British Journal of Pharmaceutical Research
7(2): 102-109, 2015, Article no.BJPR.2015.095
ISSN: 2231-2919

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Influence of *Rhizophora apiculata* Flavonoids on Chemical and Thermal Induced Nociceptive Models

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SG and KS performed plant collection, extraction and experimental studies and author AM supported docking result interpretation. Authors KS and SG developed the concept and interpretation of data for manuscript preparation. Author TR was research guide of this experimental study, provided chemicals and instruments. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJPR/2015/16732
Editor(s):
(1) Jinyong Peng, College of Pharmacy, Dalian Medical University, Dalian, China.
Reviewers:
(1) Anonymous, Iuliu Haţieganu University of Medicine and Pharmacy, Romania.
(2) James Adams, California School of Pharmacy, University of Southern, California, USA.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=1177&id=14&aid=9468

Original Research Article

Received 12th February 2015
Accepted 26th February 2015
Published 28th May 2015

ABSTRACT

Aims: *Rhizophora apiculata* is a traditional medicine used to treat pain, ulcer, and inflammation in southeast coast of India without scientific evidence. Therefore, we aimed to evaluate antinociceptive effect of *R. apiculata* to chemical and thermal induced nociceptive models.
Place and Duration of Study: Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai, Tamil Nadu, India, between June 2012 and September 2012.
Methodology: Albino mice received 10, 15, 20, or 25 mg/kg of alkaline chloroform fraction (Alk-CF) by orally and dose-dependently decreased the writhing numbers ($P<0.01$) compared to control, also potent antinociceptive agent with the involvement of opioid receptors.
Results: Myricetin was identified as a potent major component of Alk-CF using HPLC and docked with protein cyclooxygenase. Myricetin articulated more interaction and produce number of hydrogen bonds with cyclooxygenase.
Conclusion: These results suggested Alk-CF posses both peripheral and central analgesic activities with the involvement of opioid receptors might be the action of Myricetin.

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Keywords: Antinociceptive; ADME; flavonoid; HPLC; mangrove; myricetin.

1. INTRODUCTION

Pain is a direct sensorial response to pathological events such as tissue damage, injury, and inflammation but with severe pain can arise independently of any obvious predisposing causes [1]. As a result of these events associated with local release of certain chemical substances which act on the nerve terminals directly or by other forms of stimulation [2]. At present commercially available NSAIDs and narcotics analgesic agents have not been producing successful grades due to their adverse gastric lesions. In the modern world, accidents and operations are done so far then compared to earlier human lifestyle. From the past few decades, researchers focussed their interest to develop analgesic drugs which overcome the above side effects from ethnomedicinal plant derived phyto-constituents. As well as, World Health Organizations pointed out nearly 80% of populations in the developing countries have belief on floral system based drugs for their health care [3]. *R. apiculata* one of the traditional medicinal mangrove species are distributed along the southeast coast of India. Organic extracts of *R. apiculata* exhibited antimicrobial, anti-oxidant, anti-cancer and anti-malarial effect on experimental animal models [4]. Nearly 210 plants have promising analgesic activity which mediated through opioid receptors [5]. Based on the reviews, the medicinal efficacy of mangrove derived flavonoids is insufficient for the development of analgesic medications. Therefore, we aimed to evaluate the analgesic effect of *R. apiculata* flavonoids on chemical and thermal induced nociceptive models, isolate the active compound by HPLC. Furthermore, we studied molecular docking of potent lead against cyclooxygenase receptor.

2. EXPERIMENTAL DETAILS

2.1 Preparation and Extraction

Leaves of *R. apiculata* were collected from Kollidam coast, Tamil Nadu, India during January 2010 and authenticated. The voucher specimen (AUCASMB 10/2010) was deposited in the herbarium of C.A.S. in Marine Biology, Annamalai University, Parangipettai, India. About 5000 g of air-dried and powdered leaf material of *R. apiculata* was extracted with ethanol by percolation method. The obtained extract was evaporated under reduced pressure to get a viscose mass. Then, 250 g of extract was suspended in 500 mL of distilled water and was partitioned sequentially with n-hexane (5x250 mL), dichloromethane (5x250 mL), acid and alkaline chloroform fraction {pH3 (5x250 mL); pH9 (5x250 mL)} respectively. Finally, five fractions were collected and concentrated under vacuum and stored at 20°C until experiments. Each of the fractions and total extract was screened to determine the presence of alkaloids, flavonoids, terpenoids and saponins [6].

2.2 Experimental Animals

About 20 to 25 g weight of male Swiss albino mice was purchased from the Central Animal House Facility, Rajah Sir Muthiah Medical College, Annamalai University was used in this study. The animals were fed on the pellet diet (Hindustan Lever, India) water ad libitum. This study was approved by the Institutional Animal Ethical Committee of Annamalai University, India (Reg.No.169/1999/CPCSEA). Acute oral toxicity study was performed according to OECD-423 guidelines [7].

2.3 Acetic Acid Induced Writhing Test

Mice pretreated orally with vehicle 0.5% carboxy methyl cellulose (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 [8]. The number of writhing (stretching of the hind limb and abdominal constrictions) was counted for 40 min. Compared to other fractions Alk-CF indicates potent analgesic effect. Therefore, Alk-CF further studied at the 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. Number of writhes in each treated group was compared with vehicle control and expressed as percent inhibition of the writhes.

2.4 Hot Plate Test

Mice were orally treated with 0.5% CMC or pentazocine (10 mg/kg) or Alk-CF (10-25 mg/kg) intraperitoneally and placed on metal plate, the time elapsed since the appearance of reactions (latency) to the thermal stimulus (55±1°C), such

as licking of hind paw or jumping was recorded as an index of nociception [9]. The response time was noted at different time intervals, i.e. 0-60 min and the cut off time of 15s to avoid tissue damage. In another set of experiment, Naloxone (2 mg/kg) was administered 15min prior to the administration of Alk-CF or Pentazocine injection. Analgesic activity was expressed as the increase in latency time to thermal stimulus with respect to vehicle control. The results are reported as mean S.E.M analyzed with ANOVA followed by Dunnett's Multiple Range Test (Graph Pad Instat software). $P<0.01$ is considered significant. IC_{50} values were estimated by linear regression analysis.

2.5 HPLC

The major component of active Alk-CF was identified and quantified by HPLC. An extraction solvent mixture of alcohol, water and hydrochloric acid (50:20:8) and mobile phase, mixture of methanol, water, and phosphoric acid (100:100:1) was used. Accurately weighed standards of flavonoids such as Quercetin, Rutin, Kaempferol and Isorhamnetin were dissolved in methanol and obtained standard solutions 1 mg/ml. High-performance liquid chromatography (HPLC) is equipped with a 270 nm detector and a 4.6 mm × 25 cm column with the flow rate was about 1.5 ml per minute. 20 µl of each standard and the test solution into the chromatograph recorded and measured the major peaks. The percentage of each flavonoid in test fraction was calculated.

2.6 Molecular Docking

An extended PDB format termed as PDBQT file was used for coordinate files which include atomic partial charges. Auto Dock tools were used for creating PDBQT files from traditional PDB files. Three-dimensional structures of human COX1 (PDB: 1CQE), COX2 (PDB: 6COX) were obtained from Protein Data Bank. This structure was determined using X-ray diffraction. The active site of the target proteins was predicted by PDB Sum. The 2D structure of Myricetin was retrieved from NCBI PubChem Compound database in SDF format and was then converted to PDB format using OPEN BABEL 2.2.119. Optimized ligand was docked using Ligand Fit in the Auto Dock 4.0. ADME/T toxicity properties were calculated by online ADME predictor.

3. RESULTS

3.1 Antinociceptive Effect

Total yield of extraction was obtained about 37.5% from which five major fractions were separated. Total extract and n-hexane were considered to confirm phenolics, saponins, and terpenoids while alkaloid was indicated in dichloromethane fraction. Acid chloroform and alkaline chloroform fraction (Alk-CF) strongly possess alkaloids and flavonoids respectively. The extract and fractions of *R. apiculata* caused reduction in abdominal writhes in a dose-dependent manner. Compare with total extract, n-hexane, DCM and acid chloroform fraction, Alk-CF indicates potent analgesic effect. Pentazocine (10 mg/kg) was used as a standard drug which exhibited significant inhibitory response. The present study demonstrates the most significant pain reduction of Alk-CF in acetic acid-induced model (Table 1) might be the action of flavonoids preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition.

Alk-CF and pentazocine were extensively and dose-dependently delayed the time of response of mice to thermal stimulation as compared to untreated animals (Fig. 1).

The Alk-CF might be because of prolongation of latency must be acting centrally. The antinociceptive response of the Alk-CF was significantly antagonized by naloxone, an opioid receptor antagonist. The observed results indicate that the extracts poss both peripheral and central antinociceptive activities with the involvement of opioid receptors.

3.2 HPLC Analysis

Mangrove derived metabolites, especially phenolic compounds are largest and ubiquitous groups which could make the plant material useful for potential anti-oxidant, anti-diabetic and some of them involved in the drug development process. HPLC results evidenced that the Alk-CF of *R. apiculata* contains six flavonoids. The retention time of standards rutin, quercetin, myricetin, kaempferol, luteolin, and isorhamnetin were found to be 2.210, 3.710, 4.010, 8.763, 9.773 and 12.223. The retention time of rutin, quercetin, Myricetin, kamferol, luteolin and isorhamnetin in *R. apiculata* was found to be 1.993, 3.767, 4.653, 8.443, 9.427 and 12.107 which are matching with standard retention values respectively (Fig. 2).

Table 1. Effect of Alk-CF of *R. apiculata* in acetic acid-induced writhes in mice

Treatment	Dose (mg/kg)	Writhing	Inhibition (%)
Control	10ml	67.05 ±0.28	
Pentazocine	10	10.91 ±0.23*	81.4
Pentazocine + Naloxone	10+2	36.42 ±0.65	45.1
Alk- CF	10	25.24 ±4.25*	57.58
	15	19.32 ±3.42*	68.73
	20	13.35 ±6.36*	72.54
	25	10.25 ±4.12*	79.51
Alk-CF + Naloxone	25+2	39.75 ±2.31	41.5

*Values are reported as mean ± SD for group of 6 animals. The data was analyzed by ANOVA followed by Dunnett's test. Statistically significant *p<0.01 more significant values from control Alk-CF of R. apiculata*

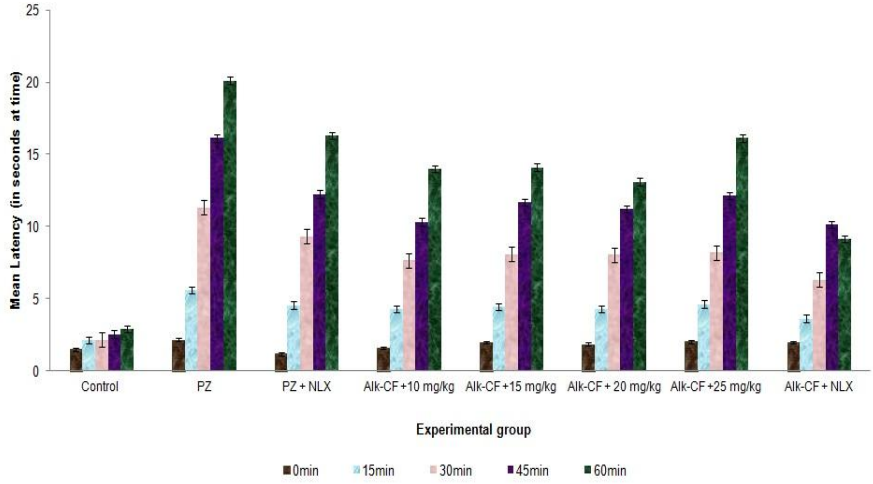

Fig. 1. Effect of potent Alkaline Chloroform fraction in hot plat test

*AlkCF- Alkaline Chloroform Fraction; PZ –Pentazocine; NLX- Naloxone. Values are reported as mean ± SD for group of 6 animals. The data was analyzed by ANOVA followed by Dunnett's test. Statistically significant *p<0.05 and **P<0.01 more significant values from the control*.

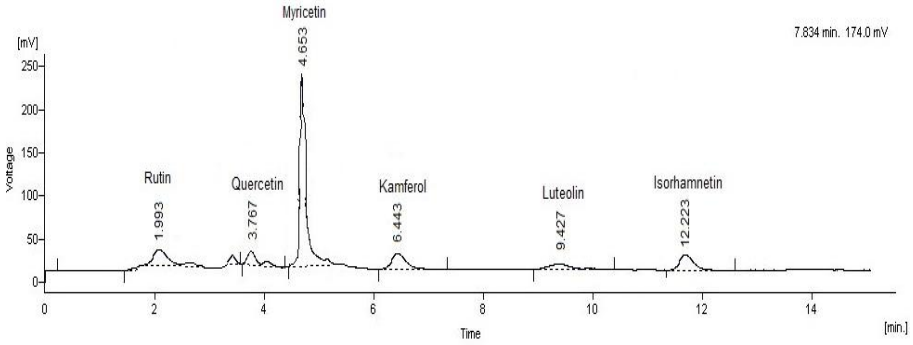

Fig. 2. HPLC chromatogram of flavonoids from *R. apiculata*

Then the amount of rutin, quercetin, Myricetin, kaempferol, luteolin and isorhamnetin in *R. apiculata* was found to be 4.5, 5.6, 71.2, 7.6, 5.3 and 4.2% w/v respectively. The identified flavonoids confirmed with HPLC-MS (data not shown). Significant antinociceptive activity of Alk-CF indicated might be the presence of Myricetin which was evidenced by analytical techniques.

3.3 Molecular Docking

R. apiculata derived the flavonoid-rich fraction evaluated on their analgesic property with cyclooxygenase receptor protein (COX) in the first time of computer-aided drug design. Natural ligand Myricetin was subjected to docking analysis with COXs using Autodock 4.0. Grid parameters were set as mentioned earlier and spacing between grid points was 0.375Å. The simulations were complete after that the docked structures were analyzed and the interactions were observed with the amino acids (Ala-Alanine; Asp- GLN-Glutamine; ASN- Asparagine; GLU-glutamic acid; Gly-Glycine; HIS-Histidine; Met-Methionine; Ile-Isoleucine; Lys-Lysine; Phe-Phenylalanine; Pro-Proline; SER-Serine; Trp-Tryptophan; Tyr-Tyrosine; Thr-Threonine; Val-Valine;) present in the target protein with hydrogen and oxygen bond energy (HE and OE). Interactions and the binding distance between the donors and acceptors of hydrogen bond were measured for the best conformers. Interaction free energies are crucial to analyzing binding propensities in proteins. While the problem of computing binding free energies remains open, approximate estimates have become very useful for filtering potential binding complexes. The ligand molecules were selected based on the low binding energies and identified as potential therapeutic action having sources. The active sites present in the COX 1 receptor protein as Tyr38, Tyr 402, Thr70, Thr212, Ala199, Val447, Tyr55, Asn410, Pro72, Lys211, Thr206, Asp450, Gln406, Ile74, His386, Trp387, Ser530, Pro67, Trp77, Ala202, Met413, Val116, Leu295, Phe210, COX 2 receptor protein as Glu67, Arg216, Val447, Ala199, Ser38, Glu140, Leu391, Val295, Pro40, Asn144, Phe200, Arg513, Tyr55, Tyr147, Thr212, Gly526, Asn68, Gln454, Lys211, His90, Phe220, Gln203and Ser353. An ADME/T toxicity property of the lead compound Myricetin was presented in Table 2.

R. apiculata derived Myricetin docking simulation with COX-1 A chain produced six clusters with binding energy -8.1 kcal / mol at 3rd run has six hydrogen bond interactions. Myricetin into COX-1 B chain produced six clusters with binding energy -9.83 kcal/mol at 3rd run has five hydrogen bond interactions (Fig. 3).

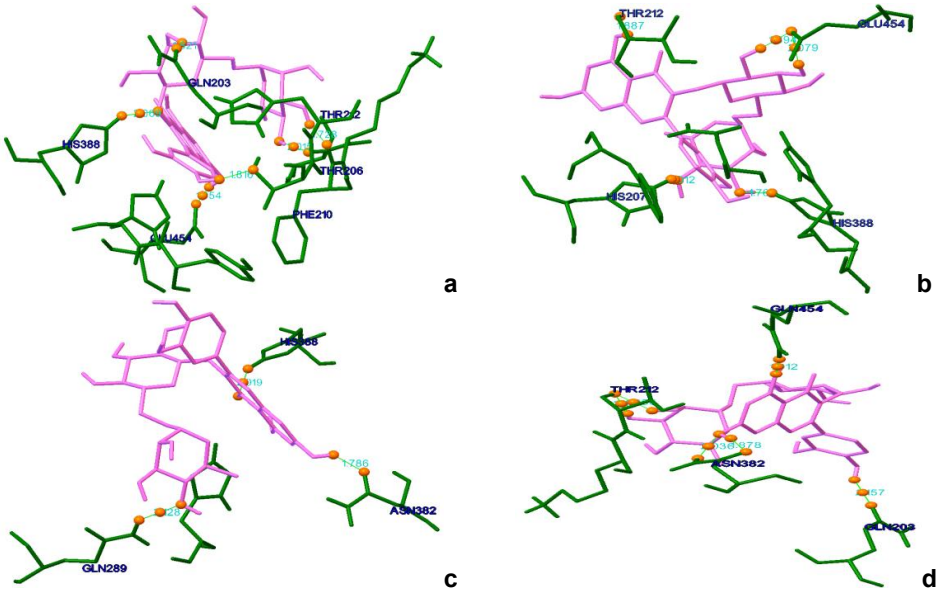

Fig. 3. Molecular docking of Cyclooxygenase receptor against ligand Myricetin

(a) COX I (Chain A) docked with Myricetin; (b) COX I (Chain B) docked with Myricetin; (c) COX II (Chain B) docked with Myricetin; (d) COX II (Chain B) docked with Myricetin. The receptor interactions with ligand are represented by Pink, Green, Gray and Orange colors respectively. Pink color represents ligand and green color protein residues, with hydrogen bond represented by Orange color

Table 2. ADME/T study of lead molecule Myricetin

S. No.	ADME properties	Myricetin
1	2DFast polar surface area	Good absorption
2	Aqueous solubility Level	Good
3	Blood Brain Barrier penetration	Undefined
4	CYP2D	Inhibitor
5	Hepatotoxicity	Non-toxic
6	HIA	Good
7	Carrier Protein Binding Level	< 90 %

Hydrogen bond distance between the donor and acceptor atoms was found to be higher in HIS 388 was, 2.069 and GLU454 was 2.079 in chain A and B respectively. Docking simulation of Myricetin into COX-2 A chain produced six clusters with binding energy -5.72 kcal/mol at 3rd run have three hydrogen bond interactions. Myricetin also, docking with COX-2 B chain produced six clusters with binding energy -7.46 kcal / mol at 3rd run has six hydrogen bond interactions (Fig. 3). Hydrogen bond distance between the donor and acceptor atoms was found to be higher in HIS 214 was, 2.167 and GLN 203 were 2.157 in chain A and B respectively.

4. DISCUSSION

The drug development field, researchers around the world used floral populations for cost-effective, low side effect and nontoxic medicinal product development. Traditionally, potential mangrove species *R. apiculata* used a fuel source, mosquito repellent, anti-oxidant, anti-cancer and malarial activity but there was no report on pharmacological evaluation of its analgesic effect. Ethanol concentration is an important factor affecting extraction yield of flavonoids, which was set at 20, 40, 60, 80 and 100%, respectively [10]. Fig. 1 shows the result of flavonoids extracted from leaves. When ethanol concentration increased from 20-60%, the extraction yield increased significantly. Further increase of ethanol concentration 80% were only affected by the slight increase of extraction yield. In addition, when ethanol concentration increased from 80 - 100%, however, the extraction yield slightly decreased. It was in accordance with existed studies [11]. The outcome of the present investigation clearly demonstrates that isolated Alk-CF of *R. apiculata* showed significant antinociceptive effect through *In vitro* and *in silico* approaches. Santanu sannigrahi [12] reported flavonoids in ethyl acetate fractions of *E. fluctuans* which significantly decreased the number of acetic acid-induced writhes in mice and increased reaction time in a hot plate method. Alk-CF showed significant analgesic action but higher analgesic activity was at 200 mg/kg b.wt. Several flavonoids also elicit an analgesic effect through the opioid system. Thirugnanasambandam [13] suggested a possible role for calcium in the analgesic action of flavonoids as with that of morphine. The acetic acid induced writhing method was found effective to evaluate peripherally active analgesics. Acetic acid induced writhing in mice ascribed visceral pain, therefore efforts are needed to screen analgesic drugs, which reducing the number of writhings will render analgesic effect preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition [14]. In our findings, significant pain reduction of Alk-CF in acetic acid induced mice model might be due to the action of flavonoids with the prostaglandin pathways. Beta-endorphin is one of the essential proteins secreted from its precursor proopiomelanocortin by the pituitary gland, in response to physiological stress conditions. They are bound to mu-opioid receptors to reduce the pain of patients under surgical conditions [15]. The analgesic effects of myricetin may be related to the action on the beta-endorphin secretion. Hagenacker et al., [16] mentioned the myricetin reduced the neuropathic pain through induction of protein kinase C, that decreases the voltage of activated calcium channel in dorsal ganglia neurons of experimental rats. The flavonoids of *Rhizophora mucronata* and *Excoecaria agallocha* and exhibited potent analgesic activity though peripherally acting mechanisms similar to the diclofenac sodium were reported [17,18]. Hot plate method is considered to be selective for the drugs acting centrally. Ibironke and Ajiboye [19] stated that an agent that causes a prolongation of the hot-plate latency using this test must be acting centrally. Therefore, the Alk-CF must have a central activity and diclofenac sodium (10 mg/kg) also presented a longer latency time than the control group in the hot plate test in a dose-related manner. At 60min, 200 mg/kg, p.o.

administration of the Alk-CF the latency period of action was found to be 16.08±0.02. Alk-CF showed significantly (P<0.01) analgesic effect in both the hot plate and tail immersion tests which implicating spinal and supraspinal analgesic pathways. The flavonoids inhibited cyclooxygenase and lipoxygenase which are involved in the initiation stage of inflammatory reactions [20]. An *in silico* findings of the present study supports the above statement through action of Myricetin docking with COX-1 and 2 inhibitor.

5. CONCLUSION

R. apiculata derived flavonoid expressed as an analgesic agent evidenced with animal and computational models. The present investigation concludes that *R. apiculata* derived flavonoid expressed as potent central and peripheral antinociceptive agent with the involvement of opioid receptors. The lead compound Myricetin will be evaluated further in human volunteers for formulation mangrove derived natural analgesic agent.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

This study was approved by the Institutional Animal Ethical Committee of Annamalai University, India (Reg.No.169/1999/CPCSEA).

ACKNOWLEDGEMENTS

The authors are gratefully acknowledged by the authorities of Annamalai University for providing all support during the study period.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=1177&id=14&aid=9468