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Phytochemical Characterization and Solvent Fraction Depending *in vitro* Antioxidant Activities of *Cassia absus, Gymnema sylvestre, Nigella sativa and Piper nigrum*

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Abstract: *This study was aimed to phytochemically characterize and assess the antioxidant activities of 70% methanol extract and subsequent fractions of Cassia absus (L.) seeds, Gymnema sylvestre (L.) whole plant, Nigella sativa (L.) seeds, and Piper nigrum (L.) seeds. Powdered plant materials were extracted with 70% methanol and further fractionated with ethyl-acetate, n-butanol and the residual aqueous fraction. Phytochemical analysis was performed to detect different phytocompounds. Mineral compositions were quantified, and total phenolic and flavonoid contents were determined. The antioxidant potential of methanol extracts and fractions was assessed in vitro through estimating DPPH radical and superoxide anion scavenging activities and reducing power assay. Extraction yields ranging highest of N. sativa methanol extract (30.42±1.49%) and lowest of P. nigrum ethyl-acetate fraction (4.58±0.61%) were obtained. Results revealed that methanol extracts and fractions of selected plants contain phytocompounds such as alkaloids, flavonoids, phenols, glycosides, tannins, terpenoids, saponins, carbohydrates, fats and fixed oils. The mineral analysis showed considerable quantities of calcium (C. absus methanol extract: 372.454±3.633 mg/100g), magnesium (G. sylvestre methanol extract: 131.045±1.346 mg/100g), and zinc (N. sativa methanol extract: 36.019±0.284 mg/100 g) in all fractions while minor quantities of manganese, copper and cobalt were also found. Methanol extracts showed considerably higher total phenolic (N. sativa methanol extract: 179.71±2.14 mg GAE/g) and flavonoid (N. sativa methanol extract: 189.18±3.17 mg CE/g) contents compared to other fractions, and subsequently exhibited pronounced scavenging activities on DPPH* (N. sativa methanol extract: 23.8 µg/mL) and superoxide radicals (N. sativa methanol extract: 24.9 µg/mL) and had potent reductive abilities (N. sativa methanol extract: 1.123±0.038 O.D.). Conclusively, C. absus, G. sylvestre, N. sativa and P. nigrum possess significant nutritive properties and could be used as natural antioxidant sources to prevent oxidative stress-associated diseases.*

Keywords*: antioxidants, Cassia absus, Gymnema sylvestre, Nigella sativa, Piper nigrum, polyphenols*

1. Introduction

The use of medicinal plants as therapeutic remedies is as old as human civilization and people have been continuing to rely on them for primary healthcare needs [1]. Approximately 80% population of underdeveloped or developing countries still depends on natural-sourced products to cure their acute or chronic ailments [2]. It is currently estimated that more than 0.4 million plant species have been discovered. Compounds derived from natural sources have a significant contribution to the discovery of novel chemical entities. A multi-disciplinary approach is involved in the drug discovery process and is dependent upon many disciplines such as ethnobotany, biology, phytochemistry and chemical separation techniques. At present, a considerable proportion (around 87%) of therapeutic agents is directly or indirectly derived from nature [3].

The generation of free radicals occurs throughout cellular metabolic activities. However, multiple antioxidant defense mechanisms effectively neutralize these free radicals and the body maintains the oxidation and anti-oxidation balance [4]. Oxidative stress is assumed to be the underlying cause of various human diseases. Oxidation of macromolecules like proteins, lipids and damage to DNA is

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attributed to be the root cause of many life-threatening ailments [5]. Antioxidants are compounds that protect cellular components against the damaging effect of reactive oxygen species (ROS) by scavenging them or reducing their production [6]. Dietary antioxidants are mostly extracted from natural sources. Moreover, plant-derived antioxidants are gaining more attention from researchers due to carcinogenicity and harmful effects on the liver and lungs associated with synthetic antioxidants [7,8].

Natural source-based antioxidants such as polyphenols alleviate oxidative stress and have been proven anti-microbial, anti-inflammatory, immunomodulatory, anti-aging, anti-asthmatic, anti-cancer properties. Also, polyphenols showed neuroprotective, cardioprotective and hepatoprotective activities [9]. The bioavailability of these phytocompounds depends on the type or source of polyphenols and their interaction with human microbiota. Several studies showed that polyphenols may indirectly improve the health status of patients through modulating gut microbiota that may reduce intestinal and systemic inflammation by improving metabolic parameters [10].

Cassia absus L., also known as Chakshu, is a part of the Fabaceae family. It is an annual plant that can be found in Sri Lanka, India, Pakistan and across the tropical regions of the world. *C. absus* seeds are traditionally used in the treatment of inflammation, asthma, bronchitis, leucoderma, conjunctivitis, hypertension, renal stones, irritable bowel syndrome, hepatic diseases and tumors [11]. Plant seeds have a cathartic effect and local application in paste form effectively treats headache and skin infections [12]. *Gymnema sylvestre* L., recognized as Gurmur, is included in the family Apocynaceae and is a wild herb found in Asia, Africa and Australia. Its traditional uses include antiviral, antibiotic, anti-cancer, gastroprotective, hepatoprotective, blood glucose and lipid-lowering potential [13]. *Nigella sativa* L. (family Ranunculaceae) is one of the several nutrient-rich medicinal plants. The plant has been widely used as a flavoring agent and spice in the preparation of food products. In Asia, Africa and Europe, *N. sativa* seeds have a wide range of traditional therapeutic uses such as in diabetes, chronic pain, inflammation, paralysis, digestive tract related disorders, hypertension and infections. Topical preparations containing *N. sativa* seeds are directly applied to treat blisters, orchitis, nasal abscesses, eczema and swollen joints [14,15].

Piper nigrum L., famous as King of spices, has been commonly used to impart its flavor and enhance the taste of other ingredients to dishes. *P. nigrum*, a member of the Piperaceae family, is among the ancient flowering plants which are grown in tropical regions. Apart from its culinary benefits, it has also been renowned due to its traditional medicinal uses include anti-microbial, anti-inflammatory, anti-depressant, anti-apoptotic, anti-diarrheal, antipyretic, antioxidant, anti-spasmodic, anti-mutagenic, anti-dysentery, and anti-colic activities [16,17].

Keeping in view the traditional uses, the present study was designed to phytochemically characterize and evaluate comparative antioxidant activities of *C. absus*, *G. sylvestre*, *N. sativa* and *P. nigrum* extracts *in vitro*.

2. Materials and methods

2.1. Chemicals and reagents

Reference chemicals: ascorbic acid, catechin and gallic acid of highest purity from Sigma-Aldrich®, Reagents: 2,2-diphenyl-1-picrylhydrazyl, aluminum trichloride, Folin-Ciocalteu reagent, Solvents: methanol, *n*-butanol and ethylacetate and all other chemicals of analytical grade were purchased from UniChem® and Merck®. All glassware used was thoroughly washed with double distilled water and cleaned by following standard protocols.

2.2. Plant materials

Gymnema sylvestre (Ghurmar boti) whole plant, seeds of *Cassia absus* (Chaksu), *Nigella sativa* (Kalonji) and *Piper nigrum* (Kali mirch) purchased from the local herbal store were identified by taxonomist of Department of Botany, University of Agriculture, Faisalabad, Pakistan, and herbarium No. *Cassia absus*; 21213, *Gymnema sylvestre*; 21214, *Nigella sativa*; 21215 and *Piper nigrum*; 21216 were allotted for future reference.

2.3. Extract preparation

Dried plant materials were coarsely powdered by a mechanical grinder to achieve maximum extraction. About 100 g of each powdered plant material was macerated in 500 mL of 70% methanol for 72 h at room temperature, with shaking at regular intervals. The extract was firstly filtered through a muslin cloth and then filtered through Whatman paper no. 1 and concentrated by using a rotary evaporator (Hei-VAP, Heidoph Rotacool®, Germany). This process was repeated three times and the combined percentage of yield was calculated. Then, a 70% methanol sample was kept aside and the remaining concentrate of 70% methanol was mixed with water and ethyl-acetate, *n*-butanol and aqueous fractioning were performed [18]. Dry concentrated extracts were stored in airtight containers at -4°C for further analysis. The percentages of extract yield were calculated by the following formula:

$$\text{Extraction yield (\%)} = [\text{Weight (g) of dry soluble solid/Weight (g) of sample}] \times 10 \quad (1)$$

2.4. Qualitative phytochemical screening

Extracts were subjected to phytochemical analysis for the presence of alkaloids, carbohydrates, flavonoids, fats and fixed oils, glycosides, phenols, saponins, steroids, tannins and terpenoids by following standard protocols [19, 20].

2.5. Mineral composition

AOAC, (1990) method was followed to prepare dry extract samples for the detection and quantification of different elements i.e. manganese (Mn), nickel (Ni), zinc (Zn), cadmium (Cd), calcium (Ca), cobalt (Co), lead (Pb), magnesium (Mg), copper (Cu) by using atomic absorption spectrophotometer [21]. About 10 mL mixture of nitric acid: perchloric acid (7:3) added to 1 g of each extract and heated at 180-200°C until white fumes appeared. The final volume was made up to 100 mL by adding distilled water.

2.6. Total phenolic content

The Folin-Ciocalteau's method was used for the estimation of total phenolic content of extracts [22]. In short, each extracted sample (50 mg) was diluted with 7.5 mL of double distilled water and mixed with 0.5 mL of Folin-Ciocalteau reagent. After incubating at 37°C for 10 min, 1.5 mL of 20% w/v Na_2CO_3 was added to sample mixtures, further heated on the water bath for 20 min and cooled immediately in an ice bath. The absorbance of resulting blue-colored complex reaction mixtures was measured at 765 nm using a spectrophotometer (Shimadzu®, Japan). Different concentrations of gallic acid as standard in methanol were prepared for the calibration curve ($y=0.0116x+0.0927$; $R^2=0.9955$).

2.7. Total flavonoid content

Total flavonoid content was determined using aluminum chloride with the help of colorimetric method [22]. Briefly, a weighed quantity of each sample was mixed with 0.3 mL of 5% $NaNO_2$ solution and 4 mL of distilled water. After 5 min, 0.3 mL of 10% $AlCl_3$ was added and 2 mL of 1 M NaOH was mixed after incubating for 6 min and further diluted with 2.4 mL of distilled water. Absorbance was measured at 510 nm and flavonoid content was determined by using a catechin calibration curve ($y=0.0027x+0.1609$; $R^2=0.9938$).

2.8. *In vitro* antioxidant activity

For the assessment of antioxidant activities, methanol extracts and fractions of selected plants were dissolved in methanol (95%) to prepare 1mg/mL concentration of samples and further reconstituted to made concentration-dependent dilutions. Ascorbic acid as a standard was used for comparison.

2.8.1. DPPH* radical scavenging assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed by following a previously prescribed method [23]. Ascorbic acid as reference and extract samples of different concentrations (25 to 300 µg/mL) were dissolved in methanol. About 2 mL of reference and sample dilutions were mixed with freshly prepared 0.5 mL of 0.002% DPPH methanolic solution, incubated for 15 min at 37ºC and absorbencies were measured at 517 nm using a spectrophotometer. All values were taken in triplicate and DPPH percentage inhibition was calculated using the given formula:

$$\text{Scavenging activity (\%)} = [(A_{Control} - A_{Sample})/A_{Control}] \times 100 \quad (2)$$

$A_{Control}$=Absorbance of Control/Blank without extracts, A_{Sample}= Absorbance of extracts or standard.

IC_{50} values of all extracts were calculated to determine 50% inhibition of DPPH* in comparison to standard.

2.8.2. Superoxide radical scavenging assay

The Beauchamp and Fridovich method [24] was adopted to determine the antioxidant activity of methanol extracts and fractions of plants through their superoxide anion radical scavenging ability. The reaction mixture comprised of 500 µL of PO_4 buffer (50 mM, *p*H 7.6), 100 µL of nitroblue tetrazolium (0.5 mM), 250 µL of phosphor-methozine sulfate (20 mM) and 300 µL of riboflavin (50 mM) was prepared and then 1 mL of each sample was added. Sample solutions were placed under a fluorescent lamp for 20 min to trigger the reaction and absorbance was taken at 560 nm. The scavenging activity (%) was calculated as:

$$\text{Scavenging activity (\%)} = [(1 - A_{Sample})/A_{Control}] \times 100 \quad (3)$$

$A_{Control}$=Absorbance of Control/Blank without extracts, A_{Sample}= Absorbance of extracts or standard.

IC_{50} values of all extracts were calculated to determine 50% inhibition of superoxide anion radical in comparison to ascorbic acid as standard.

2.8.3. Reducing power assay

Reducing power of a test sample is its ability to convert Fe^{+3} to Fe^{+2}. The appearance of a blue-colored complex indicates the conversion to Fe^{+2} and absorbance can be measured at 700 nm using a spectrophotometer [25]. In short, 2 mL of ascorbic acid (standard) and plant extracts of various concentrations were added to 2mL of 1% potassium ferricyanide and 2 mL of 0.2 M phosphate buffer (*p*H 6.6). The mixture was heated for 30 min at 45ºC. Then, 2 mL of trichloroacetic acid was added, centrifuged for 10 min at 3000 rpm and the upper layer was collected. Further, 2 mL of distilled water, 0.4 mL of freshly prepared ferric chloride (0.1% w/v) were mixed with 2 mL of supernatants, and after 10min, absorbance was measured at 700 nm. A higher absorbance value of the reaction mixture indicates higher reducing power of extract. The test was performed in triplicates and results were averaged.

2.9. Statistical analysis

Data were analyzed using Graphpad Prism® (ver. 6.0) and results were presented as mean±SEM of triplicates determinations.

3. Results and discussions

3.1. Extraction yields

Various parameters including solvents, sample to solvent ratio, method, temperature and time influence the efficiency and extraction yield [26]. The percentage extraction yields of methanolic extracts and fractions obtained from *C. absus*, *G. sylvestre*, *N. sativa* and *P. nigrum* are mentioned in Table 1. The percentages of extractable phytocompounds varied from 4.58±0.61% to 30.42±1.49%.

Methanolic extract of *N. sativa* gave the highest yield of 30.42±1.49%, whereas the ethyl-acetate fraction of *P. nigrum* gave the lowest yield (4.58±0.61%). The differences in extraction yields of plants are attributed to varying concentrations of bioactive phytocompounds and their different solubility profiles [27].

Table 1. Percentage yields of methanolic extracts and solvent fractions

Extract	Extraction yield (%)			
	C. absus	***G. sylvestre***	***N. sativa***	***P. nigrum***
Methanol extract	29.26 ± 1.76^{aA}	25.56 ± 1.31^{aA}	30.42 ± 1.49^{aA}	23.50 ± 1.70^{aB}
Ethyl-acetate fraction	8.62 ± 0.83^{cA}	5.49 ± 0.48^{cB}	9.63 ± 0.80^{bA}	4.58 ± 0.61^{bB}
n-Butanol fraction	13.71 ± 0.71^{bA}	11.71 ± 1.00^{bB}	12.64 ± 0.96^{bA}	8.41 ± 1.00^{bC}
Aqueous fraction	6.83 ± 0.64^{cC}	8.70 ± 0.81^{bB}	10.52 ± 0.77^{bA}	9.17 ± 0.71^{bA}

Values are expressed as mean±SEM (n=3). Small letters (a-c): $p<0.05$ significant difference between different solvent fractions of the same plant; Capital letters (A-C): $p<0.05$ significant difference between same solvent type in different plants.

3.2. Estimation of phytochemical constituents

Qualitative phytochemical analysis of *C. absus*, *G. sylvestre*, *N. sativa* and *P. nigrum* methanol extract and different fractions have been shown in Table 2. Phytocompounds present in plants are responsible for pharmacological activities such as alkaloids have been known for antimalarial, antihypertensive, anticancer activities, as well as isolated pure alkaloids, have been used as antibacterial, antispasmodic and analgesic agents [28,29]. Flavonoids and phenols present in plants considerably contribute to antioxidant, anti-inflammatory and anticancer activities, owing to their reaction oxygen species (ROS) scavenging potential [30]. Saponins possess an antioxidant potential and are known to lower cancer risks, modulate blood lipids and improve blood glucose response [31]. Plants possessing tannins have been used to treat hemorrhoids and wounds and also used as diuretics, antidiarrheal, astringents and against gastric and duodenal tumors [28,32]. Terpenoids have shown anti-malaria, anti-ulcers, and anti-cancer activities [33]. Glycosides have been used as flavoring agents in pharmaceutical products and have anti-carcinogenic activity [34]. Phytocompounds like steroids have therapeutic effectiveness as cardiotonic, anti-microbial and insecticidal properties. Moreover, they are useful in enhancing nitrogen retention in osteoporosis [35].

Table 2. Phytochemical screening of *C. absus*, *G. sylvestre*, *N. sativa and P. nigrum* methanol extracts and different fractions

Extract	Alkaloid	Carbohydrate	Flavonoid	Fats and Fixed oils	Glycoside	Phenol	Saponin	Steroid	Tannin	Terpenoid
CAME	+	+	+	+	+	+	+	+	+	+
CAEF	+	+	+	+	+	+	+	+	+	+
CABF	-	+	+	+	+	+	+	-	-	-
CAAF	+	+	+	-	-	-	-	-	-	-
GSME	+	+	+	+	+	+	+	-	+	-
GSEF	-	+	+	-	+	+	+	-	-	-
GSBF	-	-	+	+	-	-	-	-	+	-
GSAF	+	+	-	-	-	-	+	-	+	-
NSME	+	+	+	+	+	+	+	+	+	+
NSEF	-	-	-	+	+	-	-	+	-	-
NSBF	-	-	-	-	+	+	+	-	+	+
NSAF	+	+	+	-	-	-	-	-	+	-
PNME	+	+	+	+	+	+	+	-	+	-
PNEF	+	+	-	-	-	+	+	-	+	-
PNBF	-	+	+	-	+	+	-	-	-	-
PNAF	+	+	-	-	-	+	+	-	+	-

(+) Present, (-) Absent. Here, *C. absus*: CAME, CAEF, CABF, CAAF, *G. sylvestre*: GSME, GSEF, GSBF, GSAF, *N. sativa*: NSME, NSEF, NSBF, NSAF, and *P. nigrum*: PNME, PNEF, PNBF, PNAF are methanol extract, ethyl-acetate, *n*-butanol and aqueous fractions, respectively.

3.3. Estimation of mineral contents

The mineral compositions of methanol extract and organic/aqueous fractions are shown in Table 3. Results showed the considerably high amount of manganese in GSME (13.024±0.087 mg/100 g), zinc in NSME (36.019±0.284 mg/100 g), calcium in CAME (372.454±3.633 mg/100g), magnesium in GSME (131.045±1.346 mg/100 g) and copper in GSME (15.961±0.093 mg/100 g). Results revealed that selected plants contain significant quantities of minerals including Mn, Zn, Ca, Mg and Cu. These minerals are an important part of a diet and exist in the body at low percentages. Thus, these plants could be a potential source of major and trace minerals that are required for the normal functioning of human body [36].

Table 3. Mineral contents in methanol extracts and fractions of *C. absus, G. sylvestre, N. sativa and P. nigrum*

Plant Extract	Contents (mg/100 g)								
	Mn	**Ni**	**Zn**	**Cd**	**Ca**	**Co**	**Pb**	**Mg**	**Cu**
CAME	0.190 ± 0.027^{aC}	ND	2.492 ± 0.034^{aB}	ND	372.454 ± 3.633^{aA}	0.092 ± 0.018^{aD}	ND	7.786 ± 0.051^{aB}	0.012 ± 0.001^{aD}
CAEF	0.046 ± 0.011^{bC}	ND	0.897 ± 0.029^{bB}	ND	122.781 ± 1.240^{cA}	0.021 ± 0.001^{cB}	ND	2.374 ± 0.031^{cC}	ND
CABF	0.042 ± 0.010^{cC}	ND	0.733 ± 0.024^{cC}	ND	162.788 ± 1.493^{bA}	0.045 ± 0.006^{bB}	ND	3.011 ± 0.034^{bC}	ND
CAAF	0.079 ± 0.026^{bC}	ND	0.586 ± 0.018^{dC}	ND	89.727 ± 0.792^{dA}	0.013 ± 0.002^{dC}	ND	1.793 ± 0.027^{dC}	ND
GSME	13.024 ± 0.087^{aA}	0.110 ± 0.012^{aA}	33.081 ± 0.471^{aA}	0.010 ± 0.001^{aA}	121.812 ± 1.298^{aD}	0.613 ± 0.037^{aA}	ND	131.045 ± 1.346^{aA}	15.961 ± 0.093^{aA}
GSEF	4.161 ± 0.072^{aA}	0.010 ± 0.001^{cA}	12.017 ± 0.525^{bA}	ND	43.233 ± 0.415^{bB}	0.131 ± 0.034^{dA}	ND	34.587 ± 0.333^{cB}	3.963 ± 0.067^{cA}
GSBF	3.788 ± 0.055^{bA}	ND	5.050 ± 0.079^{cB}	ND	33.090 ± 0.314^{cC}	0.174 ± 0.004^{cA}	ND	47.042 ± 0.520^{bA}	5.916 ± 0.082^{bA}
GSAF	2.135 ± 0.030^{cA}	0.040 ± 0.011^{bA}	12.811 ± 0.057^{bA}	ND	34.066 ± 0.334^{cC}	0.273 ± 0.031^{bB}	ND	34.382 ± 0.320^{cB}	4.891 ± 0.048^{bA}
NSME	1.013 ± 0.072^{aB}	ND	36.019 ± 0.284^{aA}	0.028 ± 0.001^{aA}	189.032 ± 1.510^{aC}	0.299 ± 0.027^{bB}	ND	127.436 ± 1.248^{aA}	1.978 ± 0.092^{aB}
NSEF	0.033 ± 0.000^{dC}	ND	9.176 ± 0.031^{cA}	ND	63.989 ± 0.677^{cB}	0.096 ± 0.011^{cA}	ND	57.649 ± 0.474^{bA}	0.764 ± 0.023^{bB}
NSBF	0.516 ± 0.041^{bB}	ND	17.332 ± 0.271^{bA}	ND	71.034 ± 0.698^{bB}	0.034 ± 0.010^{dB}	ND	39.428 ± 0.402^{cA}	0.599 ± 0.042^{cB}
NSAF	0.260 ± 0.018^{cB}	ND	7.088 ± 0.240^{dB}	ND	44.323 ± 0.419^{dB}	1.326 ± 0.083^{aA}	ND	27.903 ± 0.246^{dB}	0.330 ± 0.021^{dB}
PNME	1.531 ± 0.030^{aB}	0.020 ± 0.001^{aB}	1.739 ± 0.027^{aB}	0.030 ± 0.001^{aA}	278.181 ± 2.476^{aB}	0.068 ± 0.013^{aC}	ND	121.551 ± 1.216^{aA}	4.038 ± 0.211^{aB}
PNEF	0.453 ± 0.029^{cB}	0.010 ± 0.001^{bA}	0.398 ± 0.026^{cB}	0.012 ± 0.001^{bA}	139.184 ± 1.297^{bA}	0.019 ± 0.001^{cB}	ND	37.492 ± 0.357^{cB}	1.281 ± 0.080^{bB}
PNBF	0.716 ± 0.037^{bB}	ND	0.899 ± 0.076^{bC}	ND	64.975 ± 0.619^{cB}	0.036 ± 0.002^{bB}	ND	29.763 ± 0.196^{dB}	0.973 ± 0.071^{cB}
PNAF	0.242 ± 0.017^{dB}	ND	0.374 ± 0.029^{cC}	ND	53.376 ± 0.495^{dB}	ND	ND	49.235 ± 0.411^{bA}	0.212 ± 0.010^{dB}

Values are expressed as mean±SEM (n=3). Here, *C. absus*: CAME, CAEF, CABF, CAAF, *G. sylvestre*: GSME, GSEF, GSBF, GSAF, *N. sativa*: NSME, NSEF, NSBF, NSAF, and *P. nigrum*: PNME, PNEF, PNBF, PNAF are methanol extract, ethyl-acetate, *n*-butanol and aqueous fractions, respectively. Small letters (a-d): $p<0.05$ significant difference between different solvent fractions of the same plant; Capital letters (A-D): $p<0.05$ significant difference between same solvent type in different plants.

3.4. Estimation of total phenolic and flavonoid contents

The total phenolic and flavonoid contents of methanol extracts and solvent fractions of selected plants are shown in Figure 1. Results of total phenolic contents showed a wide variation in fractions, ranging from 13.61±1.25 mg GAE/g of CABF to 179.71±2.14 mg GAE/g of NSME, indicating higher phenolic content of NSME and PNME (179.71±2.14 and 143.91±3.21 mg GAE/g of sample). Total flavonoid content was determined through a calorimetric method, using catechin equivalent as standard. Results of the present study demonstrated greater flavonoid content in NSME and CAME (189.18±3.71 and 127.71±2.28 mg CE/g of sample). The amount of total flavonoid content of plants studied using

various solvents ranged from 6.79±0.68 mg CE/g of PNBF to 189.18±3.71 mg CE/g of NSME. Polyphenols are gaining increased attention from researchers due to their potential biological activities. The antioxidant capacities of polyphenols, particularly lipid peroxidation inhibition and free radicals scavenging, are pharmacologically most important [37]. Flavonoids are widely distributed phyto-compounds that have been established as health-enhancing plant compounds owing to antioxidant chelating or scavenging attributes [38].

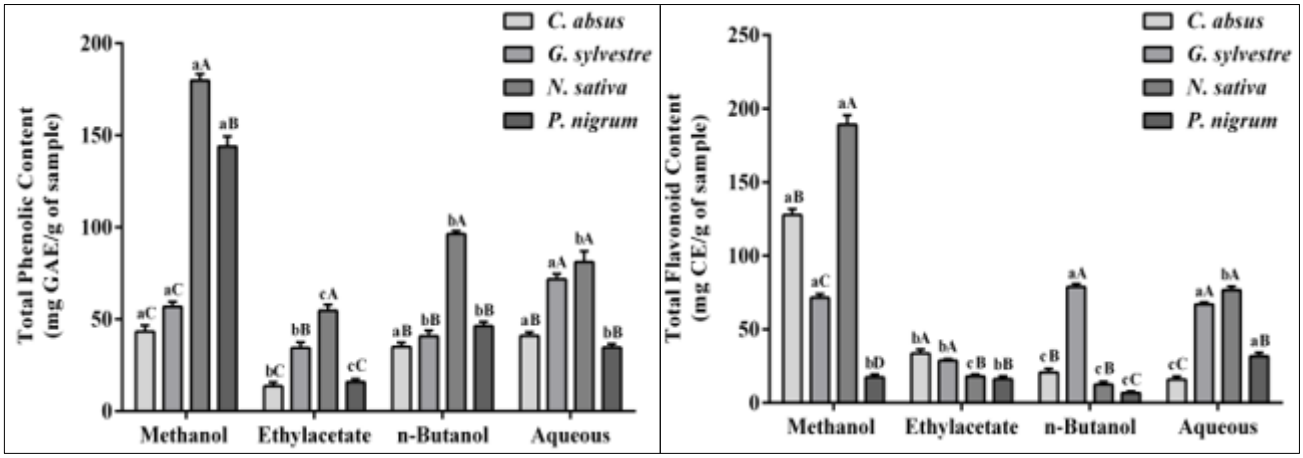

Figure 1. Total phenolic and flavonoid contents of methanol extracts and fraction of plants. *C. absus*: CAME, CAEF, CABF, CAAF, *G. sylvestre*: GSME, GSEF, GSBF, GSAF, *N. sativa*: NSME, NSEF, NSBF, NSAF, and *P. nigrum*: PNME, PNEF, PNBF, PNAF are methanol extract, ethyl-acetate, *n*-butanol and aqueous fractions, respectively. Small letters (a-c): $p<0.05$ significant difference between different solvent fractions of the same plant; Capital letters (A-D): $p<0.05$ significant difference between same solvent type in different plants. Values are presented as mean±SEM (n=3)

3.5. DPPH* scavenging activity

Antioxidant activity of foods and medicinal plants has been commonly determined by using DPPH* scavenging assay. The methanolic solution of DPPH* undergoes a reduction in the presence of H^+-donating antioxidant, appeared as a color change from purple to yellow, which is measured at 517 nm [39]. The scavenging effect of methanol extracts and fractions of *C. absus*, *G. sylvestre*, *N. sativa* and *P. nigrum* have been shown in Figure 2. Results showed the scavenging activity in the following order *C. absus*: CAME>CAAF>CABF>CAEF, *G. sylvestre*: GSME>GSAF>GSEF>GSBF, *N. sativa*: NSME> NSAF>NSEF>NSBF, and *P. nigrum*: PNME>PNAF>PNBF>PNEF, respectively. The IC_{50} values, as mentioned in Table 4, depicted that DPPH* scavenging activities of NSME (23.8 µg/mL) and GSAF (26.2 µg/mL) are close to ascorbic acid (17.3 µg/mL). Meanwhile, IC_{50} values of GSBF and PNEF were found above 300 µg/mL. It shows that *N. sativa* acts as a potent antioxidant while *C. absus* and *G. sylvestre* possess moderate antioxidant ability and relatively less antioxidant potential of *P. nigrum* was observed.

3.6. Superoxide anion scavenging activity

Figure 3 shows the *in vitro* superoxide anion scavenging activity of extracts and fractions determined by the riboflavin-NBT-PMS system. Flavins reduction generates superoxide radicals in the presence of light, subsequently causes NBT reduction and forms a blue-colored formazan [40]. In this study, methanol extracts and fractions exhibited potent scavenging activity against superoxide radicals. A concentration-dependent inhibition of blue formazan formation was observed and the highest scavenging potential of NSME was found close to ascorbic acid while GSBF showed the lowest scavenging activity. The scavenging activities were in the following pattern of *C. absus*: CAME>CAAF>CAEF>CABF, *G. sylvestre*: GSAF>GSME>GSEF>GSBF, *N. sativa*: NSME>NSAF>NSBF>NSNSEF, and *P. nigrum*: PNME>PNBF>PNAF>PNEF, respectively. The IC_{50} values of extracts and fractions presented in Table

4 show the IC_{50} of NSME (24.9 µg/mL) close to ascorbic acid (20.6 µg/mL) while GSBF revealed IC_{50} above 300 µg/mL. These findings indicated that *C. absus, G. sylvestre, N. sativa and P. nigrum* possess a considerable inhibitory effect on superoxide radical generation in comparison to ascorbic acid.

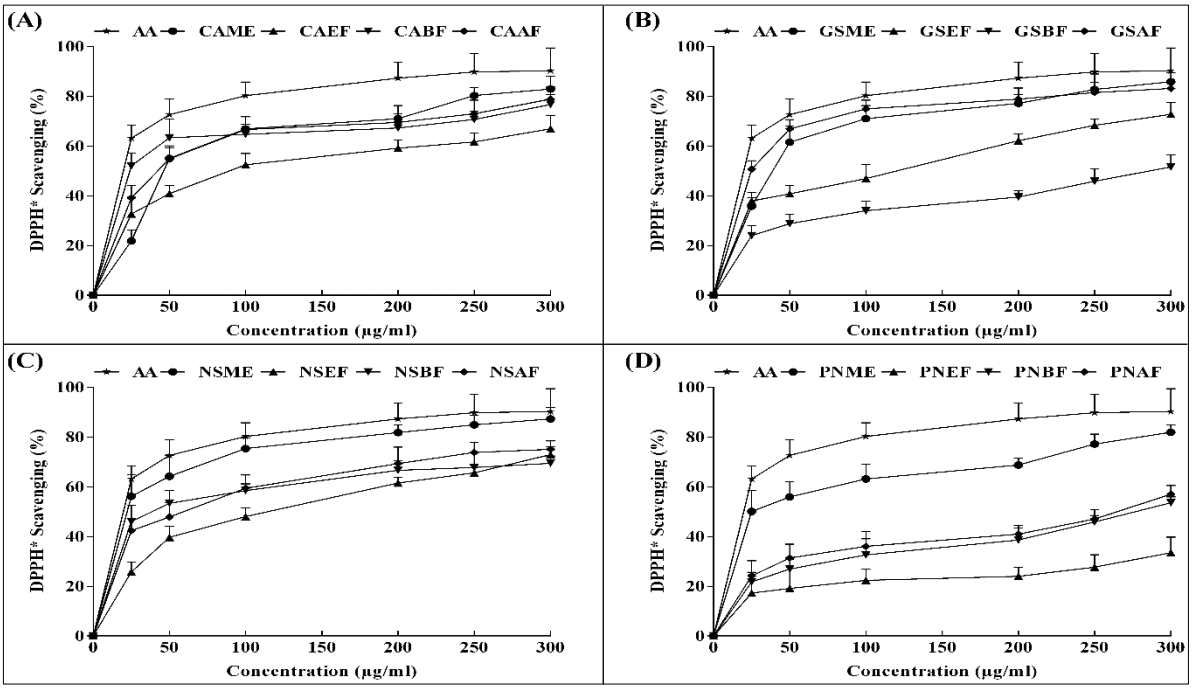

Figure 2. DPPH* scavenging activities of **(A)** *C. absus*: CAME, CAEF, CABF, CAAF, **(B)** *G. sylvestre*: GSME, GSEF, GSBF, GSAF, **(C)** *N. sativa*: NSME, NSEF, NSBF, NSAF, and **(D)** *P. nigrum*: PNME, PNEF, PNBF, PNAF are methanol extract, ethyl-acetate, *n*-butanol and aqueous fractions, respectively and Ascorbic acid (AA). Values are presented as mean±SEM (n=3)

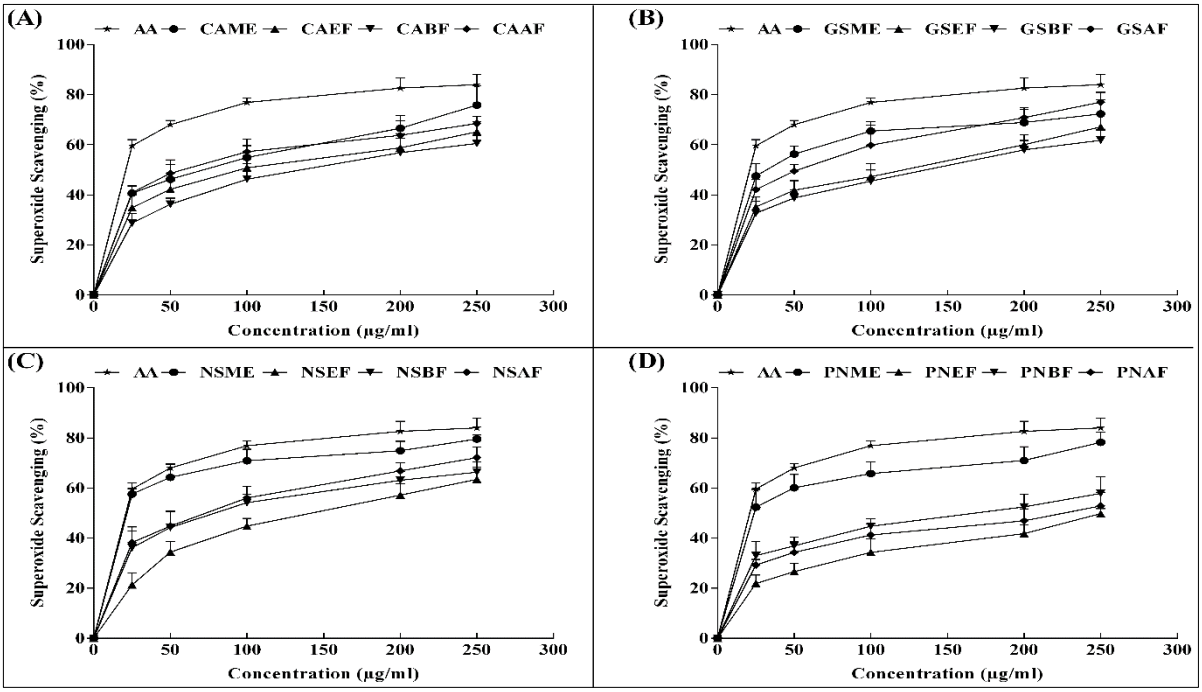

Figure 3. Superoxide anion scavenging activities of **(A)** *C. absus*: CAME, CAEF, CABF, CAAF, **(B)** *G. sylvestre*: GSME, GSEF, GSBF, GSAF, **(C)** *N. sativa*: NSME, NSEF, NSBF, NSAF, and **(D)** *P. nigrum*: PNME, PNEF, PNBF, PNAF are methanol extract, ethyl-acetate, *n*-butanol and aqueous fractions, respectively and Ascorbic acid (AA). Values are presented as mean±SEM (n=3)

Table 4. Antioxidant effect (IC_{50}) on DPPH* radical and superoxide radicals of methanol extract and solvent fractions of plants

Antioxidant assay	Plant extract					Ascorbic Acid
		C. absus	*G. sylvestre*	*N. sativa*	*P. nigrum*	
DPPH* scavenging IC_{50} (µg/mL)	Methanol extract	58.4^{bA}	39.9^{cB}	23.8^{cC}	37.8^{cB}	17.3
	Ethylacetate fraction	97.1^{aB}	83.8^{bB}	99.7^{aB}	$>300^{aA}$	
	n-Butanol fraction	34.2^{cC}	$>300^{aA}$	52.2^{bC}	269.1^{bB}	
	Aqueous fraction	46.7^{bB}	26.2^{cC}	54.4^{bB}	264.7^{bA}	
Superoxide scavenging IC_{50} (µg/mL)	Methanol extract	60.5^{cA}	40.1^{cB}	24.9^{cC}	32.4^{cB}	20.6
	Ethylacetate fraction	91.5^{bC}	92.2^{bC}	128.2^{aB}	292.3^{aA}	
	n-Butanol fraction	124.4^{aB}	$>300^{aA}$	77.2^{bC}	140.6^{bB}	
	Aqueous fraction	62.2^{cB}	50.5^{cB}	65.3^{bB}	163.9^{bA}	

Values are expressed as mean±SEM (n=3). Small letters (a-c): $p<0.05$ significant difference between different fractions of the same plant; Capital letters (A-C): $p<0.05$ significant difference between same solvent type in different plants.

3.7. Reducing power activity

The reducing power activities of methanol extracts and fractions of plants in comparison to ascorbic acid, determined by measuring the conversion of Fe^{+3} to Fe^{+2}, are presented in Figure 4. Extracts containing reductants exhibit antioxidant action through donating H-atom and halt the free radical chain. In the present study, *N. sativa* extract and fractions showed relatively high reductive ability in following order NSME>NSAF>NSBF>NSEF than *C. absus*: CAME>CAAF>CABF>CAEF, *G. sylvestre*: GSAF> GSME>GSBF>GSEF and *P. nigrum*: PNME>PNBF>PNAF>PNEF, respectively. Results revealed that NSME (1.123±0.038 O.D. at 300 µg/mL) exhibit the highest reducing power while the lowest reducing power of CAEF (0.436±0.029 O.D. at 300µg/mL) in contrast to ascorbic acid (1.548±0.042 O.D. at 300 µg/mL) was observed. Antioxidant activity of extract has been believed due to peroxides breakdown, reductive capacity on metals, binding of heavy metal ion catalysts, and radical scavenging [41]. Reducing power activities of extracts and fractions of plants increased with increasing the concentrations, similarly to antioxidant activities.

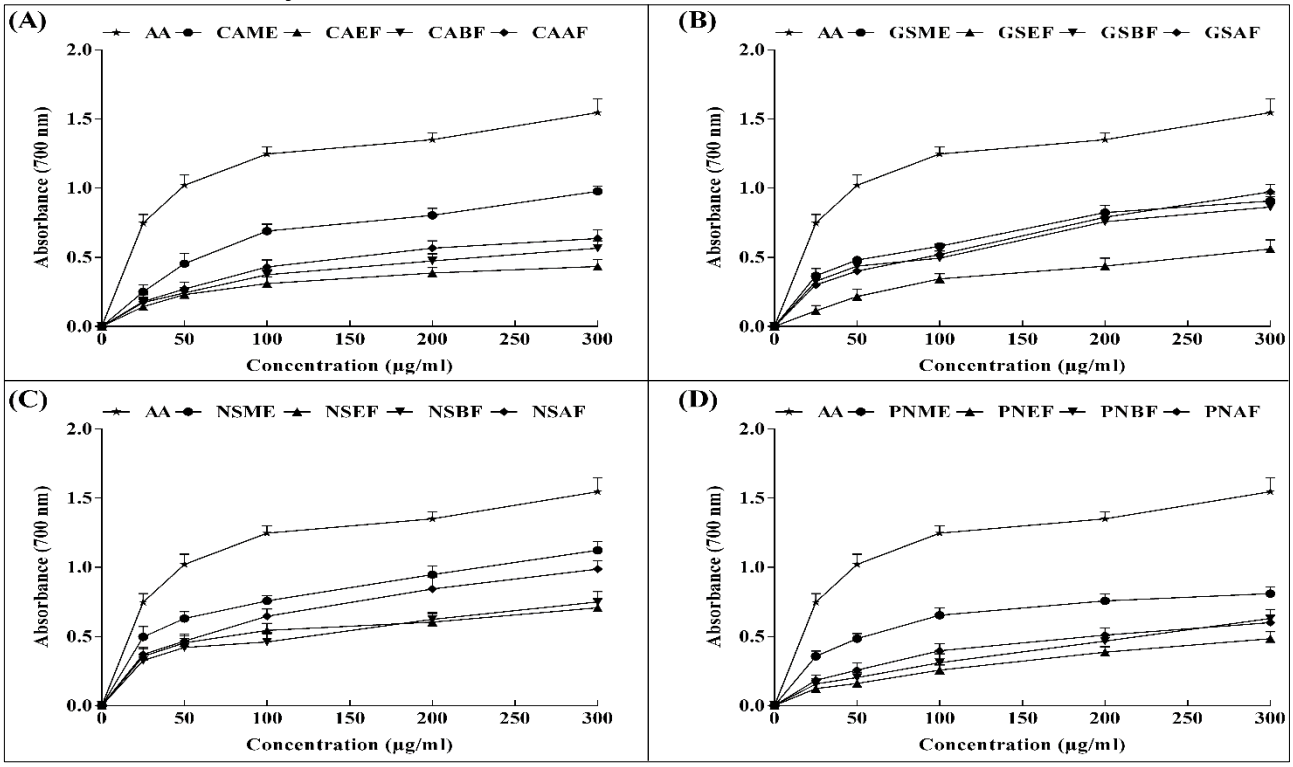

Figure 4. Reducing power activities of **(A)** *C. absus*: CAME, CAEF, CABF, CAAF, **(B)** *G. sylvestre*: GSME, GSEF, GSBF, GSAF, **(C)** *N. sativa*: NSME, NSEF, NSBF, NSAF, and **(D)** *P. nigrum*: PNME, PNEF, PNBF, PNAF are methanol extract, ethyl-acetate, *n*-butanol and aqueous fractions, respectively and Ascorbic acid (AA). Triplicate values are presented as mean±SEM

4. Conclusions

This study revealed the presence of bioactive phytocompounds, minerals, phenolic and flavonoids in plant materials analyzed. The *in vitro* antioxidant activities for four solvent extracts of *C. absus*, *G. sylvestre*, *N. sativa* and *P. nigrum* were assessed and solvent effects studied in the present study demonstrated the higher phenolic and flavonoid extraction efficiency of methanol, while ethyl-acetate extraction showed the lowest polyphenol contents. Methanolic extracts exhibited the highest *in vitro* antioxidant activities followed by aqueous, *n*-butanol and ethyl-acetate fractions. Medicinal plants are a vital source of phytocompounds that have a potential impact and beneficial effects on general health. Studies like the present investigation are progressively characterizing bioactivities of herbal products and enhancing their applications in healthcare.

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