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Research Article

Design, Synthesis, Anti-inflammatory and Antibacterial Activity of Eugenol Incorporated 1, 3, 4 - Oxadiazole Derivatives

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ABSTRACT
Eugenol is an essential oil extracted and isolated from *Syzygium aromaticum* by hydrodistillation and column chromatography respectively. The isolated eugenol was identified by TLC using n-hexane: ethyl acetate (7:3) as mobile phase. It was quantified by HPTLC and concentration of eugenol present in the sample was found to be 83.1 mg/g. This eugenol is used as a starting material for the synthesis of semisynthetic derivatives bearing 1,3,4-oxadiazole motif. The synthetic protocol involves 4 steps as depicted in methodology section. In first step, there is a condensation between eugenol and ethyl chloroacetate to yield compound (2). Second step describe the formation of acetohydrazide (3) from acetate (2) in the presence of hydrazine hydrate. Step 3 reaction involves formation of Schiff bases (4a-4g) in the presence of benzaldehyde/ substituted benzaldehyde. In the final step 4 there is a cyclization of (4a-4g) with Chloramine-T resulting in the formation of substituted 1,3,4-oxadiazole derivatives (5a-5g). All the synthesised compounds were characterized and confirmed by physical data (m.p., TLC) and spectral studies (IR, ^{1}H NMR). Synthesised compounds were evaluated for possible anti-inflammatory and antibacterial activity. The compound 5b bearing electron withdrawing nitro group possess better anti-inflammatory and antibacterial activity compared to standard eugenol tested.

KEYWORDS: Clove bud; eugenol, 1, 3, 4-oxadiazole; anti-inflammatory; antibacterial

INTRODUCTION

Inflammation and bacterial infections are the major threat to the health sector and important cause of mortality worldwide. The use of synthetic anti-inflammatory and antibacterial drugs are known to cause drastic toxic effect such as gastric ulcer and intolerance, inhibition of platelet function, inhibition of induction of labour, alteration in renal function, hypersensitivity reaction, nausea, fever, allergic reactions, including photodermatities and anaphylaxis *etc.,*[1]. Also the anti-inflammatory drugs are devoid of antibacterial properties hence the synthesis of a drug possessing anti-

inflammatory and antibacterial property is the need of the hour in order to counteract the inflammatory diseases with bacterial infections. Such diseases include ulcerative colitis, crohn's, [2] rheumatoid arthritis [3], and alzheimer's [4] *etc.,* The use of known antibacterial structural scaffolds to afford new bioactive derivatives has not been the strategy employed for these diseases. Therefore, new chemical entities synthesis is the main focus when searching for new drug leads with antibacterial and anti-inflammatory activities. The principle active constituents of plants are having potential medicinal values. Natural products are the excellent sources of new bioactive compounds since they show innovative structures that may be used as natural pharmacophores to structural derivatizations. Eugenol is one such known phytomedicine [5,6].

Eugenol is a volatile, an essential oil and important natural allyl phenolic compound, which can be obtained from the variety of medicinal plants such as clove, cinnamon, tulsi, turmeric, pepper, ginger, thyme, basil, lemon balm and nut mug *etc.,* However for its commercial use mainly extracted from the clove [7].

Clove is naturally grown in Indonesia and Malacca islands. It is belonging to the *Eugenia aromaticum/ Syzygium aromaticum/ Eugenia caryophyllata* specie, magnoliophyte division, myrtoideae subfamily and myrtaceae family. The percentage in the yield of clove oil varies based on the part from which it is extracted. *i.e.* bud oil (60-90 %), leaf oil (82-88 %) and steam oil (90-95 %). It has strong phenolic smell and acrid taste [8,9].

Clove oil can be extracted from either of the methods such as hydro distillation method, microwave-assisted extraction method, supercritical carbon dioxide extraction method, ultrasound assisted extraction method *etc.,* Extracted oils are isolated by chromatographic technique. The isolated eugenol is characterized by the physical data (m.p., b.p. and TLC) and spectral studies such as IR, ^{1}H NMR, ^{13}C NMR, GC-MS, HPTLC and Mass. Literature survey reveals eugenol has numerous potent biological activities such as antifungal, anti-inflammatory, anaesthetic, analgesic, hypotensive, antioxidant, antitumor, antiparasitic, antidepressive, antiatherogenic, antiseptive, antiviral, antibacterial, antipyretics, antinociceptive, antiplatelet aggregation, anticoagulation, antidiarrheal, antiulcer, antihypoxia, anti-oxidant, , hypotensive , anti-parasitic and anti-depressive *etc.,* Eugenol is used in combination with other drugs to treat various condition of analgesia. These combinations are available in the form of cream/lotion, mouth wash, massage oil, burners and vaporizers *etc.,* at specific brand name [10,12].

1,3,4-oxadiazole is a five-membered heterocyclic nucleus having an oxygen atom and two nitrogen atoms. Its derivatives are known for a diverse pharmacological profiles which includes anti-inflammatory, antibacterial, antifungal, antiviral, anti-tubercular, anticonvulsant, hypoglycemic, anti-allergic, vasodilator, analgesic, anthelmintic, anticancer, antiviral, antioxident, haemolytic anti-proliferative activities, antiparkinsonian, cytotoxic, anticancer and ulcerogenic *etc.,* Numerous 1, 3, 4-oxadiazole bearing marketed drugs are used in various clinical conditions [13].

By considering all the above facts of eugenol and 1,3,4-oxadiazoles, in the present study an attempt is made to design and synthesize semi synthetic drug candidates with anti-inflammatory and antibacterial properties which are devoid of side effects by incorporating 1,3,4-oxadizoles to eugenol. Therefore, these semi-synthesised molecules will be the potential anti-inflammatory and antibacterial molecules and this will be a foot step in the process of drug discovery via natural compounds.

MATERIALS AND METHOD

General experiments

Chemicals required for the present study were purchased from S.D. Fine Chem. Ltd. Qualigens, Spectrochem Pvt. Ltd, CDH, Merk, Rankem, Sigma Aldrich fine Chemicals as synthetic grade and used without purification. Purity of the compounds including the intermediates was checked by TLC using pre coated silica gel plates (604 GF 254 Merck) with n-hexane: Ethyl Acetate (7:3) as mobile phase. The detection of the spots was done by observing the plates under Arico UV cabinet at 254 nm. Recovery of solvent from the synthesized compound was carried out by using Buchi rotavapor R-210. Melting points of the synthesized compounds were recorded by using Sigma melting point apparatus. IR spectra were recorded on Bruker spectrophotometer. ^{1}H NMR spectra were recorded on Brucker Spectrospin-400 MHz spectrophotometer using $CDCl_3$ as

solvent, TMS as an internal standard. Spectra were obtained from IISc Bengaluru.

Collection and Authentication of Clove Buds

Clove buds were purchased from **M/S Amruth Kesari,** Bengaluru. Authentication of the buds was done by Regional Ayurveda Research Institute for Metabolic Disorders, Uttarahalli, Manavarthekaval, Bengaluru. The sample of bud is preserved for further reference in our laboratory. The authenticated clove buds were used for the extraction of clove oil using Clevenger's apparatus by hydrodistillation method.

Extraction of Clove oil from Clove buds [10,12]

15g of dried, freshly grounded clove buds were transferred into a 500 ml of two necked RBF and soaked the grounded cloves for 15 Minutes with 300 ml of distilled water. Porcelain chips were added to avoid bumping. The Clevenger's apparatus was set up for hydrodistillation and heated using heating mantle at a temperature of about 50-70 °C for 6-8 h. During the process of distillation, small fraction of distilled water was added to the distillation flask to maintain the original volume in the distillation flask. Once the distillate turns colorless, stopped the process of distillation. Collected emulsion was extracted with 3 x 15 ml of dichloromethane (methylene chloride) and sodium sulphate was added to remove the moisture. Separation of clove oil from dichloromethane was carried out using rotary Evaporator. The collected clove oil was Identified by chemical test, TLC and HPTLC.

TLC and Chemical test: Clove Oil was treated with neutral ferric Chloride Solution; appearance of green color indicates presence of eugenol. TLC was carried out by using n-hexane: ethylacetate as mobile phase in the ratio of 7:3. The R_f value was found to be 0.65 and spots was visualized in UV Chamber at 254 nm against standard eugenol received as a gift sample from natural remedies, Bengaluru. Based on the observations of chemical test and TLC, the extract was subjected to isolate eugenol.

Isolation of Eugenol [9,10]

Column chromatography was used for the purification of extracted clove oil. 230-400 mesh size of silica gel was used as stationary phase. Pre-activated silica gel was used for the preparation of slurry with n-hexane. With vigorous stirring, Slurry was poured in to the dried, cotton plugged (50 cm X 3 cm) glass column while slight tapping so that particles get settled uniformly through out the column. After 1/10th of the column was packed with slurry, the oil was added without disturbing the silica pack and allowed the bed to adsorb followed by mobile phase and then packed with wetted cotton dipped in n-hexane. To this, different grades of mobile phase such as 5%, 10%, 15% was prepared using n-hexane: ethyl acetate and added in fraction of 100 ml. Eluted fractions were collected in 50 ml test tube and presence of eugenol was confirmed by comparing with the standard using TLC. Solvents were recovered by rotary evaporator. This isolated Eugenol was characterized by TLC, IR, and HPTLC. The obtained R_f value was 0.64 and b.p. was found to be 253.5°C.

Percentage yield

84 ml of clove oil was extracted from the 500 g of clove buds by hydrodistillation method using Clevenger's apparatus and 68 ml of eugenol was isolated from clove oil by using Column chromatography.

Therefore, $\%\text{Yield of eugenol from clove oil} = \frac{\text{Amount of Eugenol isolated (ml)}}{\text{Amount of Clove oil extracted (ml)}} \text{x}100$

$= \frac{68}{84} \text{x}100 = 80.95\%\text{v/v}$

Quantification of eugenol [8]

10 mg of standard eugenol was dissolved in 10 ml of methanol (1 mg/ml). From this stock solution, concentrations of 50, 100, 150, 200 and 250 µg/ml were prepared for 5 levels of standards. 150 mg/ml solution of eugenol was used as samples. The standard eugenol (4 µl) ranging from 200-1000 ng per spot and the samples 150 mg/ml solution of eugenol to 1000 µg per spot was applied on the TLC plate. The spotted plates were developed and scanned in Scanner 3 at 254 nm. The Calibration curve was constructed by plotting average peak area and height versus the concentration of eugenol by the software itself. It also provides regression equation and concentration of eugenol in samples. The values are shown in Fig 1**.**

Substance: eugenol @ 254 nm

Regression via height: Linear Y = 33.69 + 0.06677 * X r = 0.98699 sdv = 5.38
Regression via area: Linear Y = 1130 + 3.286 * X r = 0.97550 sdv = 8.72

Track	Vial	Rf	Amount	Height	X(Calc)	Area	X(Calc)	SampleID/Remark
1	1	0.64	200.00 ng	47.77		1755.88		
2	2	0.62	400.00 ng	57.03		2276.22		
3	3	0.62	600.00 ng	74.98		3265.09		
4	4	0.64	800.00 ng	91.88		4062.51		
5	5	0.66	1000.00 ng	97.12		4148.69		
6	6	0.68		89.18	831.00 ng	3655.62	768.58 ng	

Fig 1: Quantification of eugenol in *Syzygium aromaticum (L.)* by HPTLC method.

Calibration Curve

The LOD and the LOQ were calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve according to the formulas: LOD = 3.3 (SD/S) and LOQ = 10 (SD/S). The standard deviation of the response was determined based on the standard deviation of y-intercepts of regression lines.The calibration plot of eugenol using different concentrations (200-1000ng) versus peak height showing the regression values y = 0.06677x +33.69(y= mx + c) r= 0.98699 and SD= 5.38.The calibration plot of eugenol using different concentrations (200-1000 ng) versus peak area showing the regression values y = 1130X+3.286(y= mx + c) r = 0.9755 and SD= 8.72. and Statistical data of LOD & LOQ presented in Table 1.

Table 1: Statistical data of LOD & LOQ of eugenol by HPTLC

Eugenol	LOD (ng)	LOQ (ng)
With respect to Height	265.57	805.7
With respect to Area	8.7371	26.53

The R_f value of eugenol was found to be 0.64±0.023. The UV spectrum as shown in Fig 2 also justifies that the test samples with that of standard eugenol.The area and height of the peaks obtained are sharp and there is an increase in height and area with increase in concentration of eugenol. This indicates the standard eugenol used for quantitative analysis is of good quality. The samples showed the peaks at R_f value 0.68 indicating the presence of eugenol in the samples and concentration of eugenol present in the sample was found to be 83.1mg/g.

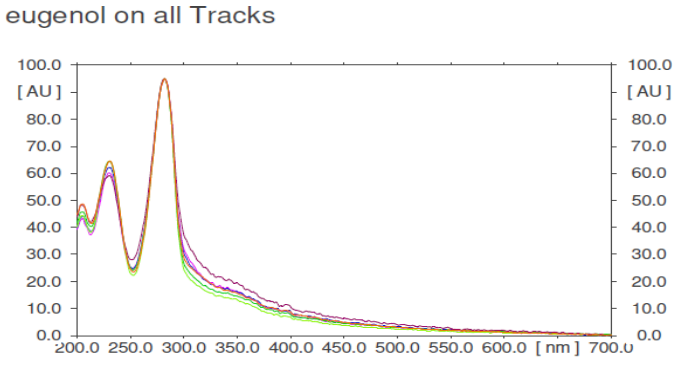


Fig2: UV Spectrum of eugenol in all the six spots

Synthesis of ethyl [2-methoxy-4-(prop-2-en-1-yl) phenoxy] acetate (2)

Reflux condenser was fixed to a clean, dry 500 ml two necked RBF. Added 16.42 ml (0.1 mol) of eugenol dissolved in 19.29 ml (0.47 mol) of acetonitrile. To this mixture 6.9 g (0.05 mol) of Potassium carbonate was added to absorb the released HCl during the process of reaction. Separately dissolved 24.52 ml (0.2 mol) of ethyl chloroacetate in 19.29 ml (0.47 mol) of acetonitrile

was added to the above mixture and refluxed for 24 h at 90°C. The progress of the reaction was monitored by TLC using n-hexane:ethyl acetate (7:3) as mobile phase. After the completion of the reaction, it was poured in to 200 ml of distilled water and stirred well. The organic layer was separated and subjected to evaporation using rotavapour at temp of 82 °C. Obtained reddish coloured liquid was transferred into a petriplates and was allowed to cool to get the half white coloured product. [15] Half white; yield: (75.5%), M.F: $C_{14}H_{18}O_4$; m.p. 85 ^{0}C; R_f: 0.75; IR:(KBr, cm^{-1}); 1032.29 (C-O-C), 3001.71 (CH Stretching), 2975.84 (CH Alkane Stretching), 1510.89 (C=C aromatic), 1638.13 (C=C alkene), 1757.34 (Ester).

Synthesis of 2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy] acetohydrazide (3)

12.5 g (0.05 mol) of compound (2) obtained from the previous step was dissolved in 10.12 ml (0.22 mol) of alcohol in a two necked 100 ml RBF equipped with magnetic bead on a magnetic stirrer and fitted with a reflux condenser. To the above solution triple the quantity of hydrazine hydrate 7.5 ml (0.15 mol) was added and refluxed for 24 h. The progress of the reaction was monitored by TLC using n-hexane: ethyl acetate (7:3) as mobile phase. After completion of the reaction, ethanol was evaporated to yield the product. **[16,17]** Cream colour; yield: (78.5), M.F: $C_{12}H_{16}O_3N_2$; m.p. 60.2 ^{0}C; R_f: 0.22; IR:(KBr, cm^{-1}); 1011.63 (C-O-C), 3040.34 (CH Stretching), 2982.50 (CH Alkane Stretching), 1517.26 (C=C aromatic), 1631.08 (C=C alkene), 1721.51 (C=O), 3443.36 (NH stretching), 1598.09 (NH bending).

Synthesis of Substituted *N*'-benzylidene-2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy] acetohydrazide (4a-4g)

Equimolar quantity 2.36 g (0.01 mol) of 2-[2-methoxy-4-(prop-2-en-1yl) phenoxy] acetohydrazide (3) and different substituted benzaldehyde were dissolved in ethanol and refluxed for 12 h. The progress of the reaction was monitored by TLC using n-hexane:ethyl acetate (7:3) as mobile phase. After completion of the reaction, ethanol was evaporated to obtain the product. [6,17]

4a: *N*'-[(*E*)-(3-chlorophenyl) methylidene]-2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy] acetohydrazide .

Compound (3) (0.01 mol, 2.36 g), p-chloro benzaldehyde (0.01 mol, 1.4 g). Yellow shining; yield: (67), M.F: $C_{12}H_{16}O_3N_2$; m.p. 152 ^{0}C; R_f: 0.12; IR:(KBr, cm^{-1}); 1012.10 (C-O-C), 2960.07 (CH alkane Stretching), 3160.26 (CH aromatic Stretching), 1488.53 (C=C aromatic), 1654.22 (C=C alkene), 1723.72 (C=O), 3231.14 (NH stretching), 1591.56 (NH bending), 1285.49 (C-N), 778.27 (C-Cl).

4b: 2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy]-*N*'-[(*E*)-(3nitrophenyl) methylidene acetohydrazide.

Compound (3) (0.01 mol, 2.36 g), p-nitro benzaldehyde (0.01 mol, 1.51 g). Shining reddish yellow; yield: (72.2), M.F: $C_{12}H_{16}O_3N_2$; m.p. 159 ^{0}C; R_f: 0.9; IR:(KBr, cm^{-1}); 1070.87 (C-O-C), 2979.65 (CH Alkane Stretching), 3108.20(CH aromatic Stretching), 1594.98 (C=C aromatic), 1647.98 (C=C alkene), 1706.24 (C=O), 3237.25 (NH stretching), 1342.25 (C-N), 1519.62 (NO_2).

4c: *N*'-[(*E*)-(3-bromophenyl) methylidene]-2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy] acetohydrazide.

Compound (3) (0.01 mol, 2.36 g), p-bromo benzaldehyde (0.01 mol, 1.22 g) .Yellowish brown; yield: (69), M.F: $C_{12}H_{16}O_3N_2$; m.p. 143 ^{0}C; R_f: 0.16; IR:(KBr, cm^{-1}); 1022.92 (C-O-C), 3067.89 (CH Stretching), 2955.28 (CH alkane Stretching), 3057.80 (CH aromatic Stretching), 1611.00 (C=C alkene), 1707.08 (C=O), 3333.89 (NH stretching), 1326.10 (C-N), 619.64 (C-Br).

4d: *N*'-[(*E*)-(3-hydroxyphenyl) methylidene]-2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy] acetohydrazide

Compound (3) (0.01 mol, 2.36 g), p-hydroxy benzaldehyde (0.01 mol, 1.22 g). yellowish brown ; yield: (65.44), M.F: $C_{12}H_{16}O_3N_2$; R_f: 0.37; IR:(KBr, cm^{-1}); 1072.97 (C-O-C), 3063.11 (CH Stretching), 2957.87 (CH Alkane Stretching), 3063.11 (CH aromatic Stretching), 1658.50 (C=C alkene), 1722.79 (C=O), 3331.89 (NH stretching), 1256.96 (C-N), 3290.39 (OH).

4e: 2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy]-*N*'-[(*E*)-phenylmethylidene] acetohydrazide

Compound (3) (0.01 mol, 2.36 g), Benzaldehyde (0.01 mol, 1.06 g) .Yellow; yield: (58), M.F: $C_{12}H_{16}O_3N_2$; R_f: 0.19; IR:(KBr, cm^{-1}); 1077.77 (C-O-C), 3063.73 (CH aromatic Stretching), 2958.11 (CH Alkane Stretching), 1597.31 (C=C alkene),

1719.94 (C=O), 3157.12 (NH stretching), 1597.31 (NH bending), 1257.04 (C-N).

4f: 2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy]-*N*'-{(*E*)-[3-(*N*, *N* dimethylamino) phenyl] methylidene} acetohydrazide

Compound (3) (0.01 mol, 2.36 g), p-dimethyl amino benzaldehyde (0.01 mol, 1.49 g). Yellowish orange; yield: (70.3), M.F: $C_{12}H_{16}O_3N_2$; m.p. 165 ^{0}C; R_f: 0.52; IR:(KBr, cm^{-1}); 1072.41 (C-O-C), 2956.79 (CH alkane Stretching), 3161.43 (CH aromatic stretching), 1646.13 (C=C alkene), 1723.36 (C=O), 3432.403443.36 (NH stretching), 1223.33 (C-N).

4g: *N*'-[(*E*)-(3-fluorophenyl) methylidene]-2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy] acetohydrazide

Compound (3) (0.01 mol, 2.36 g), p-fluro benzaldehyde (0.01 mol, 1.06 g) .Cream colour; yield: (52), M.F: $C_{12}H_{16}O_3N_2$; R_f: 0.41; IR:(KBr, cm^{-1}); 1070.75 (C-O-C), 2980.37 (CH Alkane Stretch, 3117.84 (CH aromatic Stretching), 1651.84 (C=C alkene), 1721.81 (C=O), 3233.68 (NH stretching), 1598.96 (NH bending), 1281.96 (C-N), 1374.53 (C-F).

Synthesis of Substituted 2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy]-5-phenyl-1, 3, 4-oxadiazole (5a-5g).

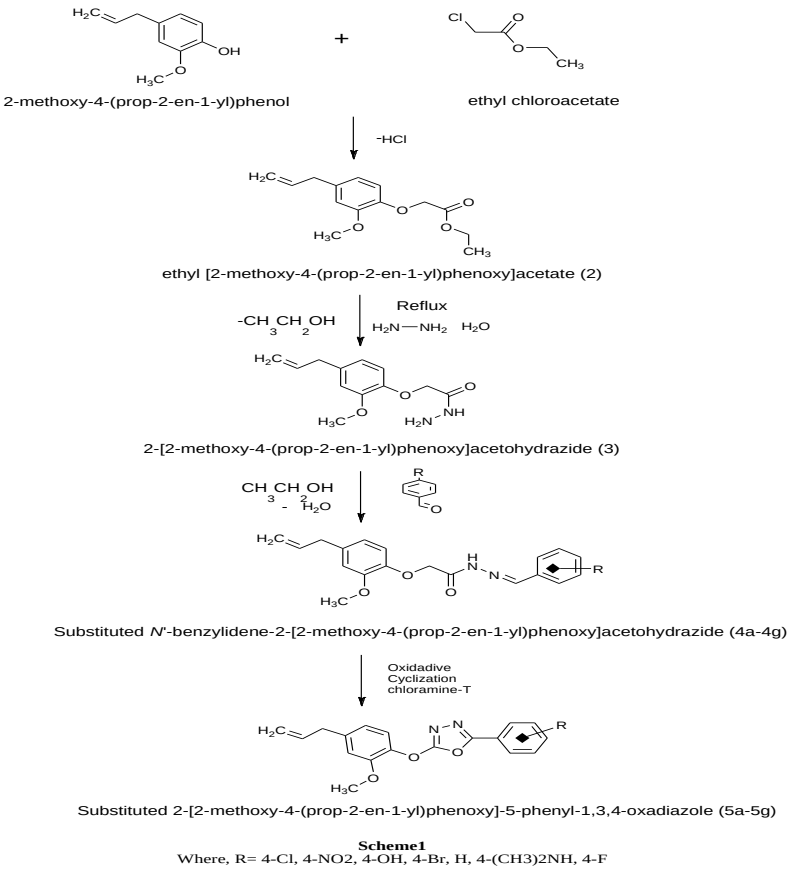
Separately, Equimolar quantity 1.7 g (0.005 mol) of substituted benzylidene-2-[2-methoxy-4-(prop-2-en 1-yl)phenoxy]acetohydrazide and 1.4 g (0.005 mol) Chloramine-T was dissolved in 10 ml of ethanol and both the solution were mixed and refluxed for 12 h at 80^{o}C. The progress of the reaction mixture was monitored by TLC using n-hexane: ethylacetate (7:3) as mobile phase. After completion of the reaction, ethanol was evaporated and thus obtained product collected. (**Scheme 1:**)

5a: 2-(3-chlorophenyl)-5-[2-methoxy-4-(prop-2-en-1-yl) phenoxy]-1, 3, 4-oxadiazole

Compound 4a (0.005 mol, 1.79 g), Chloramine-T (0.005 mol, 1.40 g) .Yellow powder; yield: (66.3), M.F: $C_{18}H_{15}O_3N_2Cl$; m.p. 148 ^{0}C; R_f: 0.24; IR:(KBr, cm^{-1}); 1080.29 (C-O-C), 2921.78 (CH alkane Stretching), 3126.78 (CH aromatic Stretching), 1522.43 (C=C aromatic), 1661.70 (C=C alkene), 3355.98 (NH stretching), 1595.29 (NH bending), 1301 (C-N), 665.83 (C-Cl); ^{1}H NMR($CDCl_3$ δppm): 3.85 (3H, O-CH_3 singlet), 4.63 (3H, CH_2=CH- triplet), 7.31 & 8.18 (2H & 1H, aromatic doublet & singlet respectively), 7.8 (4H, aromatic attached to oxadizole ring doublet), 1.24 (2H, -CH_2, doublet).

5b:2-[2-methoxy-4-(prop-2-en-1-yl)phenoxy]-5-(3-nitrophenyl)-1,3,4-oxadiazole

Compound 4b (0.005 mol, 1.68 g), Chloramine-T (0.005 mol, 1.40 g). yellow; yield: (67.6), M.F: $C_{18}H_{15}O_5N_3$; m.p. 163 ^{0}C; R_f: 0.62; IR:(KBr, cm^{-1}); 1084.20 (C-O-C), 2982.26 (CH Alkane Stretching), 3124.40 (CH aromatic Stretching), 1520.93 (C=C aromatic), 1594.31 (C=C alkene), 3238.39 (NH stretching), 1322.56 (C-N), 1461.54 (NO_2); ^{1}H NMR($CDCl_3$ δppm): 3.56 (3H, O-CH_3 singlet), 4.44 (3H, CH_2=CH- triplet), 7.32& 8.11 (2H & 1H, aromatic doublet & singlet respectively), 7.7 (4H, aromatic attached to oxadizole ring doublet), 1.36 (2H, -CH_2, doublet).



Scheme1
Where, R= 4-Cl, 4-NO2, 4-OH, 4-Br, H, 4-(CH3)2NH, 4-F

Scheme 1
Where, R= 4-Cl, 4-NO_2, 4-OH, 4-Br, H, 4-$(CH_3)_2$NH, 4-F

5c: 2-(3-bromophenyl)-5-[2-methoxy-4-(prop-2-en-1-yl) phenoxy]-1, 3, 4-oxadiazole

Compound 4c (0.005 mol, 2 g), Chloramine-T (0.005 mol, 1.40 g). brown; yield: (58), M.F: $C_{18}H_{15}O_3N_2Br$; R_f: 0.34; IR:(KBr, cm^{-1}); 1030.42 (C-O-C), 2960.21 (CH Alkane Stretching), 1513.96 (C=C aromatic), 1600.45 (C=C alkene), 3353.81 (NH stretching), 1333.94 (C-N), 661.60 (C-Br); ^{1}H NMR($CDCl_3$ δppm): 3.35 (3H, O-CH_3 singlet), 6.34 (3H, CH_2=CH- triplet), 7.26& 7.59 (2H & 1H, aromatic doublet & singlet respectively), 7.76 (4H, aromatic attached to oxadizole ring doublet), 1.29 (2H, -CH_2, doublet).

5d: 3-{5-[2-methoxy-4-(prop-2-en-1-yl)phenoxy]-1,3,4-oxadiazol-2-yl}phenol

Compound 4d (0.005 mol, 1.7 g), Chloramine-T (0.005 mol, 1.40 g). Reddish brown; yield: (69), M.F: $C_{12}H_{16}O_3N_2OH$; R_f: 0.37; IR:(KBr, cm^{-1}); 1016.51 (C-O-C), 3067.40 (CH Stretching), 2960.91 (CH alkane Stretching), 1311.73 (C=C aromatic), 1602.23 (C=C alkene), 3354.58 (NH stretching), 1259.15 (C-N), 3259.97 (OH); ^{1}H NMR($CDCl_3$ δ ppm): 3.56 (3H, O-CH_3 singlet), 4.44 (3H, CH_2=CH- triplet), 7.32& 8.11 (2H & 1H, aromatic doublet & singlet respectively), 7.98 (4H, aromatic attached to oxadizole ring doublet), 1.27 (2H, -CH_2, doublet); 4.7 (OH, 1H Singlet).

5e: 2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy]-5-phenyl-1, 3, 4-oxadiazole

Compound 4e (0.005 mol, 1.62 g), Chloramine-T (0.005 mol, 1.40 g). yellowish colour; yield: (48.8), M.F: $C_{18}H_{16}O_3N_2$; R_f: 0.82; IR:(KBr, cm^{-1}); 1035.96 (C-O-C), 3047.63 (CH Stretching), 2979.38 (CH Alkane Stretching), 1592.33 (C=C aromatic), 1623.19 (C=C alkene), 3323.22 (NH stretching), 1597.46 (NH bending), 1145.06 (C-N); ^{1}H NMR ($CDCl_3$ δppm): 3.65 (3H, O-CH_3 singlet), 4.51 (3H,

CH_2=CH- triplet), 6.76 & 8.01 (2H & 1H, aromatic doublet & singlet respectively), 7.81 & 7.3 (2H & 3H, aromatic attached to oxadizole ring doublet & triplet respectively), 1.27 (2H, -CH_2, doublet).

5f: 3-{5-[2-methoxy-4-(prop-2-en-1-yl)phenoxy]-1,3,4-oxadiazol-2-yl}-*N*,*N*-dimethylaniline

Compound 4f (0.005 mol, 1.8 g), Chloramine-T (0.005 mol, 1.40 g).Orange colour; yield: (66.3), M.F: $C_{18}H_{22}O_3N_3$; R_f: 0.48; IR:(KBr, cm^{-1}); 1151.51 (C-O-C), 3066.88 (CH Stretching), 2928.38 (CH Alkane Stretching), 1593.16 (C=C aromatic), 1662.29 (C=C alkene), 3299.82 (NH stretching), 1365.28 (C-N), ^{1}H NMR($CDCl_3$ δppm): 3.85 (3H, O-CH_3 singlet), 4.63 (3H, CH_2=CH- triplet), 7.29 & 7.63 (2H & 1H, aromatic doublet & singlet respectively), 7.75 (4H, aromatic attached to oxadizole ring doublet), 1.29 (2H, -CH_2, doublet).

5g: 2-(3-fluorophenyl)-5-[2-methoxy-4-(prop-2-en-1-yl)phenoxy]-1,3,4-oxadiazole

Compound 4a (0.005 mol, 1.7 g), Chloramine-T (0.005 mol, 1.40 g) .Buff colour; yield: (45.5), M.F: $C_{18}H_{15}O_3N_2F$; R_f: 0.41; IR:(KBr, cm^{-1}); 1073.79 (C-O-C), 3064.80 (CH Stretching), 2921.46 (CH Alkane Stretching), 1598.51 (C=C aromatic), 1651.84 (C=C alkene), 3357.22 (NH stretching), 1555.24 (NH bending), 1668.24 (C=N), 1155.53 (C-N), 1014.05 (C-F); ^{1}H NMR($CDCl_3$ δppm): 3.86 (3H, O-CH_3 singlet), 4.63 (3H, CH_2=CH- triplet), 6.72 & 8.17 (2H & 1H, aromatic doublet & singlet respectively), 7.92 (4H, aromatic attached to oxadizole ring doublet), 1.25 (2H, -CH_2, doublet), 3.02 (6H , N-CH_3 singlet).

BIOLOGICAL ACTIVITY

Anti-inflammatory activity

Human red blood cell stabilization method *(in-vitro)* [18]

Preparation of HRBC Suspension:

Blood was collected from healthy rats which are not administered with any NSAIDS for 2 weeks prior to the experiment. The collected blood was mixed with equal volume of sterilised alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and0.42% NaCl in water) and centrifuged at 3,000 RPM. The packed cells were washed with isosaline (0.85%, pH 7.2) and a 10% (v/v) suspension was made with isosaline.

Preparation and Estimation

Various concentrations of synthesized compounds were prepared (5, 10, 20, 40, 80 and 100 µg/ml) using DMSO and to each concentration 1 ml of phosphate buffer (0.15M, pH 7.4), 2 ml of hyposaline (0.36%) and 0.5 ml of HRBC suspension were added. Incubated at 37^{0}C for 30 min and centrifuged at 3,000 RPM for 20 min. The haemoglobin content in the supernatant solution was estimated spectrophotometrically at 560 nm. Eugenol of various concentrations (5, 10, 20, 40, 80 and 100 µg/ml) was used as reference standard and a control was prepared omitting the drugs.

The percentage of haemolysis was calculated using the formula

$$\% \text{ haemolysis} = \frac{OD1}{OD2} X100$$

Where, OD1= optical density of test sample/ absorbance

OD2 = optical density of control/absorbance

Carrageenan induced rat paw edema method [17,19]

Wistar albino rats and Swiss albino mice of either sex weighing between 150-200 g and 20-30 g respectively were procured from the animal house of the drug technical laboratory, Bengaluru, Karnataka. The experiments were conducted as per the guidelines of CPCSEA, Chennai, India (185/CPCSEA) and Institutional ethical committee clearance no. (DCD/GCP/20/E.C/ADM/2018-2019) dated 04.06.2018 As per OECD guidelines number 425 the dose of 200 mg/kg (1/10th of 2000 mg/kg) was selected for evaluation of anti-inflammatory activity. [35] The suspension of the synthesized compounds was prepared by using 2 % acacia in distilled water and administered orally to overnight fasted albino rats and after one hour, 0.1 ml of 1% carrageenan in 0.9% saline was injected subcutaneously in to the right hind paw. The paw was marked with ink at the level of lateral malleolus and immersed in mercury up to the mark. The initial paw volume was measured and recorded as 0 h reading. Thus the hind paw volume was measured at 1st h interval up to 3rd h of experiment. The difference between paw volumes at various time intervals indicates the edema volume due to Inflammation. The %

inhibition produced by the drug and derivatives were calculated by following formula.

$$\% \text{ inhibition of edema } = 100 - \frac{Vt}{Vc} X100$$

Where, Vc =The average paw volume in control group
Vt=The average paw volume in treated group

Antibacterial activity: cup plate method [5,6,20]

The synthesized compounds were screened for their antibacterial activity by cup plate method. The response of the organism to a synthesized compound was compared with response to eugenol of known concentration. This determination indicates whether the organism is sensitive or resistant or intermediate to the compounds. In general, the organism sensitive means infection treatable with normal dosage of the drug (an antibiotic) intermediate means infection may respond to therapy with higher dosage, resistant means unlikely to respond to the drug at the usual dosage. *Staphylococcus aureus* gram positive and *Pseudomonas aeruginosa* gram negative organism were choosed for screening antibacterial activity of the synthesized compounds.

Preparation of subculture

Subculture was prepared by the serial dilution method to obtain the pure culture of microorganism. 2.1 g of nutrient agar was dissolved in 54 ml of distilled water by heating. 9 ml of nutrient agar was added to each six culture tubes and were named as 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6. Culture tubes were sterilized by autoclave for 121 ^{0}C at 15 lb/inch^2 for 15 min. After sterilization, 1 ml of microorganism was added to the 10^1 sterilized liquid medium and shaked well. From this 1 ml of microorganism was removed and it was added to 10^2 fresh sterile liquid medium. Processes were repeated up to 10^6 sterile liquid medium. Finally, inoculated culture tubes were incubated at 37±1°C for 12 h.

Procedure

Muller Hinton Agar media was prepared by dissolved 7 g of Muller Hinton Agar in 180 ml of distilled water by heating under stirring. Then media was sterilized by autoclave for 121 ^{0}C at 15 lb/inch^2 for 15 min. The pH was maintained at 25 ^{0}C to 7.4±0.2. 60 ml of Muller Hinton Agar media was poured into the sterilized Petri plates and allowed to solidify. Sterilized cotton bud was dipped into a bacterial culture and swiped on solidified agar Media. On solidification was bored with the help of the 8 mm diameter and the analogues synthesized were added to the bore at 800 µg/ml (48 µg/ 60 µl) concentration. Eugenol was also placed in all the plates for comparison at 800µg/ml (48 µg / 60 µl). The culture plates were incubated at 37±1 ^{0}C. The plates were observed after 8 h of incubation and continued to observe for 12 h. Zone of inhibition were measured and compared with eugenol.

RESULT

Chemistry

Isolated Eugenol upon reaction with ethyl chloroacetate in the presence of acetonitrile gave ethyl [2-methoxy-4-(prop-2-en-1-yl) phenoxy] acetate (2). This on further reaction with hydrazine hydrate yielded 2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy] acetohydrazide (3). Further acetohydrazide (3) made to react with benzaldehyde/ substituted benzaldehyde to yield Schiff's bases of Substituted *N*'-benzylidene-2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy] acetohydrazide (4a-4g). These Schiff base compounds were then cyclized in the presence of Chloramine-T as promoter and obtained the series of 1,3,4-oxadiazoles derivatives that is Substituted 2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy]-5-phenyl-1, 3, 4-oxadiazole (5a-5g). The derivatives were obtained in appreciable yield which were purified by recrystallization from ethanol. The purity of derivatives were confirmed by single spot on TLC plates. Spot was detected by UV chamber at 254 nm. The synthesized compounds were characterized by IR and ^{1}H NMR. All the synthesized compounds show characteristic absorption peak in IR and ^{1}H NMR spectra. The synthesized compounds were screened for their anti-inflammatory (*in-vivo* and *in-vitro*) and antibacterial activity.

***in-vitro* anti-inflammatory activity**

The synthesized compounds were evaluated for their *in-vitro* anti-inflammatory activity by membrane stabilization method at different

concentrations. All the newly synthesized compounds were found to possess excellent anti-inflammatory activity than eugenol. Among the studied compounds electron withdrawing group substituted 5b showed highest anti-inflammatory activity. The results are presented in Table 2. The *in-vitro* studies are in correlation with the *in-vivo* studies.

Table 2: Percentage haemolysis activities of 5a-5g and Positive control (eugenol)

	% Haemolysis							
Concentration	**PC**	**5a**	**5b**	**5c**	**5d**	**5e**	**5f**	**5g**
5	98.02	96.7	96.84	96.7	97.5	96.9	97.13	96.87
10	95.7	93.4	93.1	93.63	94.6	94.1	94.53	93.88
20	91.8	88.6	86.8	87.5	90.8	86.5	89.71	87.76
40	82.5	73.8	70.7	74.8	77.85	74.2	79.16	75.52
80	67.4	47	44.2	48.7	55.8	55.72	58.64	54.68
100	60.5	34.6	29.7	36.2	47.2	42.2	51.59	38.93

***in-vivo* anti-inflammatory activity:**

In the present study, the newly synthesized compounds were screened for their *in-vivo* anti-inflammatory activity in experimental model of carrageenan induced paw edema method. 200 mg/kg doses were given orally to the 150-200 g body weight rats. Treatment with synthesized compounds shows decreased inflammation of rat paw edema when compared to the standard eugenol. This indicates that all the compounds possess good anti-inflammatory activity than eugenol. Among the studied compounds electron withdrawing group substituted 5b showed highest anti-inflammatory activity. The results are presented in Table 3. The *in-vivo* studies are in correlation with the *in-vitro* studies.

Table 3: Data showing the mean edema of rats at various time intervals

Treatment group	**Paw volume with inflammation**			**%inhibition**
	At 0th hr (ml)	**At 1st hr (ml)**	**At 3rd hr (ml**	
Positive control	0.65±0.01	0.78±0.01	0.98±0.02	-
Eugenol	0.63±0.01	0.61±0.01	0.58±0.01	40.8
5a	0.54±0.01	0.43±0.02	0.31±0.01	68.37
5b	0.52±0.01	0.35±0.01	0.26±0.01	73.46
5c	0.57±0.01	0.47±0.01	0.39±0.02	60.2
5d	0.60±0.01	0.53±0.02	0.48±0.01	48.97
5e	0.59±0.01	0.51±0.01	0.45±0.01	54.08

*Each value represented the mean ± SEM 6 for each group. *p is 0.0353 (on way ANOVA) and is found to be significant (< 0.05).*

Anti-bacterial activity

The newly synthesized compounds (800 µg/ml) were screened for *in-vitro* antibacterial activity by cup plate method against gram-positive *Staphylococcus aureus* and gram-negative *Pseudomonas aeruginosa*. DMSO was used as control. Anti-bacterial activity of tested compounds was compared with standard eugenol

and zone of inhibition measured in terms of mm. All the synthesized compounds were exhibited excellent antibacterial activity. Among those electron donating substitution (5a, 5b, 5c, 5g) and compound without substitution showed good antibacterial activity when compared to electron donating substitution (5d, 5f). However, the compound **5b** showed highest antibacterial activity. The results are presented in Table 4.

Table 4: Zone of inhibition (in mm) data of synthesized compounds (5a-5g)

Compounds	Zone of inhibition in mm at Conc. of 48 µg / 60 µl	
	Staphylococcus aureus	***Pseudomonas aeruginosa***
Control (DMSO)	0	0
Eugenol	16	18
5a	36	42
5b	45	46
5c	36	42
5d	29	27
5e	33	30
5f	23	24
5g	34	36

DISCUSSION

The Clove buds were purchased from the Amruth kesari, Bengaluru and authenticated by Regional Ayurveda Research institution for Metabolic Disorders, Bengaluru. Authenticated clove buds were used for the extraction of clove oil by hydro distillation using Clevenger's apparatus. Yellow viscous clove oil obtained was used to isolate eugenol by gradient elution of column chromatography using silica gel mesh size 230-400 as stationary phase and different ratio of hexane: ethyl acetate as mobile phase. The isolated eugenol was confirmed by TLC and R_f value was found to be 0.64. The percentage purity of eugenol was determined by HPTLC and concentration of eugenol present in the sample was found to be 83.1 mg/g. The structure of eugenol was identified by the presence of OH, C-O-C, CH stretch, CH alkane stretch, CH aromatic stretch, C=C aromatic and C=C alkene at 3525.40, 1032.35, 3003.86, 2938.13, 3076.78, 1510.91 and 1606.84 respectively in IR spectra. Identified eugenol was further used for the synthesis of semisynthetic derivatives of 1,3,4-oxadiazole.

Eugenol was used as a starting material for the synthesis of 1,3,4-oxadiazole derivatives (5a-5g) by a series of reaction (scheme). Eugenol (1) was made to react with ethyl chloroacetate to obtain ethyl [2-methoxy-4-(prop-2-en-1-yl) phenoxy] acetate(2). This structure was identified by the elimination of OH group of eugenol at 3525.40 cm^{-1} and appearance of ester group at 1757.34 cm^{-1} peak in its IR spectra.

The compound (2) was reacted with triple the quantity of hydrazine hydrate in the presence of alcohol to form 2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy] acetohydrazide (3). The structure was confirmed by the appearance of –NH stretch of acetohydrazide at 3443.36 cm^{-1} and 1598.09 cm^{-1} in its IR spectra. This hydrazide (3) was treated with benzaldehyde/ substituted benzaldehyde [4-Cl, 4-NO_2, 4-Br, 4-OH, 4-$(CH_3)_2NH$, 4-F] to yield corresponding Substituted *N*'-benzylidene-2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy] acetohydrazide (4a-4g). Appearance of C=N stretch at 1360-1080 cm^{-1} in their IR spectra.Compounds (4a-4g) undergoes oxidative cyclization in the presence of Chloramine-T to form respective Substituted 2-[2-methoxy-4-(prop-2-en-1-yl) phenoxy]-5-phenyl-1,3,4-

oxadiazole(5a-5g). The appearance of sharp peak at 1360-1080 cm^{-1} corresponds to C=N and peak at 1300-1000 cm^{-1} for CO in 1,3,4-oxadiazole ring identified their formations.

Further the derivatives formed were confirmed by ^{1}HNMR. It showed a singlet at δ 3.3.-3.8 (3H, -OCH_3) indicates the presence of methoxy group. Triplet at 4.5-6.5 (3H CH_2=CH-) indicate the presence of allyl group. Doublet (6H) and singlet (1H) at 6.5-8.5 indicates the presence of aromatic ring. Doublet at 1.2-1.6 (2H –CH_2-) indicate -CH_2- attached to CH. All these facts confirm the formation of compounds (5a-5g).

In-vivo anti-inflammatory activity was carried out for the newly synthesised compounds (5a, 5b, 5c, 5e, 5f) by carrageenan induced paw edema method. Treatment with synthesized compounds shows decreased inflammation of rat paw edema when compared to the standard eugenol. From these results we can say that all the compounds possess good anti-inflammatory activity.

Also compounds 5a-5g were screened for their possible anti-inflammatory activity by HRBC method. Among the tested compounds electron withdrawing group bearing (Cl, NO_2, Br, F) compound (5a, 5b, 5c, 5g) and without substitution (5e) at para position of phenyl ring attached to 1,3,4-oxadiazole motif at concentration of 5, 10,20,40,80 and 100 µg/ml doses showed better anti-inflammatory activity when compared to the eugenol and electron donating substitution (5d, 5f) at the same concentration. Where the electron donating substitution showed less anti-inflammatory activity when compared to electron withdrawing substitution and more anti-inflammatory activity when compared to standard eugenol. From these results we can conclude that the compounds with either electron withdrawing or electron donating group at para position has less hemolysis activity (more membrane stability) and hence greatest anti-inflammatory activity when compared to eugenol.

Antibacterial activity was performed by cup plate method. The zone of inhibition was compared with eugenol and expressed in mm. the synthesized compounds were screened at 800 µg/ml (48 µg/60µl) against gram positive *Staphylococcus aureus* and gram negative *Pseudomonas aeruginosa*. All the newly synthesized compounds shows highest zone of inhibition against organisms tested when compared to standard eugenol. From these results we can conclude that both electron withdrawing and donating substituted compounds influence zone of inhibition means compounds have antibacterial property.

CONCLUSION

The results of anti-inflammatory (*in-vivo* and *in-vitro*) and antibacterial activity reveal that the compounds 5a, 5b, 5c, 5f with electron withdrawing substitution Cl, NO_2, Br, F at Para position of phenyl attached to 1,3,4-oxadiazole and compound 5e without substitution have maximum anti-inflammatory and antibacterial activity compared to electron donating substitution and standard eugenol. Among this electron withdrawing group (NO_2) bearing compound 5b shows the highest anti-inflammatory and antibacterial activity. Electron donating group bearing compounds 5d and 5f showed less anti-inflammatory and antibacterial activity when compared to electron withdrawing compounds and more activity when compared to standard eugenol. From these results of anti-inflammatory and antibacterial activity finally it can be concluded that all the newly synthesized compounds having synergistic activity. However further studies need to be carried out with respect to their toxicity profiles in order to exploit them for clinical uses.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this research article.

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