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Synthesis of eugenol derivatives and its anti-inflammatory activity against skin inflammation

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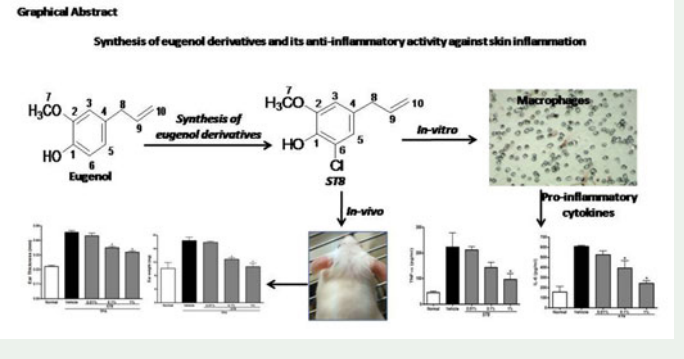
ABSTRACT

Eugenol is a phytochemical present in aromatic plants has generated considerable interest in the pharmaceutical industries mainly in cosmetics. A series of eugenol esters (ST1-ST7) and chloro eugenol (*ST8*) have been synthesized. The structures of newly synthesized compounds were confirmed by ^{1}H and ^{13}C NMR and mass spectrometry. In an effort to evaluate the pharmacological activity of eugenol derivatives, we explored its anti-inflammatory potential against skin inflammation using *in-vitro* and *in-vivo* bioassay. Synthesized derivatives significantly inhibited the production of pro-inflammatory cytokines against LPS-induced inflammation in macrophages. Among all derivatives, *ST8* [Chloroeugenol (6-chloro, 2-methoxy-4-(prop-2-en-1-yl)-phenol)] exhibited most potent anti-inflammatory activity without any cytotoxic effect. We have further evaluated the efficacy and safety in in-vivo condition. *ST8* exhibited significant anti-inflammatory activity against TPA-induced skin inflammation without any skin irritation effect on experimental animals. These findings suggested that *ST8* may be a useful therapeutic candidate for the treatment of skin inflammation.



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1. Introduction

Essential oils and its constituents have received special attention as a source of potentially useful bioactive compounds in pharmaceutical and cosmeceutical industries due to their pharmacological properties (Polzin et al. 2007). Eugenol, 2-methoxy-4-(2-propenyl) phenol is a phytochemical obtained from *Syzigium aromaticum, Ocimum Sanctum* and Perry (Daniel et al. 2009), widely known as clove it is native to tropical Asia (Polzin et al. 2007) and cultivated in different regions of the world (Agra et al. 2008). Eugenol is used in folk medicine for its biological properties, such as antimicrobial, antifungal, antioxidant (Chainy et al. 2000; Zhang et al. 2017), anesthetic, antiseptic (Daniel et al. 2009), anticarcinogenic (Zheng et al. 1992), anti-nociceptive, anti-inflammatory activities (Fonsêca et al. 2016), antiviral, and antiallergic (Kim et al. 2001) effects. It is also used in the food industry as a flavoring agent (Hatami et al. 2010). Eugenol derivatives synthesized by esterification reactions in the hydroxyl group with different carboxylic acids as well as semi-synthetic derivatives of eugenol through catalytic oxychlorination has shown potential antibacterial activities (da Silva et al. 2018; Pinheiro et al. 2018). Eugenol isolated from aromatic crops has been reported for promising pharmacological activities and this has prompted us to synthesize its derivatives to investigate its therapeutic effect. In the present study, we have synthesized the eugenol esters (ST1-ST7) and chloro eugenol (*ST8*) to examine its therapeutic effect on inflammatory response with special emphasis in skin inflammation using in-vitro and in-vivo bioassays. Skin is well known for its functional role as a protective physical barrier and dynamic organ that has some other recognized functions, such as endogenous homeostasis, metabolism, and sensory input. In addition, skin actively participates in Immunological regulatory processes and inflammatory responses (Bos 1997). Inflammation is a key molecular mechanism which includes skin disorders (Kong and Xu 2018), inflammatory skin diseases have a significant impact on the quality of life of human being and represent an enormous financial trouble. However, some inflammatory or immunological reactions lead to chronic inflammation processes, such as psoriasis or to intolerable skin inflammation conditions, such as contact dermatitis, which requires medication (Rauh et al. 2011). During inflammation, the inflammatory region is infiltrated with mononuclear cells such as monocytes, macrophages and lymphocytes, producing a wide range of Inflammatory mediators, such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and cyclooxygenase-2 (COX-2) play important roles in inflammatory diseases (Libby et al. 2002; Bertolini et al. 2004). Atopic dermatitis (AD) is a chronic or chronically relapsing, pruritic inflammatory skin disease (Kim et al. 2018). Topical application of TPA has been used to screen for topically applied anti-inflammatory steroids and non-steroid agents and events of the inflammatory processes such as edema, cell infiltration, and proliferation. Our results demonstrated that, among all synthesized derivatives, *ST8* [Chloroeugenol (6-chloro, 2-methoxy-4-(prop-2-en-1-yl)-phenol)] exhibited most potent anti-inflammatory activity without any cytotoxic effect in the in-vitro study. We have further evaluated the efficacy and safety in *in-vivo* condition. *ST8* exhibited significant anti-inflammatory activity against TPA-induced skin inflammation without any skin irritation effect on experimental animals.

2. Results and discussion

2.1. Chemistry

An ester is a chemical compound derived from an acid or its chloride (organic or inorganic) in which at least one –OH (hydroxyl) group is replaced by an –O–alkyl (alkoxy) group. Esters of eugenol derivatives were prepared from respective acid chlorides reagent such as acetyl, propinyl, valeryl, isovaleryl, palmitoyl, lauryl, benzoyl in presence of pyridine and sulphuric acid at room temperature or heating as per preparation required of different derivatives. Isolation of eugenol (EUG) and its synthetic approach of all eugenol derivatives such as eugenyl valerate (ST1), eugenyl isovalerate (ST2), eugenyl palmitate (ST3), eugenyl propionate (ST4), eugenyl acetate (ST5), eugenyl laurate (ST6), eugenyl benzoate (ST7) and chloroeugenol (*ST8*) are well written in experimental section of the paper.

2.2. Pharmacology

All the synthesized eugenol derivatives at 0.01, 0.1 and 1% concentration were evaluated for their anti-inflammatory status against the production of pro-inflammatory cytokines (TNF- α, IL-6) using ELISA technique in LPS-induced inflammation in macrophage cells. Production of pro-inflammatory cytokines was significantly ($p < 0.05$) increased in vehicle-treated LPS-stimulated cells when compared with normal un-stimulated cells. All the derivatives exhibited inhibition of pro-inflammatory cytokines production when compared with vehicle-treated LPS-stimulated cells (Supplementary file Table S1). Among all derivatives, *ST8* (Chloroeugenol) possessed most potent derivatives by significant inhibition of TNF-α, and IL-6 without any cytotoxic effect as assessed by MTT assay. Several previous studies reported that molecules derived from essential oil reduce the production of inflammatory mediators by inhibiting the activation of the inflammatory signaling pathway (Maurya et al. 2014). These pro-inflammatory cytokines are the mediators of various chronic inflammation linked diseases including skin inflammation (Maurya et al. 2018). To substantiate the physiological function of *ST8*, a most potent derivative; we have further evaluated the therapeutic efficacy and safety profile *in-vivo* system using TPA-induced skin inflammation and primary skin irritation study in mice and rabbits respectively. Topical application of *ST8* on TPA-induced skin inflammation in the ear of mice exhibited the significant reduction in ear thickness, edema, ear weight (Figure 1) as well as pro-inflammatory biomarkers in ear tissue homogenate (Figure 2) in a dose-dependent manner. Experimental evidence has shown that exposure of skin to 12-O-tetradecanoyl phorbol-13-acetate (TPA) induces a pleiotropic tissue response encompassing a strong inflammatory reaction similar to that observed in skin diseases (Kondo et al. 2000). Our observations are similar to the previous findings that essential oil can reduce the severity of skin inflammation (Boukhatem et al. 2013; Blaskovic et al. 2014). Edema and increased skin thickening are the indicative parameters of increased vascular permeability, edema and swelling within the dermis, and proliferation of the epidermal keratinocytes during skin inflammation process (De Vry et al. 2005). It is widely recognized that the secretions of cytokines by keratinocytes in response to injury are key mediators of the cutaneous inflammatory response (Murphy et al. 2000). Primary skin irritation study in rabbits revealed that *ST8* is safe for topical application on skin. Previous reports also concluded that essential oils from aromatic plants are not

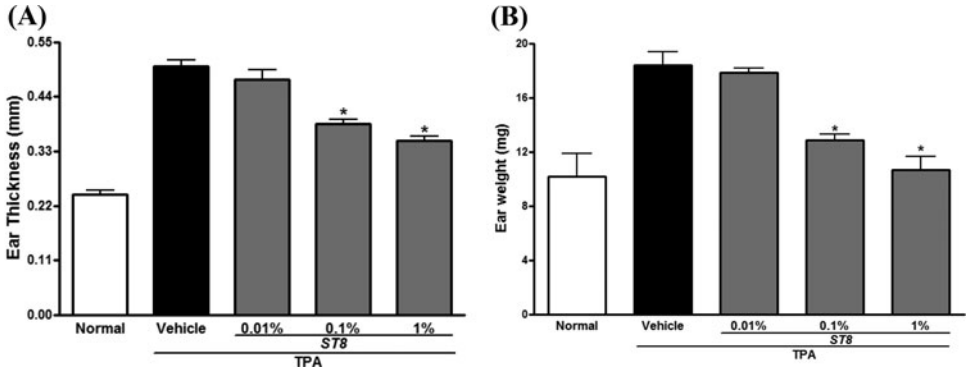

Figure 1. Dose-response effect of ST8 on TPA-induced inflammatory ear swelling in BALB/c mice (A) Change in ear thickness (B) Ear weight. Results are Mean ± SEM; n = 6; * $P < 0.05$ (Vehicle vs Treatment).

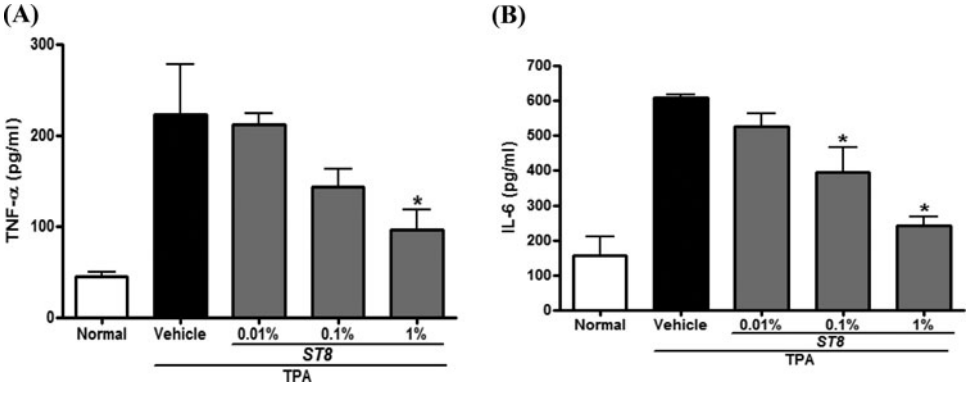

Figure 2. Effect of ST8 on inflammatory mediators on TPA-induced ear inflammation in BALB/c mice (A) Ear homogenate TNF-α and (B) Ear homogenate IL-6. Data are expressed as Mean ± SEM; n = 4; * $P < 0.05$ (Vehicle vs Treatment).

irritating to mammalian skin and safe for topical application (Yadav et al. 2013; Liu et al. 2015). Skin Irritation test was conducted to determine the primary skin irritation on rabbit skin. 72 h after the application of *ST8* on rabbit skin. Significant erythema and edema formation were not observed in *ST8* treatment site compared to the vehicle-treated site. Pigmentation, blood oozing, and rough skin were also not observed in both the control and treatment site. According to Federal Hazardous Substances Act (FHSA) regulations, a material with a PII of less than 5.00 is generally not considered a primary irritant to the skin. The PII result concluded that the application of *ST8* is not irritant to the rabbit skin.

3. Experimental section

3.1. Chemistry

3.1.1. Distillation & isolation of eugenol

Eugenol was isolated from the essential oil of *Ocimum sanctum* variety CIM-Ayu having eugenol content of about 54-60%. 200 kg of fresh herb of the plant was steam

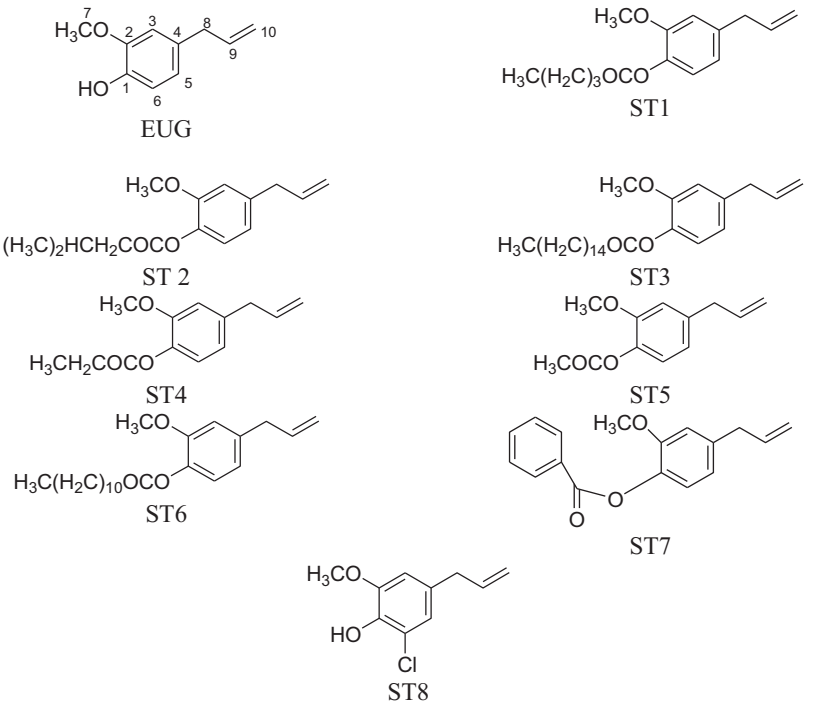

Figure 3. Structure of eugenol derivatives.

distilled in a boiler operated steam distillation unit to obtain 750 ml of essential oil. The oil was treated with anhydrous sodium sulphate to remove any moisture in the oil. 500 ml of the oil was then fractionated in a packed fractionating column of 40 mm dia x 1200 mm height packed with hyflux wire mesh packing. Fractionation was carried out under the vacuum of 5-8 mm Hg. Fractionation data is given in Table S2. Four major fractions were collected in which 253 ml eugenol of 97.2% purity was obtained which was further chromatographed over silica gel using hexane as the eluting solvent to achieve 99% purity of eugenol. The purity of eugenol was confirmed by GC. The isolated eugenol was stored under sub-zero temperatures till further experiments.

3.1.1.1. Synthesis of esters and chloro eugenol. Structure of esters and chloro derivatives of eugenol are depicted in Figure 3 and spectroscopic data and spectral figures of ^{1}H and ^{13}C NMR are given in the Supplementary file.

Eugenyl valerate (ST1): To a solution of eugenol 10 g (60.97 mmol), valeryl chloride 25 g (150.33 mmol) and 1 ml pyridine added was left at room temperature for overnight and then refluxed over the water bath for 1 hr. The reaction mixture was cooled to room temperature, diluted with water and extracted with chloroform (3 x 25 ml). The organic layer was washed with water, dried over sodium sulphate and evaporated to dryness. The crude reaction product was purified by a silica gel column and eluted with hexane/chloroform as a liquid (purity 97.5%); Yield 72%.

Eugenyl isovalerate (ST2): In a round bottom flask, 10 gm (60.97 mmol) of eugenol was dissolved in chloroform. To it isovaleryl chloride 25 gm of (150.33 mmol)

and 5 ml pyridine added. The reaction mixture was refluxed over a water bath for 2 hr. The progress of the reaction was monitored using TLC in hexane ethyl acetate. After the completion of the reaction, the reaction mixture was worked up using water and chloroform. The organic layer was then separated, dried over anhyd. sodium sulphate and concentrated till dryness. The crude reaction product was purified by silica gel column and eluted with hexane/ethyl acetate as a liquid (purity 96.5%), Yield 79.0%.

Eugenyl palmitate (ST3): Eugenol 10 g (60.97 mmol), palmitoyl chloride 25 g (90.90 mmol) and 5 ml pyridine was taken in a round bottom flask and refluxed for 1 hr. The reaction mixture was cooled to room temperature, diluted with water and extracted with chloroform (3 x 25 ml). The organic layer was washed with water, dried over sodium sulphate and evaporated to dryness. The crude reaction product was purified by silica gel column and eluted with hexane/ethyl acetate as a liquid (purity 98.5%); Yield (69%), Liquid.

Eugenyl propionate (ST4): 10 g (60.97 mmol) of eugenol was taken in a round bottom flask, 10 g (108.08 mmol) of propinyl chloride was added to the solution of Eugenol. In this mixture, 10 ml of pyridine was added dropwise. After the addition of pyridine the reaction mixture began to solidoify. The reaction mixture was left at room temperature for 2 hrs. After completion of the reaction, Reaction mixture was diluted with water and extracted with chloroform (3 x 25 ml). Organic layer was washed with water, dried over sodium sulphate and evaporated to dryness. The crude reaction product was purified by silica gel column and eluted with hexane/ethyl acetate as a liquid (purity 98.5%); Yield 78%.

Eugenyl acetate (ST5): To a solution of Eugenol 5 g (30.48 mmol), acetyl chloride 12.5 g (126.58 mmol) and 2.5ml of sulphuric acid were added. The reaction mixture was then refluxed for 2-3 hr. the progress of the reaction was monitored using TLC. After the completion of the reaction, the reaction mixture was cooled to room temperature, diluted with water and extracted with chloroform (3 x 25 ml). The organic layer was washed with water, dried over sodium sulphate and evaporated to dryness. The crude reaction product was purified by a silica gel column and eluted with hexane/ethyl acetate as a solid (purity 98.1%); Yield 79%.

Eugenyl laurate (ST6): Eugenol 5 g (30.48 mmol), lauryl chloride 15 g (73.17 mmol) and 2.5ml of sulphuric acid was taken in a Round bottom flask and refluxed for 2-3 hrs. After the completion of the reaction, the reaction mixture was cooled to room temperature, diluted with water and extracted with chloroform (3 x 25 ml). The organic layer was washed with water, dried over sodium sulphate and evaporated to dryness. The crude reaction product was purified by a silica gel column and eluted with hexane/ethyl acetate as a liquid (purity 97.3%); Yield 74%.

Eugenyl benzoate (ST7): To a solution of eugenol 5 g (30.48 mmol), benzoyl chloride 15 g (60.14 mmol) was added. To this reaction mixture 2.5ml of sulphuric acid was added dropwise and the reaction mixture was refluxed for 2- 3 hrs. After the completion of the reaction, the reaction mixture was worked up using Water and Chloroform. The crude reaction product was purified using a column chromatography in a silica gel (60-120 mesh) which yielded a pure solid compound in hexane/ethyl acetate; Yield 71%.

Chloroeugenol (ST8): In a round bottom flask, 10 g (60.97 mmol) of eugenol was dissolved in 20 ml of carbon tetrachloride. 8 g (59.25 mmol) of sulphuryl chloride was added to this reaction mixture dropwise. The reaction mixture was left at room temperature for 11-12 hrs and the progress of the reaction was monitored using TLC. After the completion of the reaction, the reaction mixture was diluted with water and extracted with chloroform (3 x 25 ml). The organic layer was washed with water, dried over sodium sulphate and concentrated under reduced pressure. The crude reaction product was purified by a silica gel column and eluted with hexane/ethyl acetate as a liquid (purity 96.5%); Yield 78%.

3.2. Pharmacology

3.2.1. In-vitro *study*

The macrophage cells were isolated from the peritoneal cavities of female Swiss albino mice after an intra-peritoneal injection of 1.0 mL of 1% peptone (BD Biosciences, USA) 3 days before harvesting. Culture macrophage cells were pre-treated with 0.01, 0.1 and 1% of synthesized derivatives of eugenol (ST1-8). The cells were stimulated with LPS (0.1μg/mL) for 24 h and collected supernatants were immediately frozen at -80°C. Pro-inflammatory mediators (TNF-α, and IL-6) were quantified from the supernatant using ELISA method according to the manufacturer's instructions (BD Biosciences, USA). The values of TNF-α and IL-6 were expressed as pg/mL. *ST8*, a most potent lead were further evaluated check its effect on cytotoxicity using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay.

3.2.2. In-vivo *study*

BALB/c Mice (25–35 g) and New Zealand white rabbits were used for efficacy and toxicity study, respectively. Experiments were performed according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

After 1 week of adaptation, the mice were randomly divided into five groups of four mice each. Topical inflammation was induced in the experimental mice except for the normal group of mice by the topical application of TPA (2.5μg). *ST8* was dissolved in acetone and administered topically for 30 minutes before application of TPA. Acetone alone was applied as the vehicle to normal and vehicle-treated mice group. The experimental animals were divided as follow

Group 1: Normal control

Group 2: Vehicle control + TPA

Group 3: *ST8* (0.01%) + TPA

Group 4: *ST8* (0.1%) + TPA

Group 5: *ST8* (1%) + TPA

TPA-induced skin inflammation was maximum at 6 hours. The area and degree of ear inflammation were assessed in order to calculate a macroscopic score of ear inflammation. A macroscopic score between 0 and 7 was attributed to all mice ears. Score 0-1 meant no skin inflammation. Skin inflammation was scored between 2-3 when very slight, 4-5 when moderate, and 6-7 when severe as described by Maurya et al. 2018 (Supplementary file; Figures S3 and S4). Edema was expressed as the increase in ear thickness due to the inflammatory challenge. Ear thickness was measured before and after the induction of the inflammatory response by using an electronic digital micrometer (Aerospace Instruments).

At 6 hours, when TPA-induced inflammation was maximal, animals were euthanized by ether asphyxiation and 1 cm diameter punch of ear tissue wet weight was taken for quantification of inflammatory mediators from ear tissue homogenate. Production of pro-inflammatory cytokines (TNF-α and IL-6) were quantified from tissue homogenate using commercially available mouse specific enzyme immune assay (EIA) kits (BD Biosciences, USA) as per the manufactures instruction. To study the skin toxicity effect, primary skin irritation study was performed for the topical application of *ST8* in four healthy rabbits (Observations were made at 1, 4, 24, 48 and 72 h to assess individual erythema and edema. The primary irritation index (PII) was determined using the following formula; PII = Test site score − Control site score.

4. Statistical Analysis

Data were expressed as Mean ± SEM. For statistical analysis, one-way ANOVA followed by Tukeys test was used. Probability (P) values less than 0.05 were considered significant.

5. Conclusion

The results of the study demonstrated that topical application of eugenol and its derivatives are effective as an anti-inflammatory agent against TPA-induced skin inflammation and among all derivatives, *ST8* [Chloroeugenol (6-chloro, 2-methoxy-4-(prop-2-en-1-yl)-phenol)] was most potent. This study suggests the suitability of eugenol and its derivatives in skin care formulations for pharmaceutical purposes with special reference to skin inflammation.

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Disclosure statement

The authors declare that there is no conflict of interests regarding the publication of this paper.

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