

**PROSTAGLANDIN-SYNTHESIS INHIBITORY ACTIVITY
FROM THE LEAVES OF *SIPHONCHILUS AETHIOPICUS*
USED IN THE TREATMENT OF DYSMENORRHOEA**

BY

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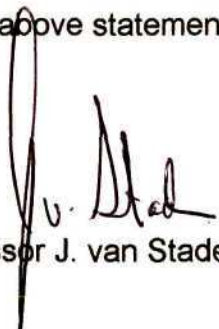
My husband and family for their continual belief in me and their never failing support.

DECLARATION

I hereby declare that this thesis, unless otherwise acknowledged to the contrary in the text, is the result of my own investigation, under the supervision of Professor J. van Staden, Department of Botany, University of Natal, Pietermaritzburg.


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We certify that the above statement is correct.


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ABSTRACT

Plants used by southern African traditional healers for the treatment of menstrual pains were screened for prostaglandin-synthesis inhibitors and the ability to reduce isolated uterine muscle contraction using the cyclooxygenase (Cox-1) and *in vitro* uterine bioassays respectively. Prostaglandins are synthesized from arachidonic acid and the enzyme that drives this reaction is cyclooxygenase. The excessive production of prostaglandins by the myometrium and endometrium induces uterine contractions. Inhibition of cyclooxygenase and hence of the prostaglandin biosynthetic pathway may lead to relief of menstrual pain.

Nine plants used by traditional healers for menstrual pains were assayed for cyclooxygenase inhibitory activity. Several plant extracts exhibited high inhibitory activity in the assay. The highest activities were obtained with ethanolic extracts of *Siphonochilus aethiopicus*, *Cenchrus ciliaris* and *Solanum mauritianum*. None of the ethanolic plant extracts were able to relax or reduce the contractions of a precontracted guinea pig uterus. Bioassay guided fractionation was used in an attempt to isolate the active compound(s) from the leaves of *Siphonochilus aethiopicus*. Isolation techniques employed were serial solvent extraction, bulk extraction, silica gel, Sephadex LH20 column, and high pressure liquid chromatography. Biological activity was followed through each purification step using the cyclooxygenase bioassay as a guide with respect to anti-inflammatory activity.

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ABBREVIATIONS

dpm - Disintegrations per minute

TLC - Thin layer chromatography

HPLC - High pressure liquid chromatography

NSAID - Non-steroidal anti-inflammatory drug

UV - Ultra violet

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Traditional medicine : A global perspective

The use of medicinal plants as a source for relief from illnesses can be traced back over five millennia to written documents of the early civilisations in China, India and the Near East, but it is doubtless an art as old as mankind (HAMBURGER and HOSTETTMANN 1991).

Today traditional medicine is one of the most important systems providing primary health care to people living in developing countries. It is estimated that up to 80% of the 5200 million people in the world live in developing countries and depend almost exclusively on traditional medicine for their health care needs (FARNSWORTH 1994). The fact that traditional medicine provides for the health care needs of such a large percentage of the world's population means that this practice must be recognised and given careful consideration by western drug development programmes (COX 1994, FARNSWORTH 1994).

It is well known that plants are chemical factories *par excellence*. The number of biologically active chemicals isolated from plants runs into thousands, with many being added to the list each year (STREAK 1995). Fieldwork exploring the medicinal uses of plants by indigenous peoples in remote parts of the world, coupled with the introduction of sophisticated assays able to determine whether plant extracts exert a biological

effect, has facilitated the discovery of bioactive molecules made by plants (COX and BALICK 1994).

At least 25% of the prescription drugs issued in the USA and Canada are known to contain bioactive compounds that are derived from, or modelled after, plant products (FARNSWORTH 1984). Higher plants have yielded many useful drugs to alleviate medical problems. In 1985, 119 metabolites isolated from plants were being used globally as drugs (FARNSWORTH, AKERELE, BUGEL, SOEJARTO, GUO 1985). Well known examples of plant-derived medicine include quinine, morphine, codeine, aspirin, atropine, reserpine and cocaine (VAN WYK, VAN OUDSTHOORN and GERICKE 1997). Most of these drugs were discovered following leads provided by traditional healers in various parts of the world (COX 1994, STREAK 1995). About 75% of these drugs are reported to have the same or related use as the plant from which they were discovered (FARNSWORTH 1994). They are reportedly extracted from only about 90 plant species (FARNSWORTH, AKERELE, BUGEL, SOEJARTO 1985).

The potential of higher plants as sources for new drugs remains, however, relatively unexplored. Among the estimated 500 000 plant species, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller (HAMBURGER and HOSTETTMANN 1991).

1.2 The use of traditional medicine by the black population of South Africa

Since ancient times, traditional healing has been an integral part of the black African culture (LEE 1996). According to World Health Organisation estimates, traditional healing provides for the primary health care needs of a large majority of the black population in South Africa (JÄGER, HUTCHINGS and VAN STADEN 1996), especially where health care systems are lacking (VEALE, FURNACE and OLIVER 1992). It is estimated that up to 80% of the Zulu population seen by medical practitioners also consult with traditional healers (JÄGER, HUTCHINGS and VAN STADEN 1996). In a country where the ratio of medical doctors to citizens is 1 : 17 500 people (SAVAGE 1985), the value of traditional healing cannot be over estimated.

Because of these statistics, and the importance of the role played by traditional healers within the community, modern health organisations are gradually recognising the need healers fulfil within the community. The Medical Association of South Africa published a report on their findings, while the Interim Medical and Dental Council and the KwaZulu Health Services are equally supportive (LEE 1996). There is a move towards the integration of traditional medicine into the official health care system. This correlates well with the philosophy that underlies the Governments Reconstruction and Development Plan (JÄGER, HUTCHINGS and VAN STADEN 1996). A scientific evaluation of plants and methods of treatment used by traditional healers is essential before traditional medicine can be incorporated into the official health care system.

1.3 Differing worldviews : Traditional vs Western medicine

South Africa is blessed with a rich cultural diversity that is reflected in the formal and informal systems of medicine that is presently being practised in different parts of the country (VAN WYK , VAN OUDTSHOORN and GERICKE 1997). The informal oral - tradition medical system of the Khoi San, the Nguni and the Sotho speaking people are passed on by word of mouth from one generation to the next. The formal system of medicine is well documented and was introduced over the last three hundred years to this country by Europeans and other settlers, and is exemplified by today's modern western medicine (VAN WYK, VAN OUDTSHOORN and GERICKE 1997).

Western and traditional African medicine are based on very different approaches: western biomedicine on a technical and analytical approach, while traditional medicine takes a holistic approach where disease or misfortune result from an imbalance between the individual and the social environment (CUNNINGHAM 1990). Western medicine may diagnose a disease in terms of a bacterial infection and treat it with antibiotics, whilst an African traditional healer will try to understand why the patient became ill and the treatment administered will address the perceived cause, usually in addition to specific remedies to alleviate the signs and symptoms of the ailment (VAN WYK, VAN OUDSTHOORN and GERICKE 1997). This difference in approach to the causality of disease is one of the reasons why demand for traditional medicine continues in the urban environment even if western biomedicine is available (CUNNINGHAM 1990).

In addition to this difference in approach there is the difference in access to traditional healers compared to medical doctors. SAVAGE (1985) reports one medical doctor for 17 500 people whereas there are reportedly one traditional healer for every 700 - 1 200 people in South Africa. For many people in South Africa dealing with health problems is a feature of daily life. Access to western type medicine however, is unattainable for most of these people. With the increasing population growth of the black population adding extra burden to the already over burdened western type medical system in South Africa the importance of the traditional healers must be realised and through training and evaluation of effective remedies, be integrated into the health care system.

1.4 African traditional healers

Culturally , Zulus believe that disease is a manifestation of disharmony between a person and ancestors (BYE and DUTTON 1991). Sorcery and medicine are intrinsically linked and it is the function of the traditional healer, who is regarded as the protector of society to act as diagnostician , apothecary and diviner (KRIGE 1981).

IWU (1993) reports that the specialized skills of a traditional healer may be acquired by three methods: firstly, by training and a long period of apprenticeship, secondly, by divine selection and in answer to a call by a powerful spirit or messenger and thirdly, as a family inheritance. The magical powers are acquired through ritual offerings and living in a prescribed manner, but the knowledge of efficacious herbs comes mainly from training and sometimes from revelation in dreams and trances.

There are basically five types of traditional healers in Africa: the diviner, or fortune teller, the herbalist, the midwife and birth attendant, the surgeon and the specialist medicine man (IWU 1993). Often however, individuals may belong to all or several groups or may restrict their practices to certain illnesses (IWU 1993). Depending on their ailment/condition patients may choose to consult with one of these healers.

The sangoma, or diviner ("isangoma" Zulu; plural: "Izangoma") consults with the spirits, using various methods of divination, such as throwing bones or going into a trance to identify the cause of disease (PUJOL 1990, BYE and DUTTON 1991). If more powerful medicine is needed, numerous magical rites can be performed. These often involve brutal methods, the aim of the sangoma being to find the cause of the symptoms at any cost (PUJOL 1990). They are also able to determine why the patient was singled out from the population to be afflicted. Treatment is dependent on superstitious belief, thus the sangoma must create a strong psychic impression in order to effect a cure (PUJOL 1990).

The herbalist ("Inyanga" Zulu; plural: izinyanga") on the other hand cures disease using plants which he/she has gathered (BYE and DUTTON 1991, IWU 1993). He possesses great knowledge about the medicinal uses of plants which is passed down from generation to generation, although uses and preparation of plants may vary considerably among different tribes (PUJOL 1990, IWU 1993, BHAT and JACOBS 1995). Plant material used by herbalists in treatments consists of roots, bulbs, tubers, rhizomes, corms, leaves and bark (PUJOL 1990).

Herbalists devote much time and personal attention to the patient, thus enabling the herbalist to penetrate deeply into the physiological and psychological state of the patient and the nature of his disease (IWU 1993).

The preparation of the plant drugs is dictated by the nature of the illness and the plant part used. The plant could be pounded or pressed and the juice applied to the affected part, especially in treatment of skin infections. Other methods for the preparation of drugs include roasting, burning, soaking, and chewing plant parts (IWU 1993). Methods of administration that may be used include purgatives, teas, infusions, ointments, inhalations, enema injections, poultices and snuffs (PUJOL 1990).

The terms "inyanga" and "sangoma" were used to refer exclusively to the herbalist and diviner respectively, however today the distinction has become blurred, with some healers practising both arts (VAN WYK, VAN OUDSTHOORN and GERICKE 1997). By law all herbalists in KwaZulu Natal are required to be registered, a prerequisite being that the person must be "skilled in herbalism" (BYE and DUTTON 1991). To be registered as a herbalist the applicant must have served an apprenticeship of seven years with a registered herbalist and be in possession of character references from the local magistrate, headman and a teacher or police officer. Application is then made to the Secretary for Health, KwaZulu Natal who in turn notifies the Herbalist Association (Nyanga National Association NNA). Officials then examine the applicant's knowledge of herbs and herbalism. Candidates are questioned orally by three established traditional healers about ailments and *muti*. The prospective healers are expected to be able to recognise traditionally used *muti* plants and explain their usage. Each

candidate is asked ninety questions and must get 60% to pass (MSOMI 1997). If successful, the applicant pays a nominal fee for a permit, allowing the herbalist to practice his trade (BYE and DUTTON 1991). LEE (1996) reports that today there are about 30 traditional healer associations throughout the country representing at least 300 000 individuals. It has become necessary for the various traditional healers associations countrywide to prevent “chancers” joining the practice if traditional healers are ever to be awarded the same recognition as doctors trained in western medicine. According to MSOMI (1997) the “Charlatans problem” is a recent phenomenon that is exacerbated by unemployment. In the past people would not claim to be *inyangas* unless they knew traditional medicine and had been trained. Today however, people are trying to earn money by claiming to be healers. Through formalising and regulating the traditional healing industry it is hoped that a *inyangas* Council will be created which will be accorded the same status as the South African Medical and Dental Council.

Indigenous systems of medicine are both dynamic and adaptive. This is illustrated by the incorporation of medicinal herbs into the *materia medica*, the use of modern medicines by some traditional healers and the interest in primary health care training programmes expressed by modern traditional healers’ associations. Together with this dynamism, appropriate official support and recognition, it is likely that traditional medicine will survive well into the next century, strengthened by modern science but not subsumed by it (VAN WYK, VAN OUDTSHOORN and GERICKE 1997).



Figure 1. Herbalists at a market in Pietermaritzburg, KwaZulu Natal.

1.5 The ethnobotanical approach to drug discovery

Virtually all of the currently used drugs derived from plants were discovered through the scientific investigation of folkloric claims of human efficacy (FARNSWORTH 1984). Such investigations are the basis of the ethnobotanical approach to drug discovery which is reported to be the most productive of the plant surveying methods (COX and BALICK 1994). The ethnobotanical approach assumes that the indigenous uses of plants can offer strong clues to the biological activities of those plants.

Ethnobotanists are reported by COX and BALICK (1994) to choose the societies they study on the basis of three criteria: Firstly, that these societies are located in a floristically diverse area, as such diversity is likely to dramatically increase the number of plants available thereby enhancing the likelihood that plants with pharmacologically active molecules will be discovered. Secondly, that the societies should have remained in the region for many generations, as groups who have resided in one region for many generations have presumably had ample opportunity to explore and experiment with the local vegetation. Thirdly, the cultures must have a tradition in which healer's pass down their plant knowledge from generation to generation, usually through apprentices. COX and BALICK (1994) state that consistent application of a given species over millennia generates information rather analogous to that produced by large scale clinical trials. Through the repeated, long term use of botanical species it can be expected that the most effective medicinal plants and those that are too toxic for use have been identified (COX and BALICK 1994).

Ethnobotanists are therefore particularly concerned with recording specialized information. That is, knowledge not necessarily shared by most other individuals in the community (COX 1994). The curative art is reported to be kept with some sanctity and secrecy, with the belief that the herbal medicines will lose their potency if revealed to other people (BHAT and JACOBS 1995). It is often no easy task to get a healer to communicate his knowledge. When this knowledge is communicated if it is to be of any use, it is essential that well documented inventories of plants reported to be used by healers be kept, detailing vernacular and scientific names of each plant, part of the plant used in treatment, details regarding the preparation of the remedy, disease or symptoms treated and dose and regime of treatment (HEDBERG 1993). Furthermore, it is essential that voucher specimens of every plant collected be prepared and properly documented with name of collector, collection number and locality. These voucher specimens should be deposited in herbaria in several parts of the world for consultation by other botanists (HEDBERG, 1993, COX and BALICK 1994, FARNSWORTH 1994).

Following collection and identification of plants, extracts are tested using a bioassay (FARNSWORTH 1994). Many assays in use today are capable of assessing the ability of an extract to influence the activity of a single enzyme involved in the biochemical interactions that underlie a disease (COX 1994). COX and BALICK (1994), however, cautions that few of the compounds exhibiting activity in laboratory tests will become new drugs, as some will turn out to be identical to, or less potent than existing agents and others too toxic for commercial use. Nevertheless demonstrating activity in a bioassay is a necessary first step in the drug development process.

Extracts that display significant activity in the bioassay are subjected to bioassay guided fractionation in order to isolate the active substance/s. The molecular structure of the active substance is determined and compared with known chemicals. If the isolated molecule is novel, or has already been found but not studied, it may be analysed further as is. In other cases a synthetic version of the molecule may be constructed and examined further as a possible drug (COX and BALICK 1994, FARNSWORTH 1994).

The demonstrated ability of ethnobotany to generate exciting leads for possible new drugs suggests that for the near future, this approach will occupy an expanding role in drug development (COX and BALICK 1994). Unfortunately the rapid disappearance of many plants, together with information as to their medicinal usage, means that ethnobotanists are in a race against time (CUNNINGHAM 1990, COX and BALICK 1992, CUNNINGHAM 1992, COX and BALICK 1994, FARNSWORTH 1994).

1.6 Conservation of medicinal plants

The exponential population growth rate observed in most developing countries has resulted in an increase in demand for traditional remedies and has placed a severe strain on already depleted natural resources. This is especially so in South Africa where the belief in traditional remedies and healers remain firm (BYE and DUTTON 1991). This urban demand for traditional medicines is reported by CUNNINGHAM (1992) to have generated a commercial trade in medicinal plants from rural source areas to urban markets and shops. The size of the national market for medicinal plants

is believed to be considerable, with an estimated trade value of at least US\$ 128 million per annum (MANDER, MANDER and BREEN, 1997). The use of medicinal plants was formerly a specialist activity of the traditional healer's who had a limited effect on plant resources (CUNNINGHAM 1992), employing groups of gatherers who were taught how to collect medicinal plants without destroying the resources (LEE 1996). Urbanization and poverty however, have produced untrained gatherers who collect and sell plants to make a living (CUNNINGHAM 1992, LEE 1996). This indiscriminate harvesting has had disastrous effects on many popular species (CUNNINGHAM 1990, BYE and DUTTON 1991, CUNNINGHAM 1992, IWU 1993, LEE 1996, VAN DUFFELEN 1996). Plants most at risk are trees that are ring barked (PUJOL 1990) and plants that are collected for their underground tubers, corms and rhizomes (CUNNINGHAM 1990, LEE 1996).

CUNNINGHAM (1990) reported that the management of traditional medicinal plant resources is probably the most complex African resource management issue facing conservation agencies, health care professionals and resource users. This is suggested to be due as much to cultural, socio economic factors as to the variety of species and plant parts used. It is proposed therefore that constructive resource management and conservation action be founded on a clear understanding of the key factors driving medicinal plant use (CUNNINGHAM 1990).

Suggestions as to conservation strategy vary greatly. These include cultivation of rare and endangered species (CUNNINGHAM 1990, 1992, WILLIAMS 1996), education programmes (LEE 1996), identification and better protection of key vegetation types in

conservation areas, the formulation of patent or pharmaceutical medicines with the same name and function as their herbal counterparts, encouraging herb traders and urban herbalists to cultivate medicinal plants and use leaves and twigs rather than roots or bark (CUNNINGHAM 1992). The Natal Parks Board now trains their staff to propagate their own medicinal plants and the Municipal Parks Department encourages traditional healers and gatherers to plant and harvest seedlings in conservation areas around the city. In Durban, KwaZulu Natal, one third of the health care service is provided by an indigenous health system. Loss of medicinal plant species threatens severe cultural, health and economic loss to millions of people, making successful collaborative conservation projects essential (O'GRADY 1997).

1.7 *Siphonochilus aethiopicus* (Zingiberaceae)

The family Zingiberaceae is well known for its spice plants. *Siphonochilus aethiopicus* (Schweinf.) B.L. Burtt, commonly known as "wild ginger" or by its' zulu names "indungulo" or "isiphephetho" is a rhizomatous herb that occurs in Africa, southwards from Senegal and Ethiopia to the northern and eastern parts of South Africa (MAVHUVKA, VAN WYK, VAN DER BANK and VAN DER BANK 1997). It is the only member of the family that is indigenous to South Africa (MAVHUVKA, VAN WYK, VAN DER BANK and VAN DER BANK 1997). The plant is deciduous with large hairless leaves developing from a small, distinctive, cone - shaped rhizome. Flowers appear at ground level in early summer and are funnel - shaped, pink and white in colour with a yellow blotch in the middle. Most plants are bisexual and have much larger flowers than female plants (VAN WYK, VAN OUDSTSHOORN and GERICKE 1997). The rhizomes

and tubers are extensively used in traditional African medicine and witchcraft : having a strong ginger smell and are crushed, mixed with warm water and drunk to relieve chest ailments (ukukhwehlela / isifuba). The plant is also sought after for the treatment of menstrual pains, toothache, rheumatism, neuralgia and malaria MANDER, MANDER, CROUCH, Mc KEAN and NICHOLS 1995). CUNNINGHAM (1990), reported that *S. aethiopicus* is listed as endangered in the wild and MANDER, MANDER, CROUCH, Mc KEAN and NICHOLS (1995) listed the plant extinct in the wild in KwaZulu - Natal. It is classified by herb traders as the third most difficult plant to acquire, after *Warburgia salutaris* and *Boweia volubilis* (CUNNINGHAM 1990) and according to O'GRADY (1997) people are prepared to pay R450/kg of "wild ginger" from the street markets. Ethanolic extracts of the leaves have been shown to inhibit prostaglandin - synthesis and in this way are likely to be effective in reducing pain and inflammation (LINDSEY, JÄGER, RAIDOO and VAN STADEN 1999) If traditional healers could be encouraged to use the leaves of this plant as a substitute for rhizomes there may be a chance to increase the numbers of this plant in the wild. No published information is available on the chemistry and pharmacology of wild ginger.

1.8 The uterus and dysmenorrhoea

Dysmenorrhoea is a menstrual disorder experienced by a large percentage of the world's reproductive female population. DINGFELDER (1981) reports that dysmenorrhoea causes an estimated 140 million lost work hours annually. Primary dysmenorrhoea is defined by KENNEDY (1997), as colicky, low abdominal pain during menstruation occurring predominantly in young women in the absence of disease

such as endometriosis. It is more than just painful cramps; it is a system complex and it is probable that a common etiology underlies nearly all the symptoms (DINGFELDER 1981). It appears to start with the onset of the ovulatory cycle shortly after the menarche, worsening during the first day or two. This ischaemic pain results from an excess production of prostaglandins which stimulate excess uterine contractions (COLLIER, LONGMORE, and HODGELLS 1995).

Both the force and the frequency of contractions of uterine muscle vary greatly during the menstrual cycle. RANG and DALE (1987), report that the non - pregnant human uterus shows weak spontaneous contractions during the first part of the cycle. At about the 14th day, larger more prolonged contractions can be recorded. During menstruation strong coordinated contractions similar to those observed in the pregnant uterus during parturition are experienced.

According to ELDER (1983) and RANG and DALE (1987), the endometrium and the myometrium of the uterus have significant prostaglandin synthesizing capacity, particularly in the second, proliferative phase of the menstrual cycle. The vasoconstrictor prostaglandin, PGF_2 , is generated in particularly large amounts and is thought to be implicated in the ischaemic necrosis of the endometrium which precedes menstruation. DAWOOD (1981) has shown that the amount of prostaglandin $\text{F}_{2\alpha}$ released per hour in the menstrual fluid can be directly correlated with the fluctuating symptoms and intensity of pain occurring during that time in the patient. The vasodilator prostaglandins, PGE_2 and the prostacyclin, are also generated by the uterus. In addition to their vasoactive properties the E and F type prostaglandins cause

contractions of both the pregnant and the non - pregnant uterus (RANG and DALE 1987). It is widely believed that prostaglandins E produce a contraction of smooth muscle by increasing free-cytoplasmic calcium concentration (VILLAR, D'OCON and ANSELMI 1985). According to CARSTENS (1974) and AL-KHALIL, AFIFI and AQEL (1991) the source of activator Ca^{2+} may be extracellular or intracellular, however the sources and mechanisms of calcium mobilization and sequestration in uterine myometrium are not completely defined. ANWER, HOVINGTON and SANBORN (1989) report that uterine contractants increase the influx of calcium, inhibit uptake and stimulate the release of calcium from uterine microsomes, and inhibit membrane $(Ca+Mg)ATPase$, whilst relaxants increase calcium uptake into uterine microsomes and potentiate calcium efflux from myometrial cells. ANDERSSON (1988) suggests that PGE_1 acts essentially by increasing the intracellular calcium concentration by translocation of membrane bound calcium from surface microvesicles or sarcoplasmic reticulum. It is suggested that intracellular calcium stores are not the primary target for $PGF_{2\alpha}$ in the human myometrium, but rather that transmembrane calcium influx sensitive to calcium antagonists is an important step in the activation. RUBANYI and CSAPO (1977) arrived at a similar conclusion, studying the effects of $PGF_{2\alpha}$ on the rabbit uterus.

DAWOOD (1981) proposes that the pain associated with dysmenorrhoea can be attributed to three mechanisms which are all mediated by the effect of prostaglandins on the pelvic tissues. Firstly, increased and abnormal production of and release of, endometrial prostaglandins give rise to increased and/or abnormal uterine contractility, thereby producing pain. Such uterine activity reduces blood flow and causes ischaemia

or hypoxia leading to pain or further compounding the pain. Finally, cyclic endoperoxides, the intermediates in the biosynthesis of prostaglandins, as well as relative increases in prostaglandin E₂ in the presence of high prostaglandin concentrations have direct pain-producing properties by sensitization of the pain fibres to mechanical and chemical stimuli such as bradykinin and histamine.

Dysmenorrhoea has been successfully treated with non-steroidal anti-inflammatory drugs which act to inhibit the synthesis of these prostaglandins by blocking an enzyme, cyclooxygenase, which converts arachidonic acid to the precursors of the various prostaglandins (ELDER 1983). This results in a reduction of uterine contraction and hence pain (RANG and DALE 1987), with minimal associated side effects (DINGFELDER 1981). DAWOOD (1993) suggests that NSAIDs can be classified as either type I or type II inhibitors. The type I inhibitors suppress prostaglandin biosynthesis by inhibiting the enzyme cyclooxygenase, which is required for conversion of arachidonic acid to cyclic endoperoxides. By contrast the type II inhibitors such as phenylbutazone and oxyphenbutazone, suppress prostaglandin production through inhibition of endoperoxide isomerase and reductase. Thus this inhibition occurs at some point after the formation of cyclic endoperoxides in the arachidonic acid cascade.

KENNEDY (1997) reports that non-steroidal anti-inflammatory drugs and combined oral contraceptives are the most commonly used treatments despite increasing reluctance to prescribe combined oral contraceptives from an early age because of possible long term side effects.

DINGFELDER (1981) reported that prostaglandin inhibitors eliminated significant pain

in 70% - 80% of women suffering from primary dysmenorrhoea. COLLIER, LONGMORE and HODGELLS (1995), reported that the NSAID mefenamic acid has been of great value in the treatment of dysmenorrhoea. BUDOFF (1979) cites an excellent 84% response to treatment with mefenamic acid (Ponstel). Phenylbutrazone, and indomethacin all have potent anti-prostaglandin activity but their side effects which may even be fatal preclude their use in many women (DINGFELDER 1981).

The choice of prostaglandin inhibitor to treat dysmenorrhoea should only be made after diagnosis of primary dysmenorrhoea has been confirmed (DINGFELDER 1981). Such a diagnosis should be made only after complete and thorough history - taking and physical examination have eliminated recognisable cause of secondary dysmenorrhoea such as congenital duplications and anomalies, pelvic inflammatory disease, adnexal pathology, and endometriosis. A trial of a prostaglandin inhibitor may then be prescribed (DINGFELDER 1981). ELDER (1983), proposed that improved treatment will come only from more knowledge of the biochemical control of menstruation and the subsequent development and use of drugs that are more specific inhibitors of the synthesis of the appropriate prostaglandins. Ranking of NSAIDs for their potency in pelvic and reproductive tissues, such as the myometrium and endometrium, will provide another dimension in the selection of NSAIDs for gynecologic use.

1.9 Prostaglandin pharmacology

Prostaglandins are responsible for the sensation of pain and inflammation. Prostaglandin biosynthesis begins with the conversion of arachidonic acid to two

endoperoxide intermediates, first prostaglandin G_2 and then prostaglandin GH_2 . The reactions are catalysed by the same enzyme, prostaglandin endoperoxide synthase, which has two components, cyclooxygenase and peroxidase. In some cells prostaglandin GH_2 is converted to thromboxanes (TXA) and prostacyclin (PGI_2) (BOHINSKI 1987).

There are at least 14 naturally occurring prostaglandins. They are named in a complex fashion dictated by their chemical structure. There are six "primary" natural prostaglandins (PGE_1 , PGE_2 , PGE_3 , $PGE_{1\alpha}$, $PGF_{2\alpha}$, $PGF_{3\alpha}$) and eight natural prostaglandins that are derived metabolically from these (LAURENCE and BENNETT 1980). Drugs that are able to inhibit the cyclooxygenase enzyme are able to prevent the formation of prostaglandins and can be used for the treatment of pain and inflammation (NASIRI, HOLTH, BJORK, 1993). Drugs having this pharmacological action are known as non-steroidal anti-inflammatory drugs (NSAID), (RANG and DALE 1987). MANTRI and WITIAK (1994) report that inhibition of prostaglandin -synthesis has been demonstrated in microsomal enzyme preparations, many cell types and tissues and a variety of animal and human tissues. It was found that cyclooxygenase can be inhibited by three different mechanisms: (1) Reversible competitive inhibition; (2) mechanism based or time-dependent inactivation (irreversible inhibition); and (3) reversible noncompetitive inhibition. Drugs such as aspirin, indomethacin, ibuprofen and paracetamol are NSAID's and are effective in treating pain, dysmenorrhoea and inflammation (RANG and DALE 1987). If a plant extract can inhibit the cyclooxygenase enzyme then no synthesis of prostaglandins will occur and we have a potential plant extract that may be developed into a drug for treating pain.

It has recently been discovered that the enzyme cyclooxygenase consists of two isozymes, COX-1 and COX-2 (VANE 1994, HERSCHMANN 1996, VANE and BOTTING 1996). COX-1 is constitutively expressed whereas COX-2 seems to be induced during inflammation. All cyclooxygenase inhibitors seem to inhibit both COX-1 and COX-2, but with different selectivity. Nearly all NSAIDs on the market today, such as aspirin, ibuprofen and indomethacin, are COX-1 inhibitors. The assay system that is used in this study tests for COX-1 activity. This is a reasonable approach for an initial screening of traditionally used medicinal plants. But plant extracts with high activity or isolated compounds should also be evaluated for COX-2 inhibitory activity, when such an assay system becomes available.

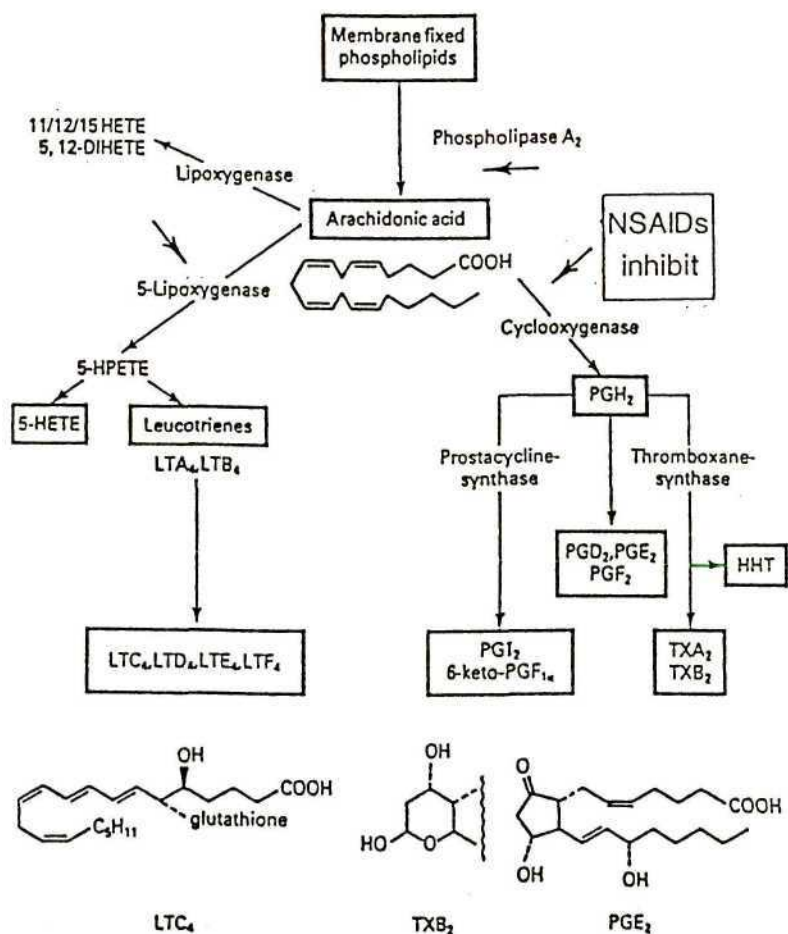


Figure 2. Prostaglandin biosynthetic pathway (RANG and DALE 1987).

1.10 Drug development in South Africa

Great potential exists within South Africa to scientifically investigate claims made by traditional healers as to the uses of plants, with a view to testing efficacy and safety of these plants as well as determining the potential use of plant extracts in future drugs.

In South Africa lists of plants used in the treatment of pain and inflammation have been constructed by HUTCHINGS (1989) and revised by HUTCHINGS and VAN STADEN (1994). These lists were completed after interviews with traditional healers as well as through literature surveys. WATT and BREYER - BRANDWIJK (1962), GELFAND, MAVI, DRUMMOND and NDEMERA (1985), HUTCHINGS, HAXTON-SCOTT, LEWIS and CUNNINGHAM (1996), PUJOL (1990) and MANDER, MANDER, CROUCH, M^cKEAN and NICHOLLS (1995) all provide information as to which plants are used by southern African traditional healers in the treatment of dysmenorrhoea. No scientific evidence is given in this literature as to the efficacy of these plants in treating dysmenorrhoea. Table 1 lists those plants that were found to be used in the treatment of dysmenorrhoea and outlines parts of plants used and method of administration, where this literature is available.

1.11 Aims of this study

The aims of this study were:

1. To screen plants reputedly used in the treatment of dysmenorrhoea for the presence of prostaglandin-synthesis inhibitors and for uterine relaxing activity, and
2. To attempt to isolate and identify the active compound/s from one plant exhibiting high prostaglandin-synthesis inhibitory activity.

Table 1. Medicinal plants used by southern African traditional healers in the treatment of dysmenorrhoea.

Plant name and family	Plant part used	Administration
<i>Bersama tysoniana</i> Oliv. (Melianthaceae)	bark	snuffed
<i>Cenchrus ciliaris</i> L. (Poaceae)	underground runners	decoction
<i>Combretum erythrophyllum</i> (Burch) Sond. (Combretaceae)	leaves	-
<i>Combretum platypetalum</i> Sond. (Combretaceae)	leaves	-
<i>Combretum zeyheri</i> Sond. (Combretaceae)	bark	powdered bark introduced into the vagina
<i>Indigofera arrecta</i> Hochst.exA.Rich. (Leguminosae)	root	juice from peeled root is drunk
<i>Gunnera perpensa</i> L. (Haloragaceae)	-	-
<i>Pentanisia prunelloides</i> Walp. (Rubiaceae)	leaves roots	Leaf poultice applied to the abdomen Decoction of root used to relieve pain

Table 1 continued

Plant name and family	Plant part used	Administration
<i>Rhoicissus tridentata</i> (Vitaceae)(L.f)Wild and Drum.	roots and tubers	-
<i>Scilla natalensis</i> Planch. (Liliaceae)	bulb	bulb is boiled and drunk as a decoction
<i>Senecio coronatus</i> (Thunb.)Harv. (Solanaceae)	leaves	-
<i>Solanum mauritianum</i> Scop. (Solanaceae)	leaves	snuffed
<i>Siphonochilus aethiopicus</i> (Schweinf) B.L Burt. (Zingiberaceae)	rhizome leaves	decoction of rhizome drunk to relieve menstrual pain leaves chewed during menstruation to relieve pain
<i>Vangueria infausta</i> (Burch.)Sond. (Rubiaceae)	leaves	decoction of leaves
<i>Ziziphus mucronata</i> Willd (Rhamnaceae)	leaves	leaf poultice applied to relieve pain

CHAPTER TWO

MATERIALS AND METHODS

2.1 Ethnological data

A list of plants used by traditional healers for the treatment of dysmenorrhoea was compiled from surveys done by WATT and BREYER-BRANDWIJK (1962), GELFAND, MAVI, DRUMMOND and NDEMERA (1985), PUJOL (1990), MANDER, MANDER, CROUCH, McKEAN and NICHOLLS (1995) and HUTCHINGS, HAXTON-SCOTT, LEWIS and CUNNINGHAM (1996)

2.2 Plant material

From the list compiled, nine plants were collected for screening for prostaglandin-synthesis inhibitors. The botanical names, plant part used, method of administration, voucher specimen numbers and collection sites for plant material are given in Table 2.

2.3 Extraction of plant material

Plant material was dried in an oven at 50°C and subsequently stored at room temperature in brown paper bags until extraction. Five hundred mg of dried, ground material was extracted with 5 ml of water or ethanol in an ultrasound bath for 30 minutes. The extracts were filtered and taken to dryness. The residues were resuspended in water or ethanol giving a 20 mg residue/ml ethanol and 2.5 mg

residue/ml water respectively.

2.4 Screening in the cyclooxygenase bioassay

2.4.1 Preparation of cyclooxygenase enzyme

Sheep seminal vesicles from freshly slaughtered animals were obtained from the Cato Ridge abattoir. Fifty grams of seminal vesicles were cut into smaller pieces and ultraturred with 150 ml 0.1 M potassium phosphate, 1 mM EDTA. The homogenate was centrifuged at 4000 g for 15 minutes. The supernatant was further centrifuged at 17000 g for 10 minutes. The microsomal pellets were isolated by ultracentrifugation at 100000 g for one hour. The microsomal pellet was resuspended in 0.1 M potassium phosphate and adjusted to 10 mg protein /ml, using a standard BSA curve constructed at 0.2; 0.4; 0.6; 0.8; 1.0 and 1.2 mg/ml respectively. Aliquots of 10, 100 and 1000 μ l were placed in Eppendorf tubes and frozen at -70°C (JÄGER, HUTCHINGS and VAN STADEN 1996).

2.4.1.1 Optimization of bioassay

2.4.1.1a Testing different enzyme concentrations

A range of enzyme concentrations were tested in the cyclooxygenase bioassay (100; 10; 1; 0.8; 0.6; 0.4; 0.2; 0.1 and 0% respectively). The incubation time in the water bath, once 14 C-arachidonic acid had been added, was 10 minutes. From results

obtained, a possible enzyme concentration to use in the cyclooxygenase bioassay was determined.

2.4.1.1b Determining optimal incubation time

Enzyme concentrations of 0.5; 0.25 and 0.125% respectively, were tested in the cyclooxygenase bioassay and incubated for 0; 2; 4; 6; 8; 10; 16 and 20 minutes respectively. From results obtained, a suitable enzyme concentration and incubation time was determined.

2.4.2 Establishing an indomethacin standard curve

Indomethacin solutions of 10^{-3} M to 10^{-9} M were assayed for activity in the cyclooxygenase bioassay. From results obtained, a standard curve was constructed.

2.4.3 Cyclooxygenase bioassay

The Cox-1 bioassay was performed as described by JÄGER, HUTCHINGS and VAN STADEN (1996). Cyclooxygenase was prepared from sheep seminal vesicles, as outlined in Section 2.4.1 above. Cyclooxygenase enzyme solution and co-factor solution (L-adrenaline and reduced glutathione, 0.3 mg/ml each in 0.1 M Tris buffer, pH 8.2) were mixed in a ratio of 1:5 and incubated on ice for 15 minutes. Sixty μ l of this enzyme/co-factor solution was added to 20 μ l of sample (20 μ l of aqueous solutions; 2.5 μ l of ethanolic extract + 17.5 μ l of water). Twenty μ l of 14 C-arachidonic acid (16

Ci/mole, 30 μ M) were added and the assay mixture incubated at 37 °C for 10 minutes. The reaction was terminated by adding 10 μ l of 2N HCl. A background was kept in the ice bucket. Four μ l of a 0.2 mg/ml carrier solution of unlabelled prostaglandins (PGE₂:PGF₂ 1:1 v/v) was added.

The prostaglandins were separated from the unmetabolized arachidonic acid by column chromatography. Silica gel in eluent 1 (hexane:dioxane:acetic acid 350:50:1) was packed to a height of 3 cm in Pasteur pipettes. One ml of eluent 1 was added to the samples and this mixture was applied to the columns. The arachidonic acid was eluted from the column with 4 ml of eluent 1 and discarded. Labelled prostaglandins were eluted with 3 ml of eluent 2 (ethyl acetate:methanol 85:15 v/v). Four ml of scintillation fluid was added to each vial and the radioactivity measured using a Beckman LS 6000LL scintillation counter. Percentage inhibition of test solutions was calculated by comparing the amount of radioactivity present in the sample to that in the solvent blank, using the following formula:

$$\text{Inhibition (\%)} = 1 - \left[\frac{\text{dpm sample} - \text{dpm background}}{\text{dpm blank} - \text{dpm background}} \right] \times 100$$

In order to determine the validity of the assay-system an ethanolic indomethacin standard solution was assayed each time samples were tested in the assay. Assays were performed in duplicate.

2.5 Uterine bioassay

The uterine bioassay was performed at the Medical School, University of Natal, Durban in the laboratories of Dr Raidoo.

The ethanolic extracts were dissolved in distilled water at a concentration of 10 mg/ml. With the exception of the *Vangueria infausta* extract, all the extracts required heating in a 45°C water bath in order to dissolve them. One hundred μl (1000 μg) of ethanolic extracts were made up to 1 ml with 900 μl Tyrodes solution to produce a final working concentration of 1 $\mu\text{g}/\mu\text{l}$. Aliquots of this were added directly to the organ bath. The plant extracts were of neutral pH.

Two cm longitudinal strips of guinea pig uterine smooth muscle tissue was suspended from a Harvard Smooth muscle transducer into a muscle chamber and immersed in 10 ml Tyrodes solution. The lower end of the muscle strip was weighted to produce gentle stretching of the muscle. Oxygen and carbon-dioxide (95%:5%) was bubbled through the solution to maintain a good oxygen tension. The muscle chamber was immersed in a water bath maintained at a temperature of 37°C. The system was allowed to stabilise for 30 minutes.

To test the function of the system and to serve as an internal standard, 1-5 μg doses of acetylcholine (positive control, 1 $\mu\text{g}/\mu\text{l}$) was added to the muscle chamber to observe the effect on muscle contraction. The effect was observed until maximal muscle contraction was achieved after which the Tyrodes solution was drained and new

solution added. The muscle contractions were allowed to return to baseline before plant extracts were tested.

Uterine activity of the plant extracts was analysed using 3 methods:

1. Initially, 100 μl (100 μg) aliquots of extract were added to the muscle bath at 2 minute intervals, up to a maximum of 500 μl or until a muscle response (increase or reduction in baseline contraction) was observed to determine whether the plant extract had direct effects on uterine function;
2. Thereafter, an initial dose of acetylcholine was added, first with the plant extract in the muscle bath and after subsequent washing. A comparison of this response with the initial effect helped to elucidate whether the plant extract sensitised or desensitised the uterine muscle to acetylcholine; and
3. The muscle was pre-contracted with 0.01 IU oxytocin and 250 μl aliquots of extract was added at 5 minute intervals, up to a maximum of 1000 μl , to determine the ability of the plant extract to relax contracted uterus muscle.

2.6 Serial extraction of *Siphonochilus aethiopicus* leaves

Leaves (2.78 g) were ground and serially extracted in a small Soxhlet apparatus with 150 ml of hexane, ethyl acetate, ethanol and water respectively. Each solvent extract was subsequently taken to dryness under vacuum at 30°C, and resuspended in ethanol

to give a concentration of 20 mg/ml. Each extract was tested using the cyclooxygenase bioassay to determine in which solvent the active compound(s) occurred.

2.7 Thin layer chromatographic systems

2.7.1 TLC of solvent extracts from serial extraction

Fifty μl of each solvent extract was placed on three individual TLC plates (0.25 mm, 20 x 5 cm) and separated using a solvent system of hexane:ethyl acetate (3:1). TLC plates were removed from the tank and the solvent front marked. The plates were allowed to dry. Bands visible to the naked eye were marked using a soft pencil. Those bands visible under UV-light (254 nm and 366 nm) were also marked. TLC plates were not stained. The respective TLC plates were divided into five zones based on compound separation. Silica gel from each zone was scraped from the glass backing and suspended in 1 ml methanol. Solutions were placed in a ultrasound bath for 1 minute, and then filtered using a Millipore filter (0.45 μm). Extracts were dried down and resuspended in 50 μl ethanol. Each of the five zones of the respective solvent extracts were tested for activity in the cyclooxygenase bioassay.

2.8 Bulk extraction

Dry, ground *Siphonochilus aethiopicus* leaves (273 g) were extracted in 2.5 litres of hexane in a large Soxhlet apparatus for 15 hours. The bulk extract was filtered through Whatman No. 1 filter paper in a Buchner funnel and then through a Millipore filter

(0.22 μm). The extract was taken to dryness under vacuum, yielding 8.02 g of residue. Residue (4.8 g), left over from a previous attempt to isolate active compounds from the leaves of *Siphonochilus aethiopicus*, which had been extracted a year earlier in the manner described above and stored in the dark at 5°C, was tested for activity in the cyclooxygenase bioassay. As there was only a 49% decrease in activity after one year of storage this extract subsequently underwent the same isolation procedures as the fresh extract and prostaglandin-synthesis inhibitory activity was followed through each step. This was done to enhance the potential to extract the active compound(s) further.

2.9 Column chromatography

Two hundred grams of silica powder (Merck) was suspended in hexane:ethyl acetate (3:1) and quickly poured into a large column (4x30 cm). The silica was left to settle for 20 minutes. Excess solvent standing over the silica gel in the column was allowed to run out. Dried hexane bulk extract (3.22 g) was dissolved in hexane and loaded onto the column. The extract was allowed to penetrate the column before being eluted with hexane:ethyl acetate (3:1). Fractions of 5 ml were collected using a microfraction collector (Gilson Model 203). After 130 tubes had been collected the solvent system was changed to hexane:ethyl acetate (1:1) and after a further 170 tubes, to 100% ethyl acetate, and a further 90 fractions collected.

The procedure was repeated with the remaining bulk residue (4.2 g).

2.10 Viewing and staining TLC plates

For all subsequent TLC plates run, the following protocol was followed once TLC plates had been removed from the tank, and their solvent fronts marked: Firstly, all visible bands were marked. Those bands visible under UV-light (254 nm and 366 nm) were marked. The plates were then stained with 5% *p*-anisaldehyde in 5% ethanolic sulphuric acid, and heated at 110°C for 5 minutes. All new bands were marked.

2.11 Testing column fractions on TLC plates

Ten μl , from every fifth tube collected by fraction collection from the silica column, was spotted onto a TLC plate (0.2 mm, 10 x 20 cm). Compounds were separated with hexane:ethyl acetate (3:1). This was repeated for both of the silica columns run as described in Section 2.9 above. Fractions containing similar compounds were combined. The respective combined fractions (referred to as samples) were taken to dryness and resuspended in ethanol to give a concentration of 20 mg/ml. Samples were tested for activity in the cyclooxygenase bioassay.

Samples A; B; C; D; E; G; H; and I from the two silica columns were combined based on similarity of compound separation (Figure 8). Samples H and I were combined based on the fact that they yielded a similar chromatographic profile after TLC.

2.12 Sephadex column chromatography

2.12.1 Small Sephadex column using methanol as eluent

Twenty five grams of Sephadex LH20 was suspended in methanol for 12 hours. This mixture was then poured quickly into a small column (2.5 x 28 cm). The Sephadex LH20 was left to settle for 20 minutes. The reservoir was filled with methanol which was left to run through the column for 40 minutes. One hundred and twelve mg of combined sample H and I was loaded onto the column and was eluted with 100 % methanol. Eighty 1 ml fractions were collected.

Ten μ l from tubes collected was spotted onto TLC plates (0.2 mm, 10 x 20 cm) as indicated in Figure 9. TLC plates were placed in tanks containing hexane:ethyl acetate (3:1). Developed TLC plates were viewed and stained as described in Section 2.10.

On the basis of compound separation it was decided to combine those fractions having similar compounds. Samples were taken to dryness and resuspended in ethanol to give a concentration of 20 mg/ml. Samples were tested for activity in the cyclooxygenase bioassay.

2.12.2 Large Sephadex LH20 column using methanol as eluent

Three hundred and seventy five mg of combined sample H and I were loaded onto a large column (2.5 cm x 64 cm) containing 75 g of Sephadex LH20 suspended in

methanol. Fractions of 1 ml were collected.

The remaining part of the combined sample H and I (480 mg) was processed in the same way.

Ten μ l from tubes collected was spotted onto TLC plates (0.2 mm, 10 x 20 cm) as indicated in Figure 11. TLC plates were developed in a tank containing hexane:ethyl acetate (3:1). TLC plates were viewed and stained as described in Section 2.10. On the basis of compound separation it was decided to combine those fractions having similar compounds. Samples 1 to 9, from the small Sephadex column and the large Sephadex columns, used to separate combined sample H and I (855 mg), were combined. All samples were taken to dryness and stored in the dark at 5°C until further use.

2.13 High pressure liquid chromatography

2.13.1 HPLC conditions

A Varian 5000 Liquid Chromatograph fitted with a Hypersil 5 ODS analytical column (25 cm x 4.6 mm) and connected to a UV-detector set at 240 nm was used. The absorbance of the UV-detector was set at 0.2 and the flow rate was 1 ml/minute. A chromatogram was recorded with a linear recorder/integrator.

2.13.2 HPLC of sample 3 (gradient)

Sample 3 was resuspended in acetonitrile to give a concentration of 2 mg/ml. Sixty μg of sample 3 was injected onto the column and separated using the following programme. A gradient elution programme starting in acetonitrile:water (10:90) and running into acetonitrile:water (100:0) over 30 minutes, followed by a 10 minute wash in 100% acetonitrile. One ml fractions were collected in a fraction collector. Fractions were taken to dryness and fractions 5; 6; 7; 14 to 19; 22 to 26 and 36 to 39 were tested separately for activity in the cyclooxygenase bioassay.

In order to unequivocally determine where active compound/s were eluted, 200 μg of sample 3 was then injected onto the column and separated using the programme described in Section 2.13.1. Fractions of 1 ml were collected and taken to dryness and then resuspended in ethanol. Fractions 4; 5; 10 to 19; 21 to 26 and 31 to 40 were tested for activity using the cyclooxygenase bioassay.

2.13.3 HPLC of sample 4 (gradient)

Sample 4 was resuspended in acetonitrile to give a concentration of 2 mg/ml. Sixty μg of sample 4 was injected onto the HPLC and the programme as described in Section 2.13.2, run. Fractions of 1 ml were collected and taken to dryness. Fractions 3; 4; 11 to 19; 22; 23; 26 to 32 and 36 to 40 were tested separately for cyclooxygenase inhibition.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Optimization of the cyclooxygenase bioassay

3.1.1 Testing of different enzyme concentrations

The results of testing different enzyme concentrations in the cyclooxygenase bioassay are given in Figure 3. A concentration of 0.4% gave a value of over 5000 disintegrations per minute (dpm). This value is equivalent to approximately a third of the substrate being converted, which is the maximum level of conversion that is within linearity. A decision was taken to test concentrations slightly stronger and weaker in the cyclooxygenase bioassay on a time course basis to determine the optimal concentration of enzyme to use, and the most suitable incubation time.

3.1.2 Determining an optimal incubation time

An enzyme concentration of 0.25% gave a good linear relationship with incubation times up to 10 minutes (Figure 4). This concentration of enzyme was then used in the bioassay and incubated with arachidonic acid for 8 minutes, to be well within the linear area of the curve.

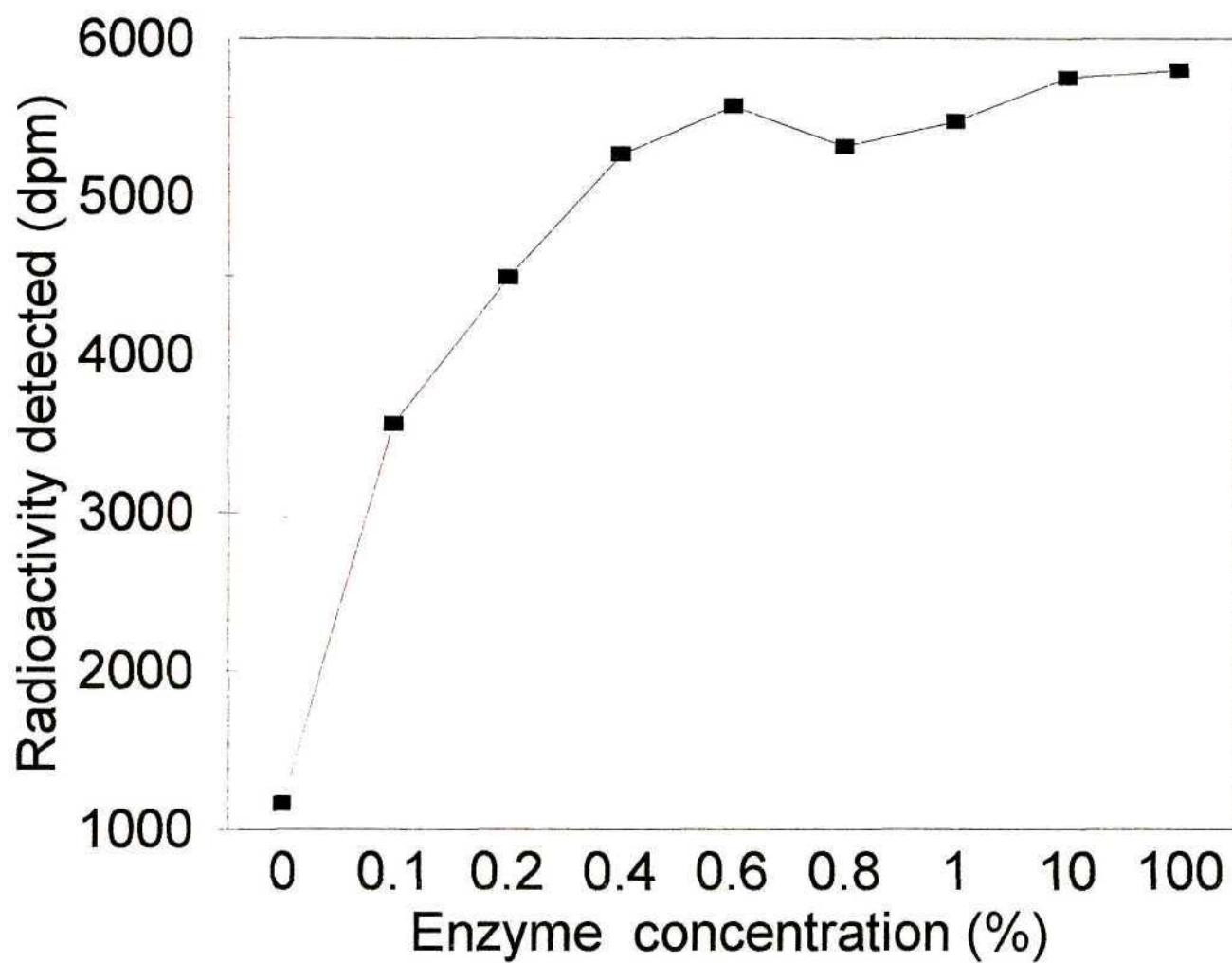


Figure 3. Effects of various enzyme concentrations on the conversion of arachidonic acid to prostaglandins in the cyclooxygenase bioassay.

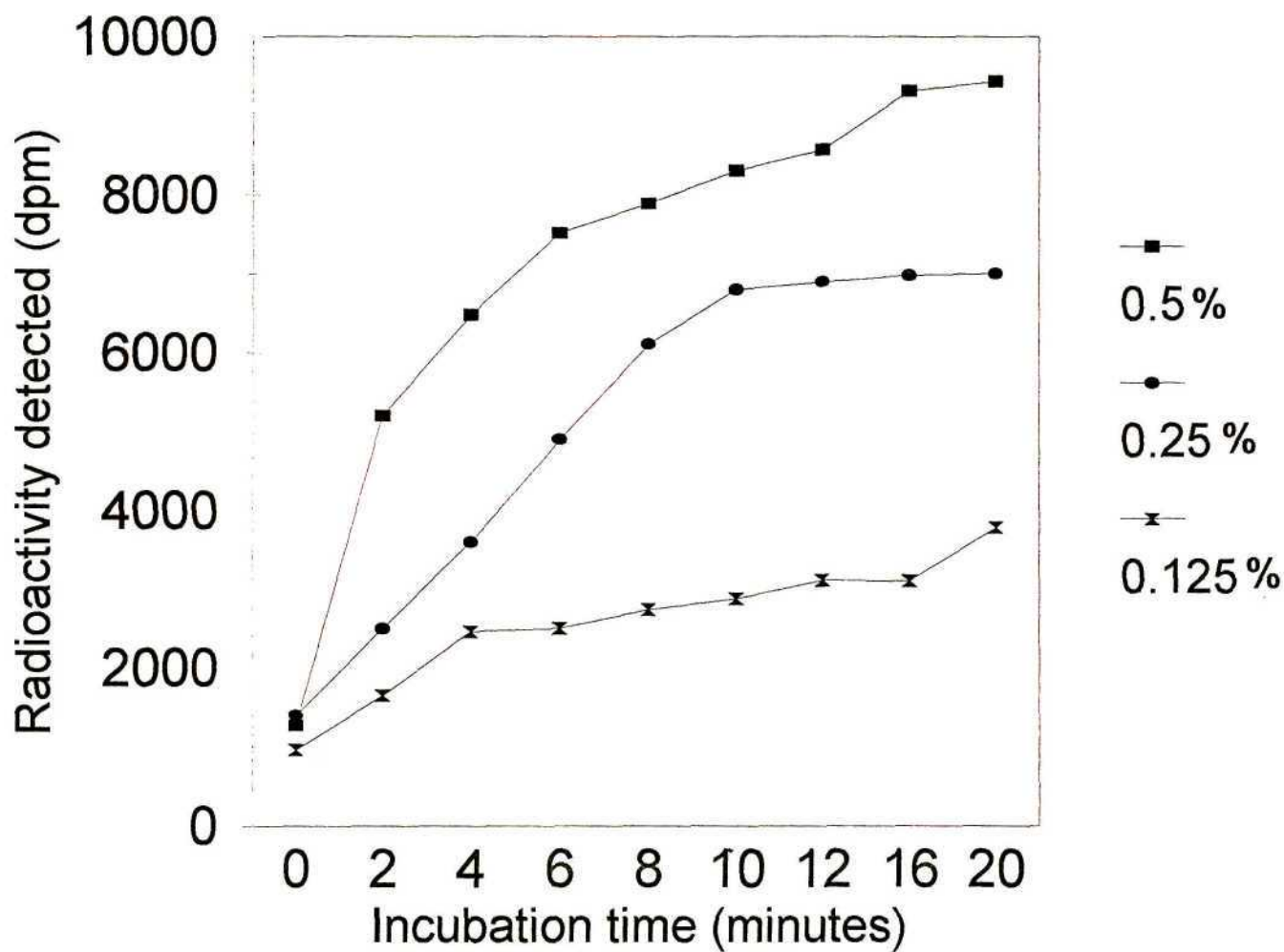


Figure 4. Effect of time of incubation and enzyme concentration (0.125 - 0.5%) on the conversion of arachidonic acid to prostaglandins.

3.2 Establishing an indomethacin standard curve

The effect of various concentrations of indomethacin on the inhibition of cyclooxygenase is given in Figure 5. For ideal assay purposes the inhibition of cyclooxygenase by the indomethacin standard should fall in the linear portion of the curve. It was decided that an inhibition in the region of 65% should be exhibited by the standard as this level of inhibition falls well within the linear portion of the curve. A concentration of 10^{-5} M, which exhibited a 63% inhibition of prostaglandin-synthesis was therefore used in all future cyclooxygenase bioassays as a standard reference.

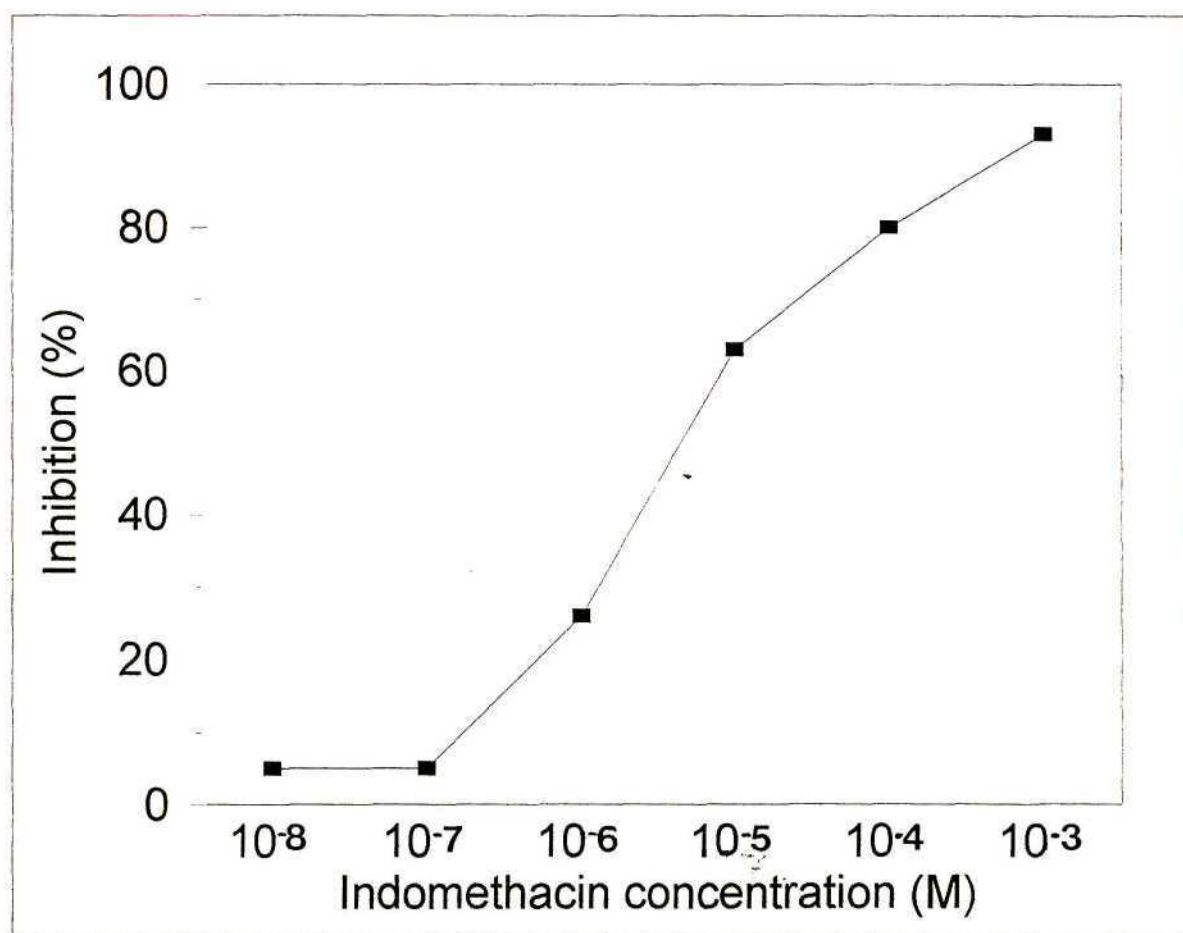


Figure 5. Standard curve for indomethacin in the cyclooxygenase bioassay.

3.3 Screening of medicinal plants for prostaglandin-synthesis inhibitors and uterine relaxing activity

There are many different methods of administration of plant remedies, most however involve crushing or grinding of material. This type of preparation is likely to improve the extraction of active compounds. The solvents that are most commonly available to traditional healers are water and ethanol, thus aqueous and ethanolic plant extracts were prepared for screening in the cyclooxygenase bioassay.

The results of the screening for prostaglandin-synthesis inhibitors are given in Table 3. The highest inhibition of cyclooxygenase was obtained with ethanolic extracts of *Siphonochilus aethiopicus*, *Solanum mauritianum* and *Cenchrus ciliaris*. In general, plant material extracted with ethanol showed higher inhibition of cyclooxygenase than did material extracted with water, except for *Vangueria infausta*, *Pentanisia prunelloides* and *Cenchrus ciliaris* material where aqueous and ethanolic extracts yielded similar results.

The indomethacin standard inhibited cyclooxygenase to a level of 67%. To classify a plant as active it was decided that the minimum inhibition by aqueous extracts must be 50% and for ethanolic extracts, 70%. This minimum inhibition criterion enabled the selection of plants for further studies. Using this criterion, eight out of the nine plants tested could be classified as active (Table 3).

It is important to note that plant extracts not exhibiting high activity in the

cyclooxygenase bioassay may not necessarily lack the ability to relieve menstrual pain. The active compound/s may act at other sites in the pain process.

The results of the investigation of the physiological actions of the ethanolic extracts on isolated strips of uterine smooth muscle are given in Table 4. All extracts except *Combretum erythrophyllum*, caused a mild contraction of the relaxed uterus.

Contraction of the uterus during menstruation is caused in part by prostaglandins. Several of the extracts tested showed high inhibition of cyclooxygenase, and should therefore prevent uterine contraction by blocking the biosynthesis of prostaglandins. With the exception of the *C. erythrophyllum* extract, none of the ethanolic extracts were able to reverse the contractions of the precontracted uterus (Table 4). This indicates that the main effect of the extracts is as prostaglandin-synthesis inhibitors. The contractory effect of the plant extracts seems to contradict the aim of reducing menstrual pains by preventing uterine contractions, but the inhibitory effect on prostaglandin biosynthesis might be the overriding effect, rendering the tested plant extracts useful in treating dysmenorrhoea.

Little information is available with respect to the chemical composition of the plants tested. Three species of *Combretum* were screened in this study and found to be active. It is generally accepted that when active compounds are found in one species, more species of the same genus will contain active compounds of a similar nature. Compounds isolated from *Combretum* species thus far include alkaloids, tannins, flavonoids (*C. micranthum*) and amino acids (*C. zeyheri*); substituted phenathrenes

from various heartwoods; a series of unique stilbenes and their glycosides and macrocyclic lactones known as combretastatins (*C. caffrum* and *C. kraussii*); and a variety of triterpenoid acids and their saponins, mainly of the cycloartane (*C. molle*) and oleanne (*C. imberbe*) type (VEROTTA and ROGERS 1996). The plant *Curcuma longa*, belonging to the family Zingiberaceae, the same family as *Siphonochilus aethiopicus*, contains curcumin which has anti-inflammatory activity (HAUNG, LYSZ, FERARRO, ABID, LASKIN and CONNEY 1991). The rhizomes of *Zingiber cassumunar*, a medicinal ginger plant, yielded curcuminoids which exhibit anti-inflammatory activity. This anti-inflammatory activity is due to the inhibition of arachidonic acid metabolism (MASUDA and JITOE 1994). *Solanum mauritianum* contains solasodine (DREWES and VAN STADEN 1995), a compound with known anti-inflammatory activity (LEWIS 1989). This may explain why the ethanolic extract from this plant was highly active. Sterols are found in the leaves of *Vangueria infausta* and peptide alkaloids in the bark and leaves of *Ziziphus mucronata* (HUTCHINGS, HAXTON-SCOTT, LEWIS and CUNNINGHAM 1996).

From the results obtained by screening the plant extracts in the cyclooxygenase bioassay (Table 3) it was decided to attempt to isolate the active compound(s) from the leaves of *Siphonochilus aethiopicus* (Schweinf.)B.L Burt., as they exhibited a high percentage inhibition of prostaglandin-synthesis, and leaf material could easily be obtained from Silverglen Nursery in Durban. It would also be good if this renewable plant part could be utilized rather than the rhizome which is capable of, and useful for, vegetative reproduction. In this way destructive harvesting could be minimized.

Table 2. Medicinal plants collected for screening.

Plant name and family	Plant part used	Administration	Voucher specimen number	Collection site
<i>Cenchrus ciliaris</i> L. (Poaceae)	underground runners	decoction	CORCORAN 1 UN	1
<i>Combretum erythrophyllum</i> (Burch) Sond. (Combretaceae)	leaves	-	BCROGERS 53 WARD	2
<i>Combretum platypetalum</i> Sond. (Combretaceae)	leaves	-	BCROGERS 51 WARD	2
<i>Combretum zeyheri</i> Sond. (Combretaceae)	bark	powdered bark introduced into the vagina	BCROGERS 52 WARD	2
<i>Pentanisia prunelloides</i> Walp. (Rubiaceae)	leaves roots	leaf poultice applied to the abdomen Decoction of root used to relieve pain	CORCORAN 2 UN	3
<i>Solanum mauritianum</i> Scop. (Solanaceae)	leaves	snuffed	JÄGER 26 UN	3
<i>Siphonochilus aethiopicus</i> (Schweinf) B.L Burt. (Zingiberaceae)	rhizome leaves	decoction of rhizome drunk to relieve menstrual pain leaves chewed during menstruation to relieve pain	McGAW 2 UN	5
<i>Vangueria infausta</i> (Burch.)Sond. (Rubiaceae)	leaves	decoction of leaves	CORCORAN 3 UN	3
<i>Ziziphus mucronata</i> Willd (Rhamnaceae)	leaves	leaf poultice applied to relieve pain	JÄGER 25 UN	3

Voucher specimens: UN, Herbarium of the University of Natal Pietermaritzburg. WARD, Herbarium of the University of Durban-Westville. Collection sites: 1 Mkuzi game reserve, Zululand; 2 University of Durban Westville, Westville; 3 Hilton, Pietermaritzburg; 4 Warick street market, Durban; 5 Silverglen Nature Reserve, Durban.

Table 3. Prostaglandin-synthesis inhibition by extracts from medicinal plants.

Plant name	Plant part tested	% Inhibition	
		H ₂ O	EtOH
<i>Cenchrus ciliaris</i>	underground runners	89	99
<i>Combretum erythrophyllum</i>	leaves	26	90
<i>Combretum platypetalum</i>	leaves	74	87
<i>Combretum zeyheri</i>	bark	83	62
<i>Pentanisia prunelloides</i>	leaves	83	87
	roots	71	79
<i>Siphonochilus aethiopicus</i>	rhizome	0	46
	leaves	26	93
	root tubers	54	83
	stem	0	43
<i>Solanum mauritianum</i>	leaves	0	96
<i>Vangueria infausta</i>	leaves	85	82
<i>Ziziphus mucronata</i>	leaves	0	87

Indomethacin standard 67 ± 7 %

Table 4. Physiological actions of ethanolic extracts on isolated strips of uterine smooth muscle.

Plant name	Baseline effect on uterine activity	Effect of plant extract on acetylcholine induced uterine function	Ability to relax an oxytocin induced contracted uterus
<i>Cenchrus ciliaris</i>	+	0	0
<i>Combretum erythrophyllum</i>	0	-	0
<i>Combretum platypetalum</i>	+	0	0
<i>Combretum zeyheri</i>	+	0	0
<i>Pentanisia prunelloides</i>	+	0	0
<i>Siphonochilus aethiopicus</i>	+	++	0
<i>Solanum mauritianum</i>	+	0	0
<i>Vangueria infausta</i>	+	0	0
<i>Ziziphus mucronata</i>	+	++	0

KEY 0 no response
 + contraction
 - inhibition of acetylcholine smooth muscle contractility

3.4 Isolation of active compounds from *Siphonochilus aethiopicus* leaves

A summary of steps used in the isolation of the active compound(s) is given in Figure 6.

Bulk extraction of 273 g dry, ground *Siphonochilus aethiopicus*

leaves with hexane by Soxhlet extraction

8.02 g residue



Silica gel columns separated with hexane:ethyl acetate

A B C D E F G H I



100 % activity. Combined, yielded 1.07 g residue



Sephadex LH20 column fractionation with methanol



1 2 3 4 5 6 7 8 9

92 % activity



Fractionation by HPLC

Figure 6. Summary of steps taken in the attempted isolation of active compound(s) from leaves of *Siphonochilus aethiopicus*.

3.4.1 Serial extraction

The amounts of residue produced in each of the solvents used in the serial extraction, together with the prostaglandin-synthesis inhibitory activity of each of the respective solvent extracts are given in Table 5.

Table 5. Amount of residue yielded following serial extraction of 2.78 g *Siphonochilus aethiopicus* leaves with different solvents and the inhibitory activity of the respective solvent extracts as determined using the cyclooxygenase bioassay.

Solvent used	Residue (mg)	Inhibition (%)
Hexane	80	94
Ethyl acetate	120	86
Ethanol	260	76
Water	210	69

Indomethacin standard ($2 \times 10^{-5}\text{M}$) 66%

All of the extracts exhibited some activity in the cyclooxygenase bioassay. The hexane extract exhibited the greatest amount of activity and it apparently extracted more of the active compound(s) than the other solvents. The water extract exhibited the lowest activity in the cyclooxygenase bioassay. It was not used in any further extractions.

This serial extraction was essentially a cold extraction. The extracted compounds were however, boiled in the solvent compartment of the Soxhlet apparatus. As each of the solvent extracts exhibited activity in the cyclooxygenase bioassay it is possible to

conclude that the active compounds must be heat stable.

3.4.2 TLC of solvent extracts from serial extraction

A solvent system of hexane:ethyl acetate (3:1) gave good separation of the compounds (Figure 7). It was therefore used for further TLC. The results of the TLC separation of solvent extracts from serial extraction using a solvent system of hexane:ethyl acetate (3:1) and the prostaglandin-synthesis inhibitory activity of each zone are given in Figure 7.

Hexane appeared to extract most lipophilic compounds (zone 5), although these exhibited little activity. The fact that hexane extracts contain inactive lipophilic compounds means that the hexane extract would be partly contaminated with these compounds. The original crude hexane extract did however, exhibit the greatest activity (Table 5). The activity appeared to decrease in the ethyl acetate and ethanol extracts with respect to zones 1 to 4. On the basis of these observations it was decided to use hexane as the solvent for bulk extraction.

3.5 Bulk extraction of *Siphonochilus aethiopicus* leaves

Bulk extraction of 273 g of leaves, using hexane as the solvent, yielded 8.02 g of residue.

The residue (4.8 g) of the bulk extraction that had been extracted a year earlier

exhibited 51% activity in the cyclooxygenase bioassay. It is possible to conclude from this that some of the active compounds may have broken down, but the extract did still contain considerable activity.

HEXANE:ETHYL ACETATE (3:1)

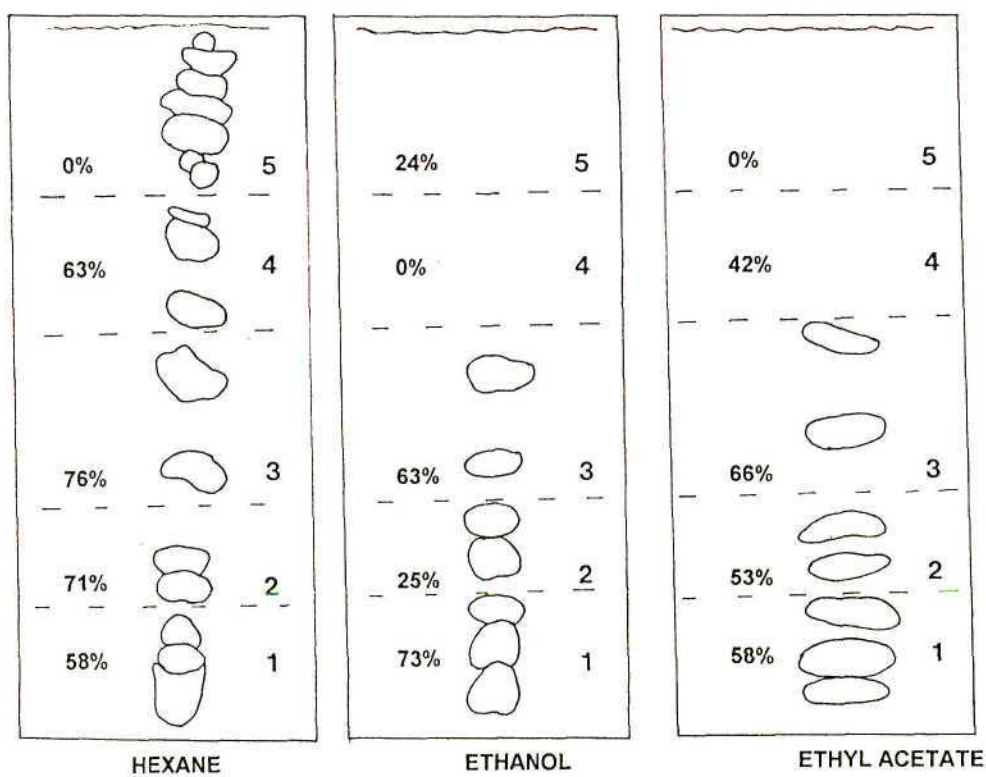


Figure 7. TLC separation of extracts from serial extraction and prostaglandin-synthesis inhibitory activity associated with each zone.

3.6 Silica gel column chromatography

The residue obtained from the bulk extractions were fractionated using silica gel columns. Results of TLC separation of fractions collected from the silica gel columns are given in Figure 8. As indicated in Figure 8 those fractions containing similar compounds were combined.

Figure 8 shows TLC separation of fractions collected from the silica gel column and prostaglandin-synthesis inhibitory activity of each of the combined fractions. Fractions containing similar compounds were combined. Combined fractions were taken to dryness yielding 2050 mg of A; 250 mg of B; 140 mg of C; 90 mg of D; 80 mg of E; 90 mg of F; 180 mg of G; 220 mg of H and 50 mg of I. High levels of prostaglandin-synthesis inhibitory activity were found in samples B, C, D, E, F, G, H and I respectively. Samples H and I exhibited 100% inhibitory activity.

Sample A was discarded as the level of prostaglandin-synthesis inhibitory activity was low. Both samples H and I appeared to contain similar compounds (as established by TLC) and exhibited the same level of activity. Samples H and I from subsequent silica gel columns yielded similar chromatographic profiles and they were therefore combined yielding 1070 mg residue. Samples H and I exhibited high (100%) prostaglandin-synthesis inhibitory activity and it was decided to concentrate all further isolation procedures on this combined extract.

HEXANE:ETHYL ACETATE (3:1)

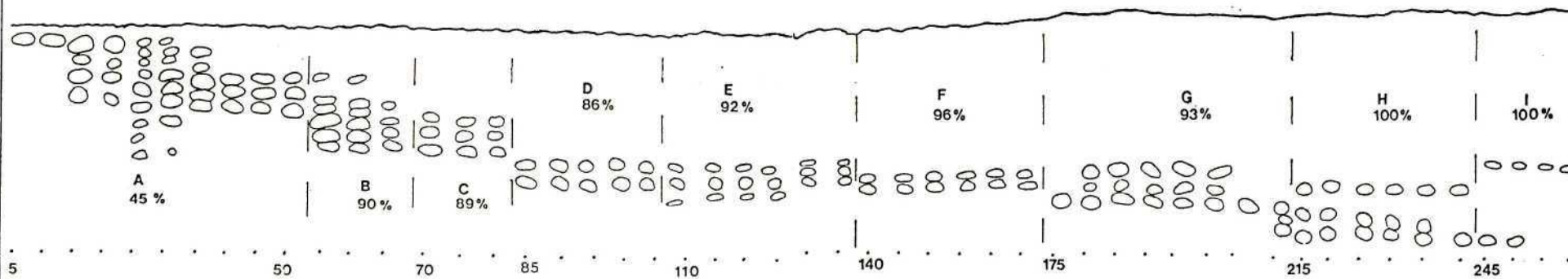


Figure 8. TLC separation of fractions collected from the silica gel column and prostaglandin-synthesis inhibitory activity of each of the combined fractions. Indomethacin standard 65%.

3.7 Sephadex column chromatography

3.7.1 Small Sephadex column using methanol

Results of TLC separation of fractions collected from the small Sephadex column and prostaglandin-synthesis inhibitory activity of combined fractions are given in Figure 9. This solvent system gave some compound separation. Fractions containing similar compounds were combined as shown in Figure 9. Samples were taken to dryness yielding 40 mg of 1; 20 mg of 2; 10 mg of 3; 10 mg of 4; 20 mg of 5; 20 mg of 6; 40 mg of 7; 10 mg of 8 and 20 mg of 9 respectively. Samples 3 and 4 yielded the highest activity in the cyclooxygenase bioassay. Consequently further large, Sephadex columns were run using methanol as the solvent. It was hoped that an increase in column length would increase compound separation.

3.7.2 Large Sephadex column using methanol

Results of TLC separation of fractions collected from the Sephadex column, used to separate 375 mg of combined sample H and I, are given in Figure 10. Some compound separation was achieved and fractions showing similar compound separation as indicated by TLC were combined. The pattern of compound separation was very similar to that obtained for the fractions eluted with methanol from the small Sephadex column (Figure 9). Four hundred and eighty mg of combined sample H and I was processed in the same manner and reproducible results obtained. Fractions containing similar compounds were combined. Samples combined from all columns

were taken to dryness yielding 400 mg of 1; 40 mg of 2; 121 mg of 3; 130 mg of 4; 70 mg of 5; 50 mg of 6; 40 mg of 7; 50 mg of 8 and 60 mg of 9. As the highest activity in the cyclooxygenase bioassay was found in samples 3 and 4, it was decided to continue isolation techniques with these samples.

HEXANE:ETHYL ACETATE (3:1)

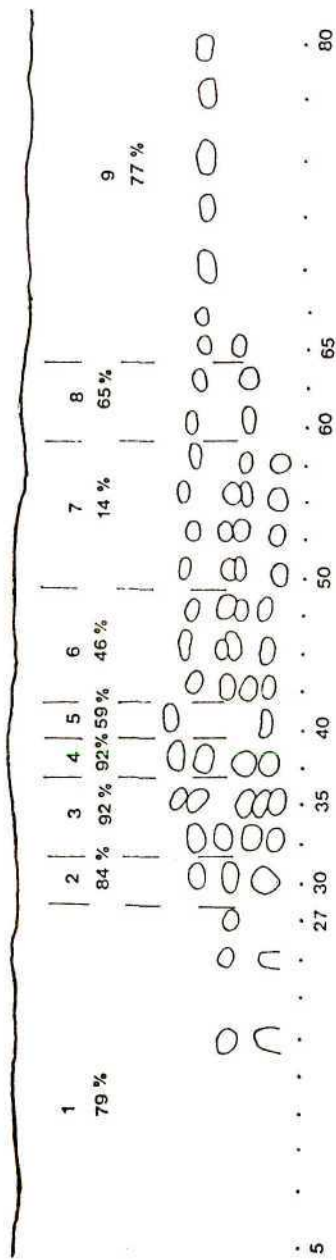


Figure 9. TLC separation of fractions collected from a small Sephadex column eluted with methanol and the cyclooxygenase activity associated with each of the combined fractions. Indomethacin standard 65%.

HEXANE:ETHYL ACETATE (3:1)

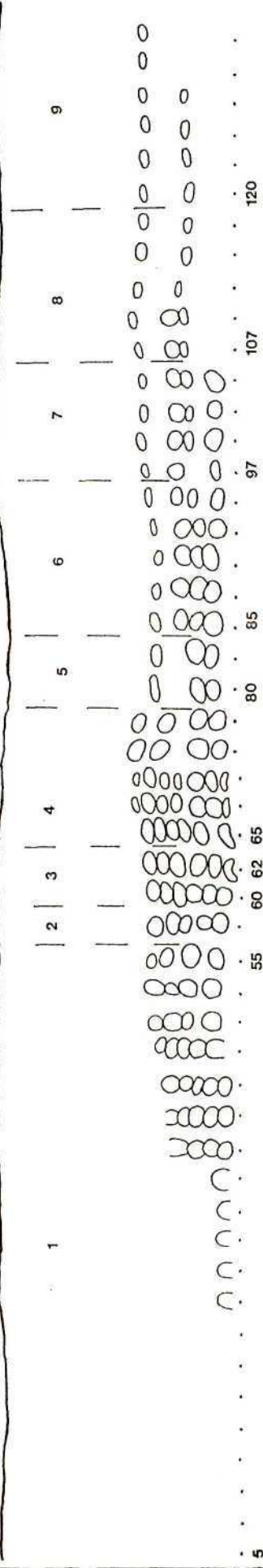


Figure 10. TLC separation of fractions from combined sample H and I (375 mg) separated on a large Sephadex column eluted with methanol.

3.8 High pressure liquid chromatography

3.8.1 HPLC of sample 3 (gradient)

A gradient elution programme starting at acetonitrile:water (10:90) and running into acetonitrile:water (100:0) over 30 minutes followed by a 10 minute wash in 100% acetonitrile gave reasonable separation of compounds. Unfortunately base line separation was not achieved and thus fractions were not pure and contained a number of compounds. Based on the presence/absence of UV-peaks it was decided to test those fractions yielding UV-peaks, for cyclooxygenase activity. Results of this activity are given in Figures 11 and 12 respectively.

Where 60 μg of sample 3 was injected onto the column (Figure 11), the greatest cyclooxygenase activity was found in fractions 36 to 39. This activity corresponded to a large UV-peak detected on the chromatogram. Two hundred μg of sample 3 was then injected onto the column and the same programme used to obtain separation of compounds (Figure 12). The highest cyclooxygenase activity was found in fractions 31 to 40. This pattern of activity corresponded to that obtained for the earlier run sample. This activity corresponded to a large UV-peak obtained at 35 to 39 minutes, and a small UV-peak that was not detected on the earlier chromatogram (Figure 11) at 30 to 32 minutes.

3.8.2 HPLC of sample 4 (gradient)

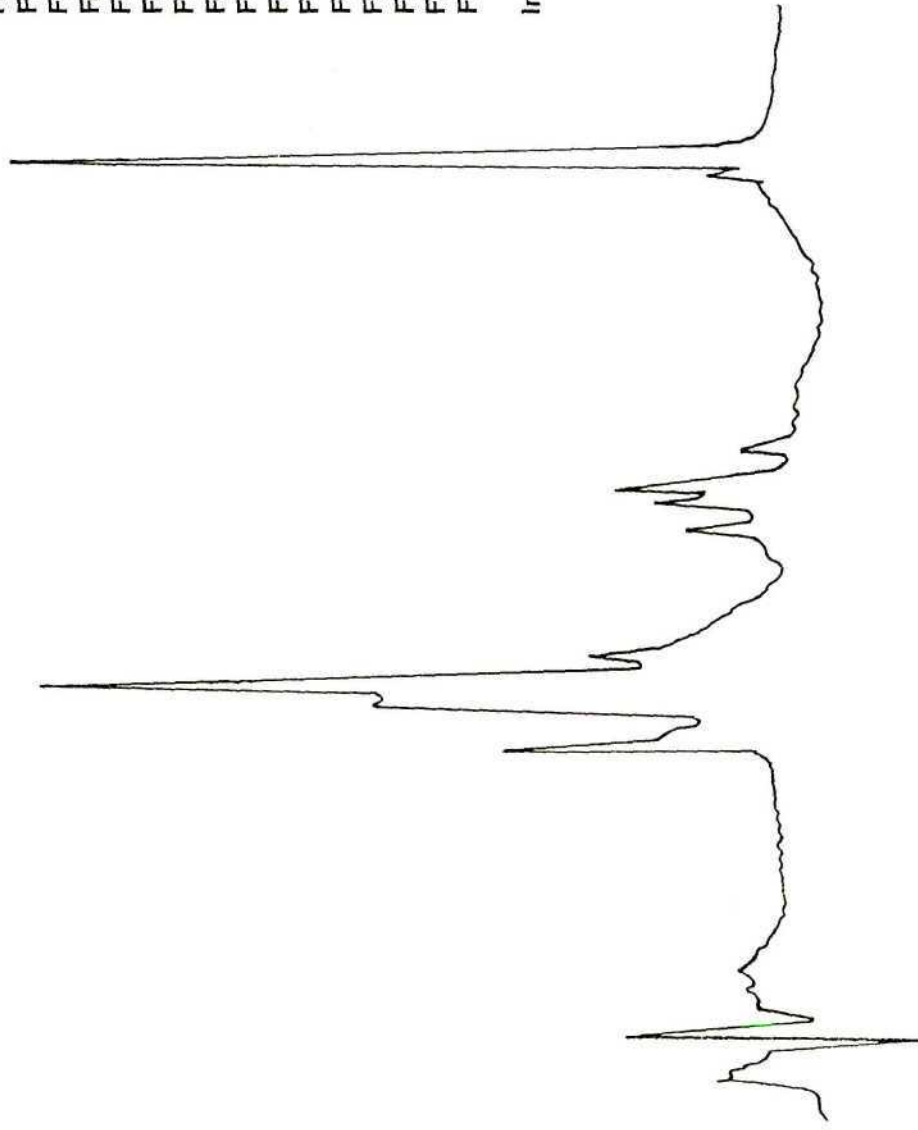
A gradient elution programme starting in acetonitrile:water (10:90) and running into acetonitrile:water (100:0) over 30 minutes followed by a 10 minute wash in 100% acetonitrile gave adequate separation of compounds. (Figure 13) Separation of compounds was better than that obtained for sample 3. Based on the presence/absence of UV-peaks it was decided to test those fractions corresponding to UV-peaks for activity in the cyclooxygenase bioassay. The highest activity was found in fractions 30 to 37 (Figure 13). The activity present in fractions 30, 31 and 32 corresponded to a UV-peak in this region. It is possible that the active compound(s) in this region are the same as observed for sample 3, although sample 4 appeared to contain a far greater amount of this compound(s) than did sample 3. The activity for fractions 38, 39 and 40 corresponded to a larger UV-peak, however, activity here was less than that present in similar fractions obtained for sample 3. It is possible that less of the active compound(s) that was eluted at this time was present in sample 4 than in sample 3.

Further HPLC is necessary to separate the active compounds in this sample. Possibly through the use of an isocratic programme running in acetonitrile one may better separate active compounds and thus obtain purer fractions that would allow for successful NMR and GC-MS.

FLOW RATE 1ml/minute

- Fraction 5 0%
- Fraction 6 0%
- Fraction 7 0%
- Fraction 14 15%
- Fraction 15 20%
- Fraction 16 15%
- Fraction 17 1%
- Fraction 18 18%
- Fraction 19 28%
- Fraction 22 0%
- Fraction 23 25%
- Fraction 24 15%
- Fraction 25 30%
- Fraction 26 37%
- Fraction 36 55%
- Fraction 37 49%
- Fraction 38 50%
- Fraction 39 56%

Indomethacin standard 64%



0 30 40
10A:90W 100A:0W 100A:0W

0
10A:90W

Figure 11. HPLC chromatogram of sample 3 (60 μg injected onto column)

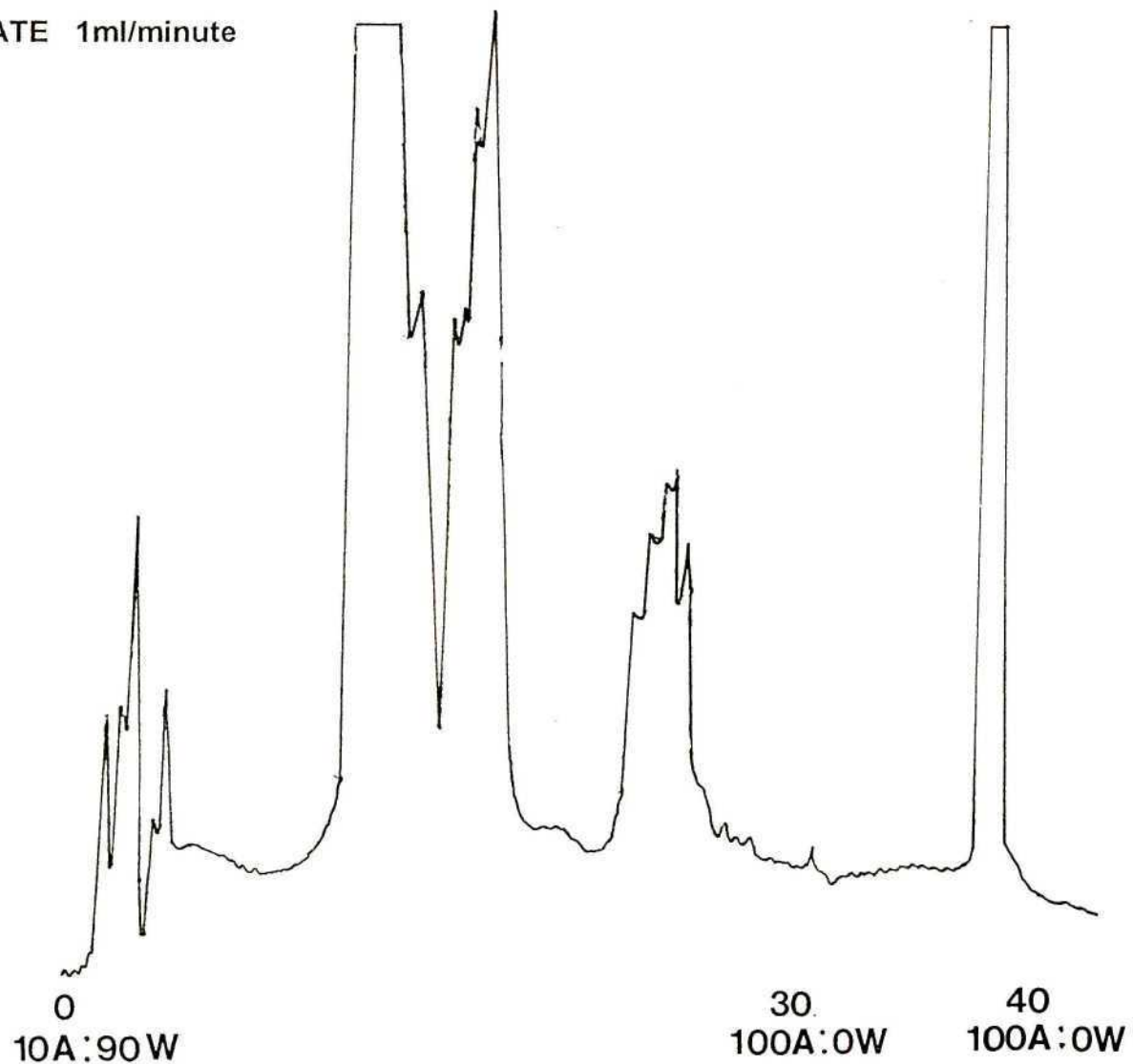
A - acetonitrile; W - water

Eluent programme: Time 0 minutes : acetonitrile:water (10:90)

Time 30 minutes:acetonitrile:water (100:0)

Time 40 minutes:acetonitrile:water (100:0)

FLOW RATE 1ml/minute



Fraction 4	0%
Fraction 5	15%
Fraction 10	0%
Fraction 11	0%
Fraction 12	0%
Fraction 13	0%
Fraction 14	0%
Fraction 15	0%
Fraction 16	0%
Fraction 17	0%
Fraction 18	0%
Fraction 19	0%
Fraction 21	0%
Fraction 22	22%
Fraction 23	20%
Fraction 24	23%
Fraction 25	34%
Fraction 26	40%
Fraction 31	94%
Fraction 32	100%
Fraction 33	82%
Fraction 34	81%
Fraction 36	72%
Fraction 37	82%
Fraction 38	81%
Fraction 39	65%
Fraction 40	58%

Indomethacin standard 67%

Figure 12. HPLC chromatogram of sample 3 (200 μg injected onto column)

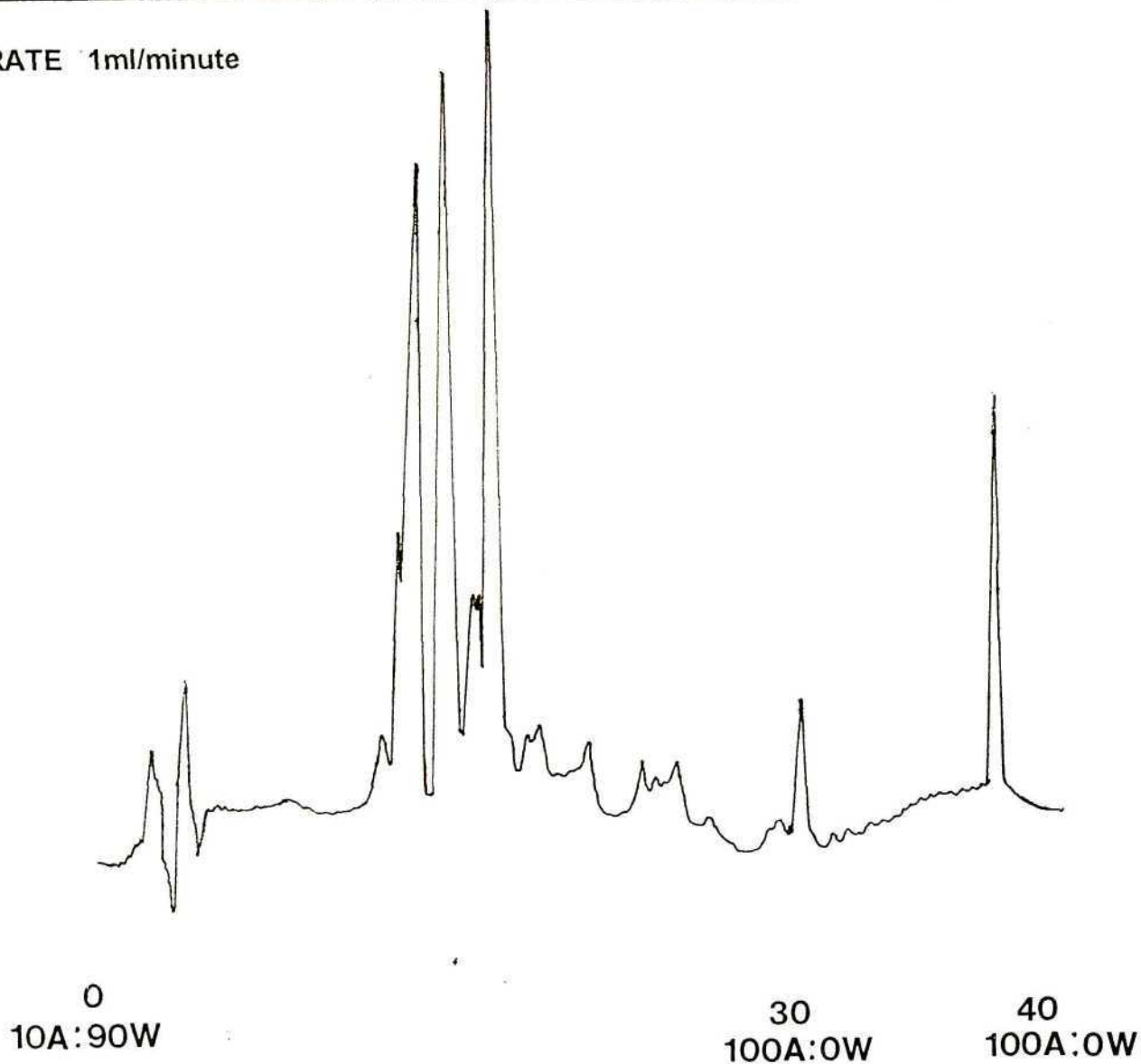
A - acetonitrile; W - water

Eluent programme: Time 0 minutes : acetonitrile:water (10:90)

Time 30 minutes:acetonitrile:water (100:0)

Time 40 minutes:acetonitrile:water (100:0)

FLOW RATE 1ml/minute



Fraction 3	0%
Fraction 4	0%
Fraction 11	0%
Fraction 12	0%
Fraction 13	0%
Fraction 14	0%
Fraction 15	0%
Fraction 16	0%
Fraction 17	0%
Fraction 18	0%
Fraction 19	0%
Fraction 22	0%
Fraction 23	0%
Fraction 26	0%
Fraction 27	16%
Fraction 28	16%
Fraction 29	39%
Fraction 30	51%
Fraction 31	43%
Fraction 32	40%
Fraction 36	42%
Fraction 37	46%
Fraction 38	32%
Fraction 39	22%
Fraction 40	11%

Indomethacin standard 65%

Figure 13. HPLC chromatogram of sample 4 (60 μ g injected onto column)

A - acetonitrile; W - water

Eluent programme: Time 0 minutes : acetonitrile:water (10:90)

Time 30 minutes:acetonitrile:water (100:0)

Time 40 minutes:acetonitrile:water (100:0)

3.9 Cyclooxygenase inhibitory activity of the year-old extract

The year-old extract was processed in the same manner as the fresh extract and the cyclooxygenase inhibitory activity followed through each isolation step. Activity was present in the same fractions as the fresh extract indicating that it is likely that the active compound(s) are the same as those in the fresh extract. As this activity was present a year after the initial bulk extraction was performed, it is possible to conclude that the active compound(s) are stable over time.

3.10 Final comments

Most of the plants screened in this study exhibited cyclooxygenase inhibitory activity. The high number of active plants indicates that over time traditional healers have selected for plants with activity and emphasise the considerable importance of the ethnobotanical approach.

Generally ethanolic extracts gave higher cyclooxygenase inhibitory activity than the aqueous extracts. None of the ethanolic plant extracts were able to relax or reduce the contractions of the precontracted guinea pig uterus indicating that the inhibitory effect on prostaglandin-synthesis might be the overriding effect, rendering the tested plant extracts useful in treating dysmenorrhoea. An attempt was made to isolate the active compound(s) from the leaves of *Siphonochilus aethiopicus*. A hexane solvent was found to extract the active compounds, indicating that the compounds must be lipophilic in nature. Using bioassay guided fractionation it was possible to follow the biological

activity through a series of isolation steps. A possible HPLC programme was developed to separate the active compounds. However, further HPLC is necessary to obtain good enough separation of compounds to allow for successful purification for NMR and GC-MS.

The high cyclooxygenase inhibitory activity present in the leaves of *Siphonochilus aethiopicus* serves to confirm the use of this plant by traditional healers in the treatment of dysmenorrhoea. This study showed that ethanolic extracts of leaves of *Siphonochilus aethiopicus* inhibit prostaglandin-synthesis to a greater level than that of rhizomes. It is hoped that this information may be used to persuade traditional healers to substitute leaves for rhizomes and in this way give these plants a chance to increase in the wild. It is further possible to conclude that there are a number of active compounds present in the leaves of this plant and that many of these compounds are stable over time.

The demonstrated ability of ethnobotany in the discovery of new drugs suggests that for the near future it will occupy an expanding role in drug development. Ethnobotanists can capture remaining knowledge, however, they are in a race against time as plant knowledge seems to be disappearing as fast as the medicinal plants.

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