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Evaluation of antioxidant, cytoprotective and genoprotective potential of *Syzygium aromaticum* (L.) hydrolyzed extracts

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Abstract

Plants of medicinal values have been used to cure many diseases since ancient times. Various bioactive ingredients of such medicinal plants serve to hinder the progression of many infectious diseases. The current study was designed to evaluate the antioxidant, anti-haemolytic and genoprotective activities of a selected medicinal plant i.e. *Syzygium aromaticum*. Extraction with aqueous methanol in variable concentrations *viz*; 50%, 70% and 100 % to squeeze out the loosely bound phenolic compounds from plant cells; while strongly bound bioactives were recovered by hydrolyzed extraction proceeded by mineral acid, HCl (0.01*N*, 0.1*N*, 0.5*N*) and an alkali, NaOH (0.01*N*, 0.1*N*, 0.5*N*). Results demonstrated that methanol (100%) was able to extract highest number of phenolic compounds, as depicted by TPC assay (75.44±0.70 mg GAE/g extract) which were responsible for highest antioxidant potential by neutralizing DPPH free radicals (77.15±1.93%) and Fe^{3+} ion reducing capacity (72.55±2.49 mg AAE/g extract). Acidic hydrolyzed extraction gave even higher phenolic contents (93.12±0.72 mg GAE/g extract) while alkaline hydrolysis (84.22±0.24 mg GAE/g extract) was not quite satisfactory. Acidic hydrolyzed extract (0.1*N*) presented highest cytoprotective potential (95.15±1.44%) by preventing H_2O_2 induced oxidative damage to human red blood cells, and highest anti-mutation in selected bacterial strains (98.26±0.48%). Acidified hydrolyzed extract was then, subjected to HPLC phytochemical characterization that indicated the presence of twenty bioactive compounds belong to variable classes of phytochemicals. These results demonstrated that *S. aromaticum* could have potent medicinal and therapeutic applications.

Keywords: Hydrolyzed extracts, *Syzygium aromaticum*, Antioxidant, Cytoprotective, Genoprotective.

Full length article **Muhammad Yasir*, e-mail: muhammad.yasir@chem.uol.edu.pk Doi # https://doi.org/10.62877/6-IJCBS-24-26-20-6

1. Introduction

Interest in medicinal plants is getting increased day by day with exploration of wide variety of medicinal plants. Folk medicinal plants have been used to cure many human diseases since ancient times. A large number of phytochemicals have been examined to be bioactive and potent against different aliments and serve to protect against many infectious diseases [1].

Plant based medicines are more effective to treat many fetal diseases because of minimal or no side effects. Many herbal plants have been explored for their phytochemical constituents and numerous bioactive compounds have been identified. In recent times, modern phytochemists mainly focus on conventional folk plants which have been the major ingredients of folk herbal formulations [2].

Condiments are complex mixture of spices, herbs and aromatic plants which are regarded as pungent vegetables. Their primary function is seasoning of food as well as to contribute piquancy to food products and drinks. They are necessary components of our food and comprise of essential micro-nutrients that play some important metabolic functions in living bodies. Many of such plants are composed of different bioactives which may serve as vitamins and pre-vitamins in human bodies. Most of the natural compounds observed in diverse plant spices hold antimutagenic, antimicrobial and anticancer ability against harmful cancerous cells [3-4]. Due to antibacterial and antifungal characteristics, clove is used as preservative in food, beverages and bakery items. The spoilage in ground beef and cattle meat is mostly controlled by clove [5]. Such findings conclude that this spice not only used to beautify the most effective taste and fragrance of the meals; additionally also offer numerous medicinal and food safety advantages [6]. Reactive oxygen species include numerous highly reactive molecules comprising of free radicals such as hydroxyl (OH), superoxide anion (O_2^-) and neutral molecules like hydrogen peroxide (H_2O_2). Such reactive species not only result from normal oxidative metabolism in living organisms but also extensively produced during oxidative stress and under the effect of environmental toxins and ionizing radiations [7-8].

Oxidative stress is a condition where such ROS are not properly counteracted by natural antioxidative defense system of an individual, which may lead to numerous health issues including neurodegenerative diseases, cardiovascular disorders, aging and cancer. The balance between body's natural antioxidant capacity and production of ROS during respiratory metabolic functions is very crucial to avoid multiple diseases. When such natural balance is disturbed by environmental factors, it may result in damaging of health, which can be supported by taking diet rich with such bioactive compounds having antioxidant properties [8-9]. Antioxidants are such compounds which protect cells against degenerative effects of reactive oxygen species (ROS) which serve by quenching free radicals, donating protons or serving as chelating agents to neutralize such reactive molecules [10-11]. *Syzygium aromaticum*, a common medicinal spice, belongs to family *myrtaceae*. It is native to Indonesia; also harvested in India, Pakistan, Sri Lanka and Tanzania. It grows up to 10-20 meter, leaves are oval in shape and crimson flowers in groups of numerous terminal clusters. It is mostly used as food flavoring agent; as a medicine for treatment of asthma and different allergies; and as antiseptic to cure dental complications. It also presents antioxidant properties along with anti-carcinogenic traits. It has been used as an aroma therapy-oil, mouth sterilizer and painkiller [12-13]. Phytochemical analysis depicts the presence of numerous bioactive compounds in *S. aromaticum* oil which include many phenolic compounds like flavonoids and phenolic acids. *S. aromaticum* oil has been found to be very potent for relieving the rheumatoidic pains and inflammations [14]. *S. aromaticum* is also used as a carminative agent. It has ability to stop vomiting because it promotes the increased release of HCl in stomach, which then serves for antimicrobial and antibacterial properties [15]. Its powder after mixing with honey, is applied for the treatment of acne. Eugenol, one of major phytochemicals of *S. aromaticum*, has been found to minimize the harmful effects of environmental wastes and food toxins that can cause the digestive tract cancer. It also helped to cure athlete's foot and nail fungus; mosquito repellent and reduces memory losses [16-17].

2. Materials and methods

2.1. Sample collection and extraction

S. aromaticum flower buds were obtained from herbal market, Faisalabad, Pakistan, washed, shade dried and ground to fine powder. Powdered samples were extracted with different solvents to obtain fundamentally two types of extracts. Free phenolic compounds were isolated using aqueous methanol in variable concentrations (25%, 50%, 75%, and 100%), while bound bioactives were set free by hydrolytic extraction that was carried out using mineral acid, HCl (0.1*N*, 0.5*N*, and 1.0*N*) and alkali, NaOH (0.1*N*, 0.5*N*, 1.0*N*). The samples were extracted by shaking, in a sample:solvent ratio 1:10, in an orbital shaker (Gallenkamp, UK) for 24 hours at room temperature. All extracts were concentrated using vacuum rotary evaporator (Rotary Evaporator N-1001, EYELA, Tokyo, Japan) and stored at 4ºC, for further analysis [18].

2.2. Total phenolic contents (TPC)

Folin-Ciocalteu reagent was applied to determine the total phenolic contents of all *S. aromaticum* extracts that were expressed as Gallic acid equivalents (GAE). Briefly, 0.5 mL sample solution (0.1 mg/mL) was mixed with 2 mL sodium carbonate (7.5% *w/v*) and 2.5 mL Folin–Ciocalteu reagent (10 % *v/v*). After incubating for 30 min at room temperature, the absorbance of the final reaction mixture was recorded at 765 nm using a Shimadzu 160-UV-Vis spectrophotometer and concentration of phenolics was calculated from gallic acid calibration curve and expressed as milligrams of GAE per gram of dry extract [19].

2.3. DPPH free radical scavenging activity

Free radical scavenging potential of *S. aromaticum* extracts was evaluated using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) a reported procedure of Safari and Asbchin, [20]. Extract samples of variable concentrations in methanol (250, 125, 62.5, 31.25, and 15.625 µg/mL) were mixed with 1 mL of 0.1mM methanolic DPPH solution. After 30 min incubation period at room temperature, their absorbance was recorded at 517 nm.

Inhibition percentage of DPPH free radical was calculated as follows:

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

Where A_c and A_s indicate the absorbance of blank and sample solution, respectively.

IC_{50} (effective dose of 50% inhibition) was obtained from a plot of percentage inhibition verses extract concentration [21].

2.4. Reducing power assay

The reducing power of extracts was determined following ferric ion reducing assay. Briefy, ethanolic extract (0.5 mL) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1 % potassium ferricyanide. The mixture was incubated at 50°C for 20 min, and then 2.5 mL of trichloroacetic acid (10 %) was added to this mixture, centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with 0.5 mL of distilled water

and 0.5 mL of 0.1 % $FeCl_3$ and the absorbance was measured at 700 nm which corresponded to reducing power [22].

2.5. Cytotoxic screening and cytoprotective activity

All *S. aromaticum* extracts were preceded through cytotoxic screening by evaluating their hemolytic potential. Freshly obtained blood samples ($3cm^3$) were added in heparinized tubes to avoid coagulation, gently mixed and centrifuged for 5 min at 850*g*. The supernatant was poured off and RBCs were washed with chilled (4ºC) phosphate buffer saline (PBS) solution. The washed RBCs were counted by heamacytometer and their count was maintained to 7.068 x 10^8 cells/mL. Each plant extract (20µL) was taken in eppendorfs, 180 µL diluted blood cell suspension was then added and incubated for 35 minutes at 37ºC. After, the tubes were placed on ice for 5 minutes and centrifuged for 5 minutes. After centrifugation, 100 µL supernatant was taken from the tubes and diluted with 900 µL chilled PBS and placed on ice. After this, 200 µL mixtures from each eppendorfs was added into 96 well plates. 0.1% triton X- 100 solution was taken as a positive control and phosphate buffer saline (PBS) as a negative control. The absorbance was measured at 576 nm with a BioTeK, µ-Quant UV/VIS spectrophotometer (BioTek, Winooski, VT, USA).

Haemolytic activity was quantified using the formula:

$$\% \text{ Haemolysis} = (A_x - A_0/A_{std} - A_0) \times 100$$

where A_x, A_{std} and A_0 represent the absorbance of sample, positive and negative control, respectively. A higher percentage of haemolysis by plant extract indicates its greater cytotoxicity [23].

The inhibitory action of plant extracts which showed lesser cytotoxicity was measured by adding H_2O_2 solution (0.5 cm^3) to aliquots having erythrocytes and plant extracts, to carry out oxidative damage of lipid membranes of erythrocytes. The inhibition to haemolysis was measured by the following formula:

$$\text{Inhibition (\%) to Haemolysis} = (1\text{-}As/Ac) \times 100$$

Where *As* is the absorbance of aliquots having erythrocytes, plant extract and H_2O_2 while *Ac* is the absorbance of aliquots having erythrocytes and H_2O_2 with no plant extract [24-25].

2.6. Genoprotective assay

Ames test was performed on micro-titer plate for genotoxic screening and afterward genoprotecive poential. Briefly, each plant extract was mixed (in five different vessels) with distilled water, two mutant strains (*S. typhimurium* TA100 and *S. typhimurium* TA98), two standard mutagens (potassium dichromate $K_2Cr_2O_7$ and sodium azide NaN_3) and reagent mixture (D-glucose, D-biotin, Davis-Mingioli salt, *L*-histidine and bromocresol purple) at amounts as shown in Table 1.

The plates were sealed in plastic bags and incubated at 37°C for 4 days. The blank plate was observed first and the rest of plates were read only when all wells in blank were colored purple indicating the assay was not contaminated. The background, standard, and test plates were scored visually and all yellow, partial yellow and turbid wells were scored as positive wells while purple wells were scored as negative. The extract was considered toxic to the test strain if all wells in the plate showed purple coloration. For an extract to be mutagenic, the number of positive wells had to be more than twice the number of positive well in the background plate [26-27].

The antimutagenic effect (%) was calculated using the following formula:

$$\text{Genoprotection (\%age)} = \left[1 - \frac{\text{Mutants of plate 4} - \text{spontaneous Mutants}}{\text{Mutants of plate3} - \text{Spontaneous Mutants}}\right] \times 100$$

2.7. Analysis of phenolic compounds by HPLC

S. aromaticum extracts were chemically characterized by RP-HPLC for different bioactives, following a modified procedure of Butsat and Siriamornpun (2016). Extract samples were filtered through a 0.45µm PTFE syringe tip filter using a 20µL sample loop. A RP-HPLC system equipped with a Shimadzu PDA detector, reverse phase (RP C-18) column (Interstsil ODS-5 µm x 4.6 mm x 250 mm), a quaternary pump, and an online vacuum degasser, was used for the separation, identification and quantification of phenolic compounds. The flow rate was set at 0.8 mL/min at 37ºC with a gradient flow of two mobile phases. Solvent A was prepared by water: acetonitrile: trifluoroacetic acid at 90:10:0.1% (v/v) and solvent B at 10:90:0.06% (v/v). Elution was performed using the following gradient: 0-10 min: 10-35% B, 10-20 min: 35-42 % B and 20-30min: 42-100% B. After each run, the system was reconditioned for 10 min before analysis of the next sample. The detection was carried out at 280 nm for hydroxybenzoic acid derivatives and flavanols, at 320 nm for hydroxycinnamic acid derivatives and 360 nm for flavonols. Qualitative analysis was performed by retention time while quantitative study by measuring the peak areas [28].

2.8. Statistical analysis

The data obtained were analyzed as mean±SD of three parallel determinations. These means were analyzed by one way analysis of variance (ANOVA) using Minitab 2000 version 13.2 (Minitab Inc. Pennsyivania, USA) [29].

3. Results and Discussions

3.1. Extract yield

Extraction is most important process to evaluate natural bioactive phytochemicals from plant tissues. These phytochemicals may serve as antibacterial, antioxidant and anticancerous agents. It has been found that 100% methanol extract showed highest extract yield (65.44±0.70%) for *S. aromaticum* among non-hydrolyzed extraction (Table 2). The variation in extract yield relates to the variation in polarities of extracting solvents [30]. Among hydrolyzed extraction, extract yield linearly increases with increasing concentration of extracting agent (70.14±0.17% by 1.0M HCl and 75.98±0.31% by 1M NaOH), because high concentration of acid and alkali release more and more bound bioactive compounds from cellular organelles.

3.2. Total phenolic contents

One of the largest class of natural antioxidants constitute by phenolic compounds. The flavor, taste, color and texture of plants are mainly controlled by their phenolic contents. These phenolic compounds combat free radicals which may trigger chain reactions that lead to numerous degenerative diseases. Thus, such phytochemicals serve as potent antioxidants, antimutagenic and genoprotective agents [31]. *S. aromaticum* have been supposed to contain high phenolic contents as shown by its strong antioxidant potential. Therefore, bioactivities of *S. aromaticum* which

include free radical scavenging, inhibition of hydrolytic and oxidative enzymes, anti-inflammatory action, reduction in blood lipid and glucose levels and to boost human immunity are associated with high phenolic content of this specie [32].

Table 2 summarizes the comparison of phenolic contents of different extracts of *S. aromaticum*. It was found that 100% methanolic extract exhibited highest phenolic contents (92.40±1.09 mg GAE/g extract) superior to 70% methanolic extract of *S. aromaticum* (81.04±0.78 mg GAE/g extract) as found by Sultana et al. [18]. Among hydrolyzed extraction, alkaline hydrolysis released more phenolic compounds from plant tissues as shown by high phenolic contents (131.33±0.59 mg GAE/g extract) of alkaline hydrolysis as compared to acidic hydrolysis (112.60±1.1 mg GAE/g extract) as well as reported by Sultana et al., (2014) using 1.0*N* acidified methanol (115.33±2.98 mg GAE/g extract). Increasing concentration of extractants in the solvents used, has amplifying effect on phenolic contents.

3.3. Free radical scavenging activity

DPPH free radical scavenging potential is an important assay to evaluate antioxidant potential of a plant extract. Free radicals are produced by oxidation, which cause lot of damage in living organisms. Bioactive compounds of plant origin were capable to capture these radicals were examined in this assay. 1,1-Diphenyl picryl hydrazyl a stable free radical, is violet in color. The plant extract act as antioxidant and neutralize it to a yellow-colored non-radical molecule i.e. diphenylpicryl hydrazine. The antioxidant species present in plant extract donate a proton or accept an electron from free radical to achieve such inhibition of free radicals [33-35].

Table 3 demonstrates the results for antioxidant activities of different *S. aromaticum* extracts by DPPH assay. It was found that 100% methanol extract of *S. aromaticum* exhibited highest free radical scavenging (67.15±1.93%) amongst non-hydrolyzed extraction while hydrolyzed extraction carried out using 1.0*N* NaOH exhibited highest free radical scavenging (79.47±1.71%) activity which was found nearly comparable to that of 50% acetone extract of clove stem (79.50%) as determined by Mashkor [36], thus showing that alkaline hydrolysis yielded highest number of antioxidants from *S. aromaticum* tissues.

The concentration of plant extracts required to scavenge 50% of free radicals i.e. IC_{50} values were also evaluated. Again, 100% methanolic extract showed maximum DPPH scavenging with lowest IC_{50} (35.1±0.24 µg/mL) among non-hydrolyzed extracts which was quite comparable to that of *S. aromaticum* buds (34.10 µg/mL) reported by Li et al. [37]. Acidified hydrolyzed extraction exhibited intermediate antiradical potential with intermediate value of IC_{50} (33.02±0.94 µg/mL) while highest radical combating potential was represented by alkaline hydrolyzed extraction with minimum IC_{50} (31.17±2.01 µg/mL).

3.4. Reducing power assay

Reducing power assay involves the reduction of ferric ions by phytochemicals present in plant extract to ferrous state which accompanied by a change in color of the reaction mixture that can be quantified spectrometrically. The increase in absorbance, in this assay, indicates an increase in reducing power. The phenolic compounds present in plant extracts serve as reducing agent and help to reduce Fe^{3+} to Fe^{2+} state. A standard reducing agent used as positive standard, is ascorbic acid. However, many phenolic compounds present in different spices are even more potent reducing agents compared to ascorbic acid [38].

Table 3 also presents the results for antioxidant activity of different *S. aromaticum* extracts in terms of their reducing power. It was found that hydrolyzed extraction by 1.0*N* NaOH exhibited highest reducing power (69.69±1.13 mg AAE/g extract) followed by acidic hydrolyzed extraction (66.34±1.24 mg AAE/g extract). Among non-hydrolyzed extraction, 100% methanolic extract exhibited maximum value of reducing power (62.55±2.49 mg AAE/g extract) as it extracted highest number of phenolic contents.

3.5. Cytoprotectivity

Cytotoxic effects of all *S. aromaticum* extracts were determined in terms of haemolytic potential, in order to carry out screening prior to cytoprotective activity. Many plants contain numerous beneficial medicinal compounds, but sometimes harmful compounds such as saponins and some toxic metabolites also present in plant extracts. Therefore such plants extracts, cannot be recommended for pharmacological applications, in spite of high concentration of therapeutic components. The toxic compounds destroy various lipid molecules present in cell membrane, thereby rupturing the cell membrane hence destroying erythrocytes and other cells in living organisms. Smaller percentage heamolytic activity means lesser the quantity of toxic chemicals present in plant extracts. Thus plant extracts having high %age haemolysis cannot be used in medicinal formulations [39-42]. It was found that all extracts of *S. aromaticum* showed very low cytotoxicity (less than 4%) as compared to H_2O_2 in haemolytic assay.

Thus, inhibitory action of all extracts against H_2O_2-generated cytotoxicity has been evaluated and presented in Table 4. Both types of hydrolyzed extracts presented higher anti-haemolytic potential (90.28±1.58%) than non-hydrolyzed extracts (68.28±2.00%). Acidified hydrolyzed extracts showed cytoprotective activity lesser than those of alkaline-hydrolyzed extracts which presented quite comparable protection against cytotoxiciy to standard antioxidants i.e. ascorbic acid (90.92±2.12%) and gallic acid (87.22±2.24%).

For all extracts, the half maximal inhibitory concentration (IC_{50}) was also calculated. Actually, it is the concentration (µg/mL) of plant extract required to inhibit the 50% haemolysis of RBCs. Alkaline-hydrolyzed extracts exhibited lower IC_{50} (58.44±0.84 µg/mL) than those of acidic-hydrolyzed (102.95±1.22 µg/mL) and non-hydrolyzed extracts (138.42±1.56 µg/mL) showing higher anti-haemolytic potential of hydrolyzed extracts, which is quite close to reference standards i.e. Ascorbic acid (56.50±0.56µg/mL) and gallic acid (66.45±1.46µg/mL).

3.6. Genoprotective activity

All *S. aromaticum* were further evaluated for their genoprotective effects. H_2O_2 in the presence of ultra-violet radiation can generate $^{\cdot}OH$ that is able to produce variety of free radical species through propagation, resulting in the interruption of DNA structure. Primarily $^{\cdot}OH$ radical reacts with DNA backbone (nitrogenous bases) thus, opening the super-coiled arrangement of DNA into open circular or linear forms [43].

Table 1. Set-up of Ames' test for mutagenic and anti-mutagenic assay.

Plate No.	Treatment	Volume added (mL)				
		Mutagen Standard	Herbal extract	Reagent mixture	Deionized Water	*Salmonella* test strain
1	Blank	___	___	2.5	17.5	___
2	Background	___	___	2.5	17.5	0.005
3	Standard Mutagens	0.1	___	2.5	17.4	0.005
4	Samples for Mutagenic Test	___	0.005	2.5	17.5	0.005
5	Samples for Antimutagenic Test	0.1	0.005	2.5	17.4	0.005

Table 2. Extract yield (%age) and total phenolic contents (mg GAE/g extract) of all *S. aromaticum* extracts.

Sr. No.	Extraction Mode	Solvent concentration	Extract Yield[A]	TPC[B]
1	Non-Hydrolyzed	50% Methanol	33.03±0.11 ^{de}	45.70±1.05 ^{e}
		70% Methanol	52.44±0.21 ^{c}	78.60±1.09 ^{de}
		100% Methanol	65.44±0.70 ^{b}	92.40±1.09 ^{c}
2	Acidic-Hydrolyzed	0.1*M* HCl	28.99±0.30 ^{e}	44.6±1.10 ^{e}
		0.5*M* HCl	48.13±0.26 ^{cd}	98.7±1.10 ^{c}
		1.0*M* HCl	70.14±0.17 ^{ab}	112.60±1.1 ^{b}
3	Alkaline-Hydrolyzed	0.1*M* NaOH	29.75±0.15 ^{e}	51.21±1.08 ^{e}
		0.5*M* NaOH	53.25±0.22 ^{c}	96.03±1.62 ^{b}
		1.0*M* NaOH	75.98±0.31 ^{a}	131.33±0.6 ^{a}

Values are mean ± SD of three replicates.
[A] % (w/w dry biomass).
[B]Total phenolic contents expressed as mg gallic acid equivalents (GAE)g^{-1} dry extract.

Table 3. DPPH scavenging potential (%age) and reducing power (mg AAE/g extract) of all *S. aromaticum* extracts.

Sr. No.	Extraction Mode	Solvent concentration	DPPH Scavenging[A]	IC_{50}[B]	Reducing Power[C]
1	Non-Hydrolyzed	50% Methanol	52.18±2.01 ^{c}	41.4±1.04 ^{d}	41.85±1.15 ^{d}
		70% Methanol	59.28±1.90 ^{b}	38.3±1.24 ^{c}	48.88±1.83 ^{c}
		100% Methanol	67.15±1.93 ^{a}	35.1±0.24 ^{b}	62.55±2.49 ^{b}
2	Acidic-Hydrolyzed	0.1*M* HCl	44.28±1.76 ^{d}	42.02±1.07 ^{d}	40.28±1.20 ^{d}
		0.5*M* HCl	59.40±1.80 ^{b}	36.23±1.24 ^{bc}	51.25±0.82 ^{b}
		1.0*M* HCl	72.31±1.86 ^{ab}	33.02±0.94 ^{a}	66.34±1.24 ^{a}
3	Alkaline-Hydrolyzed	0.1*M* NaOH	43.27±2.16 ^{c}	41.02±1.16 ^{d}	41.59±1.44 ^{d}
		0.5*M* NaOH	60.23±1.14 ^{b}	35.23±2.12 ^{b}	52.31±1.24 ^{b}
		1.0*M* NaOH	79.47±1.71 ^{a}	31.17±2.01 ^{a}	69.69±1.13 ^{a}

Values are mean ± SD of three replicates.
[A] % scavenging
[B]µg/mL
[C]Reducing Power expressed as mg ascorbic acid equivalents (AAE)g^{-1} dry extract.

Table 4. Anti-haemolytic (%, IC_{50}) and genoprotective activity (%) of optimized variety of *S. aromaticum* extracts.

Extraction Mode	Solvent Concentration	Anti-haemolytic Activity			Genoprotective Activity			
					TA 98		TA 100	
		% Inhibition	IC_{50} (µg/mL)		Mutants	% Genoprotectivity	Mutants	% Genoprotectivity
				Background	8	---	12	---
				$K_2Cr_2O_7$	92	---	---	---
				NaN_3	---	---	94	---
Non-Hydrolyzed	50% Methanol	62.21±1.78^{d}	221.44±2.24^{e}		37^{d}	65.48±1.37^{c}	43	62.20±1.22^{de}
	70% Methanol	64.58±2.04^{d}	168.46±2.78^{e}		33^{c}	70.24±0.69^{b}	40	65.85±1.86^{d}
	100% Methanol	68.28±2.00^{c}	138.42±1.56^{d}		32^{b}	71.43±1.37^{ab}	37	69.51±1.22^{c}
Acidic-Hydrolyzed	0.1*M* HCl	78.59±1.56^{b}	105.45±3.2^{cd}		34^{c}	69.05±0.69^{b}	38	68.29±1.41^{c}
	0.5*M* HCl	79.78±1.88^{ab}	103.65±2.45^{c}		31^{b}	72.62±0.69^{ab}	35	71.95±1.05^{b}
	1.0*M* HCl	79.98±1.42^{ab}	102.95±1.22^{c}		30^{b}	73.81±0.69^{ab}	33	74.39±0.78^{b}
Alkaline-Hydrolyzed	0.1*M* NaOH	82.21±1.19a^{b}	86.45±1.22^{bc}		31^{b}	72.62±0.98^{ab}	35	71.95±1.22^{b}
	0.5*M* NaOH	84.58±1.42^{a}	78.14±0.98^{b}		29^{ab}	75.0±0.97^{a}	32	75.61±0.78^{b}
	1.0*M* NaOH	90.28±1.58^{a}	58.44±0.84^{a}		26^{a}	78.57±1.38^{a}	29	79.27±1.48^{a}
Ascorbic Acid		90.92±2.12^{a}	56.50±0.56^{a}		25^{a}	79.76±0.97^{a}	28	80.49±0.66^{a}
Gallic Acid		87.22±2.24^{a}	66.45±1.46^{a}		27^{a}	77.38±1.36^{a}	30	78.05±0.61^{a}

Values are mean ± SD of three replicates.

Different letters in each column represent significant differences ($p \leq 0.05$).

IC_{50} values were calculated by linear regression analysis.

The antimutagenic effect was considered as 'strong' when the percentage of mutagenic inhibition was more than 40%, moderate' when the percentage of inhibition was 25 – 40% and 'weak' when percentage of inhibition of mutagenecity was less than 25%.

Table 5. HPLC profile of bioactive compounds of Alkaline-hydrolyzed extract of *S. aromaticum*.

Peak No.	RT(min)	Proposed Compounds	Molecular Formula	Concentration (µg/mL)
1	2.273	Ethanol	C_2H_6O	2.1352
2	2.847	Quercetin	$C_{15}H_{10}O_7$	17.9756
3	3.887	Unknown	_____	_____
4	4.813	Gallic acid	$C_7H_6O_5$	21.2183
5	5.833	Unknown	_____	_____
6	6.167	Ascorbic acid	$C_6H_8O_6$	34.1913
7	7.667	Unknown	_____	_____
8	10.487	Unknown	_____	_____
9	11.787	Unknown	_____	_____
10	12.633	Caffeic acid	$C_9H_8O_4$	19.4182
11	14.487	Benzoic acid	$C_7H_6O_2$	12.6924
12	18.533	Catechin	$C_{15}H_{14}O_6$	27.1713
13	20.973	Chrysin	$C_{15}H_{10}O_4$	11.1925
14	25.693	Cinamic acid	$C_9H_8O_4$	3.5316
15	27.253	Unknown	_____	_____
16	30.100	Orientin	$C_{21}H_{20}O_{11}$	80.1413
17	31.593	Chebulic acid	$C_{14}H_{12}O_{11}$	30.1342
18	37.207	Rosmeric acid	$C_{18}H_{16}O_8$	40.1281
19	51.627	Gallocatechin	$C_{15}H_{14}O_7$	22.1912
20	57.520	Chicoric acid	$C_{22}H_{18}O_{12}$	25.1231

At first, all clove extracts were screened by genotoxicity assay to investigate their mutagenic potential. All extracts exhibited quite low values of %age mutagenicity (6.0 – 9.0%) quite comparable to the literature report of Grozdanova et al. [44] for natural deep eutectic solvents (NADES) based extracts of *Sideritis scardica* and *Plantago major*. When all extracts were investigated for their genoprotective activity, similar results were obtained as for other biological activities. Genoprotective potential was observed in the order:

Alkaline-hydrolyzed>Acid-hydrolyzed>non-hydrolyzed

Alkaline-hydrolyzed extracts presented highest percentage gene protection (78.57±1.38%) which is quite comparable to standard reference compounds, gallic acid (77.38±1.36%) and ascorbic acid (79.76±0.97%). Acid-hydrolyzed extracts also exhibited considerable high genoprotection (73.81±0.69%), which is quite close to already published results of Pavlović et al. [45] for anti-genotoxic effects of ethanolic extracts of *Ocimum basilicum* and *Rosmarinus officinalis* against *Salmonella typhimurium* TA1535 and genoprotective activity on DNA plasmid. All extracts exhibited almost comparable genoprotection against both selected bacterial strains (TA 98 and TA100).

3.7. Chemical characterization

Reverse phase HPLC analysis with gradient elution was applied for chemical characterization of bioactive phytochemicals present in alkaline-hydrolyzed extracts of *S. aromaticum.* Chromatogram showed eighteen peaks; fourteen of them were identified with by using reference standard compounds, belonging to phenolic acids and flavonoids and tabulated in Table 5.

3.7.1. Phenolic acids

Some of identified peaks represent phenolic acids, i.e. peak 4 represented gallic acid; peak 10 stood for caffeic acid, a hydroxycinnamic acid; peak 11 showed benzoic acid while peak 14 for cinnamic acid. Some of larger phenolic acids were also identified. The Peaks 17, 18, 20 showed chebulic acid, rosmeric acid and chicoric acid, while peak 19 represented gallocatechin, a conjugate of gallic acid and catechin. Quantitative analysis represented rosemeric acid as most abundant (40.1281 µg/mL) among phenolic acids whereas cinnamic acid at lowest concentration (3.5316 µg/mL).

3.7.2. Flavonoids

Peak 2 represented quercetin, a flavonol, peak 12 showed catechin, a flavan-3-ol, peak 13 indicated chrysisn, a flavone; while peak 16 exhibited orientin, a water-soluble flavonoid. Quantitative analysis represented that orientin is highly abundant (80.1413 µg/mL) among flavonoids while chrysin at lowest concentration (11.1925 µg/mL).

4. Conclusions

The current project was aimed to explore the bioactive compounds of *S. aromaticum* responsible for its outstanding biological activities. Extraction of clove buds was carried out by hydrolyzed extraction with HCl and NaOH and non-hydrolyzed extraction with methanol. Bioactivities such as antioxidant potential, cytoprotective and genoprotective activities of all *S. aromaticum* extracts were investigated. Clove extracts expressed high values of DPPH scavenging and reducing powers along with considerable potential of protecting genetic make-up and living cells against induced oxidative damage to human erythrocytes. Phytochemical characterization by RP-HPLC exhibited the presence of thirteen bioactive compounds present in variable concentrations in hydrolyzed extracts of this spice, which are responsible for pronounced bioactivities of its extracts.

References

[1] J. Živković, M. Ilić, K. Šavikin, G. Zdunić, A. Ilić, D. Stojković. (2020). Traditional use of medicinal plants in South-Eastern Serbia (Pčinja District): Ethnopharmacological investigation on the current status and comparison with half a century old data. Frontiers in Pharmacology. 11: 1020.

[2] B. Akbari, N. Baghaei-Yazdi, M. Bahmaie, F. Mahdavi Abhari. (2022). The role of plant-derived natural antioxidants in reduction of oxidative stress. BioFactors. 48(3): 611-633.

[3] R. Kaur, A.K. Nagpal, J.K. Katnoria. Antimutagenic potential of pollen grains of some medicinal plant species. Internaional Journal of Pharmacy and Pharmaceutical Sciences. 8(7): 232-236.

[4] S. Bajwa. Potential antimicrobial, anti-glycation and antioxidant properties of bioactive compounds of Indian spices and herbs extracted using ultrasound-assisted extraction process. Manchester Metropolitan University, 2020.

[5] T. Wankhede. (2015). Evaluation of antioxidant and antimicrobial activity of the Indian clove Syzygium aromaticum L. Merr. and Perr. Int Res J Sci Eng. 3(4): 166-172.

[6] B. Basgedik, A. Ugur, N. Sarac. (2014). Antimicrobial, antioxidant, and antimutagenic activities of Gladiolus illyricus. Journal of Pharmacy & Pharmacognosy Research. 2(4): 93-99.

[7] M.A. Cardoso, H.M. Gonçalves, F. Davis. (2023). Reactive oxygen species in biological media are they friend or foe? Major In vivo and In vitro sensing challenges. Talanta. 260: 124648.

[8] K. Jomova, R. Raptova, S.Y. Alomar, S.H. Alwasel, E. Nepovimova, K. Kuca, M. Valko. (2023). Reactive oxygen species, toxicity, oxidative stress, and antioxidants: Chronic diseases and aging. Archives of toxicology. 97(10): 2499-2574.

[9] E.O. Olufunmilayo, M.B. Gerke-Duncan, R.D. Holsinger. (2023). Oxidative stress and antioxidants in neurodegenerative disorders. Antioxidants. 12(2): 517.

[10] Z. Xiang, Q. Xue, P. Gao, H. Yu, M. Wu, Z. Zhao, Y. Li, S. Wang, J. Zhang, L. Dai. (2023). Antioxidant peptides from edible aquatic animals: Preparation method, mechanism of action, and structure-activity relationships. Food chemistry. 404: 134701.

[11] B.R. Silva, J.R. Silva. (2023). Mechanisms of action of non-enzymatic antioxidants to control oxidative stress during in vitro follicle growth, oocyte maturation, and embryo development. Animal reproduction science. 249: 107186.

[12] T. Adebayo. (2023). antibacterial activities of the leaf extracts of *Syzygium aromaticum, Nelsonia*

canescens and Parkia biglobosa on Staphylococcus aureus and Streptococcus pyogenes. repository.futminna.edu.ng

[13] M. Dhanislas, S. Sampath, M. Shamya, J. Joseph, M. Yasasve, M.Z. Ahmed, A.S. Alqahtani, S. Kazmi, P. Asaithambi, A. Suresh. (2023). Green synthesis of biofabricated silver nanoparticles from Syzygium aromaticum seeds: spectral characterization and evaluation of its anti-mycobacterial activity, cytotoxicity assessment on zebrafish embryo and Artemia salina. Materials Technology. 38(1): 2269358.

[14] D. Anita, S. Avtar, M. Ritu. (2015). Antioxidants of clove (Syzygium aromaticum) prevent metal induced oxidative damage of biomolecules. International Research Journal of Pharmacy. 6(4): 273-278.

[15] A. Valizadeh, A.A. Khaleghi, H. Alipanah, E. Zarenezhad, M. Osanloo. (2021). Anticarcinogenic effect of chitosan nanoparticles containing syzygium aromaticum essential oil or eugenol toward breast and skin cancer cell lines. BioNanoScience. 11(3): 678-686.

[16] A.A.-F. Mostafa, M.T. Yassin, A.A. Al–Askar, F.O. Al-Otibi. (2023). Phytochemical analysis, antiproliferative and antifungal activities of different Syzygium aromaticum solvent extracts. Journal of King Saud University-Science. 35(1): 102362.

[17] P.C. Frohlich, K.A. Santos, J. Ascari, J.R. dos Santos Refati, F. Palú, L. Cardozo-Filho, E.A. da Silva. (2023). Antioxidant compounds and eugenol quantification of clove (Syzygium aromaticum) leaves extracts obtained by pressurized liquid extraction and supercritical fluid extraction. The Journal of Supercritical Fluids. 196: 105865.

[18] B. Sultana, F. Anwar, M. Mushtaq, M. Aslam, S. Ijaz. (2014). In vitro antimutagenic, antioxidant activities and total phenolics of clove (Syzygium aromaticum L.) seed extracts. Pakistan journal of pharmaceutical sciences. 27(4).

[19] M.A. Johari, H.Y. Khong. (2019). Total phenolic content and antioxidant and antibacterial activities of Pereskia bleo. Advances in Pharmacological and Pharmaceutical Sciences. 2019.

[20] M. Safari, S. Ahmady-Asbchin. (2019). Evaluation of antioxidant and antibacterial activities of methanolic extract of medlar (Mespilus germanica L.) leaves. Biotechnology & biotechnological equipment. 33(1): 372-378.

[21] S. Khatun. Analysis of nutritional composition, in vitro antioxidant and antidiabetic effect of three chenopodium species. Chattogram Veterinary and Animal Sciences University Chattogram-4225, Bangladesh, 2020.

[22] S. Viturat, M. Thongngam, N. Lumdubwong, W. Zhou, U. Klinkesorn. (2023). Ultrasound-assisted formation of chitosan-glucose Maillard reaction products to fabricate nanoparticles with enhanced antioxidant activity. Ultrasonics Sonochemistry. 97: 106466.

[23] S. Aichour, C. Mouffouk, M. Benkhaled, H. Haba. (2023). Phytochemical composition, antioxidant, cytotoxic, haemolytic and antibacterial activities of aerial parts of Rhamnus alaternus. Herba Polonica. 69(3): 15-28.

[24] H. Ayed, R. Zebsa, R. Merabet, M. Bouacha, S. Benosmane, M. Hamdikene, N.E.H. Harrez, O. Khaled, I. Maalala, B. Khanich. (2024). Phytochemical analyses and in vitro evaluation of antioxidant, antihemolytic and antibacterial activity of methanolic extract of aerial parts of marjoram (Origanum majorana L). International Journal of Environmental Studies. 1-18.

[25] A.A.S.A. Gupta, A. Rawat, T. Parihar, N. Pandey, V. Kumar. Relative Phenolic Profile, ROS Scavenging, and Anti-haemolytic Potential of Polarity-Driven Peel Bioactive Compounds of Distinct Malus species Indigenous to Kashmir.

[26] E. Zeiger. (2023). Determination of a positive response in the Ames Salmonella mutagenicity assay. Environmental and Molecular Mutagenesis. 64(4): 250-258.

[27] D.N. Large, N.A. Van Doorn, S.C. Timmons. (2023). Cancer and chemicals: A research-inspired laboratory exercise based on the Ames test for mutagenicity. Biochemistry and Molecular Biology Education. 51(1): 103-113.

[28] A. Yousif, W. Hassan. (2023). HPLC analysis and antifungal activity of some plant extracts against decay apple fruits. Iraqi Journal of Agricultural Sciences. 54(1): 291-302.

[29] M. Fouad, M. Badawy, A. El-Aswad, M. Aly. (2023). Experimental modeling design to study the effect of different soil treatments on the dissipation of metribuzin herbicide with effect on dehydrogenase activity. Current Chemistry Letters. 12(2): 383-396.

[30] M. Yasir, B. Sultana, M. Amicucci. (2016). Biological activities of phenolic compounds extracted from Amaranthaceae plants and their LC/ESI-MS/MS profiling. Journal of functional foods. 26: 645-656.

[31] S. Losada-Barreiro, C. Bravo-Diaz. (2017). Free radicals and polyphenols: The redox chemistry of neurodegenerative diseases. European journal of medicinal chemistry. 133: 379-402.

[32] I.A.M. Ahmed, E.E. Babiker, F.Y. Al-Juhaimi, A.E.-D.A. Bekhit. (2022). Clove polyphenolic compounds improve the microbiological status, lipid stability, and sensory attributes of beef burgers during cold storage. Antioxidants. 11(7): 1354.

[33] J. Flieger, M. Flieger. (2020). The [DPPH●/DPPH-H]-HPLC-DAD method on tracking the antioxidant activity of pure antioxidants and goutweed (Aegopodium podagraria L.) hydroalcoholic extracts. Molecules. 25(24): 6005.

[34] V. Nurmazela, R. Ridwanto, Z. Rani. (2022). Antioxidant activity test of Barangan Banana Hump's ethanol extract (Musa Paradisiaca (L.)) with DPPH (1, 1 Diphenyl-2-Picrylhydrazyl) method. International Journal of Science, Technology & Management. 3(5): 1478-1483.

[35] J. Rumpf, R. Burger, M. Schulze. (2023). Statistical evaluation of DPPH, ABTS, FRAP, and Folin-Ciocalteu assays to assess the antioxidant capacity

of lignins. International Journal of Biological Macromolecules. 233: 123470.

[36] I. Mashkor. (2015). Evaluation of antioxidant activity of clove (Syzygium aromaticum). Int. J. Chem. Sci. 13(1): 22-30.

[37] X. Li, J. Xu. (2016). Chemical composition and antioxidant activities of the oleoresin from clove buds (). Eur. J. Food Sci. Technol. 4: 16-24.

[38] D. Cvitković, P. Lisica, Z. Zorić, M. Repajić, S. Pedisić, V. Dragović-Uzelac, S. Balbino. (2021). Composition and antioxidant properties of pigments of mediterranean herbs and spices as affected by different extraction methods. Foods. 10(10): 2477.

[39] R.R.R. Kannan, R. Arumugam, P. Iyapparaj, T. Thangaradjou, P. Anantharaman. (2013). In vitro antibacterial, cytotoxicity and haemolytic activities and phytochemical analysis of seagrasses from the Gulf of Mannar, South India. Food chemistry. 136(3-4): 1484-1489.

[40] Z. Liu, W. Gao, S. Jing, Y. Zhang, S. Man, Y. Wang, J. Zhang, C. Liu. (2013). Correlation among cytotoxicity, hemolytic activity and the composition of steroidal saponins from Paris L. Journal of ethnopharmacology. 149(2): 422-430.

[41] M. Zubair, K. Rizwan, U. Rashid, R. Saeed, A.A. Saeed, N. Rasool, M. Riaz. (2017). GC/MS profiling, in vitro antioxidant, antimicrobial and haemolytic activities of Smilax macrophylla leaves. Arabian Journal of Chemistry. 10: S1460-S1468.

[42] S. Jaiswal, N. Mansa, M.P. Prasad, B.S. Jena, P.S. Negi. (2014). Antibacterial and antimutagenic activities of Dillenia indica extracts. Food Bioscience. 5: 47-53.

[43] N. Sharma, R. Bhardwaj, S. Kumar, S. Kaur. (2011). Evaluation of Bauhinia variegata L. bark fractions for in vitro antioxidant potential and protective effect against H2O2-induced oxidative damage to pBR322 DNA. Afr J Pharm Pharmacol. 5(12): 1494-1500.

[44] T. Grozdanova, B. Trusheva, K. Alipieva, M. Popova, L. Dimitrova, H. Najdenski, M.M. Zaharieva, Y. Ilieva, B. Vasileva, G. Miloshev. (2020). Extracts of medicinal plants with natural deep eutectic solvents: Enhanced antimicrobial activity and low genotoxicity. BMC chemistry. 14: 1-9.

[45] M. Oalđe Pavlović, S. Kolarević, J. Đorđević, J. Jovanović Marić, T. Lunić, M. Mandić, M. Kračun Kolarević, J. Živković, A. Alimpić Aradski, P.D. Marin. (2021). A study of phytochemistry, genoprotective activity, and antitumor effects of extracts of the selected Lamiaceae species. Plants. 10(11): 2306.