Comparison of anti-inflammatory and analgesic effects of artocarpin-rich *Artocarpus heterophyllus* extract and artocarpin

Rizwan Rashid Bazmi[1], Muhammad Asif[2], Hafiza Sidra Yaseen[3], Pharkphoom Panichayupakaranant[1,4]

Abstract

Introduction: The present study was carried out to evaluate the in vivo anti-inflammatory and analgesic effects of artocarpin-rich *Artocarpus heterophyllus* wood extract (ARE) and its major bioactive compound, artocarpin. Methods: ARE was prepared using a green microwave extraction coupled with column chromatography using polystyrene/divinylbenzene resin. The extract obtained was standardized to contain artocarpin content of 49.6% w/w using HPLC method. The effects of ARE and artocarpin were analyzed using acute and chronic paw edema anti-inflammatory models in Wister albino rats and the acetic acid induced writhing and formalin induced analgesic models in Swiss albino mice. CAM assay was performed to evaluate the antiangiogenic potential of extract and pure compound. Results: The results indicated that ARE and artocarpin exhibited time and dose-dependent anti-inflammatory and anti-nociceptive responses. ARE at a dose of 200 mg/kg (equivalent to artocarpin 99.2 mg/kg) and artocarpin (100 mg/kg) significantly reduced ($p < 0.05$) paw edema development and pain. In addition, ARE (200 mg/kg) and artocarpin (100 mg/kg) produced comparable ($p < 0.001$) anti-inflammatory and nociceptive effects as the standard drugs, indomethacin and diclofenac sodium (10 mg/kg). At the lowest doses of ARE (50 mg/kg) and artocarpin (25 mg/kg), a smaller decrease in paw swelling was observed relative to 100 mg/kg ARE and 50 mg/kg artocarpin treatment groups ($p > 0.001$) throughout the study. Findings of CAM assay showed potent antiangiogenic activity of ARE and artocarpin Conclusion: Together these findings suggested that ARE and artocarpin are effective in ameliorating inflammatory and promoting analgesic effects. Therefore, ARE and artocarpin hold high promise as alternative anti-inflammatory and analgesic therapeutics and worthy of further investigations.

Key Words: Analgesic; anti-inflammatory; artocarpin; *Artocarpus heterophyllus*; angiogenesis

Significance | Development of natural anti-inflammatory and analgesic agent.

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Introduction

Inflammation represents a complex natural defensive reaction of host body against destructive stimuli, such as radiations, infections and immunological reactions (Chen et al., 2018). Various biochemical mediators can provoke inflammation, including cytokines (IL-6, IL-1 and TNF-alpha), nitric oxide, cyclooxygenases, neuropeptides (substance P and CGRP), growth factors (FGF and PDGF) and neurotransmitters, ultimately affecting normal body function (Mondal, Maity, & Bishayee, 2019)

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Inflammation represents a complex natural defensive reaction of host body against destructive stimuli, such as radiations, infections and immunological reactions (Chen et al., 2018). Various biochemical mediators can provoke inflammation, including cytokines (IL-6, IL-1 and TNF-alpha), nitric oxide, cyclooxygenases, neuropeptides (substance P and CGRP), growth factors (FGF and PDGF) and neurotransmitters, ultimately affecting normal body function (Mondal, Maity, & Bishayee, 2019). Generally, inflammation is signaled by erythema, swelling, pain and loss of functionality. Thus, inflammatory response is considered to be the origin of all types of pain, including acute and chronic, central or peripheral, neuropathic or nociceptive (Zacher et al., 2008). Pain is the unpleasant sensation and emotional experience resulting from diseases, trauma, and injury. The pathophysiological of pain is a complex phenomenon wherein a wide array of inflammatory mediators plays a central role in the emergence of nociception. Pain is the most frequent symptom in chronic inflammatory conditions, including rheumatoid arthritis, diabetic neuropathy, cancer and infection (Ronchetti, Migliorati, & Delfino, 2017). Recently, it has been demonstrated that several herbal extracts have promising attributes with regards to the treatment of chronic inflammation and pain (Asif, Saleem, Saadullah, Yaseen, & Al Zarzour, 2020).

Identification and purification of plant-derived bioactive compounds is crucial to understanding and validating their pharmacotherapeutic potentials (Jagtap & Bapat, 2010). Generally, a pure active compound possesses stronger pharmacological effect than its crude plant extracts. However, the large-scale use of pure compounds in the phytomedicine industry is rarely successful due to high cost of purification, which often requires many steps, as well as being energy-intensive, time-consuming and tedious. Moreover, the purification process may require large volumes of toxic and volatile solvents, ultimately increasing the potential adverse effects on human health and the environment. Recently, enriched herbal extracts with high contents of the major active compounds have been proposed to overcome or obviate these limitations. In addition, a global trend in the preparation of bioactive-rich herbal extract for commercial purposes is currently shifting towards a green extraction method propelled by improved safety profile, environmental friendliness, and high-quality of the extracts (Panichayupakaranant, 2017).

Artocarpus heterophyllus Lam. or jackfruit (family Moraceae) is widely distributed in Southeast Asia and Brazil and cultivated in dry and cold climates. It has been reported that A. heterophyllus possesses anti-inflammatory, analgesic, antimicrobial, anti-oxidant and antidiabetic properties. These biological and pharmacological effects were largely attributed to its high content of flavones, especially artocarpin, which happens to be the major active compound (Hari, Revikumar, & Divya, 2014). The present study, therefore, used artocarpin as a bioactive marker for preparation of artocarpin-rich extract (ARE) from A. heterophyllus woods using a green microwave extraction coupled with a simple one-step fractionation on reversed phase copolymer styrene-divinylbenzene (Diaion® HP 20) column while the previous studies have reported on extracts of *Artocarpus heterophyllus* prepared by conventional extraction techniques (Shirajum et al, 2015) with lower contents of active constituents of artocarpin. The extract was then evaluated for its anti-inflammatory and analgesic activity compared with the pure marker compound, artocarpin which demonstrated more pronounced effects as compared to prior reported studies.

Materials and Methods

Plant materials and chemicals

Dried A. heterophyllus heartwood powder was purchased from Charoensuk Pharma Supply (Nakhon Pathom, Thailand). Ethanol was obtained from High Science Ltd. (Hat-Yai, Thailand). Methanol (HPLC grade) was purchased from RCI Labscan (Bangkok, Thailand). Diaion® HP 20, absolute ethanol, carrageenan, acetic acid, and formaldehyde were purchased from Sigma-Aldrich (Steinheim, Germany). Purified water was obtained using a Milli Q system (Millipore, Bedford, MA, USA). Indomethacin and diclofenac sodium were obtained from Siza International Pvt., Ltd. (Lahore, Pakistan).

Animals

Wistar albino rats (150-200 g body weight) and Swiss albino mice (100-150 g body weight) were purchased from University of Agriculture, Faisalabad, Pakistan. All animal handling protocols were approved by the Institutional Review Board of Government College University Faisalabad, Pakistan (Study number: 19750 and reference number: GCUF/ERC/2150).

Preparation of ARE

The heartwood powder (600 g) was extracted with ethanol (3 L) using a microwave extraction with the following parameters: electric power of 900 Watts, frequency of 2,450 MHz, at a temperature of 70°C, for 15 min. Subsequently, the extract was filtered through a filter paper and the marc was re-extracted under the same conditions. The pooled ethanol extract was then applied to a Diaion® HP-20 column (1 kg resin was packed in a 100 cm 8 cm i.d. column) equilibrated with 50% v/v ethanol. The column was eluted with 50%, 60%, 70%, and 80% v/v ethanol, respectively. Based on TLC analysis (silica gel F254, hexane/ethyl acetate; 60/40), 7.5 g of ARE was obtained from the 80% v/v ethanol pooled fractions after solvent evaporation under reduced pressure at 45°C.

Purification of artocarpin

Artocarpin was purified from ARE using silica gel column chromatography. ARE (2 g) was loaded on a silica gel column. The column was eluted using a step-gradient elution of ethyl acetate in

hexane solutions starting from 10%, 15% and 20% ethyl acetate. Fraction numbers 71 to 180 eluted with 20% ethyl acetate were pooled and evaporated under reduced pressure to obtain 510 mg yellow crystal, which was identified as artocarpin based on its 1H and 13C NMR data (Septama & Panichayupakaranant, 2015).

Quantitative HPLC analysis of artocarpin

The content of artocarpin in ARE was determined using an HPLC method previously described by Septama and Panichayupakaranant (2016) with minor modifications. The HPLC system consisted of a binary HPLC pump (Shimadzu 33236, Tokyo, Japan) equipped with an autosampler (Shimadzu 03224, Tokyo, Japan), a photodiode array detector (Shimadzu 12623, Tokyo, Japan) and a Discovery® C18 column (5 µm, 4.6 mm × 150 mm, Supelco, PA, USA). The column was eluted with a mixture of methanol and water using a gradient elution system as follows: 0-8 min, 60:40; 8-27 min, 80:20; 27-35 min, 60:40, v/v, at a flow rate of 1 mL/min. The peak of artocarpin was detected at a wavelength of 285 nm. The calibration curve of artocarpin was established at five concentrations (100-6.25 µg/mL) to obtain the linear equation, Y = 83499X - 11508 (R2 = 0.9999). The analysis was performed in triplicate.

Acute anti-inflammation assay

Acute anti-inflammatory effects of ARE and artocarpin were determined using the carrageenan- and serotonin-induced paw edema models. For both acute anti-inflammatory models, Wister albino rats were divided into 6 groups (n = 6), consisting of those orally treated with distilled water (10 mL/kg, negative control), ARE (50, 100 and 200 mg/kg), artocarpin (25, 50 and 100 mg/kg) and indomethacin (10 mg/kg, standard). One hour after treatment, 0.1 mL of 1% w/v freshly prepared carrageenan or serotonin solution was injected subcutaneously in the sub- plantar surface of left hind paw of rats in each group. A digital vernier caliper was used for measuring the paw volume at intervals of 0, 1, 2, 3, 4, 5 and 6 h (Yaseen et al., 2020). The percentage inhibition was calculated as follow:

Chronic anti-inflammation assay

Chronic anti-inflammatory effect of the samples was evaluated using the formalin induced paw oedema assay and cotton pellet induced granuloma model (Saleem et al., 2020). The animals, Wister albino rats were divided into six groups (n = 6). Group 1 served as a negative control and was orally administered 10 mg/kg distilled water, while groups 2 - 6 served as treatment groups and were orally administered ARE (50, 100 and 200 mg/kg), artocarpin (25, 50 and 100 mg/kg) and indomethacin (10 mg/kg), respectively. Percentage inhibition was calculated using the same formula given in the acute models. Anti-granuloma effect of ARE, artocarpin and indomethacin was determined using the method described by (Asif, Saadullah, et al., 2020). On 14th day in cotton pellet induced granuloma assay, rats were sacrificed, and blood collected for biochemical and hematological analysis.

Formalin induced licking assay

Swiss albino mice (100-150 g body weight) were divided into six groups having six animals each. The assay was performed as previously described by (Hacimuftuoglu et al., 2006). Briefly, ARE (50, 100 and 200 mg/kg), artocarpin (25, 50 and 100 mg/kg) and the standard drug (diclofenac sodium, 10 mg/kg) was administered orally for 30 min before the injection. The duration that the animals spent licking their right hind paw was evaluated for analgesia and this was expressed as percentage inhibition of the time spent in licking, which was determined for each experimental group as follow:

Acetic acid-induced writhing assay

The assay was performed according to the previous report by (Omeh & Ezeja, 2010) with slight modifications. Reduction in writhing number compared to the control group was evaluated for analyzing analgesia effect of ARE (50, 100 and 200 mg/kg), artocarpin (25, 50 and 100 mg/kg) and diclofenac sodium (10 ml/kg). This was expressed as percentage inhibition of writhing, which was determined for each experimental group as follow:

Antiangiogenic activity

Ex-vivo chicken chorioallantoic membrane assay was performed following reported methods to evaluate the antiangiogenic activity of extract and pure compound (Asif, Saleem, Saadullah, Yaseen, & Al Zarzour, 2020).

Statistical analysis

Data was presented as mean ± standard error of mean (SEM) and statistical variations among data was evaluated by one-way ANOVA followed by Post Hoc Tuckey's test through GraphPad Prism Software. $p < 0.05$ will be considered significant statistically.

Results

Standardization of ARE

Based on quantitative HPLC analysis, ARE was standardized to contain 49.6 ± 0.14% w/w artocarpin before being subjected to evaluation of its anti-inflammatory and analgesic effects.

Anti-inflammatory effects of ARE and artocarpin in Wister albino rats

The anti-inflammatory effects of ARE and artocarpin in two acute models (carrageenan and serotonin) are shown in Table 1 & 2. In both models, control group indicated a continuous increase in oedema development during the entire 6-hour study. In carrageenan model, ARE and artocarpin showed a time-dependent anti-inflammatory effect. Post treatment of 1st and 2nd hour, ARE

Table 1. Anti-inflammatory effects of ARE and artocarpin in carrageenan induced acute anti- inflammatory paw edema model in Wister albino rats. Values are mean ± SEM of ARE and artocarpin (n = 6), where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and ns = $p > 0.05$. A green color indicates significant analysis between 50, 100 and 200 mg/kg of ARE treatment groups whereas a red color compares between artocarpin 25, 50 and 100 mg/kg treatment groups. A brown color compares between ARE (200 mg/kg) and indomethacin (10 mg/kg), and a purple color compares between artocarpin (100 mg/kg) and indomethacin (10 mg/kg).

Time (hour)	ARE (50 mg/kg)	ARE (100 mg/kg)	ARE (200 mg/kg)	Artocarpin (25 mg/kg)	Artocarpin (50 mg/kg)	Artocarpin (100 mg/kg)	Indomethacin (10 mg/kg)
1st	0.9 ± 0.00	19.5 ± 2.90 ***	26.5 ± 2.43 *ns*	3.4 ± 0.08	14.2 ± 2.57*	25.3 ± 2.58 *	35.5 ± 2.87 *ns*/*ns*
2nd	4.4 ± 0.03	21.3 ± 3.48 ***	31.8 ± 3.51 *ns*	8.1 ± 0.02	19.4 ± 2.32 *ns*	32.9 ± 2.82 *ns*	40.2 ± 3.04 *ns*/*ns*
3rd	7.6 ± 0.01	25.5 ± 1.84***	42.1 ± 3.90 **	9.6 ± 0.06	30.4 ± 2.74 ***	34.2 ± 3.59 *ns*	45.2 ± 3.10 *ns*/*ns*
4th	11.3 ± 0.07	37.8 ± 3.07 ***	45.7 ± 3.03 *ns*	13.0 ± 0.03	45.9 ± 4.33 ***	47.1 ± 5.92 *ns*	51.6 ± 3.31 *ns*/*ns*
5th	14.3 ± 0.03	55.5 ± 5.38 ***	58.0 ± 2.66 *ns*	15.0 ± 0.01	55.0 ± 2.89 ***	61.8 ± 6.59 *ns*	64.4 ± 4.10 *ns*/*ns*
6th	15.7 ± 0.09	76.5 ± 0.94***	84.3 ± 1.07 *	16.1 ± 0.03	71.7 ± 2.26***	87.3 ± 2.23 ***	92.6 ± 2.24 */*

Table 2. Anti-inflammatory effects of ARE and artocarpin in serotonin induced acute anti- inflammatory paw edema model in Wister albino rats. Values are mean ± SEM of ARE and artocarpin (n = 6), where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and ns = $p > 0.05$. A green color indicates significant analysis between 50, 100 and 200 mg/kg of ARE treatment groups whereas a red color compares between artocarpin 25, 50 and 100 mg/kg treatment groups. A brown color compares between ARE (200 mg/kg) and indomethacin (10 mg/kg), and a purple color compares between artocarpin (100 mg/kg) and indomethacin (10 mg/kg).

Time (hour)	ARE (50 mg/kg)	ARE (100 mg/kg)	ARE (200 mg/kg)	Artocarpin (25 mg/kg)	Artocarpin (50 mg/kg)	Artocarpin (100 mg/kg)	Indomethacin (10 mg/kg)
1st	1.5 ± 0.01	21.1 ± 3.16 ***	29.6 ± 3.13 *ns*	3.8 ± 0.05	21.2 ± 2.80 ***	30.0 ± 2.95 *ns*	36.3 ± 2.99 *ns*/*ns*
2nd	3.3 ± 0.05	25.1 ± 2.22 ***	31.5 ± 2.61 *ns*	5.1 ± 0.01	24.4 ± 2.34 ***	33.5 ± 2.92 *	39.2 ± 2.92 *ns*/*ns*
3rd	5.4 ± 0.06	29.3 ± 2.43 ***	36.2 ± 4.38 *ns*	6.1 ± 0.03	26.0 ± 2.00 ***	38.3 ± 4.08 *ns*	45.3 ± 3.00 *ns*/*ns*
4th	8.8 ± 0.01	33.9 ± 1.97***	47.3 ± 3.24 *	9.3 ± 0.04	32.5 ± 2.07 ***	43.2 ± 3.18 *ns*	52.9 ± 2.91 *ns*/*ns*
5th	10.7 ± 0.03	42.2 ± 2.65 ***	52.9 ± 3.20 *ns*	13.1 ± 0.06	45.1 ± 5.01***	58.9 ± 3.78 *	64.6 ± 2.16 *ns*/*ns*
6th	11.1 ± 0.03	70.0 ± 2.43 ***	80.2 ± 5.12 *ns*	14.8 ± 0.05	65.3 ± 3.92 ***	84.4 ± 2.25 *ns*	91.0 ± 3.17 *ns*/*ns*

Table 3. Anti-inflammatory effects of ARE and artocarpin in formalin induced paw edema in Wister albino rats. Formalin induced paw edema model (% Inhibition). Values are mean ± SEM (n = 6), where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and ns = $p > 0.05$. A green color indicates significant analysis between ARE (100 mg/kg) and ARE (200 mg/kg), a red color compares between artocarpin (50 mg/kg) and artocarpin (100 mg/kg), a brown color compares between ARE (200 mg/kg) and indomethacin (10 mg/kg), and a purple color compares between artocarpin (100 mg/kg) and indomethacin (10 mg/kg)

Days	Ctriall	ARE (100 mg/kg)	ARE (200 mg/kg)	Artocarpin (50 mg/kg)	Artocarpin (100 mg/kg)	Indomethacin (10 mg/kg)
1st	0.0 ± 0.0	9.07 ± 2.05 *ns*	9.92 ± 2.23 *ns*	2.77 ± 1.53 *ns*	11.3 ± 2.47 *ns*	27.8 ± 4.11 ***/***
2nd	0.0 ± 0.0	6.19 ± 1.96 *ns*	12.8 ± 2.75 *ns*	3.76 ± 1.88 *ns*	10.8 ± 2.31 *ns*	33.6 ± 5.88 ***/***
3rd	0.0 ± 0.0	23.7 ± 3.14 *ns*	18.7 ± 2.21 *ns*	16.0 ± 2.22 *ns*	21.7 ± 0.74 *ns*	35.4 ± 3.22 ***/***
4th	0.0 ± 0.0	23.0 ± 2.69 *ns*	25.5 ± 2.01 *ns*	22.1 ± 1.99 *ns*	24.3 ± 1.83 *ns*	43.7 ± 3.00 ***/***
5th	0.0 ± 0.0	24.6 ± 3.57 *ns*	27.7 ± 3.00 *ns*	21.7 ± 2.52 *ns*	22.9 ± 2.31 *ns*	51.6 ± 3.73 ***/***
6th	0.0 ± 0.0	38.6 ± 3.57 *ns*	32.9 ± 2.45 *ns*	29.4 ± 2.74 *ns*	30.2 ± 2.30 *ns*	65.0 ± 3.80 ***/***
7th	0.0 ± 0.0	43.3 ± 3.72 *ns*	46.2 ± 4.00 *ns*	35.3 ± 3.53 *ns*	40.8 ± 2.14 *ns*	78.2 ± 4.68 ***/***
8th	0.0 ± 0.0	47.4 ± 5.15 *ns*	52.3 ± 3.94 *ns*	41.7 ± 6.03 *ns*	52.9 ± 2.85 *ns*	83.9 ± 2.74 ***/***
9th	0.0 ± 0.0	49.7 ± 5.23 *ns*	64.2 ± 4.35 *ns*	54.5 ± 6.31 *ns*	66.9 ± 3.31 *ns*	85.7 ± 2.10 */*
10th	0.0 ± 0.0	70.6 ± 1.64 *ns*	83.4 ± 2.00 *ns*	74.2 ± 0.83 *ns*	84.0 ± 0.48 *ns*	92.1 ± 1.20 ***/***

Cotton pellet induced gratriala model (% Inhibition).

Groups name	Ctriall	ARE (100 mg/kg)	ARE (200 mg/kg)	Artocarpin (50 mg/kg)	Artocarpin (100 mg/kg)	Indomethacin (10 mg/kg)
% Inhibition	0.0 ± 0.0	63.3 ± 2.56**	75.7 ± 1.31**	68.4 ± 1.83 *ns*	75.2 ± 2.94 *ns*	83.0 ± 1.48*/*

Table 4 Anti-analgesic activity of ARE and artocarpin in acetic acid induced writhing and formalin-induced paw licking model in Swiss albino mice. Values shown are mean ± SEM of ARE and artocarpin in acetic acid and formalin induced paw edema study (n = 6). (Where * = p < 0.05, ** = p < 0.01, *** = p < 0.001 and ns = p > 0.05 respectively). Green color stars show the comparison between ARE 50, 100 & 200 mg/kg, while the comparison between ARE (200 mg/kg) with diclofenac (10 mg/kg) show by brown colour stars. The red color stars show the comparison between artocarpin 25, 50 & 100 mg/kg, while the comparison between artocarpin (100 mg/kg) with diclofenac 10 mg/kg is indicated by purple color stars, respectively.

	% Inhibition	
Group name	Acetic acid induced writhing model	Formalin-induced paw licking model
Control	0.0 ± 0.0	0.0 ± 0.0
ARE (50 mg/kg)	7.0 ± 0.01	11.4 ± 0.90
ARE (100 mg/kg)	69.5 ± 3.32 ***	62.6 ± 0.971***
ARE (200 mg/kg)	73.5 ± 3.51*	70.0 ± 1.14***
Artocarpin (25 mg/kg)	8.9 ± 0.01	14.1± 0.33
Artocarpin (50 mg/kg)	62.5 ± 4.67***	60.4 ± 0.740***
Artocarpin (100 mg/kg)	81.8 ± 3.01**	72.5 ± 1.23***
Standard (Diclofenac 10 mg/kg)	89.1 ± 1.98 */*	77.0 ± 0.513*/*

Table 5. Global metrics and segment characteristics evaluation in CAM assay

Groups	**Vessel density [%]**	**Total vessel length network [px]**	**Total branching points**	**Total nets**	**Total segments**	**Mean segment length [px]**	**S.D segment length**
Control (Before treatment)	16.1 ± 0.1	216650.3 ± 5.50	86.0 ± 2.64	6.66 ± 0.57	181.6 ± 7.63	1205.3 ± 6.11	1287.3 ± 5.68
Control (after treatment)	17.6 ± 0.45	242775.3 ± 9.50***	125.0 ± 5.0***	2.33 ± 0.57***	243.6 ± 5.50***	991.6 ± 3.05***	trial6 ± 14.2 ***
Artocarpin 100 µg/mL (Before treatment)	15.6 ± 0.36	86540.0 ± 580.3	46.66 ± 1.52	11.66 ± 0.57	119.0 ± 4.0	730.0 ± 5.00	754.3 ± 5.50
Artocarpin 100 µg/mL (after treatment)	9.63 ± 0.47***	75024.0 ± 7.54***	28.33 ± 3.05***	12.33 ± 1.15ns	88.66 ± 1.15***	852.3 ± 7.50***	627.0 ± 2.64***
Artocarpin 200 µg/mL (Before treatment)	18.0 ± 1.0	200239.0 ± 10.01	77.66 ± 2.51	5168.3 ± 19.03	164.0 ± 3.60	1199.3 ± 10.50	1222.0 ± 8.73
Artocarpin 200 µg/mL (after treatment)	5.0 ± 0.2***	37149.0 ± 10.14***	19.0 ± 1.0***	6.0 ± 1.00***	48.1 ± 1.00***	775.0 ± 4.58***	764.6 ± 5.03***

ARE 100 µg/mL (Before treatment)	15.0 ± 1.0	69848.0 ± 15.0	46.3 ± 1.52	8.66 ± 0.57	113.3 ± 1.52	538.3 ± 6.02	559.6 ± 4.50
ARE 100 µg/mL (after treatment)	5.2 ± 0.26***	40946.0 ± 8.50***	25.0 ± 1.0***	7.66 ± 1.52ns	61.3 ± 1.52***	649.3 ± 6.02***	638.0 ± 7.54 ***
ARE 200 µg/mL (before treatment)	22.1 ± 1.02	284627.3 ± 18.1	183.3 ± 4.16	7.0 ± 1.0	360.0 ± 5.56	792.6 ± 6.50	673.3 ± 6.65
ARE 200 µg/mL (after treatment)	13.4 ± 0.51***	187182.7 ± 8.02***	101.0 ± 1.0***	4.66 ± 0.57***	217.3 ± 2.51***	863.3 ± 3.51***	845.6 ± 4.50***

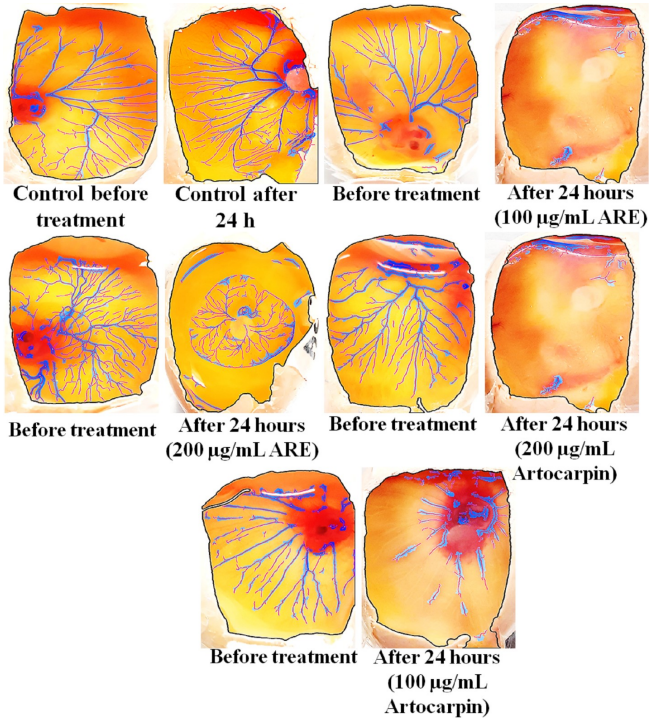

Figure 1. Antiangiogenic effects of ARE and atrocarpin in ex vivo CAM assay. Analysis of images was done through online WIMASIS software (Onimagin Technologies SCA, Spain)

200 and 100 mg/kg produced almost similar anti-inflammatory effect ($p > 0.05$) having no statistically significant difference between both concentrations, while artocarpin 100 mg/kg exhibited significant ($p < 0.05$) reduction in paw edema than 50 mg/kg artocarpin during 1st hour of the study. From the 3rd hour onwards, 200 mg/kg ARE showed better ($p < 0.001$) antiedematous effect than ARE 100 mg/kg. During this period, comparison between 50 mg/kg and 100 mg/kg artocarpin yielded higher inhibition of paw edema at higher concentration, though the increase was only modest ($p > 0.05$). Additionally, comparison of high dose ARE (200 mg/kg) and artocarpin (100 mg/kg) with the standard (indomethacin 10 mg/kg), exhibited an inhibitory effect on paw swelling with no significant difference ($p > 0.05$) almost throughout this 6-hour study. Notably, it was observed that paw oedema was significantly reduced in the ARE 100 mg/kg and artocarpin 50 mg/kg treated animals, and this reduction was also significant ($p < 0.001$) when compared with animal groups administered the lowest dose, ARE (50 mg/kg) and artocarpin (25 mg/kg) in across the entire study period (Table 1).

Data obtained from the anti-inflammatory effect of ARE and artocarpin in serotonin induced paw edema model is presented in Table 2. After 1–3-hour post serotonin administration, for animals treated with ARE (100 vs 200 mg/kg) compared to those treated with artocarpin (50 vs 100 mg/kg), a dose independent reduction in percentage inhibition of paw edema was observed with no significant difference ($p > 0.05$) between both groups. From 4 to 6 h, 200 mg/kg of ARE mostly showed similar level of inhibition of edema when compared with 100 mg/kg ARE ($p > 0.05$). With regards to the anti-inflammatory effect of the pure bioactive, artocarpin, the compound clearly attenuated paw edema formation. However, on increasing the concentration from 50 to 100 mg/kg only a modest increase in the inhibitory effect was observed towards edema formation ($p > 0.05$). At the lowest dose of artocarpin (25 mg/kg) as well as the enriched extract, ARE (50 mg/kg), only minimal decrease in paw swelling was recorded when compared to the artocarpin 50 mg/kg and ARE 100 mg/kg treatment groups ($p > 0.001$) from start to end of activity (Table 2). Therefore, the lowest dose of artocarpin (25 mg/kg) and ARE (50 mg/kg) were not investigated in the chronic inflammatory models. Most importantly, when ARE at 200 mg/kg or artocarpin at 100 mg/kg was compared with the standard drug (indomethacin10 mg/kg) for the complete study period (1-6 h), no significant difference was found in their anti-inflammatory activity ($p > 0.05$).

Anti-inflammatory effects of artocarpin and ARE in formalin induced arthritis model

Data obtained from the formalin induced arthritis model is shown in Table 3. At 1 to 10 days of formalin administration, ARE 200 mg/kg produced comparable anti-edematous effect to ARE 100 mg/kg, having no significant difference ($p > 0.05$) statistically. Moreover, artocarpin 100 mg/kg treated animals also exhibited non significantly different enhanced ($p > 0.05$) anti- inflammatory effects as compared to 50 mg/kg artocarpin throughout the 10-day study. Similarly, 1st to 8th day of formalin administration, 200 mg/kg of ARE and 100 mg/kg of artocarpin exhibited highly substantial ($p < 0.001$) reductions in inflammation as compared to the standard drug (indomethacin). In addition, on the 9th day, 200 mg/kg ARE and 100 mg/kg artocarpin showed significant ($p < 0.05$) anti-inflammatory effect when compared with the standard drug indomethacin (10 mg/kg), while 200 mg/kg ARE and artocarpin 100 mg/kg was developed better inhibition in edema development with no significant difference ($p > 0.05$) (Table 3).

Findings on the anti-edematous effects of ARE and artocarpin in cotton pellet induced granuloma model are presented in Table 3. The percentage inhibition in ARE (100 mg/kg and 200 mg/kg), artocarpin (50 mg/kg and 100 mg/kg) and indomethacin (10 mg/kg) treated groups was 63.3 ± 2.56, 75.7 ± 1.3, 68.4 ± 1.83, 75.2 ± 2.94 and 83.0 ± 1.48 respectively. ARE 200 mg/kg showed considerable ($p < 0.01$) reduction in granuloma formation as compared to ARE 100 mg/kg. When the anti-inflammatory effect induced by 100 mg/kg artocarpin was compared with that produced by 50 mg/kg artocarpin, no significant difference ($p > 0.05$) was found. Expectedly, indomethacin (10 mg/kg) was found to exhibited significantly higher ($p < 0.05$) inhibitory effect on granuloma formation relative to 200 mg/kg ARE and 100 mg/kg artocarpin (Table 3).

Acetic acid induced writhing and formalin induced paw licking analgesic model in Swiss albino mice

Data on the antinociceptive effect of ARE and artocarpin is shown in Table 4. In acetic acid induced writhing model, ARE 100 and 200 mg/kg produced almost similar effect, having no significant difference ($p > 0.05$). However, 100 mg/kg ARE treatment group produced significantly comparable ($p < 0.001$) pain relieving activity when compared with ARE 50 mg/kg treatment group. While on the other hand, 100 mg/kg artocarpin showed more efficient ($p < 0.01$) analgesic effect than 50 mg/kg artocarpin. Furthermore, 100 mg/kg artocarpin showed notable increase ($p < 0.001$) in nociceptive reflexes as compared to 25 mg/kg artocarpin. Comparison of diclofenac-10 mg/kg with ARE 200 mg/kg revealed significant ($p < 0.05$) reduction of abdominal reflexes of pain in standard treated animals (Table 4).

Data obtained from the formalin-induced licking model indicated that higher doses of both test drugs (200 mg/kg ARE and 100 mg/kg artocarpin) had very high ($p < 0.001$) antinociceptive activity than lower doses (100 mg/kg ARE and 50 mg/kg artocarpin), respectively. Noteworthy, 100 mg/kg ARE and 50 mg/kg artocarpin showed significant augmentation ($p < 0.001$) in pain relieving effect of animals than the lowest doses of ARE (50

mg/kg) and artocarpin (25 mg/kg). Moreover, 100 mg/kg artocarpin, 200 mg/kg ARE and 10 mg/kg diclofenac demonstrated significant reduction ($p < 0.05$) in licking rate throughout the study (Table 4).

Antiangiogenic activity

Data in Table 5 showed dose related anti-angiogenic effect in both pure artocarpine compound and artocarpine rich extract treatment groups. The value of vessel density, total vessel length, total branching points, total nets, total segment and total segment length was significantly ($p < 0.001$) decreased when 100 verses 200 µg/mL dose of pure artocarpine and ARE were compared through statistical software. However, in control group remarkable increase was observed in all above-mentioned parameters after 24 hours of treatment ($p < 0.001$) (Figure 1).

Discussion

The present study was conducted to evaluate the anti-inflammatory and antinociceptive attributes of ARE and artocarpin using various acute and chronic in vivo models. Generally, inflammation acts as a primary protective response against harmful stimuli, including pathogens, damaged cells, toxic compounds and radiations (Medzhitov, 2010). However, uncontrolled inflammation enhances leukocytes recruitment at inflamed area, contributing to the occurrence of chronic pathologies like arthritis, diabetes mellitus, cancer, Alzheimer, atherosclerosis, wrinkles, impotence, myocardial infarctions and autoimmune diseases (Zhou, Hong, & Huang, 2016). In this study, carrageenan was used to induce inflammation in vivo. Carrageenan is a chemical agent which induces acute inflammation in the rat paw edema model in three successive time- dependent stages. In the first stage, following 1-2 hours of carrageenan administration, proinflammatory mediators such as histamine become activated. The second stage, within 2-3 h, is due to bradykinins and serotonin, while the third stage had been attributed to prostaglandins and TNF-α (Kumar, Gupta, & Singh, 2016) Herein, investigations based on the carrageenan- induced experimental model revealed significant antiedematous effect of ARE and artocarpin on the autacoid system.

Serotonin or 5-hydroxytryptamine, as earlier alluded, is a key pro-inflammatory mediator. This oedemogen elicits its inflammatory action by acting on the surrounding vasculature and tissues. In particular, serotonin induces vasodilation and enhances vascular permeability via increasing the levels of nitric oxide and vascular endothelial growth factor (VEGF). The increased space formed between the endothelial cells creates a path through which macromolecules and fluids leak freely into the surrounding tissues which leads to edema (Yong et al., 2013). Furthermore, we also observed a dose-dependent inhibitory effect of ARE and artocarpin on animal paw edema with respect to time. Both ARE and its principal bioactive, artocarpin, suppressed the amount of inflammatory mediators at wound site, and the observed effect was similar to established pharmacological actions of the standard drug, indomethacin.

The formalin-induced chronic paw edema model is widely used to evaluate the antiarthritic, antiproliferative and anti-inflammatory effects of test drugs. Formalin produces chronic inflammation by adopting a biphasic response. The initial episode involves neurogenic pain (immediate response of formalin) while the subsequent stage entail inflammatory reactions modulated by inflammatory mediators such as prostaglandin, serotonin, histamine, bradykinin and cytokines (interleukin-1 beta, interleukin-6, TNF-α) and nitric oxide as well (Arzi, Olapour, Yaghooti, & Karampour, 2015). In this study, we found that ARE and artocarpin exhibited efficient antiedematous effects, and the outcomes were similar to standard marketed product.

The cotton pellet-induced granuloma formation model is characterized by transduative, exudative and proliferative phases of chronic inflammation. Granuloma development, in particular, involves three phases. The inflammatory phase starts after three hours of cotton pellets implantation and this is characterized by vascular permeability and prompt spillage of fluid from blood vessels. After 3 – 72 h, the proliferative phase begins, and mainly involves protein leakage from nearby blood vessels. The proliferative stage continues for 3 – 6 days, during which proinflammatory cytokines and chemokines are secreted, leading granulation tissue formation (Kumar et al., 2016). Along with the proinflammatory mediators, secretion of reactive oxygen species and lysosomal enzymes also contribute to granuloma formation (Ashok et al., 2010). In the present study, dose-related significant reduction of both wet and dry weigh of the ARE (200 mg/kg) and artocarpin (100 mg/kg) treated animals were noted. This was suggestive that the anti- inflammatory activity of these agents was considerable at the transudative and proliferative phase of the inflammatory process.

Furthermore, the antinociceptive potential of ARE and artocarpin was also assessed by performing acetic acid abdominal contraction and formalin induced paw licking test. Acetic acid is chemical agent which is reputed for its irritability and pain inducting effect. Due to transduction of pain signals towards central nervous system numerous endogenous chemical agents mainly prostaglandins (PGE2 and PGF2α) that have increased sensitivity for nociceptors are released, causing irritation (Gupta et al., 2015). Findings from the present research work affirm that ARE and artocarpin possess comparable analgesic activity. Additionally, it was also clear that higher doses of both test drugs showed significant reduction in reflexive pain signs than control. Formalin-induced paw licking response is two phasic reactions

wherein early phase comprises of direct stimulation of nociceptors and mainly involves substance P and bradykinin as irritant. The late phase depend upon progression of peripheral inflammation due to serotonin, nitric oxide and prostaglandins along with neurological alterations (Antonisamy et al., 2017). Data of present study indicated that ARE and artocarpin have comparable and significant capacity to suppress pain.

The novelty of the present study is that it is 1st study to be carried out on rich- extract of *Artocarpus heterophyllus* which are prepared by green extraction technique i-e microwave assisted extraction (MAE) and this extract contain higher contents of active compound about 49.6% of artocarpin analyzed by HPLC and the pure compound artocarpin also isolated by using green solvent from rich extract of the said plant which is also 99.9% pure, while previous data (Shirajum et al, 2015) on this plant mentioned the use of extracts prepared by using conventional extraction techniques. In present study, the anti-inflammatory and analgesic effects of rich extract and pure compound are also more pronounced as compared to extracts prepared by conventional techniques as methanolic extract of *Artocarpus heterophyllus* used for determination of anti-inflammatory and analgesic potential of this plant.

Conclusion

Together these findings suggested that ARE as well as artocarpin are effective in ameliorating inflammatory and promoting analgesic effects. Therefore, ARE and artocarpin hold high promise as alternative anti-inflammatory and analgesic therapeutics and worthy of further investigations.

Author Contributions

Panichayupakaranant, P. and Dr. M. Asif contributed to conception and design of the research. Bazmi, R.R., Dr. M. Asif and Panichayupakaranant, P. conducted the experiments, analyzed data, and prepared the manuscript.

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Competing financial interests

The author(s) declare no competing financial interests.

Supplementary Information

Please download supplementary information online.

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