CHEMICAL AND PHYSIOLOGICAL STUDIES ON ARISTOLOCHIA PETERSIANA KLOTZ

A DISSERTATION SUBMITTED TO THE SCHOOL OF NATURAL SCIENCES OF THE UNIVERSITY OF ZAMBIA IN PART-FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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By

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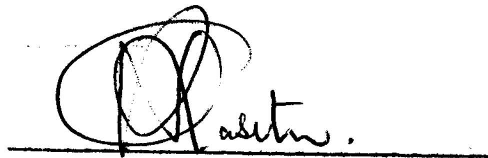

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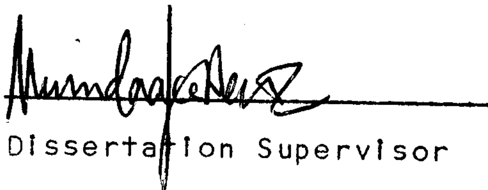

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DECLARATION

It is hereby declared that this dissertation is my own work and that it has not been previously submitted for degree purposes here or any other University.

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A B S T R A C T

The plant 'Ntulula' (*Aristolochia petersiana* klotz) native to Zambia has been used in Zambian folk-lore medicine as an abortifacient. This work presents the Physiological and Chemical studies on this plant. Preliminary experiments confirmed the abortifacient activity of *A petersiana* klotz in both pregnant white albino rats and timed pregnant Swiss webster mice (CDI). The plant was also shown to regulate fertility in female white albino rats and to stimulate isolated rat uterus. In subsequent work, the plant was screened for biologically active compounds. An unknown ninhydrin reactive material suspected to be the active compound was detected. Aristolochic acid was detected and characterised for the first time in this species of the Aristolochiaceae.

CHAPTER I

INTRODUCTION

In the past, the importance of plant derived medicines was often underestimated. Although plant derived drugs like morphine and quinine were known, most drugs that have been used in medicine are of synthetic type. Research efforts in finding new drugs have mainly involved synthesis of derivatives of the already existing drugs.

Recent research activities in the field of natural products have been directed at finding new biologically active compounds of plant origin. In the developing countries, interest in these research activities has been for economic reasons in terms of self reliance. The Health Ministry in Zambia has recognised the importance of plant derived medicines by setting up an Association of Traditional Herbalists, which is aimed at incorporating indigenous medicines into modern medical practice. The importance of these research activities must therefore be viewed from two closely related facts:

(a) In many parts of the world there is still a strong patronage of traditional healers despite the presence of modern medical expertise.

(b) Up to 1960, a study of clinically prescribed drugs showed that over half of these drugs contain compounds of natural origin (12). In 1967, a similar analysis of 1.05×10^9 prescriptions dispensed in community pharmacies showed that 25% of them contained one or more active principles of plant origin (13).

Among a broad range of pharmacologic activities possessed by biologically active plant constituents are the antineoplastic, antimalarial, insecticidal and androgenic, estrogenic and related activities. Quite recently the androgenic, estrogenic and related activities have become a fertile area for immediate development. With the current world population problem, there has been a rising need to provide oral contraceptives, which are less expensive and acceptable. The synthetic oral contraceptives available now, although very effective have some side effects (43b) and therefore have a bad reputation. It is thought that most people especially in the rural areas still prefer to use folk-lore antifertility agents (44). However, it is thought that a good proportion of women who practice these folk-lore methods end up conceiving. New research in this field has been directed towards finding oral contraceptives of direct plant origin and which are based on folk-lore knowledge and practice. Such contraceptives if incorporated with modern medicine may be more acceptable and presumably less expensive.

The World Health Organisation is currently encouraging and financing work in various Universities aimed at isolating fertility regulating compounds from plants. The problem associated with this work has been the choice of candidate plants to be studied out of the hundreds of species distributed all over the world. WHO has computerised data (43a) which investigators can use for selection of plants. This avoids duplication on those plants which have already been studied and whose active constituents are already known. Investigators select a plant whose priority rank is high on the computer list. A plant is ranked high if:

(a) From indigenous medical practice its plant extract most times affects the claimed physiological activities; has a long history of human use so that toxicity problems would be expected to be minimal; and has a wide use geographically.

(b) Reports on _in vitro_ bioassay studies suggest uterine stimulant effect or some other activities which could be correlated with the claimed effects of the plant.

(c) Plant extracts on _in vivo_ studies reported to affect the various phases of the reproductive cycle in laboratory animals which relate to anovulatory, abortifacient, anti-implantation and embroyotoxic effects.

Once a plant is chosen for study, the claimed effects are first justified. Indigenous preparations are duplicated and tested on animal systems according to the WHO Task Force protocol (43a). Chemical analysis follow where positive results are obtained in the animal tests.

Comprehensive literature (7,12,13,26,34) extending through 1973 is available on plants which are known as antifertility agents, abortifacients, ecbolics, oxytoxics and emmenagogues. Farnsworth et al (12) cited that of these plants, a total of two hundred and twenty two species are known in folk-lore as contraceptives and/or interceptives and one hundred and forty five species are reported to have been screened for antifertility effects in laboratory animals or humans. Further, very few, indeed active antifertility principles have been isolated from them. Only five active antifertility principles from higher plants are reported (12) to have been purified. These are M-xylohydroquinone (2,6-dimethylhydroquinon), Lithospermic acid, Coronaridine, Rutin and Rottlerin. Only three antifertility agents have been reported (12) as uncharacterised. Farnsworth et al (12) also cited that five hundred and sixty five species of these plants are known in folk-lore as abortifacients, ecbolics or emmenagogues. Of these species, two hundred and twenty

five have been shown to demonstrate stimulant response when tested against uterine muscle either in vivo or in vitro and active compounds have been isolated from one hundred and ninety eight species. Of these isolated compounds, one hundred and forty eight have been characterised and twenty four are uncharacterised. However, no literature has indicated that extracts of these plants or the isolated active principles have been proved as effective abortifacients. In some plants, the active principles have demonstrated toxicity when used in bioassays at high dose levels or cytotoxicity, such that the abortifacient activity has been explained in terms of toxicity or cytotoxicity (12,13). This has tended to limit further research into these plants as fertility regulators. Some known bioassays, in which plants were studied for fertility regulating activities and in some cases active principles isolated, are those of Saha, Savin and Kasinathan (34), Guerra and Andrade (20), Gran (16,17,18) and Lipton (24). Saha, Savin and Kasinathan (34) carried out bioassays on seven Indian indigenous plants to determine the oxytoxic properties of extracts from these plants. Guerra and Andrade (20) showed that Gossypium herbaceum L and Ruta graveolens inhibit implantation in pregnant **rats.** Gossypol, a cytotoxic agent was isolated from Gossypium herbaceum L (20) and it was believed that

the agent was responsible for the plant's effect on the reproduction. Gran (17) isolated serotonin and an oxytoxic polypeptide from 'Kalata-Kalata' (*Oldenlandia affinis*). Lipton (24) also isolated an oxytoxic glycoside, albitocin from *Albizia gummifera* and later showed that the glycoside acts on the uterus of a variety of species *in vivo* and *in vitro*.

Species of the Aristolochiaceae have been used and are still used in fertility regulation in Europe, America, Asia and Africa (12,14,37). However, very little research has been done on the fertility regulating activities of the Aristolochiacoae. Only five or six species have been rigorously chemically studied (14). Up to 1970, five compounds namely Aristolochic acid, Magnoflorin, Aristolactam, l-Curine and Debilic acid (14) were reported to have been isolated in all the species, which were studied. Of late only the sesquisterpene reported by Pakrashi and Shaha (30) has shown to be active in fertility regulation. In the Aristolochiaceae, bioassays have been conducted on only three species. These are those of Pakrashi and Shaha (30) on *Aristolochia Indica* linn, Angeles et al (2) on Aristolochic acid isolated from *A tangala* Cham and Saha, Savin and Kasinathan (34) on *A bracteata* Retz. There has however been a general tendency to attribute the fertility regulating activities of the Aristolochiaceae to the

presence of Aristolochic acid. It is thought that Aristolochic acid due to its cytotoxicity may be responsible for the fertility regulating activities of this family. This has tended to limit further research into the Aristolochiaceae. However, the family has for a long time been used in folk-lore for fertility regulation in humans. Cytotoxicity effects from their extracts must therefore be minimal. Further, the work of Pakrashi and Shaha (30) has revealed that other antifertility agents may be present in the Aristolochiaceae.

In this work, Aristolochia petersiana klotz (Aristolochiaceae) was studied. To date, no studies on this plant have been done. The scope of this work was to detect and isolate Aristolochic acid in A petersiana klotz and then test the remaining fractions for physiological activities. It was hoped that the assumption that Aristolochic acid is the fertility regulating agent of the Aristolochiaceae would be reversed, if the remaining fractions were physiologically active.

CHAPTER 2

B I O A S S A Y   S T U D I E S

Aristolochia petersiana klotz (Aristolochiaceae) was identified and authenticated by the Royal Botanical Gardens (Edinburgh). A specimen has been deposited with the Herbarium in the Department of Biology of the University of Zambia. Members of the Aristolochiaceae mainly occur in the tropical and warm temperate zones. Most species are climbing shrubs, poisonous and bitter tasting. Principal constituents of these species are alkaloids, Aristolochic acid, phenolic esters and ethers volatile oils and flavonoids. Aristolochic acid, a cytotoxic compound is present in most species of the genus *Aristolochia* that have been studied. The genus *Aristolochia* is the principal genus and has 350 speçies (40) of which three are found in Zambia, distributed mainly in the Eastern, Southern and Western Provinces (10).

Studies on the physiological properties of *A petersiana* klotz were undertaken because of the folk-lore reputation of the plant in Zambia as an abortifacient - hence its local name in Southern Province 'Ntulula' (37). Elsewhere members of the Aristolochiaceae are used medicinally and are reported to affect physiological activity in a manner related to fertility regulation (7,26,34). Pakrashi and Shaha (30) in a similar study have isolated an anti-implantatic and interceptic sesquisterpene

from Aristolochia indica linn, a species used in India to induce abortion.

In this work, preliminary experiments confirmed the folk-lore reputation of A petersiana klotz as an abortifacient and in addition it was also shown that the plant has fertility regulating properties. Physiological experiments were performed both in vivo and in vitro. These results were confirmed in experiments conducted at Harvard Medical School following the WHO Task Force protocol (43a).

Traditional practice suggested that roots and stems of A petersiana klotz are each capable of inducing abortion. The folk-lore practice is to soak either the roots or the stems in warm water, although most people use mainly the roots. A cupful of the water extract is then drunk and this is sufficient to induce abortion in a pregnant woman.

The extracts used in these studies were made by soaking ground or powderised roots and stems in water for forty eight hours, with constant stirring, filtering the suspension to obtain a clear filtrate. White mature albino rats were used as test animals in the initial exploratory experiments and Swiss webster mice (CDI) were used in the confirmatory experiments.

2.1 In vivo exploratory experiments

Two *in vivo* experiments were performed: One experiment tested the activity of *A petersiana* klotz as an abortifacient and the other experiment was to establish whether the plant is active in fertility regulation.

In the first *in vivo* experiment pregnant white albino rats were administered both orally and by intraperitoneal injection with the aqueous plant extract. In both cases, the rats aborted within few hours of administration of the extract. The second *in vivo* experiment used twelve male and twelve female mature white albino rats. These were randomly selected and placed in cages, in pairs of one female and one male per cage. The paired rats were arranged in three groups A, B and C of four pairs each. In group A the female rats were injected intraperitoneally every alternate day with normal saline solution. In group B the female rats received aqueous extract of root and stem by intraperitoneal injection every alternate day. The male rats of group C received a similar treatment to the female rats of group B. The results of the experiment are tabulated in table 2.1.

Table 2.1 In vivo experiment to test the activity of A petersiana klotz in regulating fertility in white albino rats.

Group	Group treatment	Mating pair 1x♀ + 1x♂	Delivery time (days)	Number of young per litter
A	♀ rats injected intraperitoneally with normal saline on alternate days	1	29	8
		2	-*	-
		3	28	7
		4	29	9
B	♀ rats injected intraperitoneally with aqueous extract on alternate days	1	NIL**	NONE
		2	NIL**	NONE
		3	NIL**	NONE
		4	NIL**	NONE
C	♂ rats injected intraperitoneally with aqueous extract on alternate days	1	29	6
		2	30	7
		3	27	5
		4	28	6

* ♀ rat died on 19th day of experiment

** experiment continued up to 60th day

In group A one female rat died and the remaining three rats delivered on average each eight young per litter, the average day of delivery was the twenty ninth day. In group B the female rats did not show any signs of conception over a period of sixty days. In group C the female rats delivered on average each six young per litter and the average day of delivery was the twenty ninth day.

2.2 In vivo confirmatory experiments to the first in vivo exploratory experiment

These were performed at the Harvard Medical School using the procedure which departed slightly from that recommended by the WHO Task Force (43a). Unoestrogenised timed pregnant Swiss webster mice (CDI) were used instead of rabbits. The mice were obtained from Charles River Laboratories, Boston, Massachusettes. For these mice the gestation period was said to be twenty one days and this was longer than the normal gestation period for mice (nineteen to twenty days (Schwartz, (35)). In all cases mice were kept for at least one day prior to dosing in order to acclimatise them.

The first experiment used eight timed pregnant mice each of average weight 37.1 g. These were obtained on their fifteenth day of pregnancy and on the nineteenth day of pregnancy, which was two days before expected parturition, the mice were each administered orally with less than 0.3 cm^3 aqueous solution. This dosage was about 78 mg of dried plant extract per kg weight of live mice. Table 2.2 gives the results of the experiment. Two mice died accidentally as a result of the extract being administered into the lungs and the remaining six mice delivered live foetuses in three to nine hours of administration of aqueous extract.

The above experiment was repeated two further times using mice in their fifteenth and fourteenth days of pregnancy in order to investigate whether the plant induces abortion in mice in the middle stage of pregnancy. These experiments also aimed at establishing the gestation period on account of the differences in the gestation period quoted by the Charles River Laboratories and Schwartz.

Table 2.2 *In vivo* experiment to test the abortifacient activity of *A petersiana* klotz using Swiss webster mice (CDI).

Mouse	Weight of mouse when received on the 15th day of pregnancy (g)	Weight of mouse at time of oral administration of aqueous extract on the 19th day of pregnancy (g)	Results and Comment
a	36	54.5	Aborted within 25 hours of administration
b	36	50.0	Aborted within 8 hours of administration
c	38	47.5	Aborted within 9 hours of administration
d	38	55.5	Aborted within 3 hours of administration
e	37	47.5	Aborted within 3 hours of administration
f	37	50.2	Aborted within 3 hours of administration
g	36	-*	-*
h	39	-*	-*

Mice administered orally with 0.3 cm^3 aqueous extract, dosage being about 78 mg dried plant extract per kg weight of live mice.

* Mice died during administration of extract.

In the first experiment, seven timed pregnant mice were obtained in their thirteenth day of pregnancy. On the fifteenth, sixteenth, seventeenth and eighteenth days, three mice (mice a,b and c) were *each administered orally with 0.3 cm^3 of aqueous extract* and one mouce (mouse d) was administered with the same vehicle of normal saline solution by the same route. On the fifteenth day, one mouse (mouse e) was injected intraperitoneally with 0.3 cm^3 of the same aqueous extract as the above and another mouse (mouse f) received 0.3 cm^3 normal saline solution by the same route. The seventh mouse (mouse g) was a control. Dosage of 0.3 cm^3 of aqueous extract corresponded to 62.55 mg of the dried plant matter per mouse or 1251 mg of dried plant matter per kg body weight of live mice. Table 2.3a gives the results of this experiment. These results were inconclusive because only mouse e which received the water extract by intraperitoneal injection aborted within twenty four hours of administration of extract. The remaining mice gave the following results: Mice a,b and c lost each on average 3.0 g in weight, mouse c died on day nineteen while mice a and b delivered dead young on day twenty.

Table 2.3a. *In vivo* experiment to test for abortifacient activity in aqueous extract of *A petersiana* klotz using Swiss webster mice (CDI) in their 15th day of pregnancy.

Mouse	Weight of live mouse when received on the 13th day of pregnancy (g)	Weight of live mouse at time of administration of aqueous extract (g)	Treatment	Result and Comment
a	35.0	43.0	0.3 cm^{3*} administered orally on the 15th, 16th 17th and 18th days of pregnancy at the same hour	All three mice lost each on average 3.0g in weight. Mice a and b delivered dead young on the 20th day and mouse c died on the 19th day
b	35.0	41.0		
c	38.0	46.0		
d	37.0	44.0	0.3 cm^3 saline solution administered orally on the 15th, 16th, 17th and 18th days of pregnancy at the same hour.	Delivered 10 young on day 20
e	37.5	43.0	0.3 cm^{3*} administered by intraperitoneal injection	Aborted 9 foetuses within 24 hours of administration
f	35.0	40	0.3 cm^3 saline solution administered by intraperitoneal injection	Delivered a normal young on day 20
g	35.4	44.1	control, no dosage	Delivered a normal young on day 20

* 0.3 cm^3 = 62.55 mg of dried plant extract

In the second experiment nine timed pregnant mice were obtained in their twelveth day of pregnancy. On the fourteenth day, five mice (mice a,b,c,d and e) were each administered intraperitoneally with 0.3 cm^3 (62.55 mg) aqueous dried plant extract, two mice (mice f and g) were administered by the same route with 0.3 cm^3 (39.21 mg) freshly prepared aqueous dried plant extract and mouse h received normal saline solution intraperitoneally. The nineth mouse (mouse i) was a control. Table 2.3b gives the results of this experiment. Mice h and i each delivered a live young on day twenty one, while mice f and g aborted foetuses on the fourteenth day and died within few hours of abortion. Of the mice a,b,c,d and e, mouse a died during administration of extract, mice b and c aborted and then died in twenty four hours of intraperitoneal injection of aqueous extract and mice d and e did not abort, but looked sickly and died on the fifteenth day. The gestation period for mice was found to be twenty one days.

Table 2.3b. *In vivo* experiment to test for abortifacient activity in aqueous extract of [illegible] klotz using Swiss webster mice (CD1) in their 14th day of pregnancy.

Mouse	Weight of live mouse on the 12th day of pregnancy (g)	Weight of live mouse at the time of administration of aqueous extract on the 14th day of pregnancy (g)	Treatment	Result and Comment
a	37.0	39.3	0.3 cm^3* aqueous extract administered by intraperitoneal injection	Mouse died during administration
b	34.5	35.1		Aborted foetus and died in 24 hours of administration
c	39.2	41.5		Aborted foetus, but sickly looking in 24 hours of administration
d	38.6	40.2		Not aborted, sickly looking and died on day 15
e	41.4	42.7		Not aborted, died on day 15
f	36.8	38.3	0.3 cm^3** freshly prepared aqueous extract administered by intraperitoneal injection	Aborted 10 foetuses on day 14, died on day 15
g	36.5	38.6		Aborted 1 foetus and died on day 14
h	28.8	30.6	0.3 cm^3 saline solution administered by intraperitoneal injection	Delivered a live young on day 21
i	43.9	45.0	control, no dosage	Delivered a live young on day 21

* 0.3 cm^3 = 62.55 mg dried plant matter

** 0.3 cm^3 = 39.21 mg dried plant matter

2.3 In vitro experiment

The in vitro experiment to test the extracts of A petersiana klotz for uterotonic activity were conducted in the laboratories of the School of Medicine at the University of Zambia. The procedure followed in this experiment departed slightly from that recommended by the WHO Task Force because unsensitised uterine tubes were used instead of sensitised ones. Sensitisation of uterine tubes was done as described in section 6.8. The tests on uterine tubes used (0.1 - 0.5) ml, occasionaly 1.0 ml aqueous extract of a mixture of minced leaf, root and stem and fractions of this extract separated on a Sephadex G100 column in the Biology Department. Rythmic uterine muscle contractures caused by the extracts were measured as trace heights on a kymograph and these trace heights were compared with those due to oxytocin used as a reference. The trace heights for oxytocin were always taken as 100%. Treatment of uterii with each extract was done several times and average values of maximum and minimum values were recorded (table 2.4). Although the trace height for a particular uterus is proportional to dosage, the maximum and minimum values varied for each specimen

Table 2.4 Uterine muscle response to plant extracts of *A petersiana* klotz.

Fraction Number	Number of runs	Percent		
		Average	Maximum	Minimum
Oxytocin (reference)	14	100	100	-
NWPE	4	32	40	15
N_2	4	41	46	33
N_3	5	39	95	7
N_4	4	33	38	28
N_2R	1	33	33	-*
N_4R	4	38	60	14

* A single run was performed on fraction.

Notes on table

1. NWPE is water extract of *A petersiana* klotz made by soxhlet extracting 155 g of dried minced leaf, root and stem in 5 litres of distilled water and concentrating to 500 ml, 1 ml NWPE = 16.9 g dry plant matter.

2. N_2 is fraction number 2 of NWPE extract fractionated on Sephadex G100 with distilled water as eluent. N_3 and N_4 are fraction numbers 3 and 4.

3. N_2R and N_4R are the same fractions as N_2 and N_4 respectively but extracted at a much earlier date.

and thus the values recorded are not statistical. The **range** may reflect differences in the physiological conditions. The duration of uterine muscle activity was in all cases one minute. A methanolic extract of A petersiana klotz also produced similar activity when tested on uterii.

2.4 Testing of Aristolochia petersiana klotz extracts for major biologically active chemical groups

On the basis of the results from the physiological experiments, it was decided to do a chemical test on the extracts of A petersiana klotz. According to the WHO Task Force protocol, a physiologically active plant extract is fractionated with concurrent testing of the extracts for activity on isolated rat uterus. In this work the absence of a kymograph made it impossible to do this, instead the plant was chemically screened for major biologically active groups likely to be responsibl for the abortifacient and fertility regulating activitie

Literature survey has revealed that the greatest majority of natural products used medicinally are alkaloidal in nature (11,12,43a). Other chemically active constituents of plants belong to two main groups, namely, acids and peptides.

Farnsworth et al (12) cited that of the two hundred and twenty two species that have been shown to elicit stimulant response against uterine muscle either *in vivo* or *in vitro*, active compounds have been isolated from one hundred and ninety eight species. Of these compounds one hundred and forty eight have been identified while the remaining are still unidentified. One hundred and twenty two of the isolated compounds are alkaloids. Apart from this information, it is known that the most effective and widely used contraceptives to date are steroids. These are of synthetic type. It was from this background that extracts of *A petersiana* klotz were tested for steroids, alkaloids, saponins, peptides and Aristolochic acid mainly for a chemotaxonomic reason. Alkaloids were tested for using the Dragendorff's reagent employing spot test analysis on paper. Phosphomolybdic acid and Mayer's reagent were also used to precipitate out the alkaloid bases. Steroids and saponins were tested for using the Libermann-Bouchard reagent for colour formation. Ninhydrin reaction for α amino acids and peptides with terminal α amino acids was investigated by spot testing on paper and by test tube spot testing. Lastly the presence of Aristolochic acid was investigated

by comparing R_f values of an authentic sample of Aristolochic acid on thin-layer and paper chromatograms. Details of reagent compositions are described in section 6.2.

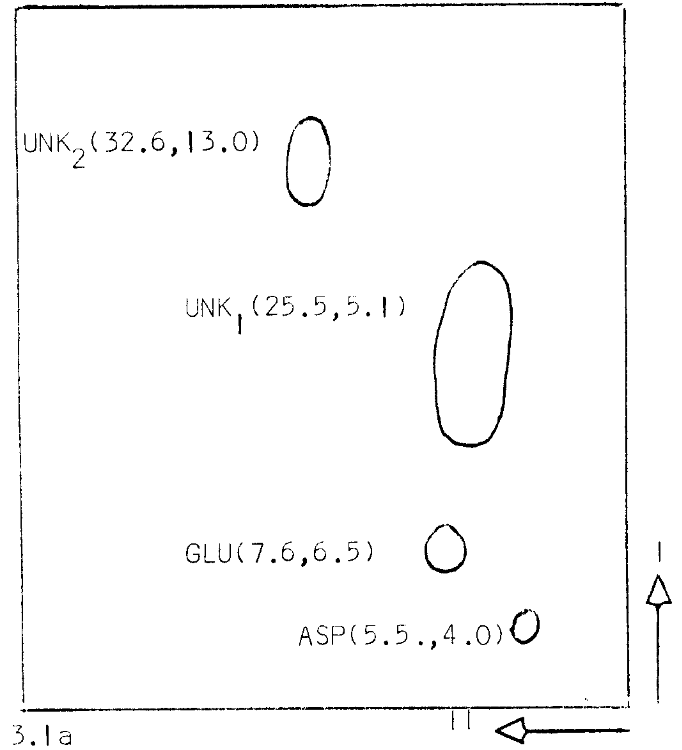
The results from these tests indicated that no isolatable amounts of alkaloids, steroids and saponins were present in A petersiana klotz. The presence of ninhydrin reactive compounds was however revealed. Aristolochic acid was also detected and its presence was later confirmed by ultraviolet and infrared spectroscopy.

CHAPTER 3

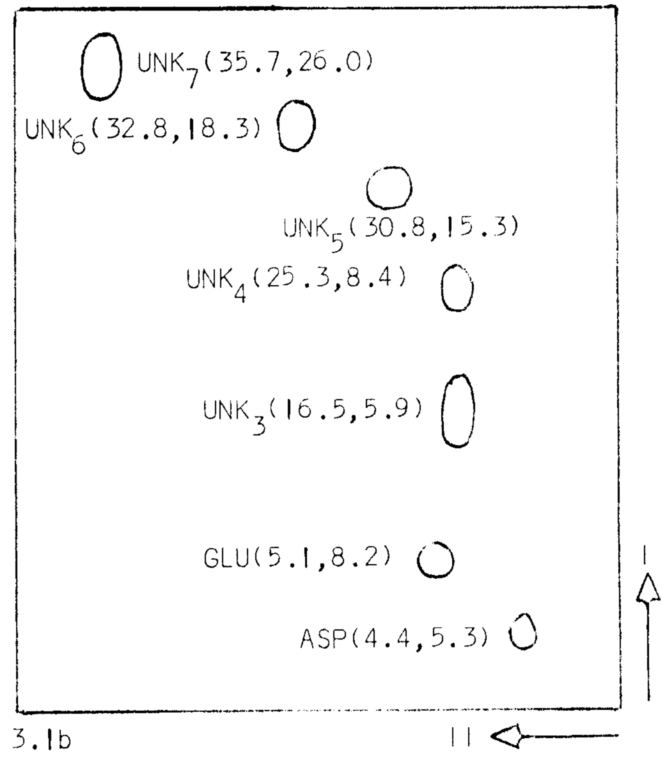
THE UNKNOWN NINHYDRIN REACTIVE MATERIAL

In an attempt to identify biologically active compound(s) from <u>Aristolochia petersiana</u> klotz, an ethanol solution of the 'residue' (figure 6.1) of a chloroform extract of dried and ground leaves of this plant was applied as a spot on filter paper and tested for a ninhydrin reaction with ninhydrin reagent. The extract gave a positive reaction with the reagent. The 'residue' was then examined to find the constituents responsible for the reaction.

The 'residue' dissolved in ethanol was applied on Whatman No 1 chromatography paper. Two dimensional descending paper chromatography was performed using as development solvents, phenol-water in an atmosphere of ammonia and butanol-acetic acid-water. The spots were detected with ninhydrin reagent. The chromatogram so obtained was compared against a standard chromatogram of known amino acids. Aspartic and glutamic acids were found to be present together with unknown compounds, which were designated as UNK_1 and UNK_2. UNK_1 appeared as a fairly intense and large spot relative to all other spots on the chromatogram (figure 3.1a). The pattern of appearence of UNK_1 on the chromatogram and its susceptibility to acid hydrolysis in 5M hydrochloric acid suggested that it may be a low



3.1a



3.1b

figures 3.1a and 3.1b

3.1a Two dimensional descending paper chromatogram of the 'residue' of a chloroform extract of leaves of A petersiana klotz.

3.1b Two dimensional descending paper chromatogram of the digest of the 'residue' of a chloroform extract of leaves of A petersiana klotz.

I Direction of first development in phenol-water (4:1, w/v) in the presence of the vapour of aqueous ammonia (S.G. 0.92) for approximately ten hours.

II Direction of second development in butanol-acetic acid-water (90:10:29, v/v/v) for approximately eight hours.

Numbers in brackets are distances of (I,II) in cm from the origin.

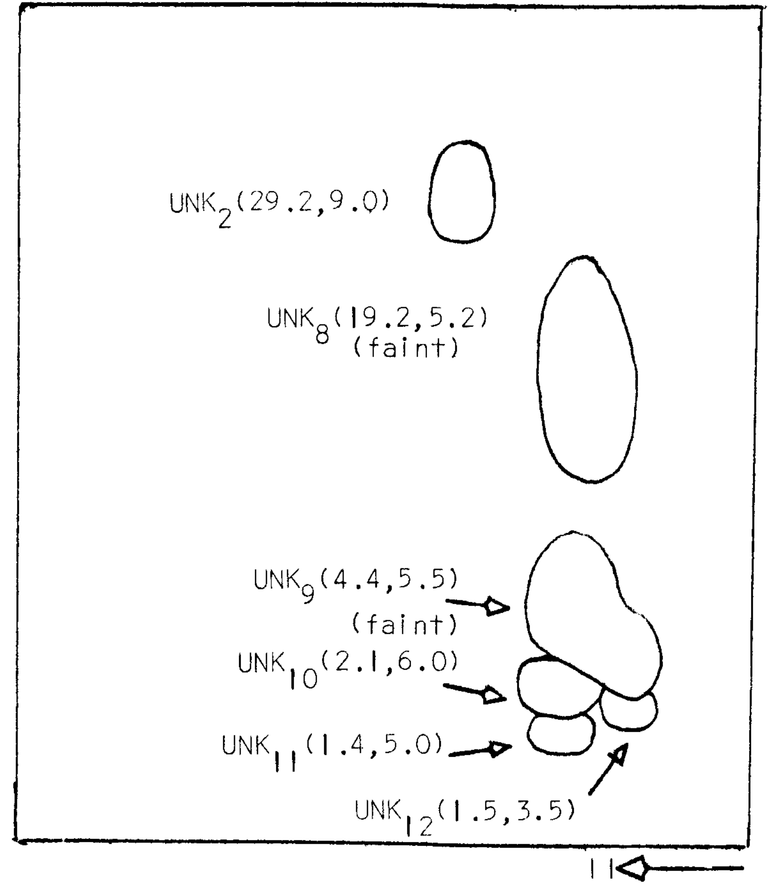


figure 3.1c

Two dimensional descending paper chromatogram of the digest of UNK_1* eluted from a 'blank' chromatogram.

I Direction of first development in phenol-water (4:1, w/v) in the presence of the vapour of aqueous ammonia (S.G. 0.92) for approximately thirty eight hours.

II Direction of second development in butanol-acetic acid-water (90:10:29, v/v/v) for approximately fourteen hours.

Numbers in brackets are distances of (I, II) in cm from the origin.

* UNK_1 is the unknown compound on figure 3.1a.

molecular weight peptide. Further analyses of extracts of root and stem of A petersiana klotz under the same chromatographic conditions also showed the presence of the unknown compounds. Experiments to confirm the presence of a peptide in A petersiana klotz were conducted and an attempt was also made to isolate the possible peptide in pure form.

There are two reasons for interest in the study of the ninhydrin reactive material. Firstly it appears to occur in a fairly large quantity relative to other fractions. Secondly and more important is that previous work (16,18) has shown that peptides are active in uterine muscle stimulation. As an example, a uteroactive oxytoxic polypeptide (17) was isolated from 'kalala - kalata' (Oldenlandia affinis), a plant native to Zaire, which is used in the Kasai Province of that country to enhance child birth. Also the strong uterine muscle stimulant oxytocin, a cyclic peptide is used in cases of difficult delivery in child birth. Thus the possible presence of a peptide in the ninhydrin reactive fraction suggests that it may be the active agent imparting fertility regulating activities of A petersiana klotz.

3.1 Extraction of the ninhydrin reactive fraction

The ninhydrin reactive fraction was isolated from the 'residue' of the chloroform fraction of leaves of A petersiana klotz on a column of Silica Gel (Kieselgel 60 grade, Merck). The 'residue' dissolved in ethanol was loaded to the column. Elution analysis was performed following a method similar to that of Kupchan and Merianos (23). Chloroform, chloroform-ethanol mixtures and 95% ethanol were used as eluents. The effluent fractions were examined for reactivity with ninhydrin by spot test analysis. Positive fractions were obtained when a chloroform-ethanol mixture (1:1 v/v) and 95% ethanol were used as eluents. Table 3.1 gives the results of the fractionation. Fraction C and D were pooled together and concentrated. The concentrate was analysed by two dimensional descending paper chromatography. The chromatographic pattern obtained was similar to that obtained for the crude 'residue' on two dimensional descending paper chromatogram, except for slight changes in position of the spots. Aspartic and glutamic acids were present together with UNK_1 and UNK_2.

Table 3.1 Fractionation of a 'residue' of minced leaves of *Aristolochia petersiana* klotz on a column of Silica Gel (Kieselgel 60 grade, Merck).

Solvent	Fraction	Effluent volume (litres)	Comment
Chloroform	A	2	Green, **ultraviolet** spectrum characteristic of chlorophylls, not reactive to ninhydrin reagent
Chloroform/ Ethanol 9:1	B	3	Yellowish Green, not reactive to ninhydrin reagent
Chloroform/ Ethanol 1:1	C	2.5	Yellowish brown, reactive to ninhydrin reagent
Ethanol	D	8	Yellow, reactive to ninhydrin reagent
Methanol	E	2.25	Reddish brown, not reactive to ninhydrin reagent.

A number of experiments were performed to confirm the presence of a peptide in the ninhydrin reactive fraction. These experiments included acid and enzyme hydrolysis of the extracts as well as chemical tests specific for a peptide bond.

3.2 (i) Acid hydrolysis

The crude 'residue' and UNK_1 eluted from a 'blank' (see experimental section 6.4i) chromatogram were separately digested in a sealed ampoule in 5M hydrochloric acid at 108°C for an hour. The hydrolysate was evaporated to dryness on a watch glass, the residue dissolved in 1 ml distilled water, evaporated to dryness once more to remove the excess acid and redissolved in minimum water. The aqueous solution was applied on Whatman No 1 chromatography paper and chromatographic conditions employed earlier were used. Figures 3.1b and 3.1c are chromatograms of the hydrolysate of the crude 'residue' and UNK_1 respectively. Comparison of these chromatograms against a chromatogram of known amino acids (38) and against figure 3.1a gave the following results:

On figure 3.1b UNK_1 and UNK_2 did not appear. Instead there appeared five new spots labelled as UNK_3, UNK_4, UNK_5, UNK_6 and UNK_7. It appeared that aspartic and glutamic acids had enhanced in intensity and could therefore be some constituent amino acids of the unknown compounds. However aspartic and glutamic acids were not detected on the chromatogram of the digest of UNK_1 (figure 3.1c) - thus indicating that they were not constituent amino acids of UNK_1. On figure 3.1c six new spots appeared instead of UNK_1. A two dimensional descending paper chromatogram of unhydrolysed UNK_1 produced two spots indicating that UNK_1 also fragments under neutral conditions. The second fragment appeared like the same compound as UNK_2 on figures 3.1a and 3.1c. The position of UNK_2 on the chromatograms indicated that it may be tyrosine or phenylalanine. However, comparison of figures 3.1a and 3.1c chromatograms with a two dimensional descending paper chromatogram of tyrosine and phenylalanine revealed that UNK_2 is neither tyrosine nor phenylalanine. That tyrosine is absent is supported by the fact that the 1-nitroso-2-naphthol test, which is a test specific for tyrosine and its derivatives, was negative.

The hydrolysis experiments described above indicated that UNK_I is unstable under acid hydrolysis and therefore it may be a peptide.

(ii) Enzyme hydrolysis

This experiment used the enzyme carboxy-peptidase A, which cleaves in succession the terminal amino acids at the carboxyl end group of peptides and proteins. An aliquot of ninhydrin reactive material taken in 0.04M veronal (diethyl barbiturate) buffer at pH 7.45 was treated with the enzyme. At time intervals of fifteen minutes during hydrolysis, aliquotes of 10 μℓ were withdrawn and examined by thin-layer chromatography. The results obtained were inconclusive because it was not known whether the spots obtained on the chromatogram were a result of hydrolysis of UNK_I or from the residual amino acids in the carboxy-peptidase A. This is owing to the fact that similar spots were observed on the thin-layer chromatogram of carboxy-peptidase A alone.

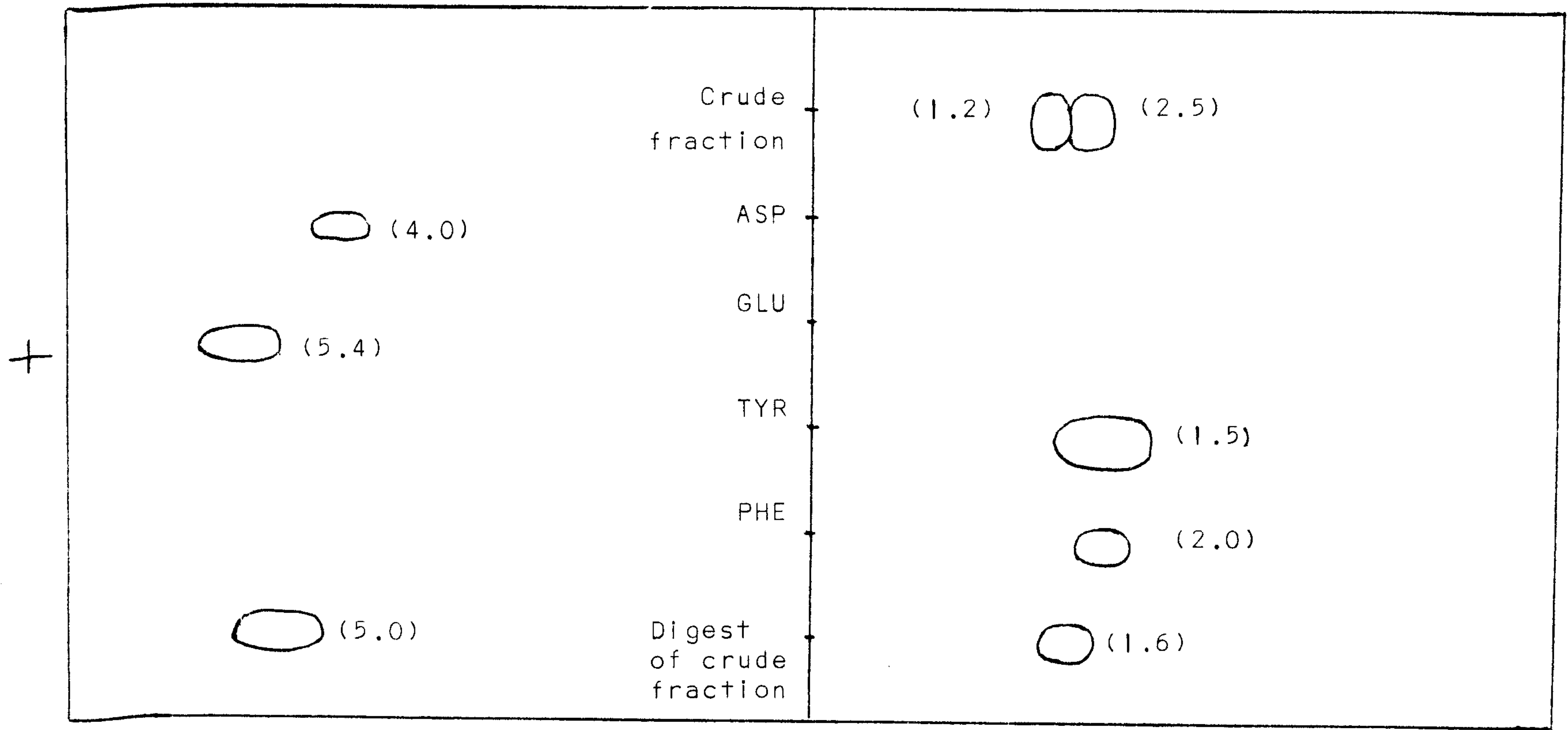
(iii) Paper electrophoresis

Paper electrophoresis was conducted on the ninhydrin reactive material, the acid digest of

the ninhydrin reactive material and the amino acids: aspartic acid, glutamic acid, tyrosine and phenylalanine. The medium for electrophoresis was pyridine-glycial acetic acid-water (10:0.4:90) buffer at pH 6.4 (see figure 3.2). The electrophoregram showed that two of the spots, one of which must be UNK_1 in the crude 'residue' are due to neutral or basic compounds because of their movement in the opposite direction to that of acidic glutamic and aspartic acids, which had moved towards the anode. The non-appearence of aspartic and glutamic acids in the crude 'residue' may be due to their low concentration (see the two dimensional paper chromatogram figure 3.1a). They would however appear stronger in the digest if they were constituent amino acids of UNK_1. Tyrosine and phenylalanine did not appear on the electrophoregram of the digest and their presence on the electrophoregram of the crude 'residue' is inconclusive.

(iv) <u>Chemical tests specific for a peptide bond</u>

The following chemical tests were performed on the ninhydrin reactive material:



<u>figure 3.2</u> Paper electrophoresis of pooled ninhydrin reactive fraction from leaves of <u>A petersiana</u> klotz, the digest of this fraction and some amino acids in pyridine-acetic acid-water (10:0.4:90) buffer at pH 6.4.

Crude fraction = pooled ninhydrin reactive fraction.

X = start point.

Numbers in brackets are distances from the start point in cm.

(a) The Biuret test which requires 1-20 mg of sample (5) is a test which is specific for peptides containing three and more amino acid constituents and for proteins.

(b) The Folin-Ciocalteau's test, which is sensitive to as little as 5 µg of sample (5) is similar to the Biuret test, except that it is also sensitive to phenolic compounds such as tyrosine.

(c) The 1-nitroso-2-naphthol test, which is a test for tyrosine and tyrosine derivatives has a limit of detection of about 1-2 µg tyrosine (1,19b,42).

(d) The chlorination tests: (i) the chlorination-heat and (ii) the chlorination-starch/iodine. These tests have sensitivities of the order of 1 µg (33,39,42). The tests are sensitive to amide bonds of peptides and proteins. The amino acids glutamic acid, phenylalanine lysine, histidine, serine and proline react to these tests as well.

The extract gave a negative reaction to the Biuret test. A 1% (w/v) sample was used but UNK_I may have been present in small amounts to be detectable by this test. The Folin-Ciocalteau's test was however positive because it requires lesser amounts of sample. The tyrosine test was negative. This test was performed to rule out the presence of tyrosine and tyrosine derivatives, which together with other phenolic compounds are also sensitive to the Folin-Ciocalteau's test. It was however not possible by the tyrosine test to rule out the presence of other phenolic compounds. The chlorination tests gave positive reactions with both the ninhydrin reactive fraction and even UNK_I on a two dimensional chromatogram. These tests are also sensitive (to the same sensitivity) to the amino acids glutamic acid, phenylalanine, leucine, lysine, histidine, serine and weakly sensitive to proline. Hence, if any of these amino acids were present in the extract, they could be responsible for the chlorination reactions. However, comparison of a two dimensional chromatogram of these amino acids with the two dimensional chromatogram of the crude 'residue' (figure 3.1a) indicated that

UNK$_I$ is not any of the above mentioned amino acids and therefore it may be a peptide.

3.3 Attempts to isolate UNK$_I$

The subsequent experiments were aimed at isolating UNK$_I$ in pure form from the ninhydrin reactive material. These experiments used column chromatography in which the following resins were employed: Sephadex G25-80, Silica Gel (Kieselgel 60 grade), Sephadex A25 and Dowex I. Experiments on columns of Sephadex G25-80, Silica Gel and Sephadex A25 described in section 6.6(i) - 6.6.(iii) were not able to isolate UNK$_I$. However the experiment performed on a small column of Dowex I separated the acidic amino acids aspartic and glutamic from the rest of the ninhydrin reactive material.

Column Chromatography on Dowex I

This experiment used the strongly basic anionic exchanger Dowex I in the acetate form. On a column of Dowex I, the amino acids and peptides which are acidic are separated from the basic and neutral ones by ion exchange. Neutral and basic amino acids (with the exception of tyrosine, tyrosine derivatives and aromatic compounds if present) and basic and neutral peptides should pass

through the column or be eluted during washings with water. Weakly acidic and strongly acidic amino acids and peptides are exchanged on the resin and can only be eluted with acetic acid.

Ninhydrin reactive material was dissolved in 0.5M acetic acid and applied to a column of Dowex I in the acetate form. Effluent fractions of each 1.2 ml were collected as soon as the sample solution was loaded to the column. Acetic acid (0.5M) was used as an eluent. One dimensional descending paper chromatography of the effluent fractions against the ninhydrin reactive fraction and the amino acids: aspartic acid, glutamic acid, phenylalanine and tyrosine was used to monitor the fractions The effluent fractions were also examined for the presence of tyrosine using the 1-nitroso-2-napthol reagent and for the presence of amide bond(s) using the chlorination tests. The details of the experimental procedure are given in section 6.6(iv).

The results indicated that UNK_1 is a weakly acidic compound because it was eluted much earlier in fractions 6 and 7 than glutamic acid (fractions 9-25) and aspartic acid (fractions 25-39). This result is consistent with the results of the electrophoresis experiment. UNK_1 migrated to the cathode in a buffer of pH 6.4. In this

buffer, glutamic and aspartic acids which are acidic migrated in the opposite direction. All fractions gave negative reactions to the 1-nitroso-2-napthol test indicating the absence of tyrosine and compounds containing tyrosine. Dowex resins are known to adsorb aromatic compounds to a great extent suggesting that if tyrosine was present, very small amounts would be eluted and small amounts would be insensitive to the 1-nitroso-2-naphthol test. Fractions 6 and 7 and 18-25 gave positive reactions to the chlorination tests. This was expected if fractions 6 and 7 contained UNK_1 and fractions 18-25 contained glutamic acid. UNK_1 had earlier been shown to be reactive to the chlorination tests and glutamic acid is also reactive to the tests.

At this stage it would have been of interest to test the whole ninhydrin reactive fraction and perhaps fractions 6 and 7 above for activity on a uterine muscle. Due to lack of chemicals, suitable equipment and animal sources, work on the isolation of UNK_1 has been held in abeyance.

CHAPTER 4

ARISTOLOCHIC ACID

Screening for Aristolochic acid in *Aristolochia petersiana* klotz was based on chemotaxonomy as the presence of Aristolochic acid generally characterises the Aristolochiaceae. At least six species among the members of this family that have been studied contain Aristolochic acid (3,7,8,22,23,41,43a). Of the six, five belong to the genus *Aristolochia* and one to the genus *Asarum*. However apart from such chemotaxonomic information the writer is not aware of any study that has shown the presence of Aristolochic acid in *A petersiana* klotz.

Aristolochic acids have a phenanthrene chromophore (figure 4.1). The acids normally occur together with other compounds possessing the phenanthrene chromophore (23).

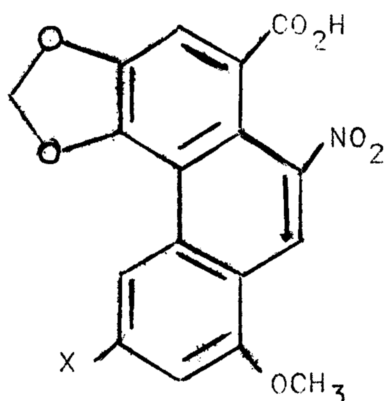


figure 4.1	Aristolochic acid I	X = H
	Aristolochic acid II	X = OH

It is not surprising therefore that Aristolochic acids exist in different natural forms through the type of substituent X attached to the chromophore. In the Aristolochiaceae the most commonly occuring Aristolochic acid is Aristolochic acid I.

Aristolochic acid I exhibits various forms of physiological activities. Of these, repression of tumor development, toxicity and cytotoxicity have been particularly well studied. Aristolochic acid I isolated from _A indica_ linn has been shown to be effective against the adecocarcinoma 755 in mice (22) just like the plant itself is known to be effective against tumor development. Kupchan and Doskotch (23) attribute the antitumor activity of _A indica_ linn to Aristolochic acid.

Aristolochia species generally have a bitter taste and are poisonous. Eeu, Reichstein and Rothschild (9) attribute this to the presence of Aristolochic acid. Experimental proof that Aristolochic acid is toxic and cytotoxic is derived from the work of Angeles et al (2). Farnsworth et al (12) include Aristolochic acid among the cytotoxic isolates of plants. In the study carried out by Angeles et al (2), Aristolochic acid isolated from _A tangala_ cham was shown to be toxic to various tissues

and internal organs of a wide range of animals and man. It is further shown that 40 mg per kg body weight Aristolochic acid induced uterine contractures in the cat. An unspecified dose of Aristolochic acid similarly caused uterine contractures in the guinea pig. The above results seem to suggest that Aristolochic acid may be the factor to which A petersiana klotz owes its abortifacient and fertility regulating properties.

4.1 Attempts to detect Aristolochic acid

Experiments to show the presence of Aristolochic acid in A petersiana klotz were based on analyses of extracts of minced leaves and minced roots and stems by thin-layer, paper and column chromatography.

(i) Thin-layer chromatography

One dimensional separation of the constituents of the extracts was performed on thin-layer plates of Silica Gel G(Merck) using acetonitrile-diethylamine-water (8:1:1) (8) in an ascending solvent front system. In this solvent, Aristolochic acid moves as a lime yellow spot at R_f 0.62. Such a spot was not observed in this experiment due to trailing.

(ii) Paper chromatography

Consequent upon the failure of ascending thin-layer chromatography to effect separation of the chemical constituents of the extracts, it was decided to employ descending paper chromatography which offers the advantage of relatively longer distance of movement of solvent front and increased separation of spots. Chromatograms were made on Whatman No I chromatography paper previously buffered at pH 9.2 with 0.05M carbonate-bicarbonate buffer. Chromatograms were developed in water-saturated n-butanol (8). In this experiment no lime yellow spot was observed at R_f 0.62, at which Aristolochic acid was expected to appear. Instead a spot appeared at R_f 0.34 on chromatography of both the acid/neutral fraction and the 'residue' fraction of minced leaves. Chromatography of an ethanol extract of minced root and stem produced a spot at R_f 0.54. These results made identification of Aristolochic acid uncertain without the aid of a "marker" of pure Aristolochic acid.

(iii) Column chromatography

Due to the inconclusive results obtained in the above attempts to identify Aristolochic acid, it was decided to use adsorption chromatography on columns of Kieselgel 60 grade (Merck) using the elution analysis technique. It was hoped that elution with various solvents which differ in polarity would separate the plant extracts into different chemical groups according to their solubilities in these solvents. Three experiments were performed on three columns employing the chromatographic procedure of Kupchan and Merianos (23). Effluent fractions were examined for the presence of Aristolochic acid by ultraviolet spectroscopy and thin-layer ascending front chromatography. Acetonitrile-diethylamine-water (8:1:1) was used to develop thin-layer chromatograms.

The 'residue' fraction of minced leaves and the extract of minced root and stem were fractionated under similar chromatographic conditions (tables 3.1 and 4.2). The acid/neutral fraction of minced leaves was also fractionated

Table 4.1 Fractionation of an acid/neutral fraction of minced leaves of *A petersiana* klotz on a column of Silica Gel (Kieselgel 60 grade, Merck).

Solvent	Fraction	Volume effluent (litres)	Comment
Chloroform	A	3	Green, ultraviolet spectrum similar to a standard spectrum of pheophytin b (28)
Chloroform	B	4.5	Brown
Chloroform/ Methanol 9:1	C	0.5	Green
Chloroform/ Methanol 9:1	D	2	Greenish brown

Table 4.2 Fractionation of an ethanol extract of root and stem of A petersiana klotz on a column of Silica Gel (Kieselgel 60 grade, Merck).

Solvent	Fraction	Volume effluent (litres)	Comment
Chloroform	A	2	Green, ultraviolet spectra similar to those of chlorophylls a and b (28)
Chloroform/ Ethanol 3:1	B	2	Yellow
Chloroform/ Ethanol 1:1	C	3	Yellow, reactive to ninhydrin reagent
Ethanol	D	12	Yellow to colourless, reactive to ninhydrin reagent

(table 4.1) under conditions similar to those used above, except that chloroform-methanol mixtures were used as eluents where chloroform-ethanol mixtures had been employed. Results from thin-layer chromatograms of the effluent fractions on both cellulose MN 300 (Marchery, Nagel and Co) and Silica Gel G(Merck) were inconclusive due to incomplete separation and trailing. Ultraviolet data revealed that the chloroform effluent fractions of tables 3.1 and 4.2 and all effluent fractions of table 4.1 were characteristic of chlorophylls a and b and did not indicate that Aristolochic acid was present. The remaining effluent fractions of tables 3.1 and 4.2 (fractions B,C,D and E) on the basis of ultraviolet data indicated that Aristolochic was absent.

4.2 Detection of Aristolochic acid

The use of pure Aristolochic acid I (Sigma), which was donated by Professor Vandendriessche (University of Ghent) made it possible to re-examine the extracts. More experiments were performed on the extract of minced root and stem following the procedures in section 4.1(i) and 4.1(ii).

The authentic sample was spotted along side the extract on a thin-layer plate and also on Whatman No I chromatography paper.

One dimensional descending paper chromatograms were developed in water-saturated n-butanol. Aristolochic acid "marker" gave a lime yellow spot at R_f 0.46, but the extract trailed. A portion from the extract at around R_f 0.46 was cut off and washed in methanol. The ultraviolet spectrum of the methanol washing gave two broad peaks around 220 nm and 248.8 nm. The absorbance at 220 nm was greater than that at 248.8 nm. The authentic sample in methanol produced sharp peaks at 220 nm and 248.8 nm with the absorbances in the reverse order. This result revealed that a compound possessing a chromophore similar to that of the authentic sample was present in the methanol washing.

To overcome the problem of trailing encountered earlier on thin-layer chromatograms, trials were first made in various solvents until the solvent acetonitrile-diethylamine-water (12:1:1) was found to give good and reproducible results. The authentic sample in this solvent gave a lime yellow spot at R_f 0.64 and the extract gave a similar spot, but at

R_f 0.59. The extract was then loaded on a preparative thin-layer plate of Silica Gel and developed in the same solvent. A lime yellow band was produced around R_f 0.59, scraped off, extracted in methanol and filtered. The filtrate was examined for the presence of Aristolochic acid by ultraviolet spectroscopy. Absorptions were observed at 223.2 nm and at 249.4 nm similar to those observed for the authentic sample of Aristolochic acid.

The root and stem fractions A,B and C of table 4.2 were also examined for the presence of Aristolochic acid by thin-layer chromatography on Silica Gel with the aid of pure Aristolochic acid used as a "marker." Fraction A which earlier had shown by ultraviolet spectroscopy that it contained the chlorophylls, now showed the presence of a lime yellow compound also around R_f 0.59. It seems from this result that the chlorophylls, which have strong absorption maxima in the same ultraviolet region, obscured the absorption due to the lime yellow compound. The acid/neutral fraction and the 'residue' of minced leaves also showed the presence of small amounts of the lime yellow compound when chromatographed with a "marker" of pure Aristolochic acid.

4.3 Extraction and purification of the lime yellow compound from fractions A and B of root and stem (table 4.2)

Fractions A and B of table 4.2 were pooled, concentrated and extracted for the total acid according to the scheme in figure 4.2. The total acid was further partitioned into fractions AB_1 and AB_2.

Fraction AB_1 was loaded on preparative thin-layer plates of Silica Gel G6 (BDH), which were developed by the ascending front technique in acetonitrile-diethylamine-water (12:7:1). Visually the chromatograms showed two bands (figure 4.3a):- a lime yellow band, band α, which appeared at R_f 0.68 and a yellowish orange band, band β, which appeared at R_f 0.36. Under long wave (λ = 365 nm) ultraviolet light, other minor components (figure 4.3b) were evident. Band α appeared with two light blue flourescent bands, bands γ and ∂ above and below it. Band β also appeared closely followed by two bands:- band ε (yellow) and band ξ (blue). Bands α and β were each scraped separately and extracted from the gel with a chloroform-ethanol mixture. The solvents were removed by evaporation at 45°C under reduced

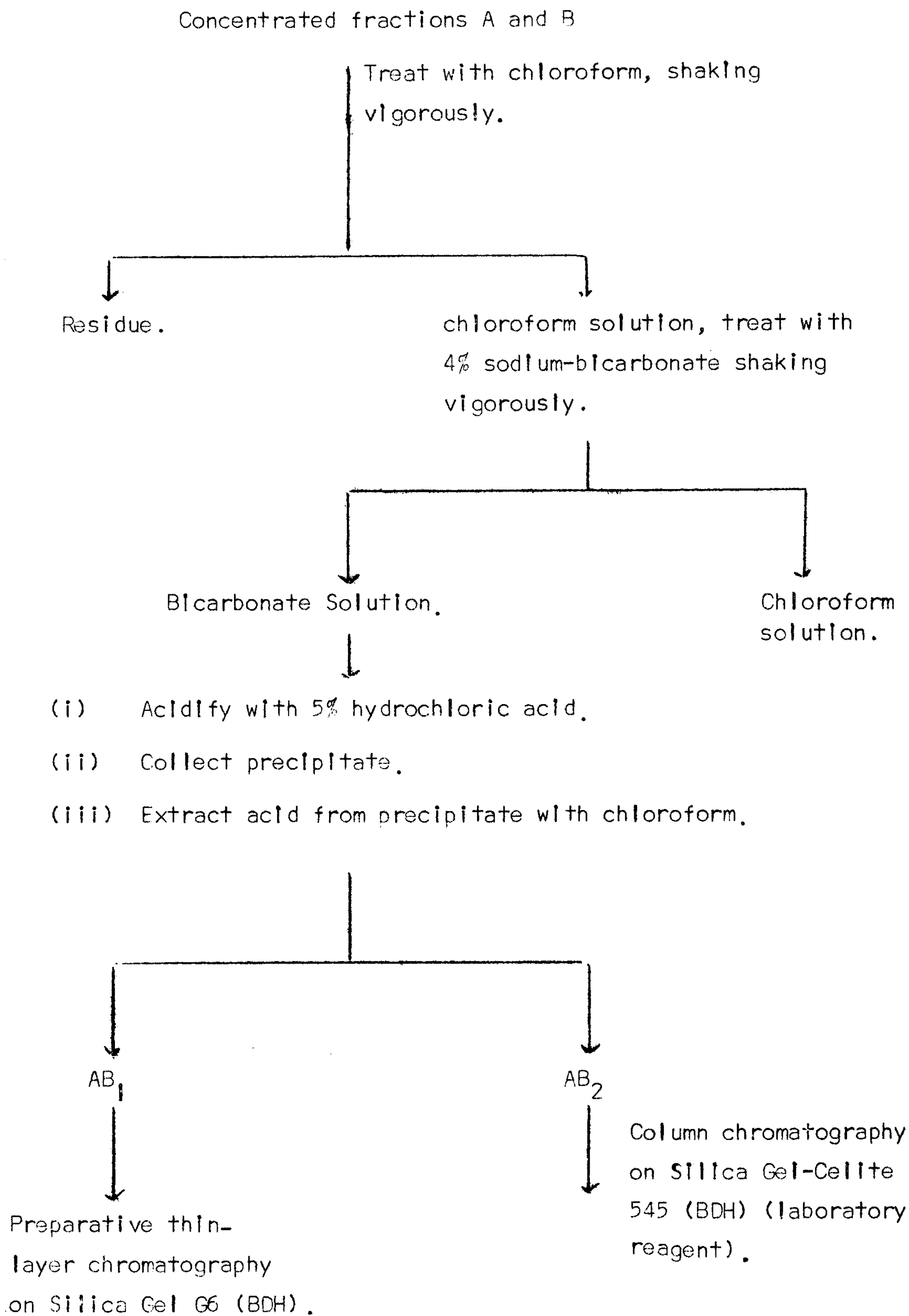
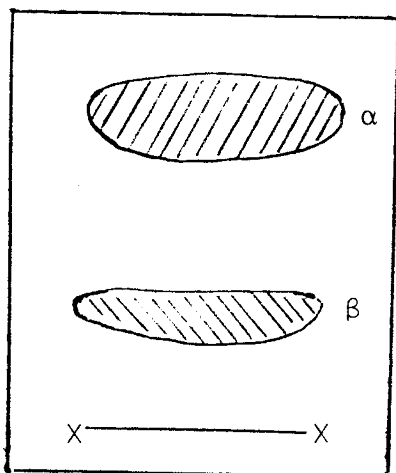
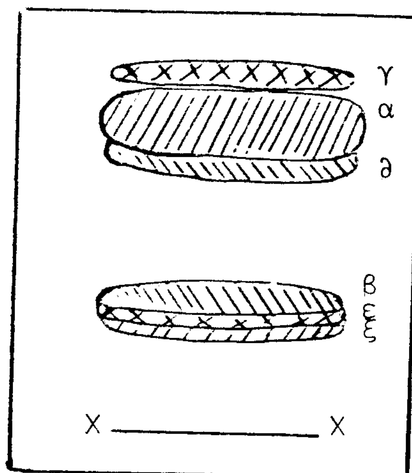


figure 4.2 Flow sheet for isolation of the total acids and the lime yellow compound of root and stem of *A petersiana* klotz. Method is similar to that of Kupchan and Doskotch (22).



4.3a



4.3b

figures 4.3a and 4.3b:

Thin-layer chromatograms of AB_1 on Silica Gel G6(BDH) in acetonitrile-diethylamine-water (12:7:1, v/v/v).

Figure 4.3a: plate observed visually.

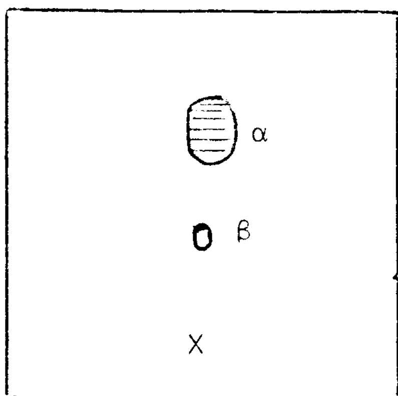
Figure 4.3b: plate observed under ultraviolet light.

Bands α and β are the lime yellow and yellowish orange compounds at R_f 0.68 and 0.36 respectively. Bands γ, ∂, ε and ξ are very minor components.

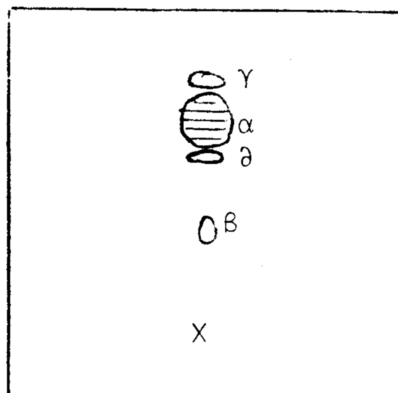
X = start point.

pressure using a Büchi rotary evaporator. Each sample was dried under vacumn. The lime yellow compound from band α was present in a larger proportion than the yellowish orange compound which formed band β. The yellowish orange compound was isolated in such a small quantity that only its ultraviolet spectrum was obtained. After several attempts to crystallise the lime yellow compound in various solvents, dimethylformamide and water was finally found to give good results.

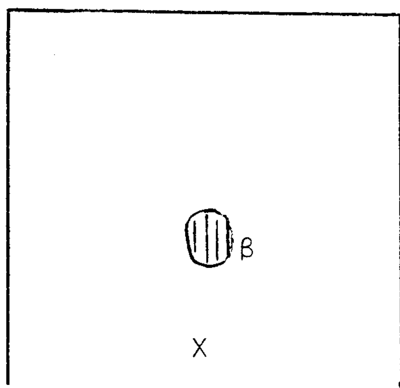
Fraction AB_2 was applied to a column of Silica Gel-Celite (4:1 w/w). Elution with chloroform resulted in the separation of two bands: A lime yellow, band $AB_{2.1}$, which emerged first, and a smaller yellowish orange band, band $AB_{2.2}$. Band $AB_{2.1}$ was eluted with chloroform and band $AB_{2.2}$ was eluted with 10% methanol in chloroform. Effluent fractions of each 100 ml were collected and these were analysed by thin-layer chromatography. It was found that band $AB_{2.1}$ contained the lime yellow compound (spot α figure 4.4a) as the main constituent. A minor yellowish orange compound (spot β) was also present. Viewing thin-layer chromatograms of fractions containing band $AB_{2.1}$ under ultraviolet



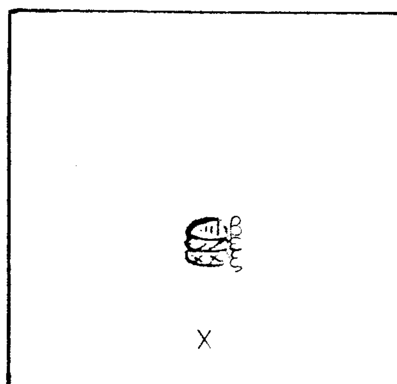
4.4a



4.4b



4.4c



4.4d

figures 4.4a, 4.4b, 4.4c and 4.4d:

Thin-layer chromatograms of effluent fractions of AB_2 on Silica Gel G6(BDH) in acetonitrile-diethylamine-water (12:7:1, v/v/v).

Figure 4.4a: chromatogram of fraction $AB_{2.1}$, plate observed visually.

Figure 4.4b: same chromatogram as figure 4.4a, but plate observed under ultraviolet light.

Figure 4.4c: chromatogram of fraction $AB_{2.2}$, plate observed visually.

Figure 4.4d: same chromatogram as figure 4.4c, but plate observed under ultraviolet light.

Spots α,β,γ,∂,ε and ξ are the same compounds of figures 4.3a and 4.3b.

X = start point.

Ultraviolet data

The ultraviolet spectra of the lime yellow compound obtained in both methanol and water were typical of a phenanthrene chromophore: absorptions at 220 nm and 251 nm. Table 4.3 gives ultraviolet data for the authentic sample of Aristolochic acid I, the lime yellow compound and the yellowish orange compound. Ultraviolet data for the yellowish orange compound suggests that it may be Aristolochic acid II (Absorptions at 220(ε 29,800), 242(37,790), 252(37,800), 292(13,850) and 325(11,300) (23).

Infrared data

The infrared data of the lime yellow compound as a mull in nujol and hexachloro-1-3-butadiene were identical with that of pure Aristolochic acid I. Table 4.4 gives the infrared data for both the authentic sample of Aristolochic acid I and the lime yellow compound. Except for a weak absorption observed at 3640 cm^{-1} in the authentic sample, the rest of the peaks were superimposable. This absorption at 3640 cm^{-1} is due to the free carboxyl OH stretching in the monomer. In the dimer it is bonded, giving rise to a broad peak between 3400 cm^{-1} and 2300 cm^{-1}. The non-appearence of an absorption around 3640 cm^{-1} in

Table 4.3 Ultraviolet data

Compound	Solvent	λ max (nm)			
Aristolochic acid I	water	220	249	308	385
	methanol	220	250	311	385
lime yellow compound from	water	223	248	313	385
A peterslana klotz	methanol	223	249	316	385
yellowish orange compound from *A peterslana* klotz	methanol	225 240 256 308 386			

Table 4.4 Infrared data

Lime yellow compound from *A petersiana* klotz (cm^{-1})	Authentic sample of Aristolochic acid (cm^{-1})	
3640 w* b**		OH free carboxylic
1690 s***	1695 s	C = O carboxyl
1600 s	1600 s	C = C Aromatic
1530 s	1525 s	$\nu_a NO_2$ substituted to aromatic
1350 s	1350 s	$\nu_s NO_2$ substituted to aromatic
1275, 1252 } s doublet	1275, 1250 } s doublet	ν_aC-O-C aryl alkyl
1155 s	1152 s	-
1045 s	1045 s	ν_sC-O-C aryl alkyl

* Weak

** broad

*** strong

the lime yellow compound may be due to concentration effects. The presence of a carboxyl moiety in the lime yellow compound is to be expected as the compound was extracted with sodium bicarbonate as its sodium salt. The presence of a carboxyl group is supported by NMR data. A broad carboxylic acid proton was identified at low field (δ = 9.6). This appeared to be the only exchangable proton when the NMR spectrum was obtained in D_2O. This evidence is further supported by the fact that the compound is irreducible by sodium borohydride. However the compound is reducible with dithionite (21) as might be expected when a nitro group is present. This latter evidence is supported by absorptions in the infrared region at 1530 cm^{-1} and 1350 cm^{-1} due to the nitro group. Absorptions at 1275 cm^{-1} and 1252 cm^{-1} indicated the presence of an aromatic alkyl C-O-C group.

Mass spectrometric data

The mass spectrum (figure 4.5) is identical with that of Aristolochic acid I (9), except for weak peaks at m/z 355 and 371. The molecular ion peak appears at m/z 341. The peak at m/z 355 and 371 may be attributed to impurities evident on thin-layer chromatograms of the sample. The impurities seem to possess the phenanthrene chromophore like Aristolochic acid. The peak at m/z 355

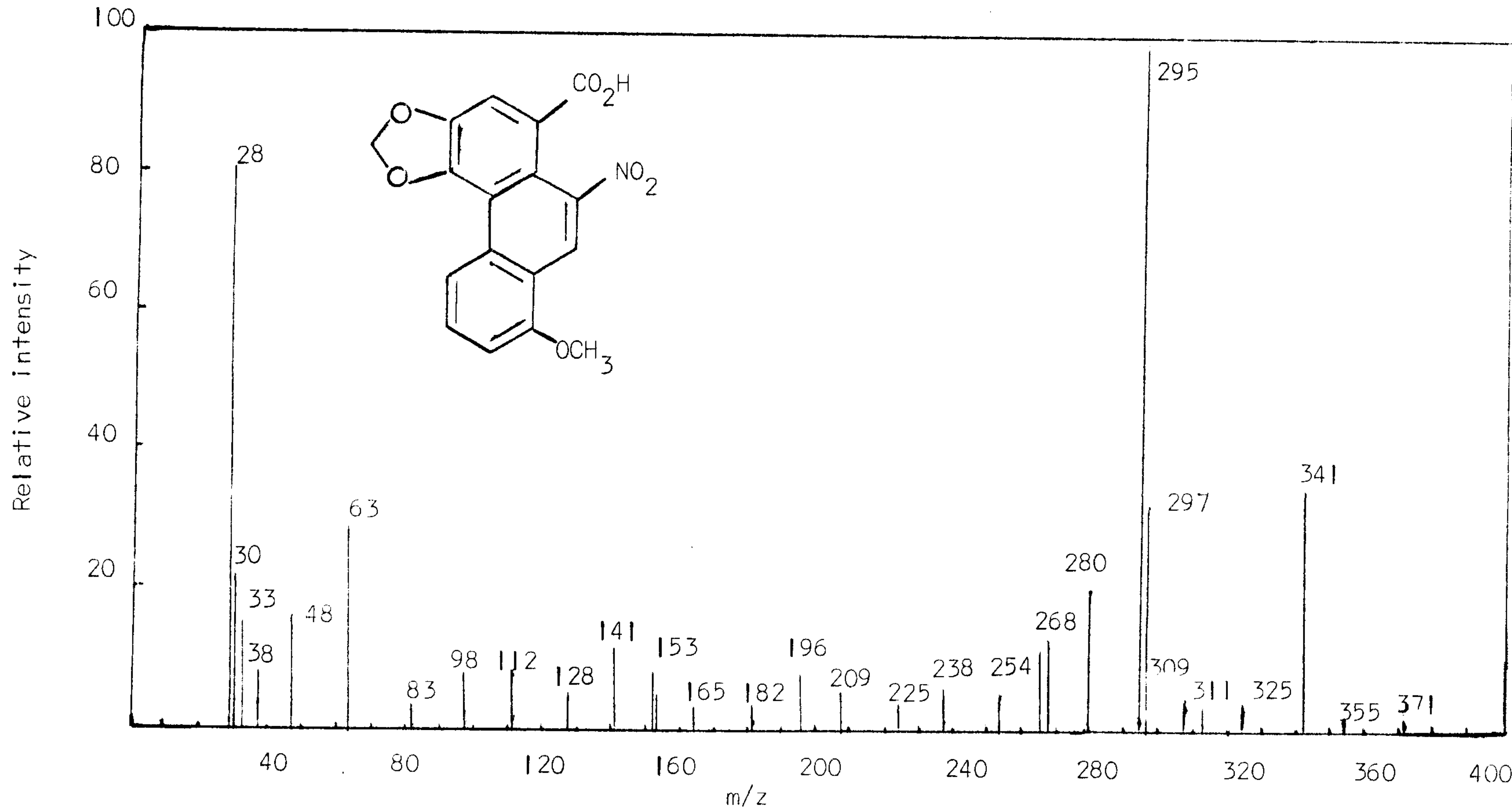


figure 4.5. Mass spectrum* of Aristolochic acid isolated from A petersiana klotz. Melting point: decomposes between 233°C and 238°C.

* spectrum was obtained by Professor Dr. P. Hemmrich (University of Konstanzy, Germany).

may be due to the yellowish orange compound, which on the basis of ultraviolet data (table 4.3) may be identified as Aristolochic acid II (molecular mass 357). This would explain the presence of a peak at m/z 309 which is absent in Aristolochic acid I (9). This peak may be due to loss of NO_2 (46 units) if the compound is indeed Aristolochic acid II. The peak at m/z 371 may be due to the second impurity, which might have a contribution to m/z 325 also due to loss of NO_2 if the compound is another form of Aristolochic acid.

The above evidence from ultraviolet, infrared, mass spectrometric and thin-layer chromatographic data strongly indicates that the lime yellow compound which is the main constituent of the total acid extractable from A petersiana klotz is Aristolochic acid I (figure 4.1). On the basis of ultraviolet data the yellowish orange compound seems to be Aristolochic acid II.

CHAPTER 5

DISCUSSION

5.1 Bioassay studies

In the preliminary and exploratory experiment (section 2.1), the folk-lore reputation of *A petersiana* klotz as an abortifacient was verified. The water plant extract when administered orally or by intraperitoneal injection to pregnant white albino rats caused abortion. In another exploratory experiment (table 2.1), the water extract was further shown to possess an antifertility activity. While the female rats of group B which were injected intraperitoneally every alternate day with the water extract did not conceive over a period of sixty days; the female rats of both groups A and C conceived. In group A, the female rats received normal saline solution by intraperitoneal injection and in group C, the male rats received a similar treatment to the female rats of group B. The difference between group B and group C is profound; the water extract is an antifertility agent in female rats but not in male rats. The difference between group A and group C is in the number of the young delivered per litter; the latter has two young less than the former. This result may not be statistically

significant as the number of experimental animals used was small.

In the _In vitro_ experiment (section 2.3), the water plant extract and fractions of this extract separated on a Sephadex G100 column were shown to stimulate uterine muscle. The extracts when applied to isolated rat uterii, caused rythmic muscle contractions, which were comparable to those of the hormone, oxytocin, a uterine muscle stimulant when applied to rat uterii. All fractions separated on Sephadex G100 column gave positive activity (table 2.4) indicating that they contained active material. This result could also mean that separation on Sephadex was poor. It is however clear that the activity of (0.1-1.0) ml of the extracts is less than 0.002 - 0.005 units of oxytocin.

The WHO Task Force protocol on indigenous plants for fertility regulation was satisfied in three _in vivo_ experiments (section 2.2). The abortifacient activity of _A petersiana_ klotz was confirmed using unsensitised Swiss webster mice in two different terms of pregnancy: the middle and late stages of pregnancy. In the first

experiment (table 2.2), the water extract caused abortion when orally administered to mice in their nineteenth day of pregnancy. This was two days before expected parturition and therefore a late stage of pregnancy. In the second and third experiments (tables 2.3a and 2.3b), the water extract was administered to mice in the fifteenth and fourteenth days of pregnancy. In both experiments, only the mice which received the water extract by intraperitoneal injection aborted. These mice also died soon after abortion - suggesting that the extract is toxic. In table 2.3a three mice received the water extract orally over four consecutive days, but they did not abort. Instead these mice lost weight and eventually one mouse died on day nineteen, while the remaining two mice delivered dead young on day twenty. These latter results following oral administration of extract to mice are inconclusive because of the following speculations:

(i) Oral administration is difficult because the plant is bitter. It is possible that the mice did not abort because they received insufficient dosage. It is to overcome this problem that oral administration of the water extract to these mice was repeated over four successive days.

(ii) It is probable that drug metabolism took place. However, drug metabolism may be ruled out because in two earlier experiments, the exploratory experiment (section 2.1) and the first confirmatory experiment (section 2.2, table 2.2), the water extract when administered orally to pregnant mice and rats caused abortion.

(iii) Lack of abortion suggests a different mechanism of action to have taken place in mice in middle term of pregnancy. Even oxytocin is not effective in the first middle **semester** of pregnancy in humans.

Related bioassays on other Aristolochiaceae are those of Pakrashi and Shaha (30), Angeles et al (2) and Saha, Savin and Kasinathan, (34). Pakrashi **and** Shaha in their studies on A indica linn isolated a sesquisterpene, which possesses interceptive and abortifacient activities. Angeles et al (2) in similar studies showed that Aristolochic acid isolated from A tangala cham is active in uterine stimulation in the cat and the guinea pig. Saha, Savin and Kasinathan (34) carried out bioassays on A bracteata Retz. Although this plant is reported by Datta and Sastry (6) to stimulate

uterii of the rat and the guinea pig, they found that the plant possesses very little oxytoxic properties.

5.2 The unknown ninhydrin reactive compound

In chapter 3, a ninhydrin reactive material, which contains an unknown ninhydrin reactive compound was isolated from A petersiana klotz. The experiments to be discussed later in this section suggested that this unknown compound may be a peptide.

Initially two dimensional descending paper chromatography of the ninhydrin reactive material revealed that one of the spots designated by UNK_I (figure 3.1a) may be a peptide. The spot was more intense and larger than any of the spots on the chromatogram. Although enzyme hydrolysis using carboxy-peptidase A (section 3.2ii), which is a test for peptides, was inconclusive and the Biuret test (section 3.2iv), which is also a test for a peptide bond, was not sensitive enough, the following tests indicated that UNK_I may be a peptide:

(i) In section 3.2i, extracts were acid digested and the hydrolysate examined by two dimensional

descending paper chromatography. A portion of the crude ninhydrin reactive fraction was digested in 5M hydrochloric acid and the hydrolysate chromatographed (figure 3.1b). UNK_1 eluted from a 'blank' (see experimental section 6.41) chromatogram was also digested in 5M hydrochloric acid and chromatographed (figure 3.1c). On both chromatograms, new spots appeared and these spots were not on the two dimensional paper chromatogram of the crude ninhydrin reactive fraction (figure 3.1a). A fourth chromatogram of the unhydrolysed UNK_1 eluted from a 'blank' chromatogram revealed that UNK_1 breaks down into two fragments even before hydrolysis. The fragments were identified as those designated by UNK_1 and UNK_2 on figure 3.1a.

(ii) UNK_1 on a chromatogram like that of figure 3.1a was detectable by the chlorination tests described in section 3.2(iv). Although the chlorination tests are sensitive to amide bonds of peptides and proteins, the amino acids glutamic acid, histidine, Leucine, Lysine,

phenylalanine, serine and proline give positive reactions to these tests as well. However, a two dimensional descending paper chromatogram of these amino acids when compared with the two dimensional chromatogram of the crude 'residue' (figure 3.1a) indicated that none of the amino acids is UNK_1.

(iii) The ninhydrin reactive fraction gave a positive reaction to the Folin-Ciocalteau's test (section 3.2 iv), which is a test for peptides, proteins as well as phenolic compounds such as tyrosine and tyrosine derivatives. The presence of tyrosine and its derivatives was however ruled out by the negative reaction given between the ninhydrin reactive fraction and the 1-nitroso 2-naphthol reagent, which is specific for tyrosine and its derivatives. It was however not possible to rule out the presence of other phenolic compounds.

On the basis of the results discussed above, the unknown compound may be a peptide. Paper electrophoresis (section 3.2 iii) and column chromatography on Dowex 1 (section 3.3 iv) suggested that UNK_1 is weakly acidic. The

chromatogram of the digest of UNK_I (figure 3.Ic) indicated that the unknown compound may be a peptide of at least six constituent amino acids.

5.3 Aristolochic acid

Of the species of the principal genera *Aristolochia* and *Asarum* that have been examined, at least seven including *A petersiana* klotz contain Aristolochic acid, a cytotoxic compound. There is a controversy over the factor responsible for the abortifacient activity of these species. It is thought that Aristolochic acid owing to its cytotoxicity may be the agent inducing abortion. Angeles et al (2) in their study of the toxicity of Aristolochic acid in a number of systems showed that the acid caused marked toxicity mostly on the kidney, spleen, liver, lungs and hematopoietic systems. Marked uterine contractures were also observed in the cat when treated with 40 mg of Aristolochic acid per kg body weight. Similar uterine contractures were observed in the guinea pig when treated with an unspecified amount of Aristolochic acid. It is realistic to say that a uterine muscle stimulant may also induce abortion. Angeles et al (2) also cited that

Aristolochic acid is a violent abortive. However, the deduction that Aristolochic acid may be the abortifacient in A petersiana klotz is complicated by the fact that bioassays were carried out with crude extracts, which contain several other constituents and only a small amount of Aristolochic acid. This supposition is justified by the fact that the yields of pure Aristolochic acid from the Aristolochiaceae species including A petersiana klotz do not exceed 800 mg per kg weight of dried plant matter. Furthermore the existing evidence that an abortifacient and interceptive sesquisterpene was isolated from A indica linn (30), a species which also contains Aristolochic acid suggests that other active compounds may be present in A petersiana klotz. The scope of isolating Aristolochic acid in A petersiana klotz was to remove it completely so that the remaining fractions could be tested for physiological activities. Negative results would indicate that Aristolochic acid is the active agent in A petersiana klotz.

CHAPTER 6

EXPERIMENTAL

6.1 General methods and materials

All chemicals and solvents used in the experiments were of analytical reagent grade except where specified. The general purpose reagent solvents were distilled prior to use. Used chloroform, methanol and ethanol were reused after distillation. Used chloroform-methanol and chloroform-ethanol mixtures were separated by fractional distillation. The chloroform fraction was then purified as recommended by Rydon and Smith (33) and the methanol and ethanol fractions were redistilled.

All solvent evaporations were carried out under reduced pressure using a rotary evaporator (Büchi Rota Vapor R). Infrared spectra were obtained at room temperature on Perkin Elmer 197 and 297 spectrophotometers. Ultraviolet spectra were obtained on a Pye-Unicam SP 800 spectrophotometer. Melting points were determined in a mineral oil bath and were uncorrected.

The following chromatographic techniques were employed: column, ascending thin-layer, descending paper and electrophoresis. Extracts were examined on Whatman No I chromatography paper and on thin-layers

of Silica Gel G(Merck), Silica Gel G6(BDH) and Cellulose MN 300 (Macherey, Nagel and Co). The following solvent systems were used for development:

A. n-Butanol-acetic acid-water (90:10:29, v/v/v) (36): The solvent was used immediately after preparation.

B. Phenol-water (4:1, w/v) in an atmosphere of ammonia (S.G. 0.92) (38): The solvent, which keeps indefinitely at room temperature was made by adding 250 ml of distilled H_2O to 1 kg of phenol and the mixture warmed slightly on a water bath until all the phenol had dissolved.

C. Water-saturated n-butanol (8): An equal volume of n-butanol and distilled H_2O were shaken in a separatory funnel, allowed to settle for sometime and the excess H_2O removed. This solvent was used immediately after preparation.

D. Acetonitrile-diethylamine-water (12:7:1, v/v/v).

E. Acetonitrile-diethylamine-water (8:1:1, v/v/v) (8). The acetonitrile and diethylamine were distilled before use. The solvents made from

these reagents were used soon after preparation.

Detection of spots on thin-layer and paper was by visual observation, observation under ultraviolet light and by staining with spray or dip reagents.

6.2 Colour reactions

(a) Alkaloids

(i) Dragendorff's reagent (29): Solution (a), 850 mg $Bi(NO_3)_3 \cdot 5H_2O$ in 40 ml H_2O + 10 ml HoAc. Solution (b), 8g KI in 20 ml H_2O. Solution (a) and (b) were mixed to make the stock solution. Spray reagent was prepared by mixing 10 ml of the stock solution with 20 ml HoAc and making up the volume to 100 ml with distilled H_2O. Alkaloids gave red or orange spots.

(ii) Mayer's reagent (29): Solution (a) 1.36g $HgCl_2$ dissolved in 60 ml distilled H_2O. Solution (b), 5.00g KI dissolved in 10 ml distilled H_2O.

The test solution was prepared by mixing solution (a) and (b) and diluting to 100 ml with distilled H_2O. The reagent was added to the solution being tested for acidified with HCl or H_2SO_4. Alkaloids gave yellow precipitates.

(iii) Phosphomolybdic acid (29): A solution of ammonium molybdate was completely precipitated at 40°C with a solution of $NaHPO_4$. The yellow precipitate was washed, suspended in water and warmed with a concentrated solution of Na_2CO_3 until completely dissolved. The solution was evaporated to dryness and the residue ignited to drive off all ammonia. If reduction (blue colour) took place, residue was moistened with nitric acid and again ignited. The test solutions were prepared by dissolving the residue in hot water with addition of nitric acid until the solution was strongly acidic. 10 parts of solution were prepared from 1 part of residue. Most alkaloids gave yellow precipitates. Easily oxidisable alkaloids reduce molybdic acid to molybdic oxide resulting in a blue colouration.

(b) Steroids and Saponins

Lieberman-Burchard reagent (Acetic anhydride-sulphuric acid (19:1, v/v)) (11): Used as a spray or dip reagent. Steroidal saponins gave blue or green colours while triterpenoid saponins give red, pink or purple colours. Colour formation is due to reduction at a carbon-carbon double bond present in the compound.

(c) Amino acids and peptides

(i) Ninhydrin reagent (0.2%, w/v ninhydrin in 95% v/v acetone in water): The solution which keeps indefinitely under refrigeration was used as a spray or dip reagent and for micro spot tests. α amino acids and peptides with terminol α amino acids gave purple to blue colours. Proline and unsaturated amino acids give yellow or green coloured spots. Reaction is at the site of the amino group.

(ii) Biuret reagent (0.1% $CuSO_4.5H_2O$ in 0.1% NaOH) (15): Used as a test tube spot test reagent, purple complexes formed between the copper in the reagent and amide bonds of peptides.

(iii) Folin-Ciocalteau's reagent (25):

Solution (a), 2% $NaHCO_3$ in 0.1M NaOH.
Solution (b), 2% sodium potassium tartrate.
Solution (c), 1% $CuSO_4.5H_2O$. The test reagent was prepared by mixing 1 ml of each of solution (b) and (c) with 100 ml of solution (a). An aliquot of peptide solution (containing about 100 mg/ml) was mixed with 10 ml of test reagent and left to stand at room temperature for 10 minutes. To the mixture was added 0.5 ml diluted (1:1,v/v) Folin-Ciocalteau's reagent (Hopkin and Williams). Peptides formed purple complexes. The phosphomolybdate and phosphotungstate in the Folin-Ciocalteau's reagent can also be reduced by phenolic compounds such as tyrosine to give purple colouration.

(iv) Chlorination tests

(a) Chlorination - Starch/iodine test (39,42):

Spots on filter paper were chlorinated with chlorine gas at the amide bond followed by treatment with a solution of 1% starch/1% potassium iodide (1:1, v/v).

A blue complex of iodine - starch was produced. Some amino acids like glutamic acid, phenylalanine, leucine, lysine, histidine and proline give positive results with this test.

(b) <u>Chlorination - heat test</u> (33): Spots on filter paper were heated at temperature above 200°C following chlorination with chlorine gas. A browning spot indicated the presence of an amide bond. This reaction is also positive for glutamic acid, phenylalanine leucine, histidine, lysine and proline.

(v) <u>1-Nitroso-2-naphthol reagent</u> (0.1% 1-nitroso-2-naphthol in acetone, w/v) (1,19b,42): Spots on filter paper were sprayed with the reagent, dried resprayed with freshly prepared 10% nitric acid and finally heated for 3 minutes at 100°C. A rosepink colouration was obtained when spots contained tyrosine or tyrosine containing compounds.

6.3 Fractionation of crude extracts

A petersiana klotz was collected from Maamba in the Southern Province between February and April. Crude extracts were obtained from ground leaves, roots and stems and separated into fractions as shown in the schemes in figures 6.1 and 6.2.

6.4 Fractionation of the ninhydrin reactive constituents

Silica Gel (Kieselgel 60 grade, Merck) was activated by heating at 150 - 200°C for one hour and cooled in a desiccator. It was suspended in chloroform and used to pack a column, 45 x 5 cm. Fraction R_4 (5.28g) was taken in minimum ethanol and loaded to the column. Elution was initially performed in chloroform followed by successive gradients of chloroform-ethanol mixtures (10%, 50% and 90% ethanol in chloroform) and finally 95% ethanol. Effluent fractions of each 250 ml were collected. Samples of 50 µℓ were withdrawn from each fraction and tested as spots on filter paper for the ninhydrin reaction. Ninhydrin reactive fractions were combined (total volume 10.5ℓ), and concentrated to 11 ml on a rotary evaporator under reduced pressure at a temperature below 45°C.

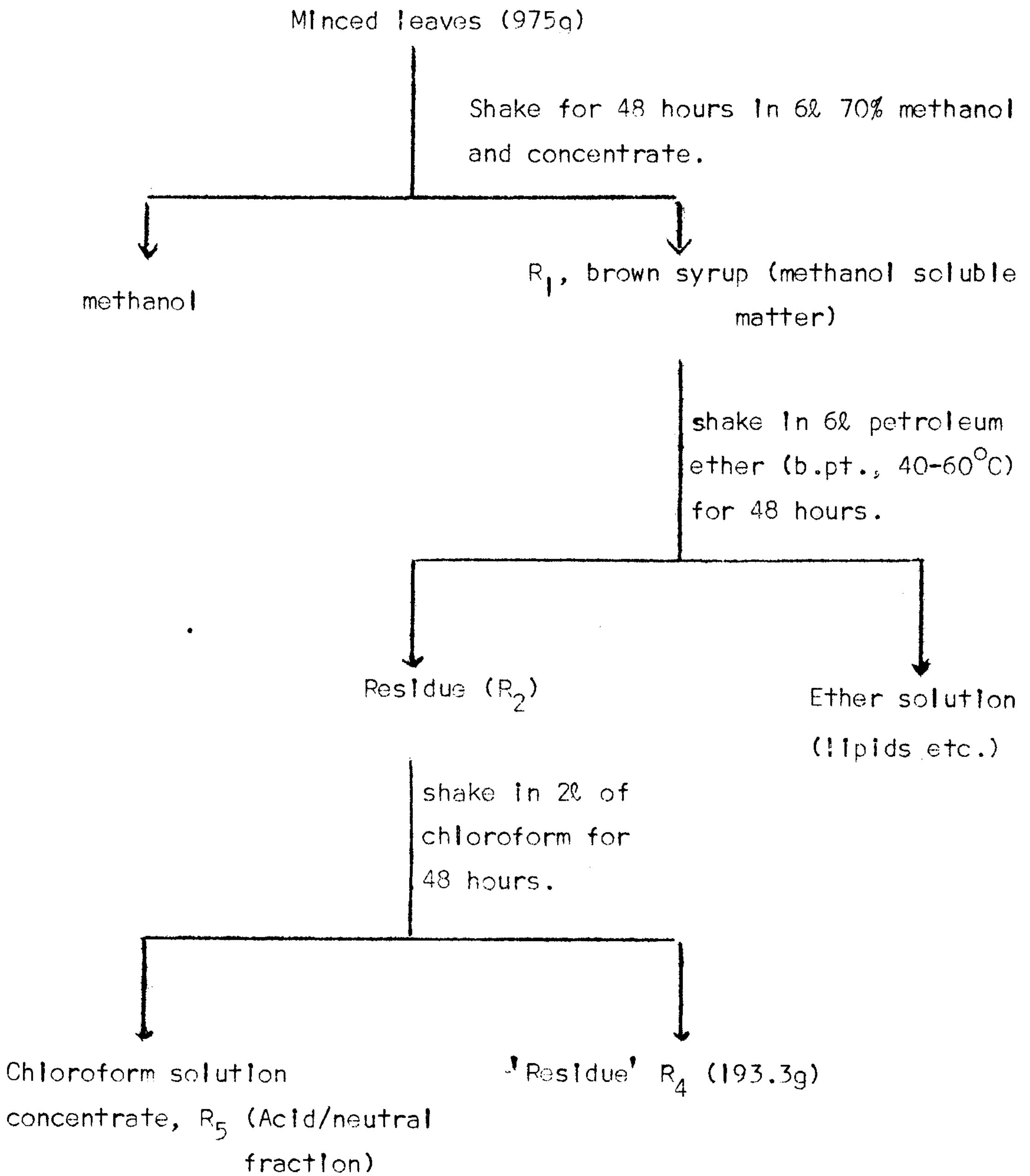


Figure 6.1: Flow chart for extraction of minced leaves of *A petersiana* klotz.

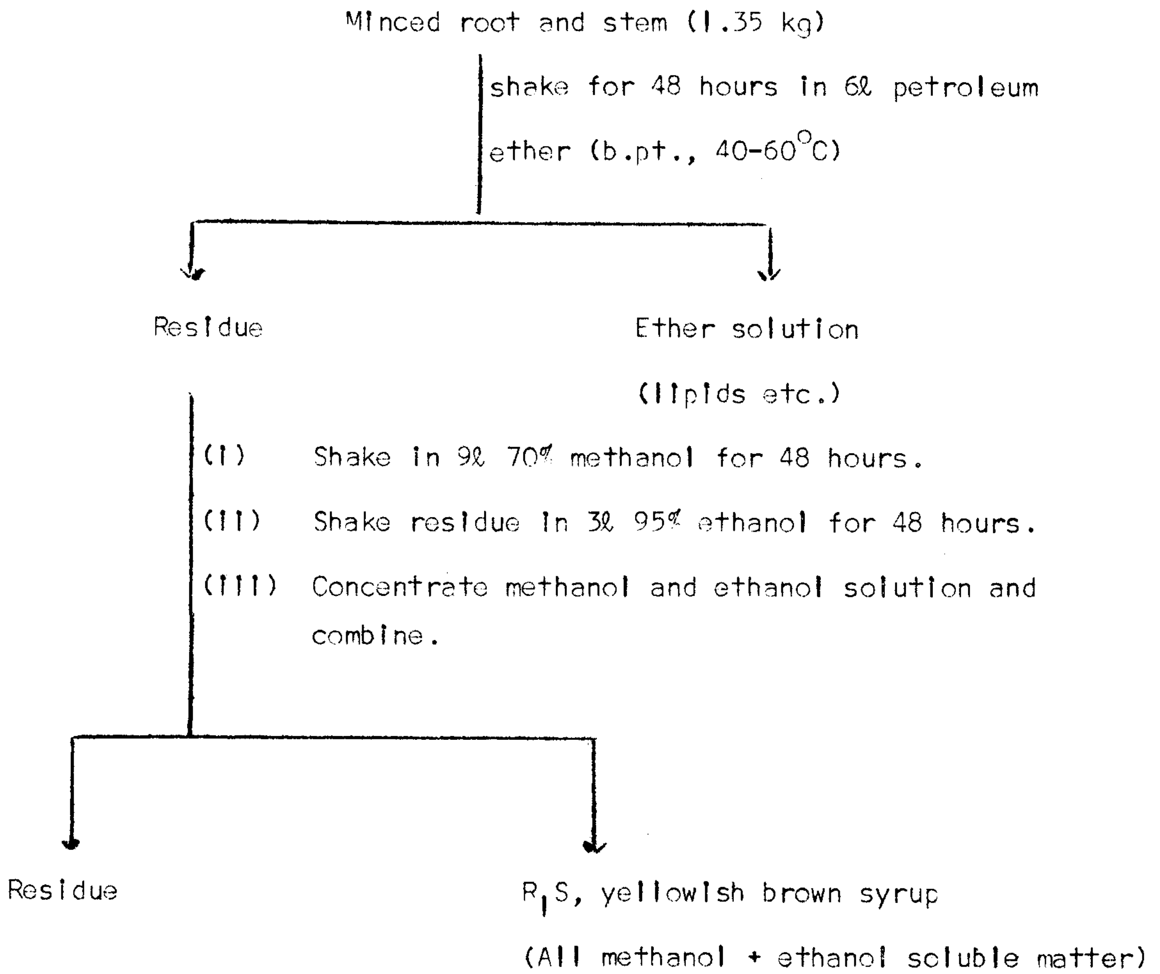


Figure 6.2: Flow chart for extraction of ground root and stem of *A petersiana* klotz.

(i) <u>Acid hydrolysis of UNK_1</u>

An ethanol solution (2 ml) of R_4 was evaporated to dryness on a watch glass at temperature below 100°C. The residue was dissolved in 2 ml of 5M HCl and digested in a sealed ampoule at 108° for one hour. The hydrolysate was evaporated to dryness on a watch glass, redissolved in 2 ml distilled water and evaporated to dryness once more. The residue was finally dissolved in minimum water and used to prepare two dimensional chromatograms on Whatman No 1 chromatography paper. Chromatograms were developed in phenol-water in the presence of the vapor ammonia (S.G. 0.92) (solvent 1) and in butanol-acetic acid-water (solvent 2). The ninhydrin reagent was used to detect ninhydrin reactive spots. Several two dimensional chromatograms of R_4 dissolved in ethanol were made on Whatman No 3 chromatography paper and developed in the same solvents as used above. One of the chromatograms was stained with ninhydrin reagent and the remaining chromatograms were designated as 'blank'.

The stained chromatogram was put over the 'blank' chromatograms and the area corresponding to the spot for UNK_I on the 'blank' chromatograms was cut off and washed with distilled water. The aqueous solution was concentrated to I ml on a rotary evaporator under reduced pressure at temperature below 45°C. A few μℓ of sample was withdrawn and used to prepare two dimensional chromatograms on Whatman No I chromatography paper. The remaining sample was transfered to a sealed ampoule and digested in 5M HCl at 108°C for one hour. The digest was used to prepare two dimensional chromatograms, which were developed under similar chromatographic conditions as the above. Similar two dimensional chromatograms of the known amino acids: glycine, serine, alanine, tyrosine, phenylalanine, leucine, valine and glutamic acid were made for comparison.

(ii) Enzyme hydrolysis

To a ml (5mg) aliquot of ninhydrin reactive material in a test tube was added I ml 0.04M veronal (diethylbarbiturate) buffer at pH 7.45 (19a). The mixture was incubated at 37° in a

water bath. 10 μℓ carboxy-peptidase A (Sigma) was pipetted and diluted to 2 ml with distilled water. Specific activity of the carboxy-peptidase A was 41 units/mg, concentration 20 mg/ml. 5 μℓ from the incubated sample mixture and 2 μℓ from the diluted enzyme were withdrawn and spotted on a Silica Gel plate as reference. To the sample mixture incubated at 37°C was added 1 ml of the diluted carboxy-peptidase A and incubation continued for one hour. During this time, 10 μℓ aliquotes were withdrawn at 15 minute intervals and spotted side by side with the reference on the plate. The plate was developed in the ascending solvent technique in phenol-water in an atmosphere of ammonia. Spots on the plate were detected with ninhydrin reagent.

(iii) Paper electrophoresis

A 20 x 18 cm strip of Whatman No 1 chromatography paper was impregnated with pyridine-acetic acid-water (10:0.4:90, v/v/v) buffer at pH 6.4 (42). Extracts were applied across the middle of the longer side of the

paper alongside "maker" amino acids: glutamic acid, aspartic acid, phenylalanine and tyrosine. Electrophoresis was performed on a glass block support for half an hour, in the cold room at 4°C, at 250 volts and a current of 60 amperes. The ninhydrin reaction was used to detect spots.

6.5 Attempts to isolate UNK_{1} by column chromatography

(i) Fractionation on Silica Gel

11 ml of the ninhydrin reactive fraction was loaded to a column (74 x 2.6 cm) of Silica Gel. Elution was started with a mixture of 50% ethanol in chloroform followed by 95% ethanol. Effluent fractions of 16 ml were collected at a rate of 60 ml/hour. Test samples of 50 μℓ were pipetted from each effluent fraction and examined as spots on filter paper for the ninhydrin reaction. The fractions were further analysed by one dimensional descending paper chromatography using butanol-acetic acid-water and by two dimensional descending paper chromatography using phenol-water in an atmosphere of ammonia and butanol-acetic acid-water. In both cases

the chromatographic patterns obtained were similar to those of R_4 on two dimensional and one dimensional descending paper chromatograms. This indicated that the eluted fractions were the same and the ninhydrin reactive material was unresolved.

(ii) Fractionation on Sephadex G 25-80

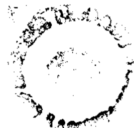
Sephadex G 25-80 (Sigma) was prepared according to Mikes (27) and packed to a 50 ml burette (22 x 1.5 cm). A portion of the ninhydrin reactive fraction in 1 ml distilled water was loaded to the column. Effluent fractions of 10 ml, which were eluted with distilled water were collected and concentrated to about 0.5 ml on a water bath. 50 μℓ aliquotes were withdrawn and spotted on thin-layer plates of cellulose MN 300 and Silica Gel G(Merck). The plates were developed in one dimension in the ascending solvent technique in butanol-acetic acid-water, dried and sprayed with the ninhydrin reagent. The results obtained indicated that separation had not been achieved because all fractions were the same.

(iii) Fractionation on DEAE Sephadex A25

DEAE Sephadex A25-120 (weakly basic anionic exchanger, (Sigma)) in the chloride form was prepared as recommended by Mikes (27) and converted to the acetate form by washing with 0.5M acetic acid. The resin was then packed to a 32 x 1 cm column in 0.1M collidine (2,4,6-trimethyl pyridine,(BDH))- acetate buffer at pH 8.55. The ninhydrin reactive material (2.35g) was dissolved in 2 ml of the collidine buffer and adjusted to pH 8.55 with ammonia. Elution was started with the same buffer followed by increasing amounts of 0.1M acetic acid. Effluent fractions of 2 ml were collected at a rate of 30 ml/hour. After fraction 46, 1M acetic acid was used to establish another pH gradient. Samples of 50 µl were withdrawn from each effluent fraction and tested as spots on filter paper for the ninhydrin reaction. All fractions gave positive reactions. One dimensional descending paper chromatography of these fractions in the solvent butanol-acetic acid-water revealed that separation had not been achieved because the same pattern was obtained for all effluent fractions.

(iv) Fractionation on Dowex I

Dowex I x 8-100 (strongly basic anionic exchanger, (Sigma)) in the chloride form was converted to the acetate form by washing with 3M sodium acetate solution (4) until the filtrate was chloride free. The anhydrous sodium acetate (BDH) (laboratory reagent) was purified prior to use by recrystallisation from aqueous ethanol. The resin was finally washed with 0.5M acetic acid and poured into a 32 x 0.5 cm column. After the column was washed with water to get rid of excess acetic acid, the ninhydrin reactive material (90 mg), dissolved in 2 ml 0.5M acetic acid, was loaded to the column. 0.5M acetic acid was used for elution. Effluent fractions of I.2 ml were collected at a rate of 6-7 ml/hour. 39 fractions were collected in all. Samples of 50 μℓ were pipetted from each fraction and tested as spots on filter paper with the ninhydrin reagent. Further 200 μℓ portions were pipetted from each fraction for application on Whatman No I chromatography paper. Aspartic acid, glutamic acid, tyrosine and phenylalanine were also spotted together



with the effluent fractions as "markers". Chromatograms were developed in one dimension by the descending solvent technique. Fractions 1-4 were blank; fractions 5-8 contained the unstable compound, UNK_1; fractions 9-25 contained glutamic acid; 26-29 contained aspartic acid. Only fraction 6 and 7 and 18-25 gave positive reactions to the chlorination tests indicating the presence of amide bond. Lack of enough of fractions 6 and 7 made it impossible to do further confirmatory experiments. An attempt to fractionate the ninhydrin reactive fraction on a larger column of Dowex 1 was unsuccessful.

6.6 Isolation of the total acid:

Fraction R_1S (29.71g) of the extract of root and stem (figure 6.2) in a separatory funnel was treated with 1.5% of chloroform and shaken vigorously. The suspension was filtered to obtain a clear chloroform solution, which was treated with 4% $NaHCO_3$ solution. The chloroform and bicarbonate layers were separated, the chloroform recycled to extract some more acids and the wine red bicarbonate

layer acidified with 5% HCl to yield a yellow precipitate. Extraction with $NaHCO_3$ was completed when no further precipitation resulted upon acidification of the bicarbonate extract. A total of 1g $NaHCO_3$ was used. The precipitate was filtered over a buchner funnel and dried in a dessicator, under reduced pressure. The yield of total acid was 219 mg.

6.7 Isolation of Aristolochic acid

(i) Preparative thin-layer chromatography:

Silica Gel G6 was spread on thin-layer plates to a thickness of 0.75 mm. The total acid taken in chloroform was applied to the plates as a streak (approximately 2.8 mg/ml was applied to each plate). Thin-layer chromatograms were developed in acetonitrile-diethylamine-water (12:7:1). After development, two bands were visually observed, with Aristolochic acid I forming a lime yellow band at R_f around 0.68. A second band, yellowish orange in colour formed at R_f around 0.36. The two major bands were scraped off separately and the compounds

extracted from the gel with chloroform-ethanol mixtures. Aristolochic acid I was crystallised from dimethyformamide-water and washed with chilled distilled water. The compound decomposed between 230°C and 238°C within the decomposition range of the authentic sample of Aristolochic acid (238-240)°C.

(ii) Fractionation on Silica Gel-Celite

75g Silica Gel-Celite (4:1) was packed into a column, 19 x 2.8 cm in chloroform. The total acid (140 mg) in minimum methanol was loaded to the column. Elution was started with chloroform and the column was resolved into two bands: a lime yellow band at the bottom, the largest, and a yellowish orange band at the top. Effluent fractions of 100 ml were collected and monitored both by ultraviolet spectroscopy and by thin-layer chromatography on Silica Gel G6. Development of thin-layer plates was in acetonitrile-diethylamine-water (12:7:1). The lime yellow band was eluted with chloroform (1.5ℓ) and the yellowish orange band with 10% methanol in chloroform (1ℓ). The

ultraviolet spectra of the middle chloroform effluent fractions (fractions 4-9) were similar to that of pure Aristolochic acid dissolved in methanol, absorptions at 223 nm and 249 nm. Thin-layer chromatography of these fractions revealed that the yellowish orange component was also present in very minute quantities. The yellowish orange component increased in amount in the successive fractions 10-22.

6.8 Bioassay studies

These were preliminary studies on *A petersiana* klotz and were performed by Professor Siamwiza (37). The studies comprised of *in vivo* experiments for testing the abortifacient and fertility regulating activities and *in vitro* experiment for testing the physiological activity on isolated rat uterus. The detailed experimental procedures of the *in vivo* experiments are given in sections 2.1 and 2.2.

In vitro experiment for testing for uterotonic activity

Leaves, roots and stems of *A petersiana* klotz were dried at room temperature and ground. Water extracts were made by soxhlet extraction of the dried

ground matter (155g) in 5ℓ of distilled water. Approximately 13g of dried ground matter was extracted with 0.5ℓ or 0.3ℓ of water in a thimble until the solvent, which is generally brown became clear. The water extract was concentrated to 0.5ℓ on a rotary evaporator, under reduced pressure at temperature below 45°C. Each ml of this extract was equivalent to about 16.9 mg of dried plant material. The experimental procedure followed closely the WHO Task Force protocol (43a) on indigenous plants for fertility regulation. The uterii from virgin female white albino rats of average weight 135g were sensitised by injecting each rat intramusculary with 0.5 mg of oestradiol benzoate dissolved in 0.5 ml peanut oil. 24-26 hours after sensitisation, rats were killed by cervical dislocation. The uterine horns were quickly removed and placed in a modified Van Dyke - Hastings solution.* $\frac{1}{3}$ of the uterine tube from each uterine horn was cut off from the ovarian end and discarded. The remaining $\frac{2}{3}$ of each horn was then separated by cutting at their point of attachment to give 2 uterine tubes. The tubes were mounted with non reactive cotton thread in a 10 ml organ bath containing the modified Van Dyke - Hastings solution at 30°C and aerated with 95% O_2 in CO_2 (v/v). The uterine tube was then attached to an isometric transducer and the muscle tension at

about 0.1g. The muscle was washed twice after attachment and allowed to equilibrate for 2 hours. If spontaneous activity was present after the 2 hour period, the muscle was discarded. If no spontaneous activity was present, acetylcholine was added to give a final concentration of 10^{-6}M. The maximum volume which was added to the bath was 0.1 ml. If contractions became constant after several contractions, the muscle was considered unsable and then washed twice using twice the bath volume. All washings used the modified Van Dyke - Hastings solution. 10 minutes after the last wash, A petersiana klotz extracts were added in volumes of (0.1 - 0.5) ml and occassionally 1.0 ml. Oxytocin, (0.002 - 0.005) units was used as a reference. Activity measurements were done using a kymograph (Electric 12 recording drum, Palmer) and readings were recorded as trace heights. The trace height of oxytoxin was always taken as 100%. In between each measurement, stimulated uterine muscle was washed until the deflector on the kymograph returned to baseline. Activity measurements of each extract were done on several uterine preparations and average values of maximum and minimum values were recorded. In all cases the duration of uterine muscle activity was 1 minute.

* Preparation of modified Van Dyke - Hastings solution.

(a) Stock solution I:

NaCl, 120.67g

$NaHCO_3$, 46.62g

KCl, 8.27g

Phenol red, 135 ml of 0.02% solution

Water was added to make 18ℓ

(b) Stock solution II, Phosphate buffer, 0.11 mole/l:

Na_2HPO_4 (anhydrous), 22.714g dissolved in hot distilled water and made up to 1ℓ

$NaH_2PO_4.H_2O$, 5.520g dissolved and diluted to 1ℓ

The two solutions were titrated to pH 7.40

(c) Stock solution III, $CaCl_2$, 0.5 mole/l:

$CaCl_2.2H_2O$, 73.515 g/l

(d) Stock solution IV, $MgCl_2$, 0.5 mole/l:

$MgCl_2.6H_2O$, 101.665 g/l

To prepare 1ℓ of the working solution, 988 ml of stock solution I was gased with 5% CO_2 in O_2 for about 20 minutes until the pH was 7.4. 10 ml of stock solution II, 1 ml of stock solution III, 1 ml of stock solution IV and 0.5g of glucose were added. The solution was ready when the glucose had dissolved.

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