Analgesic and Antioxidant Properties of Ethanolic Extract of *Terminalia catappa* L. Leaves

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**Abstract:** The aim of the present study was to investigate the analgesic and antioxidant activities of ethanolic extract of *Terminalia catappa* (TCSE) leaves obtained by soxhlet extraction. The analgesic effects of TCSE extract was studied by formalin induced pain, hot plate and tail flick tests where as antioxidant activity was evaluated by ABTS radical scavenging and metal chelating assays. In formalin test, 80 mg kg$^{-1}$ (p.o.) dose of TCSE extract inhibited both the phases (p<0.05) of animal’s noiception, but TCSE extracts (40 mg kg$^{-1}$, p.o.) inhibited only late phase. TCSE extract (80 mg kg$^{-1}$, p.o.) showed a significant (p<0.05) increase in the reaction time in hot plate test at the time interval of 60, 90 and 120 min. In contrast, both the doses of TCSE extracts did not show any analgesic effect in tail flick test, but morphine (5 mg kg$^{-1}$, s.c.) shown significantly (p<0.05) higher analgesic effect at all time intervals. TCSE extracts showed moderate ABTS free radical scavenging activity compared to standard gallic acid and higher activity compared to BHT (88.07, 96.35 and 68.76% of inhibition, respectively) but shown less ability to chelate ferrous ion. It was concluded from our studies that TCSE extracts have analgesic and anti-inflammatory properties as well as better radical scavenging ability.

**Key words:** *Terminalia catappa*, analgesic, antiinflammation, ABTS, metal chelation

**INTRODUCTION**

*Terminalia catappa* L. (*T. catappa* L.) is a Combretaceae plant which grows commonly in tropical and subtropical countries. The leaf, bark and fruit of this plant have long been used in folk medicine for antiarheic, antipyretic and haemostatic purposes in India, Philippines, Malaysia and Indonesia (Lin, 1992). *T. catappa* leaf has been reported to possess antioxidative, hepatoprotective, antidiabetic, anti-inflammatory and anti-HIV reverse transcriptase activity (Chiyau et al., 2002; Tang et al., 2004; Nagappa et al., 2003; Middleton et al., 2000; Fan et al., 2004; Tan et al., 1991). Leaves of *T. catappa* contains a number of hydrolysable tannins such as punicalagin, punicalin, chebulagic acid, corialgin, gerunin, terflavins A and B, tergalagin, gratin B (Tanaka et al., 1986), flavonoids like, isovitexin, vitexin, isoorientin, rutin (Lin et al., 2000) and triterpenoids such usric acid and 2α, 3β, 23-trihydroxyurs-12-en-28 oic acid (Fan et al., 2004).

The undesired effects of the analgesic compounds available in the market provided an opportunity for new and better analgesic compounds (Katzung, 2007). Bioactive compounds derived from plants have been utilised since from the earlier time for the various purposes including the treatment of pain. Moreover, numerous plant species were found to possess analgesic and anti-inflammatory activities (Gill et al., 2010; Jothinamivannan et al., 2010; Mills and Bone, 2000). The plants belonging to the family Combretaceae comprise of about 200 species of *Terminalia*, which are most widely used for medicinal purposes (McGaw et al., 2001).

The presence of wide number of bioactive compounds with variety of biological activities in *T. catappa* attracted us to select this plant for our analgesic study. Earlier researchers have reported the analgesic activity of aqueous juice prepared by maceration from the tender leaves of *T. catappa* to support its folklore use (Ratnasooriya et al., 2002). The amount bioactive constituents present in the plant extract depend on the method of extraction and type of solvent employed which directly influence the biological activity of the plant extracts (Hayouni et al., 2007). In this regard, our main objective was to evaluate the analgesic activity of *T. catappa* leaf ethanolic extract obtained by soxhlet extraction. Free radicals are involved during pain stimulation and antioxidants shown to reduce such pain (Kim et al., 2004; Das and Maulik, 1994). Hence, an attempt also been made to evaluate the antioxidant activity of *T. catappa* leaf extract.

**MATERIALS AND METHODS**

**Plant material:** Fresh leaves of *Terminalia catappa* were collected from the campus of Universiti Sains
Malaysia (USM), Penang, Malaysia in November 2008. The plant was authenticated by a botanist and the voucher specimen (No. 11048) was deposited in Herbarium, School of Biological Sciences, USM. Collected leaves were then washed with running water to remove the dirt and adherent then dried and grinds to get powder of 40 mesh size.

**Preparation of extract:** Hundred gram of ground sample was refluxed with 1000 mL of ethanol (99.8%) at 70°C for 48 h using soxhlet apparatus. The liquid extract was separated from solid residue by vacuum filtration through Whatman filter paper (No. 1), filtered ethanolic was then concentrated to small volume using rotary evaporator (Buchi Rotavapor R-215, Switzerland) followed by drying in freeze dryer (LABCONCO, Free Zone 6 Liter, USA) to get constant mass and kept in air tight container at 4°C until further analysis.

**Chemicals:** Potassium persulfate, 2,2-azinobis (ethylenethiourea-6-sulfinic acid) diaminonitric salt (ABTS), gallic acid, butylated hydroxytoluene (BHT), aspirin, ferrocene, formaldehyde and ethylene diamine tetraacetic acids (EDTA) were purchased from Sigma Aldrich, Germany. Morphine was obtained from Hospital USM, Kelantan, Malaysia. All solvents were used of analytical grade and purchased from Fisher Scientific (M) Sdn. Bhd, Malaysia.

**Animals:** Male Sprague Dawley rats (150-200 g) and Swiss albino mice (20-25 g) were obtained from animal house, USM. These animals were acclimatized to the laboratory one week before the experimental procedure. All animals had free access to water and food pellets and were fasted overnight prior to use. The experimental procedures were approved by Animal Ethics Committee, USM.

**Analogesic activity**

**Formalin-induced pain:** Modified method described by Santos and Calixto (1997) was used in this study. The pain was induced by injecting 0.05 mL of 2.5% formaldehyde (40%) in saline in the subplantar region of rat hind paw. Preliminary oral LD₅₀ dose of TCSE in mice was 800 mg kg⁻¹ hence rats (six in a group) were given with one-tenth LD₅₀ dose of TCSE extract (40 and 80 mg kg⁻¹, p.o.), morphine (5 mg kg⁻¹, s.c.), aspirin (100 mg kg⁻¹, p.o.) and co-solvent. The time spent in licking and biting the injected paw was considered as indicative of nociceptive behaviour was recorded. Two successive phases are involved following the injection of formalin, first phase (0 to 5 min) representing neurogenic pain and second phase (15 to 30 min) representing tonic and inflammatory pain response (Hunskaar and Hole, 1987). The percentage inhibition for each rat and each group was obtained as follows:

\[
\text{Percentage inhibition} = \left[ \frac{A_s - A_t}{A_s} \right] \times 100
\]

where, \(A_s\) is the reading of the co-solvent and \(A_t\) is the reading of samples.

**Hot plate:** The hot plate test to measure the latency response was performed according to the method described by Woolfe and Macdonald (1994), with slight modification (Santos et al., 1998). Rats were placed on the heated surface of a hot plate analgesia meter (IITC Life Science Series 8, Model PE34, Victory Blvd, Woodland Hills, CA 91367, USA) maintained at 55±0.2°C. The time taken for licking, shaking of forepaws or jumping was recorded as the index of response latency. Animals were tested before administration of samples in order to obtain the baseline latency and an automatic cut-off time of 45 sec was used in order to prevent rat paw tissue damage. Rats (six in a group) were given with TCSE extract (40 and 80 mg kg⁻¹, p.o.), morphine (5 mg kg⁻¹, s.c.), aspirin (100 mg kg⁻¹, p.o.) and co-solvent (propylene glycol: Tween 80: water = 4:1:4, v/v/v) served as control. The response latency was measured 30 min before and 30, 60, 90 and 120 min after the administration of samples.

**Tallick:** The method described by D’Amour and Smith (1941) was used to measure response latency in the tail flick test. A radiant heat analgesimeter (IITC Life Science Series 8, Model 331, Victory Blvd, Woodland Hills, CA 91367, USA) was used. Rats responded to a focused beam of light by flicking their tail. An automatic cut-off time of 10 s was used to avoid tissue damage. Animals were tested before administration of samples in order to obtain the baseline latency. Rats (six in a group) were given with TCSE extract (40 and 80 mg kg⁻¹, p.o.), morphine (5 mg kg⁻¹, s.c.), aspirin (100 mg kg⁻¹, p.o.) and co-solvent. The latency for flicking the tail was determined 30 min before and 30, 60, 90 and 120 min after the administration of samples and quantified by an average of three measurements.

**Antioxidant activity**

**ABTS radical scavenging assay:** ABTS radical scavenging activity of TCSE was determined according to the previously described procedure (Re et al., 1999). Fresh ABTS radical was prepared by adding 5 mL of a 4.9 mM potassium persulfate solution to 5 mL of a 14 mM ABTS solution and this solution was kept for 16 h in dark. This solution was diluted with methanol to yield an absorbance of 0.700±0.02 at 734 nm and the same solution was used for the antioxidant assay. The final reaction mixture (1 mL) of standard and extract comprised 950 µL of ABTS
solution and 50 μL of the extract at variable concentration (0.5-10 μg mL⁻¹). This reaction mixture was vortex for 10 sec, after 6 min absorbance was recorded at 734 nm and compared with the control ABTS solution.

ABTS radical scavenging activity of different extracts was calculated using the following formula:

\[
\text{ABTS radical scavenging activity (\%) = \left[ \frac{(A_t - A_i)}{A_t} \right] \times 100}
\]

where, \(A_t\) is the absorbance of the control and \(A_i\) is the absorbance of samples.

**Metal chelating activity:** The chelating ability of ferrous ions by TCSE extract was evaluated by the method described by Dimis et al. (1994). To the 2.5 mL of TCSE extract (0.250-2 mg mL⁻¹) and EDTA served as the positive control (0.0025-0.04 mg mL⁻¹), 0.05 mL of FeCl₃•4H₂O (2 mM) was added. The reaction was initiated by the addition of 0.2 mL of ferrozone solution (5 mM) then the mixture was vortexed and kept for 10 min at room temperature. Absorbance of the resulting solution was then measured at 562 nm against the blank prepared in the same way using FeCl₃ and methanol. Sample without extract served as the negative control. All tests were done in triplicate and the percentage inhibition was calculated using the formula used for the ABTS assay.

**Statistical analysis:** The results of antioxidant and analgesic activity were expressed as means±SD and means±SEM respectively. Statistical analysis of variance was performed with one way ANOVA, followed by Tukey’s HSD (Honestly Significant Difference) using SPSS 17 (SPSS Inc., Wacker Drive, Chicago, USA). p<0.05 were considered to be statistically significant when compared to control.

**RESULTS**

**Analgesic activity**

**Formalin-induced pain:** The effect of TCSE extract, aspirin and morphine in early phase (0-5 min, neurogenic pain) and late phase (15-30 min, inflammatory pain) of formalin test are shown in Table 1. TCSE extract significantly (p<0.05) blocked the neurogenic pain only at the dose of 80 mg kg⁻¹ and at the same dose it inhibited the inflammatory pain (46.9%, p<0.05) better than neurogenic pain (16.39%, p=0.05). TCSE extracts shown significant (p<0.05) activity during the late phase. Similarly, morphine produced significant inhibition (p<0.05) of both early and late phase. In contrast, aspirin showed significant inhibition (p<0.05) only during the late phase.

<table>
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<tr>
<th>Table 1: Effect of TCSE extract, morphine and aspirin on formalin-induced pain</th>
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<tr>
<td><strong>Total time spent licking (sec)</strong></td>
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<td><strong>Treatment</strong></td>
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<td>Aspirin</td>
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<td>*Each value represents mean±SEM in seconds for six rats treated with TCSE extracts or reference drugs. <em>p&lt;0.05, significant from control</em></td>
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<th>Table 2: Effect of TCSE extract and morphine on pain induced by hotplate</th>
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<td><strong>Latency period (min)</strong></td>
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<td><strong>Dose (mg kg⁻¹)</strong></td>
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<td><strong>Treatment</strong></td>
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<td>TCSE extract</td>
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<td>TCSE extract</td>
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<td>Morphine</td>
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<td>*Each value represents mean±SEM in seconds for six rats treated with TCSE extracts or reference drugs. <em>p&lt;0.05, significant from control</em></td>
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<th>Table 3: Effect of TCSE extract and morphine on pain using tail flick test</th>
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<td><strong>Latency period (min)</strong></td>
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<td><strong>Dose (mg kg⁻¹)</strong></td>
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<td>Morphine</td>
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<td>*Each value represents mean±SEM in seconds for six rats treated with TCSE extracts or reference drugs. <em>p&lt;0.05, significant from control</em></td>
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**Tail flick:** Results of tail flick test (Table 3) revealed that TCSE extracts did not show any analgesic effect in this test at the different intervals of time tested, whereas morphine showed significantly (p<0.05) higher analgesic activity at all the tested time interval. It was also found that the antinociceptive activity of morphine was three and two half times more than that of 80 mg kg⁻¹ TCSE extract at 30 and 60 min, respectively and was found to be almost equal at 90 min time interval.
model (formalin induced pain) and thermal models (hot plate and tail flick) of nociception in rats. The antinociceptive models used were such that both centrally and peripherally mediated effects were investigated. Antioxidant models used were to evaluate the radical scavenging and metal chelating ability of TCSE extract.

Formalin method was normally employed to elucidate the mechanism of pain and analgesia which consists of early phase (0-5 min) because of the stimulation of nociceptive receptors in the paw and the late phase probably reflects the inflammation process (Tjolsen et al., 1992; Coderre and Melzack, 1992). Centrally acting drugs inhibit both phases of formalin induced pain, whereas drugs like aspirin and which acts peripherally inhibit only the late phase (Chan et al., 1995). Oral administration of TCSE extract (80 mg kg⁻¹) inhibited the early phase of formalin test but the percentage of inhibition was four times less than that of morphine, which shows that central mechanism may be involved in the analgesic activity of TCSE extract. The second phase of formalin test is related to a peripheral inflammatory process. Both the doses (40 and 80 mg kg⁻¹) significantly (p<0.05) inhibited the late phase. Hence, it can be deduced that peripheral mechanism might also be involved in the anti-inflammatory effect. This result was in agreement with the findings of Fan et al. (2004) in which they isolated triterpenic acids which are responsible for anti-inflammatory activity from T. catappa leaf. But our results were contrary to the previous findings of Ratnasooriya et al. (2002) in which administration of macerated aqueous juice of T. catappa inhibited only the early phase not the late phase. The reason may be the extraction method and the solvent we employed might have extracted out the bioactive compounds responsible for the anti-inflammatory activity.

Hot plate test is normally employed to find out the involvement of central analgesic activity (Paulino et al., 2003). In the present study, TCSE extract showed analgesic activity at higher dose (80 mg kg⁻¹) thus, the extract may act via central mechanism. Morphine (5 mg kg⁻¹) a centrally acting analgesic produced significant (p<0.05) analgesic activity than TCSE extract (80 mg kg⁻¹). Thus, TCSE extract may have lower potency than morphine. Present findings were in agreement with the previous findings of Ratnasooriya et al. (2002) in which oral administration of macerated aqueous juice of T. catappa increased the antinociceptive reaction time.

Tail flick test is a very well known test used for discriminating morphine like analgesics from non opiate analgesics (Vogel, 2002). Both the doses of TCSE extracts (40 and 80 mg kg⁻¹) were unable to produce analgesic effect. We suspect it might be because of the dose of the TCSE extract used was too low to induce

\[\text{DISCUSSION}\]

In this study, we evaluated the potential antinociceptive activity of TCSE extract using chemical
the antinociception in this test. Present results were in agreement with the previous findings of Ratnaasooriya et al. (2002) in which oral administration of macerated aqueous juice of *T. catappa* did not exhibit analgesic effect in tail flick test.

Since, the TCSE extract inhibited both the early and late phase of formalin test and analgesic activity in hotplate test, both central and peripheral mechanism may be involved which implies that it has not only antinociceptive but also anti-inflammatory activity.

Production of oxyradicals as well as nitric oxide plays an important role in various models of inflammation (Cuzzocrea et al., 1998; Moncada et al., 1991). TCSE extract was found to possess good radical scavenging activity and may partly contribute for the anti-inflammatory activity of the extract. Certain flavonoids like quercetin were found to inhibit cyclooxygenase, 5- lipoxygenase pathways and even phospholipase A2, responsible for the anti-inflammatory response (Williams et al., 1995). A study by Filho et al. (1996) reported on the antinociceptive effect of quercetin through a central mechanism. Flavonoids may increase the amount of endogenous serotonin or may interact with 5-HT1A and 5-HT, receptors (Lee et al., 2005) which may be involved in the mechanism of central analgesic activity (Colpaert et al., 2002). Previous researchers reported the presence of several therapeutically valued flavonoids from the leaves of *T. catappa* (Lin et al., 2000). Even though TCSE extract was found to possess good radical scavenging activity, it had weak metal chelating ability in comparison with the reference drug EDTA.

**CONCLUSIONS**

Based on the results of present study, we conclude that TCSE extract possess antinociceptive activity at higher dose and anti-inflammatory activities at lower dose. This activity may be related to the presence of phytochemicals such as flavonoids, tannins and triterpenes reported in leaf extract. This study also justifies the influence of extracting solvent and the method of extraction with respect to the biological activity of the plant extract. Taking into account its potent bioactivity, the plant is an ideal candidate for progress into advanced studies.

Further studies are currently underway to isolate and characterise the bioactive compounds and their antinociceptive mechanisms.

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