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Pharmacological basis of the use of *Acorus calamus* L. in inflammatory diseases and underlying signal transduction pathways

[Bases farmacológicas del uso de Acorus calamus L. en enfermedades inflamatorias y las vías de transducción de señales subyacentes]

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Abstract: *Acorus calamus* L. is used as anti-inflammatory remedy in traditional system of medicine in Pakistan and India. This study was designed to explore the anti-inflammatory mechanism of *Acorus calamus* L. and its underlying signaling pathways. Aqueous, butanolic and n-hexane fractions of *Acorus calamus* were tested against cyclooxygenase (COX) and lipoxygenase (LOX) mediated eicosanoids production by arachidonic acid (AA). Butanolic fraction of *Acorus calamus*, but not the aqueous and n-hexane fractions, inhibited the COX mediated production of thromboxane B_2 (TXB_2) and liopxygenase product 1 (LP1) -a metabolite of LOX pathway. 12-(hydroxyeicosatetraenoic acid) HETE- another product of the LOX pathway was unaffected by all three fractions. Butanolic fraction of *Acorus calamus* showed strong inhibition against AA-induced platelet aggregation. Investigation of the underlying signaling pathways revealed that butanolic fraction inhibited phospholipase C (PLC) pathway in platelets, most probably acting on protein kinase C (PKC). Aqueous and n-hexane fractions of *Acorus calamus* were not effective against any platelet agonist. This study shows that butanolic fraction of *Acorus calamus* possesses components that inhibit AA metabolism and platelet aggregation through multiple pathways.

Keywords: *Acorus calamus*, anti-inflammatory, cyclooxygenase, lipoxygenase, thromboxane, platelets

Resumen: *Acorus calamus* L. se utiliza como remedio anti-inflamatorio en el sistema tradicional de la medicina en Pakistán y la India. Este estudio fue diseñado para explorar el mecanismo anti-inflamatorio de *Acorus calamus* L. y sus vías de señalización subyacentes. Fracciones acuosa, butanólica y de n-hexano de Acorus calamus se ensayaron frente a la ciclooxigenasa (COX) y de la lipoxigenasa (LOX) mediada por la producción de eicosanoides por el ácido araquidónico (AA). La fracción butanólica de *Acorus calamus*, pero no las fracciones acuosas y de n-hexano, inhibieron la producción de COX mediada por tromboxano B2 (TXB2) y el producto lipoxigenasa 1 (LP1) - un metabolito de la vía de LOX, 12 - (ácido hidroxieicosatetraenoico) HETE - otro producto de la ruta de LOX no fue afectado por las tres fracciones. La fracción butanólica de *Acorus calamus* mostró una fuerte inhibición contra la agregación plaquetaria inducida por AA. La investigación de las vías de señalización subyacentes reveló que la fracción butanólica inhibió la fosfolipasa C (PLC) y la vía en las plaquetas, probablemente actuando sobre la proteína quinasa C (PKC). Fracciones acuosas y de n - hexano de *Acorus calamus* no fueron eficaces contra ningún agonista de plaquetas. Este estudio muestra que la fracción butanólica de *Acorus calamus* posee componentes que inhiben el metabolismo del AA y la agregación plaquetaria a través de múltiples vías.

Palabras Clave: *Acorus calamus*, anti -inflamatorio, ciclooxigenasa, lipoxigenasa, tromboxano, plaquetas.

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Introduction

Acorus calamus L. (*Araceae*) is found in the northern areas of Pakistan, in India, Central Asia, North America and Eastern Europe. In England, it is present as a wild plant on the edges of lakes and streams (Wallis, 1985) and has become widely diffused due to cultivation. It is a perennial herb with a creeping and much branched aromatic rhizome (Kapoor, 1990).

Rhizome of *Acorus calamus* L. is widely used as anti-inflammatory agent in the Indian systems of medicine, such as Ayurveda, Siddha and Unani (Meena *et al.*, 2010). It is also frequently used as nerve tonic and as an emetic (Baquar, 1989). It is used in low grade remittent fever, in colic pain, in inflammation, in dyspepsia (Kapoor, 1990) and in diarrhea, flatulence and asthma. It is usually used as a decoction at a dose of 1-3 g (Usmanghani *et al.*, 1997) or 2-7 g as powdered rhizome (Duke, 2002).

Many pharmacological activities of the genus *Acorus* are known including *Acorus tatarinowii*, showing antidepressant (Han *et al.*, 2013) and anti-fatigue activity (Zhu *et al.*, 2012), while anticonvulsant activity is reported from *Acorus gramineous* (Chen *et al.*, 2013) and anti-melanogenic and antioxidant activities from *Acorus macropadiceus* (Huang *et al.*, 2012). However, *Acorus calamus* has been repoted for most of the activities. *Acorus calamus* extract has been used in traditional Chinese prescription due to its beneficial effects in memory disorder and on learning performance while anti-aging effect in senescence has also been reported (Nishiyama *et al.*, 1994; Zhang *et al.*, 1994).

Acorus calamus L. also finds its use in the Ayurvedic system of medicine, where it is used for the treatment of epilepsy, hysteria, insomnia, melancholia, neurosis, loss of memory and remittent fevers (Kirtikar and Basu, 1956; Agarwal *et al.*, 1956). Ethanolic extract of rhizome of this plant possesses sedative, analgesic, moderately hypotensive and respiratory depressant properties (Agarwal *et al.*, 1956). Many other activities have been reported from this plant including, antifungal (Rajput and Karuppayil, 2012), anti-inflammatory (Kim *et al.*, 2009; Tiwari *et al.*, 2010) and antispasmodic through calcium channel blockade (Gilani *et al.*, 2006). Hot water (HW), butylene glycol (BG), hexane (HE) and steam distilled (SD) extracts of *Acorus calamus* showed different cytoxicity levels evaluated in RBL-2H3 cells (Kim *et al.*, 2012a).

Studies on chemical composition of *Acorus* spp. have revealed that α- and β-asarones are the major active components (Lee *et al.*, 2010; Geng *et al.*, 2010). Ten (10) compounds were isolated by Qiao *et al.*, (2012) from the *Acorus calamus*, many of which for the first time from this genus. A bioassay-guided fractionation and chemical investigation of the MeOH extract from the rhizomes of *Acorus gramineus* resulted in the isolation and identification of thirteen phenolic derivatives (Kim *et al.*, 2012b) including two new 8-O-4'-neolignans, named surinamensinols A and B and a new phenolic compound, named acoramol. Using supercritical fluid extraction, Dang *et al.*, (2011) isolated two new compounds, (E)- and (Z)-diastereomers of α-asarone and β-asarone from *Acorus tatarinowii* Schott and purified by high-speed counter-current chromatography with a two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water (1:0.2:1:0.3, v/v).

Acorus calamus L. is used in India and Pakistan for its anti-inflammatory effects (Baquar, 1989; Kapoor, 1990). However, the underlying mechanisms of these anti-inflammatory effects are not known. Since arachidonic acid (AA) metabolites formed via cyclooxygenase (COX) and lipoxygenase (LOX) play important roles in inflammation, we hypothesized that the observed anti-inflammatory effects of *Acorus calamus* may be mediated through inhibition of COX and LOX enzymes. We further investigated the effects of *Acorus calamus* on human platelet aggregation induced by a diverse set of platelet agonists.

MATERIAL AND METHODS

All organic solvents, chemicals and reagents used in the experiments were of analytical and highest purity grade. All the chemicals are purchased from Sigma chemical company, St. Louis, USA except ^{14}C arachidonic acid which was purchased from Amersham Biosciences; acetic acid and citric acid from BDH, sodium phosphate (mono and dibasic) from Merck and m-3M3FBS was purchased from Calbiochem (La Jolla, CA, USA).

Extraction of plant material

Fresh rhizomes of *Acorus calamus* L. (2 kg) were collected from Swat District, K.P.K Pakistan, and authenticated by Dr. Humera Gul, Assistant Professor of the Department of Botany, University of Karachi, Pakistan. The plant material was ground using a

commercial grinder to obtain fine powder. Five hundred (500) grams of ground material was soaked in 5 liter aqueous methanol and left for 24 hours at 4º C. The extract was filtered using a filtration flask and whattmann paper. This extract was evaporated on a rotary evaporator under reduced pressure (-760 mm Hg) to a thick, semi-solid mass of dark brown color i.e. crude extract.

Fractionation of the crude extract

One hundred grams of the extract was dissolved in distilled water. This was then introduced in a separating funnel. *n*-hexane (50-70 ml) was then added into the same separating funnel. This mixture was shaken vigorously, regularly allowing the air to escape out. It was kept for about 30 min to let the two layers separate. The upper layer of *n*-hexane was acquired and the same procedure was repeated twice and all the *n*-hexane layers were collected and concentrated in a rotary evaporator to obtain the *n*-hexane fraction. Butanol (50 ml) was then added to the remaining layer and the same process was repeated as with *n*-hexane, finally obtaining the butanol fraction, the yield of both fractions was 23.6% and 30%, respectively, while the remaining layer was filtered using a filtration flask and whattmann paper and used in the experiments as aqueous fraction.

Arachidonic acid metabolism by human platelets

Archidonic acid metabolism and thromboxane B_2 (TXB_2) formation was studied using a Berthold T.L.C. linear analyzer and chromatography data system (Model LKB 511, Berthold, W. Germany) as described previously (Saeed *et al.*, 2007a; Saeed et al., 2007b). Human blood platelets were obtained in plastic bags containing 30-40 ml concentrated platelet rich plasma (PRP) from the diagnostic laboratory of the Aga Khan University, Karachi. The PRP was centrifuged at 1200 g for 20 min and the sedimented platelets were washed twice with an ice-cold phosphate buffer (50 mM, PH 7.4), containing NaCl (0.15 M) and EDTA (0.2 mM).

After centrifugation platelets were re-suspended in the same buffer without EDTA at the initial PRP concentration. The PRP suspension was homogenized at 4º C using a polytron homogenizer for 15 sec and the homogenate centrifuged at 1200g for 20 min. Supernatant (300 µl containing 0.4 mg protein) was incubated with 10 µg unlabelled AA and 0.1 µCi [1-^{14}C]AA in the presence and absence of test fraction. After 15 min of gentle shaking in air at 37º C the reaction was stopped by adding 0.4 ml citric acid (0.4 M) and ethyl acetate (7.0 ml). After mixing and centrifuging at 600 g for 5 min at 4º C, the organic layer was separated and evaporated to dryness under nitrogen. Residues were dissolved in 40 µl of ethanol and 20 µl was applied to silica gel G thin layer chromatography (TLC) plates. The AA, TXB_2 (a stable degradation product of TXA_2), LP1 and 12-HETE standards were plotted separately.

Solvent Systems for Developing TLC Plates

The TLC plates were developed in ether/petroleum ether [boiling range 40-60]/acetic acid (50:50:1, v/v) to a distance of 17 cm. By use of this solvent system the various lipoxygenasse products such as 12-hydroxy-eicosatetraenoic acid (HETE) were separated with TXB_2 and prostaglandins remaining at the origin. The solvent system used for the separation of TXB_2 was ethyl acetate/iso-octane/water/acetic acid (11:5:10:2, v/v upper phase). Radioactive zones were located and quantified by use of a TLC linear analyzer and chromatography data system.

Preparation of platelets

Platelet effects were studied in human platelets by taking blood via venipuncture from normal human volunteers reported to be free of medication for 7 days. Blood sample were mixed with 3.8% (w/v) sodium citrate solution (9:1) and centrifuged at 260 × g for 15 min at 20º C to obtain platelet-rich plasma (PRP). The remaining blood samples were centrifuged at 1200 × g for 10 min to obtain platelet-poor plasma (PPP). Platelet count was determined by phase contrast microscopy and all aggregation studies were carried out at 37º C with PRP having platelet counts between 2.5 and 3.0 × 10^8 ml^{-1} of plasma.

Measurement of platelet aggregation

Aggregation was monitored using dual-channel Lumi-aggregometer (Model 400 Chronolog Corporation, Chicago, USA) using 0.45 ml aliquots of PRP (Saaed *et al.*, 2007a; Saeed et al., 2007b). The final volume was made up to 0.5 ml with the test fraction. Aggregation was induced by AA (1.7 mM), ADP (2.2 µM), PAF (0.8 µM), PLC (with 30 µM m-3M3FBS), PKC (with 100 ng/ml PMA), IP_3 (30 µM). The antiplatelet effects of test fractions were studied by pretreatment of PRP with each fraction for 2 min followed by addition of platelet agonist. The resulting aggregation was recorded for 5 min after challenge by the change in light transmission as a function of time. The number of experiments (n) with each agonist is indicated in the table 1 and ranged 3-5. After

establishing the anti-platelet activity against various agonists, dose-response curves were constructed to calculate the $1C_{50}$ values.

Statistical Analysis

Statistical analysis of the results was done by Students's t test. The differences were considered significants between the means of two groups when the p value was found below 0.05 ($p < 0.05$).

RESULTS AND DISCUSSION

Inflammation is a common sign of many life-threatening pathologies (Aldridge, 2002; Zia-Ul-Haq *et al.,* 2013) and lipid peroxidation is a characteristic feature of inflammation (Cook, 2005). During inflammation oxidative metabolites of AA play an important role (Kubala *et al.*, 2010). Activation of the phospholipase A_2 in response to various stimuli releases AA, which are then metabolised by two major enzymatic pathways: COX and LOX, leading to pro-inflammatory mediators, prostaglandins (PGs) and leukotrienes (LTs), respectively (Marruchella *et al.*, 2010). PGs and LTs are potent inflammatory mediators and regulate immune responses and physiological processes such as bronchoconstriction, vasodilation and mucus secretion (Gilroy *et al.*, 1999; Kumar *et al.*, 2007).

Anti-inflammatory activity of the *Acorus calamus* has been reported a few years ago (Kim *et al.*, 2009, Tiwari *et al.*, 2010). However, the mechanism was not known. Kim *et al.*, (2009) found that *Acorus calamus* inhibited the interleukin (IL)-6 and IL-8 as well as nuclear factor κB. Most of the anti-inflammatory drugs available in the market inhibit prostaglandin synthesis and this might be the mechanism of action of *Acorus calamus* (Tiwari *et al.*, 2010). Our study, therefore, explored whether the inhibition of the prostaglandin synthesis can account for the observed anti-inflammatory effects of *Acorus calamus*.

Aqueous fraction of *Acorus calamus* was found to be completely ineffective against both COX and LOX pathways as indicated by the lack of inhibition of TXB_2, LP1 and 12-HETE. However, its butanolic fraction inhibited the COX metabolite TXB_2 and one of the LOX metabolites-LP1 (Figure 1). The other LOX metabolite -12-HETE remained unaffected. n-hexane fraction was also ineffective against COX and LOX pathways. This indicates that butanolic fraction of *Acorus calamus* contains constituents which completely inhibit the COX pathway while partially blocking the LOX pathway of AA metabolism. Butanolic fraction inhibited the formation of TXB_2 and LP1 with IC_{50} value (mg ± SEM) of 0.80 ± 0.10 and 1.05 ± 0.07 respectively (Table 1 and Figure 1). The standard, asprin, completely inhibited both COX and LOX pathways at 200 µM, and indomethacin at 500 µM while the negative control, saline was unable to significantly inhibit any pathway. Inhibition of the COX mediated formation of TXB_2 by butanolic fraction indicates that COX inhibitory components are concentrated in the butanolic fraction of *Acorus calamus*. This action of *Acorus calamus* is similar to nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, which also directly target COX. Aspirin inhibited TXB_2 with IC_{50} of 11 ± 0.21 (mg ± SEM) and LP1 with 25 ± 4.3 (mg ± EM).

It is becoming increasingly evident that blood platelets do not only exert important functions in hemostasis and thrombus formation but are also involved in atherosclerotic vascular disease (Weber, 2005). Platelets play a critical role in the initial restriction of blood loss following vascular injury by rapidly adhering to exposed subendothelial matrix components and aggregating to form a provisional plug. Since TXB_2 was inhibited by butanolic fraction, we hypothesized that it would be effective against AA-induced human platelet aggregation as AA induces platelet aggregation after it is converted by COX into TXA_2. Butanolic fraction indeed inhibited the human platelet aggregation induced by AA with IC_{50} value (mg ± SEM) of 0.08 ± 0.004 (Figure 2 and Table 1).

In this study, only the butanolic fraction of *Acorus calamus* was active against AA-induced platelet aggregation but at a concentration 10-times lower than required for inhibition of TXB_2 formation through COX pathway. This indicates the involvement of other mechanisms of platelet inhibition besides COX pathway.

Receptor for TXA_2 is a G-protein coupled receptor which primarily stimulates Gq and activates phospholipase C (PLC) (Nakahata, 2008). PLC converts phosphtidylinositol biphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG remains in the plane of the membrane to stimulate PKC while IP_3 travels through the cytoplasm to endoplasmic reticulum where it releases calcium (Berridge and Irvine, 1984; Nishizuka, 1984). Both of these effects i.e., activation of PKC and release of calcium can initiate and enhance platelet aggregation (Brass *et al.*, 1993; Heemskerk and Sage, 1997).

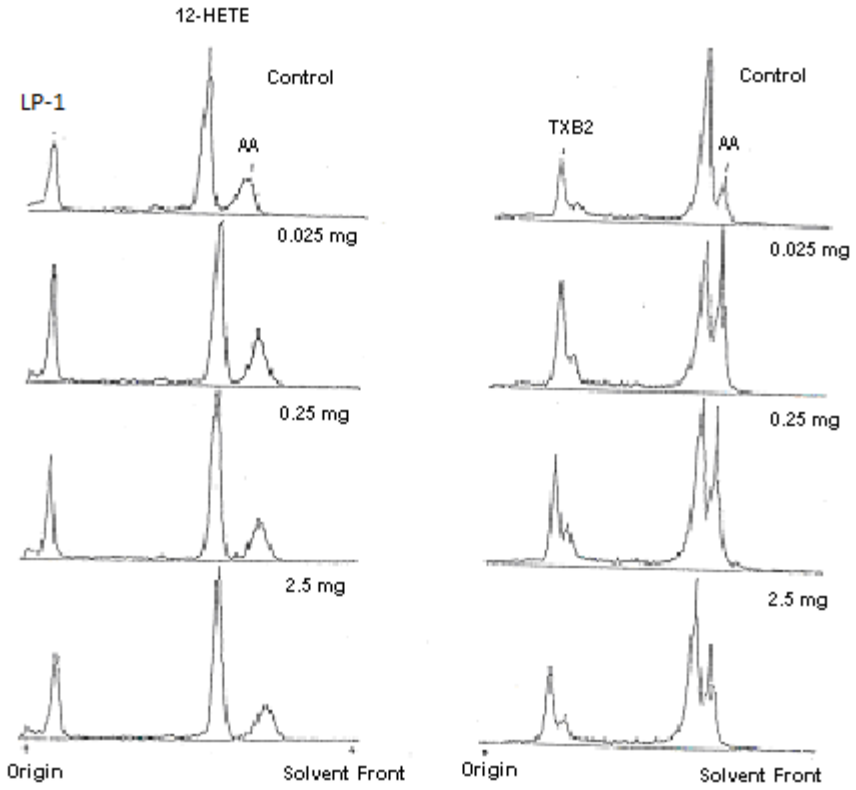

Figure 1
Figure shows the effects of butanolic fraction of *Acorus calamus* on AA metabolism suggesting dose-dependant inhibition (n = 4).

Butanolic fraction inhibited PLC-induced (30 µM m-3M3FBS) platelet aggregation with IC_{50} value (mg ± SEM) of 0.46 ± 0.13 (Figure 2 and Table 1), indicating that apart from inhibiting COX pathway butanolic fraction also inhibits PLC pathway in platelets. In order to further explore whether PLC itself is inhibited or downstream effectors are implicated, we used PMA and IP3 induced platelet aggregation to evaluate their contribution in the antiplatelet actions of the butanolic fraction. Although only 50% inhibition (IC_{50} value of 2.56 ± 0.40 (mg ± SEM)) was observed against IP3-induced (30 µM) platelet aggregation, butanolic fraction potently and completely inhibited PMA-induced (100 ng/ml) platelet aggregation (IC_{50} value 0.52 ± 0.18 (mg ± SEM). This suggests that butanolic fraction inhibits platelet aggregation by interfering not only with COX but PKC as well.

Metabolite	IC_{50} (mg ± SEM)
Against AA metabolism	
TXB_2	0.80 ± 0.10 (n = 4)
LP1	1.05 ± 0.07 (n = 4)
Against platelet aggregation	
Platelet agonist	IC_{50} (mg ± SEM)
AA	0.080 ± 0.04 (n = 5)
PLC (Induced by 3M3FBS)	0.460 ± 0.13 (n = 4)
PKC (Induced by PMA)	0.520 ± 0.18 (n = 5)
IP_3	2.560 ± 0.40 (n = 3)
PAF	0. 0.16 (n = 3)
ADP	1.200 ± 0.21 (n = 4)

Table 1
IC_{50} values of the butanolic fraction of *Acorus calamus* against AA metabolism and human platelet aggregation induced by a diverse set of agonists.

Butanolic fraction was further evaluated against PAF and ADP induced human platelet aggregation as these platelet agonists stimulate PLC downstream of their receptors. Since PAF induces platelet aggregation exclusively by binding to its receptor and stimulating PLC and subsequently PKC and IP3, it was found to be potently inhibited by the butanolic fraction with IC_{50} value (mg ± SEM) of 0.55 ± 0.16 (Figure 2 and Table 1). However, ADP induced aggregation was inhibited at much higher concentrations and complete inhibition was not observed. This might be due to the fact that ADP induces platelet aggregation by stimulating G_{12} as well.

Our study does not identify the compounds responsible for the observed pharmacological activities by the *Acorus calamus.* However, it can be safely said that compounds such as isocalamendiol, asarone, acoradin, acrogermacrone, and preisocalamendiol, etc. are not responsible for these effects as these compounds are present in highly non-polar factions such as n-hexane, ether, benzene, ethyl acetate and chloroform (Raja *et al.*, 2009) while in the current investigation, the activities were mainly observed in the butanolic fraction. Therefore, the most likely candidates responsible for these activities may be triterpenoid saponins, phenyl derivatives, dihydroxy phenols such as galangin etc as these compounds are present predominantly in alcoholic fractions of the plant (Raja *et al.*, 2009).

In this study the pharmacological activity was found to be concentrated mainly in the butanolic fraction and not in the aqueous or n-hexane fraction. This suggests that the active compounds responsible for these activities are slightly non-polar in nature. The lack of activity in the aqueous fraction is understandable as the pharmacological targets in this investigation are intracellular, and for polar compounds it is not easy to pass through the lipids of plasma membrane to get access to the intracellular targets.

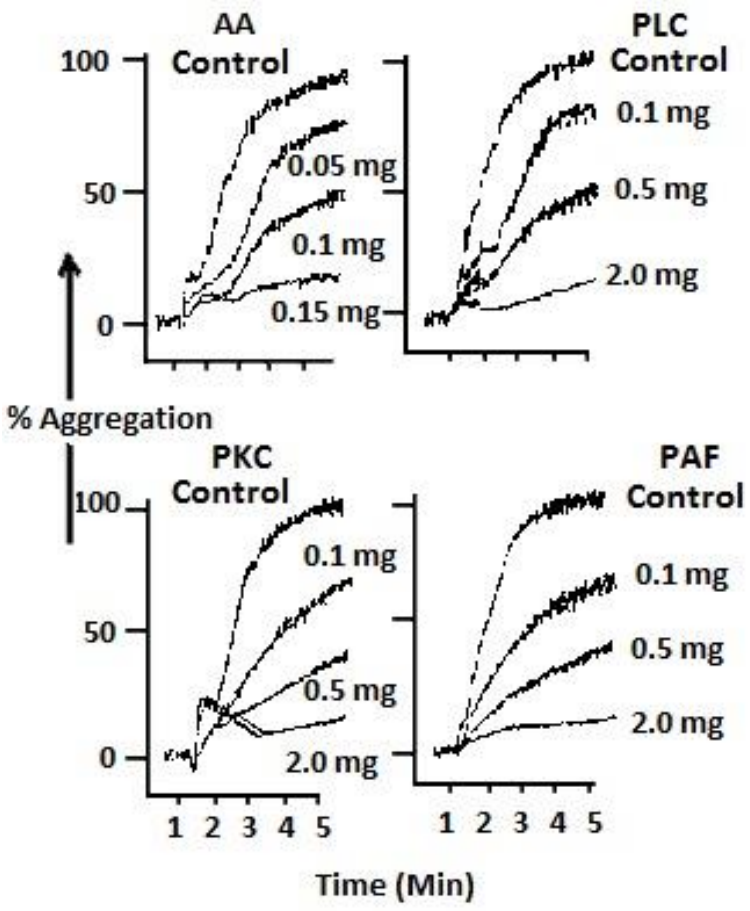

Figure 2
Butanolic fraction of the *Acorus calamus* inhibits platelet aggregation induced by various platelet agonists, (n = 4).

CONCLUSION

It appears that AA metabolism inhibitory constituents as well as platelet inhibitory constituents are concentrated in the butanolic fraction of the *Acorus calamus*. Therefore, it is likely that traditionally reported anti-inflammatory activities of *Acorus calamus* are mediated through inhibition of COX, LOX and PKC pathways. This new information provides a theoretical basis for development of new anti-inflammatory approaches targeted to COX, LOX and PKC activity. Since no effective inhibitor is currently available to block the COX, LOX and PKC pathways simultaneously, compounds isolated from *Acorus calamus* might be valuable in the future for treatment and prevention of a number of inflammatory conditions.

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