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# MODULATION OF EICOSANOIDS BIOSYNTHESES *IN VIVO* AS A MECHANISM-BASED EVALUATION OF PUTATIVE ANTI-INFLAMMATORY PLANT EXTRACTS

BY

JOHN DADZIE-MENSAH

JUNE, 2000

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I achieve nothing if I live for myself but

I achieve ALL if I live for the truth and social justice,

And love, and serve my God and humanity in all sincerity.

*JOHN DADZIE-MENSAH*

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# MODULATION OF EICOSANOIDS BIOSYNTHESES *IN VIVO* AS A MECHANISM-BASED EVALUATION OF PUTATIVE ANTI-INFLAMMATORY PLANT EXTRACTS

A THESIS SUBMITTED

BY

JOHN DADZIE-MENSAH

IN PARTIAL FULFILMENT OF THE
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A MASTER OF PHILOSOPHY DEGREE

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF SCIENCE
UNIVERSITY OF GHANA
LEGON

JUNE, 2000

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# DECLARATION

The experimental work contained in this thesis was carried out by me in the laboratories of the Department of Biochemistry and the Noguchi Memorial Institute for Medical Research both of the University of Ghana, and the findings therefrom, my exclusive contribution to the science of plant medicine.

......................................
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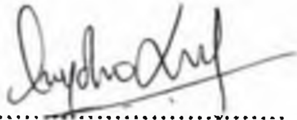

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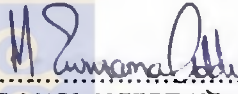

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PROF. MARIAN EWURAMA ADDY
(Supervisor)

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# DEDICATION

TO

*MY PARENTS*

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## ACKNOWLEDGEMENT

To the glory of the living God! I wish to express my heartfelt appreciation to all who helped in diverse ways to making this piece of work a success.

I am most grateful to my supervisors; Prof. Marian Ewurama Addy and Dr. Alexander Kwadwo Nyarko, whose guidance, patience and respect encouraged me to go through this exercise successfully.

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I am so grateful to my family for the encouragement, love, prayers and financial support.

I must also express my profound gratitude to the Association of African Universities for the award of a grant that contributed, in no small way, to the successful completion of this thesis.

To the management and staff of the Green Earth Organization I say, God richly bless you for allowing me the full usage of your electronic facilities, without which this work

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would have been completed with an unprecedented difficulty. I also thank the organization greatly for its financial support.

To the technical staff of the Department of Biochemistry and of the Chemical Pathology, Electron Microscopy and the Research Animal Breeding Units of the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, and to Mrs. Sarah Dwamenah Abassah and Sarah Acheampong of the Green Earth Organization, I say a big thank you for your assistance. My special appreciation goes to Dr. Addo , the head of the Research Animal Breeding Unit, for allowing me the use of the facilities at her Unit.

May God richly bless you all.

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## ABSTRACT

The eicosanoids are a group of oxygenated unsaturated 20-carbon chemical compounds produced from arachidonic acid (AA), that mediate almost every step in the inflammatory process naturally. Some of the eicosanoids are anti-inflammatory while others are pro-inflammatory.

*Desmodium adscendens*, a medicinal plant used by local herbalists to manage asthma, had been shown to be anti-anaphylactic *in vivo*, and to modulate AA metabolism *in vitro*. In this work, the anti-inflammatory properties of *D. adscendens in vivo* were examined by measuring the effect of its aqueous extract on eicosanoid production. Two other putative anti-inflammatory medicinal plants, *Parquetina sp* ('*Tina A*') and *Cassia sieberiana* ('*Kenken*') were also examined.

The effects of the extracts on phospholipase $A_2$ ($PLA_2$), the enzyme responsible for the mobilization of arachidonic acid (AA) from membrane glycerophospholipid stores to initiate the *de novo* biosyntheses of the eicosanoids were also examined.

Microsomal enzymes prepared from the lungs of both extract-treated (test) and untreated (control) male guinea pigs were used to catalyze the metabolism of arachidonic acid (AA), the natural substrate for eicosanoid synthesis, via the cyclo-, mono- and the lipoxygenase pathways to produce various eicosanoids. Reduced glutathione, GSH, was added to the reaction mixture for the cyclooxygenase pathway while NADPH was added

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for the monoxygenase pathway. For the lipoxygenase pathway no cofactor was added. The monooxygenase metabolites were not quantified due to limited resources. However, the influence of added NADPH on the production of the metabolites of the other two pathways was evaluated. All synthesized eicosanoids were assayed by ELISA.

As sources of secretory phospholipase $A_2$ ($sPLA_2$), blood samples were taken from the experimental animals and used to assess the effects of the plant extracts on $PLA_2$ activity.

The cyclooxygenase and lipoxygenase pathways were identified as good bioassay systems for assessing the anti-inflammatory properties of *D. adscendens*. The plant's extract inhibited the pro-inflammatory lipoxygenase pathway in a dose-dependent manner (68% and 98% reductions in peptidoleukotrienes syntheses for the lower and higher doses respectively). In the cyclooxygenase system, the extract enhanced the syntheses of the anti-inflammatory prostanoids; $PGI_2$ (6437% increase) and $PGE_2$ (581% increase). The effect on the synthesis of the pro-inflammatory prostanoid $PGF_{2\alpha}$ was insignificant (0.5% increase) and that on $TXA_2$ was 49% increase, both at the higher dose of the extract.

Using *D.adscendens* as a model, the anti-inflammatory effects of '*Tina A*' and '*Kenken*' were assessed using the cyclooxygenase bioassay system only. Like *D. adscendens*, the two medicinal plants also increased $PGI_2$ and $PGE_2$ production and hardly showed any

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effect on $PGF_{2\alpha}$ and $TXA_2$ syntheses. The increases in $PGI_2$ production ranged between 290% and 1417% ; those for $PGE_2$ were 57% and 78%, all at the higher doses of the extracts. The effects at the lower doses were not significant except for '*Tina A*'. In all, *D. adscendens* proved to be the best anti-inflammatory plant with respect to enhancing anti-inflammatory eicosanoids syntheses, followed by '*Tina A*'.

All three extracts inhibited phospholipase $A_2$ activity with '*Tina A*' showing a dose-dependent inhibitory effect even with a small dose difference of 1: 2.5. '*Tina A*' was the best $PLA_2$ inhibitor followed by '*Kenken*'.

The results indicate that the medicinal plants evaluated provide therapeutic relief to inflammatory disorders by the following mechanisms: (a) directly reducing $PLA_2$ activity and thus release of AA which the rate determining step in eicosanoid production, and/or by (b) enhancing $PGI_2$ and $PGE_2$ production when arachidonic acid has been released. Inhibition of peptido-leukotriene synthesis could also be said to be a good determinant of the anti-inflammatory status of *D. adscendens* in particular.

Increased synthesis of the anti-inflammatory prostanoids $PGE_2$ and $PGI_2$, and/or inhibition of phospholipase $A_2$ activity appear to be good bioassays for evaluating medicinal plants claimed to have anti-inflammatory properties. It was therefore concluded that good bioassay systems have been developed for *in vivo* evaluation of putative anti-inflammatory drugs with respect to eicosanoid biosynthesis.

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# CHAPTER ONE

## INTRODUCTION AND LITERATURE REVIEW

### General Introduction

Eighty percent of the world's population relies entirely on local medicines made almost exclusively from plants (Lewington, 1990). People belonging to the most advanced civilizations and the simplest cultures the world over have relied on plants to keep them healthy. Only recently, with the advances in synthetic chemistry have developed countries broken their dependence on cures that came almost exclusively from plants (Lewington, 1990). Though this practice of dependence on non-plant based drugs is gradually creeping into simple cultures, it may perhaps, never gain roots fully in these cultures which utilize such drugs because of the ever-increasing cost of Western medicine.

Interestingly, the complex nature of many plant compounds essential to modern medicine has made their synthesis either too expensive for chemical companies to venture into, or practically impossible. For these reasons, plants will continue to be the source of medical care for most patients of the world, particularly those in the tropics where two-thirds of all the plant species of the world exist. The tropics also have the largest resource of traditional knowledge and experience in the use of plants as medicines, possessing the greatest diversity of as yet unknown active compounds.

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The mystery surrounding the time-proven curative properties and the effectiveness of different plant extracts in the management of several diseases, including those declared incurable by Western medicine, coupled with the low treatment cost are the reasons why many countries the world over have held on to folk medicine. These countries, including Ghana, have done so inspite of the rather contemptuous attitudes in some areas of Western medicine that exist towards the use of crude plant extracts in disease management (Abbiw, 1990; Lewington, 1990). Other countries like the United States of America have increased their interest in plants as sources of raw materials for discovering and developing new pharmaceutical products (Komen, 1991).

It is estimated that between 35,000 and 70,000 different species of plants have been used as medicines by various peoples of the world with the commercial value of plant-derived drugs by Western medicine standing at about 40 billion U.S. dollars every year (Lewington, 1990). At least some 7000 different plant-derived medicinal compounds have been introduced into Western pharmacopoeia. Out of these, only 120 or so plant-based drugs, coming from just 95 plant species, are prescribed for use worldwide.

Out of the 250,000 species of flowering plants, approximately 5000 only have had their pharmaceutical potential tested in laboratories and very few have been acknowledged in the West to have any real therapeutic value (Krogsgaard-Larsen *et al*, 1984).

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The plant-based drugs prescribed for worldwide use include analgesics, anesthetics, antibiotics, anti-cancer, anti-parasitic compounds and drugs for diseases of the heart. Others are anti-inflammatory drugs, oral contraceptives, hormones, laxatives, diuretics and for ulcer treatment. These indicate that plants possess rich medicinal properties that could be exploited to treat almost all known diseases of man and animals.

The contribution of plant medicine to health care has great economic potential, with a large and rapidly growing global market for affordable and effective plant-based remedies. Ideally therefore, both Western and traditional medicine (which is plant-based) should complement each other. It is in recognition of this fact that the World Health Organization (WHO) is officially encouraging the integration of traditional medicine into the health care systems of developing countries where the majority of the population cannot afford the high cost of Western medicine.

Several plant species have been documented to be effective in the management of human diseases. These include, *Hemamelis virginiana* (Wych hazel), *Eucalyptus globulus, Cephaelis impecacuanha, Papaver somniferum, Ephedra* spp, *Catharanthus roseus* (rosy periwinkle), *Rauwolfia serpentina, Erythroxylum coca* and *Cinchona* spp. Crude extracts from these plants are used to treat a number of diseases including colds and fevers, coughs, bilharziasis, guinea worm infestations, bronchitis, pain and anxiety, cancers, hypotension and hypertension, menstrual tension, menopausal problems, psychiatric conditions and malaria. With respect to

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inflammation, a pathological condition which is the subject of study of this thesis, crude extracts from *H. virginiana* (Wych hazel), *Glycyrrhiza* spp, *Salix alba* (White willow), *Filipendula ulmaria*, *Tenacetum parthenium*, *Oenothera* spp and pineapple have been effectively used over the years to manage a broad spectrum of inflammatory diseases including rheumatoid arthritis, thrombosis, migraine and asthma (Lewington, 1990).

Scientific investigations into *Desmodium adscendens*, a medicinal plant, whose extracts are used among Ghanaians to remedy asthmatic attacks, have confirmed its efficacy. It has been shown that *D. adscendens* extracts are anti-anaphylactic both *in vivo and in vitro* (Addy and Awumey, 1984; Addy and Burka, 1988; Addy and Dzandu, 1986). The extracts also reduced the production of some spasmogens from lungs (Addy and Dzandu, 1986). Following the anti-anaphylactic effects *in vivo*, the effect of the extract on some eicosanoids biosyntheses *in vitro* were analyzed using radiochromatographic methods. The results indicated an increase in prostaglandin $E_2$ synthesis (Addy and Schwartzman, 1995) and a decrease in NADPH-dependent oxygenation of arachidonic acid (Addy and Schwartzman, 1992). The research work reported here seeks to investigate the effect of aqueous extracts from *D. adscendens* on eicosanoids biosyntheses *in vivo*. Since eicosanoids are typical spasmogens that mediate inflammation, changes in their production could be used to assay for the anti-inflammatory effects of putative medicinal plants. Using the results from *D. adscendens* studies as a model, the anti-

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inflammatory effects of extracts from *Cassia sieberiana and Parquetina sp* on eicosanoids biosyntheses will be evaluated.

**Inflammation and its mediators**

Inflammation is the local response to cell injury, involving small blood vessels, the cells circulating within these vessels, and nearby connective tissues. The response characteristically begins with hyperemia, edema, and adherence of the circulating white blood cells to the endothelial cells. The white cells then migrate between the endothelial cells of the blood vessel into the tissue to effect the necessary immunological response to the injury (Lagunoff, 1994).

Symptoms of inflammation include redness, swelling, heat and pain. Increased chemotactic movement of blood to the site of injury through the small blood vessels causes heat and redness. The loss of water from circulation to the extracellular edematous connective tissue causes edema/swellings through the engorged and distended capillaries. The collective effects of these events may lead to reduced blood flow rate in the immediate vicinity of the injury, with subsequent cessation of flow (stasis) and clotting of the concentrated blood cells in the constricted venules. This is the cause of thrombosis. Stimulation of nerve endings by agents released during the inflammatory process causes pain.

Inflammation is fundamentally a protective response whose ultimate goal is to rid the organism of both the initial cause of cell injury (e.g., microbes, toxins) and the consequences of such injury (the necrotic cells and tissues). However,

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inflammation and repair may be potentially harmful. Inflammatory reactions, for example, underly life-threatening hypersensitivity reactions to insect bites, drugs, toxins and antigens in asthmatics as well as some chronic diseases of modern times. These include rheumatoid arthritis, atherosclerosis, tuberculosis, lung fibrosis, common cold, influenza, gastroenteritis in salmonellae “food poisoning”, bacillary dysentery (acute colitis), diphtheria, pseudomembranous colitis, fibrinous inflammation, abscesses e.g. boils, ulcers and migraine (Robbins *et al*., 1994; Thier and Smith, 1981; Whaley and MacSween, 1992). The process of inflammation, both vascular and cellular, is orchestrated by an array of molecules produced locally. Many anti-inflammatory drugs function by preventing the formation of these mediators or by blocking their actions on the target cells whose behaviour is modified by the mediators (Lagunoff, 1994). These mediators include histamine, various peptides, complement components, kinins, antibodies, interleukins , and various eicosanoids.

The eicosanoids are very important in inflammatory disorders because of their various and sometimes conflicting pharmacological effects that enable them to mediate virtually every step in inflammation (Devlin, 1997; Robbins *et al*., 1994). They include leukotrienes, prostaglandins, thromboxanes, prostacyclins, lipoxins and a number of hydroperoxy and hydroxy fatty acids (Smith, 1989). These myriad pharmacological effects are often species-, sex- and tissue-dependent (Taylor and Ritter, 1986). Being natural mediators of inflammation, the eicosanoids are produced by virtually all mammalian tissues (Smith, 1987). They are local

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hormones synthesized *de novo* on induction by an appropriate stimulus and rapidly get metabolized (Smith *et al.*, 1991).

Though produced as products from the metabolism of a common substrate, arachidonic acid, some of the eicosanoids exhibit pro-inflammatory effects while others are anti-inflammatory. For example, prostaglandin $F_{2\alpha}$, thromboxane $A_2$ and the leukotrienes are pro-inflammatory while prostaglandin $E_2$ and prostaglandin $I_2$ (prostacyclin) are generally anti-inflammatory (Whaley and MacSween, 1992).

Therefore, a putative anti-inflammatory drug acting on the metabolism of arachidonic acid, may reasonably do so by either blocking a common pathway in the metabolic process, or modulate the process to yield more of the anti-inflammatory eicosanoids, and either maintain or reduce the levels of the pro-inflammatory eicosanoids.

**Biosyntheses of Eicosanoids**

***Phospholipase $A_2$ Activation***

Eicosanoids are synthesized from arachidonic acid (AA), a 20-carbon poly-unsaturated fatty acid commonly found in the *sn*-2 position of membrane phospholipids. The common glycerophospholipids with AA are phosphatidyl-serine, -inositol, -choline and phosphatidyl-ethanolamine (Smith *et al.*, 1991). Since unesterified free AA is the substrate for eicosanoid biosynthesis, the

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reactions involved are initiated by the hydrolytic release of AA from the *sn*-2 position by a stimulus-activated phospholipase $A_2$ (Murayama *et al.*, 1990; Silk et al., 1989). Alternatively, phosphatidylinositol may be degraded by phosphatidylinositol-specific phospholipase C to yield diacylglycerol, from which AA may be obtained (Smith *et al.*, 1991; Zubay, 1983).

The phospholipases $A_2$ ($PLA_2$) have been found to be either free and soluble ($sPLA_2$) (Channon and Leslie, 1990; Leslie *et al.*, 1988) or membrane-associated (Dennis, 1987; Lister *et al.*, 1988, 1989; Ross *et al.*, 1985; Ulevitch *et al.*, 1988). The activated $PLA_2$ appears to be a soluble enzyme, which becomes reversibly associated with membrane in the presence of higher $Ca^{2+}$ concentrations (Channon and Leslie, 1990).

The movement of $sPLA_2$ from solution into the membrane is essential to bring it to close proximity to the glycerophospholipid whose AA is to be hydrolyzed. Such phospholipids are normally in the vicinity of the membrane associated AA metabolizing enzymes for the rapid metabolism of the AA when released (DeWitt *et al.*, 1981; Rollins and Smith, 1980; Smith, 1986, 1987). Though some calcium-independent phospholipases have been characterized (MacDonald and Maxey, 1998; Smith *et al.*, 1991), it has been observed that almost all phospholipases operate optimally at higher $Ca^{2+}$ concentrations (Smith *et al.*, 1991). For example, the activity of phospholipase $A_1$ does not necessarily require $Ca^{2+}$ but its hydrolytic effect is enhanced by $Ca^{2+}$ and charged amphipaths because of their role in altering

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the surface charge on the substrate micelle or membrane (Gurr and Harwood, 1991).

Most of the external stimuli for $PLA_2$ activation therefore, operate by elevating intracellular calcium ion concentration (Smith *et al*, 1991). $PLA_2$ activities are present in both the soluble and membrane-associated fractions of cellular preparations (Kramer *et al.*, 1986). The bulk of AA metabolizing enzyme activity, in general, is associated with endoplasmic reticulum (DeWitt *et al.*, 1981;Rollins and Smith, 1980; Smith, 1986).

The free AA is rapidly metabolized into specific eicosaniods via any of three principal enzyme catalyzed pathways, namely, the cyclooxygenase, lipoxygenase, and monooxygenase (epoxygenase) pathways (Smith, 1989). All the membrane-associated enzymes for the metabolisms of AA are already present and active (Gurr and Harwood, 1991). The release of AA is thus said to be the rate-limiting step. The biosynthesis of the eicosanoids is thus thought to be regulated, at least acutely, at the level of arachidonic acid release (Bettazoli *et al.*, 1990; Dennis, 1987). Therefore, an anti-inflammatory drug acting against eicosanoid biosynthesis may do so at the level of AA release through the inhibition of the activity of $PLA_2$.

Although the substrate is the same (AA) the concentration and availability of enzymes and coenzymes for the three pathways differ in different tissues, giving rise to various metabolites in the different tissues. These metabolites exhibit

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different physiological and pharmacological effects on various organs and tissues, and therefore, defects in AA metabolism could lead to different diseases associated with different organs and tissues (Dunn, 1976; Samuelsson, 1983;Schibouta *et al.*, 1981).

The cyclooxygenase pathway yields the prostanoids; prostaglandins, prostacyclins and thromboxanes, while leukotrienes and lipoxins are formed via one or more lipoxygenase reactions (Samuelsson *et al.*, 1987; Smith, 1989), and lipid epoxides and diols are formed through cytochrome P-450-dependent reactions of the epoxygenase pathway (Capdevila *et al.*, 1990; Fitzpatrick and Murphy, 1989; Laniado-Schwartzman *et al.*, 1988). All enzymes of the arachidonate cascade are inactivated during catalysis. A summary of the oxygenative metabolism of AA is shown in figure 1.

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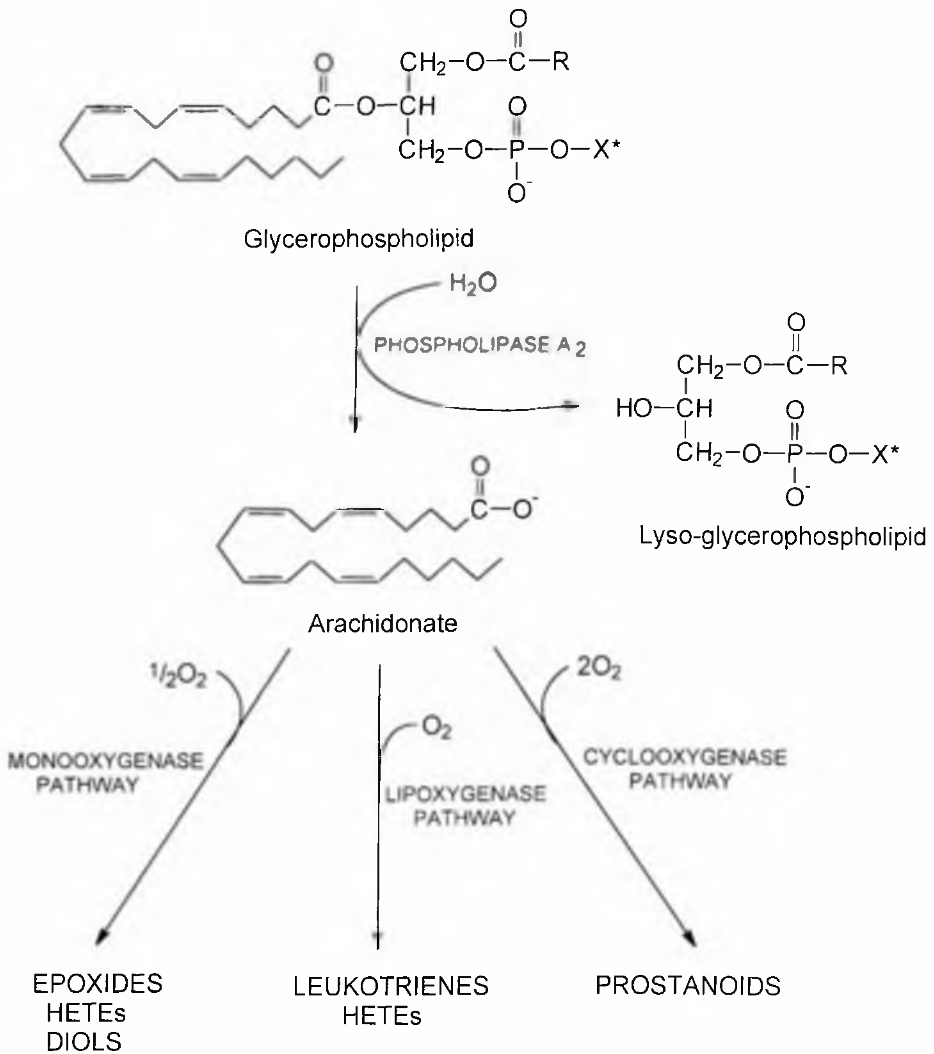
**Figure 1. Arachidonic acid release and oxygenative metabolism**
(Huber *et al.*, 1993)




X* = choline, ethanolamine, inositol or serine

R = Alkyl group

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***The Cyclooxygenase Pathway***

This pathway involves the incorporation of two moles of molecular oxygen ($2O_2$) into a mole of arachidonic acid by the cyclooxygenase activity of the bifunctional enzyme, prostaglandin endoperoxide synthase (PES). The unstable initial product formed, prostaglandin $G_2$ ($PGG_2$), is reduced by the peroxidase activity of PES to the stable prostaglandin $H_2$ ($PGH_2$). The conversion of $PGG_2$ to $PGH_2$ requires two moles of reduced glutathione (GSH) as cofactor. The resulting $PGH_2$ undergoes further reduction or rearrangement to yield what are considered to be the biologically active prostanoids: prostaglandin $D_2$ ($PGD_2$), prostaglandin $E_2$ ($PGE_2$), prostaglandin $I_2$ ($PGI_2$), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane $A_2$ ($TXA_2$) (Smith *et al.*, 1991)

The mechanism of conversion of $PGH_2$ to the various prostanoid products varies from one product to the other. While $PGE_2$, $PGD_2$, $PGI_2$ and $TXA_2$ are synthesized from $PGH_2$ by non-oxidative rearrangements, $PGF_{2\alpha}$ is formed from $PGH_2$ by a net two-electron reduction by the endoperoxide reductase (PGF synthase), which uses NAPDH as a reducing agent (Bergstrom *et al.*, 1963; Dunn *et al.*, 1978; Watanabe *et al.,* 1985). In contrast, $PGE_2$ is reported to be formed non-oxidatively from $PGH_2$ by the catalytic action of PGH-PGE isomerizing enzyme, PGE synthase, which does not consume the GSH coenzyme in contrast to the NADPH consuming function of PGF synthase (Moonen *et al.*, 1982; Ogino *et al.*, 1977; Tanaka *et al.,* 1987). However, nonenzymic formation of PGE from $PGH_2$ at relatively faster rates has also been reported (Hamberg and Samuelsson, 1973; Nugteren and

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Hazelhof, 1973). Conversion of $PGH_2$ to $PGD_2$ is by catalysis of PGD synthase which may either be GSH-dependent (Christ-Hazelhof and Nugteren, 1982; Ujihara *et al.*, 1988; Urade *et al.*, 1987) or GSH-independent (Urade *et al.*, 1985).

The conversion of $PGH_2$ to $PGI_2$ involves PGI synthase activity, which is high in vascular endothelial cells and in both vascular and non-vascular smooth muscle cells (DeWitt *et al.*, 1983; Smith *et al.*, 1983). $PGI_2$ is hydrolyzed to the stable and physiologically inert product, 6-keto-$PGF_{1\alpha}$ with an approximate half-life of 2 minutes (Salmon and Flower, 1982; Whaley and MacSween, 1992). The amount of $PGI_2$ produced from the metabolism of AA can therefore be quantified indirectly by measuring the amount of 6-keto-$PGF_{1\alpha}$ present in the medium.

The conversion of $PGH_2$ to $TXA_2$ is catalysed by TXA synthase. TXA is very unstable with a half-life of 30 seconds. It is hydrolysed to the stable product $TXB_2$ which lacks appreciable biological activity (Haurand and Ullrich, 1985; Nusing *et al.*, 1990; Zubay, 1984). The amount of the biologically active $TXA_2$ resulting from the metabolism of AA can, therefore, be estimated by measuring the amount of the inactive stable product, $TXB_2$ present. Details of the cyclooxygenase pathway are shown in figure 2.

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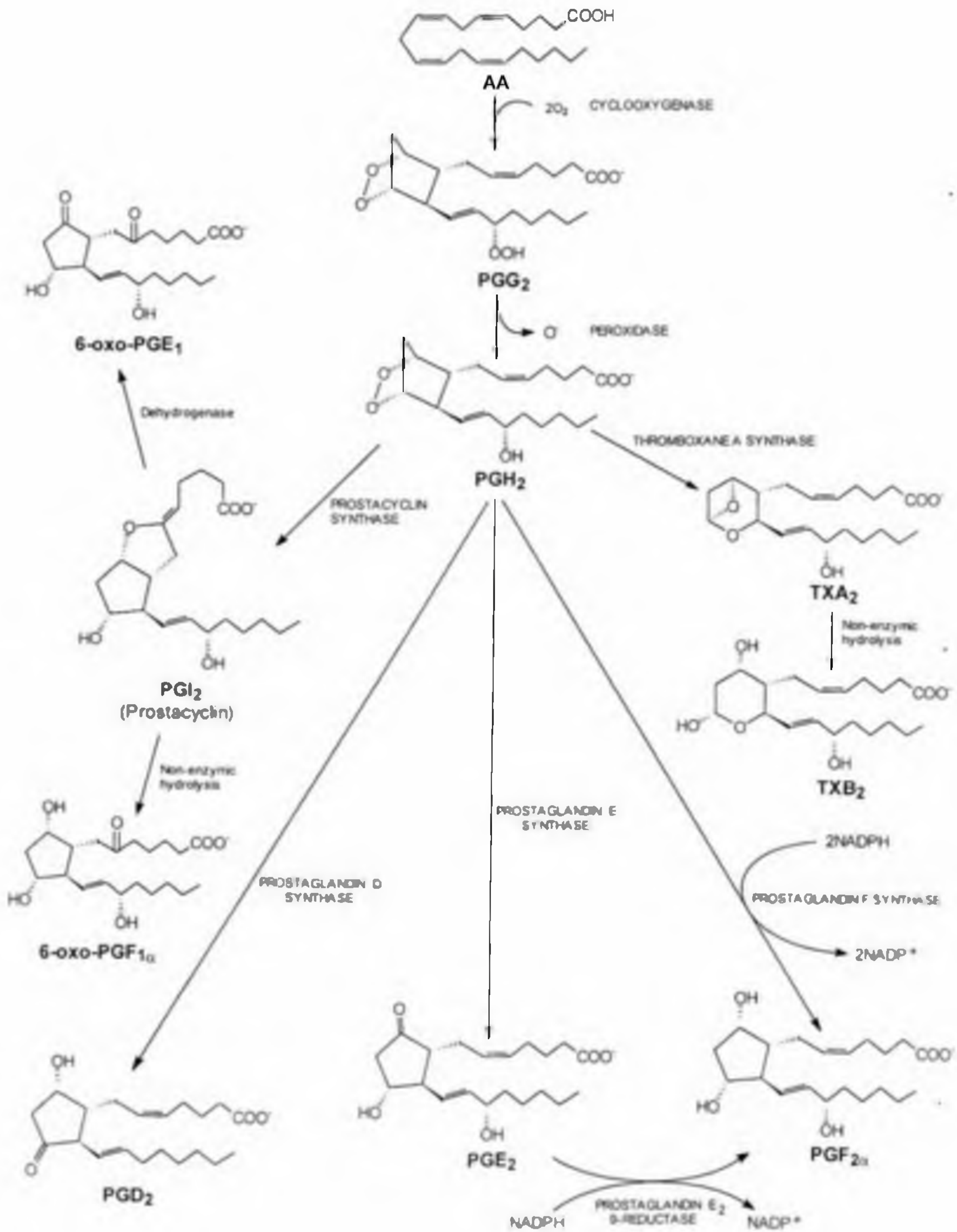
**Figure 2. Cyclooxygenase pathway for prostanoid biosynthesis**
(Huber *et al*., 1993; Taylor and Ritter, 1986)



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***The Lipoxygenase Pathway***

The conversion of unbound arachidonic acid (AA) to the leukotrienes involves an initial enzyme-catalyzed incorporation of one mole of molecular oxygen ($O_2$) into a molecule of AA.

The hydroperoxy substitution of AA by lipoxygenases may occur at position 5, 12 or 15 to produce the hydroperoxyeicosatetraenoic acids (HPETEs). The 5-HPETE is the major lipoxygenase product in basophils, polymorphonuclear (PMN) leukocytes, macrophages, mast cells and any organ undergoing an inflammatory response (Devlin, 1997). The HPETEs undergo dehydration among many other reactions, to produce the epoxy fatty acids. The epoxy eicosatrienoic acids and their metabolic products are the leukotrienes. The 5-HPETE is responsible for leukotriene production and is important in neutrophils, eosinophils, monocytes, mast cells and keratinocytes as well as lung, spleen, brain and heart (Gurr and Harwood, 1991).

In the formation of leukotrienes, the epoxide intermediate, leukotriene $A_4$, is converted enzymatically by hydration to leukotriene $B_4$ and to leukotriene $C_4$ ($LTC_4$) by addition of reduced glutathione (GSH). $LTC_4$ is further metabolized to leukotrienes $D_4$ and $E_4$ by the successive elimination of glutamyl residue by glutamyl transferase catalysis to yield $LTD_4$, and further elimination of glycine from $LTD_4$ by a dipeptidase to yield $LTE_4$ (Samuelsson, 1983). Details of the lipoxygenase pathway are shown in figure 3.

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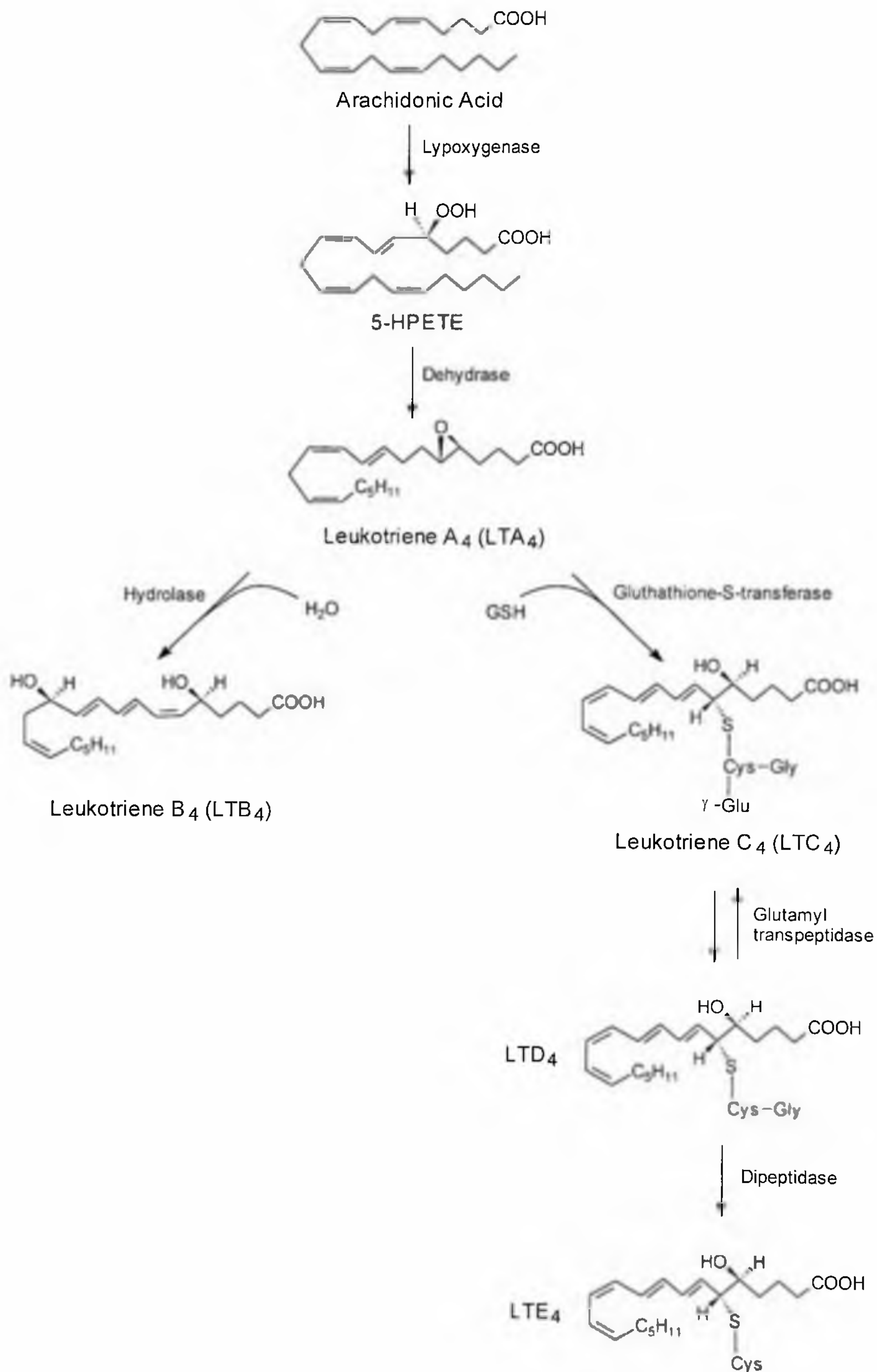
Figure 3: Lipoxygenase pathway of arachidonic acid metabolism for leukotriene synthesis (Samuelsson, B., 1983)




DHTs and HETEs other than the 5 derivatives are also produced (not shown)

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***The Monooxygenase/Epoxygenase Pathway***

There are three known reactions in this third pathway, all of which involve oxygenation of the AA using the NADPH-dependent monooxygenase enzyme. One atom of oxygen is incorporated into a molecule of AA while NADPH reduces the other atom to water. The enzyme complex consists of a flavoprotein reductase and cytochrome P-450 already present in the membranous subcellular structures of the cell as electron acceptors in microsomal electron transfer from NADPH to oxygen (White and Coon, 1980).

The different actions in this pathway lead to the formation of various products, including different regiospecific isomers of epoxy-eicosatrienoic acid (EET): 5,6-, 8,9-, 11,12- and 14,15-EET. Other products are the 19- and 20-hydroxyeicosatetraenoic acids (19- and 20-HETE) and various regioisomeric monohydroxyeicosatetraenoic acids (HETEs). The EETs produced are rapidly hydrolyzed to their corresponding dihydroxyeicosatrienoic acids (DHTs) by epoxide hydrolases (Chacos *et al.*, 1983). Details of the monooxygenase pathway are shown in figure 4.

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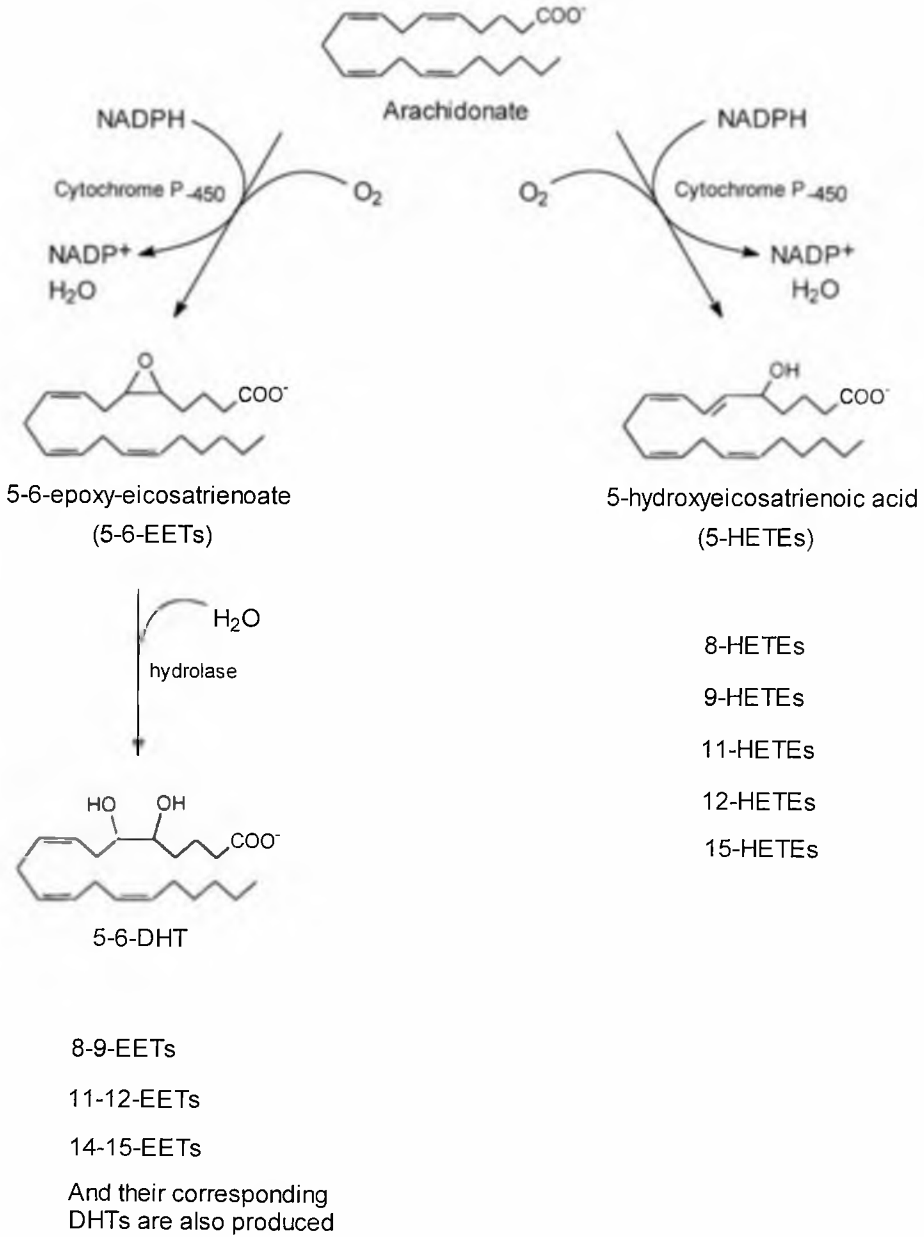
**Figure 4: Monooxygenase pathway of Arachidonic Acid Metabolism**
(Huber *et al.*, 1993)



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**Eicosanoid function in inflammation**

Unlike most chemical messengers, the eicosanoids are not stored in cells but instead, are synthesized and released immediately in response to a stimulus (Devlin, 1997). They exert a range of profound biological activities including effects on smooth muscle contraction/relaxation, inhibition or stimulation of platelet aggregation, bronchoconstriction/dilation and vasoconstriction/dilation (Gurr and Harwood, (1991).

Typical responses of a cell to eicosanoids include changes in intracellular concentration of cAMP (Sonnenburg and Smith, 1988), cGMP (Zubay, 1984) and $Ca^{2+}$ (Negishi *et al.*, 1989) upon binding of the eicosanoid to appropriate cell membrane surface receptors (Smith, 1989). However, thromboxane $A_2$ ($TXA_2$) is reported to cause an increase in cytosolic $Ca^{2+}$ by acting as $Ca^{2+}$ ionophore (Zubay, 1984).

The modulation of cell function by the eicosanoids during inflammation has been reported to be influenced by the relative levels of these secondary messengers (Whaley and MacSween, 1992). Agents like prostaglandin $E_2$ ($PGE_2$) and prostaglandin $I_2$ ($PGI_2$), which increase cAMP levels with resultant activation of cAMP dependent protein kinases, reduce inflammation (anti-inflammatory). In contrast, agents like prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane $A_2$ ($TXA_2$), which increase cytosolic $Ca^{2+}$ ion levels through the elevation of cGMP concentrations,

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enhance inflammatory cell function (pro-inflammatory) (Whaley and MacSween, 1992).

$PGE_2$ and $PGI_2$ are described as being strongly anti-inflammatory (pro-inflammatory in edema) with $PGI_2$ proving much more potent than $PGE_2$ in most systems (Samuelsson, 1983). They act as vasodilators, inhibitors of platelet aggregation, smooth muscle relaxants, relaxers of coronary arteries and preventors of platelet binding to arterial walls (Robbins *et al.*, 1994; Samuelsson, 1983; Zubay, 1984). They are thus opposite in biological function to $TXA_2$ which is an unstable platelet aggregating, smooth muscle contracting, serotonin releasing and vasoconstrictor substance (Gurr and Harwood, 1991; Samuelsson, 1983; Zubay, 1984). Similarly, $PGE_2$ and $PGI_2$ are antagonistic in function to $PGF_{2\alpha}$ which is a pro-inflammatory substance that causes smooth muscles to contract (Devlin, 1997).

The monohydroxyeicosatetraenoic acids (HETES) particularly 5-HETE and leukotriene $B_4$ ($LTB_4$), work synergistically to cause adhesion and chemotactic movement of leukocytes, and stimulate aggregation, lysosomal hydrolytic enzyme release, adenylate cyclase, polymorphonuclear neutrophils (PMNs) degradation and generation of superoxide in neutrophils. They thus function as pro-inflammatory substances (Devlin, 1997; Samuelsson, 1983).

Leukotrienes $LTC_4$, $LTD_4$ and $LTE_4$, collectively referred to as the peptido-leukotrienes, are humoral agents usually released from the lung tissue of asthmatic

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subjects exposed to specific allergens. They cause slowly evolving, but protracted contraction of smooth muscles in the airways and gastrointestinal tract, and enhance capillary permeability. They are thus referred to as slow-reacting substances of anaphylaxis (SRS-A). By these effects, they play a pathological role in immediate hypersensitivity reactions (Devlin, 1997; Samuelsson, 1983). They are thus pro-inflammatory in function. Generally therefore, the anti-inflammatory effects of $PGE_2$ and $PGI_2$ antagonize the pro-inflammatory functions of the peptido-LTs, $PGF_{2\alpha}$ and $TXA_2$ that cause bronchial asthma and other hypersensivity related diseases.

Products from the monooxygenase pathway have a wide range of biological activities (Capdevila *et al.*, 1983; Schlondorff *et al.*, 1986; Snyder *et al.*, 1983). The 5,6-epoxyeicosatrienoic acid (5,6-EET) is reported to be a potent vasodilator and a relaxer of arterial rings (Schwartzman *et al.*, 1987).

**Anti-inflammatory drugs**

The effectiveness of a therapeutic agent in the management of inflammatory disorders might, therefore, be based on its ability to modulate arachidonic acid metabolism in favour of the anti-inflammatory substances. It may also be based on its ability to inhibit the $PLA_2$ enzyme responsible for the mobilization of AA, or its inhibition of the prostaglandin endoperoxide synthetase enzyme that possesses the cyclooxygenase activity. In this connection it is known that the non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin (acetylsalicylic acid), ibuprofen,

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indomethacin and phenylbutazone, block prostaglandin production by inhibiting the cyclooxygenase enzyme. (Flower, 1974; Mizuno *et al* 1982; Van der Ouderaa *et al.*, 1980; Vane and Botting, 1987). Aspirin irreversibly inhibits the enzyme by acetylating the side-chain hydroxyl group of a seryl residue (Ser 530) on the enzyme (DeWitt and Smith, 1988), while the others inhibit cyclooxygenase by binding non-covalently to it (Devlin, 1997). The steroidal anti-inflammatory drugs like hydrocortisone, prednisone, and betamethasone, block prostaglandin synthesis by inhibiting phopholipase $A_2$ activity so as to interfere with the mobilization of AA (Delvin, 1997). Glucocorticoids induce the synthesis of a protein, lipocortin (macrocortin, lipomodulin), which inhibits phospholipase $A_2$ activity (Robbins *et al.*, 1994;Whaley and MacSween, 1992).

In studying the effect of a putative anti-inflammatory drug therefore, the absolute amounts of the eicosanoids produced, or the relative amounts of anti-and pro-inflammatory prostanoids, or the activity of $PLA_2$ are some of the parameters to be measured. Drugs that are effective against inflammatory disorders are expected not to decrease the amounts of anti-inflammatory eicosanoids, nor increase the levels of pro-inflammatory eicosanoids. The $PLA_2$ activity is expected to significantly decrease in response to such drugs. Ratios of the absolute amounts of anti- and pro-inflammatory eicosanoids could also be used to assess anti-inflammatory properties of drugs.

$PGI_2$ is produced in significantly high amounts in the endothelial lining of blood vessels and it antagonizes the platelet aggregating effect of $TXA_2$, which is

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produced in high amounts by the platelets themselves during inflammation (Whaley and MacSween, 1992; Zubay, 1983). The ratio $PGI_2/TXA_2$ would therefore be a useful parameter for assessing anti-inflammatory activity. The ratio $PGE_2/PGF_{2\alpha}$ is also a useful parameter because of the direct antagonistic effects of the two prostanoids involved, which are also commonly produced together in all tissue where they are found. A ratio of $PGE_2+PGI_2/PGF_{2\alpha}+TXA_2$ could also be useful.

**Anti-inflammatory Medicinal Plants**

A number of plants have been recorded to have anti-inflammatory properties (Lewington, 1990). These include the following:

***Hemamelis virginiana (Wych hazel):*** Alcohol extracts from the leaves and bark of this plant help prevent inflammation and control bleeding. Tea prepared from the leaves and bark of the plant is drunk to alleviate colds, fevers and sore throats, and also used to wash sores and wounds. Over one million gallons of *Wych hazel* are sold in the United States alone each year.

***Glycyrrhiza sp:*** Crude extracts from the dried roots and rhizomes of *Glycyrrhiza glabra* (liquorice) is used as an expectorant and anti-inflammatory drug, and is common in cough syrups, sweets and pastilles. The main ingredient is saponin-like glycosides of which glycyrrizin is the most important.

***Salix alba* and *Filipendula ulmaria:*** The parent chemical compound of Aspirin (salicylic acid), the celebrated anti-inflammatory drug that irreversibly inhibits the

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cyclooxygenase enzyme by acetylation, was extracted from the leaves and bark of the white willow, *Salix alba,* and the perennial herb meadowsweet, *Filipendula ulmaria*

***Oenothera sp:*** Oil extracted from the evening primrose, a species of *Oenothera,* is reported for its possible remedy for arthritis, migraine, asthma, eczema, high blood pressure and premenstrual tension. The medical profession now officially recognizes primrose oil as a treatment for atopic eczema.

***Tenacetum parthenium***: Extracts from the plant have been found to be specific in the treatment of migraine, an inflammatory disease resulting from vasospasm and dilation of intracranial arteries and their branches, due to intermittent release of 5-hydroxytryptamine (serotonin) and prostaglandins (Their and Smith, 1981).

***Dioscorea sp. (the yam family):*** Many plants in the species are the source of several steroids. Products of steroidal sapogenins include cortisone and hydrocortisone, very important steroidal anti-inflammatory agents that block AA mobilization in eicosanoid biosynthesis by inhibiting phospholiphase $A_2$ activity. They are thus used to treat rheumatoid arthritis, rheumatoid fever and sciatica, which are common inflammatory disorders.

***Ananas comosus (Pineapple):*** Extracts from the stems, fruits and leaves of pineapple are reported to contain bromelain, an enzyme that breaks down proteins including fibrin which is responsible for blood clots in thrombosis, an inflammatory disorder enhanced by $TXA_2$ release.

Inspite of the successes achieved in the isolation, purification and identification of active chemical constituents of plants as well as the successful chemical synthesis of quite a reasonable number of these chemicals and their derivatives, the crude extracts from most of these plants have been found to be more potent, with less or

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no quantifiable side effects, with broader spectra of performance, and less expensive (Lewington, 1990). It has often been found that while the effect of a plant may be marked when all its compounds are used together, isolation of those thought to be responsible (active principles) do not always produce cures (Lewington, 1990).

It has therefore become increasingly important that the use and dosage of medicinal plant extracts are examined and refined to incorporate them into modern treatments as well as conduct thorough studies that will scientifically verify the claims made on them to facilitate their acceptance into future medicine.

It is for this reason that the Department of Biochemistry of the University of Ghana has, over the years, been investigating the scientific basis or the mechanism of action and the toxicology of several putative medicinal plants including those used in the treatment of malaria, diabetes mellitus, asthma and other inflammatory diseases.

One such anti-inflammatory plant that has been investigated scientifically is *Desmodium adscendens* (Sw) DC. var *adscendens* locally known as *Ananse nkatee*or *Akwanfamu* or *Nkatenkate*. *D. adscendens* is a leguminous plant belonging to the family, Papilionaceae. Both herbalists and medical practitioners at the Centre for Scientific Research into Plant Medicine (CSRPM) in Ghana use aqueous decoction from the dried stem leaves of the plant to manage asthma. It is also used for treating abdominal colic, diarrhoea, and dysmenorrhoea (Ampofo,

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1977). According to Ayensu, the plant is also used to treat constipation, ringworm, convulsions, veneral sores and for dressing wounds (Ayensu, 1978).

Other species of *Desmodium* are also known for their medicinal properties. Preparations from different parts of *Desmodium gangeticum* (Linn). DC are used to treat urinary problems, diarrhoea, chronic fever, asthma, abdominal tumors, nasal polyps, febrifuge, and catarrh (Ayensu, 1978). Preparations from the roots are also used as astringents, tonics and diuretics (Ayensu, 1978). The leaves of *Desmodium triflorum* (L.) DC. are used to induce lactation, as a remedy for diarrhoea, dysentery and convulsion. The roots are used in coughs, asthma, and also applied to wounds and abscesses. These properties are usually ascribed to the alkloidal content of the various parts of the plant. (Ghosal *et al.,* 1971; Ghosal and Bhattacharya, 1972).

Several chemical constituents have been isolated from the stem-leaves of aqueous extracts of *D. adscendens*. These include tyramine, N, N-dimethyltyramine, 3,4-dimethoxy-β-phenethylamine, salsoline, N, N-dimethyltryptamine, in addition to several unidentified indole and other minor basic components (Asante-Poku et al., 1988). Also purified and identified from the extracts are the triterpenoid glycosides dehydrosoyasaponin I (DHS-I), soyasaponin I, soyasaponin III, soyasapogenol B and E (McManus *et al.,* 1993).

Chemical structures of some of the listed isolated chemical constituents of *D. adscendens* are shown in figure 5.

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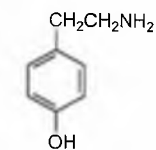
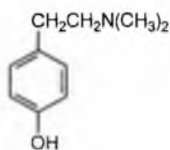
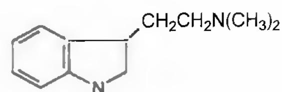
Figure 5: Some isolated chemical constituents of *Desmodium adscendens* (Asante-Poku *et al.*, 1988; McManus *et al.*, 1993)



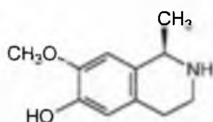

Tyramine



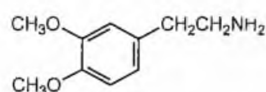

N,N-dimethyltyramine



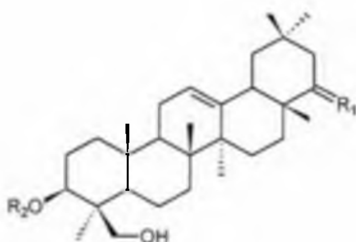

N,N-dimethyltryptamine

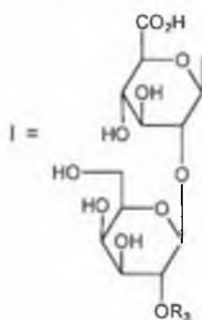


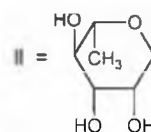

Salsoline




3,4-dimethoxy-β-phenethylamine




| Compound | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| **Soyasaponin I** | H, OH | I | II |
| **Dehydrosoyasaponin I** | O | I | II |
| **Soyasaponin III** | H, OH | I | H |
| **Soyasapogenol B** | H, OH | H | |

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Extracts from *D. adscendens* have been found to reduce anaphylactic contractions, interfere with histamine-induced and antigen-induced contractions of smooth muscles and to reduce the histamine content, as well as the amount of the amine released from lung tissue in a dose dependent fashion (Addy and Awumey, 1984; Addy and Dzandu, 1986). The extract also inhibited AA- and peptidoleukotriene-induced contractions of guinea pig tracheal spirals and lung parenchymal strips (Addy and Burka, 1988; 1989). The extracts have also been found to inhibit NADPH-dependent oxygenation of arachidonic acid (Addy and Schwartzman, 1992). As noted earlier, this third pathway of arachidonic acid metabolism produces some pro-inflammatory eicosannoids. *Desmodium adscendens* extracts were also found to activate cyclooxygenase and increase the synthesis of the anti-inflammatory prostanoid $PGE_2$ (Addy and Schwartzman, 1995). In these experiments $PGF_{2\alpha}$ also varied depending on the concentration of cyclooxygenase enzyme, presence or absence of GSH, and concentration of the *D. adscendens* extract.

Some of the experiments involving antigen-induced contractions of smooth muscle were carried out *in vivo* that is, the extracts were administered to sensitized laboratory animals from which organs or tissues containing smooth muscles were removed and challenged with antigen. The experiments on eicosanoid production, AA- and peptidoleukotriene-induced contractions were carried out *in vitro*. *In vivo* conditions may alter significantly the effective concentrations as well as the overall effect of the extracts on eicosanoid synthesis due to possible barriers to absorption and the stability of the active compounds in the gastro intestinal tract. One cannot,

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therefore easily extrapolate *in vitro* results to apply to *in vivo* conditions. However, by administering plant extracts to experimental animals and using tissues from such animals as a source of enzymes for metabolizing AA, the *in vivo* effect of the extract on the enzymes involved in eicosanoid synthesis could be evaluated.

The overall aim of this thesis therefore, was to find out if the effects of *D. adscendens* on the biosynthesis of eicosanoids from AA, which have been shown to occur *in vitro,* would occur *in vivo.* For the *in vitro* studies, the substrate, AA, was freely supplied. However, *in vivo* mobilization of AA via the activity of phospholipase $A_2$, is the rate-limiting step in the *de novo* biosynthesis of the eicosanoids. Therefore, the *in vivo* studies also included an investigation into the effect of the plant extract on $PLA_2$ activity. After establishing effects of *D. adscendens*, it was worth finding out if the effects could be used as an assay to evaluate other medicinal plants purported to have anti-inflammatory properties.

At the Center for Scientific Research into Plant Medicine, an aqueous suspension of powdered root bark of *Cassia sieberiana* (‘*Kenken*’) is used to treat abdominal colic and pains associated with the joints. Likewise, hot aqueous decoction from grated whole shoot of *Parquetina* sp (‘*Tina A*’) is used to treat asthma, and it is claimed to effect relief to patients under severe asthmatic attacks within a period of about 15 minutes (Mills-Robertson, personal communication). These plants could also be anti-inflammatory and may exert their effect by modulating eicosanoid production.

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Another aim of the work reported here was to use extracts from these two other plants, *Kenken* and *Tina A*, to verify the *in vivo* anti inflammatory assay developed using *D. adscendens*.

This thesis, therefore, contains results of experiments carried out specifically

i. to study the effect of extracts from *D. adscendens* on the *in vivo* production of eicosanoids with special reference to the relative production of pro-and anti-inflammatory eicosanoids

ii. to investigate the effect of the plant on the activity of phospholipase $A_2$

iii. to use these parameters for *D. adscendens* to assay for anti-inflammatory effects of extracts from *Cassia sieberiana ('Kenken')* and *Parkitina* sp (*'Tina A'*)

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# CHAPTER TWO

## MATERIALS AND METHODS

### MATERIALS

#### Guinea pigs

Male guinea pigs were obtained from the Noguchi Memorial Institute for Medical research (NMIMR), the Korle-Bu Teaching Hospital, the Achimota School and from the open market.

#### Medicinal Plant parts/preparations

Powdered root bark of *Cassia sieberiana* (*Kenken*), grated whole shoot of *Parquetina sp* (*Tina A*), and a preserved aqueous extract of *Desmodium adscendens,* as dispensed to patients were obtained from the Center for Scientific Research into Plant Medicine (CSRPM), Mampong-Akuapem, Ghana.

#### Chemicals and reagents

The following enzyme immunoassay kits; prostaglandin $E_2$ ($PGE_2$) (Cat # 514010), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) (Cat # 515211), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$ (Cat # 516011), peptido-leukotriene (peptido-LT) (Cat #520501), thromboxane $B_2$ ($TXB_2$) (Cat #519031) and secretory phospholipase $A_2$ ($sPLA_2$) (human synovial) (Cat # 585000), as well as secretory phospholipase $A_2$ ($sPLA_2$) activity (Cat # 765001), were obtained from Cayman Chemical Company, Ann Arbor, U.S.A.

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Arachidonic acid (5,8,11,14-eicosatetraenoic acid), reduced nicotinamide adenosine dinucleotide triphosphate (NADPH), reduced glutathione (GSH) and Bovine Serum Albumin (BSA) were obtained from Sigma Chemical Company, St. Louis, U.S.A. Folin-Ciocalteau reagent and all other chemicals of analytical or higher grade which were used, were obtained from Fluka Chemie, Switzerland.

## METHODS

### Animals and Pretreatment

Male guinea pigs weighing between 250 g and 350 g were selected. They were quarantined for, at least, two weeks in the Research Animals Unit of the NMIMR for clinical examination/observation before used. They were fed mainly on the leaves of the elephant grass (*Panicum maximum*) supplemented with pelleted animal feed. Drinking water was freely provided. Daily weight measurements were taken and plotted against time as a means of monitoring intake of food supply and detecting any possible defects resulting from handling. The guinea pigs were put in groups of five for the various treatments.

### Preparation of plant extracts

The preserved aqueous extract of *D. adscendens* prepared for the treatment of patients attending the out-patient clinic at the Centre for Scientific Research into Plant Medicine (CSRPM), was kept at 4°C and used as such without any further modification. A sample of 277.2 g of powdered *Cassia sieberiana* (*Kenken*) was

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mixed with 200ml of distilled water and stored at 4°C as stock solution. For *Parquetina* sp (*Tina A*), a sample of 9.4 g of the grated plant shoot was boiled in 150mls of distilled water for 2 minutes and strained using a muslin cloth. It was allowed to cool to room temperature and stored at 4°C. With the exception of the *D. adscendens* extract that was preserved, fresh stocks were prepared for "*Kenken*" and "*Tina A*" every five days. The quantities of plant materials used to prepare the stock solutions were estimated based on the proportions prescribed by the CSRPM for outpatients.

**Administration of plant extracts**

All three extracts were administered orally to separate groups of animals using graduated sterile syringes. Two different volumes of each of the extracts were administered to two groups of guinea pigs during the experimental period of 28 consecutive days to obtain a high and a low dose for each extract. Control groups received water in place of the extracts.

In one set of experiments three groups of five guinea pigs each, weighing between 250 g and 350 g, were used to evaluate the *in vivo* effect of *D. adscendens* extract. Animals receiving the low dose of extract were given 2 ml of the preserved extract of *D. adscendens* per day, and those receiving the high dose were given 5 ml of the same extract per day. The corresponding approximate dosages estimated for the animals are, 32.6 mg of freeze-dried extract/ kg body weight of guinea pig/day for the lower dose of 2 ml (designated as 2D), and 81.6 mg body weight of guinea

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pig/day for the higher dose of 5 ml (designated as 5D) (see **Appendix II**). The control group (designated as C) received 2 or 5 ml of water in place of the extract.

In a different set of experiments, thirty-five male guinea pigs weighing between 250 g and 350 g were separated into seven groups of five animals per group. Extracts from *D. adscendens* and two other plants, *C. sieberiana* (*Kenken*) and *Parquetina* sp. (*Tina A*) claimed to have anti-inflammatory properties, were orally administered over the experimental period of 28 consecutive days. Two groups of animals were given two different doses of each extract; a lower dose of 2 ml and a higher dose of 5 ml per day. These were respectively estimated as 32.6 mg of freeze-dried extract/kg body wt/day (2D) and 81.6 mg of freeze dried extract/kg body wt/day (5D) for *D. adscendens*; and as 111.4 mg of freeze-dried extract/kg body wt/day (designated as 2T) and 278.4 mg/kg body wt/day (designated as 5T) for '*Tina A*' extract. For '*Kenken*' however, the estimated dosages were for the fine-powdered plant material and not the freeze-dried extract: 285.3 mg of plant material/kg body wt/day (designated as 2K) and 713.3 mg of plant material/kg body wt/day (designated as 5K) respectively for the lower and higher doses. The control group, again, received water in place of the extract.

**Blood sampling and treatment**

On the 29th day, a maximum of 5 mls of blood was obtained from each animal by cardiac puncture. A sample of 100 µl of blood from each animal was quickly transferred into an eppendorf tube containing 900 µl of pre-chilled 25 mM Tris-HCl

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buffer (pH 7.5) to obtain 1:10 dilution. This sample was used for determination of concentration of secretory phospholipase $A_2$ ($sPLA_2$). The remaining blood samples in the test tubes were allowed to clot at 4°C until a separated clear serum was observed. They were then centrifuged at 1500 r.p.m for 5 minutes using a bench centrifuge (Kubota KN-70, Japan). The serum for each sample was divided into small aliquots and kept in dry sterile clean eppendorf tubes. A tube of serum for each sample was kept on ice for use on the same day for estimating $sPLA_2$ activity. The remaining tubes were kept frozen at -20°C and used within two weeks for the estimation of total protein content.

**Preparation of Microsomes**

Animals were sacrificed on day 29 by cervical dislocation. The lungs were removed and rinsed in pre-chilled 0.15 M KCl solution on ice to get rid of blood. Each tissue was transferred into, and kept in a fresh solution of 0.15 M KCl on ice before being used for preparation of microsomes.

Each tissue was finely chopped with a clean pair of scissors in a prechilled beaker kept on ice. Approximately 40 mls of pre-chilled homogenizing buffer (0.25 M sucrose-10 mM Tris-HCl, pH 7.5) were added to approximately 6 g of the chopped lung tissue, which was then homogenized using a teflon-glass homogenizer (Glas-Col® Terre Haute, U.S.A.). The homogenate was transferred into a centrifuge tube and centrifuged at 10,000 *g* for 20 minutes at 4°C using a high-speed refrigerated centrifuge (Model 20PR-52D, Hitachi Koki Co., Ltd., Japan). The supernatant fraction was centrifuged at 105,000 *g* for 60 minutes at 4°C using a preparative

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ultracentrifuge (Model 80P-7, Hitachi Koki Co., Ltd., Japan). The microsomal pellet was homogenized in 0.1 M potassium phosphate buffer (pH 7.6) using a volume of buffer, not exceeding 3 mls, for a yield from a lung tissue weighing approximately 6 g. The resuspended microsomal preparation from each lung tissue was divided into small aliquots, dispensed into 1.5 ml capacity eppendorf tubes and stored at -80°C until ready for use. Microsomes were used within a maximum of three months.

**Protein determinations**

Two different methods for protein determination were employed:

***i. Folin-Lowry method***

A volume of 5mls of alkaline copper sulphate solution was added to 1ml of test sample diluted to 1:10 with 0.1 M potassium phosphate buffer (pH 7.4). The mixture was allowed to incubate at 40°C on a water bath for 15 minutes. A volume of 0.5 ml of commercial Folin-Ciocalteau reagent (1:2 dilution) was then added to the mixture with rapid mixing. The final mixture was allowed to stand for 30 minutes at room temperature. Absorbance at 750 nm was read against a blank containing 1 ml of the buffer instead of the 1 ml 1:10 buffer-diluted sample. The protein concentrations were estimated from a standard curve of absorbances against corresponding concentrations of five serial dilutions from 1 mg/ml stock solution of Bovine Serum Albumin (BSA). Dissolution and serial dilutions of BSA were made with 0.1 M potassium phosphate buffer (pH 7.4).

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*i. Biuret method*

A volume of 3 mls of Biuret reagent was added to 2 mls of test sample diluted 1:10 (for microsomal samples) and 1:100 (for serum samples) with 0.2 M NaOH solution and thoroughly mixed. The mixture was incubated at 37°C for 10 minutes, cooled to room temperature and the absorbance at 540 nm read against a blank containing 2 mls of 0.2 M NaOH in place of the diluted sample. The protein concentrations were estimated from a standard curve prepared with five serial dilutions from a stock solution of 5 mg/ml Bovine Serum Albumin (BSA). Dissolution and dilutions of BSA were made with 0.2 M NaOH solution.

**Eicosanoid Biosynthesis**

A total volume of 1ml reaction mixture consisted of microsomes (0.92 mg/ml protein), 0.5 mM of either GSH or NADPH freshly prepared, and freshly prepared 15 μM arachidonic acid (AA) in 0.1 M potassium phosphate buffer (pH 7.4). All solutions/suspensions of the reaction mixture were maintained on ice before use. The mixture, without AA, was placed in a water bath with a shaker, maintained at 37°C, allowed to incubate for two minutes, before the addition of the substrate, 100 μl of freshly prepared 150 μM AA, to start the reaction. The reaction was terminated after 5 minutes for the cyclooxygenase reaction, in which GSH was the coenzyme, and after 15 minutes for the monooxygenase reaction with NADPH as the coenzyme. This was done by the addition of 100 μl of 1 M citric acid to bring the pH to < 3. The reaction mixture was quickly removed, kept on ice or stored in

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the refrigerator, and used within 8 hours for the determination of the type and amount of eicosanoids produced. Just before use, the pH of the mixture was adjusted upwards to pH 7.4 - 7.6 by adding 100 μl of 5M NaOH solution and mixing well by shaking. Reaction mixtures with no coenzyme were similarly treated.

**Quantitative Estimation of the Eicosanoids**

The assay is based on competition between the specific eicosanoid (X) and an X-acetylcholinesterase conjugate (X-tracer) for a limited amount of X monoclonal antibody. Because the concentration of the tracer is held constant while concentration of X varied, the amount of the tracer that was able to bind to the monoclonal antibody would be inversely proportional to the concentration of the specific eicosanoid (X) in the well. This antibody-X complex got bound to a goat anti-mouse polyclonal antibody already coated on the inner walls of the wells.

The wells of the microtitre plates were rinsed once with wash buffer. Fifty microlitres (50 μl) of the reaction mixture was pipetted into a well to which 50 μl of the tracer and 50 μl of the antibody were added. This was done in duplicates for each sample. The plates were covered with plastic film and incubated for 18 hours at 4°C or room temperature as prescribed for a particular eicosanoid kit. After the incubation period, the wells were washed five times with wash buffer to remove any unbound reagent. The buffer was removed from the wells by inverting the plate and shaking to remove the last drops. Two hundred microlitres (200 μl) of

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Ellman's reagent which contains the substrate for acetylcholinesterase (acetylcholine) were added to each well and the plate covered with plastic film. The plates were allowed to develop in the dark for 60-120 minutes depending on the type of eicosanoid kit, and the extinction read at 405-450 nm. The intensity of the yellow colour resulting from the acetylcholinesterase activity, determined spectrophotometrically, is proportional to the amount of tracer bound to the well and inversely proportional to the amount of free eicosanoid (X) in the reaction mixture. Appropriate standards were included in the determination of the various eicosanoids. Also included were various incomplete reaction mixtures containing all but cofactor, or AA or microsomes. All resuspended reagents from the ELISA kits were kept at 4°C until used. A special CAYMAN ELISA microplate reader software was used to convert the absorbance values to the corresponding concentrations in pg/ml. The various eicosanoids evaluated were prostaglandins $E_2$, $F_{2\alpha}$, 6-keto $F_{1\alpha}$ (for prostacyclin), thromboxane $B_2$ (for thromboxane $A_2$) and the peptidoleukotrienes.

**Assay for $sPLA_2$ Activity**

This assay is based on the following principles: upon hydrolysis of the thioester bond at the *sn*-2 position by $PLA_2$, free thiols react with DTNB to give a coloured product. Thus, the higher the rate of colour development, the higher the rate of release of free thiols, and the more active the $PLA_2$ catalysing the hydrolysis. This assay was used for free or secretory $PLA_2$ in serum.

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Ten microlitres (10 ul) of undiluted serum sample, 10 ul of 5,5[1]- dithiobis (2-nitrobenzoic acid) (DTNB), and 5 ul of assay buffer (25mM Tris-HCl, pH 7.5 containing 10 mM $CaCl_2$, 100 mMKCl, 0.3 mM Triton X-100, and 1 mg/ml BSA) were pipetted into wells and mixed by shaking the plate. Each sample was run in duplicate. Blank wells contained assay buffer in place of sample. Bee venom $PLA_2$ replaced sample in the positive control wells. The reactions were initiated by the addition of 200ul of reconstituted substrate (diheptanoylthio-phosphatidylcholine) solution. The contents of the wells were mixed by careful shaking. The absorbances at 405 nm were read at 10 different time intervals starting from the $2^{nd}$ to the $50^{th}$ minute after the introduction of substrate.

Straight-line curves of absorbances against time were plotted for each specific sample well. The slope $\Delta A_{405}$, that is the rate of substrate breakdown, was determined from the line of best fit for each plot including plots obtained from the blank wells. Duplicate values for the slopes determined for each sample, control and the blank were averaged and the value for the blank, subtracted from those of the samples and positive control. The $sPLA_2$ activity for each sample was then calculated from the following formula:

$$sPLA_2 \text{ Activity} = \frac{\text{Slope}}{*\epsilon} \times \frac{*V_t}{*v} \times \text{sample dilution}$$

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*€ = extinction coefficient for DTNB at 405nm (adjusted for solution path length in the well)=$10.0mM^{-1}$

*$V_t$ = total volume (ml) of mixture in well

*v = volume (ml) of sample in well

$$sPLA_2\ Activity = \frac{\Delta A_{405}/min}{10.0\ mM^{-1}} \times \frac{0.225ml}{0.01ml} \times 1$$

$$= \mu mol/min/ml$$

**Assay for Plasma $sPLA_2$ Amounts**

This immunometric assay is based on a double-antibody "sandwich" technique. Each of the wells of the microtitre plate is coated with a monoclonal antibody specific for secretory phospholipase $A_2$ ($sPLA_2$ capture antibody). Any $sPLA_2$ introduced into the well gets bound to the antibody. An acetylcholinesterase: Fab[1] conjugate (AChE: Fab[1]), which binds selectively to a different epitope on the $sPLA_2$ molecule, is introduced and it binds to the $sPLA_2$ molecule forming a "sandwich" immobilized inside the wells. Excess reagent is washed away and the concentration of the analyte determined by measuring the enzymic activity of the AChE on addition of Ellman's reagent containing the substrate for AChE. The product is coloured and can be measured spectrophotometrically. The intensity of

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the colour is directly proportional to the amount of bound conjugate, which, in turn, is proportional to the concentration of the $sPLA_2$.

Secretory phospholipase $A_2$ Enzyme Immunoassay kit was used to assay for the amount of plasma $sPLA_2$ as follows: the wells of the microtitre plates were rinsed once with reconstituted wash buffer. Hundred microlitres (100 ɥl) each of serially diluted sPLA standard solutions and blood samples diluted 1:10 with 25 mM Tris-HCl buffer (pH 7.5) were pipetted into the wells. One hundred microlitres (100 ɥl) of acetycholinesterase: $sPLA_2$ Fab[1] conjugate was added to each of the wells except the ones labelled blank. The plate was then covered with plastic film and incubated overnight (18 hrs) at 4°C. The wells contents were discarded and rinsed five times with reconstituted wash buffer. A volume of 200 ɥl of reconstituted Ellman's reagent was added to each well, the plate was covered with a plastic film and allowed to develop in the dark for at least 60 minutes. The absorbances of the yellow colour developed were read at 405-450 nm on a microplate spectrophotometer (WF043 Denley "We" Scan, England)

Secretory Phospholipase $A_2$ ($sPLA_2$) concentrations in pg/ml were estimated from a standard curve obtained by plotting absorbances against concentration of serially diluted $sPLA_2$ standards. Average absorbances for duplicate readings for each sample and standards were used.

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**Statistical Analysis**

Analysis of variance (ANOVA) was used for all statistical analyses because three or more treatment conditions were compared for each set of experiments. Statistical significance was calculated at $p < 0.05$.

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# CHAPTER THREE

# RESULTS

## A. EICOSANOID BIOSYNTHESIS

### I: Effect of *Desmodium adscendens* extract

Eicosanoids produced using microsomes prepared from lungs of guinea pigs treated with or without aqueous extracts from *Desmodium adscendens* are presented in this section.

The group of guinea pigs which did not receive any extract, the control group, is designated C, the group which received a lower dose of the extract is designated 2D and that which received a higher dose of the extract is designated 5D.

When GSH was used as a cofactor, there was an overall increase of prostanoids compared to the controls except for $PGF_{2\alpha}$. Prostaglandin $E_2$ production in the 2D group was not significantly different from that for the control; its production in the 5D group was comparatively higher than in both the C and 2D animals. The production of 6-keto-$PGF_{1\alpha}$ in the 2D was significantly higher than that in the control group. Its production in the 5D group was very high compared to both the C and 2D groups. Extract administration at both doses, did not affect $PGF_{2\alpha}$ production levels. Although production of $TXB_2$ in both 2D and 5D animals were

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significantly higher than in the control group. There was no statistically significant difference between the two values (Table A1).

## TABLE A1

## Eicosanoid production in the presence of GSH as a coenzyme

***Values are mean + SEM, n=5***

| Type and amount of prostanoid (ng/ml) | | | | |
|---|---|---|---|---|
| **Sample** | **$PGE_2$** | **6-Keto-$PGF_{1\alpha}$** | **$PGF_{2\alpha}$** | **$TXB_2$** |
| C | 2.5±0.7 | 0.03±0.004 | 1.02±0.22 | 3.20±0.48 |
| 2D | 2.7±0.4 | *0.06±0.010 | 1.30±0.39 | *6.30±2.10 |
| 5D | $*^{+}$17.0±5.0 | $*^{+}$1.9±0.020 | 1.02±0.35 | *4.80±1.10 |

* _ significantly different from control
\+ _ significantly different from corresponding 2D value
C = control : no extract administered
2D = lower extract dosage: ~32.6mg/kg body wt/day
5D = higher extract dosage: ~81.6mg/kg body wt/day

**see appendix II**

The data expressed as percentage increases over the controls are shown in figures 6 and 7. As indicated in figure 6, higher percentage increases were recorded for 6-keto-$PGF_{1\alpha}$ and $TXB_2$ compared to the increases in $PGE_2$ and $PGF_{2\alpha}$ for the 2D

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treatments. On administration of a higher dose of extract (5D) however, very high percentage increases over the controls were recorded for both $PGE_2$ and 6-keto-$PGF_{1\alpha}$ (Figure 7).

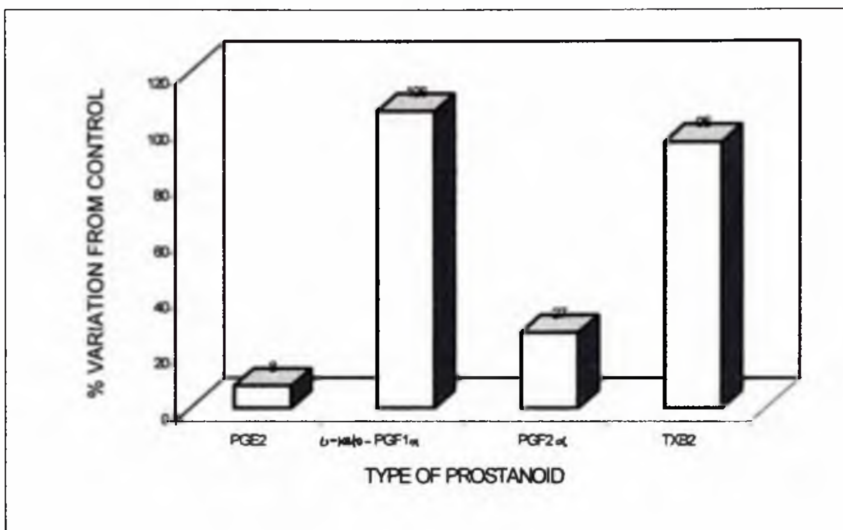


**Fig. 6: Effect of *D. adscendens* extract on prostanoid production with GSH as cofactor expressed as % change over control; dose = 2ml of preserved extract/animal/day (2D).**

2D = lower extract dosage: ~32.6mg/kg body wt/day

**see appendix II**

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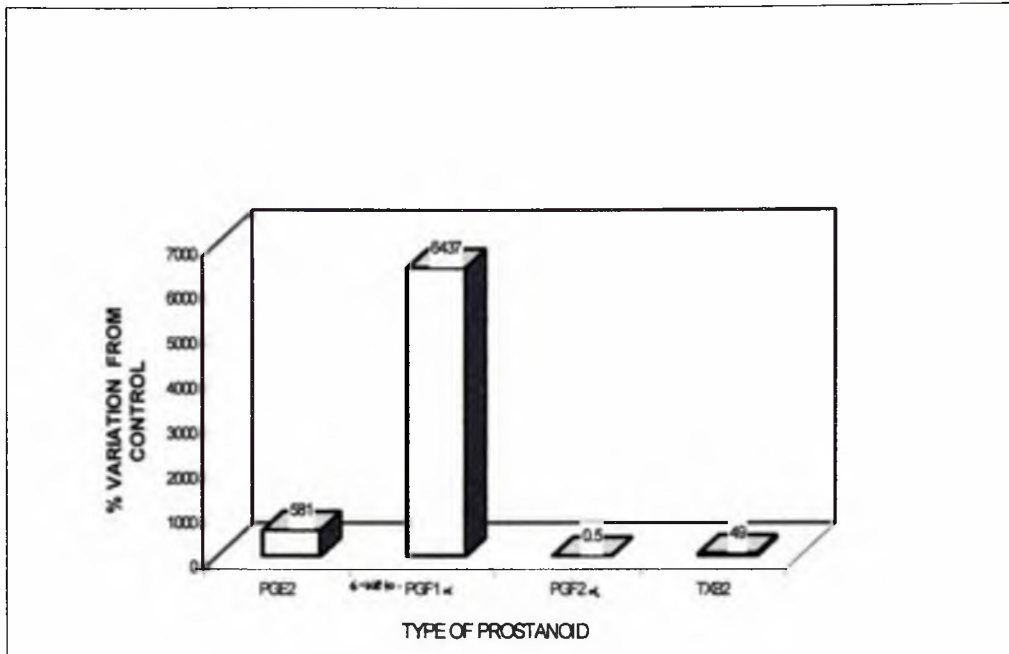


**Fig. 7: Effect of *D. adscendens* extract on prostanoid production with GSH as cofactor expressed as % change over control; dose = 5ml of preserved extract/animal/day (5D).**

5D = higher extract dosage:~ 81.6mg/kg body wt/day

**see appendix II**

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With NADPH as the coenzyme for eicosanoid production, the monooxygenase pathway was to be favoured. However, the unavailability of appropriate ELISA kit did not allow for the evaluation of products via this pathway. Instead, the effect of adding NADPH on the cyclooxygenase pathway was examined by quantifying the prostanoids produced.

The amount of $PGE_2$ produced in response to the lower dose of the extract (2D) indicated significant increase over the control, but that in response to the higher dose (5D) did not indicate a significant difference from the control. The amounts of 6-keto-$PGF_{1\alpha}$ produced with both doses were the same, and significantly higher than the control value. Significant increase in $PGF_{2\alpha}$ production over the control was recorded for the lower dose but not for the higher dose. Significant increase in $TXB_2$ over the control was recorded for the lower dose. The 5D value was not statistically different from the control value, but significantly lower than the value obtained at the lower dose (Table A2).

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# TABLE A2

## Prostanoid Production in the Presence of NADPH as a Coenzyme

*Values are + SEM, n=5*

| | Type and amount of prostanoid (ng/ml) | | | |
|---|---|---|---|---|
| **Sample** | **$PGE_2$** | **6-Keto-$PGF_{1\alpha}$** | **$PGF_{2\alpha}$** | **$TXB_2$** |
| C | 4.6±0.9 | 0.044±0.006 | 0.60±0.09 | 2.70±0.66 |
| 2D | * 7.6±1.3 | *0.058±0.009 | *0.80±0.10 | *4.90±0.86 |
| 5D | +4.8±0.7 | *0.058±0.014 | 0.66±0.14 | +2.80±0.61 |

* _ significantly different from control
+ _ significantly different from corresponding 2D value
C = control: no extract administered
2D = lower extract dosage: ~32.6mg/kg body wt/day
5D = higher extract dosage:~ 81.6mg/kg body wt/day

**see appendix II**

Results from reaction mixtures with no cofactor indicated tremendous variations in prostanoids amounts for the 2D treatments compared to their control levels. There was no significant difference in the amount of $PGF_{2\alpha}$ produced; the amount of $TXB_2$ declined significantly, while $PGE_2$ and 6-keto-$PGF_{1\alpha}$ showed significant increases at 2D. All prostanoid amounts recorded for 5D animals were very low compared to their corresponding values in both the 2D and C groups (Table A3).

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# TABLE A3

## Prostanoid production without the addition of a coenzyme

***Values are mean + SEM, n=5***

| Type and amount of prostanoid (ug/ml) | | | | |
|---|---|---|---|---|
| **Sample** | **$PGE_2$** | **6-Keto-$PGF_{1\alpha}$** | **$PGF_{2\alpha}$** | **$TXB_2$** |
| C | 9.2±1.2 | 32.0±3.4 | 0.36±0.05 | 400±70 |
| 2D | * 14.0±1.4 | *61.0±6.5 | 0.36±0.03 | *200±32 |
| 5D | *+$5.4x10^{-4}$ ±$5.3x10^{-5}$ | *+$5.8x10^{-4}$ ±$6.1x10^{-5}$ | *+$3.6x10^{-4}$ ± $4.7x10^{-5}$ | *+$1.5x10^{-2}$ ±$2.6x10^{-3}$ |

* _ significantly different from control
\+ _ significantly different from corresponding 2D value
C = control: no extract administered
2D = lower extract dosage: ~32.6mg/kg body wt/day
5D = higher extract dosage:~ 81.6mg/kg body wt/day

**see appendix II**

The ratios of anti- to pro-inflammatory prostanoids were expressed as $PGE_2/PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}/TXB_2$ to assess if the effect of the plant extract would be better expressed in this manner.

The lower dose of *D. adscendens* did not affect the $PGE_2/PGF_{2\alpha}$ ratio in order of magnitude for all three different reaction mixtures, that is, when GSH and NADPH were used as cofactors and when no cofactor was used. For the higher dose of

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extract, when GSH was employed as the cofactor, there was an increase in this ratio: an increase one order of magnitude greater than the control value (TableA4). A similar trend was observed for the $PGE_2$ production alone.

**TABLE A4**

**Ratio of anti- to pro-inflammatory prostanoids ($PGE_2/PGF_{2\alpha}$) compared to $PGE_2$ only in the presence of GSH**

| Sample | $PGE_2/PGF_{2\alpha}$ | $PGE_2$ (ng/ml) |
|---|---|---|
| C | 2.45 | 2.5 |
| 2D | 2.07 | 2.7 |
| 5D | 16.62 | 17 |

C = control: no extract administered
2D = lower extract dosage: ~32.6mg/kg body wt/day
5D = higher extract dosage: ~81.6mg/kg body wt/day
**see appendix II**

When GSH was used as a cofactor, the ratio 6-keto$PGF_{1\alpha}/TXB_2$ was not changed in response to the lower dose of the extract. However, with a higher dose, there was an increase of two orders of magnitude over the control (Table A5). The trend was the same when production of only 6-keto-$PGF_{1\alpha}$ was considered.

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## TABLE A5

**Ratio of anti- to pro-inflammatory prostanoids (6-keto-$PGF_{1\alpha}/TXB_2$) compared to 6-keto $PGF_{1\alpha}$ only in the presence of GSH**

| Sample | 6-keto $PGF_{1\alpha}/TXB_2$ | 6-keto $PGF_{1\alpha}$ (ng/ml) |
|---|---|---|
| C | $8.90 \times 10^{-3}$ | $3 \times 10^{-2}$ |
| 2D | $9.39 \times 10^{-3}$ | $6 \times 10^{-2}$ |
| 5D | $3.91 \times 10^{-1}$ | 1.9 |

C = control: no extract administered
2D = lower extract dosage: ~32.6mg/kg body wt/day
5D= higher extract dosage:~ 81.6mg/kg body wt/day

**see appendix II**

The effect of *D. adscendens* on the synthesis of peptido-leukotrienes with different cofactor is shown in table A6. The data, presented as percentage changes over the control values are shown in figure 8.

When GSH was used as a cofactor, the amount of peptido-leukotrienes synthesized for both the lower (2D) and higher (5D) doses of the extract were not significantly different from each other (i.e. no dose effect), but both were significantly higher than the control value. A higher percentage increase was observed for 2D than for 5D.

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When NADPH was used as the cofactor, the amount of leukotrienes synthesized in response to extract administration was significantly higher than the control value only at the higher dose of the extract (5D). The values, expressed as percentage changes over the control, is presented in figure 8, showing virtually no change in 2D but a high percentage increase in 5D.

When no cofactor was added, the amount of peptido-leukotrienes produced was much greater than when cofactor was added. In contrast to the reaction mixtures with cofactors, the amount of peptido-leukotrienes were reduced in response to administration of the extract when no cofactor was added. The reductions in the amount of peptido-leukotrienes produced, which were statistically significant, occurred in a dose dependent manner.

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# TABLE A6

## Effect of *D. adscendens* on peptido-leukotriene level in the presence and absence of cofactors

Values are mean ± S. E. M., n=5

| | Cofactor used (values quantified in pg/ml) | | |
|---|---|---|---|
| **Sample** | **GSH** | **NADPH** | **NO COFACTOR** |
| C | $2.53x10^2$±24.35 | 77±8.49 | $2.85x10^5$±$3.74x10^4$ |
| 2D | *$5.92x10^2$±77.85 | 82±6.13 | *$9.19x10^4$±$7.25x10^3$ |
| 5D | *$4.87x10^2$ ±87.87 | *+140±24.71 | *+$5.08x10^3$ ±$4.90x10^2$ |

* - significantly different from control
\+ _ significantly different from lower dose

C = control: no extract administered
2D = lower extract dosage: 2ml of preserved extract/animal/day ~32.6mg/kg body wt/day
5D = higher extract dosage: 5ml of preserved extract/animal/day ~ 81.6mg/kg body wt/day

**see appendix II**

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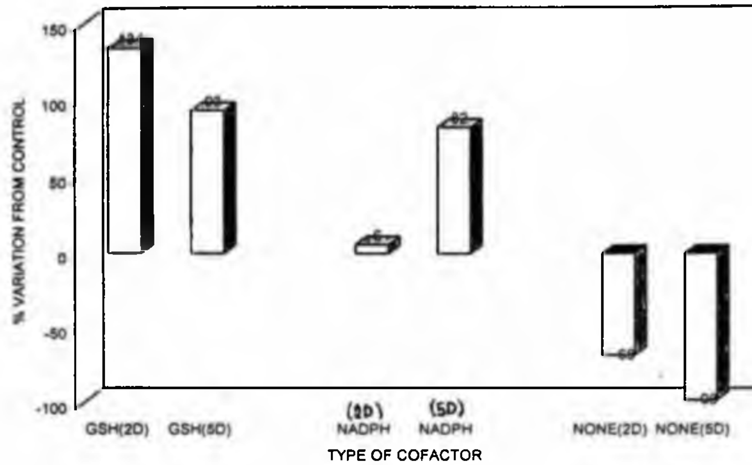


**Fig. 8: Effect of D. adscendens on peptido-leukotriene production expressed as % change over control on addition of different cofactors; doses of extract represented by 2D and 5D.**

2D = lower extract dosage: 2ml of preserved extract/animal/day ~32.6mg/kg body wt/day

5D = higher extract dosage: 5ml of preserved extract/animal/day ~ 81.6mg/kg body wt/day

**see appendix II**

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## II: Effect of other medicinal plants

*D. adscendens* was used as positive control to which *Cassia sabriena* (*kenken*) and *Parquetina* sp (*Tina A)* were compared. Extracts from the three plant materials were administered in two doses to the experimental animals daily for 28 consecutive days before the animals were sacrificed, and microsomes, as source of enzymes, prepared from their lungs. The reaction mixtures contained GSH as coenzyme.

For convenience, the different aqueous extracts from *D.adscendens*, *Tina A* and *kenken* have been respectively represented by D, T and K. The numbers 2 and 5 preceding any of the letters, represent the lower and higher dosages of the extracts respectively. The results are presented in Table A7.

At a higher dose (5D), *D. adscendens* extract caused significant increases in the amounts of $PGE_2$ and 6-keto-$PGF_{1\alpha}$ synthesized over their corresponding lower dose (2D) and control (C) values. The 2D values in turn, were higher than the control values for both eicosanoids, the increase being significant only for 6-keto-$PGF_{1\alpha}$.

Similar trends with respect to the effect of *D.adscendens* on the synthesis of $PGE_2$ and 6-keto-$PGF_{1\alpha}$ were observed for *kenken* but not for *Tina A*. In the case of *Tina A*, significant increases in $PGE_2$ and 6-keto-$PGF_{1\alpha}$ productions at both dose levels

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over their control values were observed. The amount of 6-keto-$PGF_{1\alpha}$ produced in 2T and 5T were not significantly different from each other.

With respect to the synthesis of $PGF_{2\alpha}$ and $TXB_2$, none of the two dose levels for all three extracts caused any significant change from the control value except for a significant reduction in $PGF_{2\alpha}$ value at the higher dose of *D. adscendens* (5D), and a significant increase in $TXB_2$ produced at the lower dose.

Effect of the extracts on $PGE_2$ and 6-keto-$PGF_{1\alpha}$ production as percentage changes over the controls are shown in figure 9. Generally, percentage increases for 6-keto-$PGF_{1\alpha}$ are higher compared to corresponding $PGE_2$ increases. For 6-keto-$PGF_{1\alpha}$, the % increase for the higher dose of *D. adscendens* (5D) is the most pronounced; there is no significant difference between 2T and 5T (for *Tina A*) whereas there is a significant difference for *kenken* (2K and 5K).

Ratios of anti- to pro-inflammatory prostanoids expressed as $PGE_2/PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ /$TXB_2$ for all three extracts are shown in Table A8. Extracts effect on 6-keto-$PGF_{1\alpha}$ /$TXB_2$ were more pronounced than for $PGE_2/PGF_{2\alpha}$.

The lower dose of *D. adscendens* did not seem to affect the control value of the ratio 6-keto-$PGF_{1\alpha}$ /$TXB_2$. However, the lower doses of '*Tina A*'and '*Kenken*' caused appreciably high increases. All three extracts effected very large increases in the ratio at their higher doses with *D. adscendens* indicating the highest effect

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followed by '*Tina A*'. A similar trend was observed when only the absolute values of the anti-inflammatory 6-keto-$PGF_{1\alpha}$ were considered, except that the lower dose of *D. adscendens* effected some appreciable increase in this case (Table A8).

For the ratio $PGE_2/PGF_{2\alpha}$, the higher doses of all three extracts caused increases in the range of about twice as much the control value, but the lower dose values were not any different from the control value (Table A8). The trend was the same when production of $PGE_2$ only was considered (Table A7).

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# TABLE A7

## Effect of different plant extracts on types and amounts of prostanoids produced in the presence of GSH as cofactor

***Values are means ± S. E. M, n=5***

| | Type and amount of prostanoid (pg/ml) | | | |
|---|---|---|---|---|
| **sample** | **$PGE_2$** | **6-keto-$PGF_{1\alpha}$** | $PGF_{2\alpha}$ | **$TXB_2$** |
| C | 0.17±0.01 | 2.08±0.38 | 2.89±0.51 | $9.09\times10^{3}$±0.00 |
| 2D | 0.18±0.03 | *3.01±0.51 | 3.50±0.55 | *$1.77\times10^{4}$±$7.76\times10^{3}$ |
| 5D | *$^{+}$0.28±0.05 | *$^{+}$31.49±4.24 | $^{+}$2.53±0.40 | $^{+}$$9.09\times10^{3}$±0.00 |
| 2T | *0.22±0.04 | *8.67±0.49 | 2.98±0.59 | $9.09\times10^{3}$±0.00 |
| 5T | *$^{+}$0.30±0.03 | *9.29±0.53 | 2.63±0.41 | $9.09\times10^{3}$±0.00 |
| 2K | 0.18±0.02 | *3.21±0.47 | 2.49±0.56 | $9.09\times10^{3}$±0.00 |
| 5K | *$^{+}$0.27±0.04 | *$^{+}$8.08±0.64 | 2.75±0.67 | $9.09\times10^{3}$±0.00 |

* _ significantly different from control value
\+ _ significantly different from lower dosage value
C = control: no extract administered
2D= lower extract dose for *D.adscendens:* ~32.6mg/kg body wt/day
5D= higher extract dose for *D.adscendens:* ~ 81.6mg/kg body wt/day
2T= lower extract dose for *Tina A* : ~111.4 mg/kg body wt/day
5T= higher extract dose for *Tina A*: ~278.4 mg/kg body wt/day
2K= lower extract dose for *Kenken* : ~285.3 mg/kg body wt/day
5K= higher extract dose for *Kenken* :~ 713.3 mg/kg body wt/day

**see appendix II**

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# TABLE A8

## Ratio of anti-to pro-inflammatory prostanoids for different plant extract compared to anti-inflammatory prostanoids alone

| sample | **$PGE_2/PGF_{2\alpha}$** | **6-keto-$PGF_{1\alpha}/TXB_2$** |
|---|---|---|
| C | $5.90 \times 10^{-2}$ **(0.17)** | $2.28 \times 10^{-4}$ **(2.08)** |
| 2D | $5.22 \times 10^{-2}$ **(0.18)** | $1.70 \times 10^{-4}$ **(3.01)** |
| 5D | $1.09 \times 10^{-1}$ **(0.28)** | $3.47 \times 10^{-3}$ **(31.49)** |
| 2T | $7.43 \times 10^{-2}$ **(0.22)** | $9.55 \times 10^{-4}$ **(8.67)** |
| 5T | $1.16 \times 10^{-1}$ **(0.30)** | $1.02 \times 10^{-3}$ **(9.29)** |
| 2K | $7.35 \times 10^{-2}$ **(0.18)** | $3.54 \times 10^{-4}$ **(3.21)** |
| 5K | $9.75 \times 10^{-2}$ **(0.27)** | $8.90 \times 10^{-4}$ **(8.08)** |

Figures in parentheses represent only **$PGE_2$** or **6-Keto-$PGF_{1\alpha}$** measured in pg/ml
C = control: no extract administered
2D= lower extract dose for *D. adscendens:* ~32.6mg/kg body wt/day
5D= higher extract dose for *D.adscendens*:~81.6mg/kg body wt/day
2T= lower extract dose for *Tina A* : ~111.4 mg/kg body wt/day
5T= higher extract dose for *Tina A*: ~278.4 mg/kg body wt/day
2K= lower extract dose for *Kenken* : ~285.3 mg/kg body wt/day
5K= higher extract dose for *Kenken* : ~713.3 mg/kg body wt/day

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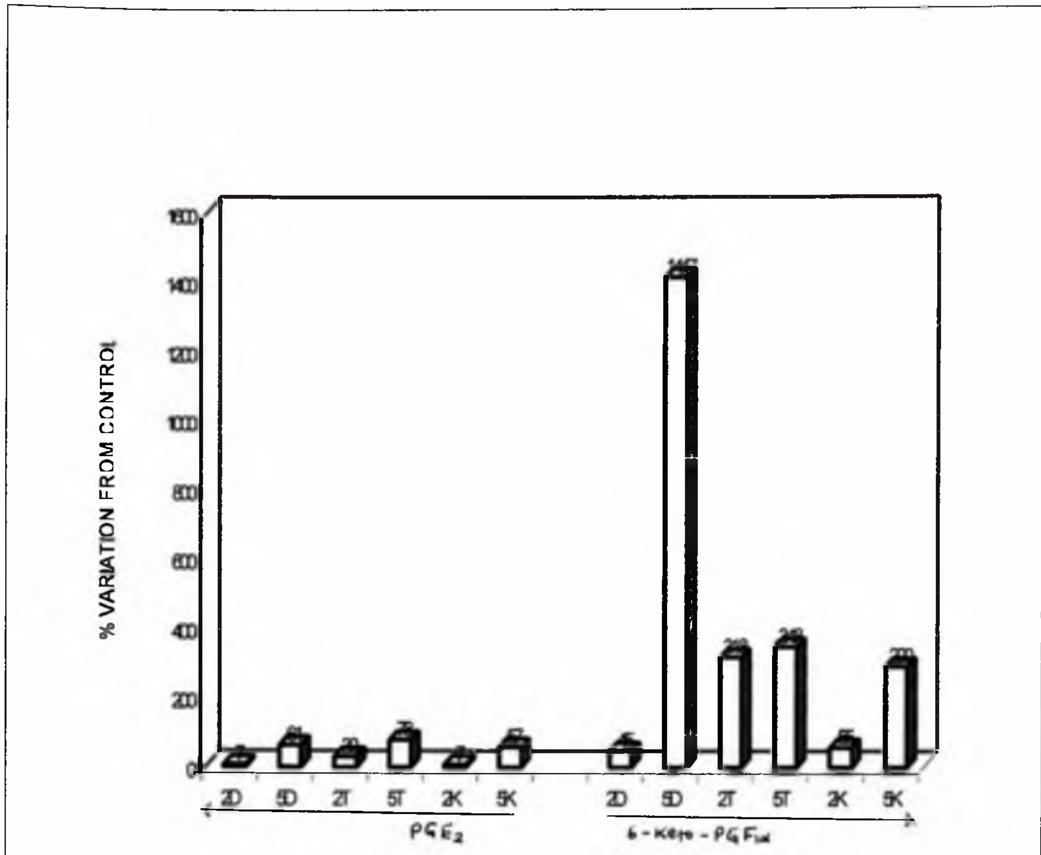


**Fig. 9: Effect of *D. adscendens* (D), *Tina A* (T) and *Kenken* (K) extracts on anti-inflammatory prostanoid production expressed as % change over control;doses represented by 2D, 5D, 2T, 5T, 2K, 5K**

2D= lower extract dose for *D.adscendens:* ~32.6mg/kg body wt/day
5D= higher extract dose for *D.adscendens*:~81.6mg/kg body wt/day
2T= lower extract dose for *Tina A* : ~111.4 mg/kg body wt/day
5T= higher extract dose for *Tina A*: ~278.4 mg/kg body wt/day
2K= lower extract dose for *Kenken* : ~285.3 mg/kg body wt/day
5K= higher extract dose for *Kenken* : ~713.3 mg/kg body wt/day

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## B: PHOSPHOLIPASE $A_2$ ACTIVATION

Groups of guinea pigs receiving aqueous extracts of either *Desmodium adscendens*, *Parquetina* sp. *(Tina A)* or *Cassia sieberiana* (*Kenken)* are designated D, T and K respectively. The numbers 2 and 5 preceding any of the letters represent the lower and higher doses of the extracts administered. C represents the control group that received no extracts.

**Enzyme activity**

Oral administration of the different plant extracts in two doses resulted in significant decreases in $sPLA_2$ activity with respect to the basal (control) level. The extent of inhibition was about the same for both the lower (2D) and higher (5D) doses of *D. adscendens* extracts. The same was true for *Kenken* which had a greater inhibitory effect. *Tina A* extracts exerted similar inhibitory effect which was dose-dependent, that is, a significant difference was observed between enzyme activity for the two doses. The extent of inhibition of $sPLA_2$ activity was greater for *Tina A* at the higher dose than for *D. adscendens* and *Kenken* (Table B1).

**$sPLA_2$ protein**

The higher dose of *D. adscendens* effected a significant increase in $sPLA_2$ protein level over the control value but the lower dose did not. None of the other extracts administered at the two dose levels caused significant change in the amount of $sPLA_2$ protein (Table B2).

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**Total serum protein**

There was no significant change in total serum proteins in response to any of the three extracts administered (Results not shown because they were of no analytical interest)

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# TABLE B1

## Effect of different plant extracts on serum secretory phospholipase $A_2$ activity.

*Values are mean + SEM n=5*

| Serum sample (enzyme source) | Activity (nmol/min/ml) | Percentage decrease in activity |
|---|---|---|
| Control | 27.02±7.62 | – |
| 2D | *16.68±4.50 | 38.3 |
| 5D | *17.36±0.95 | 35.8 |
| 2T | *16.53±2.99 | 38.8 |
| 5T | *+ 11.94±2.10 | 55.8 |
| 2K | *14.26±4.10 | 47.2 |
| 5K | *13.08+3.30 | 51.6 |

* _ significantly different from control
+ _ significantly different from lower dose market
C = control: no extract administered
2D= lower extract dose for *D. adscendens:* ~32.6 mg/kg body wt/day
5D= higher extract dose for *D. adscendens*: ~81.6 mg/kg body wt/day
2T= lower extract dose for *Tina A* : ~111.4 mg/kg body wt/day
5T= higher extract dose for *Tina A*: ~278.4 mg/kg body wt/day
2K= lower extract dose for *Kenken* : ~285.3 mg/kg body wt/day
5K= higher extract dose for *Kenken* : ~713.3 mg/kg body wt/day

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## TABLE B2

### Effect of different plant extracts on serum secretory phospholipase $A_2$ protein.

*Values are mean + SEM n=5*

| Plasma sample | Amount ofsPLA$_2$ (pg/ml) | Percentage change |
|---|---|---|
| Control | 77.86 ± 7.24 | _ |
| 2D | 74.26 ± 3.65 | -4.6 |
| 5D | *+94.28 ± 8.99 | 21.1 |
| 2T | 80.00 ± 6.75 | 2.8 |
| 5T | 80.20 ± 9.40 | 3.0 |
| 2K | 70.02 ± 14.36 | -10.1 |
| 5K | 67.14 ± 11.64 | -13. 8 |

* - significantly different from control
+ - significantly different from lower dose value
C = control: no extract administered
2D= lower extract dose for *D. adscendens:* ~32.6mg/kg body wt/day
5D= higher extract dose for *D. adscendens*: ~81.6mg/kg body wt/day
2T= lower extract dose for *Tina A* : ~111.4 mg/kg body wt/day
5T= higher extract dose for *Tina A*: ~278.4 mg/kg body wt/day
2K= lower extract dose for *Kenken* : ~285.3 mg/kg body wt/day
5K= higher extract dose for *Kenken* : ~713.3 mg/kg body wt/day

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# CHAPTER FOUR

## DISCUSSION AND CONCLUSION

The eicosanoids play very important and myriad pharmacological roles including those associated with inflammatory processes. The fact that some eicosanoids are pro-inflammatory while others are anti-inflammatory makes them a unique class of chemical compounds that are important in almost all inflammatory studies.

The anti-inflammatory effects of *Desmodium adscendens* with respect to eicosanoid biosynthesis *in vitro* had been investigated. The results indicated increased production of the anti-inflammatory prostaglandin $E_2$ ($PGE_2$) and varying effects on the levels of the pro-inflammatory prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in response to an extract of the plant ( Addy and Schwartzman, 1995). Since in its therapeutic use to manage asthma, an extract of this plant is administered orally, it was worth investigating whether the results obtained *in vitro* could also be obtained *in vivo*.

The overall aim of the study reported in this thesis therefore, was to study the effect of aqueous extract of *Desmodium adscendens in vivo*, specifically, its effects on the biosyntheses of eicosanoids, with special reference to the relative production of pro- and anti-inflammatory eicosanoids.

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To this end, an extract of *D. adscendens* was administered orally to guinea pigs, the experimental animals, for its effect *in vivo*, before tissues were removed for evaluation in *in vitro* reaction systems. The previous *in vitro* work did not include evaluation of the extract's effect on the activity of phospholipase $A_2$ ($PLA_2$), the enzyme involved in the rate-determining step in eicosanoid biosyntheses. This was not possible in *in vitro* studies where arachidonic acid (AA) is supplied exogenously. Therefore, the *in vivo* study also aimed at examining the effect of the extract on $PLA_2$ activity.

These experiments were done in an attempt to establish a meaningful bioassay that can be used to evaluate the efficacy of putative anti-inflammatory medicinal plants. Since the effects of *D. adscendens in vitro* on prostanoid biosynthesis were known, the strategy was to evaluate these effects of *D. adscendens in vivo* and then use the results obtained as a model. The parameters obtained for *D. adscendens* were therefore used as a basis/model to evaluate other putative anti-inflammatory medicinal plants such as *Cassia sieberiana* ('*Kenken*') and *Parquetina sp* ('*Tina A*'). Herbalists in Ghana use these two medicinal plants therapeutically to manage abdominal colic and asthma respectively.

The bioassay involved the effects of the plants on types of eicosanoids biosynthesized and, on the activity of $PLA_2$. Two different dose levels were selected for each plant used. They were administered orally to the guinea pigs over a period of 28 consecutive days before blood samples were drawn and/or, lung

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tissues removed for the preparation of microsomes. Blood samples were used for the determination of $PLA_2$ enzyme activity or $PLA_2$ protein concentration, and the microsomes were used for eicosanoids biosyntheses *in vitro*. A typical reaction mixture for eicosanoid biosynthesis contained a phosphate buffer, arachidonic acid (AA) as substrate, microsomes as sources of enzymes, and with or without a specific cofactor to represent the different metabolic pathways of AA metabolism.

The following eicosanoids were assayed by ELISA: - prostaglandin $E_2$ ($PGE_2$) and prostacyclin ($PGI_2$), assayed as 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$), are both anti-inflammatory eicosanoids. Other eicosanoids assayed are prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), thromboxane $A_2$ ($TXA_2$) assayed as thromboxane $B_2$ ($TXB_2$), and the peptido-leukotrienes (LTs) all of which are pro-inflammatory. The classification of the eicosanoids as either pro- or anti-inflammatory is primarily based on their reported functional roles with respect to asthma, an inflammatory disease (Watkins, 1989).

Arachidonic acid (AA) is a common substrate for three major metabolic pathways namely, cyclooxygenase, lipoxygenase and monooxygenase pathways. Any one of these pathways may be favoured at the expense of the others depending on the type of tissue and the cofactor present. The lung tissue is known to possess the full complement of enzymes required for eicosanoids biosyntheses via all three pathways (Watkins, 1989). Therefore, the only probable restriction to any of the pathways would be that of availability and type of cofactor. For example, the

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addition of GSH to the reaction mixture favoured the cyclooxygenase pathway. This pathway yields both pro- and anti-inflammatory eicosanoids, which are collectively referred to as prostanoids. The prostanoids occur in a near balance of antagonistic pairs with respect to pro- and anti-inflammatory effects.

Results of previous *in vitro* work during which microsomes were pre-incubated with plant extract before the addition of AA indicated that, *D. adscendens'* anti-inflammatory effect was not that of blocking the cyclooxygenase pathway altogether, but by actually activating the enzyme to increase the syntheses of prostaglandins, tilting the balance in favour of the anti-inflammatory $PGE_2$ (Addy and Schwartzman, 1995). In that study, the effects of the extract on other prostanoids were not evaluated. Similar results have been obtained in this *in vivo* study with respect to the effect of *D. adscendens* on the production of pro- and anti-inflammatory eicosanoids.

The amounts of the various eicosanoids produced from the control experiments varied. Therefore, assessing the anti-inflammatory properties of a putative drug by comparing the absolute values of the various eicosanoids produced in the presence and absence of the drug could be misleading. A better assessment would be obtained by expressing these values as percentage change over their respective controls. Results, expressed as percentage change with GSH as cofactor, indicated that very large amounts of anti-inflammatory prostanoids were produced in a dose-dependent manner. The percentage increases in levels of prostacyclin ($PGI_2$) were

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much bigger compared to prostaglandin $E_2$ ($PGE_2$). $PGE_2$ once formed may be converted to the pro-inflammatory $PGF_{2\alpha}$ by prostaglandin $E_2$-9-reductase catalysis (fig. 2). Therefore, $PGE_2$ may not be as good an anti-inflammatory substance as $PGI_2$. This presumption is substantiated by the report that $PGI_2$ is more potent than $PGE_2$ in most systems (Samuelsson, 1983). The large percentage increase in $PGI_2$ in response to *D. adscendens* therefore, points to the plant as an effective anti-inflammatory agent. This fact is further supported by the observation that the percentage change in the values calculated for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane $A_2$ ($TXA_2$), both of which are pro-inflammatory prostanoids, were relatively less significant (fig.7).

Ratios of anti- to pro-inflammatory prostanoids were used to assess if the effect of the plant extract would be better expressed as ratios. The ratios were expressed as $PGI_2/TXA_2$ and $PGE_2/PGF_{2\alpha}$. $PGI_2$ and $TXA_2$ are antagonistic to each other. $TXA_2$ is produced by platelets and it is a thrombogenic substance, while $PGI_2$ is produced by vascular endothelial cells and acts as an anti-thrombogenic agent. $PGE_2$ and $PGF_{2\alpha}$ are predominantly associated with muscular activity: while $PGF_{2\alpha}$ is a contractant, $PGE_2$ is a relaxant.

The ratios shown in tables A4 and A5 indicated trends similar to those produced when just the absolute amounts of the anti-inflammatory prostanoids were assessed. Therefore, it could be concluded that, the evaluation of *D. adscendens* as an anti-inflammatory medicinal plant with respect to its effect on prostanoid biosynthesis

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could be carried out by measuring individual anti-inflammatory prostanoids, without measuring the pro-inflammatory ones needed for calculating the ratios.

The results generally, indicated a dose-dependent enhancing effect on the biosyntheses of anti-inflammatory prostanoids in an assay system where the levels of the pro-inflammatory prostanoids remained unaffected (see table A1). These *in vivo* observations do not only confirm the *in vitro* modulatory effect on prostanoid production by *D. adscendens* (Addy and Schwartzman, 1995), but also indicate that the anti-inflammatory effect of *D. adscendens* is predominantly that of a more pronounced effect on prostacyclin ($PGI_2$) synthesis than on $PGE_2$ production.

By having an insignificant effect on the synthesis of $TXA_2$, and a pronounced affect on $PGI_2$, *D. adscendens* could have a good potential as a drug for use to manage hypertension. Prostaglanding $E_1$ and $PGI_2$ are reported to have been used with some success to provide relief in severe pulmonary hypertension and in primary pulmonary hypertension (Swan *et al.*, 1986). It is therefore suggested that those who dispense the extract could use it in the treatment of hypertension because of its potency to increase $PGE_2$ and $PGI_2$ production. Also, $PGI_2$ has been found to afford some protection against acute lung injury (Wasserman *et al.*, 1977). It is therefore encouraging to see $PGI_2$ as the anti-inflammatory prostanoid whose synthesis is most enhanced by the administration of D. *adscendens* extract *in vivo*. Enhancement of $PGI_2$ is likely to be the mode of action of the plant used to effectively manage asthma, an inflammatory disorder associated with the lungs.

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The conversion of arachidonic acid (AA) to the common intermediate, $PGH_2$ via the cyclooxygenase pathway is by the enzyme, prostacyclin endoperoxide synthase (PES). This enzyme possesses both the cyclooxygenase and peroxidase activities which are respectively involved in oxygenation and reduction reactions. Loss of peroxidase activity is correlated with loss of cyclooxygenase activity. Excess peroxides may initiate peroxidase inactivation. Presumably, peroxide-dependent inactivation involves a modification of the heme group required for the enzyme activity (Smith *et al.,* 1991). The non-steroidal anti-inflammatory drugs (NSAIDs) act by blocking the cyclooxygenase pathway via the inhibition of PES (Rome and Lands, 1975; Smith *et al.*, 1990). Aspirin, a NSAID, acts not only by inhibiting PES, but also by selectively blocking $TXA_2$ synthesis at lower concentration (Lewis *et al.*, 1983; Cairns *et al.*, 1985).

From the results presented here, *D. adscendens* is exhibiting an anti-inflammatory property via a mechanism different from that of aspirin. It is seen to enhance the production of prostanoids and therefore cannot be said to be inhibiting PES. Also from the results, *D. adscendens* effected selective enhancement of $PGI_2$ production instead of inhibiting $TXA_2$ production as in the case of aspirin. The selective enhancement of some prostanoids production by *D. adscendens* is probably due to some constituent chemical compounds in the extract, which selectively activate specific enzymes to catalyse the conversion of $PGH_2$ to specific prostanoids.

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Reduced nicotinamide adenosine dinucleotide triphosphate (NADPH) was to be used as a cofactor for the biosyntheses of eicosanoids via the monooxygenase pathway so that products of the monooxygenase pathway could be measured. Unfortunately, this could not be done due to limited resource constraints. However, the availability of extra ELISA kits for measuring prostanoids afforded the opportunity to examine the effect that NADPH could possibly have on the biosyntheses of the prostanoids, considering that the peroxidase activity of prostaglandin endoperoxide synthase (PES) is somewhat non-specific with respect to electron donors in converting $PGG_2$ to $PGH_2$ (Smith *et al.*, 1991). The peroxidase activity is however known to function optimally with GSH as the cofactor. It was also an opportunity to examine the influence of *D. adscendens* on the syntheses of the prostanoids in an assay system with NADPH because NADPH has been implicated as a cofactor in the conversion of $PGH_2$ and $PGE_2$, to $PGF_{2\alpha}$ in the cyclooxygenase pathway (fig. 2). Results from the prostanoids measurements indicated that the cyclooxygenase enzyme system was still active even with NADPH as cofactor. This, perhaps, confirmed the non-selectivity of the peroxidase moiety of the bifunctional enzyme with respect to electron donors. The results were however, inconsistent with no clear-cut (anti-inflammatory) effect by *D. adscendens* (table A2). Therefore, reactions with NADPH as the cofactor could not be selected as a model to evaluate the anti-inflammatory effects of the other two putative anti-inflammatory medicinal plants selected for the study.

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The lipoxygenase pathway has no known cofactor (fig.3). Therefore, the reaction mixtures without exogenous cofactor represented the lipoxygenase enzyme system. The microsomal preparations contained the full complement of enzymes of the three pathways for eicosanoid biosyntheses, as well as some residual endogenous cofactors. Therefore, in all the reaction mixtures containing microsomes and exogenous arachidonic acid, a certain amount of metabolites from all three pathways would be produced whether cofactor was added or not. An exogenous cofactor, when present, would only optimize conditions for a particular AA metabolic pathway. This may have accounted for the biosyntheses of the prostanoids and the leukotrienes as quantified for all three types of reaction mixtures with respect to cofactors.

Results for estimated prostanoids in the absence of exogenous cofactor were as inconsistent as they were for the assay system with NADPH as cofactor. This is because, although some prostanoids were formed, the conditions were not optimal for prostanoid biosynthesis in both situations, and therefore, made the results difficult to interpret.

Almost all products of the lipoxygenase pathway are pro-inflammatory with the leukotrienes being particularly important in asthmatic conditions for which *D. adscendens* is known to provide relief. The peptido-leukotrienes are known to cause slowly evolving but protracted contraction of lung muscles, and are thus referred to as slow reacting substances of anaphylaxis (SRS-A). They restrict the

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flexible rhythmic inhalation and exhalation of air with a consequent reduction of the air holding capacity of the lungs of patients under asthmatic attacks.

The lipoxygenase pathway produced high amounts of peptido-leukotrienes when no cofactor was present, and the effect of *D. adscendens* on their biosyntheses was pronounced (table A6, fig.8). Leukotrienes were also measured in reaction mixtures containing GSH or NADPH as cofactors. The yields were small and the effect of the extract was difficult to interpret. This could be because conditions were not optimal for leukotriene synthesis in the presence of exogenous cofactors. The small yields could also be attributed to AA being metabolized via the other pathways.

The anti-inflammatory effect of *D. adscendens* is seen here as that of inhibiting the biosyntheses of the peptido-leukotrienes in a dose-dependent manner. These *in vivo* observations, though not made under diseased conditions (the guinea pigs were normal and not sensitized), tend to suggest that the observed *in vivo* anti-anaphylactic effect of *D. adscendens* extract in previous studies (Addy and Awumey, 1984; Addy and Dzandu, 1986), could be due to inhibition of peptido-leukotriene biosynthesis, an interference of the lipoxygenase pathway.

The cyclooxygenase pathway with GSH as the cofactor was successfully used in demonstrating the anti-inflammatory activity of *D. adscendens*. Therefore this model was selected for assessing the anti-inflammatory properties of Cassia *sieberiana* (*'Kenken'*) and *Parquetina sp* (*'Tina A'*), which are used to manage

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abdominal colic and asthma respectively. Because of these therapeutic effects, the plants are suspected to be having anti-inflammatory properties also. In this set of experiments *D. adscendens* was added as a positive control.

The results for *D. adscendens* followed a similar pattern as in the first set of experiments although the increases in the anti-inflammatory prostanoids were not as pronounced as in the previous experiments. Similar to the earlier observations made on *D. adscendens*, evaluation of the anti-inflammatory effect based on individual prostanoids was as good as that based on ratios of anti- to pro-inflammatory prostanoids for all three plant materials (table A8). The pro-inflammatory prostanoids levels were not influenced by any of the three extracts except for a significant increase in $TXA_2$ ($TXB_2$) production at the lower dose of *D. adscendens*, which was similar to results obtained for the first set of experiments. The enhancing effects of all three extracts on the synthesis of prostacyclin ($PGI_2$) assayed as 6-keto-$PGF_{1\alpha}$, were greater than they were for $PGE_2$ with *D. adscendens* showing the greatest effect at its higher dose (5D) (table A7). By these observations, *D. adscendens* is perceived as the most effective anti-inflammatory plant of the three.

The release of arachidonic acid (AA) from membrane glycerophospholipids is the rate-determining step in the *de novo* biosyntheses of the eicosanoids. This is because all other enzymes required for the subsequent catalyses of AA metabolism through the monoo-, cycloo- and lipoxygenase pathways are present in catalytic

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excess. Most of these enzymes are not regulated except in the sense that the balance of their activities determines the patterns of eicosanoids, which are found in a given tissue. In the assay systems already considered, this vital step of AA release was not studied because exogenous AA was provided for the syntheses of the eicosanoids *in vitro* following the effects of the extracts *in vivo*. The experimental protocol involved subjecting the experimental animals to oral administration of plant extracts for 28 consecutive days during which the extract could modify the activities of the microsomal enzymes. Any such modifications would be reflected in the catalytic abilities to convert exogenous AA to products *in vitro*. Therefore, the *in vitro* assays reflected *in vivo* modifications of eicosanoids biosynthetic enzymes by the extracts.

The *de novo* biosyntheses of the eicosanoids in an intact living animal however, make use of endogenous AA made available through the hydrolytic action of phospholipase $A_2$ ($PLA_2$). The mobilization of AA from membrane glycerophospholipid stores by $PLA_2$ activity is thus a very crucial step in the *de novo* biosynthesis of the eicosanoids. An examination of the possible effects of the plant extracts on the activity of $PLA_2$ was therefore necessary for a better understanding of the modes of action of these anti-inflammatory plants. The steroidal anti-inflammatory drugs (SAIDs), commonly dispensed to patients with severe allergic disorders, are reported to act by inhibiting the activity of $PLA_2$ either directly, or indirectly by inducing the synthesis of some peptides which inactivate the enzyme (Samuelsson, 1983). The effects of the medicinal plants on

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$PLA_2$ activity were evaluated by measuring their effects on the activity of the serum enzyme, secretory phospholipase $A_2$ ($sPLA_2$).

Results for the specific $sPLA_2$ activity indicated that all three plant extracts reduced $sPLA_2$ activity with *'Tina A'* showing dose-dependence even with the small dose difference (i.e. 1: 2.5). Of the three plant materials, '*Tina A*' inhibited $sPLA_2$ activity the most followed by '*Kenken*' and then *D. adscendens* (table B1).

In a pathological state, more eicosanoids are formed from an increased release of arachidonic acid than in non pathological state. Therefore, the inhibition of $PLA_2$ activity is a good indication of therapeutic effectiveness. The claim of a high effectiveness of '*Tina A*' in bringing severe asthmatic attacks under control in less than 15 minutes, a claim made by its users, may primarily be due to its ability to inhibit $PLA_2$.

The regulation of AA release by $PLA_2$ activity is reported to be controlled both at the level of transcription and translation in some cells (Glasser *et al.*, 1990). An inhibition of $sPLA_2$ activity by extracts could be due to the extract suppressing $sPLA_2$ protein synthesis. Specific assay for $sPLA_2$ protein levels revealed that all the three extracts were not effective in influencing any significant change in $sPLA_2$ protein levels except the higher dose of *D. adscendens* where a slight but significant increase was observed (table B2). In spite of this significant increase,

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the extent of inhibition of $sPLA_2$ activity was statistically the same for the two dose levels of *D. adscendens*.

It could be inferred from these results that the inhibitory effects exhibited by all three extracts on the activity of $sPLA_2$ were not due to reductions in the levels of $sPLA_2$ protein. The reductions in activity might have resulted from either, the synthesis of a modified $sPLA_2$ protein with reduced activity, or extract induction of the synthesis of a protein/peptide that inhibits $sPLA_2$ activity, as in the case of the steroidal anti-inflammatory drugs (Samuelsson, 1983), or by direct inhibition. Since the assay for the $sPLA_2$ protein was immunological, a modified $sPLA_2$ protein would have reflected as a low $sPLA_2$ protein concentration in the test samples, because of reduced antigen-antibody binding due to a modified epitope that would not have been recognized by the monoclonal antibody. As the $sPLA_2$ protein levels were not significantly reduced by the plant extracts, induced synthesis of protein/peptide by the extracts, as an indirect means of reducing the activity of $sPLA_2$, or their direct inhibition of $sPLA_2$ is more plausible.

All three plant extracts have been found to exhibit multiplicity of effects on the events leading to the release of AA and its metabolism via the cyclooxygenase pathway; they inhibit $sPLA_2$ activity and elevate the levels of anti-inflammatory prostanoids. *Desmodium adscendens*, which inhibits NADPH-dependent cytochrome $P_{450}$ oxygenation of AA, was seen in this study to also inhibit the lipoxygenase pathway. It would be interesting to find out if the other two plants

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also block the formation of products of the mono- and lipoxygenase pathways of arachidonic acid metabolism, whether *in vitro* or *in vivo.*

The anti-asthmatic effect of *D. adscendens* is reported to be less curative but more preventive (prophylactic) (Addy and Awumey, 1984). Normal physiological function requires some basal syntheses of eicosanoids from endogenous arachidonic acid (AA). During anaphylaxis however, release of AA is increased through the activation of phospholipase $A_2$ by an immunologically stimulated increase in cytosolic $Ca^{2+}$ ion concentration (Roitt *et al.*, 1993). The excess AA is metabolized via the otherwise regulated lipoxygenase and monooxygenase pathways whose products are predominantly pro-inflammatory. Some of the products, such as the peptido-leukotrienes, acting as local hormones activate the cyclooxygenase enzyme, in a paracrine/autocrine fashion, making the otherwise optimally functioning cyclooxygenase enzyme system, hyperactive.

Asthmatic attacks are typical symptoms of such pathophysiological processes that result in lung injury or inflammation (Roitt *et al.*, 1993). The peptido-leukotrienes, products of the lipoxygenase pathway are reported to be highly potent in causing the characteristic symptomatic slowly evolving but protracted contraction of lung muscles associated with asthma. Thromboxane $A_2$, a product of the cyclooxygenase pathway is reported to be an even more effective agonist for the contraction of lung parenchyma strips than the lipoxygenase products (Piper and Samhuon, 1982).

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Prostaglandin $D_2$ ($PGD_2$) and $PGF_{2\alpha}$, metabolites from cyclooxygenase activity, are also bronchoconstrictors (Watkins, 1989).

The fact that both pro- and anti-inflammatory eicosanoids are required in physiologically balanced levels to maintain good health, a complete blockade of their production through absolute inhibition of $sPLA_2$ activity by any of the extracts administered would have implied a potential pathological side effect for its use. Therefore, the partial inhibition of $sPLA_2$ by all three extracts even at higher concentrations is an indication of their therapeutic tolerance. However, since there was an indication of a concentration effect for *'Tina A'* on $sPLA_2$ activity (see table B1), dosage levels for patients should be clinically prescribed.

Prostaglandin $GI_2$ is reported to alleviate the pathophysiological responses to asthmatic lung injury. This protective ability of $PGI_2$ has been attributed to its role as a stimulant of cyclic adenosine monophosphate (cyclic AMP) production, a pulmonary vasodilator and an inhibitor of platelet aggregation (Watkins, 1989). Prostaglandin $E_2$, is reported to inhibit the immunologic release of AA by increasing intracellular levels of cyclic AMP (Kuehl, 1974). The prophylactic attributes of *Desmodium adscendens* in the management of asthma could therefore, be due to its enhancing effect on the biosynthesis of $PGI_2$ and $PGE_2$ which, as stimulants of cAMP production, inhibit the antigenic release of AA and thus, prevent anaphylaxis.

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Therefore, the efficacy of *D. adscendens*, a prophylaxis for asthma, and an anti-anaphylactic agent, is predominantly due to its prevention of AA release by either the direct or indirect inhibition of $PLA_2$ activity, and its effect of increasing the production of $PGI_2$ and $PGE_2$.

*Parquetina sp* (*'Tina A'*) is used as an anti-asthmatic medicinal plant. It also increased $PGI_2$ biosynthesis and decreased $sPLA_2$ activity; but the extent was different from that of *D. adscendens*, whose effect was more on the $PGI_2$ biosynthesis and less on the $sPLA_2$ inhibition. Perhaps it is the combined effects of these two events: $PLA_2$ inhibition and increase in anti-inflammatory prostanoids biosyntheses that is important in alleviating pathological conditions like asthma.

Crude plant extracts contain not one, but many chemical compounds that are secondary plant metabolites. Presumably therefore, the multiple effects observed for the crude extracts used in this work might not have resulted from the action of a single chemical compound, but from two or more, with each contributing to produce the desired cumulative therapeutic effect. It is probably for this reason that in most instances, isolated individual chemical compounds from otherwise therapeutically effective crude extracts are found not to produce the desired effect (Lewington, 1990). This makes the application of crude plant extracts in the management of diseases interesting.

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In conclusion, the cyclooxygenase and lipoxygenase pathways of eicosanoids biosyntheses have proved to be good assay systems for evaluating the efficacy of *Desmodium adscendens* as an anti-inflammatory plant *in vivo*. In using *D. adscendens* as a model, the cyclooxygenase system was also useful in substantiating the anti-inflammatory claims of *Cassia sieberiana* ('*Kenken*') and *Parquetina sp* ('*Tina A*'). It is suggested that much more screening of medicinal plant extracts, using the established bioassay systems, should be done to include even those plants not known to be anti-inflammatory, to help build much more confidence in the developed bioassays as excellent means of evaluating putative anti-inflammatory drugs.

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# REFERERENCES

Abbiw, D.K. (1990) Useful Plants of Ghana. Intermediate Technology Publications and the Royal Botanic Gardens, U.K. pp 118-205

Addy, M.E. and Awumey, E.M.K. (1984) Effect of the extracts of *Desmodium adscendens* on anaphylaxis. *J. Ethnopharm.* **11**:283-292

Addy, M.E. and Burka, J.F. (1988) Effect of *Desmodium adscendens* fractions on antigen- and arachidonic acid-induced contractions of guinea pig airways. *Can. J. Physiol. Pharmacol.* **66**: 820-825

Addy, M.E. and Burka, J.F. (1989) Effect of *Desmodium adscendens* fraction F1 (DAF1) on tone and agonist-induced contractions of guinea pig airway smooth muscle.*Phytother. Res.* **3**(3): 85-90

Addy, M.E. and Dzandu, W. K. (1986) Dose-response effects of *Desmodium adscendens* aqueous extract on histamine response, content and anaphylactic reactions in guinea pig. *J. Ethnopharm.* **18**:13-20

Addy, M.E. and Schwartzman, M.L. (1992) An extract of *Desmodium adscendens* inhibits NADPH-dependent oxygenation of arachidonic acid by kidney cortical microsomes. *Phytother. Res.* **6**:245-250

Addy, M.E. and Schwartzman, M.L. (1995) An extract of *Desmodium adscendens* activates cyclooxygenase and increases prostaglandin synthesis by ram seminal vesicle microsomes. *Phytother. Res.* **9**:287-293

Ampofo, O. (1977) Traditional medicine: Some examples of its use. Plants that heal. *WHO chronicle.* **9**:287-293

University of Ghana http://ugspace.ug.edu.gh

Asante-Poku, S., Sakakibara, J. and Addy, M.E. (1988) Alkaloids of *Desmodium adscendens*. *Ghana Med. J.* **22**(4): 111-115

Ayensu, E.S. (1978) Medicinal Plants of West Africa. Reference Publications, Inc., Michigan. U.S.A. pp 149-152

Bergstrom, S., Ryhage, R., Samuelsson, B. and Sjovall, J. (1963) Prostaglandins and related factors : the structures of prostaglandins $E_1 F_{1\alpha}$, and $F_{1\beta}$. *J. Biol. Chem.* **238**: 3555-3563

Bettazoli, L., Zirrolli, J.A., Reidhead, C.T., Shahgholi, M. and Murphy, R.C. (1990) Incorporation of arachidonic acid into glycerophospholipids of a murine bone marrow-derived mast cell. *Adv. Prostaglandin Thromboxane Res.* **20**: 71-78

Cairns, J. A., Gent, M., Singer, J., Finnie, K.J., Froggatt, G. M., Holder, D. A., Jablonsky, G., Kostuk, W. J., Melendez, L. J., Myers, J. G., Sackett, D. L., Sealey, G. J. and Tanser, P. H. (1985) Aspirin, sulfinpyrazone or both in unstable angina. New Eng. J. Med. 313: 1369-137

Capdevila, J., Chacos, N., Falck, J.R., Manna, S., Negro-Vilar, A. and Ojeda, S.R.. (1983) Noval hypothalamic arachidonate products stimulate somatostatin release from the median eminence. *Endocrinology*. **113**: 421

Capdevila, J.H., Karara, A., Waxman, D.J., Martin, M.V., Falck, J.R. and Guenguerich, F.P. (1990) Cytochrome P-450 enzyme-specific control of the regio- and enantiofacial selectivity of the microsomal arachidonic acid epoxygenase. *J. Biol. Chem.* **265**: 10865-10871

University of Ghana http://ugspace.ug.edu.gh

Chacos, N., Capdevila, J.H., Falck, J.R., Manna, S., Martin-Wixtrom, C., Gill, S., Hammock, J. and Estabrook, R. (1983) The reaction of arachidonic acid epoxides (epoxy-eicosatrienoic acids) with a cytosolic epoxide hydrolase. *Arch. Biochem. Biophys.* **223**: 639-648

Channon, J.Y. and Leslie, C.C. (1990) A calcium-dependent mechanism for associating a soluble arachidonoyl-hydrolyzing phospholipase $A_2$ with membrane in the macrophage cell line RAW 264.7. *J. Biol. Chem.* **265**: 5409-5413

Chris-Hazelhof, E. and Nugteren, D.H. (1982) Isolation of PGH-PGD isomerase from rat spleen. *Meth. Enzym.* **86:** 77-84

Dennis, E.A. (1987) Regulation of eicosanoid production: role of phospholipases and inhibitors. *Biotechnology* 5: 1294-1300

Devlin, M.T. (1997) Textbook of Biochemistry with clinical correlations (4th edition). John Wiley and Sons, Inc., Publication, N.Y. pp 431-441

DeWitt, D.L., Day, J.S., Sonnenburg, W.K. and Smith, W.L. (1983) Concentrations of PGH synthase and $PGI_2$ synthase in the endothelium and smooth muscle of bovine aorta. *J. Clin. Invest.* **72**: 1882-1888

DeWitt, D.L., Rollins, T.E., Day, J.S., Gauger, J.A. and Smith,W.L. (1981) Orientation of the active site and antigenic determinants of prostaglandin endoperoxide (PGH) synthase in the endoplasmic reticulum. *J. Biol. Chem.* **256**: 10375-10382

University of Ghana http://ugspace.ug.edu.gh

DeWitt, D.L. and Smith, W.L. (1988) Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc. Natn. Acad. Sci.* U.S.A. **85**: 1412-1416

Dunn, M.J., Liard, J.F. and Dray, F. (1978) Basal and stimulated rates of renal secretion and excretion of prostaglandins $E_2$, $F_\alpha$, and 13,14-dihydro-15-keto-$F_\alpha$ in dog. *Kidney Int.* **13**: 136-143

Fitzpatrick, F.A. and Murphy, R.C. (1989) Cytochrome P-450 metabolism of arachidonic acid : formation and biological actions of 'epoxygenase'-derived eicosanoids. *Pharmac. Rev.* **40**: 229-241

Flower, R.J. (1974) Drugs which inhibit prostaglandin biosynthesis. *Pharmac. Rev.* **26**: 33-67

Ghosal, S., Banerje, S.K., Bhattacharya, S.K. and Sanyal, A.K. (1971) Chemical and pharmacological evaluation of *Desmodium pulchellum. Planta Medica* **21**: 398

Ghosal, S. and Bhattacharya, S.K. (1972) Desmodium alkaloids II : Chemical and pharmacological evaluation of *Desmodium gangeticum. Planta Medica* **22** : 434

Glaser, K. G., Asmis, R. and Dennis, E. A. (1990) Bacterial lipopolyssaccharide priming of $P388D_1$ macrophage-like cells for enhanced arachidonic acid metabolism: platelet-activating factor receptor activation and regulation of phospholipase $A_2$. *J. Biol. Chem.* **265**: 8658-8664.

Gurr, M.I. and Harwood, J.L. (1991) Lipid Biochemistry An introduction (Fourth edition). Chapman and Hall, London. pp 99-117

University of Ghana http://ugspace.ug.edu.gh

Hamberg, M. and Samuelsson, B. (1973) Detection and isolation of an endoperoxide intermediate in prostaglandin biosynthesis. *Proc. Natn. Acad. Sci.* U.S.A. **70**: 899-903

Haurand, M. and Ullrich, V. (1985) Isolation and characterization of thromboxane synthase from human platelets as a P-450 enzyme. *J. Biol. Chem.* **260**: 15059-15067

Huber, M., Keppler, D. and Falk, P (1993) Metabolic Pathways of the Eicosanoids, Scheme A 13 (2nd Edition), Falk Foundation, e.V. Leinenweberstra ße 5, D-79108 Freiburg i.Br., Germany.

Komen, J. (1991) Screening plants for new drugs: In Biotechnology and Development Monitor. Publish Electronic Publishing, Amsterdam. pp 4-6

Kramer, R. M., Checani, G.C., Keykin, A., Pritzker, C.R. and Deykin, D. (1986) Solubilization and properties of $Ca^{2+}$- dependent human platelet phospholipase $A_2$. *Biochim. biophys. Acta* **878**:10 394-403

Krogsgaard-Larsen, P., Christensen, S.B. and Kofod, H. (editors) (1984) Natural products and drug development. Munksgaard. Copenhagen.

Kuehl, F. A. (1974) Prostaglandins, cyclic nucleotides and cell function. *Prostaglandins*. **5**: 325-340

Lagunoff (1994) Inflammation. In: Encyclopedia of Science and Technology (8th edition) McGraw Hill Inc. New York

University of Ghana http://ugspace.ug.edu.gh


Laniado-Schwartzman, M., Davis, K.L., McGiff, J.C., Levere, R.D. and Abraham, N.G. (1988) Purification and characterization of cytochrome P-450-dependent arachidonic acid epoxygenase from human liver. *J. Biol. Chem.* **263:** 2536-2542

Leslie, C.C., Voelker, D.R., Channon, J.Y., Wall, M.M. and Zelarney, P.T. (1988) Properties and purification of an arachidonoyl-hydrolyzing phospholipase $A_2$ from a macrophage cell line, RAW 264.7. *Biochim. biopys. Acta* **963**: 476-492

Lewington, A. (1990) Plants for people. The Natural History Museum, Cromwell Road. London. pp 135-167

Lewis, H. D., Jr, Davis, J. W., Archibald, D. G., Steinke, W. E., Smitherman,T. C., Doherty, J. E., Schnaper, H.W., LeWinter, M. M., Linare, E., Pouget, J. M., Sabharwal, S. C., Chesler, E. and DeMots, H. (1983) Positive effects of aspirin against acute myocardial infarction and death in men with unstable angina: results of a Vetrans Administration cooperative study. *New Eng. J. Med.* **309**: 396-403

Lister, M.D., Deems, R.A., Watanabe, Y. Ulevitch, R.J. and Dennis, E.A. (1988) Kinetic analysis of the $Ca^{2+}$-dependent, membrane-bound macrophage phospolipase $A_2$ and the effects of arachidonic acid. *J. Biol. Chem.* **263**: 7506-7513.

University of Ghana http://ugspace.ug.edu.gh

Lister, M.D., Glaser, K. B., Ulevitch. R. J. and Dennis, E. A. (1989) Inhibition studies on the membrane-associated phospholipase $A_2$ *in vitro* and prostaglandin $E_2$ production *in vivo* of the macrophage-like P388D$_1$ cell: effect of manalide,7,7-dimethyl-5,8-eicosadienoic acid, and P-bromophenacyl bromide. *J. Biol. Chem.* **264**: 7506-7513.

MacDonald, J. and Maxey, K.M.D. (1998) A short phospholipase review. In: Cayman Currents (issue 8). Cayman chemical, U.S.A. pp 1-4

McManus, O.B., Harris, G.H., Giangiancomo, K.M., Feigenbaum, P., Reuben, J.P., Addy, M.E., Burka, J.F., Kaczorowski, G.J. and Garcia. M.L. (1993) An activator of calcium-dependent potassium channels isolated from a medicinal herb. *Biochemistry.* **32**(24): 6128-6133

Mizuno, K., Yamamoto, S. and Lands, W.E.M. (1982) Effects of non-steroidal anti-inflamotory drugs on fatty acid cyclooxygenase and prostaglandin hydroperoxidase activities. *Prostaglandins* **23**: 743-757

Moonen, P., Buytenhek, M. and Nugteren, D.H.(1982) Purification of PGH-PGE isomerase from sheep vesicular glands. *Meth. Enzym.* **86**: 84-91

Murayama, T., Kajiyama, Y. and Nomura, Y. (1990) Histamine-stimulated and GTP-binding proteins-mediated phospholipase $A_2$ activation in rabbit platelets. *J. Biol. Chem.* **265**: 4290-4295

Negishi, M., Ito, S. and Hayaishi, O. (1989) Prostaglandin E receptors in bovine adrenal medulla are coupled to adenylate cyclase via $G_i$ and to phosphoinositide metabolism in a pertussis toxin-insensitive manner. *J. Biol. Chem.* **264**: 3916-3923

University of Ghana http://ugspace.ug.edu.gh

Nugteren, D.H. and Hazelhof. E. (1973) Isolation and properties of intermediates in prostaglandin biosynthesis. *Biochim. Biophys. Acta* **326** : 448-461

Nusing, R., Schneider-Voss, S. and Ullrich, V. (1990) Immunoaffinity purification of human thromboxane synthase. *Archs Biochem. Biophys.* **280**: 325-330

Ogino, N. Miyamoto, T., Yamamoto, S. and Hayaishi, O. (1977) Prostaglandin endo peroxide E isomerase from bovine vesicular gland microsomes, a glutathione-requiring enzyme. *J. Biol. Chem.* **252**: 890- 895

Piper, P.J., and Samhoun, M.N. (1982) Stimulation of arachidonic acid metabolism and generation of thromboxane$A_2$ by leukotrienes $B_4$, $C_4$ and $D_4$ in guinea pig lung ii vitro. *Br. J. Pharmacol.* **77**: 267-275.

Robbins, S.L., Cotran, S.R. and Kumar, V. (1994) Robbins Pathologic Basis of Disease (5th edition). W.B. Saunders Company, U.S.A. pp 51-91

Roitt, I. M., Brostoff, J. and Male, D. K. (1993) Immunology (3rd edition) Mosby-Year Book Europe Limited. pp 19.1-19.5

Rollins T.E.and Smith, W.L. (1980) Subcellular localization of the prostaglandin-forming cyclooxygenase in Swiss mouse 3T3 cells by electron microscopic imuno-cytochemistry. *J. Biol. Chem.* **255**: 4872-4876

Rome, L. H. and Lands, W. E. M. (1975) Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory drugs. *Proc. Natn. Acad. Sci.* U.S.A. 72: 4863-4865

Ross, M.I., Deems, R.A., Jesaitis, A.J., Dennis, E. A, and Ulevitch, R.J. (1985) Phospholipase activities of the P388$D_1$ macrophage-like cell line. *Archs Biochem. Biophys.* **238**: 247-258

University of Ghana http://ugspace.ug.edu.gh

Salmon, J.A. and Flower, R.J. (1982) Preparation and assay of prostacylin synthase. *Meth. Enzym.***86**: 91-99

Samuelsson, B., (1983) Leukotrienes: Mediators of immediate hypersensitivity reactions and inflammation. *Science* **220**: 558-575

Samuelsson, B., Dahlen, S.E., Lindgren, J.A., Rouzer, C.A. and Serhan C.H. (1987) Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science Wash D.C.* **237**:1171-1176

Schibouta, Y., Terashita, Z., Inada, Y., Nishikawa, K., and Kikuchi S. (1981). Enhanced thromboxene $A_2$ biosynthesis in the kidney of spontaneously hypertensive rats during the development of hypertension *Eur. J Pharmacol.* **70**: 247-256

Schlondorff, D. Petty, E. Oates, J. and Levine, S.D. (1986) Inhibition of vasopressin (ADH)-stimulated water flow and adenylate cyclase in toad bladder by dihydroxyeicosatriencoic acids (DHETs), the spontaneous hydrolysis products of epoxyeicosatrienoic acids (EETs). *Clin. Res.* **34**: 700A

Schwartzman, M., Carroll, M.A., Capdevila, J., Falck, J.R. and McGiff, J.C. (1987) Vasoactivity of arachidonic acid epoxides - short communication. *Eur. J. Pharmacol.* **138**: 281-283

Smith, W.L. (1986) Prostaglandin synthesis and its compartmentation in vascular smooth muscle and endothelial cells. *Ann. Rev. Physiol* **48:** 251-262

Smith, W.L. (1987) Localization of enzymes responsible for prostaglandin

University of Ghana http://ugspace.ug.edu.gh

formation. In: Handbook of Eicosanoids : Prostaglandins and Related Lipids ( vol.1A). Willis, A. L. (ed.) CRC Press Boca Raton pp 175-184

Smith, W.L. (1989) The eicosanoids and their biochemical mechanisms of action. *Biochem. J.* **259**: 315-324

Smith, W.L., DeWitt, D.L, and Allen, M.L. (1983) Bimodal distribution of the PG $I_2$ synthase antigen in smooth muscle cells. *J. Biol. Chem.* **258**: 4922-4926

Smith, W. L., DeWitt, D. L., Shimokawa, T., Kraemer, S. A. and Meade, E. A. (1990) Molecular basis for the inhibition of prostanoid biosynthesis by nonsteroidal anti-inflammatory agents. *Stroke*, in press

Smith, W.L., Lawrence, J., Marnett and DeWitt, D.L. (1991) Prostaglandin and thromboxane biosynthesis. *Pharmac. Ther.* **49**: 153-179

Snyder, G., Capdevila, J., Chacos, N., Menna, S. and Falck, J.R.(1983) Action of leutenizing hormone-releasing hormone : Involvement of novel arachidonic acid metabolites. *Proc. Natl. Acad. Sci.* U.S.A. **80** : 3504

Sonnenburg, W.K. and Smith, W.L. (1988) Regulation of cyclic AMP metabolism in rabbit cortical collecting tubule cells by prostaglandins. *J. Biol. Chem.* **236**: 6155-6160

Swan, P. K., TibballLs, J., and Duncan, A. W. (1986) Prostaglandin $E_1$ in pulmonary hypertension. *Crit. Care Med.* **14**: 72-73

Tanaka, Y., Ward, S.L. and Smith, W.L. (1987) Immunochemical and kinetic evidence for two different prostaglandin H-prostaglandin E isomerases in sheep vesicular gland microsomes. *J. Biol. Chem.* **262**: 1374-1381

University of Ghana http://ugspace.ug.edu.gh

Taylor, G.W. and Ritter, M. (1986) Eicosanoids. In: Trends in Pharmacological Siences. Cambridge Life Sciences plc., U.K.

Their, S.O. and Smith, L.H. (1981) PATHOPHYSIOLOGY: The Biological Principle of Diseases (vol. 1). W.B. Saunders Company, U.S.A pp 1450-1452

Ujihara, M., Tsuchida, S., Satoh, K. and Urade, Y. (1988) Biochemical and immunological demonstration of prostaglandin $D_2$, $E_2$ and $F_{2\alpha}$ formation from prostaglandin $H_2$ by various rat glutathione-S-transferase isozymes. *Archs. Biochem. Biophys.* **264**: 428-437

Ulevitch, R.J., Watanabe, Y., Sano, M., Lister, M.D., Deems, R.A. and Dennis, E.A, (1988) Solubilization, purification, and characterization of a membrane-bound phospholipase $A_2$ from the $P388D_1$ macrophage-like cell line. *J. Biol. Chem.* **263**: 3079-3085

Urade, Y., Fujimoto, N. and Hayaishi, O. (1985) Purification and characterization of rat brain prostaglandin D synthetase. *J. Biol. Chem.* **260**: 12410-12415

Urade, Y., Fujimoto, N., Ujihara, M. and Hayaishi, O. (1987) Biochemical and immunological characterization of rat spleen prostaglandin D synthetase. *J. Biol. Chem.* **262**: 3820-3825

Van der Ouderaa, F.J., Buytenhek, M., Nugteren, D.H. and van Dorp, D.A. (1980) Acetylation of prostaglandin endoperoxide synthetase with acetylsalicylic acid. *Eur. J. Biochem.* **109**: 1-8

Vane, J.R. and Botting, R. (1987) Inflammation and the mechanism of action of anti-inflammatory drugs. *FASEB J.* **1**: 89-96

University of Ghana http://ugspace.ug.edu.gh

Wasserman, M. A. DuCharme, D. W., Griffin, R. L., DeGraaf, G. L., and Robinson, F.G. (1977) Bronchopulmonary and cardiovascular effects of prostaglandin $D_2$ in the dog. *Prostaglandins*. **13**: 255-270

Watanabe, K., Yoshida, R., Shimizu, T. and Hayaishi, O. (1985) Enzymatic formation of prostaglandin $F_{2\alpha}$ from prostaglandin $H_2$ and $D_2$ : purification and properties of prostaglandin F synthetase from bovine lung. *J. Biol. Chem*. **260**: 7035-7041

Watkins, W. D. (1989) Prostaglandins in Clinical Practice, Raven Press Ltd., New York. pp 45-58

Whaley, K. and MacSween, R.N.M. (1992) Muir's Textbook of Pathology ($13^{th}$ edition). Arnold, Hodder Headline Group, London. pp 15,112-149

White, R.E., and Coon, M.J. (1980) Oxygen activation by cytochrome P-450. *Ann. Rev. Biochem*. **49**: 315-356

Zubay, G.L. (1983) Biochemistry. Addison-Wesley Publishing Company, Inc., N.Y., U.S.A. pp 535-543

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# APPENDIX I

## PREPARATION OF SOLUTIONS AND REAGENTS

Ultra pure water was used for the preparation of all solutions and dilutions.

### 0.1 M Potassium phosphate buffer pH 7.4-7.6

95 mls of 0.1M $KH_2PO_4$ (m.wt. 136.09) were added to 405 mls of 0.1M $K_2HPO_4$ (m.wt. 174.18) and the volume topped up to 1L (pH 7.4). The pH was checked at 950 ml and adjusted to the appropriate pH (7.4-7.6) with drops of concentrated NaOH or HCl before the volume was made to the final mark of 1L.

### Homogenizing buffer

85.575 g of D(+)-sucrose (m.wt. 342.3) and 1.2114 g of Tris (m.wt. 121.14) were dissolved in 700 ml of ultra pure water. The pH was adjusted to 7.4-7.6 with 1 M HCl and the volume made up to1L (0.25 M sucrose-10 mM Tris-HCl)

### Plasma diluting buffer

3.0285 g of Tris (m.wt. 121.14 g) was dissolved in 940 mls of ultra pure water. The pH was adjusted to 7.5 with drops of concentrated HCl and the volume then topped up to 1L (25 Mm Tris-HCl pH 7.5)

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**Reduced glutathione (GSH)**

Required concentration in reaction mixture (1000 ɥl) was 5 Mm. 100 ɥl of GSH stock solution of concentration $m_l$ was to be included, hence,

$$m_l = \frac{5 \times 1000}{100}$$

$m_l$ = 50 mM

0.1537 g of GSH (m.wt. 307.3) was dissolved in 10 mls of 0.1M potassium phosphate buffer (pH 7.4) to give the 50mM stock solution of GSH.

**Reduced nicotinamide adenosine dinucleotide triphosphate (NADPH)**

As for GSH, 0.4167 g of NADPH (m.wt. 833.4) was dissolved in 10 mls of the phosphate buffer to give 50 mM of NADPH stock solution. 100 ɥl of this stock was included in the reaction mixture (1000 ɥl) to give a final concentration of 5 mM NADPH.

**Arachidonic acid (AA)**

Required concentration in reaction mixture was 15 ɥM, and 100 ɥl was to be included,hence

$$m_l = \frac{15 \times 1000}{100}$$

$m_l$ = 150 ɥM

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0.4898mg of AA (m.wt. 326.5) was dissolved in 10mls of 0.1M potassium phosphate buffer to give 150 µM of AA stock solution.

**Alkaline copper reagent**

i. Alkaline sodium carbonate _ 2% w/v in 0.1M NaOH

ii. Hydrated copper sulphate _ 0.5% w/v in 1% Na,K tartrate

"Alkaline reagent" was prepared fresh by mixing 50 ml of (i) and 1ml of (ii) (Plummer, 1987)*

**Biuret reagent**

3 g of hydrated copper sulphate ($CuSO_4.5H_2O$) and 9 g of sodium potassium tartrate were dissolved in 500 ml of 0.2M NaOH. 5 g of potassium iodide was added and the volume made up to 1 litre with 0.2M NaOH (Plummer, 1987)*.

**Plummer, D.T. (1987) An Introduction to Practical Biochemistry (3rd edition). McGraw-Hill* Book Company (U.K) LTD., England. pp 95-96, 159-160

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# APPENDIX II

## SAMPLE ESTIMATION OF EXTRACTS CONCENTRATIONS ADMINISTERED TO GUINEA PIGS

Aqueous extract of *Desmodium adscendens* as prepared for patients by staff at the Centre for Scientific Research into Plant Medicine (CSRPM), Mampong-Akuapem, Ghana, was administered to the guinea pigs, the experimental animals, without any modification.

Prescribed dosage for adult patients = 3 tablespoonfuls/day (~ 45 mls/day)

Average weight of an adult human being = 70 kg

Average weight of experimental guinea pigs = 300 g

Therefore, volume of extract/guinea pig/day = 0.3/70 x 45 ml
= 0.193 mls

Considering higher metabolic rate in guinea pig: rate of metabolic process is inversely proportional to body weight (Paget, 1970)*.

Rate $\alpha$ 1/body weight

An arbitrary corrective factor of **10** was chosen

Therefore, the corrected volume to be administered per guinea pig per day
= 0.193mls x **10**
~ 2mls

Estimated concentration of aqueous extract of *D. adscendens* by freeze-drying
= 4.893 mg/ml

Therefore, quantity of freeze-dried material in the 2ml of extract administered to a guinea pig weighing 300 g (0.3 kg)
= 4.893 mg/ml x 2 ml
= 9.786 mg

Hence, the dosage at 2ml of extract = 9.786 mg x 1/0.3 kg
= 32.62mg/kg body weight/day

Maximum volume of extract that can be administered to an adult guinea pig at a time is 5ml (Wagner and Manning, 1976)*

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Therefore, higher dose of extract/animal/day (5D)

= 4.893 mg/ml x 5ml/0.3 kg
=81.6 mg/kg body wt/day

**Paget, G.E (1970) Methods in toxicology. Blackwell scientific publications, Oxford, U.K. pp 90-95*

**Wagner, J. E. and Manning, P. J. (1976) The biology of the guinea pig. Academic press inc., New York. pp 13-15*

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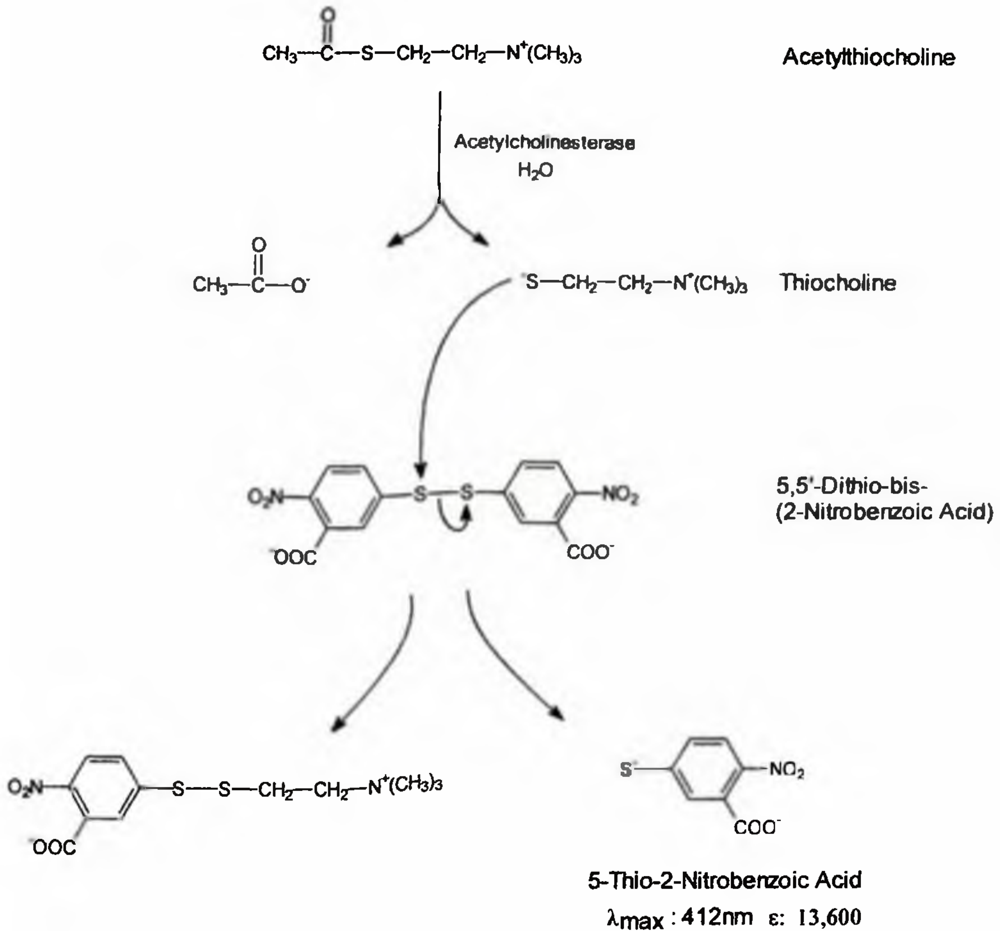
**Appendix III**

**Reaction catalysed by Acetylcholinesterase**
(Cayman Chemical, U.S.A., 1998)




5-Thio-2-Nitrobenzoic Acid
$\lambda max$ : 412nm ε: 13,600

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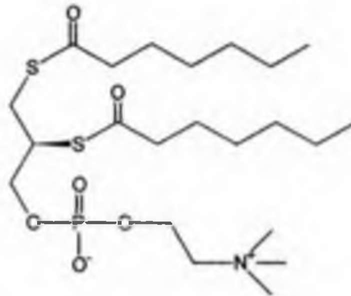
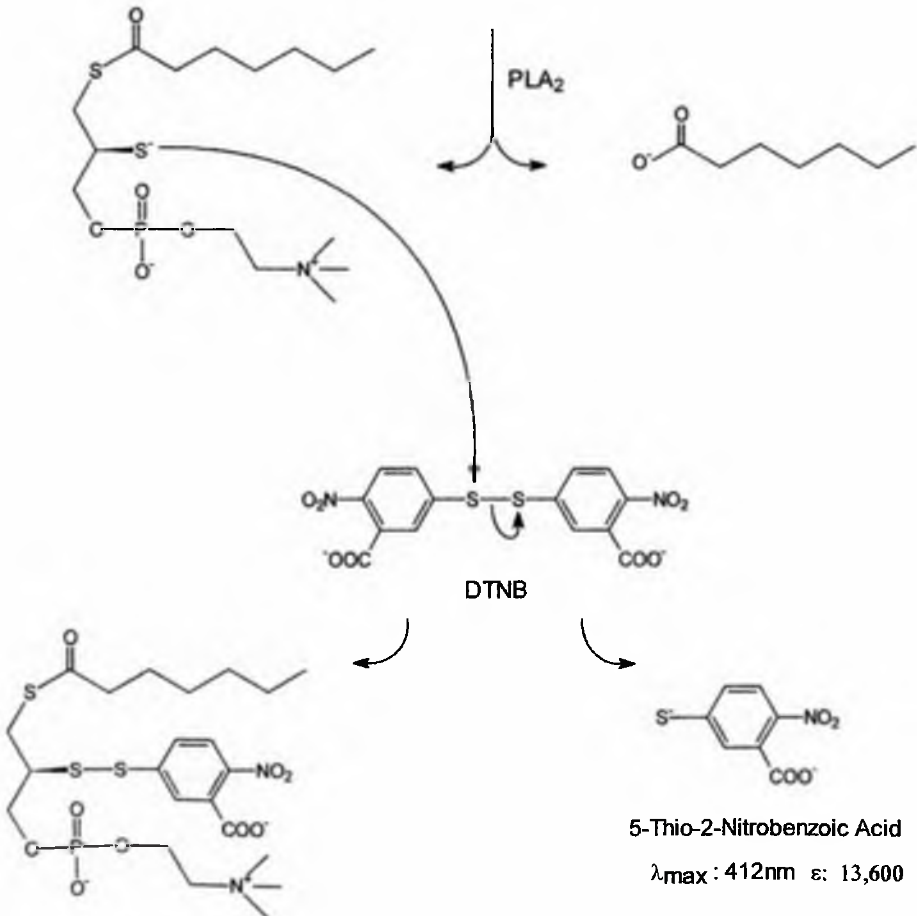
## Appendix IV

**Mechanisms involved in the assay for $PLA_2$ activity**
(Cayman Chemical, U.S.A., 1998)




Diheptanoyl Thio-PC




5-Thio-2-Nitrobenzoic Acid

$\lambda_{max}$ : 412nm ε: 13,600

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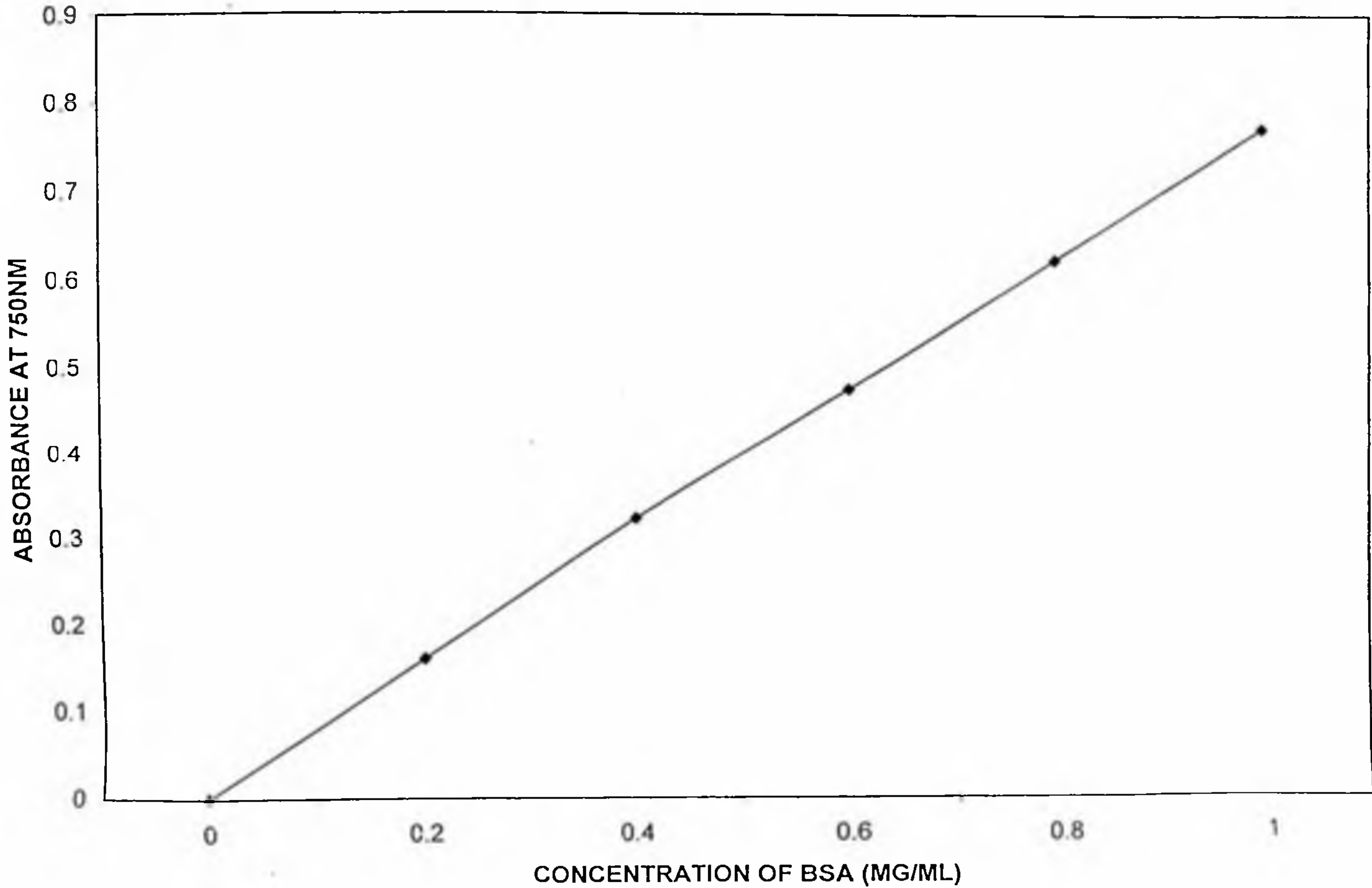
# APPENDIX V

STANDARD CURVE FOR PROTEIN ESTIMATION BY FOLIN-LOWRY METHOD



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