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Abstract

Clove oil inhibited human platelet aggregation induced by arachidonic acid (AA), platelet-activating factor (PAF) or collagen. Clove oil was a more effective inhibitor for aggregation induced by AA and PAP (IC50:4 and 6 uM respectively) than collagen (1C50: 132 uM). The in vivo experiments in rabbits showed that clove oil (50.100mg/kg) afforded 100% protection against PAP (11mg/kg, i.v.) and 70% protection against AA (2.0 mg/kg, i.v.)- induced thrombosis and shock due to pulmonary platelet thrombosis. It also inhibited thromboxane-A2 and 12-HETE production by human platelets incubated with [C14] AA. These results are indicative that clove oil is inhibitory of platelet aggregation and thromboxane synthesis and may act as anti- tbrombotic agent (JPMA 44:112, 1994).

Introduction

A diversity of pharmacological effects have been attributed to clove oil (Eugenia caryophyllata; locally known as laung). Among these effects are antibacterial, antifungal antispasmodic flavouring agent in foods, pharmaceuticals, herbal medicine and clove cigarettes[1-4]. Recently we have reported that the clove oil (eugenol) exhibits antihypertensive and spasmolytic activities in anaesthetized rats and in the in vitro studies (unpublished data). From clinical studies, evidence has accrued suggesting that clove oil is efficacious in the treatment of dental pain[5]. Because of the similarity between such effects of clove oil and the actions of non-steroidal anti-inflammatory drugs (NSAID), it has been speculated that eugenol, major constituent of clove oil may act, in part, atleast by way of inhibition of prostaglandin synthase[6]. To investigate further, the effects of clove oil on arachidonic acid (AA), we utilized two complimentary test methods. The first method determined the effect of clove oil against cyclo-oxygenase and lipoxygenase enzymes in platelets. Platelets metabolize AA through these pathways into thromboxane A2 (TXA2) and 12-hydroxyeicosatetraenoic acid (12-HETE). We used also the methods of Silver et al. [7] and Saeed et al. [8] to examine the efficacy of clove oil in the inhibition of platelet aggregation in vivo. These methods have established that intravenous injection of AA or platelet-activating factor (PAF) induces mortality in rabbits by producing diffuse pulmonary capillary platelet aggregation and shock. It was demonstrated also that pre-treatment of rabbits with aspirin was protective against such lethality[8].

Materials and Methods

A (grade 1,99% pure), nordihydroguaiaretic acid (NDGA), clove oil, acetylsalicylic acid, PAF, ADP,

collagen and carrageenan were purchased from the Sigma Chemical Co. (St. Louis Mo, USA). [14C] AA (specific activity 58.4 mCi mmol1, 5,6,7,8,9,11,12,14,15 (n)-{3H} thromboxane B2 (120 Ci mmol-1) and 12-(5)-hydroxy-[3H] eicosatetraenoic acid (12-HETE)(100 Ci mmol 1) were obtained from Amersham International (Amersham, UK). Polysorbate 80 was obtained from Pharmachemic, Antwerp, Belgium. Silica gel G thin layer chromatography (TLC) plates by Analtech, Delaware, USA. All other chemicals used were of the highest purity grade available.

Animals

New Zealand white male rabbits (3-3.5 kg) and Sprague-Dawley male rats (150-200 g), were obtained from the Laboratory Animal Unit, The Aga Khan University. They were fed a standard diet and food was with-held the night before an experiment. Water was available adlibitum.

Platelet Aggregation

Blood was taken by venepuncture from normal volunteers reported to be free of medication for 7 days. Blood samples were mixed with 3.8% (w/v) sodium citrate solution (9:1) and centrifuged at 260g for 15 min at 20°C to obtain platelet-rich plasma (PRP). The remaining blood sample was 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 37°C with PRP having platelet counts betwee 2.5 and 3.0×10^8/ml of plasma. Aggregation was monitored with a Dual-channel chronolog lumi aggregometer (model 400, Chronolog Corporation, Chicago, USA) using 450 uL samples of PRP. The PRP was pre-incubated with an appropriate amount of test compound for 1 min before challenge with the aggregating agent. The light transmission was adjusted to 0 and 100% with PRP and PPP respectively. Aggregation was induced byADP (2.2 uM), AA (0.8 uM), collagen (20 ug/ml) or PAF (0.8 p.M). The resulting aggregation was recorded and expressed as percentage inhibition compared with control at 4 mm after challenge. Test compounds were tested at 3 or 4 concentrations in duplicate. Clove oil was initially dissolved in absolute ethanol and further diluted with 0.9% NaCl. The maximum final ethanol concentration which did not interfere with the platelet aggregation was 1% (v/v).

Arachidonic acid metabolism by platelets

Human blood platelets from donors were routinely obtained in plastic bags containing 30-40 ml of concentrated PRP from the Aga Khan University Hospital Laboratory. The PRP was centrifuged at 1 , 200g for 20 mm and the sedimented platelets were washed twice with an ice-cold phosphate buffer (50 mM, pH 7.4) containing sodium chloride (0.15M) and EDTA (0.2mM). After centrifugation platelets were resuspended in the same buffer without EDTA at the initial PRP cell concentration. The PRP suspension was homogenized at 4°C using a polytron homogenizer for 15 seconds and the homogenate centrifuged at 1,200g for 20 min. 300 uL of the supernatant (containing 0.4 mg of protein) was incubated with 10 ug unlabelled AA and 0.1 mCi [1-14C]AA in the presence and absence of test compound. After 15 min with gentle shaking at 37EC the reaction was stopped by adding 0.4 mL of citric acid (0.4 M) and 7 ml of ethyl acetate. After mixing and centrifuging at 600g for 5 mm at 4°C, the organic layer was separated and evoporated to dryness under nitrogen. Residues were dissolved at 50 mL ethanol and 20 mL were applied to silica gel G (TLC) plates (Analtech, Delaware, USA). The AA, TXB2 (a stable degradation product of TXA2) and 12-HETE standards were spotted separately. The plates were developed in ether/petroleum ether (boiling range 40-60°C) acetic acid (50:50:1 by volume) to a distance of 17 cm. By use of this solvent system the various lipoxygenase products (HETEs) are separated by TXB2 and PGs remaining at the origin. The solvent system used for the separation of various PGs and TXB2 in dried organic extracts of platelet incubates as above was ethyl acetate:isooctane:water:acetic acid (11:5:10:2, v/v, upper phase). Radioactive zones were located and quantifled by use of a Berthold TLC linear analyser and chromatography data system (Model LB 511, Berthold, Germany). Protein concentration was determined by the method of Lowry et al. [10] using human serum albumin as standard. All experiments were conducted with appropriate controls. The platelet homogenate (1,200g supernatant) was boiled for 10 min, cooled and incubated with [14CJ AA

using assay conditions as described above. Inactivation of platelet enzymes by this treatment did not produce any AA metabolites (lipoxygenases product 1:LP1,, 12-HETE or TXA2). Similarly, incubation of clove oil alone with [14C] AA in the absence of platelet homogenate (1,200g supernatant), also did not result in the production of AA metabolites due to possible non-enzymatic autoxidation. Differences between control and test measurements were assessed by Student's t-test.

Arachidonic acid and/or PAF-induced mortality in rabbits

Male rabbits (NZ white 3.0-3.5 kg) were used to test the lethal effect of injected AA by the method of Silver et al. [7]. Sodium arachidonate was prepared before each injection by dissolving AA in sodium bicarbonate (0.2% w/v) to give a final concentration of 2 mg/ml. The sodium arachidonate was injected into the marginal ear view of the rabbits over a period of approximately 1 minute. In preliminary experiments we established that an i.v. dose of 2 mg/kg of AA or 11 mg/kg of PAF was required to induce consistently fatal pulmonary thrombosis in control animals. This dose of arachidonate or PAP was used in subsequent experiments with clove oil. Animals were pretreated by intraperitoneal injection of either aspirin (50 mg/kg) or clove oil (25-100 mg/kg) in 5% polysorbate 80 in distilled water 2 hours before the challenging dose of sodium arachidonate or PAF. Control rabbits received the same amount of 5% polysorbate 80 in distilled water. Each animal was used for only one experiment.

Results

The effects of clove oil on platelet aggregation in vitro induced by AA, ADP, collagen or PAF are presented in Table I.

Table I. Comparison of inhibitory effects of clove oil and aspirin on human platelet aggregation.

Compound aggregating agent	ADP	AA	IC$_{50}$* (μM) Collagen	PAF
Clove oil	NA	4.2±0.2	132±0.5	6.0±0.3
Aspirin	NA	173±22	NA	154±36

NA: Not active upto 2500 μM

*IC$_{50}$: Concentration producing 50% inhibition of platelet aggregation and values represent mean±SEM of 3-4 determination

When PRP was pre-incubated with clove oil for 1 min before challenge, platelet aggregation was inhibited in a concentration-related manner. The mean±SEM (n= 6) for inhibiting platelet aggregation by 50% against AA, collagen and PAF were 4.2±0.2, 132±5 and 6.0±0.4 uM respectively. Clove oil (up to 320 uM) however, had no effect on platelet aggregation induced by ADP. These results demonstrate that clove oil preferentially inhibited AA and/or PAF-induced aggregation as compared with aggregation induced by other agents. When compared with aspirin at IC50 levels, against AA (173±22), collagan (2791±765) and PAF (154±36 uM) clove oil was found to be 20-40 times more potent than aspirin in antiplatelet effects. In separate experiments, the effect of clove oil on te in vivo model of platelet aggregation was investigated. Silver et al. [9] have demonstrated that AA when injected into the ear vein of the rabbit causes sudden death associated with occlusive platelet aggregates in the

microvasculature of the lung. In the present study it was found that pretreatment of rabbits with clove oil (100 mg/kg) prevented the sudden death induced by AA (Table II).

Table II. Effect of clove oil on AA (2mg/kg) and PAF (11 mg/kg)- induced mortality in rabbits

Compound	Dose	Route	Survival
1. AA-control		i.v.	0/7
Clove oil	50mg/kg	i.p.	1/7
	100	i.p.	5/7
Aspirin	50	i.p.	7/7
2. PAF-control			
Clove oil	50mg/kg	i.p.	2/7
	100	i.p.	7/7
Aspirin	50	ip	.5/7

inhibited PAF- induced platelet aggregation, the effect of clove oil on PAF- induced sudden death associated with platelet agjregation in the rabbit was also investigated[10]. It was found that clove oil (100 mg/kg) afforded complete protection against PAF-induced sudden death in the rabbits (Table II). In similar experiments it was found that pretreatment of rabbits with aspirin (50 mg/kg), a known inhibitor of platelet aggregation completely protected rabbits against AA-induced death[7] and provided over 70% protection against PAF-induced sudden death in rabbits.

Effect of clove oil on platelet AA metabolism

Incubation of {1-[^{14}C] AA with homogenate (1200g supernatant fraction) of human platelets resulted in the formation of two lipoxygenase products. The mobility of one product (Rf 0.62) on a silica gel G chromatogram was similar to that of the authentic 12-HETE. The formation of a more polar lipoxygenase product (LP1, Rf 0.15) was also observed. Its absolute identification, however, remains to be determined: it may be a tri-hydroxy-eicosatrienoic acid (HETE) as described previously[10]. The synthesis of lipoxygenase products by human platelets was inhibited by clove oil (30-304 uM) and NDGA (5-50 uM), an antioxidant and a known inhibitor of lipoxygenase in a concentration-related manner (n=4) . The mean values of clove oil (±S.E.M., n-= 7) for inhibiting formation of these products by 50% (IC50) were 110±6 uM for LP and 115±9 uM for 12-HETE whereas aspirin had no effect. On the other hand, aspirin (7-700 uM) inhibited the production of TXB2 (a stable metabolite of TXA2) in a concentration-related manner The mean values (±S.E.M.; n=4) for inhibition of TXB2 by 50% were 100±4 for clove oil and 2 10±9 p.M for aspirin. These results show that clove oil is a dual inhibitor of platelet cyclooxygenase and lipoxygenase enzymes.

Discussion

Platelet aggregation is a key element in acute thrombosis, a major cause of human morbidity and mortality. Mounting evidence supports the efficacy of antiplatelet drugs like aspirin, in the treatment and prevention of this process. The recent dramatic increase in the use of herbal foods and medicines in the treatment of various ailments has led to extensive investigation of the scientific basis of the

conditions alleviated by herbal medicines. Cloves (clove oil) is known to possess many pharmacological properties. In the present study, clove oil at low doses was found to inhibit human platelet aggregation induced by AA and PAF in vitro (Table I). In vivo, studies on clove oil demonstrated beneficial effects in a model of AA and/PAF-induced sudden death in rabbits. Pretreatment of rabbits with clove oil (50-100 mg/kg) prevented the lethal effect of PAF and/or AA suggesting that clove oil acts as antagonist of PAF and AA. While the precise site of AA (through the formation of TXA2) is known to result in platelet aggregation, the mechanism of PAF-induced action in sudden death of rabbits remain to be clarified. It has been demonstrated that intravenous injection of PAF induces platelet aggregation in several animal species through activation of PAF receptors12. Thus hypercoagulability, thrombosis and microembolism may be implicated in this model of shock. Indeed, it has been shown that the prostacyclin, a potent anti-aggregating agent, displays beneficial effects in shock[1] . Similarly, antiplatelet effects of clove oil against PAF-induced aggregation observed in the in vitro studies may be responsible for its protective effect in vivo model. In conclusion, whether the inhibitory effect of clove oil on platelet aggregation are clinically relevant will depend on long term prospective studies; however, there are good reasons to suggest that clove oil may represent a new class of antithrombotic agents with dual AA and PAF antagonistic properties.

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