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INVESTIGATING THE FREE RADICAL SCAVENGING AND ACETYLCHOLINESTERASE INHIBITION ACTIVITIES OF *ELLETARIA CARDAMOMUM, PIPER NIGRUM* AND *SYZYGIUM AROMATICUM*

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ABSTRACT: Spices have been used since long to enhance the flavor and aroma of our foods. Besides, they also produce several medicinal effects and are used in treating various clinical ailments. The different phytochemicals present greatly influence the biological activities possessed by plants/spices. The present research work investigates the free radical scavenging and acetylcholinesterase (AchE) inhibition activity of different extracts of *Elletaria cardamomum, Piper nigrum* and *Syzygium aromaticum*. Total phenol, flavonoid, condensed tannins and saponin contents were also measured. Among the different extracts of spices evaluated for DPPH free radical scavenging, ethanolic extract of *S. aromaticum* exhibited the highest inhibition with IC_{50} value of 42±7.4 µg/ml. This high radical scavenging activity can be directly correlated with the presence of high total phenolic content (310±6.87 mg GAEs/g extract) possessed by the extract. The ethanolic extracts of all three spices had shown better inhibition activity against AChE than other extracts. At 500 µg/mL concentration, *S. aromaticum* and *E. cardamomum* ethanolic extracts showed 52.22±3.7 % and 52.95±2.9 % AchE inhibition respectively. These findings suggest that these spices could act as an anticholinesterase agent and also are an efficient free radical scavenger, which may be helpful in preventing or alleviating patients suffering from Alzheimer's disease.

INTRODUCTION: The foods containing antimutagenic, antibacterial, antiviral and anti-inflammatory compounds have increasingly been gaining importance since numerous studies have proved strong protective effects of these novel phytochemicals against many diseases [1, 2].

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Groups of secondary plant metabolites, antioxidant phenolics, and flavonoids are commonly found in various fruits, vegetables and herbs and they have been shown to provide a fruitful defence against oxidative stress from oxidizing agents and free radicals [3, 4, 5]. Oxidative stress is often defined as an imbalance between free radicals and antioxidant defense system. These radicals contribute to the development of many diseases (cancer, Alzheimer and diabetes mellitus etc.) and can be neutralized by antioxidants [6]. Due to toxic effects of synthetic antioxidants; [7] recent studies are focusing on their replacement with naturally occurring antioxidants derived from medicinal plants.

Alzheimer's disease (AD) is the most common form of neurodegenerative disease and characterized by memory dysfunction. Oxidative stress has been considered a mechanism involved in the pathogenesis of AD, and it has also played a major role in the aging process [8, 9]. Another mechanism is reduction of acetylcholine levels in the brain, which is the most notable biochemical change in AD [10]. Thus, cholinesterase inhibition has been one of the mainstays for treatment of AD and is considered to be a promising strategy for dementia therapy. Most of the drugs approved and licensed for the disease can cause undesirable side effects and they are largely ineffective for treating severe AD cases. Therefore, it is compulsory to search for new anti-AD drug candidates. There have been a large number of examples, which have repeatedly pointed to the need of expanding the exploration of Nature as a source of bioactive compounds that may serve as the leads, or scaffolds for further chemical elaboration [11, 12].

Spices and/or oilseeds are an important category of food that has been used since long to enhance the taste and aroma of foods. Besides imparting characteristic flavor and color to foods, they also produce several medicinal effects and hence are used in several indigenous systems of medicines [13, 14]. Few spices and oilseeds have been shown to impart several beneficial effects of which the antioxidant effect is most pronounced [15, 16]. It has been found that spices have higher antioxidant activity as compared to fruits, cereals and nuts. The active components in spices phthalides, polyacetylones, phenolic acids, flavonoids, coumarins and terpenes are reported as powerful antioxidants [17].

This study aims to evaluate the antioxidant and acetylcholinesterase inhibitory effect of different extracts obtained from three commonly used spices namely *Elletaria cardamomum, Piper nigrum* and *Syzygium aromaticum*, as well as their phenolic, flavonoid and saponin contents. Thus, the present study might supply valuable information on the phytochemical properties of these spices for nutraceutical and pharmacological industry.

MATERIALS AND METHODS:

Materials: Potassium ferricyanide, ferric chloride, Folin–Ciocalteu's reagent, trichloroacetic acid, methanol, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and methanol were purchased from Merck (Darmstadt, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), b-carotene, linoleic acid and Tween 40 were purchased from Sigma Chemical Co. (Sigma–Aldrich GmbH, Sternheim, Germany. All the standard phyto-constituents were purchased from Merck, Germany and Sigma Chemicals, USA, respectively and the solvents used were of analytical grade.

Plant materials and extraction procedure: The seeds of different plants *viz E. cardamomum*, *P. nigrum*, *S. aromaticum*, were purchased from local market in Delhi, India. All the plant materials were identified and contaminated particles were removed. The air-dried plant materials were powdered and used for extraction. Extracts were prepared using four different solvents with increasing polarity – Hexane, dichloromethane, ethanol and water. 50 g of each powdered spice was extracted/stirred in 500 ml of the solvent for 24 h with intermittent shaking. Each extracted material was vacuum filtered using Whatman filter paper and then the solvent was evaporated using rotary evaporator.

Determination of total bioactive components:

Total Phenolic content: The total phenolic content of plant extract was determined using Folin-Ciocalteu reagent [18]. In brief, to 250 µL of Folin-Ciocalteu's reagent, 10 µL of sample was added, followed by 3.5 mL of deionised water. After 3 min, 1 mL of 20% sodium carbonate was added. The mixture was vortexed and incubated at 40 °C for 40 min. It was allowed to cool in the dark. The absorbance was measured at 685 nm and all determinations were carried out in duplicates. A standard curve was obtained using various concentrations of gallic acid. The results were expressed as mg gallic acid equivalent/g dry weight of material.

Total Flavanoid Content: Total flavonoids were measured by $AlCl_3$ colorimetric assay [19]. Briefly, to 500 µL of extract and 2000 µL of distilled water, 5% of sodium nitrate was added. After 5 min, 150 µL of 10% $AlCl_3$ was added. A total of 2 000 µL of sodium hydroxide (1 mol) was added after 1 min followed by 1200 µL of distilled water. The

mixture was vortexed and incubated for 30 min. The absorbance was measured at 510 nm against a prepared blank. A yellow color indicated the presence of flavonoids. Quercetin was used as a standard to generate a standard curve. The total flavonoids content of samples were determined in duplicates and the results were expressed as mg quercetin acid equivalent/g dry weight of material.

Total condensed tannins content: The total condensed tannin content was determined by the vanillin method [20] with slight modification. Sample solution (0.5 mL) was mixed with vanillin reagent (1.5 mL, 1% in 7 M H_2SO_4) in an ice bath and then mixed well. Similarly, a blank was prepared by adding sample solution (0.5 mL) to 7 M H_2SO_4 (1.5 mL). The sample and blank absorbance were read at 500 nm after 15 min incubation at room temperature. The absorbance of the blank was subtracted from that of the sample. The total condensed tannin content was expressed as equivalents of (+)-catechin according to the equation obtained from the standard (+)- catechin graph.

Total Saponins content: The total saponins content was determined by the vanillin–sulfuric acid method [21]. Sample solution (0.25 mL) was mixed with vanillin (0.25 mL, 8%) and sulfuric acid (2 mL, 72%). The mixture was incubated for 10 min at 60◦C. Then the mixture was cooled for another 15 min, followed bythe sample absorbance measurement at 538 nm. The total saponin content was expressed as equivalents of Quillaja according to the equation obtained from the standard Quillaja graph.

Radical scavenging activity using DPPH method: The free-radical scavenging activities of the extracts were tested by their ability to bleach the stable radical DPPH. The antioxidant activity using the DPPH (1, 1-diphenyl-2- picrylhydrazyl) assay was assessed by the method of Blois [22]. The reaction mixture contained 100 μM DPPH in methanol and different concentrations (100-1000 μg/ml) of extracts. Absorbance at 517 nm was determined after 30 min at room temperature and the scavenging activity were calculated as a percentage of the radical reduction. Each experiment was performed in triplicate.

***In vitro* inhibition of AChE**: The inhibitory potency of extracts on AChE was determined using spectroscopic method of Ellman *et al.*, [23] and was expressed as IC50 *i.e*. inhibitory concentration that reduces the cholinesterase activity by 50%. Acetylcholinesterase (AChE, E.C. 3.1.1.7, from electric eel), 5,5'- dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, DTNB), acetylthiocholine iodide (ATC), butyryllthiocholine iodide (BTC), donepezil was purchased from Sigma Aldrich. Stock solutions of extracts were prepared in ethanol and diluted using 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 8.0) to afford a final concentration range between (50-500) μg/ml. Enzyme solutions were prepared by dissolving lyophilized powder in double-distilled water. The assay solution consisted of 1 mL of 0.1 M KH_2PO_4/K_2HPO_4 phosphate buffer, 25μL of AChE (0.22 U/mL) and 100 μL of various concentrations of extracts which was allowed to stand for 5 min before 100 μL of 0.01 M DTNB were added. The reaction was started by addition of 20 μL of the 0.075 M substrate solution (ATC/BTC) and exactly 2 min after substrate addition the absorption was measured at 25 °C at 412 nm. Assays were carried out with a blank containing all components except AChE in order to account for non-enzymatic reaction. Each concentration was analyzed in triplicate at 25°C.

RESULTS AND DISCUSSION:

Total Bioactive Compounds: Total bioactive compounds of *E. cardamomum, P. nigrum, S. aromaticum* extracted with different solvents (Hexane, ethyl acetate, ethanol and water) are presented in **Table 1**. Phytochemicals give plants their colour, flavour, smell and are part of a plant`s natural defense system and protect them against herbivorous insects and vertebrates, fungi, pathogens, and parasites [24]. The phytochemicals alkaloids, coumarins, flavonoids, saponins, steroids, tannins and phenols were present in different amounts in the different extracts of *E. cardamomum, P. nigrum, S. aromaticum.* The results found are in accordance with our previously reported results of phytochemical screening of these spices [25]. Plant materials rich in phenolics content are increasingly being used in the food industry because they retard oxidative degradation of lipids and improve the quality and nutritional value of food [26].

Phenolic compounds are considered secondary metabolites and these phytochemical compounds derived from phenylalanine and tyrosine occur ubiquitously in plants and are diversified [27]. Among the three spices *S. aromaticum* extracts showed highest presence of phenolics. Our results revealed that the ethanolic extract of *S. aromaticum* had higher total phenolic content (310 ± 6.87 mg GAEs/g extract), followed by DCM (242 ± 8.53 ± 9.61 mg GAEs/g extract), water (213 mg ± 4.34 GAEs/g extract) and hexane (90 ± 4.34 mg GAEs/g extract). Other two spice extracts showed variable presence of total phenolics. Among the two spices *P. nigrum* and *E. cardamomum*, aqueous extract of *P. nigrum* showed highest presence of total phenolics with 95 ± 1.56 mg GAEs/g extract.

Phenolic compounds of plants fall into several categories; chief among these are the flavonoids which have potent antioxidant activities [28]. Flavonoids are naturally occurring in plants and are thought to have positive effects on human health. Studies on flavonoidic derivatives have shown a wide range of antibacterial, antiviral, anti-inflammatory, anticancer, and anti-allergic activities [29, 30]. Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals [31] implicated in several diseases. The content of total flavonoids varied from 118 ± 14.3 mg (DCM extract) to 200 ± 3.69 mg (aqueous extract) for *E. cardamomum*, from 303 ± 8.25 (Hexane extract) to 378 ± 18.54 mg (aqueous extract) for *P. nigrum* and from 134 ± 10.3 mg (hexane extract) to 332 ± 10.32 mg (aqueous extract) of rutin equivalents/g extract for *S. aromaticum* (**Table 1**). From our results, it was apparent that the total flavonoid content was dependent on extraction solvents and their polarity. This experimental result is in accordance with previous reports suggesting that a polar solvent system is superior to a non-polar solvent system in the extraction of flavonoids [32].

The total condensed tannin content of the extracts was determined spectrophotometrically and the results are expressed as catechin equivalents (mg CEs/g extract). Among the studied spice extracts, *E. cardamomum* hexane (50 ± 1.23 mg CEs/g extract) and ethanolic (46 ± 3.12 mg CEs/g extract) extracts showed the maximum presence of condensed tannins. Compared to *E. cardamomum* and *P. nigrum*, *S. aromaticum* showed very little presence of condensed tannins in its various extracts.

Saponins are a vast group of glycosides, widely distributed in higher plants. They have pharmacological properties and are used in phytotheraphy. These compounds are believed to from the main constituents of many plant drugs and are reported many pharmacological properties. Total saponins content extracted from all the three spice extracts was found to be lowest in polar solvents. Our results are contrary to the reports in which the total saponins contents were found in greater quantity in polar solvent extracts of different plants [33, 34, 35].

Free radical scavenging activity: The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples [36]. DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen or electron donation. Substances, which are able to perform this reaction, can be considered as antioxidants and therefore radical scavengers [37].

In the present study extracts of three spices were evaluated for their free radical scavenging activity with ascorbic acid as standard compound. The IC_{50} was calculated for each spice extracts as well as ascorbic acid as standard and summarized in **Table 2** and graphically represented in **Fig. 1-3**. Free radical scavenging activity of all the spice extracts was found to be concentration dependent, as the concentration of the test compounds increases, the radical scavenging activity increases and lower IC_{50} value reflects better protective action.

The DPPH radical scavenging activity was affected by solvent used for extraction and are listed here in order from high to low: ethanol > water > ethyl acetate > hexane for *E. cardamomum* and *P. nigrum*. *S. aromaticum* ethanolic extract showed highest free radical inhibition with IC_{50} value of 42 ± 7.4 µg/ml (**Table 2**). This high radical scavenging activity can be directly correlated with the presence of high total phenolic content possessed by the extract. Results of this study

suggest that the spice extracts contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage.

Acetylcholinesterase (AchE) enzyme inhibition: AchE inhibitors are well utilized for the management of mild to moderate Alzheimer's disease, and there are several researchers focused on the search of new AChE inhibitors from the herbal resources [38]. This study examined the anticholinesterase activity of three commonly used Indian spices for the first time. The spice extracts were screened for AChE inhibitory activity using Ellman's colorimetric method in a 96-well plate. The results of AChE inhibitory activities of tested spice extracts were expressed as percentage of inhibition (**Table 3**). The ethanolic extracts had shown better activity against AChE than other extracts. At 500 µg/mL concentration, *S. aromaticum* and *E. cardamomum* ethanolic extracts showed 52.22 ± 3.7 % and 52.95 ± 2.9 % AchE inhibition respectively. While at the 50 µg/mL concentration also these two extracts exhibited 27.6 ± 1.4 % and 38.08 ± 3.6 % inhibition. All three spice extracts exhibited ~42% to ~55% inhibition of the AchE activity, which suggested that these three spices contain bioactive components with AchE inhibitory activity.

TABLE 1: TOTAL PHENOLIC, TANNIN, FLAVONOIDS AND SAPONIN CONTENT OF DIFFERENT EXTRACTS OF *ELLETARIA CARDAMOMUM, PIPER NIGRUM* AND *SYZYGIUM AROMATICUM*

Spices	Extracts	Total Phenol Content (mg GAE g^{-1})[a]	Total Condensed tannin content (mg CE g^{-1})[b]	Total flavonoid content (mg QE g^{-1})[c]	Total saponin content (mg QAE g^{-1})[d]
Elletaria cardamomum	Hexane	78 ± 2.33	50 ± 1.23	164 ± 11.2	204 ± 2.38
	DCM	38 ± 2.13	36 ± 1.78	118 ± 14.3	209 ± 4.12
	EtOH	54 ± 1.67	46 ± 3.12	157 ± 6.73	161 ± 3.23
	Aqueous	32 ± 3.12	3 ± 0.78	200 ± 3.69	24 ± 0.85
Piper nigrum	Hexane	5 ± 0.32	38 ± 1.23	303 ± 8.25	138 ± 4.32
	DCM	50 ± 0.67	23 ± 3.23	339 ± 14.01	97 ± 6.73
	EtOH	40 ± 1.12	24 ± 2.3	305 ± 12.0	105 ± 7.23
	Aqueous	95 ± 1.56	7 ± 1.01	378 ± 18.54	60 ± 1.32
Syzygium aromaticum	Hexane	90 ± 4.34	8 ± 1.67	134 ± 10.3	174 ± 3.94
	DCM	242 ± 8.53	9 ± 0.43	305 ± 8.54	75 ± 2.21
	EtOH	310 ± 6.87	10 ± 0.63	279 ± 11.98	157 ± 4.52
	Aqueous	213 ± 9.61	5 ± 0.98	332 ± 10.32	41 ± 0.29

[a] Total phenolic content expressed as Gallic acid equivalent (mg GAE g^{-1} extract). [b] Total condensed tannin content expressed as Catechin equivalent (mg CE g^{-1} extract). [c] Total flavonoid content expressed as Quercetin equivalent (mg QE g^{-1} extract). [d] Total saponin content was expressed as Quillaja equivalents (mg QAE g^{-1} extract). Values expressed are means ± SD.
DCM – Dichloro methane, EtOH - Ethanol

TABLE 2: IC_{50} VALUE (µG/ML) OF DPPH RADICAL SCAVENGING OF DIFFERENT EXTRACTS OF *ELLETARIA CARDAMOMUM, PIPER NIGRUM* AND *SYZYGIUM AROMATICUM* ON DPPH RADICAL

	Hexane Extract	DCM Extract	Ethanolic Extract	Aqueous Extract
Elletaria cardamomum	806 ± 6.4	909 ± 12.3	772 ± 8.43	101 ± 21.2
Piper nigrum	357 ± 10.9	427 ± 8.3	336 ± 15.64	535 ± 11.3
Syzygium aromaticum	312 ± 20.3	113 ± 10.5	42 ± 7.4	117 ± 18.3

Values are expressed as mean ± SD

TABLE 3: ACETYLCHOLINESTERASE (AChE) INHIBITION (%) OF DIFFERENT EXTRACTS OF *ELLETARIA CARDAMOMUM, PIPER NIGRUM* AND *SYZYGIUM AROMATICUM*

Spices	Extracts	AchE inhibition (%)			
		50 µg/ml	100 µg/ml	250 µg/ml	500 µg/ml
Elletaria cardamomum	Hexane	21.1 ± 1.2	33 ± 2.3	42.4 ± 3.8	44.8 ± 2.5
	DCM	26.1 ± 1.6	29 ± 1.5	45.2 ± 4.2	59.7 ± 5.5
	EtOH	38.0 ± 3.6	42.7 ± 4.1	47.1 ± 1.4	52.9 ± 2.9
	Aqueous	33.2 ± 3.7	39 ± 3.2	45.5 ± 1.6	50.1 ± 5.2
Piper nigrum	Hexane	10.1 ± 2.1	15.9 ± 3.7	23.1 ± 1.8	48.7 ± 3.4
	DCM	18.9 ± 1.3	35.2 ± 3.2	36 ± 4.2	42.3 ± 1.3
	EtOH	24.3 ± 1.7	31.8 ± 1.9	37.2 ± 4.2	46.8 ± 2.7
	Aqueous	29.7 ± 2.4	38.3 ± 3.1	42 ± 6.3	48.8 ± 2.1

	Hexane	24.4 ± 2.5	42.3 ± 3.2	48.5 ± 4.3	49.2 ± 4.9
Syzygium aromaticum	DCM	13.9 ± 1.5	37.5 ± 2.3	42.2 ± 1.6	44.3 ± 4.7
	EtOH	27.6 ± 1.4	48.43 ± 3.2	54.2 ± 1.8	52.2 ± 3.7
	Aqueous	38.2 ± 3.2	43.6 ± 2.3	51.8 ± 4.7	50.6 ± 2.6

Values are expressed as mean ± SD, DCM – Dichloro methane, EtOH - Ethanol

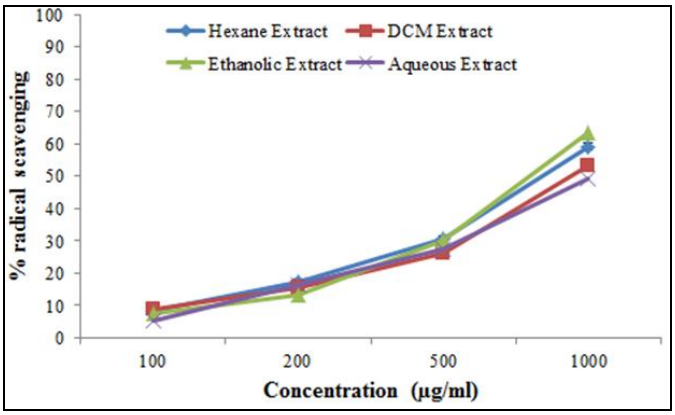

FIG. 1: SCAVENGING ACTIVITY (%) OF DIFFERENT EXTRACTS OF *ELLETARIA CARDAMOMUM*

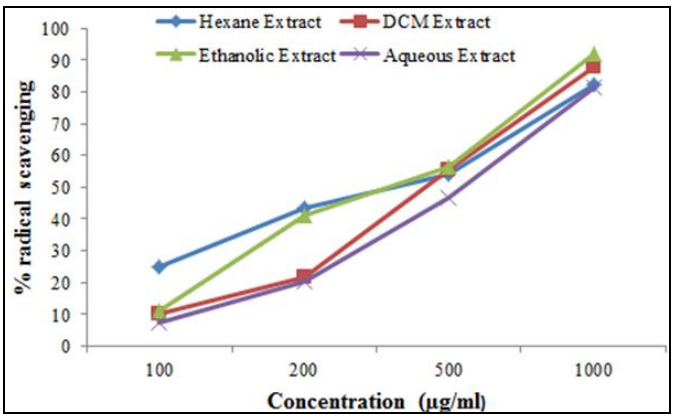

FIG. 2: SCAVENGING ACTIVITY (%) OF DIFFERENT EXTRACTS OF *PIPER NIGRUM*

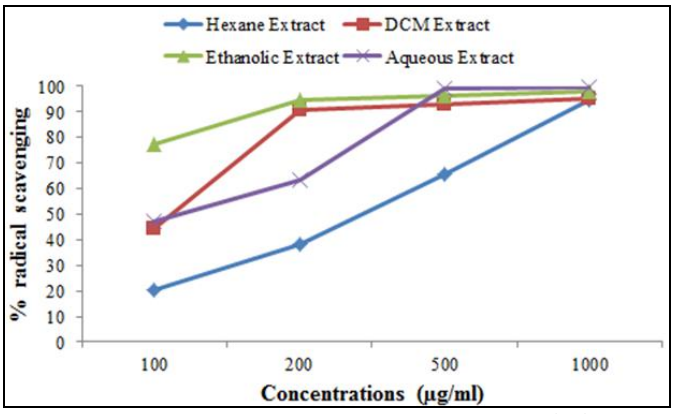

FIG. 3: SCAVENGING ACTIVITY (%) OF DIFFERENT EXTRACTS OF *SYZYGIUM AROMATICUM*

CONCLUSIONS: From this study it is evident that tested spice extracts have potential free radical scavenging properties. Moreover, ethanolic extracts of *S. aromaticum* exhibited high phenolic content which can be correlated with its high free radical scavenging and high inhibitory activity against AChE. Our results strongly suggest that these spice extracts could have great importance as therapeutic agents in preventing or slowing the progress of aging and age associated oxidative stress related degenerative diseases. Further studies are required for identification of bioactive compounds in these extracts responsible for observed antioxidant and AchE inhibitory activities.

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CONFLICT OF INTEREST: There are none to declare.

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