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STUDIES ON THE OUTER ROOT BARK OF *Kokoona ochracea* (ELM.) MERR. (FAMILY CELASTRACEAE)

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ABSTRACT

Kokoona ochracea (Elm.) Merr. (Fam. Celastraceae), one of eight species in the genus Kokoona, is a tree endemic to the Philippines, particularly to Palawan. It is locally known as "repetik" because of the crackling sound it makes when it burns. The natives use the stem bark to kindle fire. The petroleum ether extract of the outer root bark possesses anti-inflammatory activity. The active compounds may be phenolic triterpenes or friedo-oleanane triterpenoids as have been reported to occur in various members of the family. The activity of the extract is due to inhibition of protein denaturation, platelet aggregation, erythrocyte hemolysis, and the enzyme 5-lipoxygenase. The ethanol extract prevents protein denaturation and erythrocyte hemolysis and may contain phenolic compounds which may be anti-inflammatory. The petroleum ether and ethanol extracts of the outer root bark are potential sources of anti-inflammatory drugs. Further studies are indicated.

INTRODUCTION

Celastraceae, a tropical family of plants, is represented by 22 species distributed in 12 genera (Ding Hou 1962). Eight species comprise the genus *Kokoona,* distributed as follows: one in Sri Lanka and southern India, one in Burma, and six in Malaysia (Sumatra, Malay Peninsula, Borneo, and the Philippines). The six species found in Malaysia are: *K. ochracea* (Elm.) Merr. (=*Ardisia ochracea* Elm.), *K. littoralis* Laws., *K. coriacea* King, *K. sessilis* Ding Hou, *K. ovatolanceolata* Ridl., and *K. reflexa* (Laws.) Ding Hou. *Kokoona* species are distributed in lowland rainforests, dryland, swampy, or peat, rarely up to 1500 m in altitude. The bark of practically all species contains oil, burns easily, and is sometimes used for tinder.

Phytochemically, many highly characteristic compounds are known from the family Celastraceae but most of them have been found in only a few species with two exceptions (Ding Hou 1962).

All Celastraceae seem to accumulate the hexitol, dulcitol, and the all-*trans*-polyisoprenoid, gutta, in leaves and bark. These compounds may be considered as highly characteristic biochemical features of the family. Triterpenoids are probably accumulated in large amounts in leaves and bark by many Celastraceae. Lupeol, betulin, β-amyrin, and triterpenes of the friedelane series have been reported. Taxonomically more important are the red bark pigment celastrol (=tripterin), and pristimerin (=monomethylcelastrol) from the root bark of various species of different genera of the family. Compounds of chemotaxonomic importance are dulcitol, gutta, and pristimerin (R. Hegnauer through Ding Hou 1962). The bark of *Kokoona* and *Lophopetalum* species contains oil in a thin outer layer and is easily inflammable, even in a wet state (Ding Hou 1962). It is used as a fire-lighter in the forest. Other constituents reported to occur in the family include alkaloidal amines, alkaloids, sugar alcohols, saponins, cardenolides, terpenoids, and substances having antitumor activity (Trease 1989).

Kokoona zeylanica Thwaites, the species found in Sri Lanka and southern India, is the only species in the genus that has been the subject of a comprehensive phytochemical study at the University of Peradeniya in Sri Lanka (Gamlath et al. 1986, 1987, 1988, 1990; Gamlath and Gunatilaka 1988; Gunaherath and Gunatilaka 1983a, 1983b, 1983c; Gunaherath et al. 1980, 1982; Gunatilaka and Nanayakkra 1984; Gunatilaka et al. 1979, 1981, 1982, 1983). Pristimerin, a cytotoxic triterpene quinone methide, and a series of natural phenolic D:A-*friedo*-24-noroleanane triterpenoids were isolated from the hot light petroleum and hot benzene extracts of the outer stem bark (Gamlath et al. 1986, 1987; Gunaherath and Gunatilaka 1983a, 1983b, 1983c). Pristimerin has also been isolated from *K. reflexa,* a species closely related to *K. zeylanica* (Gamlath et al. 1990). The inner stem bark of *K. zeylanica* yielded friedelin and other oxygenated D:A-*friedo*-oleanane triterpenes from the hot benzene extract (Gamlath et al. 1986; Gunatilaka et al. 1979, 1981, 1982, 1983; Gunatilaka and Nanayakkara 1984). The hot ethyl acetate extract of the outer root bark yielded phenolic D:A-*friedo*-24-noroleanane triterpenoids (Gamlath et al. 1986, 1987). An investigation of the methanolic extract of the inner root bark resulted in the isolation and identification of dulcitol and (-)-4'-*O*-methylepigallocatechin (Gunaherath et al. 1982).

Kokoona ochracea (Elm.) Merr. (Fam. Celastraceae) is the species endemic to the Philippines, particularly to Palawan. It is locally known as "repetik" because of the crackling sound it makes when it burns. The natives use the stem bark to kindle fire.

At the College of Pharmacy, University of Illinois at Chicago, three new cytotoxic lupane lactones were isolated from nonpolar extracts of the stem bark of *K. ochracea* collected in the island of Palawan in the Philippines, namely, 3-oxolup-20(29)-en-30,21α-olide (ochraceolide A), 20,29-epoxy-3-oxolupan-30,21α-olide (ochraceolide B), and 3,6-dioxolup-20(29)-en-30,21α-olide (ochraceolide C) (Ngassapa et al. 1991). Ochraceolides A and C exhibited significant toxicity with cultured P-388 cells while ochraceolide B was weakly active with cultured P-388 and KB-3 cells.

The present studies were undertaken to test the outer root bark for biological activity and, if biologically active, to test for anti-inflammatory activity. If any anti-inflammatory activity is detected, initial steps will be taken to isolate the active constituent(s). If time permits, the active compound(s) will be purified and characterized.

MATERIALS

A. Plant Material

Kokoona ochracea (Elm.) Merr. is the only species found in the Philippines. The plant materials used in the Illinois study and in this report were collected in Palawan and identified by Dr. D.D. Soejarto of the University of Illinois at Chicago and Dr. D. A. Madulid of the National Museum of the Philippines. Voucher specimens of the plant (Soejarto and Madulid 6098) (Figure 1) were deposited at the herbarium of the Field Museum of Natural History, Chicago, and at the Philippine National Herbarium, Manila.

Kokoona ochracea is a tree 25-40 m in height and 20-40 cm in diameter, rarely with buttresses up to about 3 m tall (Ding Hou 1962). The tree has a brilliant yellow outer root bark which burns with a smoky flame (Figure 2). Samples of the root bark (Figure 3) were collected from a tree growing in the Irawan Valley, Tatanarom vicinity of Mt. Beaufort slope at an altitude of 200 m, northwest

of Puerto Princesa. Samples were also collected from lowland Mariwara of Southern Palawan, where the species is also found to be abundant. The trees at a lower altitude are taller and wider in diameter.

B. Brine Shrimp

Brine shrimp (*Artemia salina* Leach) eggs (Sanders Brine Shrimp Co., 3850 South 540 West, Ogden, Utah 84405, U.S.A.) were hatched in a 1-liter beaker, containing a solution of noniodized sodium chloride, AR grade, Merck, in distilled water (12-30 ppt). The eggs (1.5 g) were sprinkled into the beaker and the hatching solution was aerated and illuminated under continuous fluorescent light. After 48 hours the phototropic nauplii were collected by pipette.

C. Egg Albumin

The albumin was separated from the yolk of fresh chicken eggs. Normal saline solution was added to the albumin in small amouts, with mixing, to make 100 ml of stock solution with a concentration of 0.1 g albumin/ml of solution.

D. Porcine Blood

Porcine blood collected from freshly butchered hogs was obtained from the Manila slaughterhouse. It was mixed with 4.0% sodium citrate solution (900 ml porcine blood/100 ml sodium citrate solution) and brought to the laboratory. Only freshly collected blood was used in the study.

E. Platelet-Rich Plasma (PRP) and Erythrocytes

One liter of citrated blood was added to 200 ml of 6.0% dextran solution and the erythrocytes were allowed to sediment for one hour. Within the period of time, the majority of red cells separate from the platelet-rich plasma containing the thrombocytes and leukocytes. The erythrocytes were separated from the PRP by centrifugation at 1000 rpm for 10 min at room temperature. The PRP was carefully removed from the top of the erythrocyte pellet with a Pasteur pipette.

METHODS

A. Extraction

The powdered dried outer root bark (450.0 g) of *K. ochracea* was subjected to exhaustive extraction in a Soxhlet apparatus with a series of solvents of increasing polarity: petroleum ether, benzene, chloroform, ether, ethyl acetate, and ethanol. The different extracts were dried in a rotary evaporator.

B. Chemical Screening

Small amounts of each of the dried extracts were taken up with 95% ethanol. Any insoluble matter was solubilized with a few drops of dimethylsulfoxide (DMSO) not exceeding 1% of the total solution. The following screening tests (Harborne 1984; Simes et al. 1959) were performed on each of the prepared solutions:

1. Alkaloids - Formation of a precipitate with Mayer's and Dragendorff's reagents. A heavy dense precipitate was designated as 4+, a strong precipitate as 3+, a moderate precipitate as 2+, and a faint cloudy appearance as 1+.

2. Phenolic Compounds - Green or blue coloration with ferric chloride TS.

3. Saponins - Formation of a froth upon shaking. A froth lasting at least 30 min was designated as 1+; a froth which lasted for two h was designated 2+; for three h, 3+; and for more than four h, 4+.

4. Triterpenes and Steroids - Liebermann-Burchard (acetic anhydride-concentrated sulfuric acid) reaction. Formation of a bright purple, red, pink, or orange coloration generally indicates the presence of triterpenes and a blue or green coloration indicates the presence of steroids.

C. Brine Shrimp Bioassay (Meyer et al. 1982)

1. Sample Preparation - Samples were prepared by dissolving 20 mg of each of the dried extracts in 20 ml chloroform

(Solution A). Solution B was prepared by diluting 2.0 ml of Solution A to 20 ml with chloroform. Appropriate amounts of solution (1000 µl and 100 µl of Solution A to make concentrations of 1000 and 100 µg/ml, respectively; and 100 µl of Solution B to make a concentration of 10 µg/ml) were transferred to 10-ml vials and air-dried. Each sample was reconstituted with 100 µl of methanol and controls were prepared using only methanol. Five replicates were prepared for each dose level and for the control.

2. Bioassay - Using a pipette, ten shrimps were transferred to each sample vial containing 5 ml of artificial sea water (noniodized sodium chloride, AR grade, Merck, in distilled water, 12-30 ppt). A drop of dry yeast suspension (Red Star) was added as food to each vial. The vials were maintained under continuous illumination using a fluorescent lamp. Survivors were counted after 2, 6, 12, and 24 h and the percent deaths at each dose level and control were determined. The 24-h counts were the most useful. In cases where control deaths occurred, the data were corrected using Abbott's formula:

$$\text{Deaths (\%)} = [(\text{Sample} - \text{Control}) / \text{Control}] \times 100$$

3. LC50 Determination - LC50 and 95% confidence intervals were determined from the 24-h counts using the probit analysis method described by Finney (1971).

D. Testing for Anti-Inflammatory Activity

1. Sample Preparation - Appropriate amounts of solution (1000 µl, 750 µl, 500 µl, 250 µl and 100 µl of Solution A to make concentrations of 1000, 750, 500, 250, and 100 µg/ml, respectively; and 1000 µl, 750 µl, 500 µl, 250 µl, and 100 µl of Solution B to make concentrations of 100, 75, 50, 25, and 10 µg/ml, respectively) were transferred to clean, empty vials and the extracts were dried in air. The dried extracts were reconstituted with 100 µl DMSO. Three replicates were prepared for each dose level. Solutions of acetylsalicylic acid, USP grade, were prepared with the same concentrations as the sample solutions as a basis of comparison of activity. Three replicates were also prepared.

2. Protein Denaturation (Lewis 1989) - To each of the various concentrations (1000, 750, 500, 250, 100, 75, 50, 25, and 10 μg/ml) of the different extracts in DMSO, 4.0 ml of 1% w/v protein solution of egg albumin in saline were added. The tubes containing the solutions were warmed in a water bath at a temperature just sufficient to produce a turbidity in the tubes in about 20 min (70°C). The turbidity which was due to the thermal denaturation of the protein was measured in a spectrophotometer (Coleman-Hitachi Model 101 spectrophotometer) at 460 nm. Three replicates were prepared for each dose level and for the control (1% w/v of the albumin in saline). The percent denaturation was calculated using the equation

$$\text{Denaturation (\%)} = [(Abs_{sample}) / (Abs_{control})] \times 100$$

3. Platelet Aggregation (Born 1962; Okuyama et al. 1986) - One ml of each of the different concentrations (1000, 100, and 10 μg/ml) of extracts (prepared as described above) was added to 5 ml of PRP. Platelet aggregation was induced upon recalcification of the citrated PRP (to 3 ml of PRP, 0.04 ml of saline, and 0.04 ml 1 M calcium chloride were added) and incubation at 37°C for 30 min. Two controls were prepared: Control 1 contained 5 ml of PRP, 2 ml of saline, and 100 μl of DMSO; Control 2 contained 3 ml of PRP, 0.04 ml of saline, 0.04 ml of 1 M calcium chloride, and 100 μl of DMSO. Three replicates of each concentration and of the controls were prepared. Platelet aggregation was measured spectrophotometrically at 600 nm. The percent aggregation was calculated using the equation.

$$\text{Aggregation (\%)} = [(Abs_{control\ 1} - Abs_{sample}) / (Abs_{control\ 1} - Abs_{control\ 2})] \times 100$$

4. Erythrocyte Hemolysis (Lewis 1989) - The pellet consisting of erythrocytes obtained after centrifugation of citrated porcine blood was suspended in 0.9% w/v sodium chloride solution. Two ml of the erythrocyte suspension were added to 100 μl each of the various concentrations (1000, 750, 500, 250, 100, 75, 50, 25, 10 μg/ml) of the different extracts prepared as described above. Hemolysis was

induced by diluting with 4.0 ml distilled water the 2.0 ml suspension of erythrocytes, mixing, and allowing the mixture to stand for 30 min at 37°C. The control contained 100 µl of DMSO, 2 ml of the erythrocyte suspension, and 4.0 ml of distilled water (100% hemolysis). The amount of lysis can be determined by measuring the amount of hemoglobin (Hb) present in the supernatant after centrifugation. The amount of Hb present in the supernatant is determined spectrophotometrically (Coleman-Hitachi Model 101 spectrophotometer) at 540 nm and the percentage hemolysis calculated using the equation

$$\text{Hemolysis (\%)} = (\text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100$$

5. *In Vitro* 5-Lipoxygenase Assay

This portion of the study was performed by one of us (RAME) in the laboratory of Prof. Dr. Hildebert Wagner, Institut für Pharmazeutsche Biologie der Universität München.

Isolation of Porcine Leukocytes - Freshly extracted peripheral porcine blood (450 ml) was mixed with 50 ml of 4.0% sodium citrate solution. This suspension was added to 100 ml of 6.0% dextran solution and the cells were allowed to sediment for one h. During this period of time the majority of red cells separated from the thrombocytes and leukocytes. After centrifugation in portions of 50 ml at 1000 rpm (300 x g) for 7 min at 4°C, the pellet, which contains mainly the leukocytes, was resuspended in Tyrode solution containing per 100 ml, 0.1 g D-glucose, 0.74 mg calcium chloride, 0.02 g magnesium chloride, 0.04 g potassium chloride, and 1.75 g Tris. The centrifugation was repeated once more. After resuspending in 40 ml of isotonic 0.17% ammonium chloride solution, the cells were incubated at room temperature for 5 min to induce lysis of remaining red cells and centrifuged again for 4 min. The cells were resuspended and washed again with Tyrode solution, followed by centrifugation at 1000 rpm (300 x g) for 4 min. The pellet was taken up with 40 ml of phosphate buffer (pH 7.4) and adjusted to 15×10^6 cells/ml.

Incubation - Test substances were dissolved in ethanol and adjusted to a concentration such that the total percentage of ethanol in the incubation mixture was less than 5%. The incubation

mixture contains 2.5 ml suspension of leukocytes, 25 µl of 0.2 M calcium chloride solution, 10 µl of 0.2% of ETYA (5,8,11,14-eicosatetraynoic acid) solution in ethanol, 50 µl of ethanol with the required amount of the sample, 12 µl of 2% ionophor A in ethanol, and 10 µl of 1-^{14}C-arachidonic acid solution (50 µCi in 5 ml ethanol). A blank was also prepared to serve as the control. This mixture was incubated at 37°C for 5 min in a water bath. The reaction was stopped by adding 15 µl of 25% sodium hydroxide and 525 µl of 1% formic acid.

Extraction of Arachidonic Acid and Its Metabolites - The arachidonic acid and its metabolites, 5-HETE (5-hydroxyeicosatetraenoic acid) and LTB_4 (leukotriene B_4) were extracted twice from the incubation mixture with 4 ml ethyl acetate. The phases were separated by centrifugation at 1500 rpm (460 x g) for 15 min. The upper phases were transferred to small retorts and evaporated. Each residue was dissolved in 100 µl ethanol and subjected to analytical HPLC.

Separation of Arachidonic Acid and Its Metabolites - Arachidonic acid and its metabolites were separated by RP-HPLC using an acetonitrile-water-gradient system:

Column	:	Hibar Lichrospher 100 CH 18/2 125 x 44 mm ID, 0.5 µm (Merck)
Eluent A	:	acetonitrile/water 1/1 (v/v)
Eluent B	:	acetonitrile/water 9/1 (v/v) containing 0.1% of 1 N phosphoric acid
Gradient system	:	0% - 100% B linear in 20 min, following an isocrating eluation for 10 min
Flow	:	1 ml/min
Detection	:	HPLC-Radioactivity-Monitoring
Injection volume	:	30 µl

F. Fractionation of Active Extracts

1. Vacuum Liquid Chromatography (Hostettmann et al. 1986; Pelletier et al. 1985) - Separation of fractions was accomplished by vacuum liquid chromatography (VLC). The apparatus consists of a sintered-glass Buchner filter funnel (60 x 4 cm) into which a layer (500 cm) of TLC grade

silica gel was dry-packed. The sorbent was compressed to a hard layer under vacuum (water aspirator) with gentle tapping to remove air pockets. The vacuum was released, solvent of low polarity was poured quickly onto the surface of the adsorbent and then vacuum was reapplied. The solvent should pass through the column uniformly. If it did not, the column had to be repacked. When the solvent began to emerge from the bottom of the column, it was sucked dry and was then ready for loading. The crude extract in a suitable solvent was carefully introduced onto the surface of the column (no vacuum). Enough solvent had to be used to completely cover the top of the surface of the column. Then vacuum was applied gently to draw the sample into the packing. A thin uniform line of substrate should result at the top of the column. The column was then developed under gentle vacuum with appropriate solvent mixtures, starting with solvent of low polarity and gradually increasing the polarity, pulling the column dry between each fraction collected. After each fraction was taken, appropriate solvent was added to the top of the column without vacuum until the surface was well covered. Then vacuum was gently applied.

The column was eluted by the step gradient-elution technique with a series of solvents: hexane, benzene, chloroform, ethyl acetate, butanol, acetone, ethanol, methanol, and water. A total of 37 fractions of 100 ml each was collected and subjected to TLC on 20 x 10 cm plates. Fractions exhibiting similar TLC patterns were pooled and the solvent evaporated *in vacuo*.

Pooled fractions were again subjected to VLC (column size: 20 x 3 cm) and treated with another series of solvents (hexane, chloroform, and ethyl acetate). Nine 100 ml fractions were also collected and subjected to TLC on 20 x 20 cm plates. Fractions exhibiting similar TLC patterns were again pooled and the solvent evaporated *in vacuo*. Pooled fractions were subjected to preparative TLC.

2. Thin-Layer Chromatography - Fractions collected after VLC were all subjected to TLC using precoated plastic plates with silica gel 60 F_{254}. The samples were spotted using fine

capillary tubes. The plates were then allowed to develop in a developing chamber which was previously lined with filter paper saturated with the developing solvent (hexane: chloroform:ethyl acetate, 1:1:1). After development, the plates were removed from the chamber and air-dried. Spots were visualized under short wave UV light (254 nm) and iodine vapor.

3. Preparative Thin-Layer Chromatography (PTLC) (Hostettmann et al. 1986) - To prepare 20 x 20 cm plates with thickness approximately 1.5 nm, 200 g of Kieselgel 60 PF_{254} were homogeneously suspended in a round-bottom flask with about 480 ml of water by swirling slightly without shaking. The suspension was allowed to stand for 1 h then poured in approximately 70-ml portions onto plates. Each plate was previously enframed with adhesive strips which were removed afterwards. The plates were stored in a closed chamber for 2 days at room temperature to dry. These were activated for 2 h at 120° in a drying oven before using.

   Concentrated amounts of the pooled fractions from the 20 x 3 cm VLC column were uniformly streaked as a narrow band across the entire width of the plate. The plates were dried, then developed by a solution of hexane: chloroform: ethyl acetate (1:1:1). The bands were visualized under short wave UV light (254 nm), scraped, and the substances extracted from the adsorbent with chloroform. The extracts were volatilized to dryness.

RESULTS AND DISCUSSION

A. Extraction

From the outer root bark, yellow-orange extracts were obtained with nonpolar solvents while blood red extracts were obtained with polar solvents. The yields for each of the extracts were 4.27% for petroleum ether, 1.81% for benzene, 1.30% for chloroform, 0.54% for ether, 0.52% for ethyl acetate, and 0.14% for ethanol.

The extracts differed in color after drying to constant weight in an oven at 80°C. The petroleum ether extract was orange, the

benzene extract was yellowish brown, the chloroform extract was reddish brown, the ether extract was yellow orange, and the ethyl acetate and ethanol extracts were reddish brown in color. All of the extracts were resinous in consistency.

B. Chemical Screening

The extracts were subjected to the Liebermann-Burchard and ferric chloride tests. The nonpolar extracts gave positive results with both the Liebermann-Burchard test (orange coloration) and the ferric chloride test (green coloration) which may indicate the presence of phenolic triterpenes or possibly *friedo*-oleanane triterpenes. The polar extracts gave negative results with the Liebermann-Burchard test but gave positive results with the ferric chloride test, which may indicate the presence of phenolic compounds.

C. Brine Shrimp Bioassay

The method using brine shrimp (*Artemia salina* Leach) as a simple bioassay for natural product research determines LC50 values in μg/ml of active compounds and extracts in a brine medium (Meyer et al. 1982). The method is rapid, reliable, inexpensive, and convenient as an in-house general bioassay tool for screening and fractionation monitoring of physiologically active plant extracts. Active compounds thus obtained could then be subjected to more elaborate bioassays for specific pharmacologic activities.

The petroleum ether, benzene, and ethanol extracts were found to be active at concentrations less than 1000 μg/ml, whereas the chloroform, ether, and ethyl acetate extracts were found to be less active (Figure 4). The petroleum ether extract had an LC50 of 22 μg/ml and the benzene extract, 215 μg/ml. The ethanol extract was found to be the most potent with an LC50 of 4.0 μg/ml. The activity of the extracts was compared to that of acetylsalicylic acid (ASA), the prototype of the anti-inflammatory, analgesic, and antipyretic drugs designated as the nonsteroidal anti-inflammatory drugs (NSAIDS). ASA gave an LC50 of 0.50 μg/ml. For the purpose of this study, in comparison with the LC50 of ASA, an extract or fraction is considered active if the LC50 is $</=$ 100 μg/ml. Subsequent tests for anti-inflammatory activity were done on all extracts but attention was focused on the petroleum ether and ethanol extracts.

D. Screening for Anti-inflammatory Activity

Inflammation is caused by the subjection of living tissues to trauma of some kind which causes tissue injury (Bainton 1980; Lewis 1989). Acute inflammation is recognized by its cardinal signs of redness, edema, heat, pain, and loss of function. Acute inflammation is a defensive response to injury but it is not always beneficial. In man, inflammation occurs in vascular tissues and inflammation is primarily a local vascular response where protein-rich fluid and cells are brought to the site of injury to neutralize the damaging agent. The damaging agent can be due to a variety of causes such as mechanical damage (cuts, blows, etc.), chemical damage (acids, alkalies, toxins, etc.), radiations, burns, scalds, viruses, bacteria, parasites, insect bites and stings, and local pathology such as antigen-antibody reactions. The cellular events consist of the selective migration of blood leukocytes to the site of inflammation.

The usual methods of testing samples for anti-inflammatory activity involve the use of animals or biochemical models (Lewis 1989). There is no single model which may cover all aspects of anti-inflammatory activity. Some of the biochemical tests have been employed to screen naturally occurring compounds for effect of anti-inflammatory activity. Biochemical tests are based on the effect of anti-inflammatory drugs on cells, organelles, enzymes, and mediators of inflammation. These will explain, at least in part, their anti-inflammatory activity. It is therefore necessary to use more than one biochemical test since each one examines only a single parameter of inflammation. Known anti-inflammatory drugs do not give positive reactions to all the biochemical tests due to their different modes of action. Four biochemical models were utilized for this study in order to obtain information regarding the mode of action of the extracts and the fractions as anti-inflammatory agents. These models are acute inflammatory models (in contrast to chronic inflammatory models) and they test for ability to stabilize proteins from denaturation, to prevent platelet aggregation, to inhibit erythrocyte hemolysis, and to inhibit 5-lipoxygenase which catalyzes the formation of leukotrienes from arachidonic acid (AA, eicosatetraenoic acid).

1. Protein Denaturation - Many NSAIDS combine with proteins to stabilize them and protect them from denaturation by heat, chemicals, agitation, etc. (Lewis 1989).

In general, as the concentration of the extracts of the outer root bark of *Kokoona ochracea* increases, percentage protein denaturation decreases. The ethanol, petroleum ether, and ether extracts gave a 100% inhibition of protein denaturation at concentrations less than 1000 μg/ml (Figure 5). The effect of ASA in inhibiting protein denaturation was fairly constant with varying concentrations, although an optimum of 55% was observed at a concentration of 100 μg/ml.

2. Platelet Aggregation - Platelets are the smallest blood-borne cells and they may be regarded as a special form of leukocyte (Lewis 1986; Nachman and Weksler 1980). Platelets play two distinct roles in hemostasis and thrombosis. Firstly, they prevent leakage of red cells from blood vessels by adhering to the basement membrane in areas of minor endothelial damage. Secondly, platelets help to arrest hemorrhage after vascular injury by adhesion to the altered vascular surface, aggregation of platelets to each other, and collaboration with the blood coagulation cascade to form a hemostatic plug. Platelet aggregation is a calcium-requiring process. Platelets must adhere to each other at sites of vascular injury in order to establish an effective hemostatic plug. Thromboxane A_2 (TxA_2), one of the metabolites of AA, is a potent aggregating agent, while the phospholipid platelet factor 3, released during aggregation, aids in the activation of thrombin, which not only initiates fibrin formation, but also causes further platelet aggregation.

   The petroleum ether extract of the outer root bark showed a significant decrease in platelet aggregation as the concentration was increased (Figure 6). In the case of ASA, an increase in platelet aggregation was observed as the concentration was increased above 100 μg/ml.

3. Erythrocyte Hemolysis - The principle of this test is that isolated erythrocytes are subjected to mild stress by hypotonic lysis (Lewis 1989). This causes the membranes to rupture, thus releasing hemoglobin (Hb) into the medium. The amount of lysis can then be determined by measuring spectrophotometrically the amount of Hb present in the supernatant after centrifuging.

As the concentration of the extracts increases, there is an increase in inhibition of erythrocyte hemolysis (Figure 7). The petroleum ether, ether, ethyl acetate, and ethanol extracts showed 100% inhibition of erythrocyte hemolysis at concentrations less than 1200 μg/ml. The petroleum extract is the most active, 100% inhibition was attained at approximately 1010 μg/ml. ASA stabilizes erythrocyte membranes at low concentrations but the effect decreases at high concentrations.

4. 5-Lipoxygenase Assay - The largest proportion of arachidonic acid (AA) is found in the phospholipids of the membrane of nearly all cells (Lewis 1986). Any kind of cell injury is sufficient to activate phospholipase A_2, which results in the release of AA from membrane phospholipids. AA is oxidized by two major pathways. The first, via a cyclo-oxygenase enzyme system, leads to the formation of prostaglandins and thromboxanes. A second pathway catalyzed by the lipoxygenases results in the production of leukotrienes (LTs).

Leukocytes contain a lipoxygenase which controls peroxidation of AA in the C-5 position, and this leads to the production of 5-HETE (5-hydroxyeicosatetraenoic acid) and the dihydroxy acid, 5,12-DHETE (leukotriene B_4, LTB_4) (Higgs et al. 1982). It is now known that LTB_4, the most potent lipoxygenase product, is one of the most active endogenous chemotactic factors yet discovered (Higgs et al. 1982; Lewis 1989). Chemotactic compounds mediate the amoeboid directional movement (chemotaxis) of the white cells towards the traumatized tissue. LTB_4 has been shown to possess potent inflammatory properties in rabbit skin and it is possible that the local generation of LTB_4 is an important mechanism in the contol of vascular permeability and leukocyte accumulation in injured tissues (Higgs et al. 1982). It is likely that the source of leukotrienes in inflammation is the leukocytes themselves, and this is supported by the increase in 5-lipoxygenase metabolism in skin with elevated leukocyte infiltration. AA metabolism by leukocytes could therefore represent a central mechanism in the development and amplification of the acute inflammatory response.

For evaluating the active plant extracts by means of their ability to inhibit 5-lipoxygenase, each was incubated with 1-^{14}C-arachidonic acid and purified porcine leukocyte suspension containing the 5-lipoxygenase. Calcium ions are necessary for the production and release of LTs (Lewis 1986). LTs are not stored but formed as a result of a specific stimulus such as Ca^{++} ionophores. The acetylenic acid, 5,8,11,14-eicosatetraynoic acid (ETYA), inhibits both the lipoxygenase and cyclo-oxygenase pathways in a concentration-dependent fashion (Sams et al. 1982). At the concentration used, ETYA inhibits cyclo-oxygenase. After incubation, the AA and its metabolites, 5-HETE and LTB_4, were separated by reversed-phase HPLC.

The formation of 5-HETE and LTB_4 can be inhibited by specific inhibitors of 5-lipoxygenase. Inhibition of the formation of these metabolites by plant extracts may indicate the presence of anti-inflammatory constituents. AA exhibits a peak at a retention time of 18.56 to 19.52 min; LTB_4, at 4.24 to 5.04 min; and 5-HETE, at 11.12 to 12.08 min (Figure 8a). Disappearance of the peaks at 4.24 to 12.08 min indicates 100% inhibition of the metabolism of AA by 5-lipoxygenase (Figure 8b). Percent inhibition was calculated directly from the percent amount of unmetabolized AA present after incubation.

The 5-lipoxygenase test was used to confirm the anti-inflammatory activity of the petroleum ether and ethanol extracts which were shown to be biologically active by the brine shrimp assay. In the control test system, 44.48% unmetabolized AA remained after incubation. In the test system containing 50 µg/ml of the petroleum ether extract, 89.88% unmetabolized AA remained indicating the presence of an inhibitor in the extract. There was no inhibition of the metabolism of AA in the test system containing 20 µg/ml of the petroleum ether extract nor in the test systems containing 20 µg/ml and 50 µg/ml of the ethanolic extract. Table 1 summarizes the results of these trials.

These observations indicate that the petroleum extract contains an anti-inflammatory principle which acts by inhibiting 5-lipoxygenase, besides preventing protein denaturation, platelet aggregation, and erythrocyte hemolysis. This principle may be a phenolic triterpene or a *friedo*-oleanane triterpenoid similar to those isolated from *K. zeylanica*. The ethanol extract may possess anti-inflammatory activity since it prevents protein denaturation and erythrocyte hemolysis although it does not inhibit 5-lipoxygenase.

E. Activity-Directed Fractionation

VLC is a very simple and inexpensive method of chromatography that provides good resolution of components in a short time (Pelletier et al. 1985). It consumes small amounts of solvents, has reasonable resolution, and requires little time to carry out a separation. VLC uses reduced pressure to increase the flow rate of the mobile phase (Hostettmann et al. 1986). In contrast to methods which use pressure applied at the top of the column to increase flow rates, manipulations on the column (such as solvent changes, etc.) are easy because the head of the column is at atmospheric pressure.

The active petroleum ether and ethanol extracts were subjected to further separation of their constituents by VLC and the resulting fractions were subjected to the brine shrimp bioassay.

With the petroleum ether extract, 25 out of 37 fractions (66% of the total number of fractions) were active in the brine shrimp bioassay (LC_{50} </= 100 µg/ml). There was a significant 50% increase in potency with fractions 12 to 19 (Table 2). Fractions 12 to 19 exhibited similar TLC patterns showing nine spots with their respective Rf values: *a*, 0.046; *b*, 0.115; *c*, 0.172; *d*, 0.195; *e*, 0.264; *f*, 0.379; *g* 0.414; *h*, 0.494; and *i*, 0.552. These fractions were pooled together, designated as VLC-1-1219, dried *in vacuo* (yield = 2.63%), and subjected to VLC, eluting successively with hexane, chloroform, and ethyl acetate. Eleven fractions were obtained of which fractions 1 and 2 showed no eluted constituents by TLC. Fractions 3, 4, and 5, had similar TLC patterns showing four well-defined blue-violet fluorescent spots under short-wave UV light with Rf values corresponding to those of spots *e, g, h,* and *i* of VLC-1-1219. These fractions were pooled together and designated VLC-11-345. Fractions 6, 7, and 8 showed similar TLC patterns with four

well-defined blue-violet fluorescent spots under short-wave UV light with Rf values corresponding to spots *c, d, e,* and *f* of VLC-1-1219. These were pooled and designated VLC-11-678. Fractions 9, 10, and 11, showing three well-defined blue-violet fluorescent spots on TLC plates, with Rf values similar to those of spots *a, b,* and *c* of VLC-1-1219, were pooled together and designated VLC-11-911. Of the 11 fractions, nos. 3, 4, and 5 were found to be the most biologically active, LC50 = 12 μg/ml. These fractions were pooled, designated as VLC-11-345, and streaked on preparative TLC plates. Four bands were formed and designated as B1e, B2g, B3h, and B4i. The four bands were eluted with chloroform and the solvent was evaporated to dryness. A red-amber-colored residue was obtained from each of the eluted bands with the following yields: B1e = 0.289%, B2g = 0.287%, B3h = 0.256%, and B4i = 0.252%. Band no. 2 (Rf = 0.414) was found to be the most active with an LC50 of 10 μg/ml.

Of the total number of 13 fractions (50 ml each) of the ethanol extract, 25% were active: fractions 5, 8, and 10, with an LC50 of 51 μg/ml (Table 3).

CONCLUSIONS

1. The petroleum ether extract of the outer root bark of *Kokoona ochracea* (Elm.) Merr. (Fam. Celastraceae) possesses anti-inflammatory activity. The active compounds may be phenolic triterpenes or *friedo*-oleanane triterpenoids as have been reported to occur in various members of the family. The activity of the extract is due to inhibition of protein denaturation, platelet aggregation, ertythrocyte hemolysis, and inhibition of the enzyme 5-lipoxygenase.

2. The ethanol extract prevents protein denaturation and erythrocyte hemolysis and may contain phenolic compounds which may be anti-inflammatory.

3. The petroleum ether and ethanol extracts of the outer root bark of *K. ochracea* are potential sources of anti-inflammatory drugs. Further studies are indicated.

4. The benzene extract showed activity in the brine shrimp bioassay but its specific pharmacological action has yet to be investigated.

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Figure 1. Herbarium specimen of *Kokoona ochracea* (Elmer) Merrill (Family Celastraceae). (Voucher specimen No. 6098, Philippine National Herbarium)

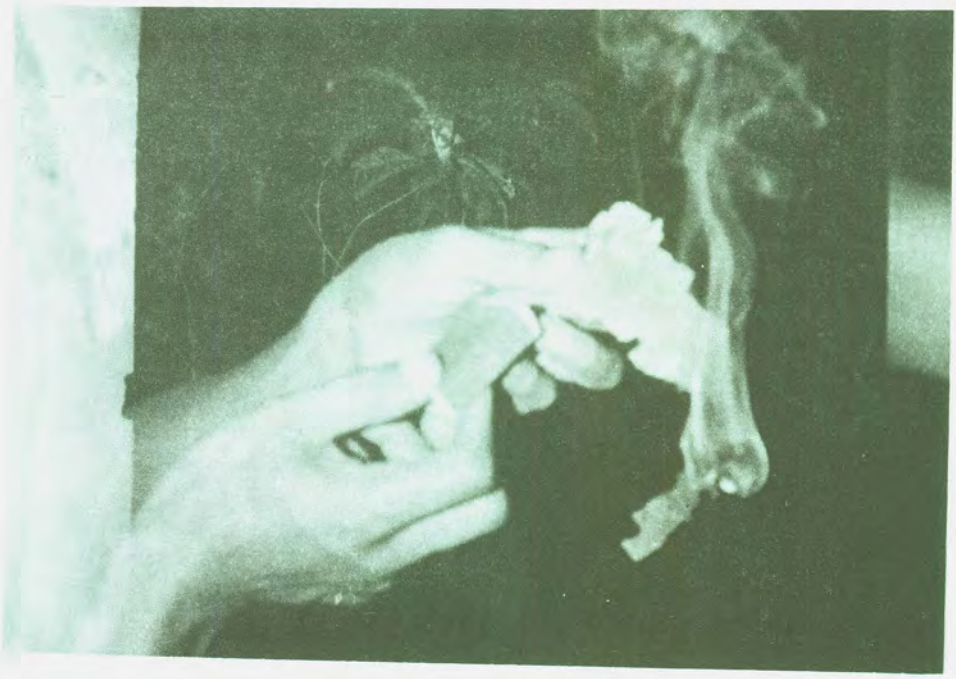
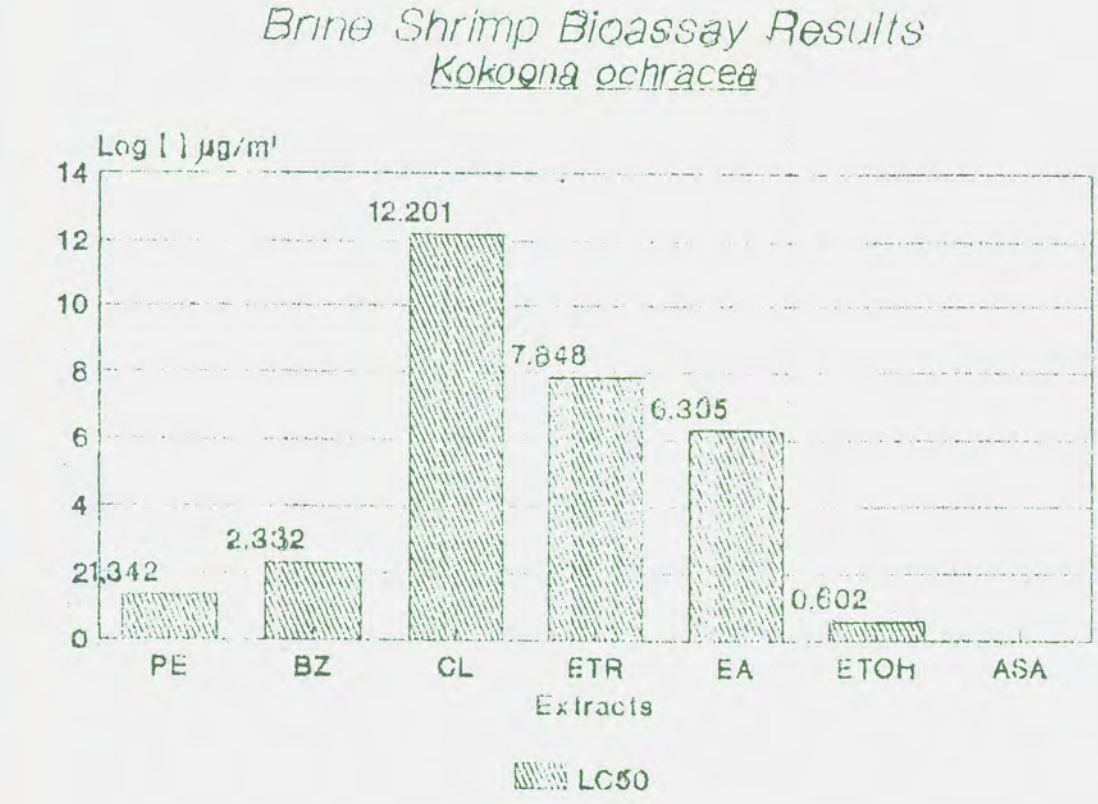


Figure 2. The root bark burns with a smoky flame.

Figure 3. Samples of broken pieces of and powdered stem and root barks. The powdered root bark is bright yellow-orange in color while the powdered stem bark is darker in color.



LC 50 (root bark extracts)

Figure 4. LC50 of the different extracts of the root bark of *K. ochracea* L. as shown in the brine shrimp bioassay.

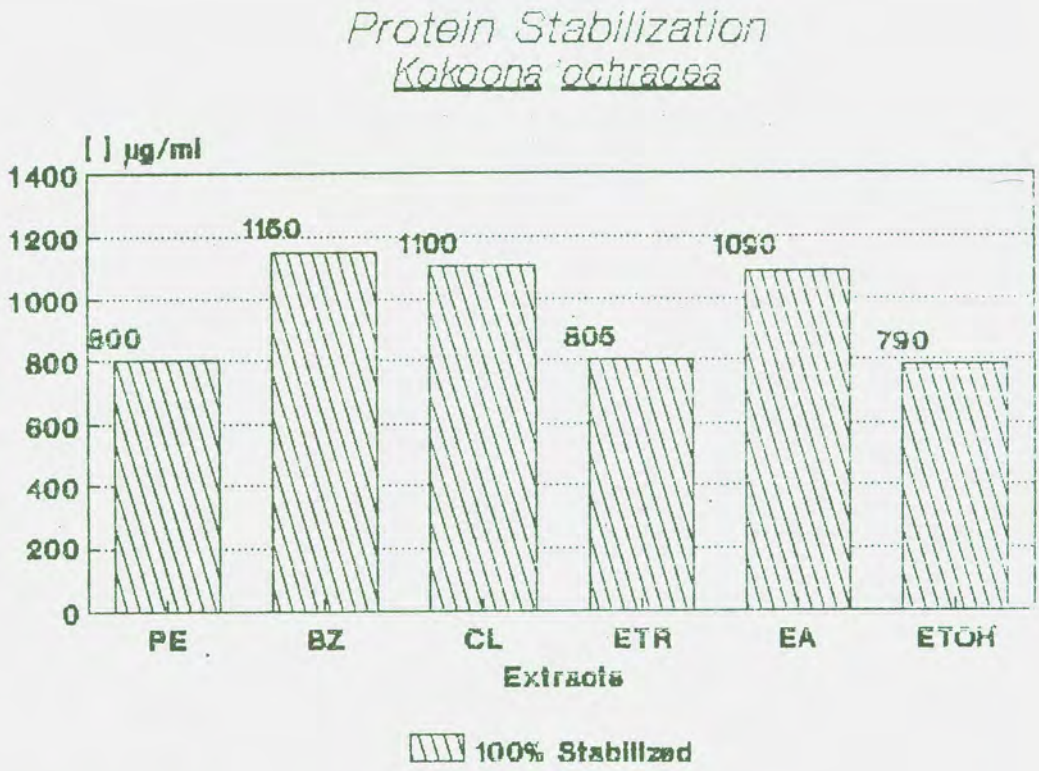


Figure 5. Effect of different root bark extracts on protein (egg albumin) stabilization.

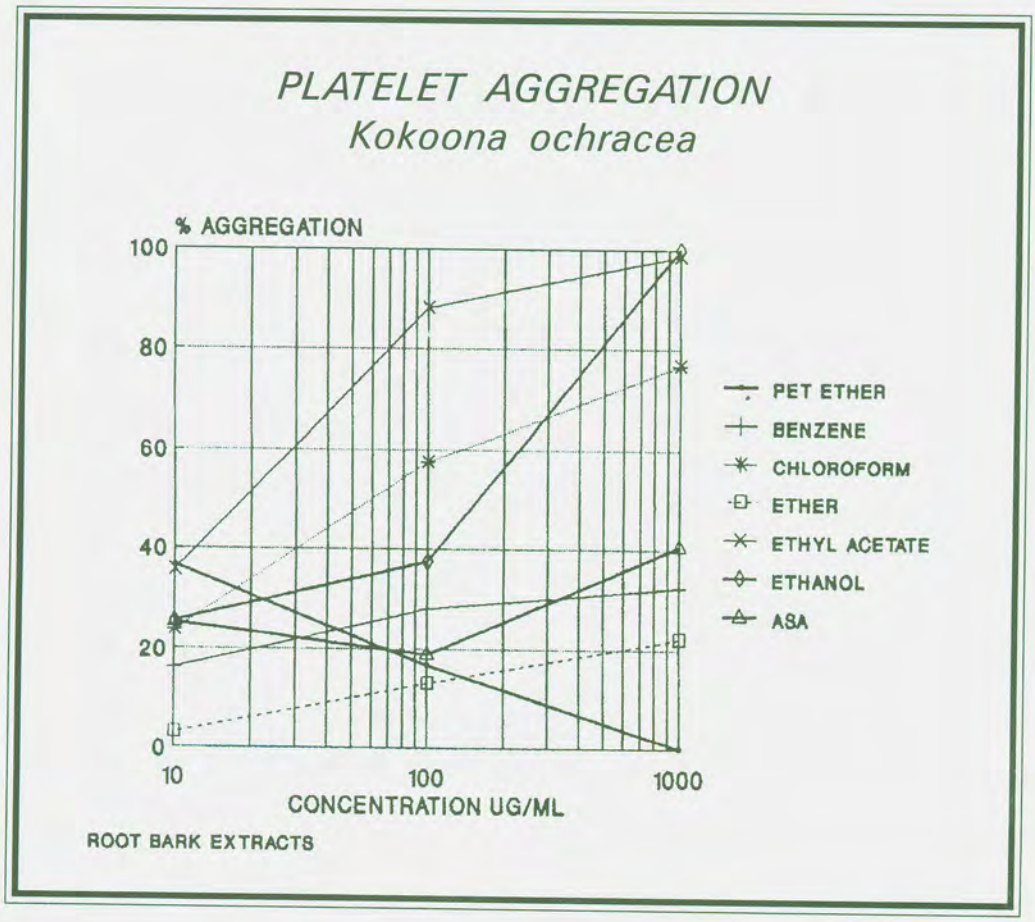


Figure 6. Effect of different root bark extracts on platelet (from porcine blood) aggregation.

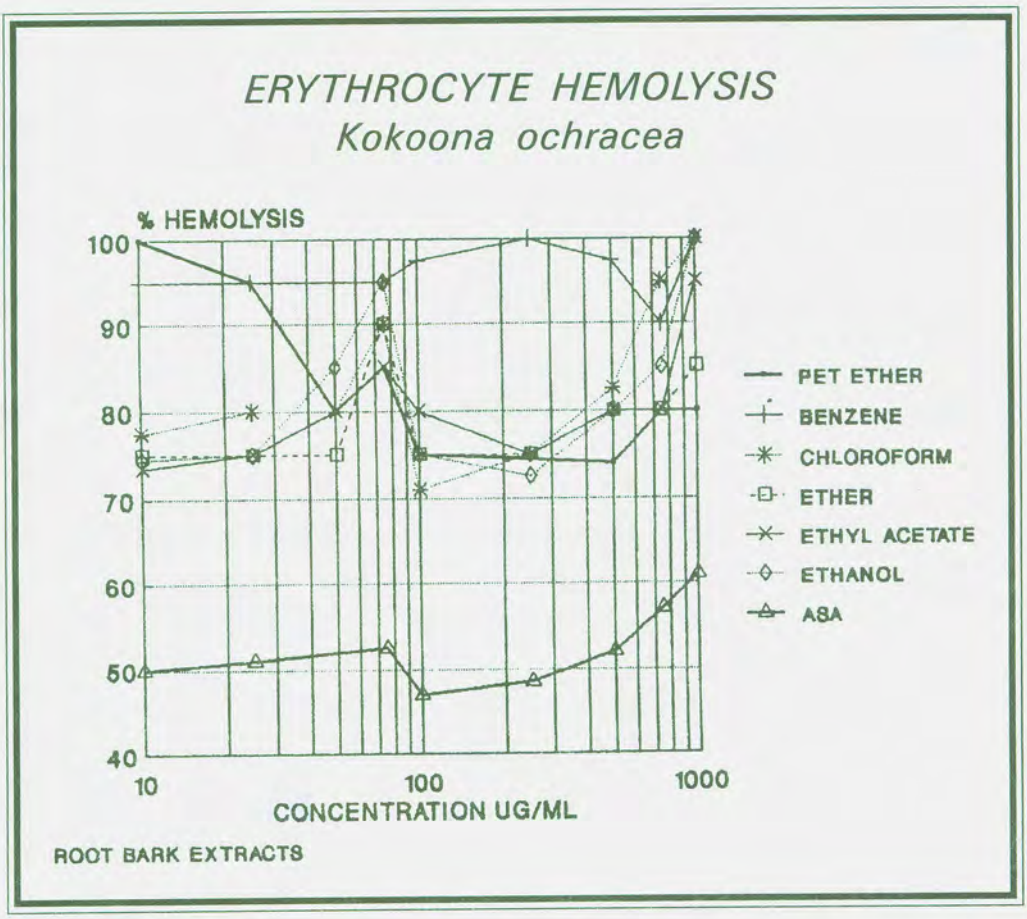
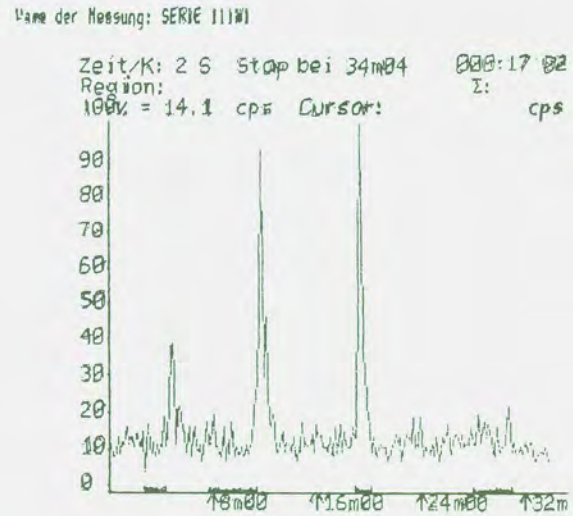
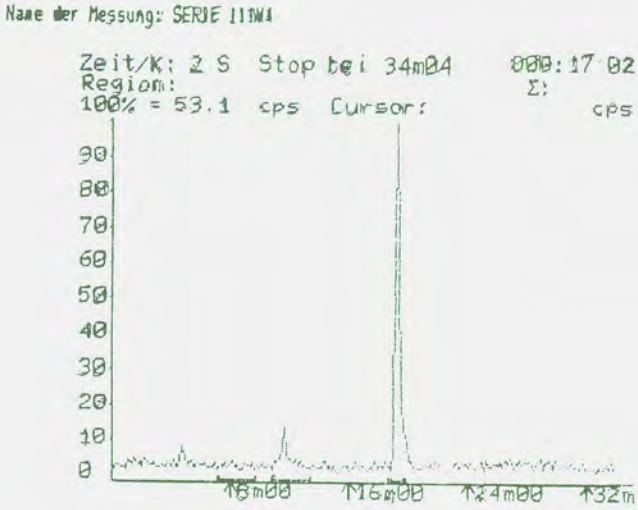


Figure 7. Effect of different root bark extracts on erythrocyte (from porcine blood) hemolysis.



(a)



(b)

Figure 8. HPLC chromatograms of arachidonic acid (AA) and its metabolites, leukotriene B_4 (LTB_4), and 5-hydroxyeicosatetraenoic acid (5-HETE). (a) AA exhibits a peak at a retention time of 18.56 to 19.52 min; LTB_4, at 4.24 to 5.04 min; and 5-HETE, at 11.12 to 12.08 min. (b) Decrease or disappearance of the peaks at 4.14 to 12.08 min indicates partial or complete inhibition of the metabolism of arachidonic acid by 5-lipoxygenase.

Table 1. Effect of the petroleum ether and ethanol extracts on the metabolism of arachidonic acid to LTB_4 and 5-HETE in the presence of the enzyme 5-lipoxygenase.

I. Control

	Compound	Peak Location From	To	Maximum	Amount %
1.	LTB_4	4m24	5m04	4m46	12.72
2.	5-HETE	11m12	12m08	11m36	42.81
3.	AA	18m56	19m52	19m18	44.48

II. Petroleum Ether Extract, 20 μg/ml

	Compound	Peak Location From	To	Maximum	Amount %
1.	LTB_4	4m16	5m04	4m48	21.78
2.	5-HETE	11m04	12m08	11m36	44.57
3.	AA	18m48	19m44	19m12	33.66

III. Petroleum Ether Extract, 50 μg/ml

	Compound	Peak Location From	To	Maximum	Amount %
1.	LTB_4	4m32	4m56	4m44	2.68
2.	5-HETE	11m12	11m52	11m40	7.44
3.	AA	18m56	19m52	19m14	89.88

IV. Ethanol Extract, 20 μg/ml

	Compound	Peak Location From	To	Maximum	Amount %
1.	LTB_4	4m24	5m04	4m48	28.84
2.	5-HETE	11m12	12m08	11m36	44.65
3.	AA	18m48	19m28	19m06	26.50

V. Ethanol Extract, 50 μg/ml

	Compound	Peak Location From	To	Maximum	Amount %
1.	LTB_4	4m24	5m04	4m50	24.65
2.	5-HETE	11m12	12m08	11m38	44.58
3.	AA	18m48	19m28	19m08	30.77

Table 2. Brine shrimp bioassay of fractions obtained from the petroleum ether extract by VLC.

Percent Deaths After 24 h				95% Confidence Interval		
Fraction No.	10 µg/mL	100 µg/mL	1000 µg/mL	LC50 µg/mL	Lower Limit	Upper Limit
3	100	0	10	6	4	10
4	0	50	10	80	96	340
5	10	0	100	25	23	28
7	0	0	20	0	0	0
8	20	30	90	30	.20	412
9	0	80	60	14	7	27
10	0	40	70	23	12	43
11	0	40	70	23	12	43
12	10	90	100	3	1	7
13	10	70	80	7	2	29
14	10	60	100	4	2	44
15	40	40	100	10	1	100
16	10	30	70	29	6	135
17	0	90	80	7	3	15
18	0	0	20	138	30	632
19	0	0	100	29	14	57
20	0	0	10	51	18	146
21	70	0	20	3	1	5
22	90	0	30	3	2	6
23	70	0	20	3	1	5
24	10	0	100	25	15	40
25	60	70	0	1	1	3
26	100	60	30	788	0	> 1000
27	0	0	0	0	0	0
28	0	60	0	0	0	0
29	0	80	80	3	0	30
30	0	30	70	6	0	1765
31	0	70	20	350	35	3479
32	0	70	20	350	35	3479
33	0	0	30	107	52	222
34	0	60	50	26	15	44
35	80	0	60	4	1	19
36	0	30	30	62	40	98
37	50	50	30	43	0	7565
ASA	60	90	100	0.48	0.02	12.76

Table 3. Brine shrimp bioassay of fractions obtained from the ethanol extract by VLC.

Percent Deaths After 24 h				95% Confidence Interval		
Fraction No.	10 µg/mL	100 µg/mL	1000 µg/mL	LC50 µg/mL	Lower Limit µg/mL	Upper Limit µg/mL
2	0	10	10	461	7	28856
3	0	0	20	139	28	685
4	0	0	0			
5	0	0	10	51	4	674
6	0	0	0			
7	0	0	0			
8	0	0	10	51	4	674
9	10	20	50	131	6	3100
10	0	0	10	51	4	674
11	10	10	10			
12	10	10	10			
13	0	10	20	106	73	154
ASA	60	90	100	0	0.02	12.8