub>3</sub>. Record the absorbance of the reaction mixture at 775 nm against the sample blank.<sup>[12]</sup>

### *In vitro* Free Radical Scavenging and Antioxidative Potential Estimation

#### 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The experimental plant extracts and standard (ascorbic acid) 0.250 ml were treated with 0.004% DPPH solution (2.5 ml) and allowed to incubate at room temperature for about 30 min (in dark) before recording the absorbance at 517 nm.<sup>[13]</sup>

#### SO<sub>2</sub> scavenging activity

The reaction mixture contained nitroblue tetrazolium (0.144 mM), NADH (0.677 mM), different dilutions of plant extract along

with rutin standard, and premenstrual syndrome (0.060 mM). All reagents were prepared using 0.1 M phosphate buffer (pH 7.4). Absorbance was measured spectrophotometrically at 560 nm after incubation of the test tubes for 3 min.<sup>[14]</sup>

### ***OH*<sup>-</sup> scavenging activity**

The consolidated reaction mixture constituted ethylenediaminetetraacetic acid (10 mM), FeSO<sub>4</sub>·7H<sub>2</sub>O (10 mM), 2-deoxyribose (10 mM), plant extracts and mannitol as a standard, 0.1 M phosphate buffer (pH 7), and H<sub>2</sub>O<sub>2</sub> (10 mM), to make up a total final volume of 1.8 ml. Reaction tubes were kept for incubation at 37°C (for 1 h). Later on, 0.5% thiobarbituric acid (TBA – 1 ml) and ice-cold trichloroacetic acid (TCA – 2.8% in 0.025 M NaOH) were mixed. The reaction mixture was incubated at a temperature of 80°C for about 30 min, then the absorbance was recorded at 532 nm.<sup>[15]</sup>

### ***NO*<sub>2</sub> radical scavenging activity**

The consolidated reaction mixture (3 ml) composed of sodium nitroprusside (SNP; 10 mmol/L), 0.1 M phosphate buffer (pH 7.4), and experimental plant extracts along with rutin. Test tubes were allowed to incubate at 25°C for about 150 min. Post-incubation, the reaction mixture (0.5 ml) was mixed with 1 ml of sulfanilic acid reagent (i.e., 0.33% in 20% GAA) and kept at room temperature for nearly 5 min. Then, 1 ml of 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride was mixed. After 30 min, the absorbance was recorded at 564 nm.<sup>[16]</sup>

### **Ferric reducing antioxidative power assay**

The stock solution consisted of 300 mM acetate buffer (pH 3.6) and 10 mM tripyridyltriazine in 40 mM HCl and 20 mM FeCl<sub>3</sub>·6H<sub>2</sub>O. For preparing fresh ferric reducing antioxidant power (FRAP) reagent, all the components were mixed in the 10:1:1 ratio. The working solution was heated to attain the temperature of 37°C. All the plant extract samples and standard (FeSO<sub>4</sub>·7H<sub>2</sub>O) were treated with FRAP reagent (2.85 ml) and allowed to incubate for about 30 min (in dark). Later on, absorbance was recorded at 593 nm.<sup>[17,18]</sup>

### **Total reducing power assay**

The plant extract sample and rutin (standard) were treated with 0.2 M phosphate buffer (pH 6.6) and potassium ferricyanide (1%). The samples were further incubated for about 20 min at 50°C before the addition of 10% TCA. Later on, the upper layer of the reaction tube (2.5 ml) was procured and treated with FeCl<sub>3</sub> (0.1%) after diluting the reaction mixture by addition of 2.5 ml distilled water. The absorbance of the reaction test tubes was recorded at 700 nm.<sup>[19]</sup>

### **Total antioxidant capacity (TAC)**

The plant extract was treated with TAC solution (1 ml) prepared by addition of 0.6 M sulfuric acid, NaH<sub>2</sub>PO<sub>4</sub> (28 mM), and ammonium molybdate (4 mM) in the ratio of 1:1:1. The reaction mixture was further incubated at 95°C for about 90 min before recording absorbance at 695 nm.<sup>[20]</sup>

Calculations:

Experimental results were calculated and expressed as milligram (mg) standard equivalents/grams of dry weight of plant material using formula:

$$C = c \cdot V/m$$

where, *C* = contents of experimental plant extract (mg/g); *c* = content of phytoconstituents in experimental plant sample that is extrapolated from standard curve (mg/ml); *V* = volume of the sample (ml), *m* = weight of the experimental plant extracts (g).

## **RESULTS AND DISCUSSION**

### **Quantitative Assays**

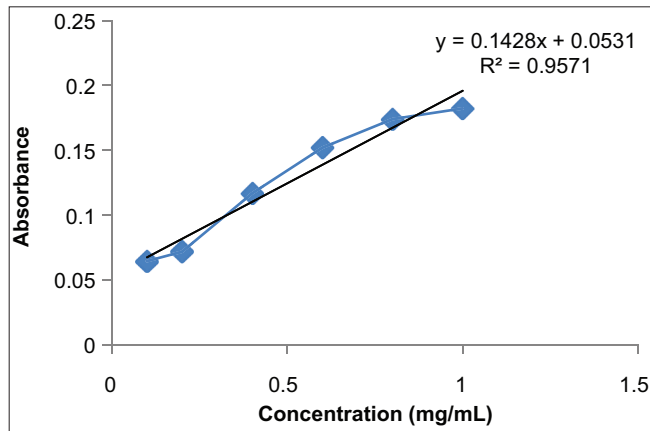
Secondary metabolites of *P. cineraria* stem bark such as phenols, flavonoids, proanthocyanidins, tannins, and saponins in various extracts were analyzed [Table 1]. Total content in

**Table 1:** The parameters analyzed on different extracts of stem bark of *Prosopis cineraria*

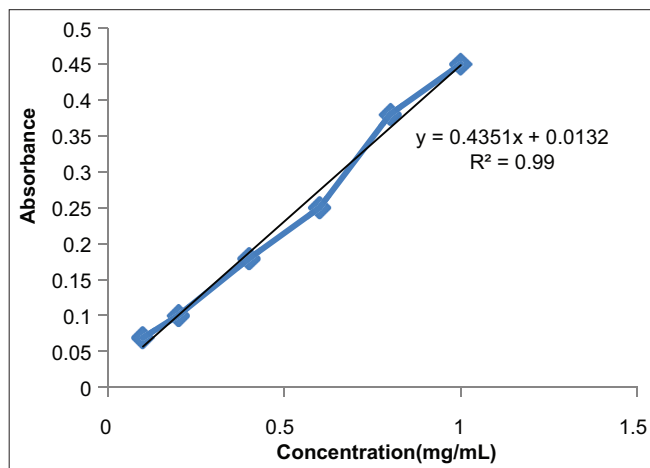
Parameter evaluated	Extracts of stem bark of <i>Prosopis cineraria</i>					
	Petroleum ether	Chloroform	Ethyl acetate	Ethanol	Hydroethanol	Aqueous
Total phenolic content (mg GAE/g)	168.03±0.01	178.01±0.04	183.02±0.15	221.15±0.21	233.09±0.12	210.14±0.11
Total saponin content (mg SE/g)	-	-	-	65.16±0.23	76.19±0.21	79.09±0.09
Total flavonoid content (mg QE/g)	87.03±0.11	115.17±0.31	91.04±0.23	165.89±0.51	177.90±0.47	143.12±0.87
Total proanthocyanidin content (mg CE/g)	-	-	-	9.12±0.01	12.07±0.05	7.78±0.03
Total tannin content (mg TAE/g)	191.09±0.51	204.12±0.03	198.03±0.12	241.01±0.07	253.13±0.03	213.02±0.04

All values are expressed in mean±SD, GAE: gallic acid equivalent, SE: saponin equivalent, CE: Catechin equivalent, QE: Quercetin equivalent, TAE: tannic acid equivalent, SD: standard deviation

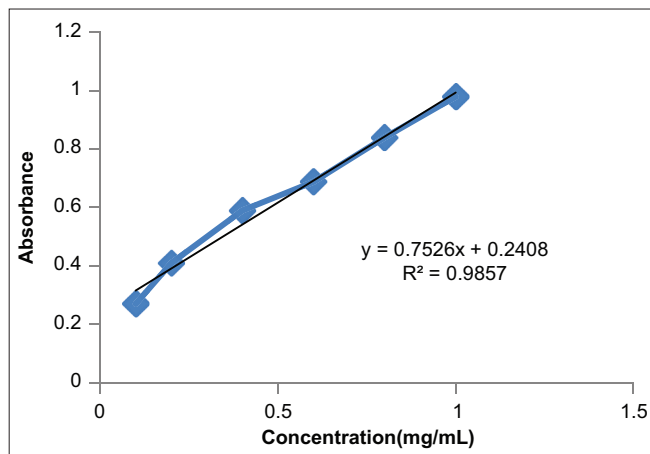
different sequential fractions of *P. cineraria* stem bark was estimated as GAE using regression equations extrapolated from the standard curve. Regression equations used for estimation of total phenols ( $y=0.142x+0.053$ ,  $R^2=0.95$ ) as per the standard curve [Figure 1], flavonoids ( $y=0.435x+0.013$ ,  $R^2=0.99$ ) as



**Figure 1:** Standard curve of gallic acid for phenol content determination

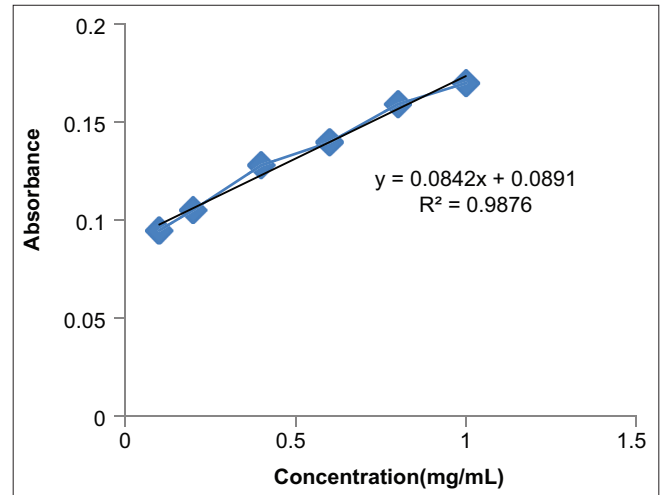


**Figure 2:** Standard curve of quercetin for flavonoid content determination

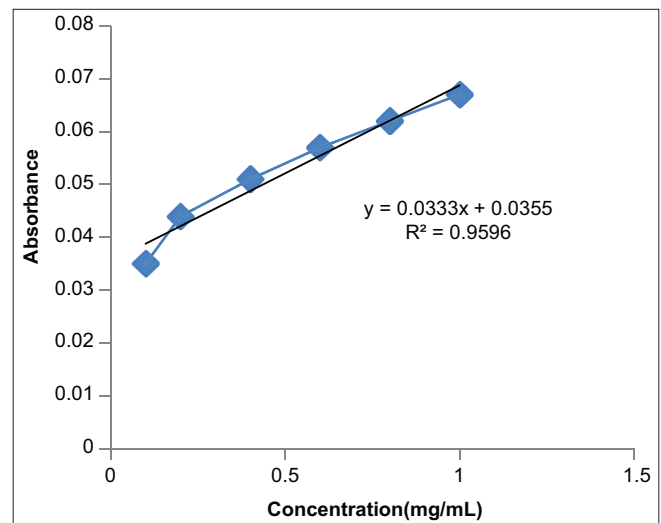


**Figure 3:** Standard curve of catechin for proanthocyanidin content determination

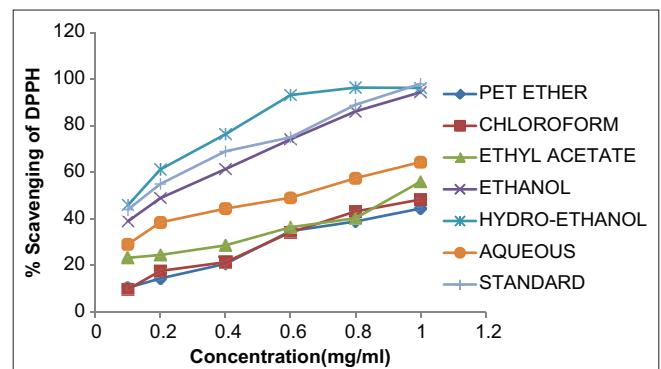
calculated in the standard curve [Figure 2], proanthocyanidins ( $y=0.752x+0.024$ ,  $R^2=0.98$ ) as per the calculation in the standard curve [Figure 3], tannins ( $y=0.084x+0.089$ ,  $R^2=0.98$ ) as calculated in the standard curve [Figure 4] and saponins



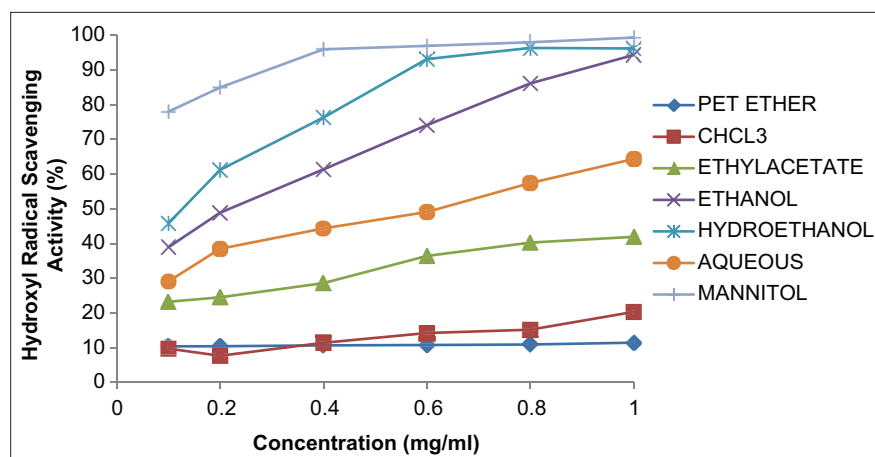
**Figure 4:** Standard curve of tannic acid for tannin content determination



**Figure 5:** Standard curve of sapogenin for saponin content determination



**Figure 6:** 2,2-Diphenyl-1-picrylhydrazyl scavenging potential of various extracts of *Prosopis cineraria*



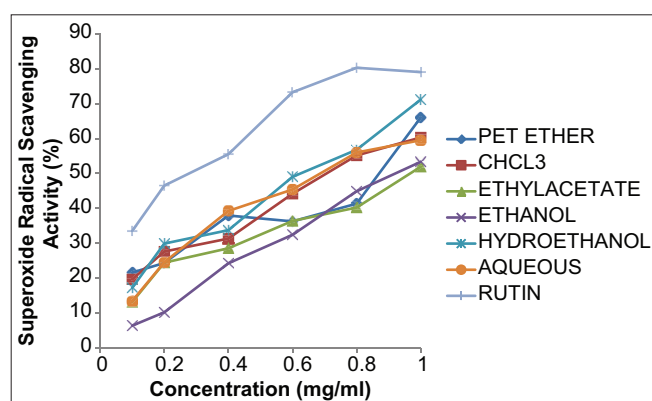
**Figure 7:** Hydroxyl radical scavenging potential of various extracts of *Prosopis cineraria*

( $y=0.033x+0.035$ ,  $R^2=0.95$ ) as calculated in the standard curve [Figure 5]. Hydroethanol extract is rich in these components in comparison to other extracts. Table 1 shows that petroleum ether and ethyl acetate extract possess very low TFC. Proanthocyanidin content was present in hydroethanolic extract, other extracts contain diminished amount of proanthocyanidin. Literature highlighted that various phytochemicals (phenols, tannins, flavonoids, and saponins) show antimicrobial, anticarcinogenic, and antimutagenic potentials.<sup>[21]</sup> Petroleum ether extract possesses very less amount of all components in comparison to the other extracts. Results showed that the polar fractions (ethanol, aqueous, and hydroethanol) possess more amounts of phytochemicals necessary for the free radical scavenging and antioxidant potential as compared to the non-polar fractions. This might be the reason for the hydroethanol extract to possess more phenols, flavonoids, and other phytochemicals in comparison to the other all extracts. Hydroxyl groups present in phenols render the capacity of free radical scavenging. These are very important for the treatment of various diseases such as cardiovascular diseases because phenols inhibit the process of oxidation of unsaturated lipids. The presence of phenols and flavonoids indicates that this plant might be used for antimicrobial, anti-inflammatory, antioxidant, antipyretic, and analgesic activities.<sup>[22,23]</sup> These findings suggested that *P. cineraria* is an important medicinal plant for the treatment of various ailments.<sup>[24]</sup> *P. cineraria* also contain saponins that can bind to cholesterol and enable the formation of insoluble complex that is excreted through the bile, thereby prevents the reabsorption of cholesterol.<sup>[25]</sup> *P. cineraria* is also helpful in wound healing and this is due to the presence of tannin content which plays a significant role in wound healing. The presence of tannins in *P. cineraria* strongly supports their use as a phytomedicine.<sup>[26]</sup> Thus, all these phytoconstituents are known to possess medicinal as well as pharmacological activities.<sup>[27]</sup>

## Antioxidant Activity

### DPPH radical scavenging activity

DPPH is among the most stable free radical even at room temperature. DPPH is used to testify the potential of



**Figure 8:** Superoxide radical scavenging potential of various extracts of *Prosopis cineraria*

compound as free radical scavenger of hydrogen donor and to explore the antioxidant potential of *P. cineraria*. The mode of the action of DPPH free radical scavenging activity is a result of neutralization of DPPH free radical by plant extract due to the transfer of an electron or hydrogen ion. All plant extracts revealed a good DPPH radical scavenging activity, as shown in Figure 1. DPPH scavenging activities of various fractions of *P. cineraria* depend on the concentration of the extracts. Maximum DPPH scavenging activity and minimum  $IC_{50}$  value were shown by hydroethanol extract after the standard (ascorbic acid), which contained the highest amount of phenol, flavonoid, tannin, and saponin. Petroleum ether extract showed minimum scavenging activity and maximum  $IC_{50}$  value as well as less amount of phenolic content. The ability to scavenge 50% of DPPH was found to be  $IC_{50}$   $0.12 \pm 0.05$  mg/ml for hydroethanol <ethanol ( $0.234 \pm 0.03$  mg/ml) <aqueous ( $0.595 \pm 0.02$  mg/ml) <ethyl acetate ( $0.926 \pm 0.11$  mg/ml) <chloroform ( $1.01 \pm 0.87$  mg/ml) <petroleum ether ( $1.12 \pm 1.14$  mg/ml). According to these results, each extract of *P. cineraria* demonstrated concentration-dependent antioxidant activity. Experimental research establishes that ascorbic acid, flavonoid, tannin, and aromatic amines can reduce and decolorize DPPH by their hydrogen donating capability. Results also proved this because hydroethanol extract showed maximum amount of phenol,



flavonoid, and tannin and also showed maximum DPPH scavenging activity [Figure 6].

### Hydroxyl radical scavenging activity

The plant extracts were found to exhibit tremendous antioxidant activity against hydroxyl radical with the IC<sub>50</sub> values ranging from 0.64 to 3.7 mg/ml compared to mannitol. Hydroethanol extract was experimentally proved to possess the highest antioxidant activity with an IC<sub>50</sub> value of 0.64 mg/ml after the standard mannitol. Petroleum ether extract exhibits low level of antioxidant activity in comparison to the other extracts. The ability to scavenge 50% of hydroxyl radical was found to be IC<sub>50</sub> 0.64 ± 0.14 mg/ml for hydroethanol <aqueous (0.757 ± 0.08 mg/ml) <ethanol (0.854 ± 0.09 mg/ml) <chloroform

(1.2 ± 0.19 mg/ml) <ethyl-acetate (1.96 ± 0.16 mg/ml) <petroleum ether (3.7 ± 0.06 mg/ml). Hydroxyl radicals are the most reactive and can render severe tissue damage and injury. The free radical scavenging potential of the plant extracts relies on the presence of phenols, tannins, flavonoids, and saponins [Figure 7].

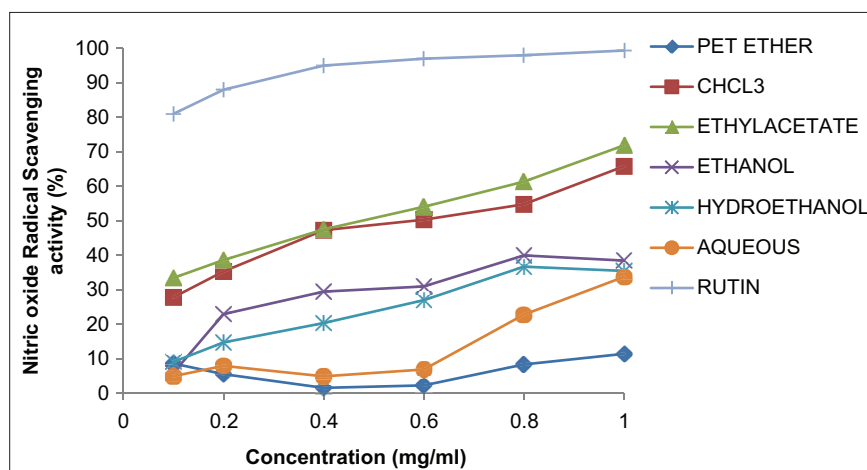
### Superoxide anion radical scavenging potential

The extracts of the studied medicinal plant *P. cineraria* were found to exhibit strong antioxidant activity against superoxide anion radical with the IC<sub>50</sub> values from 0.54 to 3.4 mg/ml compared to rutin. Hydroethanol extract was found to have the potent antioxidant activity with lower IC<sub>50</sub> value (0.54 mg/ml) in comparison to standard and other extracts. Petroleum

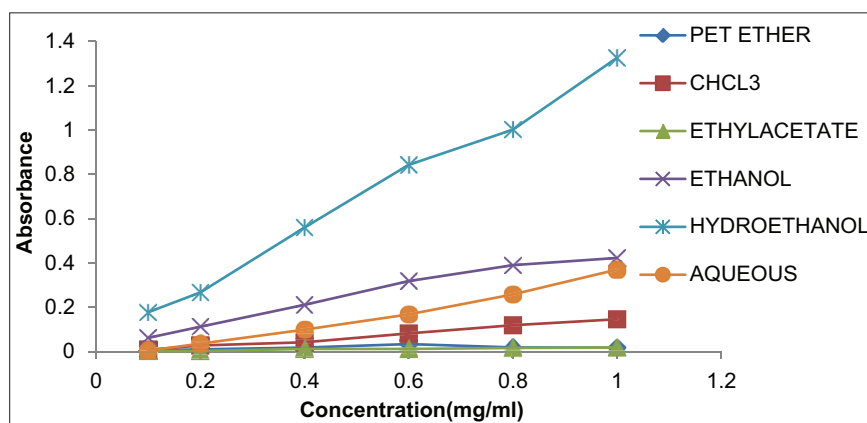
**Table 2:** Parameters evaluated for different extracts of stem bark of *Prosopis cineraria*

Parameter evaluated	Extract of stem bark of <i>Prosopis cineraria</i>					
	Pet ether	Chloroform	Ethyl acetate	Ethanol	Hydroethanol	Aqueous
FRAP activity (µM Fe[II] ions/mg dried plant extract)	2.67±0.01	4.51±0.09	3.78±0.14	27.09±0.61	34.09±0.11	19.03±0.04
Total antioxidant capacity (mg GAE/g)	233.09±0.86	350.01±0.51	312.04±0.38	919.04±0.54	974.09±0.04	816.03±0.01

FRAP: Ferric reducing antioxidant power, GAE: gallic acid equivalent



**Figure 9:** Nitric oxide radical scavenging potential of various extracts of *Prosopis cineraria*



**Figure 10:** Reducing potential of various extracts of *Prosopis cineraria*

ether extract exhibits low level of antioxidant activity in comparison to the other extracts. The ability to scavenge 50% of superoxide anion was found to be  $IC_{50}$   $0.54 \pm 0.09$  mg/ml for hydroethanol <ethanol ( $0.674 \pm 0.08$  mg/ml) <aqueous ( $0.727 \pm 0.17$  mg/ml) <chloroform ( $1.72 \pm 0.14$  mg/ml) <ethyl acetate ( $2.3 \pm 0.18$  mg/ml) <petroleum ether ( $3.4 \pm 0.14$  mg/ml). Superoxide anions are important biologically as they can lead to the formation of singlet oxygen and hydroxyl radicals but when overproduced; then, they prove to be very detrimental to cellular components by triggering the generation of other more potent ROS.<sup>[28]</sup> Hence, it could be presumed that the hydroethanol extract of *P. cineraria* acts as a potential therapeutic agent for the treatment of oxidative damage and prevents the effects of excessive superoxide anion production in the human body [Figure 8].

### Nitric oxide radical scavenging potential

The experimental plant extracts were found to exhibit strong antioxidant activity against nitric oxide radical with the  $IC_{50}$  values ranging from 0.076 to 0.33 mg/ml compared to rutin. Hydroethanol extract was unraveled to possess the highest antioxidant activity with the  $IC_{50}$  value of 0.076 mg/ml, after the standard rutin. However, petroleum extract exhibits low level of antioxidant activity in comparison to the other extracts. The ability to scavenge 50% of nitric oxide was found to be  $IC_{50}$   $0.076 \pm 0.21$  mg/ml for hydroethanol <ethanol ( $0.089 \pm 0.02$  mg/ml) <aqueous ( $0.094 \pm 0.08$  mg/ml) <chloroform ( $0.15 \pm 0.01$  mg/ml) <ethyl acetate ( $0.26 \pm 0.31$  mg/ml) <petroleum ether ( $0.33 \pm 0.14$  mg/ml) [Figure 9].

### Reducing power activity

Reducing power activity estimates the electron-donating capacity of the plant extracts.<sup>[29]</sup> The presence of reducers in the plant extract serves as indicator of excellent antioxidant capacity. Reducing power of the various extracts of *P. cineraria* was increased with an increase in its concentration. According to the results, hydroethanol extract showed maximum reducing power in comparison to the other extracts. Petroleum ether extract showed minimum reducing power. Reducing capacity of the experimental plant indicates its antioxidant activity. Hence, plants reducing capacity and antioxidant activity are directly correlated to each other. *P. cineraria* stem bark extract showed high reducing capacity as well as high antioxidant activity [Figure 10].

### FRAP assay

FRAP is a useful method to determine the capability of the experimental plant extracts to transfer electron to Fe(III). All sequential extracts of *P. cineraria* were found to exhibit FRAP activity [Table 2]. Hydroethanol extract demonstrated a higher FRAP activity than other extracts, while petroleum ether demonstrated minimum FRAP activity. The higher value of FRAP indicates the greater antioxidant activity. Based on the experimental findings, the hydroethanol extract was found to be most effective in comparison to the other extracts.

### TAC

TAC of different sequential fractions was calculated using regression equations obtained from standard graph. All extracts showed tremendous TAC [Table 2]. Hydroethanol extract of *P. cineraria* possesses maximum TAC in comparison to the other extracts.

## CONCLUSIONS

The present research work unfolded and showed that all extracts of *P. cineraria* stem bark (petroleum ether, chloroform, ethyl acetate, ethanol, hydroethanol, and aqueous) are rich in various phytochemicals, namely, phenols, flavonoids, tannins, and saponins. Hydroethanol extract of *P. cineraria* was found to exhibit maximum phytochemicals and antioxidative potential in contrast to all other extracts (petroleum ether, chloroform, ethyl acetate, ethanol, and aqueous). It could be concluded that *P. cineraria* can be further studied for the isolation and structural characterization of natural antioxidative entities as it was explored that *P. cineraria* could be a storehouse of natural antioxidants for combating ROS injury. Moreover, *in vivo* studies are also needed to be conducted so as to determine the underlying mechanism of action of these phytoconstituents in alleviating oxidative stress-induced damages.

## ACKNOWLEDGMENTS

The authors are grateful and wish to acknowledge the support provided by the authorities of the Banasthali Vidyapith, Rajasthan for conducting experimental research work.

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**Source of Support:** Nil. **Conflicts of Interest:** None declared.