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# Antinociceptive Activity of *Syzygium Aromaticum* Linn. Flower Buds (Clove) Extract and its Fractions

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**Summary***: Syzygium aromaticum* (*Myrtaceae*) flower buds (clove) are traditionally known to possess antimicrobial, antiprotozoal, antiviral and other activities, including the antinociceptive action. Scientific evidence also suggests the antileishmanial, antiherpetic and anti-HIV activities of the buds. This paper reports the antinociceptive activity of *S. aromaticum* flower buds, reinforcing its use in decreasing the pain. The present study was designed to confirm the analgesic activity of *S. aromaticum* extract and seven of its fractions to reveal the common belief in its painkilling effects. We chose two thermal nociception assays (i) hot–plate test (ii) and tail-flick method as our experimental techniques. Both of these methods are well established to screen anti-nociceptive activities in new molecules. The standard drug indomethacin (5 mg/kg) given by intra-peritoneal route was used in the study for comparison. The study has shown that the methanolic extract (SA-EXT) and its active fractions possess anti-nociceptive activity ($p<0.05$) in the models of nociception used.

**Key words:** *Syzygium aromaticum*, Clove, Anti-nociceptive activity, Thermal nociception assays

## Introduction

Existing therapy for pain such as the use of non-steroidal anti-inflammatory drugs (NSAIDs) and opiates often produce harmful effects (specially the development of tolerance through opiates) making these drugs unsuitable for chronic pain management. The pharmacological effects of NSAIDs are variable depending upon various clinical conditions. The available analgesic drugs are either too potent for use (e.g. meloxicam, ketorolac used in severe pains) or exhibit weak analgesic activity (e.g. paracetamol having anti-pyretic action). Moreover, few of the drugs have limited use in therapeutics (e.g. diflunisal), since they are useful in specified pains only, such as dental pains. Additionally, various side effects such as increased GI ulceration, worsening of bleeding, hypertension, nephrotoxicity, increased respiratory allergy etc. are also associated with the use of NSAIDs (1-4). All these provide sufficient rationale to explore new drugs for pain. Plants having therapeutic effects are known sources for new chemical entities with possible curative effects since long [5-6]. Exploration of plants with conventional pain relieving effects is therefore a reliable way to find new analgesic drugs [7].

*Syzygium aromaticum* (Linn.) Merr. & L. M. Perry (Syn. *Caryophyllus aromaticus, Eugenia aromatica, E. caryophyllata*) commonly known as clove tree belongs to the family *Myrtaceae*, and is endemic in the North Moluccas (Indonesia). Dried unopened floral buds, known as cloves, are used as spice. Clove has been used for the treatment of various human ailments showing cardiotonic, antimalarial, anticancer, aphrodisiac, antioxidant, antihistaminic, antirheumatic, antineuralgic, antibiotic and spasmolytic activities [8]. It has also been used as counterirritant, stomachic, antiemetic, and vermifuge. Eugenol is the active constituent of clove oil that has been utilized in dentistry since long and acts as germicide, antiseptic, local analgesic and local anesthetic when applied in tooth decay [9]. Eugenol also possesses stimulating expectorant effects against respiratory problems such as phlegm production and bronchial infections [10-18]. Previous phytochemical investigation [19] of *S. aromaticum* have resulted in the isolation of pentacyclic triterpenoids e.g. oleanolic acid, oleanolic acid lactone, 2$\alpha$-hydroxy oleanolic acid, 11-oxo-oleanolic acid [20], polyphenols e.g. ellagic acids and ellagitannins [21], steroids e.g. $\beta$-sitosterols, stigmasterol and $\beta$-sitosterol glucoside [22], chromones e.g. eugenin and eugenitin [21], flavanoids e.g. nigricin and kampferol [20, 23]. Clove oil is a complex mixture of variety of compounds including mono and sesquiterpenes, alcohol, aldehyde, esters, acetophenone etc such as eugenol and caryophyllene [24]. Pharmacological reports revealed that oleanolic acid possesses antidiabetic activity [25]. Few more clove bioactive constitutes such as kaempferol, rhamnoctrin and myricetin compounds possess growth inhibitory activity against oral pathogens [23]. Eugeniin, casuarictin, 1,3-di-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-$\beta$-D-glucopyranose, and tellimagrandin I and two chromones isobiflorin and biflorin possess inhibitory activity on the syncytia formation [26]. A study showed that clove extract is the basic component of formulations used as analgesic and anti-inflammatory agents to promote circulation when applied externally [27].

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Additional studies revealed that the clove oil diminished the chronic hyperglycemia-induced oxidative tissue damage and cataract formation in the eye lense of rats that was resulted due to continuous hyperglycemic condition [28], in addition to strong analgesic and anti-inflammatory activities *in vivo* [29-30]. Studies focused on investigating the cytotoxicity of *S. aromaticum* reported it to be safer in mice as treatment with up to 50 mg/kg clove oil did not show abnormal symptoms in general health, since it did not decreased the food intake. Further, no deaths were observed in toxicological studies reported. These data indicated that the clove oil shows safer toxic profile since the $ED_{50}$ of clove oil was far below the $LD_{50}$ value [30]. These findings need further experiments to elucidate the underlying mechanisms involved in the pharmacological effects of clove oil. However, according to our understanding no organized work has been carried out on the anti-nociceptive activity of *S. aromaticum* flower buds that can reinforce its conventional use to decrease the pain. The present study was designed to confirm the analgesic activity of *S. aromaticum* extract (SA-EXT) and its fractions to reveal the common belief in its painkilling effects (e.g. in rheumatic conditions, toothache and inflammation) by using thermally-induced nociception assays i.e. Hot-plate and tail-flick methods in which time to paw-withdrawal and tail-withdrawal is measured.

**Experimental**

*Plant Material*

*Syzygium aromaticum* (dried flower buds; Clove) was purchased from a market of Karachi. The plant specimen [Voucher no. KUH-GH 01] authenticated by the taxonomist, Dr. Jan Alam, Department of Botany, University of Karachi, Pakistan and specimen was submitted to the same department.

*Extraction and Isolation*

*S. aromaticum* flower buds (5 kg) were crushed and extracted five times with methanol at room temperature according to the method described earlier [20]. The syrupy residue obtained on removal of the solvent under vacuum was partitioned into ethyl acetate (SA-EAR) and water (SA-MAQ). The ethyl acetate residue after usual work up was divided into petroleum ether soluble (SA-PES), ether soluble (SA-ES), ethyl acetate soluble (SA-EAS), acetone soluble (SA-AS) and methanol soluble (SA-MS) fractions.

*Determination of Antinociceptive Activity*

We used heat as pain stimulus to determine analgesic action of clove flower buds extract and its fractions by means of two common tests i.e. hot-plate and tail-flick tests [31-33].

*Standard Drug and Test Samples*

Indomethacin (purchased from Sigma Chemical Co., MO, USA) was used as standard analgesic for the comparison of the measured activities of the test extracts and fractions. The test extracts and fractions were dissolved in 0.01N NaOH to make a stock solution of 0.5 mg/ml. Animals were given single doses of standard drug and test samples according to their weight. The test samples and standard drug were administered intraperitoneally (*i.p.*) on the day of experiment.

*Animals*

All *in vivo* experiments were performed in accordance with the international guidelines for the care and use of laboratory animals and in agreement with the Institutional Animal Care, Use and Standards Committee [34]. Male mice weighing 20-30 g of NMRI strain (a well-established model/strain for testing anti-nociception activity) were allowed for a time of 3-4 days for acclimatization with the experimental environment before starting the protocol. The animals followed 12 hours light and dark cycle in a temperature and humidity controlled room (22 ± 2 °C; 53 ± 3%).

*Tests for Analgesic Activity*

*I) Paw-Withdrawal Latency (Hot-plate test)*

The hot plate test involves higher brain function, and is considered to be a supraspinally organized response [35]. The advantage of the hot plate test is that, it can be applied repeatedly in the same animals over a short period of time (2–3 h) without causing tissue injury. Total of 72 animals were divided into 12 groups with n=6 per group. Details of animal groups are provided in Table-1. Animals were placed onto the hot-plate at 55 ± 1 °C to record the response time having cut off time of 30 seconds [36]. The response time of experimental animal to the heat stimulus was measured as the time interval of placement of mice on hot plate and the start of paw licking or jumping. Pre-drug treatment reading (with cut- off value of 30 sec) was recorded before the extracts, fractions or drug administration i.e. (0 min readings). Post-drug reaction time was repeatedly measured at various time intervals i.e. 15, 30, 60 and 120 min after drug administration by using the same protocol. To avoid biased results, the observation was done by a person unknown to the treatment given to animals. To further reduce animal suffering, the cut-off value for response to heat stimulus was set at 15seconds. Increase in the latency to response was the ultimate target to achieve by treated animals as compared to control animals. The graphs showed the mean values of each group.

Table-1: Experimental groups details used in the study.

| Experimental groups | Details of groups | Route of administration | doses | Comments |
|---|---|---|---|---|
| GpIa, | Normal Control | ---- | Without treatment | To check general acclimatization and health |
| GpIb | Saline control | *i.p.* | 10ml/kg | 0.9% NaCl |
| GpIIa, | Indomethacin | *i.p* | 5mg/kg | 0.1N NaOH |
| GpIIb | Indomethacin | *i.p.* | 5mg/kg | 0.1N NaoH |
| GpIIc | Indomethacin | *i.p.* | 5 mg/kg | 0.1N NaOH |
| GpIII | SA-EXT | *i.p* | 100mg/kg | 0.9% NaCl |
| Gp IV | SA-AS | *i.p* | 100mg/kg | 0.9% NaCl |
| Gp V | SA-MS | *i.p* | 100mg/kg | 0.9% NaCl |
| Gp VI | SA-MAQ | *i.p* | 100mg/kg | 0.9% NaCl |
| GpVII | SA-EAR | *i.p* | 100mg/kg | 2.5% DMSO |
| GpVIII | SA-EAS | *i.p* | 100mg/kg | 2.5% DMSO |
| GpIX | SA-PES | *i.p* | 100mg/kg | 2.5% DMSO |

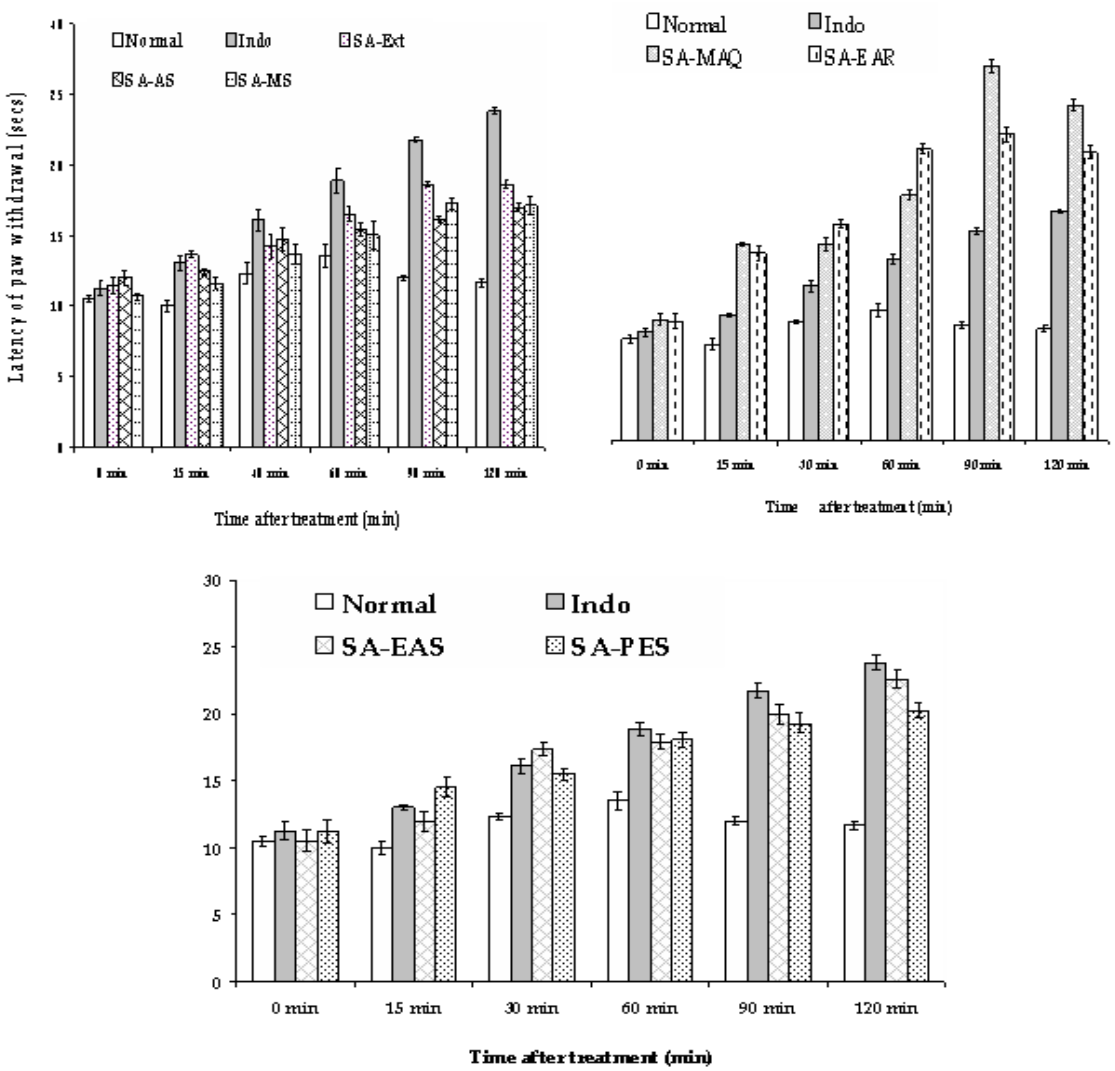


Fig. 1: Time course of the antinociceptive effect of extract SA-EXT and its fractions in the hot-plate test (sec). On Y-axis: Latency to paw withdrawal (sec) is shown while on x-axis: time after treatment is mentioned (min). Each value (mean ± S.E.M, n= 6) represents the time until the mice showed a paw withdrawal response. Significant results are shown as: SA-MAQ=($p< 0.02$) & SA-EAR = ($p<0.03$) while SA-AS a nd SA-MS fractions = ($p<0.05$). However, SA-PES & SA-EAS showed similar results as of indomethacin standard.

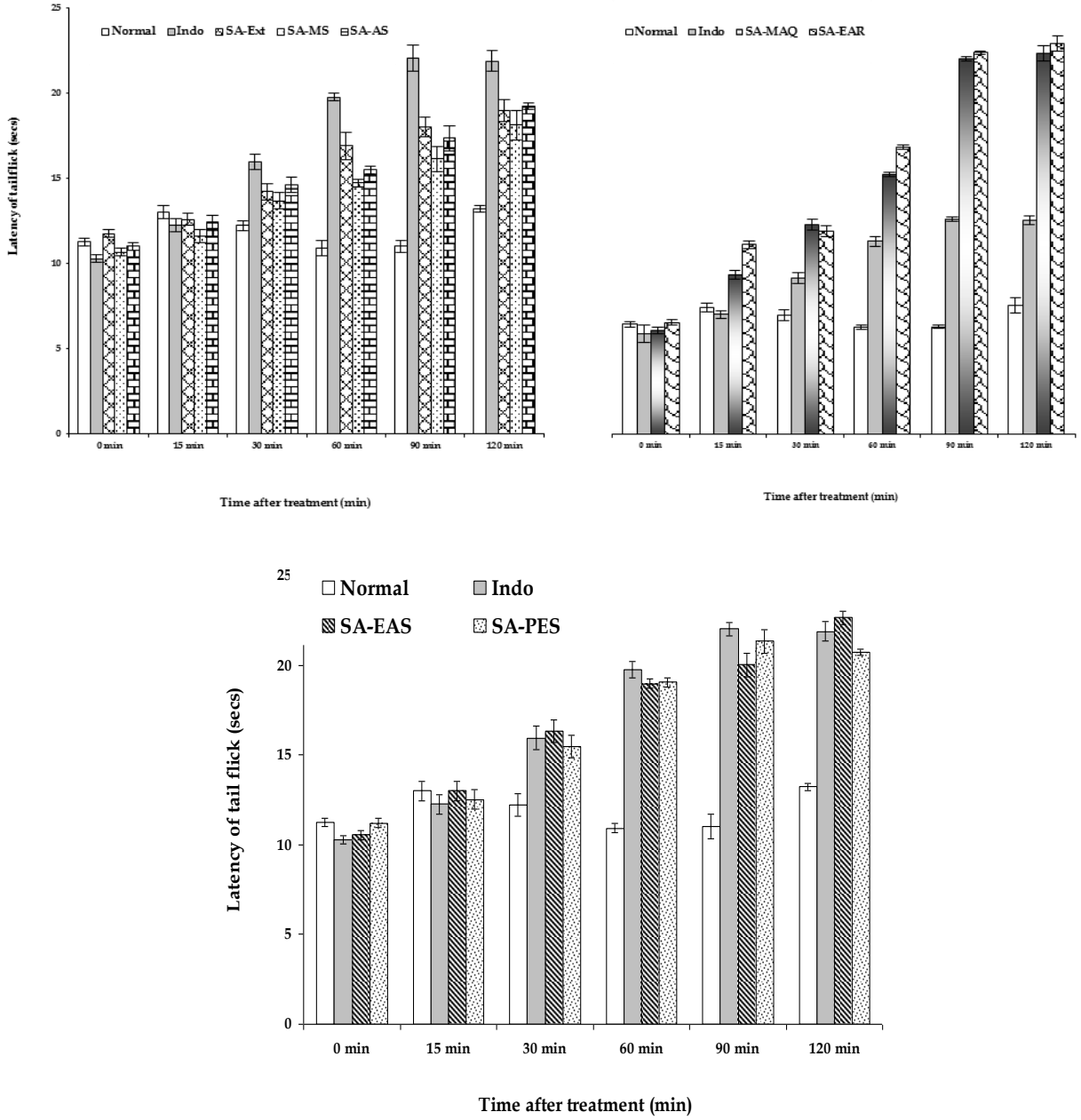


Fig. 2: Time course of the antinociceptive effect of extract (SA-EXT) and its fractions in the tail flick test (sec). On Y-axis: Latency to tail flick (sec) is shown while on x-axis: time after treatment is mentioned (min). Each value (mean ± S.E.M, n= 6) represents the time until the mice showed a tail withdrawal response. Significant results are shown as: SA-MAQ= ($p< 0.02$) & SA-EAR = ($p<0.03$) with onset of action at 15 min. only, while SA-AS and SA-MS fractions = ($p<0.05$). However, SA-PES & SA-EAS showed similar results as of indomethacin i.e. onset of action is 30 minutes.

*II) Tail-flick Assay:*

This is a method commonly used for tail withdrawal of animals from hot water (55 °C). The animals were marked on tail at around 3.5 cm (the part of tail to be dipped) in hot water. The time to withdraw tail (sec) was observed in seconds with a stop time of 10 seconds before administration of any drug or vehicle so that no tissue will be damaged. After having reading before treatment (i.e. 0 minute), the response-time was noted repetitively at 15, 30, 60 and 120 minutes after each administration. The principle for analgesia was post-drug latency which was more than double the pre-drug average latency. Tail flick latency difference

(TFLD) or mean increase in latency after drug administration was used to point out the analgesia. TFLD for analgesic activity was estimated by subtracting the pre-drug reading from the post-drug readings.

*Statistical Analysis*

All data were expressed as the Mean ± S.E.M and analyzed by student's *t*-test using SPSS v. 20. Results having *p* value = less than 0.05 were said to be significantly different.

**Results and Discussion**

In the present study, we employed nociception models of thermal-induced pain for the assessment of analgesic action of the test samples (Fig. 1 and 2). It was observed that methanolic extract (SA-EXT) of clove flower buds exhibits mild analgesic action though its key fractions SA-EAR ($P < 0.03$) and SA-MAQ ($P < 0.02$) demonstrate significantly marked activity compared to control group in paw withdrawal /hot-plate test. The subfractions of SA-EAR i.e. SA-PES and SA-EAS, have revealed analgesic action equivalent to the standard drug indomethacin. Other fractions that are more polar in nature i.e. SA-AS and SA-MS, have also shown mild analgesic action as there is not any significant difference for initial 60 minutes and then a delayed significant response ($p = < 0.05$)was noted till 90-120 minutes of observation protocol of paw-withdrawal latency test. However, in tail flick method, SA-EAR and SA-MAQ have shown significant analgesic effects of quite similar pattern as in other hot-plate test. As the analgesic effect was started at 15 minutes and was increased with time and peak effect was achieved at 120 minutes of observation. Inadequate amounts of the pure compounds available in lab, restricted us to do further study for their antinociceptive activity.

In the light of the findings, this study has shown that the methanolic extract (SA-EXT) and its active fractions possess significant anti-nociceptive activity in the model of nociception used. Our results are almost similar to the standard drug indomethacin in both the heat stimuli tests i.e. tail flicking and paw withdrawal responses. However, the graphs clearly show that the subfractions of methanolic extract of clove flower buds i.e. SA-EAR and SA-MAQ have increased analgesic potential than the standard one. Our results support its use in folk medicine for pain management. In hot- plate test, licking of paw and jumping are the two important parameters to comprehend "analgesic effect" and "escape response" from closed test environment respectively. Testing materials that alter nociceptive threshold may increase the latency to licking and / jumping showing (analgesic effect) or reduction in escape response. At present, only speculation can be made based on obtained results that observed activity may either be peripherally or centrally mediated. Though, Schuler et al., 2001 have reported that NSAIDs such as aspirin and ibuprofen are usually less active in this test than more powerful analgesics such as opioids and indomethacin. Hence, this report supports our selection of the standard drug "Indomethacin" in our study. Rationale for this statement is that anti-nociceptive activity is usually ascribed to stimulation of $GABA_B$ receptors in upper body centers. This activation is associated to an increase in GABA levels within the thalamus. The significance of the role of $GABA_B$ receptors in pain mechanisms is reinforced in earlier knockout mice studies in which pain threshold was increased due to the absence of the $GABA_B$ receptor genes [37]. Furthermore, as described in a report by National Academies Press, Washington, on "Recognition and alleviation of pain in Laboratory animals" published in 2009, it is believed that the fast acting unmyelinated A-delta fibres are responsible for nociception in stimulus evoked pain models rather than the slow acting myelinated C-fibres. It is not possible to dissect out the pharmacologically valuable most effective anti-nociceptive component of these samples because we have not employed any of the pure compounds isolated from clove oil which further necessitates the detailed investigation to understand the underlying mechanism as well.

**Conclusion**

In the light of the findings, this study has shown that the methanolic extract (SA-EXT) and its active fractions possess anti-nociceptive activity in the model of nociception used. The results of our study show potent analgesic effects of flower buds of clove and our earlier study on the clove buds oil have provided technical basis of its use as analgesic in common day medicine. We claim this because the active petroleum ether soluble fraction SA-PES, contains the components that are present in oil. However, detailed mechanistic studies require further investigation to identify a single component having this analgesic activity.

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