

EVALUATION OF BIOLOGICAL ACTIVITIES OF THE ESSENTIAL OIL AND MAJOR COMPONENT OF *SYZYGIUM AROMATICUM*

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ABSTRACT

Hydro distilled essential oil from buds of *Syzygium aromaticum* was investigated for antioxidant and enzyme inhibition activities. GC-MS analysis of the oil indicated eugenol (80.0%) as the major constituent followed by caryophyllene (10.27%) and eugenol acetate (5.03%). Thin layer chromatography of the essential oil yielded eugenol confirmed by comparison of spectral data (EI-MS, ¹H and ¹³C-NMR) with the literature. Reactive oxygen species (ROS), xanthine oxidase (XO), acetylcholine esterase (AChE) inhibition, lipid peroxidation by thiobarbituric acid (TBA) and ferric thiocyanate (FTC) methods, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays were carried out on the essential oil and the major component. The IC₅₀ values for eugenol showed significant potential against enzyme inhibition and antioxidant activities while the essential oil of *S. aromaticum* was found moderately active.

Key words: *Syzygium aromaticum*, essential oil, eugenol, ROS, xanthine oxidase, acetylcholine esterase.

INTRODUCTION

The free radicals which produced in biological systems are responsible for oxidation of cell lipids and DNA damage. They may cause serious diseases such as cancer, coronary arteriosclerosis and diabetes mellitus (Dorman *et al.*, 2000). Dietary antioxidants may be effective in prevention of oxidative damage (Farag *et al.*, 1989). Many scientists have focused on the medicinal and edible plants to discover natural antioxidants as they show an important role in protecting human health (Bauer *et al.*, 1997).

Xanthine oxidase (XO) plays a major role in catalyzing the oxidation of hypoxanthine to xanthine which finally forms uric acid (Saiful *et al.*, 2012). Excessive accumulation of uric acid *in vivo*, characterized by the deposition of uric acid in the joints leads to severe inflammatory arthritis called acute gout (Apaya *et al.*, 2011). Superoxide radicals and hydrogen peroxide produced during the re-oxidation of xanthine oxidase and other reactive oxygen species (ROS) contribute to the oxidative stress on the organism and are involved in many pathological processes such as inflammation, atherosclerosis, cancer and aging (Cos *et al.*, 1998). Although allopurinol is used to treat hyperuricemia but associated with several adverse side effects.

The acetylcholine esterase (AChE) is a biologically important enzyme that hydrolyzes acetylcholine (ACh), a neurotransmitter considered to play role in the pathology of Alzheimer's disease (Shahwar *et al.*, 2013). One of the most important approaches for treatment of this disease involves the

enhancement of acetylcholine level in brain using AChE inhibitors (Shahwar *et al.*, 2012).

S. aromaticum commonly known as clove, is a tropical perennial plant of family Myrtaceae. It is widely grown in India, Madagascar, Sri Lanka, Indonesia and the south of China. In folklore medicine, the buds of this plant have been traditionally used for the treatment of toothache (Dorman *et al.*, 2000). The essential oil of the buds is reported to possess antimicrobial, cytotoxic, antioxidant, antiinflammatory and anaesthetic properties (Nassar *et al.*, 2007). The current study was carried out on the essential oil and major component of *S. aromaticum* from Pakistan. Standard protocols were used to explore the XO and AChE inhibition potential of essential oil and major component of *S. aromaticum* while the antioxidant activity was investigated through ROS, DPPH, FTC, TBA and FRAP methods. Pakistan.

MATERIALS AND METHODS

Chemicals: Xanthine oxidase, allopurinol, xanthine, hydroxylamine, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, sulfanilic acid, ethylenediamine tetra acetate (EDTA), butylated hydroxytoluene (BHT), gallic acid, 2, 4, 6-tripyridyl-s-triazine (TPTZ), 1,1-diphenyl-2-picrylhydrazyl (DPPH), acetylthiocholine iodide, 5,5-dithiobis [2-nitro benzoic acid (DTNB) were purchased from Merck (Germany) and sigma aldrich.

Plant material: Fresh buds of *S. aromaticum* were purchased from local market of Lahore.

Extraction of essential oil: The buds of *S. aromaticum* were dried under shade and milled to powder form (1 Kg)

were subjected to steam distillation using Dean Stark apparatus. The oil was separated, dried over anhydrous sodium sulphate, filtered and kept in vials at 4 °C (% yield = 2.1% w/w).

GC/MS analysis: Gas chromatography/mass spectroscopy analysis of essential oil was carried out using a Shimadzu GCMS-QP-2010 system, with DB-5 MS capillary column (30 m X 0.25 mm; 0.25 μmeter film thickness). Carrier gas was helium with a flow rate of 1 ml/min. The GC oven temperature was programmed from 50- 260 °C at 4°C/min rise. 1 μl diluted sample (1: 100 in acetonitrile, V/V) was injected manually in the split less mode. Mass spectrometry was carried out in EI mode at 70 eV. The constituents were identified by matching their mass spectra in the NIST Library.

Thin layer chromatography (TLC): The essential oil of *S. aromaticum* was subjected to TLC to specify the active components (Erasto *et al.*, 2004). Pre-coated TLC aluminium sheets of silica gel were developed in the system of dichloromethane: petroleum ether (20: 80, v/v) for the purification of major component.

ROS inhibition assay: Superoxide levels were measured by the nitrite method (Cos *et al.*, 1998). 0.6 mL of xanthine oxidase (6.25 mU/mL) in a phosphate buffer solution (pH = 7.5, 20 mM) was added to initiate the test solutions containing 300 μl of each xanthine (0.05 mM), hydroxylamine (200 mM) and EDTA (200 mM), 150 μl of essential oil (1 mg/ml) in methanol in various concentrations and 750 μl phosphate buffer solution (pH = 7.5, 20 mM). The mixture was incubated for 30 min at 37 °C followed by 0.1 mL of HCl (0.58 M). The uric acid production was measured at 290 nm. To detect superoxide, 750 μl sulfanilic acid (200 mM), 300 μl *N*-(1-naphthyl)-ethylenediamine dihydrochloride (2 mg/ml) in 2 normal (*N*) H₂SO₄, was added drop wise in ice bath. The absorbance was measured at 550 nm.

Acetylcholine esterase activity: The reaction mixture contained 2.8 ml of (100 mM) Tris-HCl buffer (pH 7.8), 30 μl of DTNB, 150 μl (50-250 μg/ml) of test sample and 100 μl of acetyl choline esterase. The reaction was initiated by adding 30 μl acetyl thiocholine and monitored at 412 nm after 30 min. Hyoscine was used as the positive control. (Shahwar *et al.*, 2013).

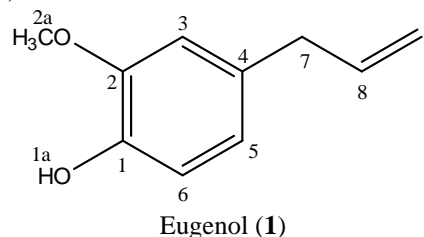
Antiradical activity by DPPH method: 1 ml of methanolic solution of each test sample (100-500 μg/ml) was added to 1 ml of 0.02 % methanolic solution of DPPH. The solution was rapidly mixed and kept at room temperature for 30 minutes in dark. The absorbance was measured at 517 nm while BHT was used as the reference standard (Erasto *et al.*, 2004).

Ferric thiocyanate (FTC) method: 1 mg/ml of test solution, 4.1 ml of 2.5% linolenic acid, 8.0 ml of 0.05M phosphate buffer (pH 7.0) and 3.9 ml of water were

placed in dark at 40 °C. 0.1 ml of this solution was misted with 9.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 0.02M ferrous chloride in 3.5% HCl. Absorbance was measured at 500 nm. BHT was used as the positive control while the mixture without sample was used as the negative control (Onay *et al.*, 2006).

Thiobarbituric acid (TBA) method: 1 mg/ml of test solution, 4.1 ml of 2.5% linolenic acid, 8.0 ml of 0.05M phosphate buffer (pH 7.0) and 3.9 ml of water were placed in dark at 40 °C. 1 ml of this solution was misted with 2 ml of 20% trichloroacetic acid and 2 ml of 0.67% 2-thiobarbituric acid. The mixture was placed in a boiling water bath for ten min. After cooling it was centrifuged at 3000 rpm for 20 min. Absorbance of supernatant was measured at 532 nm. BHT was used as the standard reference (Khan *et al.*, 2010).

FRAP assay: The stock solutions included 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl, 20 mM FeCl₃.6H₂O and 300 mM of acetate buffer, pH 3.6 (3.1 g sodium acetate trihydrate in 16 ml glacial acetic acid and the volume was made upto 1 L with distilled water). The FRAP working reagent was freshly prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ and 2.5 ml FeCl₃.6H₂O. 0.1 percent of sample (150 μL) was allowed to react with 2850 μL of the FRAP reagent for 30 min in the dark and the absorbance of the colored product was measured at 593 nm. The data was expressed relative to values obtained from calibration curve of FeSO₄.7H₂O and expressed as equivalent to FeSO₄.7H₂O (Gourine *et al.*, 2009).



Eugenol (1); EI-MS *m/z* (rel. int. %): [M]⁺ 164 (100), 149 (30), 137 (16), 131 (19), 103 (21), 77 (23), 55 (19) and 39 (11). ¹H-NMR (CDCl₃, 400 MHz) : 3.32 (2H, d, *J* = 9.4, H-7), 3.86 (3H, s, H-2a), 5.09 (2H, d, *J* = 9.4, H-9), 5.98 (1H, m, *J* = 8.8, H-8), 6.58 (1H, s, H-3), 6.76 (1H, d, *J* = 9.4, H-5) and 6.88 (1H, d, *J* = 9.4, H-6). ¹³C-NMR (CDCl₃, 100 MHz) : 40.2 (C-7), 57.9 (C-2a), 110.9 (C-9), 114.5 (C-3), 115.8 (C-6), 122.3 (C-5), 133.1 (C-4), 136.7 (C-8), 143.6 (C-1) and 147.2 (C-2).

RESULTS AND DISCUSSION

The volatile oil of the buds of *S. aromaticum* was obtained with a yield of 2.1%. The GC-MS analysis of the volatile oil indicated nine components (Table 1, Figure 3). Eugenol was found as the major constituent

(81.0 %). The other identified components are caryophyllene (10.27 %) and eugenol acetate (5.03 %). The major compound of the essential oil was isolated by thin layer chromatography (TLC) and confirmed by comparison of spectral data (EI-MS, ^1H and ^{13}C -NMR) with the literature as eugenol (1).

Superoxide scavenging activity exhibited by essential oil and its major component was determined by nitrite method (Cos *et al.*, 1998). ROS percent inhibition exhibited by volatile oil and eugenol was $89.7 \pm 1.0\%$ ($\text{IC}_{50} = 41.4 \pm 1.2 \mu\text{g}$) and $84.6 \pm 1.4\%$ ($\text{IC}_{50} = 57.4 \pm 3.1 \mu\text{g}$), respectively (Figure 1, Table 2). Moreover *S. aromaticum* essential oil $85.1 \pm 2.2\%$ ($\text{IC}_{50} = 61.9 \pm 2.1 \mu\text{g}$) and eugenol $81.2 \pm 3.0\%$ ($\text{IC}_{50} = 43.5 \pm 2.6 \mu\text{g}$) exhibited significant xanthine oxidase inhibition activity at 150 $\mu\text{g/ml}$ as compared to the reference standard allopurinol $67.9 \pm 1.1\%$ ($\text{IC}_{50} = 96.4 \pm 2.0 \mu\text{g}$). The results are summarized in Table 2.

Acetylcholine (ACh), a neurotransmitter, has been implicated to play an important role in cerebral cortical development and modulation of cognitive performance (Shahwar *et al.*, 2013). AChE inhibition potential was more significant in eugenol with $80.3 \pm 3.1\%$ inhibition as compared to *S. aromaticum* essential oil with $75.8 \pm 1.2\%$ inhibition. While hyoscine was used as the reference standard which showed $83.6 \pm 1.5\%$ inhibition (Table 2). The % inhibition potential of eugenol and the essential oil was $89.4 \pm 0.4\%$ ($\text{IC}_{50} = 3.7 \pm 0.3 \mu\text{g}$) and $81.2 \pm 0.2\%$ ($\text{IC}_{50} = 4.5 \pm 0.4 \mu\text{g}$) respectively in the DPPH method as shown in Table 2, Figure 1. The reference standard BHT exhibited $94.8 \pm 1.1\%$ inhibition. Both eugenol and the pure essential oil showed moderate activity in the TBA assay with $52.8 \pm 0.9\%$ and $56.7 \pm 1.0\%$ respectively. No significant results were obtained in the FTC assay (Table 3, Figure 2). The reducing capacity towards Fe^{2+} of the volatile oil of *S. aromaticum* and eugenol (1) were comparable with Fe^{2+} equivalents of $327 \pm 0.8 \mu\text{M}$ and $323 \pm 1.3 \mu\text{M}$, respectively (Table 3, Figure 3).

The present results demonstrated that eugenol, a phenolic compound which is present in major quantity in the essential oil of *S. aromaticum* possess effective enzymatic and antioxidant capacity due to the presence of a conjugated double bond and a hydroxyl group in its structure. The antioxidant activity of eugenol towards

ROS and lipid peroxidation can be attributed to the presence of phenolic group which is capable of capturing an alkyl radical by donating a hydrogen atom of the phenolic hydroxyl group. A comparison of the inhibitory effect on XO with the inhibitory effect on the generation of superoxide anion radical (O_2^-) from the hypoxanthine-XO system revealed that the inhibition of O_2^- generation by eugenol is due to its radical-scavenging activity while inhibitory activity is due to the presence of binding sites in eugenol for XO. Similarly AChE inhibition activity is also correlated with the availability of proper region in the molecule to bind with AChE. Therefore, it is suggested that further studies to explore the drug-protein binding nature is necessary to verify these results.

In the present study evaluation of ROS, xanthine oxidase, acetylcholine esterase inhibition effect and antioxidant capacity of the essential oil and eugenol from *S. aromaticum* was carried out. Furthermore, GC-MS analysis was used for the determination of the chemical composition of essential oil of *S. aromaticum* of Pakistan region. The results suggested that the presence of eugenol plays an important role for the bioactivities of essential oil from *S. aromaticum*.

Statistical analysis: The results are presented as mean \pm standard deviation of three replicates using Microsoft Excel 2003.

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Table 1. Chemical composition of the essential oil of *S. aromaticum*.

Compounds	%	^a Identification method
Undecane	0.19	MS
Anethol	1.55	MS
Eugenol	81.00	MS
Caryophyllene	10.27	MS
–caryophyllene	1.21	MS
Rimantadine	0.04	MS
Bergamotene	0.13	MS
eugenol acetate	5.03	MS
Deltacadinene	0.31	MS

^aMass Spectral similarity with NIST MS library database Version 2.0

Table 2. ROS, XO, AChE and DPPH activity of essential oil and major component of *S. aromaticum*.

Sample	ROS		XO		AChE		DPPH	
	% ^a Inhibition	IC_{50} (μg)	% ^a Inhibition	IC_{50} (μg)	% ^a Inhibition	IC_{50} (μg)	% ^b Inhibition	IC_{50} (μg)
Essential oil	84.6 ± 1.4	57.4 ± 3.1	85.1 ± 2.2	61.9 ± 2.1	75.8 ± 1.2	80.9 ± 1.8	81.2 ± 0.2	4.5 ± 0.4
Eugenol	89.7 ± 1.0	41.4 ± 1.2	81.2 ± 3.0	43.5 ± 2.6	80.3 ± 3.1	74.4 ± 1.9	89.4 ± 0.4	3.7 ± 0.3
Hyoscine	-	-	-	-	83.6 ± 1.5	69.4 ± 1.0	-	-
Gallic acid	73.6 ± 1.6	87.4 ± 2.7	77.2 ± 1.6	67.9 ± 2.2	-	-	86.2 ± 0.7	3.8 ± 0.5
Allopurinol	-	-	67.9 ± 1.1	96.4 ± 2.0	-	-	-	-
BHT	-	-	-	-	-	-	94.8 ± 1.1	1.2 ± 0.7

% inhibition ^aat 150 $\mu\text{g/ml}$, ^bat 500 $\mu\text{g/ml}$.

Table 3. Lipid peroxidation and FRAP assay of essential oil and major component of *S. aromaticum*.

Sample	Lipid peroxidation (% Inhibition ^c)		FRAP ^d
	TBA	FTC	
Essential oil	56.7±1.0	27.2±0.4	327±0.8
Eugenol	52.8±0.9	28.4±1.2	323±1.3
Gallic acid	-	-	335±1.1
BHT	51.2±1.4	36.1±0.8	-

% Inhibition^c at 5 mg/ml, ^dequivalent to FeSO₄.7H₂O (uM).

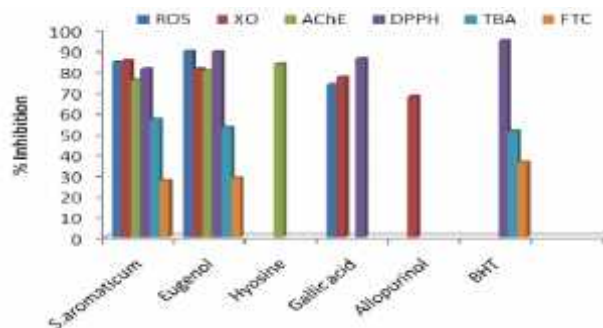


Fig. 1. ROS, XO, AChE, DPPH, TBA and FTC activities of the essential oil and major component of *S. aromaticum*.

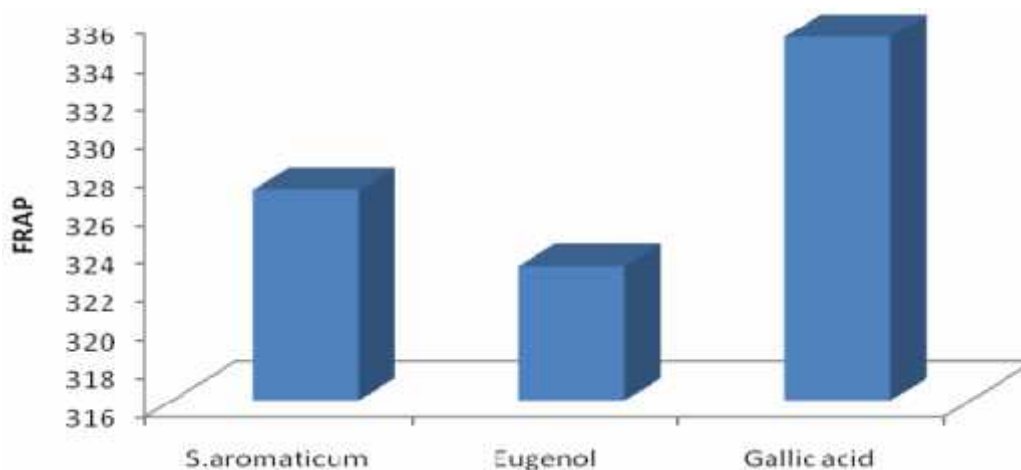


Fig. 2. FRAP activity of essential oil and major component of *S. aromaticum*.

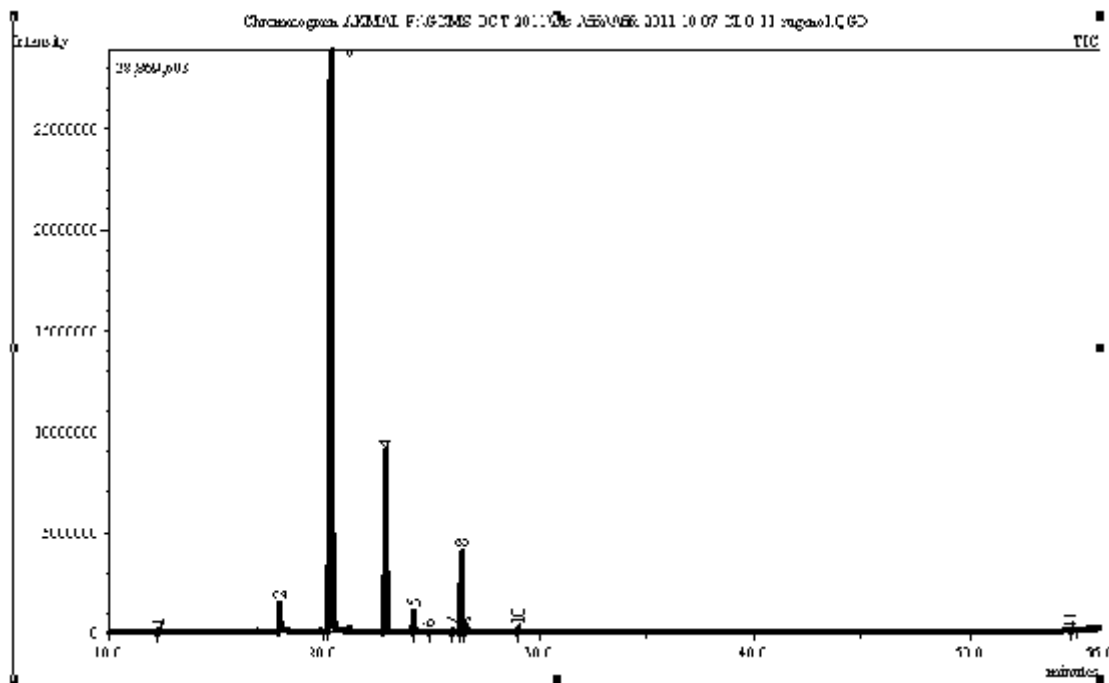


Fig. 3. GC-MS chromatogram of the essential oil of *S. aromaticum*.

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