Letter to the Editor

Anti-Nociceptive Effect in Mice of Thillai Flavonoid Rutin

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We investigated the anti-nociceptive effect of *Excoecaria agallocha* (*E.agallocha*) against chemically and thermally induced nociception, Albino mice received a dose of 10, 15, 20, or 25 mg/kg of alkaline chloroform fraction (Alk-CF) of *E.agallocha* by oral administration. Compared with controls, Alk-CF decreased the writhing numbers (P<0.01) in a dose dependent manner. Further we determined that, Alk-CF contained, a potent compared to control, also potent anti-nociceptive agent that acted via opioid receptors and using HPLC, identified this compound as Rutin. Docking simulation demonstrated that Rutin interacted strongly with cyclooxygenase, forming a number of specific hydrogen bonds. In conclusion we have identified peripheral and central anti-nociceptive activities of *E.agallocha* that involve opioid receptor, and in which the active compound is Rutin.

The human environment contains a large number factor responsible for pain which is a sensory modality that often represents the only symptom for the diagnosis of variety diseases. Moreover the administration of synthetic drugs to the treatment of pain is in some cases associated with side effects, such as the induction of gastric lesions by non steroidal anti-inflammatory drugs (NSAIDs)[1]. Plant-based drugs have recently been garnering increased attention and in particular those derived from mangroves, which are used by coastal fishing communities have been both toxicologically and medicinally validated[2]. Mangroves contain a variety of phenolic compounds with biological activity, which includes flavonoids and their glycosylated derivatives performed physiological roles, also act as catalysts and regulators in photo-phosphorylation[3] as well as more than 100 alkaloids. *Excoecaria agallocha* (*E. agallocha*) commonly termed as Thillai, is an ever green mangrove of Euphorbiaceae family, which is distributed along the southeast coast of India. Although the extracts of leave and bark of this plant have been used for the treatment of chronic pains, rheumatism, leprosy, paralysis, inflammation and ulcers[4], it active compound has not yet been identified. Accordingly, the aim of the study was to identify the antinociceptive compounds in the extracts of *E. agallocha* in animal models and to evaluate its efficiency in *in silico* docking with cyclooxygenase (COX) receptor.

Leaves of *E. agallocha* were collected from Kollidam coast, Tamil Nadu, India during January 2011. The vouchered specimen (AUCASMB 63/2011) was deposited in the herbarium of C.A.S. in Marine Biology, Annamalai University, Parangipettai, India. 5 kg of air-dried, powdered leaf was extracted with ethanol using a percolation method. The obtained extract was evaporated under reduced pressure to generate a viscous mass. 250 g of extract was suspended in 500 mL of distilled water and partitioned sequentially with n-hexane (5×250 mL), dichloromethane (DCM) (5×250 mL), to generate acid (pH3, 5×250 mL) and alkaline Alk-CF (pH9, 5×250 mL) chloroform fractions. Five fractions were collected and concentrated under vacuum and stored at 20 °C until experiments. Both the total extract and fractions were screened to determine the presence of alkaloids, flavonoids, terpenoids, and saponins[5].

Swiss albino mice of either sex (20-25 g) were used for experiment. The acute oral toxicity study was performed according to OECD-423 guidelines. Animals were fasted for 4 h with free access to water only. Isolated fractions and total extract were suspended in a 0.5% carboxy methyl cellulose (CMC) solution and administered orally at initial doses of 1 to 2000 mg/kg after which mortality was assessed for 3 d[6]. A dose was considered toxic when

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mortality was observed in 2/3 or 3/3 animals. Total and n-hexane extract were non-toxic upto 1 g/kg, whereas, diethyl chloromethane (LD50=750 mg/kg), acid chloroform (LD50=250 mg/kg) and alkaline chloroform fraction (LD50=250 mg/kg) were all toxic. These results guided the selection of 25 mg/kg as higher dose to be used for determining the analgesic potential of *E. agallocha* extracts.

Mice were pre-treated orally with vehicle 0.5% CMC, total extract (100 mg/kg) and n-hexane, DCM, acid and alkaline fraction (5 mg/kg of each) respectively before 30 min of intraperitoneal (i.p.) injection of 1% acetic acid[7]. The number of writhing events (stretching of hind limb and abdominal constrictions) was counted for 40 min. Compared with other fractions Alk-CF had the most potent analgesic effect, and used in subsequent studies, at a dose of 10-25 mg/kg. Pentazocine (10 mg/kg) was administered as a positive control. Naloxone (2 mg/kg) was administered 15 min prior to the Alk-CF (25 mg/kg) or Pentazocine (10 mg/kg) injection. The number of writhing events in each treated group was compared with vehicle control and expressed as percent inhibition of the writhing events.

Mice were treated orally with 0.5% CMC or pentazocine (10 mg/kg) by intraperitoneally or Alk-CF (10-25 mg/kg) and placed on metal plate. The time elapsed until the appearance of reactions (latency) to the thermal stimulus (55±1 °C), such as licking of hind paw or jumping was recorded as the index of nociception[8]. The response time was noted at time intervals from 0-60 min with the cut off time of 15 s to avoid tissue damage. In another set of experiment, Naloxone (2 mg/kg) was administered 15 min prior to the oral administration of Alk-CF or pentazocine injection. Analgesic activity was recorded as the increase in latency time after thermal stimulus relative to vehicle control. The results were reported as mean±S.E.M analyzed by ANOVA followed by Dunnets Multiple Range Test (Graph Pad Instat software). $P<0.01$ was considered significant. IC_{50} values were estimated by linear regression analysis.

High performance liquid chromatography was used to identify and quantify the active component of Alk-CF. The extraction solvent was mixture of analytical grade alcohol, water and hydrochloric acid (Hi Media, India) (50:20:8) and the mobile phase was a mixture of methanol, water and phosphoric acid (Hi Media, India) (100:100:1). The flavonoid standards Quercetin, Rutin, Kaempferol and Isorhamnetin were weighed and dissolved in methanol to generate standard solution of 1 mg/mL. 22 mL of Alk-CF was added to 78 mL of extraction solvent and refluxed on a water bath for 35 min, after which 20 mL of methanol was added, followed by sonication for 30 min. The residue filtrate was washed with methanol and used for the analysis. HPLC was equipped with a 270 nm detector and a 4.6 mm × 25 cm column with a flow rate of 1.5 mL per min. 20 µL of each standard and the test solution was injected into the column and the major peaks were recorded. The percentage of each flavonoid in the test fraction was calculated.

The three dimensional structures of human COX 1 (PDB: 1CQE) and COX 2 (PDB: 6COX) were obtained from the Protein Data Bank (PDB). Auto Dock Tools was used to create PDBQT files from traditional PDB files. The 2D structure of Rutin was retrieved from the PubChem database. The optimized ligand was docked using Ligand Fit in Auto Dock 4.0[9].

The total yield from the extraction was approximately 37.5% from which five major fractions were separated. Total extract and n-hexane contained phenolics, saponins and terpenoids, while alkaloid was indicated in the dichloromethane fraction. The acid chloroform and Alk-CF fractions contained higher amounts of alkaloids and flavonoids, respectively. Both the total extract and fractions of *E.agallocha* reduced in abdominal writhing events in a dose-dependent manner. Compared with total extract and the n-hexane, DCM and acid chloroform fraction, the Alk-CF had more potent anti-nociceptive effect (Figure 1).

Pentazocine (10 mg/kg) was used as a positive control of anti-nociceptive effect. The analgesic effect of Alk-CF in the acetic acid and hot plate models were confirmed (Tables 1&2). The present study demonstrates that the pain reduction by Alk-CF in the acetic acid induced model (Table 1) might be attributable to the inhibition by flavonoids of prostaglandin synthesis, a peripheral mechanism of pain inhibition. In addition compared with control animals, Alk-CF effectively delayed the time of response of mice to thermal stimulation (Table 2) in a dose dependent manner. We speculate that because of its prolongation of latency Alk-CF was acting centrally. The anti-nociceptive effect of Alk-CF was significantly antagonized by naloxone, an opioid receptor antagonist. Collectively these results indicate that Alk-CF possessed both peripheral and central anti-nociceptive activities that involved opioid receptors.