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Correlation of total phenolic content and antioxidant activity of *Salvadora persica* root extracts

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Abstract
The accumulation of free radicals is known to contribute to various oxidative stress-related disorders, including diabetes, arthritis, neurodegenerative diseases, cardiovascular diseases, and cancer. As synthetic antioxidants are associated with side effects, there is a growing interest in identifying new naturally occurring antioxidant compounds. In this particular study evaluation of antioxidant activity of *Salvadora persica* root extracts using different in *vitro* assays was done. Various solvent extracts were assessed for their ability to scavenge DPPH free radicals, superoxide radicals, and hydroxyl radicals, as well as metal chelating activity. Additionally calculation of the reducing power and determination of the total phenolic content of the five different extracts at varying concentrations was carried out. The methanolic extract demonstrated the highest antioxidant activity in the various assays, and the total phenolic content in the methanolic extract was measured to be 44.83mg/g Gallic acid equivalent. The study revealed a positive correlation between the total phenolic content and antioxidant activities, suggesting that phenolic acids may play a crucial role in the antioxidant activity of the plant. Based on these findings, it can be concluded that *S. persica* possesses noteworthy antioxidant activity and has the potential to serve as therapeutic agent in preventing the progression of oxidative stress related degenerative disorders

Keywords: Free radicals, *Salvadora persica*, oxidative stress, DPPH radical, Superoxide radical

Introduction
Generation of free radicals through endogenous factors (aerobic respiration), and exogenous factors such as smoking, stress, ionizing radiation, and environmental pollution is obvious in biological systems. Under normal metabolic conditions their generation is regulated by various enzymatic and non- enzymatic pathways. During disease state when immune system is compromised their build-up can lead to lipid peroxidation, enzyme inactivation and DNA damage resulting in various pathological conditions, including atherosclerosis, stroke, arthritis, neurodegenerative disorders, diabetes, cardiovascular diseases, cancer and aging. Antioxidants by possessing redox properties have the ability to neutralize free radicals by donating electrons and terminating chain reactions thus show great potential as therapeutic agents in management of free radical mediated oxidative stress [1, 2, 3].
Although synthetic antioxidants like BHA, BHT, and PG exhibit potent antioxidant properties, their use is limited due to mutagenesis, toxicity, high cost, and limited accessibility. As a result, there is a search for novel antioxidants that are safe, easily available, and cost-effective. Medicinal plants, which have been traditionally used in various systems of medicine, offer promising alternatives as antioxidants. These plant based antioxidants show great promise as electron donors or metal chelators, thereby preventing the generation of free radicals. These are secondary metabolites, such as flavonoids and other polyphenolic compounds produced naturally in the roots, leaves, stems, and bark of plants and have been used in various drug formulations for treatment of chronic diseases [4, 5].
Salvadora persica, commonly known as miswak, peelu, toothbrush tree, is a native plant found in various regions including Africa, Iran, Pakistan, India, Sri Lanka, and Middle Eastern countries. It is an evergreen shrub capable of growing under extreme conditions, from very dry environments to highly saline soils. All parts of the plant, including flowers, fruits, leaves, bark, seeds, stems, and roots, have been utilized in the treatment of various disorders related to different physiological systems in humans. Its use as a toothbrush by many communities has been scientifically proven to be effective in preventing tooth decay, even without additional tooth cleaning methods. Extensive phytochemical analysis has identified the presence of carbohydrates, flavonoids, terpenes, sterols, alkaloids, and glycosides in *S. persica*. Organic sulfur compounds, elemental sulfur, fluoride, calcium, phosphorus, silica, and ascorbic acid have also been detected in small amounts. Numerous biological effects of *S. persica* have been reported, including antibacterial, antiviral, antifungal, antidiabetic, anti-plasmodial, antioxidant, and even antiulcer effects [6, 7, 8]. This research intends to investigate the potential antioxidant properties of *S. persica* roots extracts.

Material and methods
Roots of *Salvadora persica* were collected from Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana and authenticated with accession number 10786 at FRI, Dehradun. The collected leaves were dried in the shade and ground into a fine powder using a mechanical grinder. The dried powdered plant material was subjected to extraction using five different solvents (petroleum ether, benzene, chloroform, methanol, and water) through the cold percolation method. The obtained extracts were concentrated under vacuum using a rotary evaporator at 40°C and stored at 4°C for further use.
For the determination of total phenolic content and antioxidant activity, various concentrations of these extracts (ranging from 0.1 mg/ml to 0.5 mg/ml) were employed. The total phenolic content in the plant extracts was estimated using the Folin-Ciocalteau method, as described by Singleton and Rossi [9], and expressed as mg/g gallic acid

equivalents (GAE) by constructing a standard gallic acid calibration curve. The DPPH free radical scavenging activity of the different extracts was assessed by their ability to decolorize stable DPPH radicals, following the method outlined by Lee *et al*. [10]. The superoxide scavenging ability was measured according to the procedure described by Liu et al. [11], on the basis of reduction of nitroblue tetrazolium in the test solution and compared with standard BHT. To test hydroxyl radical scavenging activity of the extracts method established by Kunchandy and Rao [12] was employed that depends on their capacity to inhibit hydroxyl radical generation in the Fenton reaction. Chelation of metal ions by the extracts was evaluated by inhibition of Ferrozine-Fe^{2+} complex formation, according to the method of Dinis *et al*. [13]. The reducing power assay was conducted using the potassium ferricyanide method proposed by Yen and Duh [14]. An increase in absorbance at 700 nm with increasing concentration indicated an increase in reducing power.

All the analyses were performed in triplicate, and the data was presented as mean ± standard deviation. Percentage inhibition was calculated using the formula:

% inhibition = (Acontrol - Asample or standard) / Acontrol x 100,

For statistical analysis, one-way analysis of variance (ANOVA) followed by Duncan's multiple range test was conducted with level of significant at $p < 0.05$. The correlation between total phenolic content (TPC) and antioxidant assays was assessed using Pearson's correlation coefficient.

Result & Discussion

Total phenolic content (TPC)

Phenolic compounds are secondary metabolites produced by plants and are widely distributed throughout the plant kingdom. They play essential roles in various physiological processes and contribute to the plant's defence mechanisms against environmental stresses. The determination of TPC is important because phenolic compounds are known to possess diverse biological activities, including antioxidant, anti-inflammatory, anticancer, and antimicrobial properties [15]. Measuring the TPC provides valuable information about the potential health benefits and bioactive potential of a plant or its extracts. Phenolic content in different extracts is depicted in figure 1. Methanolic extract has highest phenolic content (44.83mg/g GAE) followed by chloroform, petroleum ether, aqueous and benzene extracts.

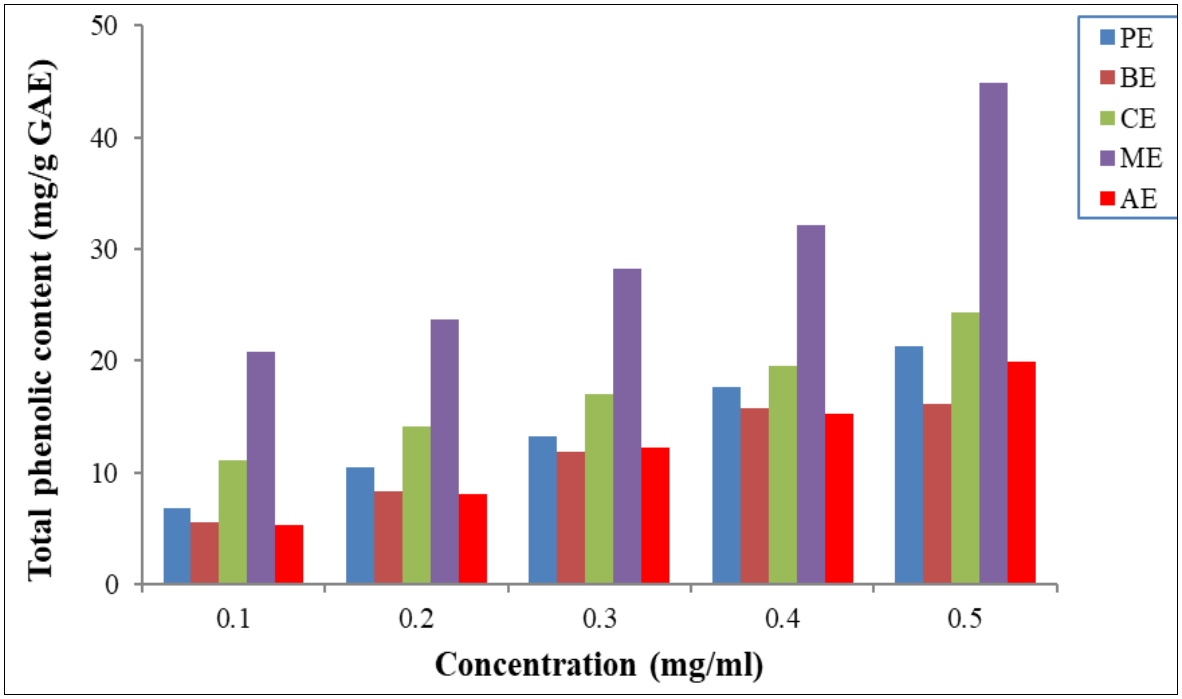

Fig 1: Determination of Total phenolic content (TPC) of *S. persica* roots extracts; PE-Petroleum ether extract; BE-Benzene extract; CE-Chloroform extract; ME- Methanol extract; AE- Aqueous extract

DPPH radical scavenging activity

The DPPH assay is widely accepted method to assess the free radical scavenging capacity of natural antioxidants due to its simplicity and short testing time. DPPH radical produces a violet-colored solution in ethanol, and upon accepting an electron converted into a stable diamagnetic molecule (DPPH-H) resulting in discoloration of solution with maximum absorption occurring at 517 nm. The greater the bleaching of the violet color to yellow, the more effective the extract is at neutralizing the radicals. Numerous studies have reported the ability of plant extracts to scavenge DPPH radicals in *vitro* [16,17]. Ascorbic acid was employed as a standard compound (at concentrations ranging from 10 µg/ml to 50 µg/ml) to determine the antioxidant activity. Table 1 presents the scavenging effect of different concentrations of solvents and ascorbic acid on the DPPH radical. The methanolic extract displayed the highest scavenging potential, followed by aqueous, benzene, petroleum ether, and chloroform extracts. The IC_{50} values of the of ascorbic acid was significantly lower than different extracts suggesting that the plant extracts had weaker DPPH radical scavenging abilities compared to the reference compound.

Table 1: DPPH free radical scavenging activity (%) of root extracts of *Salvadora persica*

Root extracts							
Concentration (mg/ml)	**PE**	**BE**	**CE**	**ME**	**AE**	**Conc. (µg/ml) of AS**	**AS**
0.1	8.35±0.64^{e}	10.32±1.12^{e}	9.84±0.26^{e}	22.06±0.71^{e}	29.27±0.48^{e}	10	20.84±0.62^{e}
0.2	14.67±0.19^{d}	14.47±0.38^{d}	12.75±0.31^{d}	37.29±0.18^{d}	32.26±0.79^{d}	20	38.63±0.40^{d}
0.3	22.14±0.51^{c}	20.85±0.13^{c}	20.56±0.35^{c}	50.07±0.82^{c}	38.44±0.50^{c}	30	75.17±0.60^{c}
0.4	26.38±0.47^{b}	28.31±0.28^{b}	21.23±0.39^{b}	65.22±0.84^{b}	44.14±0.25^{b}	40	80.28±0.12^{b}
0.5	30.63±0.13^{a}	36.97±0.54^{a}	28.24±0.78^{a}	72.41±0.77^{a}	52.67±0.48^{a}	50	84.80±0.66^{a}

Values are expressed as mean±S.D., (n=3). Values with in the column not sharing common superscript letters (a-e) differ significantly at p<0.05 by Duncan's multiple range tests. PE- petroleum ether, BE- Benzene extract, CE- Chloroform extract, ME- Methanol extract, AE- Aqueous extract, AS- Ascorbic acid

Superoxide radical scavenging activity

Superoxide anion production in biological systems as a byproduct of aerobic respiration is a common phenomenon. Superoxide itself can initiate lipid peroxidation or can lead to generation of highly reactive hydroxyl radical in presence of iron via Haber-Weiss process [18]. In the current study both the extracts and the standard compound BHT (Butylated hydroxytoluene) displayed a concentration-dependent decrease in absorbance at 560 nm, indicating their ability to quench superoxide radicals in the reaction mixture (Figure 2). Methanolic extract exhibited the highest activity, followed by the benzene, aqueous, petroleum ether, and chloroform extracts. The percentage inhibition of superoxide radicals by the methanolic extract at 0.5 mg/ml concentration was 70.81%. The IC_{50} value of the methanolic extract was determined to be 0.32 mg/ml, while that of BHT was 0.27 mg/ml, suggesting that methanolic extract has a weaker inhibitory effect on superoxide radicals compared to BHT.

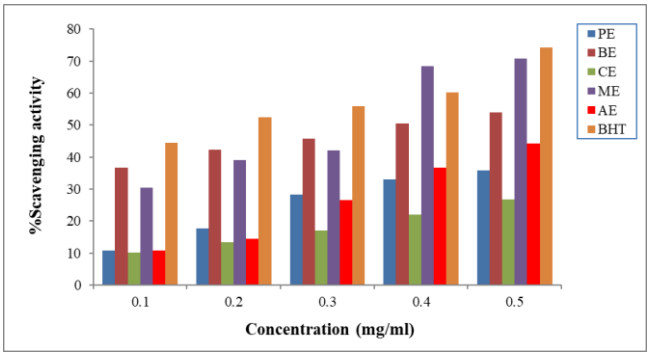

Fig 2: Determination of Superoxide radical scavenging activity (%) of *S. persica* roots extracts; PE-Petroleum ether extract; BE-Benzene extract; CE-Chloroform extract; ME- Methanol extract; AE- Aqueous extract; BHT- Butylated hydroxytoluene

Hydroxyl radical scavenging activity

Hydroxyl radicals are highly reactive and short-lived free radicals that can cause significant damage to biological molecules, including DNA, proteins, and lipids. The hydroxyl radical scavenging activity of plant extracts serves as an important parameter to evaluate their ability to counteract the damaging effects of hydroxyl radicals, highlighting their potential as natural sources of antioxidants for various health applications [19]. The hydroxyl radical scavenging activity of plant extracts was assessed by measuring the inhibition of deoxyribose degradation to malondialdehyde by hydroxyl radicals. Various extracts of *S. persica* showed a dose-dependent reduction in hydroxyl radical generation. The methanolic extract exhibited the highest percentage inhibition, ranging from 19.74% (at 0.1 mg/ml) to 52.17% (at 0.5 mg/ml) (Figure 3). The scavenging activity of the different extracts followed the order: methanol > benzene > chloroform >aqueous > petroleum ether extracts. The IC_{50} value was determined to be 0.48 mg/ml for the methanolic extract and 0.09 mg/ml for ascorbic acid.

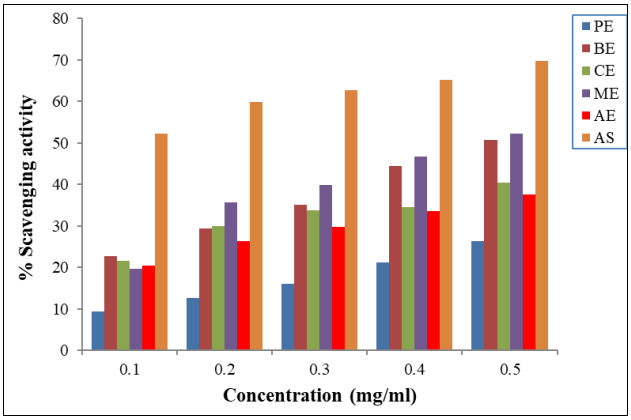

Fig 3: Determination of Hydroxyl radical scavenging activity (%) of *S. persica* roots extracts; PE-Petroleum ether extract; BE-Benzene extract; CE-Chloroform extract; ME- Methanol extract; AE- Aqueous extract; AS-Ascorbic acid

Metal Chelating activity

In biological systems, the presence of transition elements can lead to the production of reactive oxygen species through Fenton and Haber-Weiss reactions. These reactive oxygen species can cause oxidative damage to biomolecules and contribute to various diseases. Metal chelation can disrupt the redox chemistry involving these metal ions, reducing their ability to generate free radicals and protecting against oxidative stress [20]. In the current study presence of metal chelating agents in the plant extract compete with ferrozine for binding to iron (II) ions, leading to a decrease in color intensity measured spectrophotometrically at 562nm. The metal chelating activity of various extracts and the standard chelating agent EDTA are compared in Figure 4. The methanolic extract exhibited the highest effectiveness in chelating metal ions, followed by the benzene, aqueous, petroleum ether and chloroform extracts. The mean IC_{50} value of the methanolic extract was 0.37 mg/ml which was comparable to standard EDTA (0.32 mg/ml).

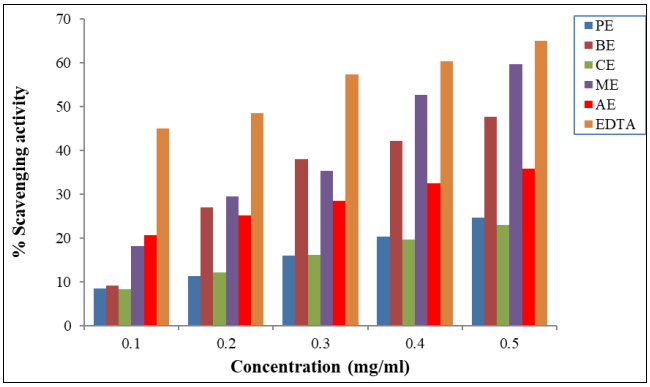

Fig 4: Determination of Metal chelating activity (%) of *S. persica* roots extracts; PE-Petroleum ether extract; BE-Benzene extract; CE-Chloroform extract; ME- Methanol extract; AE- Aqueous extract; EDTA- Ethylenediamine tetra acetic acid

Reducing power assay

The reducing power assay is a simple and rapid method that provides a qualitative and quantitative assessment of the antioxidant potential of plant extracts. Plant extracts rich in antioxidant compounds, such as phenolics, flavonoids, and other reducing agents, have the ability to donate electrons and can reduce ferric ions to ferrous ions (Fe^{2+}) followed by a color change, which can be measured spectrophotometrically. By doing so, they can neutralize reactive species and inhibit oxidative damage caused by free radicals. This reduction reaction is indicative of the reducing power activity of the plant extract [21]. Figure 5 depicted the reductive potential of reference compound and plant extracts at different concentration. Methanolic extract showed highest reducing ability followed by chloroform, benzene, petroleum ether and aqueous extracts at 0.5mg/ml concentration but lower than ascorbic acid. These results suggest that methanolic extract has the capacity to neutralize free radicals and can contribute to *Salvadora persica* medicinal properties.

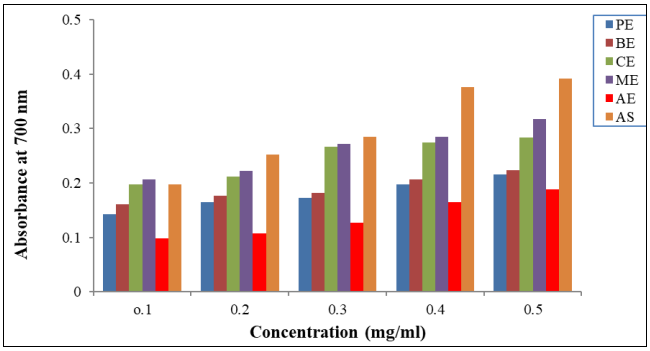

Fig 5: Determination of Reducing power assay of *S. persica* roots extracts; PE-Petroleum ether extract; BE-Benzene extract; CE-Chloroform extract; ME- Methanol extract; AE- Aqueous extract; As- Ascorbic acid

Correlation between total phenolic content and antioxidant activity

Plant phenolics refer to a diverse group of secondary metabolites produced by plants. They are characterized by the presence of one or more phenolic rings and can be further classified into various subclasses, including flavonoids, phenolic acids, tannins, stilbenes, and lignans. The presence of phenolic compounds in plant extracts is often associated with higher antioxidant activity, as phenolics can effectively quench free radicals and inhibit oxidative stress. The correlation between phenolics and antioxidant activity has been demonstrated in numerous studies using various in *vitro* assays [22,23]. In case of methanolic extract of *Salvadora persica* significant correlation between the total phenolic content and various antioxidant assays was observed. The value of correlation coefficients for DPPH, superoxide, hydroxyl and metal chelating activity were 0.914773, 0.892272, 0.877505, and 0.933867, respectively, with a p-value of less than 0.05, indicating a strong relationship between total phenolic content and antioxidant assays (Table 2). This suggests that the presence of phenolic compounds may contribute to the antioxidant activity observed in the methanolic extract of *S. persica*. Previous studies have identified various phenolic compounds in *S. persica*. Phenolic acids and flavonoids, including gallic acid, hydroxybenzoic acid, chlorogenic acid, cinamic acid, catechin, rutin, and myricetin, have been isolated from different fractions of *S. persica* [24]. The stem of *S. persica* contains flavonoids rutin and quercetin, while Salvadourea, kaempferol, quercetin, rutin, quercetin glycosides Benzylisothiocynate, and n-hexadecanoic acid was reported from its roots [25, 26]. γ-tocopherol and α-tocopherol, were detected from its seed oil, both having antioxidant activity similar to vitamin E [27]. These compounds are known for their antioxidant properties. This correlation highlights the importance of phenolic compounds as natural antioxidants and supports the utilization of plant-based phenolics for their potential health benefits in combating oxidative stress-related disorders.

Table 2: Determination of correlation between total phenolics and antioxidant activity

Antioxidant activity	Total phenolic content	
	r	R^2
DPPH assay	0.914773	0.836
Superoxide radical scavenging assay	0.892272	0.796
Hydroxyl radical scavenging assay	0.877505	0.77
Metal chelating assay	0.933867	0.872

*significance at 95% confidence level

Conclusion

Based on the in *vitro* assays employed in this study, methanolic extract of *Salvadora persica* possesses ability to inhibit free radical generation, demonstrate metal chelation properties, and exhibit reducing power. The presence of phenolic compounds in the extract may be responsible for these activities, as supported by the significant correlation observed between the in *vitro* antioxidant assays and the total phenolic content. Further extensive research is required to isolate and identify the specific antioxidant components present in the methanolic extract of *S. persica*.

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