Comparative Analysis of Different Bioactivities of *Curcuma longa*, *Nigella sativa* Seeds, and *Camellia sinensis* Extracted by Four Different Methods: A Green Way to Reduce Oxidative Stress

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Abstract Medicinal use of plants is as old as human history. *Curcuma longa*, *Nigella sativa* seeds, and *Camellia sinensis* have been widely used in various remedies since ages. In this study, the effect of extraction method on different bioactivities and phytochemical constituents of *Curcuma longa*, *Nigella sativa* seeds, and *Camellia sinensis* were evaluated and compared using single solvent system (50% ethanol). Plant extracts were prepared by percolation-assisted extraction, sonication-assisted extraction, microwave-assisted extraction, and polyphenol extraction. Following phytochemical screening, extracts were screened for antioxidant activity, antihemolytic activity, osmotic tolerance, and osmotic fragility. All plant extracts showed good phytochemical content irrespective of extraction method. However, activities *in vitro* antioxidant assays were dependent on plant as well as on extraction methods. Promising results were observed for antihemolytic activity against hydrogen peroxide-treated erythrocytes. Overall, *Camellia sinensis* exhibited the highest bioactivities followed by *Curcuma longa* and *Nigella sativa* seeds.

Keywords: *Camellia sinensis*, erythrocytes, antihemolytic activity, free radicals, antioxidant, Pakistan

Introduction

Oxygen is the fundamental element of life, but it is also notorious in nature as it produces free radicals, which are produced during physiological processes. They are continuously neutralized by antioxidants. However, an imbalance in oxidants and antioxidants leads to oxidative stress that is not only detrimental to biomolecules but also causes progression of various diseases. Therefore, minimizing oxidative stress will be the helpful in treatment of diseases (1).

Today, plants are explored for new drugs and pharmaceutical products. They are naturally enriched with phytochemicals, which are easy to isolate (2). Owing to several concerns in using synthetic drugs, it can be replaced by naturally occurring plant antioxidants. Plants stop the propagation of free radical chain reaction by donating a hydrogen atom. They overcome oxidative stress through various interconnected mechanisms depending on their phytoconstituents (3). Therefore, adapting the green way of using plants for reducing the oxidative stress is the need of the hour. In addition to antioxidant and other biological properties, plants have been explored for antihemolytic activity to treat anemia-related disorders as well.

Erythrocytes are the most abundant cells in the human body. Oxidation depletes membrane protein contents, deforms red blood cells (RBCs), and disturbs micro-constituents that lead to their lysis, releasing hemoglobin, which can be easily measured by a spectrophotometer. Simplicity, availability, and ease of isolation make erythrocytes membrane an excellent model for research (4).

In this study, three plants namely *Curcuma longa* (locally known as Haldi), *Nigella sativa* seeds (locally known as Kalonji), and *Camellia sinensis* (green tea) were studied. Recently, *Curcuma longa* has been employed in many clinical trials, and it appears non-toxic even at a dose of 500 µg/mL (5). Tea is the most widely consumed beverage after potable water (6). Among varieties of tea, green tea leaves are a rich source of catechins, flavonols, flavanones, and phenolic acids that aid in inhibition of oxidation of organic molecules (1). *Nigella sativa* seeds or black seeds are routinely used as spice in Asian communities, and very little is known about their activities (7). This study aims to identify natural alternatives to treat disorders associated with hemolysis by reducing oxidative stress and to determine the effect of extraction systems on the functionality of phytochemicals.

Materials and Methods

Reagents and instrument All buffers and solutions were prepared in autoclaved distilled water. Glass apparatuses were heat sterilized at 180°C for 2–3 h. Proper precautionary measures were taken while handling potentially toxic substances. All chemicals were purchased from MP Biochemicals, Merck, or HiMedia laboratories. Standards, e.g. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), rutin, and ascorbic acid, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Optical densities for all assays were measured by an Epochmicroplate reader101 (Biotek Instruments, Winooski, VT, USA) and a double beam Cecil Aquarius CE 7200 UV/Visible spectrophotometer.

Plant collection and extract preparation Plant materials such as *Curcuma longa* (CL), *Nigella sativa* seeds (NS) and *Camellia sinensis* (CS) were purchased from a local market in Lahore, Pakistan. They were dried under ambient conditions and grounded to a powdered form. Four extraction methods namely percolation-assisted extraction (PAE), sonication-assisted extraction (SAE), microwave-assisted extraction (MAE), and polyphenol extraction (PPE) were used. Fifty percent ethanol (v/v) was used as an extraction solvent in a ratio of 1:20 (w/v). A portion of homogenized plant extract was dried at 40–50°C, and the percentage of extraction yield was calculated using the following equation: %Yield=(Weight of dried sample extract/Initial weight of sample)×100.

Percolation-assisted extraction (PAE): The dried powdered material was infused in 50% ethanol for 24 h at room temperature under constant shaking (200 rpm), filtered through Whatman No. 4 filter paper, concentrated with a rotary vacuum at 35–40°C, and stored in a dry, cool place until further use (8).

Sonication-assisted extraction (SAE): A modified method was used for sonication-assisted extraction. The dried powdered material was infused in 50% ethanol for 15 min before it was exposed to a sonicator at a rate of 20 kHz for 60 min at 20–35°C. After filtration through Whatman No. 4 filter paper, the filtrate was concentrated and stored in the same manner as described above (3).

Microwave-assisted extractions (MAE): The dried powdered material was pre-leached in 50% ethanol for 30 min before treatment. A household microwave oven (200 V/50 Hz, DW+80, Dawlance) was used at 500–700 W irradiation for 5 min as follows: 45 s until the desired temperature (85–90°C) was achieved, 10 s to heat, and 10 s to power off for cooling and so on until the pre-set extraction time was attained. Following treatment, the mixture was filtered through Whatman No. 4 filter paper, concentrated, and stored in the same manner as described before (9).

Polyphenol extraction (PPE) For PPE, the dried powdered material

was infused in the extraction solvent (methanol, acetone, and water in the ratio of 3.5:3.5:3) and subjected to constant shaking for 30 min. It was extracted twice, and like other extracts, it was filtered, concentrated, and stored as described earlier (10).

Twelve extracts (four extracts for each plant) used in this study were *Curcuma longa* percolation-assisted extract (CPAE), *Curcuma longa* sonication-assisted extract (CSAE), *Curcuma longa* microwave-assisted extract (CMAE), *Curcuma longa* polyphenol extract (CPPE), *Nigella sativa* seeds percolation-assisted extract (NPAE), *Nigella sativa* seeds sonication-assisted extract (NSAE), *Nigella sativa* seeds microwave-assisted extract (NMAE), *Nigella sativa* seeds polyphenol extract (NPPE), *Camellia sinensis* percolation-assisted extract (SPAЕ), *Camellia sinensis* sonication-assisted extract (SSAE), *Camellia sinensis* microwave-assisted extract (SMAE), and *Camellia sinensis* polyphenol extract (SPPE).

Phytochemical screening

Total flavonol (TF) content: Total flavonol content was determined by a previously described method (8). Rutin was used as a standard, and a calibration curve was prepared (0.015–0.5 mg/mL). Briefly, 2 mL plant extract or a standard was mixed with 2 mL 2% aluminum trichloride and 6 mL 5% sodium acetate and incubated for 2.5 h at 20°C. After incubation, absorbance was read at 440 nm, and TF was calculated in rutin equivalents (RE) using the following formula:

$$X=C \cdot V/m,$$

where

X=Flavonol content, mg/g plant extract in rutin equivalents;

C=Concentration of rutin solution, established from the calibration curve, mg/mL;

V=Volume of plant extract, mL

m=Weight of plant extract, g

Total flavonoids (TFDs) content: Total flavonoid content was determined by an aluminum chloride colorimetric method (11). A rutin calibration curve was prepared ranging from 12.5 to 100 µg/mL. Briefly, 0.5 mL plant extract or a standard with 1.5 mL methanol, 0.1 mL 10% aluminum chloride, 0.1 mL 1 M potassium acetate, and 2.8 mL distilled water were mixed and allowed to stand at room temperature for 30 min. Absorbance was measured at 415 nm, and TFDs were calculated in rutin equivalents (RE) using the same formula used for total flavonols.

Determination of total phenolic (TP) content: Total phenolic content was determined by the modified Folin-Ciocalteu colorimetric method (12). Gallic acid was used as a standard with concentrations ranging from 0.02 to 0.3 mg/mL. Briefly, 1 mL standard or plant extract and 5 mL Folin-Ciocalteu reagent (1:10 diluted with distilled water) were mixed. After 5 min, 4 mL 1 M sodium carbonate was added and incubated for 30 min at 20°C. Absorbance of blue coloration was measured at 765 nm, and the contents were calculated in gallic acid equivalents (GAE) using a similar formula used for total flavonols.

***In vitro* antioxidant screening**

DPPH radical scavenging activity: The radical scavenging effect of stable DPPH radical was measured and compared to that of Trolox, rutin, and ascorbic acid (13). DPPH free radical is reduced in the presence of plant antioxidants. When an antioxidant donate hydrogen atom or electron, the purple color of DPPH radical disappears. A stock solution of DPPH was prepared by mixing 24 mg DPPH in 100 mL methanol and stored at 20°C until used. For the working solution, 10 mL of stock solution was diluted with 45 mL methanol to attain an absorbance of 1.1 ± 0.02 at 517 nm. An Assay was performed by mixing 50 μ L sample with 150 μ L DPPH solution and kept in dark for 30 min at room temperature. Disappearance of the initial purple color is directly linked with the ability of the extract to scavenge the DPPH free radical. Scavenging activity was calculated by the following formula: % of scavenging activity = $(A_c - A_i) / A_c \times 100$, where A_c is the absorbance of pure DPPH (control) and A_i is the absorbance of DPPH in the presence of extracts. Trolox was used as a standard. The scavenging activity of plants was expressed as μ M calculated from Trolox calibration curve. The concentration required to scavenge 50% of DPPH radicals (IC_{50}) was also calculated using regression analysis.

DPPH radical scavenging activity was also determined qualitatively by TLC as described previously (14). The standard or sample (10 μ L) was loaded individually at single spot on a silica-coated plate and air dried. This was repeated until a suitable amount was spotted. The plate was developed by butanol, acetic acid, and distilled water in the ratio of 40:50:10, sprayed with 0.2% of DPPH methanolic solution, and dried for 30 min in the dark at room temperature. Yellow spots against purple background indicate positive antioxidant activity. The intensity of color depends on both the amount and the nature of antioxidant compounds in the sample.

Reducing assays

ABTS assay: In the presence of antioxidants, ABTS radical reduces to a relatively stable non-radical form. For the ABTS assay, a modified method was followed (13). For the ABTS radical cation stock, equal quantities of 7 mM ABTS and 2.5 mM potassium persulfate were mixed and incubated in the dark for 12-16 h. $ABTS^{++}$ was prepared freshly for every assay. The working solution was prepared by diluting the stock solution with a suitable amount of ethanol to achieve an absorbance of 0.70 ± 0.02 at 734 nm. Fifteen microliters of standard or sample mixed with 285 μ L $ABTS^{++}$ was placed in the dark for 30 min at room temperature. The extent of decolourization was read at 734 nm and calculated as percentage inhibition of ABTS radical using the formula used for DPPH assay. Trolox was used as standard and IC_{50} was also calculated.

Ferric cyanide (Fe^{3+}) reducing antioxidant power (FRAP) assay: The reducing ability of the standard and samples were determined by the FRAP method (15). This assay entails the reduction of Fe^{3+} to Fe^{2+} in the presence of antioxidants. To 1 mL of plant extract or standard of varying concentration (10-100 mg/L), 2.5 mL 0.2 M phosphate buffer

(pH 6.6) and 2.5 mL of 1% potassium ferricyanide were added and incubated for 20 min at 50°C. Later, 2.5 mL 10% trichloroacetic acid was added, and the solution was centrifuged at 1,500 $\times g$ for 10 min. After centrifugation, 2.5 mL supernatant was mixed with 2.5 mL distilled water and 0.5 mL 0.1% $FeCl_3$. After 10 min, absorbance was measured at 700 nm against phosphate buffer blank. The absorbance value is directly proportional in reducing the ability of antioxidants. Ascorbic acid was used as a standard.

Other bioactivities

Antihemolytic activity: Antihemolytic activity of ethanolic extracts was performed (16). Approval for this study was obtained from the Ethics Committee at the School of Biological Sciences, the University of the Punjab in Lahore, Pakistan. Blood samples were obtained from healthy volunteers after consent and collected in EDTA vials. The blood samples were centrifuged at 2,000 $\times g$ for 10 min at 4°C to remove plasma and buffy coat. The pellet was washed thrice with phosphate buffer saline (PBS) pH 7.4 so that supernatant became colourless. The erythrocyte pellet was diluted with PBS to make a 4% suspension. Briefly, 0.5 mg/mL of plant extract or a standard was mixed and the final volume was adjusted to 5 mL with PBS followed by incubation for 30 min at 37°C. To the reaction mixture, 0.5 mL of H_2O_2 was added and centrifuged at 3,000 $\times g$ for 10 min. The absorbance of the reaction mixture was read at 540 nm. The concentration of H_2O_2 was adjusted to bring about 90% hemolysis after 240 min. For the control, 2 mL cell suspension was mixed with 2 mL distilled water to bring about 100% hemolysis. Ascorbic acid was used as a standard. Inhibitory activity of extracts was measured as percentage hemolysis as follows: percentage hemolysis = $(A_c - A_s) / A_c \times 100$, where A_c is absorbance of the control and A_s is absorbance of the sample.

Osmotic tolerance of human erythrocyte: Blood samples were centrifuged for 10 min at 2,000 $\times g$ at 4°C. The supernatant was discarded and the pellet was washed with PBS three times (pH 7.4). The cell pellet was divided into four equal portions; the first three portions were incubated with H_2O_2 (10 mM) in PBS with or without plant extract or a standard at constant shaking (200 rpm) for 3 h at 37°C. The reaction mixture was centrifuged and the supernatant was discarded. Cells were exposed to a hypotonic solution (85% PBS) for 5 min and centrifuged at 1,500 $\times g$ for 10 min at 4°C. The last portion of erythrocyte pellet was completely hemolyzed by dissolving it in distilled water. Absorbance of all supernatants were read at 540 nm and expressed as percentage of complete hemolysis (17).

Osmotic resistance (fragility) of human erythrocyte: The effect of plant extract on osmotic stability of human erythrocytes was also investigated. Briefly, 1 mL of NaCl solution (0-1%) in deionized water or NaCl solution (0-1%) in plant extract or a standard were incubated for 15 min at 37°C and then 25 μ L blood was added. It was mixed by inverting the tube several times and incubated for 30 min at 37°C. The reaction mixture was centrifuged at 1,300 $\times g$ for 10 min and supernatant was used to measure the lysis of erythrocytes at 540 nm

Fig. 1. Phytochemical screening: Flavonoids, flavonol, and phenolic contents (mg/g dry weight of plant). CL, *Curcuma longa*; NS, *Nigella sativa* seeds; CS, *Camellia sinensis*; PAE, percolation-assisted extraction; SAE, sonication-assisted extraction; MAE, microwave-assisted extraction; PPE, polyphenol extraction

(4). The percentage hemolysis was calculated by the following equation: percent hemolysis = $(A_s/A_c) \times 100$.

Statistical analysis All experiments were performed in triplicate and data was analysed using SPSS software (v. 20). Results were represented as mean $\pm$ standard deviation. The IC_{50} values were calculated by linear regression dose response curve.

Results and Discussion

Consumable foods rich in non-toxic phytochemicals increase resistance and healing to infection by boosting the immune system. They have multiple functions as compared to synthetic drugs that usually have a single function (18). The nature, polarity, and chemical behavior of functional groups present in plant extracts are actually responsible for their diverse outcomes (5).

Effect of solvent A number of solvents have been used for recovery of phytochemicals. Ethanol is safe to use because it is non-toxic and can be mixed with water in different ratios. However, the recovery of phytochemicals depends on solubility of phenolics and polarity of solvent used. Therefore, it is difficult to establish standard extraction method. Different factors for example liquid solid ratio, extraction solvent, solvent composition, extraction time, and temperature affect the extraction. Different studies have shown that 20:1 (mL:g) ratio and 50% ethanol in water (v/v) provide good extraction results as a number of phenols are soluble in ethanol/water solvent (15). Increased attention towards plant antioxidants demands an easy, rapid, sensitive, and reliable method that should not be time consuming (1).

Effect of extraction method Considering the different extraction

methods used in this study, the difference in phytochemical constituents and other activities of extracts was not much obvious. However, the methods used were time-saving when compared to 24 h extraction time required in conventional percolation method. Sonication method was adapted for the first time for plant extraction, and it gave good results. The time of extraction with MAE, SAE, and PPE was about 300-, 24-, and 20-fold longer than that with PAE. Thus, these methods can easily reduce the extraction time. In addition, pre-leaching effectively increased the extraction, and consequently, its constituents were increased.

Extraction yield The percentage extraction yield of ethanolic extracts showed that *Camellia sinensis* had the highest extraction yield of $55.1 \pm 0.5\%$ followed by *Curcuma longa* with $49.4 \pm 0.9\%$ and *Nigella sativa* seeds with $4.3 \pm 1.6\%$. Hot water extraction had 42% yield, while methanolic extracts had 41-45.5% yield (19,20). Previous studies showed extraction yield of 10-13% (21,22) for *Curcuma longa* and 7.5-32% for *Nigella sativa* seeds (7,23).

Phytochemical screening Phenolics, flavonoids, and flavonols content are shown in Fig. 1. Phenolic contents were measured using the photometric Folin-Ciocalteu reagent. In the presence of plant phenolics, components of Folin-Ciocalteu's reagent mainly phosphomolybdic and phosphotungstic acid reduce to blue-colored molybdenum and tungsten oxides in an alkaline solution (sodium carbonate). The intensity of blue colour is directly proportional to the number of phenolic groups present in extracts. In the present study, phenolic contents ranged from 12.3 ± 0.5 mg GAE/g in SSAE to 6.2 ± 1.5 mg GAE/g in SMAE. Total phenolic contents in decreasing order were as follows: SSAE > CPPE > SPAE > SPPE > CMAE > CPAE > CSAE > NMAE > NPAE > NSAE > NPPE > SMAE. The flavonoids content, unlike phenolic and flavonols, varied greatly from 25.9 ± 1.1 mg RE/g in CPPE to 1.0 ± 0.3 mg RE/g in NPAE. Flavonoids are considered as the largest

Table 1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of plant extracts. Low IC₅₀ represents high antioxidant activity. Variation in coloration depends upon the presence of reductants in samples. (unit: µg/mL)

Plant extract	DPPH scavenging activity (%) under different concentrations used								IC ₅₀
	7.8	15.6	31.3	62.5	125.0	250.0	500.0	1000.0	
SSAE ²⁾	27.5±1.1 ¹⁾	38.8±0.1	41.9±1.1	46.5±1.1	57.5±1.1	64.9±0.9	64.3±1.1	64.9±1.1	113.5±0.9
SMAE	1.9±0.3	44.3±0.2	53.6±1.1	61.9±1.5	66.6±0.5	70.8±1.1	-	-	22.9±0.8
SPPE	22.6±0.2	35.1±0.7	44.6±1.2	57.8±1.4	64.1±0.7	65.1±1.4	69.5±1.0	70.5±1.2	64.7±1.1
SPAE	22.8±0.1	21.6±1.1	24.0±1.3	27.8±1.1	37.5±1.5	52.3±1.6	86.7±1.1	96.4±0.5	303.5±1.1
NSAE	20.2±0.5	24.4±1.5	24.7±1.4	27.0±1.3	28.6±1.7	30.7±1.3	-	-	896.0±1.2
NMAE	21.3±0.1	37.5±1.1	38.7±1.5	40.8±1.1	43.4±1.6	43.7±1.1	46.2±1.7	47.9±0.7	1123.0±1.5
NPPE	16.6±0.6	18.6±0.9	19.8±0.7	22.4±1.0	29.8±0.5	44.4±1.2	54.3±1.6	69.7±0.6	534.9±0.9
NPAE	41.1±0.7	48.5±0.7	51.7±0.4	58.4±0.9	61.0±1.1	64.0±1.4	65.5±1.8	66.4±1.1	41.8±1.1
CSAE	22.1±0.8	23.3±0.5	24.4±0.9	26.3±0.5	31.1±1.7	37.4±0.9	43.3±0.7	-	430.0±0.9
CMAE	22.4±0.9	29.3±0.6	31.4±1.1	34.8±0.1	36.5±1.8	43.0±1.7	46.9±0.5	48.7±1.4	510.1±1.1
CPPE	35.1±1.5	38.5±1.4	38.6±1.3	38.8±1.5	40.9±1.2	41.3±0.4	46.4±0.9	69.3±1.9	459.6±1.6
CPAE	47.4±1.1	53.7±0.2	57.3±1.0	60.9±0.7	60.1±1.4	61.0±1.4	63.4±0.4	89.7±1.8	330.5±1.3
SA	30.4±1.1	32.8±0.5	37.9±0.6	49.1±1.2	64.5±1.1	70.9±1.4	76.8±0.4	87.7±0.9	96.0±0.9
SR	28.1±0.9	32.8±0.4	43.6±1.1	59.0±1.1	62.2±0.9	63.2±0.8	66.8±0.5	68.2±2.1	64.5±1.1
ST	23.5±0.5	23.2±0.5	27.9±1.7	34.5±0.9	49.0±0.8	63.4±0.6	69.3±0.7	81.3±1.7	164.6±1.3

¹⁾Mean±standard error.

²⁾SSAE, *Camellia sinensis* sonication-assisted extract; SMAE, *Camellia sinensis* microwave-assisted extract; SPPE, *Camellia sinensis* polyphenol extract; SPAE, *Camellia sinensis* percolation-assisted extract; NSAE, *Nigella sativa* seeds sonication-assisted extract; NMAE, *Nigella sativa* seeds microwave-assisted extract; NPPE, *Nigella sativa* seeds polyphenol extract; NPAE, *Nigella sativa* seeds percolation-assisted extract; CSAE, *Curcuma longa* sonication-assisted extract; CMAE, *Curcuma longa* microwave-assisted extract; CPPE, *Curcuma longa* polyphenol extract; CPAE, *Curcuma longa* percolation-assisted extract; SA, Ascorbic acid; SR, Rutin; ST, Trolox

naturally occurring phenols and are considered more potent than vitamins (18). Total flavonoids contents in decreasing order were as follows: CPPE>CSAE>CPAE>SSAE>SPAE>SPPE>CMAE>SMAE>NMAE>NPPE>NSAE>NPAE. Interestingly, flavonols such as phenolics did not vary much, being highest with 15.5±1.1 mg RE/g in CPAE and lowest with 14.5±0.9 mg RE/g in NPAE. Overall, high contents were present in *Camellia sinensis* followed by *Curcuma longa* and *Nigella sativa* seeds, but *Curcuma longa* showed more total flavonoids content as compared to other extracts. Phytochemical contents varied independently with different extraction techniques used in this study. None of the single extraction methods were responsible for the highest phytochemicals. In *Curcuma longa*, phenolics and flavonoids were highest in PPE and flavonols were highest in PAE. However, in *Nigella sativa* seeds, all these were highest in MAE, and in *Camellia sinensis*, they were highest in SAE and PPE. Phytochemical activities depend on various factors, including extraction method, nature of phenolic compounds, and assay method employed. Extraction methods are important for accurate quantification of antioxidant contents and capacity (24).

In vitro antioxidant screening The antioxidant system involves complex mechanisms against oxidative stress that cannot be explained well by a single antioxidant method. Therefore, different mechanisms—namely reducing activity, scavenging activity, antihemolytic activity, osmotic tolerance, and osmotic fragility were performed to give a comprehensive overview. A number of methods are used to determine the antioxidant activity, which produces different results for all activities mainly due to different mechanisms involved,

reaction conditions required, and reagents used (25). Radical scavenging methods are based on inhibition of oxidized products that may otherwise initiate chain reactions. The scavenging activities vary greatly among extracts as well as among extraction method, showing that these are related to reducing mechanisms (2).

DPPH radical scavenging activity Scavenging activities of antioxidants are analyzed conventionally by DPPH radical. When antioxidants donate hydrogen atoms or electrons, the purple colored DPPH radical in an alcoholic solution reduces to a yellow colored α , α -diphenyl- β -picrylhydrazine. The higher the number of available hydroxyl groups in a sample, the higher the number of DPPH molecules reduced. Therefore, absorption at 517 nm represents the amount of residual DPPH. This assay is short, sensitive, and easy to perform and considered as a valid test for determining the overall antioxidant activity (13). The increase in inhibition rate with the increase in the concentration of sample showed higher scavenging activity and hence higher antioxidant activity. The quenching was observed at a single point after 30 min. IC₅₀ was also calculated using regression analysis of dose dependence; the lower the IC₅₀, the more potent the substance. Rutin, ascorbic acid, and Trolox, used as reference compounds, have IC₅₀ of 64±1.1, 96±0.9, and 164.6±1.3 µg/mL, respectively (Table 1). Among different extracts tested, SMAE was the most potent with an IC₅₀ of 22.9±0.8 µg/mL, and least active was NMAE with IC₅₀ of 1123.0±1.5 µg/mL. All extracts have comparable activity to the reference compounds except that of *Curcuma longa* extracts, which had IC₅₀ value ranged from 330.5±1.3 µg/mL in CPAE to 510.1±1.1 µg/mL in CMAE. Extracts in descending order were

ranked as SMAE (22.9±0.8 µg/mL)>NPPE (41.8±1.1 µg/mL)>SPPE (64.7±1.1 µg/mL)>SSAE (113.5±0.9 µg/mL)>SPAE (303.5±1.1 µg/mL)>CPAE (330.5±1.3 µg/mL)>CSAE (430±0.9 µg/mL)>CPPE (459.6±1.6 µg/mL)>CMAE (510.1±1.1 µg/mL)>NPPE (534.9±0.9 µg/mL)>NSAE (896±1.2 µg/mL)>NMAE (1123.0±1.5 µg/mL). On TLC plates, yellow spots of different intensities against purple background were observed, indicating the presence of antioxidant compounds in plant extracts. The brightest were observed for *Camellia sinensis*, followed by *Nigella sativa* seeds. TLC is an instant qualitative method that is easy to perform and gives a quick view of the antioxidant potential of substance.

Reducing assays

ABTS assay: In the presence of antioxidants, ABTS radical reduces to a relatively stable, non-radical form. For the ABTS assay, a modified method was followed (13). The extent of decolorization was read at 734nm and calculated as percentage inhibition of ABTS radical using the formula used for the DPPH assay (Table 2). Trolox was used as a standard, and IC₅₀ was calculated.

FRAP assay: In the presence of plant antioxidants, ferric cyanide complex reduces to the ferrous form, and the intensity of Perl's Prussian blue coloration reflects the amount of Fe²⁺ produced. It represents the reducing activity of plant extract and hence its antioxidant activity. Due to its simplicity and high reproducibility, it is widely used to determine the overall antioxidant activity of extracts. In our study, good antioxidant activity of extracts was observed; even *Camellia sinensis* extracts showed higher activity as compared to the activity of ascorbic acid used as reference compound as shown in

Table 2. 2,2'-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) scavenging activity of plant extracts. Radical scavenging methods are based on inhibition of oxidized products that may otherwise initiate chain reactions. The higher the percentage, the higher the antioxidant activity.

Plant extract	ABTS scavenging activity (%) at different time intervals (100 µg/mL)	
	30 min	60 min
CMAE ²⁾	61.2±1.3 ¹⁾	65.5±0.7
CSAE	84.7±0.8	79.7±1.0
CPPE	73.6±0.4	85.5±0.8
CPAE	91.6±0.7	91.9±1.1
NMAE	65.8±2.1	70.1±1.5
NSAE	50.5±1.9	60.7±1.1
NPPE	59.7±0.4	68.9±0.7
NPAE	22.8±0.8	29.4±0.9
SMAE	55.3±0.7	48.5±1.0
SSAE	86.1±1.1	81.3±0.4
SPPE	75.6±1.4	70.2±1.5
SPAE	75.0±1.3	75.4±0.9
SR	28.1±1.7	32.8±1.5
ST	23.5±1.2	23.2±1.1

¹⁾Mean±standard error.

²⁾CMAE, *Curcuma longa* microwave-assisted extract; CSAE, *Curcuma longa* sonication-assisted extract; CPPE, *Curcuma longa* polyphenol extract; CPAE, *Curcuma longa* percolation-assisted extract; NMAE, *Nigella sativa* seeds microwave-assisted extract; NSAE, *Nigella sativa* seeds sonication-assisted extract; NPPE, *Nigella sativa* seeds polyphenol extract; NPAE, *Nigella sativa* seeds percolation-assisted extract; SMAE, *Camellia sinensis* microwave-assisted extract; SSAE, *Camellia sinensis* sonication-assisted extract; SPPE, *Camellia sinensis* polyphenol extract; SPAE, *Camellia sinensis* percolation-assisted extract; SR, Rutin; ST, Trolox

Table 3. Reducing power of plant extracts. High absorbance (nm) indicates strong antioxidant activity.

Plant extract	Concentration (µg/mL)				
	20	40	60	80	100
CMAE ²⁾	0.26±1.2 ¹⁾	0.36±0.5	0.40±0.4	0.42±0.4	0.71±0.1
CSAE	0.49±0.9	0.71±0.3	0.87±1.4	1.15±1.1	1.26±0.3
CPPE	0.32±0.8	0.33±1.1	0.73±1.6	0.75±0.2	0.76±0.4
CPAE	0.31±0.4	0.39±1.5	0.47±1.4	0.64±0.3	0.62±0.4
NMAE	0.96±0.1	1.26±0.4	2.06±1.5	2.18±0.1	1.80±1.1
NSAE	0.76±0.3	1.01±0.2	1.18±1.1	1.72±1.1	1.88±0.8
NPPE	0.50±0.1	0.53±0.1	0.56±0.4	0.78±0.4	0.96±0.4
NPAE	0.28±0.7	0.54±0.8	0.68±0.3	0.79±0.3	0.83±0.1
SMAE	2.34±0.9	2.61±0.6	2.70±0.4	4.10±1.5	4.70±0.7
SSAE	1.99±1.1	2.58±0.7	3.62±0.5	4.70±1.4	4.58±0.9
SPPE	2.55±1.5	2.85±1.1	4.06±0.4	4.43±1.2	4.46±1.3
SPAE	4.18±1.4	4.39±1.2	4.46±0.6	4.65±1.5	4.88±1.5
AA	0.94±1.6	1.48±1.6	1.86±0.7	2.23±0.8	2.65±1.1

¹⁾Mean±standard error.

²⁾CMAE, *Curcuma longa* microwave-assisted extract; CSAE, *Curcuma longa* sonication-assisted extract; CPPE, *Curcuma longa* polyphenol extract; CPAE, *Curcuma longa* percolation-assisted extract; NMAE, *Nigella sativa* seeds microwave-assisted extract; NSAE, *Nigella sativa* seeds sonication-assisted extract; NPPE, *Nigella sativa* seeds polyphenol extract; NPAE, *Nigella sativa* seeds percolation-assisted extract; SMAE, *Camellia sinensis* microwave-assisted extract; SSAE, *Camellia sinensis* sonication-assisted extract; SPPE, *Camellia sinensis* polyphenol extract; SPAE, *Camellia sinensis* percolation-assisted extract; AA, Ascorbic acid

Fig. 2. Antihemolytic activity of plant extracts (%). The higher the percentage, the higher the activity. At five intervals, hemolysis at 540nm was measured: before H₂O₂ addition and 1, 2, 3, and 4 h after H₂O₂ was introduced in erythrocytes. All extracts except NSAE and NPAE showed a decrease in activity. CMAE, *Curcuma longa* microwave-assisted extract; CSAE, *Curcuma longa* sonication-assisted extract; CPPE, *Curcuma longa* polyphenol extract; CPAE, *Curcuma longa* percolation-assisted extract; NMAE, *Nigella sativa* seeds microwave-assisted extract; NSAE, *Nigella sativa* seeds sonication-assisted extract; NPPE, *Nigella sativa* seeds polyphenol extract; NPAE, *Nigella sativa* seeds percolation-assisted extract; SMAE, *Camellia sinensis* microwave-assisted extract; SSAE, *Camellia sinensis* sonication-assisted extract; SPPE, *Camellia sinensis* polyphenol extract; SPAE, *Camellia sinensis* percolation-assisted extract

Table 3. A positive correlation was observed between concentration and absorbance; the higher the concentration, the higher the absorbance, but it was not true for all cases. A sudden decrease was observed in SMAE and CSAE. At 100 µg/mL, *Camellia sinensis* extracts showed higher activity, followed by *Nigella sativa* seeds and *Camellia sinensis*. At 100 µg/mL, SAE showed more potent reducing values as compared to others, but in *Camellia sinensis*, it was PAE > MAE > SAE > PPE. Overall, SAE showed more promising results as compared to others.

Antihemolytic activity: Erythrocytes are most common cells in human body. Due to their physiological and morphological characteristics, they have been extensively studied, particularly in drug delivery (2,16). In this study, erythrocyte suspension was prepared from normal blood samples. Antihemolytic activity of extracts was measured against hydrogen peroxide-induced erythrocytes (Fig. 2). Hydrogen peroxide is safe to use, and it can cross cell membranes easily by increasing hydroxyl radicals in cell (3). Readings were taken before and after hydrogen peroxide was added. An increase in inhibition rate was observed in NSAE and NPAE when hydrogen peroxide was added. Plant extracts showed different haemolytic pattern, and there was a positive correlation between inhibitory activity and concentration of extract up to a certain concentration (12,500-50,000 µg/mL) after which abrupt changes in reading were observed. Extracts in addition to antioxidant potential also showed promising antihemolytic activities. Consistent inhibition values were found with *Nigella sativa* seeds extract, while only SAE extracts showed decent activity in *Camellia sinensis* and *Curcuma longa* extracts. Continuous exposure to high oxygen tension during respiration with presence of high fatty acid content make erythrocytes a vulnerable site for peroxidation. Exposure to oxidants leads to generation of free radicals that can be potentially harmful to membranes (17).

Antihemolytic activity can be attributed to high content of flavonoids because they bind to RBC membranes and inhibit lipid peroxidation and thus prevent oxidative hemolysis (18). RBC hemolysis is a sensitive method for evaluating antihemolytic properties of medicinal plants.

Table 4. Osmotic fragility of erythrocytes in isotonic solution. With increase in sodium chloride concentration, cells become fragile.

Plant extracts	NaCl concentration (%)			
	0	0.25	0.5	0.75
CMAE ²⁾	100	97.12±0.9 ¹⁾	59.6±0.8	33.6±1.3
CSAE	100	90.6±0.5	79.55±0.4	22.7±0.5
CPPE	100	95.9±1.1	80.6±0.2	50.6±1.1
CPAE	100	94.5±1.2	85±1.1	55.9±1.4
NMAE	100	90.5±1.1	59.15±1.5	47.5±1.7
NSAE	100	58.9±0.2	56.4±1.7	17.6±0.8
NPPE	100	94.5±0.5	58.6±1.4	21.18±1.2
NPAE	100	72.54±0.4	50.6±1.6	37.2±1.5
SMAE	100	92.4±1.3	88.5±1.5	79.1±1.4
SSAE	100	93.3±1.4	90.01±1.4	80.56±1.4
SPPE	100	93.1±1.7	89.5±1.1	39.6±1.5
SPAE	100	90.4±1.1	55.9±0.5	34.6±1.4
SA	100	97.2±1.5	85.9±0.7	69.6±1.7
ST	100	98.1±1.4	94.1±0.4	21.3±1.1

¹⁾Mean±standard error.

²⁾CMAE, *Curcuma longa* microwave-assisted extract; CSAE, *Curcuma longa* sonication-assisted extract; CPPE, *Curcuma longa* polyphenol extract; CPAE, *Curcuma longa* percolation-assisted extract; NMAE, *Nigella sativa* seeds microwave-assisted extract; NSAE, *Nigella sativa* seeds sonication-assisted extract; NPPE, *Nigella sativa* seeds polyphenol extract; NPAE, *Nigella sativa* seeds percolation-assisted extract; SMAE, *Camellia sinensis* microwave-assisted extract; SSAE, *Camellia sinensis* sonication-assisted extract; SPPE, *Camellia sinensis* polyphenol extract; SPAE, *Camellia sinensis* percolation-assisted extract; SA, Ascorbic acid; ST, Trolox

Fig. 3. Osmotic tolerance of human erythrocytes. Exposure of erythrocytes to hypotonic solution decreases the hemolysis that is an indication of oxidative stress. Plant treatment increased the osmotic tolerance when compared to the control (H₂O₂ only with no plant extract). CMAE, *Curcuma longa* microwave-assisted extract; CSAE, *Curcuma longa* sonication-assisted extract; CPPE, *Curcuma longa* polyphenol extract; CPAE, *Curcuma longa* percolation-assisted extract; NMAE, *Nigella sativa* seeds microwave-assisted extract; NSAE, *Nigella sativa* seeds sonication-assisted extract; NPPE, *Nigella sativa* seeds polyphenol extract; NPAE, *Nigella sativa* seeds percolation-assisted extract; SMAE, *Camellia sinensis* microwave-assisted extract; SSAE, *Camellia sinensis* sonication-assisted extract; SIAE, *Camellia sinensis* polyphenol extract; SPAE, *Camellia sinensis* percolation-assisted extract

Table 5. Pearson correlation between phytochemicals, antioxidant activity, and antihemolytic activity

	Flavonoids	Flavonols	DPPH	ABTS	Antihemolytic
Phenol	0.65* ¹⁾ (0.02)	0.50 (0.1)	0.28 (0.38)	0.57 (0.06)	0.35 (0.27)
Flavonoids		0.60* (0.04)	0.54 (0.07)	0.68* (0.02)	0.71* (0.01)
Flavonols			0.13 (0.70)	0.76** ²⁾ (<0.0)	0.70*(0.01)
DPPH				0.1 (0.73)	0.37 (0.24)
ABTS					0.66*(0.02)

¹⁾*, Correlation is significant at the 0.05 level (2-tailed).

²⁾**, Correlation is significant at the 0.01 level (2-tailed).

Osmotic tolerance of human erythrocyte: Positive effects of plant extracts were observed for osmotic tolerance of erythrocytes. The presence or absence of plant extracts marked a difference in osmotic tolerance as shown in Fig. 3. Plant-treated cells showed more osmotic tolerance as compared to cells without plant extracts. A different pattern was observed, being highly positive in case of NPPE (35.3±0.3%) and highly negative in case of CSAE (19.4±1.1%). *Nigella sativa* seed extracts were most effective among all, and most *Camellia sinensis* extracts showed negative values but were still less than the control.

Osmotic resistance (fragility) of human erythrocyte: None of the extracts showed activity less than 50% as shown in Table 4. Compared to the standard ascorbic acid, all extract showed competitive osmotic fragility, and NPAE showed more resistance to osmotic fragility. Osmotic fragility is defined as the sensitivity of RBCs to change in osmotic pressure. In this assay, resistance of erythrocytes to lysis in the presence or absence of plant extracts was measured against oxidative stress by exposing them to the decreasing concentration of isotonic saline solution. It is actually the measurement of hemoglobin released during lysis when exposed to saline solution (4,16).

High nutritional and bioactive contents make local plants valuable to explore. The impact of extraction methods on their activities has

increased research efforts to devise more techniques for their extraction, separation, and identification. Sonication-assisted extraction and microwave-assisted extractions were efficient as compared to other extraction methods. Exploring plant extracts for activities other than their antioxidant potential is propitious. These plants can be explored further to use as an effective measure against oxidative stress, especially in hemolysis-related disorders. On the whole, *Camellia sinensis* was most effective in different *in vitro* assays followed by *Curcuma longa* and *Nigella sativa* seeds.

Correlation findings: Significant correlation was observed among phenols and flavonoids (p -value=0.02), flavonoids and flavonols (p -value=0.04), flavonoids and ABTS scavenging activity (p -value=0.02), flavonols and ABTS (p -value=0.004), flavonoids and antihemolytic activity (p -value=0.01), flavonols and antihemolytic activity (p -value=0.01), and ABTS and antihemolytic activity (p -value=0.02) (Table 5). These results are consistent with previous studies, where a strong correlation between antioxidant and phenolic contents was reported (26-29); however, some studies showed poor or non-linear relation (30,31).

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