Evaluating the Medicinal Properties of Mandravasarotra (*Cinnamosa sida*) Through Chemical and Pharmacological Screening

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. "Evaluating the Medicinal Properties of Mandravasarotra (*Cinnamosma sida*) Through Chemical and Pharmacological Screening"

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Acknowledgements

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. "Evaluating the Medicinal Properties of Mandravasarotra (*Cinnamosma sida*) Through Chemical and Pharmacological Screening"

Abstract

I worked for four weeks at the University of Antananarivo (Ankatso), spending two weeks each in the chemistry and pharmacology laboratories. There I ran a total of eight experiments or "screens" with an alcoholic extract of the medicinal plant Mandravasarotra. This plant is found mainly in northwestern Madagascar, and is often used to stimulate contractions for women having difficulty giving birth. The plant tested positive for three secondary metabolites: tannins, steroids and flavanoids. In the pharmacological tests, the plant exhibited anti-inflammatory properties, and high toxicity in doses exceeding 10ml/kg. Together, conjectures may be made between what is known of the present secondary metabolites, and the physiological effects manifested. More work is necessary to know the exact nature of the classes of chemical compounds found, and their relation to the exhibited physiological manifestations.

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Introduction/Background

Ethnobotany is a unique area of research that concerns itself with learning how people incorporate and assign value to various local plants into their culture. Often researchers seek to learn more about cultures in developing countries, where people involve themselves intimately with their environment. In fact, ethnobotany is a widely ranging field which incorporates six diverse disciplines: botany, ethnopharmacology, anthropology, ecology, economics and linguistics. (Martin 3) With these six disciplines ethnobotanists develop research questions and goals following four basic formats, as outlined by Gary Martin, author of a manual of ethnobotany. The first research format is to uncover and record the "botanical knowledge" held by a particular ethnic group. The second format is to evaluate and quantify the use and management of botanical resources. The third format is to assess via a series of experiments benefits derived from the plants that are used for both subsistence and profit. The fourth approach is to engage in an applied project where the researcher actively searches for solutions and ways to increase the value that an ethnic group receives from their "ecological knowledge of resources."

This project is designed according to the third format, wherein a series of experiments were conducted to discover the medicinal properties of the plant Mandravasarotra. This type of investigation falls under the sub-discipline of phytochemistry. Phytochemistry is defined as "the study of the distribution of both primary and secondary metabolites in the plant world, guides the search for not only new plant compounds but also new sources of known drugs". (Martin 69) As with ethnobotany, phytochemistry involves an array of disciplines including: nutritional chemistry, natural products chemistry, chemical ecology, ethnopharmacology and pharmacognosy. Also

according to Martin, nutritional chemists measure the quantities of specific nutrients found in edible animals and plants in order to discover the extent to which they fulfill dietary requirements. Natural products chemists seek to find ways to apply biological molecules into industrial materials. For example they work with latex to develop rubber or essential oils to produce perfumes. Chemical ecologists study how biological compounds affect relationships plants and animals, especially that between plants and insects. Ethnopharmacologists are primarily concerned with describing the medicinal properties in remedies used by local people, paying special attention to plant selection, preparation and administration. It has both field and lab components and is a mixture of chemistry, biology and anthropology. Pharmacognosy is the branch of phytochemistry this project entails. It is the study of naturally occurring compounds that can be used medicinally or otherwise. It comprises of both chemical and biological studies.

The factor which ties these vastly differing research fields together is their work with secondary metabolites. These diverse biomolecules are found in every plant on earth. The beginning of their production occurs when a plant intakes micro and macronutrients. Micronutrients consist of certain metallic elements which exist in the soil. These include copper, manganese, zinc, iron and cobalt. Macronutrients consist of a variety of molecules which are derived from a number of sources. These include hydrogen, carbon and oxygen, which come from air and water; potassium, phosphorus and nitrogen, which come from the soil; and amino acids, sugar and carbohydrates, which are synthesized from photosynthesis.

Primary metabolites are derived from a combination of all these components. Primary metabolites are uniform in structure and are found in every plant [and animal] on earth. They comprise of carbohydrates, proteins, fats and nucleic acids (DNA/RNA).

Through a number of processes, the primary metabolites produce secondary metabolites which are "distributed throughout the plant kingdom in characteristic patterns". (Martin 60) At one time it was believed that these chemicals were merely waste products of physiological processes. Now it is known that this vast array of molecules aid in a plant's adaptation to its environment by enhancing various aspects of competition, defense and attraction. For it is now known these chemicals lend plants their individual smells, colors, flavors and medicinal properties

Perhaps one of the most crucial tools available to a .pharmacognosist is that of screening. However, before this may occur there is often some previous collaboration with an ethnopharmacologist. There are three steps which occur in this partnership: sampling/plant selection, screening and bulk collection. For the sampling of the local plants, an ethnopharmacologist will follow one of three methods. He will sample randomly, taking widely of whatever plant is most available; he will take plants only from species that are known to house a certain chemical; or he will take plants already being used by local people for medicinal purposes, usually narrowing it down to plants used for treatments of specific ailments. (Martin 72) For a pharmacognosist, selection of a plant involves performing extensive background research on various compounds and plants of choice. Thi might include speaking with an ethnopharmacologist, about a particular plant, or plants containing certain properties or compounds. After this is done, "screening is performed to decide which species are apt to yield the most promising results before performing an extensive investigation". (Martin 72)

One last step must be taken before screening can begin. The ethnopharmacologist must present the pharmacognosist's laboratory with a voucher specimen. This preserved

form of the plant(s) to be tested has been called the "link between western knowledge, and traditional knowledge". (Martin 28) Though fresh plants are desirable, it is highly impractical as they most surely will wilt, become desiccated or moldy before reaching the laboratory causing the chemical composition to be altered significantly. (Martin 85) Therefore, plants are carefully sun dried in the field to preserve them, and then they are carried to lab to be tested by the pharmacognosist. Once in the laboratory, the plant is chopped into tiny pieces, and then ground into a coarse powder. In this state the plant is ready to be screened for its chemical and medicinal properties.

There are two subtypes of screening. The first is chemical screening, which seeks to identify the presence of the major families of secondary metabolites present in the plant. To screen the plant chemically, either the powder itself or an alcoholic extract is exposed to a number of reagents, per a given set of procedures. "The results are visual; a solution turning color, forming a precipitate (a solid material that forms within the extract), or some combination of the two". (Martin 77) The second type of screening is a pharmacological screen. These experiments are designed to uncover how the chemicals present in the plant affect the physiological processes within animals. Unlike chemical screening, the nature of the results from pharmacological screens can vary greatly from test to test. Generally, all pharmacological screens are comprised of a control set of animals, and then three sets of animals containing varying doses of the plant extract. Screens (or assays) which may be performed include those testing for toxicity, anti-inflammatory and anti-bacterial properties. The results of each screen are known as a "data set". Once all the data is gathered the researcher "organizes it, structures a database, protects it, analyzes it and then presents it". (Martin 10)

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For this project, the plant *Mandrvasarotra* was chosen, by the acclaimed ethnobotanist, Dr. Nat Quansah, whose tireless work to bridge modern and traditional medicinal practices, earned him the prestigious Goldman Environmental Prize in 2001. In our first interviews discussing the nature of my work, I learned that my particular plant was an herb of the species *Cinnamosma sida.* One of the plant's major uses, in its native location, is to stimulate contractions in the uterine walls of women having difficulty with labor, by a preparation of its stems and leaves. In my background research on the plant I discovered Dr. Henri Randrianasolonjanahary, who has done extensive research with *Cinnamosma fragrans*, which is in the same family as my plant. It is found predominantly in the fishing village of Antsanitia, which is located on the northwestern coast of Madagascar in the region of Majunga. Through his research he has discovered many of the reasons why Mandrvasarotra is an integral part of *fanafody gasy* or Malagasy pharmaceuticals. Depending on the preparation of the plant, it may be used to treat fevers, as a diuretic or integrated into religious ceremonies. In addition, its bark is used to treat intestinal parasites; and its balsam (a type of fragrant sap) is used to treat coughs and dysentery. The combined knowledge of the medicinal uses of these plants has aided in the interpretation of some of the results of the various screens.

Altogether, eight separate screens were performed with the plant at the chemistry and pharmacology laboratories located at the University of Antananarivo (Ankatso). Six were chemical and two were pharmacological. I worked with three professors, Dr. Amélie Ramarisololalao, Dr. Fanantenanirainy Randimbivolona and Dr. Patricia Quansah as well as the masters' students who worked in their respective laboratories. In each of the chemical screens, a test was performed to detect the presence of a single class of compounds. In the pharmacological screens an extracted form of the plant was injected into

mice to determine specific physiological effects. Before any screening could commence it was necessary to isolate the secondary metabolites from the plant. Afterwards this liquid was incorporated by several means to evaluate the medicinal properties and chemical composition of Madravasarotra.

The first class of molecules screened for were the flavanoids. This family is dichotomized by two parent molecules flavane and flavone. They differ slightly in structure, (flavonoids have two doubly bonded sites with oxygen and within the second ring, where flavanoids have methyl groups at these locations). However each molecule performs greatly differing tasks within the plant. Each has an isomorph and a morph with a hydroxide group (OH), rendering it an alcohol. Flavonoids are synthesized from a reaction between a phenol having an adjacent hydroxide side chain and COOH group. The two condense to produce a second ring. Then, using oxygen as a link, a molecule of phenol is added to the second ring. The flavonoids are in a family of three ringed compounds that produce pigments in flowers; the flavonoids produce the color yellow. (Langeheim 110) The flavanoids are also synthesized to have this third ring, and often are further synthesized to adopt a form know as catéchétique. This is when two hydroxide groups are added to the third ring. (See Appendix A)

The flavanoids are used as antioxidants in plants, and because they are water soluble are easily absorbed into the human body and may be used in the same manner as well as help prevent heart disease and cancer. (jayateas.com) They are found in abundance in teas; in green teas they are simple catechins and in black teas they are the complex flavanoids theaflavins and thearubins. (jayateas.com) These compounds have been shown to inhibit the oxidation of LDL, prevent platelet aggregation (improving blood vessel function) and have

anti-inflammatory properties. (jayateas.com) However, at high doses, flavanoids have been shown to be toxic in lab animals and humans, affecting the liver, kidneys and intestines. (sciencedaily.com)

The second class of molecules screened for were the alkaloids. Perhaps no other group of secondary metabolites have been more sought out or revered or feared by man. Being as they are nerve exciting chemicals they are the active principle in soma, the mushroom religiously revered in the ancient Indian text Rig Veda, peyote, a small cactus in the Mexican desert known for its hallucinogenic properties, opium, which is derived from the seed of the poppy plant, and it is the active ingredient in nicotine, derived from the tobacco plant. (Martin 425) These powerfully addicting dugs have also had an effect on Malagasy culture. The following two anecdotes demonstrate how strong their effect can be:

> "Our ancestors not only forbade the eating of tobacco, but they cursed the one who did so. The origin of this taboo is as follows: long, long ago there was a terrible famine in our village. Several of the inhabitants suffered death through lack of food. Those who were dependent of tobacco sold the little food they had in order to get tobacco leaves. They suffered, as well as their wives and children. When the authorities of the village saw this misery, they pronounced the following imprecation (*nanao tsitsika*): As far as our children and decedents are concerned, let them have no future, may they never get what they need for their living, whether they go Northwards, Southwards, Eastwards or Westwards, let them be destroyed." (Ruud 74)

The second tale is as follows:

> "The smoke of hemp-seed is such a strong narcotic that it dulls the man completely. He becomes slack and lazy and has no feeling of responsibility. He becomes short tempered, capricious and beats his wife and children. He involves himself in quarrels and fights, so that the village council will have to take steps against him. As long as

> nothing disastrous occurs, nothing is done, but as soon as the smoking leads to disaster in one way or another, they agree to make the smoking of hemp a taboo." (Ruud 75)

As demonstrated by the documented change in behaviors above, alkaloids have serious effects on the brain. This is because they work on the nerve endings in the nervous systems of animals. (Martin 426) Undoubtedly, they do not have this effect in plants as plants do not have nervous systems. Alkaloids, though they are not proteins, are synthesized from amino acids and there are over 5,000 varieties of alkaloids known to man. (Martin 426)

In plants, alkaloids are bitter tasting and therefore deter predators, they might also be poisonous and therefore deter predators and they serve as storehouses of nitrogen. (Martin 69) Alkaloids are alkaline in solution and are found as organic acids in plants. This is because the nitrogen, in the form of ammonia (NH_3), is condensed with an amino acid to become incorporated into a carbon rich ring. Subsequently, one of the hydrogen's attached to the nitrogen is hydrolyzed and replaced by an organic radical. (Langeheim 425) The indole alkaloids are especially known to stimulate the brain. This is because they closely resemble nerve stimulator, serotonin, and inhibit the effects of the enzyme that removes the amino group ($NH_{2)}$ group from serotonin after which, would normally arrest its function. (Langeheim 435) These alkaloids are found predominantly in the plant families of Apocynaceae, Leguminosae, Rubiaceae and Euphorbiaceae, and comprise one fourth of all known alkaloids. (Langeheim 435)

The third group of compounds screened for were the tannins. Tannins are "astringent phenolics" found in fruits as persimmons and cause a puckering effect in the

mouth. (Langeheim 330) When fruits ripen the tannins are polymerized onto the cell walls thereby lessening the puckering effect. Also, this triggers a change in pigment causing the green chlorophyll to break down; exposing the xanthophylls, carotenes, lycopenes (all produce the color yellow) and the anthocyanins (produces the colors red/purple). Also, this polymerization of tannins stimulates the synthesis of volatile essential oils which enhance the fruits aroma and flavor. (Langeheim 330) Tannins are also present in the skin of red grapes and give red wines their deep color and characteristic "dry" flavor. (Langeheim 368)

In addition, tannins are concentrated in the "rich, inner layers of bark". (Langeheim 410) Here they work to protect the plant against invading insects. They are able to inhibit the proteases and the digestive enzymes found in the insects intestines. (Langeheim 536) Industrially tannins are revered for their staining and strengthening abilities. Tannins extracted from bark are often incorporated into inks. They are most often incorporated into leather production, because they can bond with collagen, thereby strengthening and making the leather tougher and resistant to tear. (Langeheim 410)

Some tannins are synthesized through a condensation of gallic acid and a sugar. This combination produces a molecule known as gallic tannin. In a similar process, ellagic acid combines with a sugar to produce ellagic tannin. (Ramarisololalao) Also, as in the case of the catéchétique flavanoids, a sugar may be attached to a molecule of catéchol (a phenolic ring with a second hydroxide side chain) to produce a "*tannin catéchétique*". (Ramarisololalao) (See Appendix B)

The next class of compounds screened for were the terpenes. They are the largest group and most structurally varied of all the secondary plant metabolites. (Langeheim 455) In general, terpenoids are structured into chains or rings derived from a C_5 isoprene

building block, and may be reduced or oxidized to form alcohols, ketones, aldehydes and acids. (Langeheim 455) The intricate chain of synthesis begins with the molecule pyruvate (used most often in respiration). This is transformed to create the molecule Acetyl Coenzyme A (acyl CoA), three of which are subsequently condensed to form mevalonic acid which contains six carbons. This then undergoes three reactions involving phosphorylation and decarboxylation to give the activated C_5 isoprene. To this, two different morphs of isopentenyl pyrophosphate (IPP) condense, and then lose a pyrophsphate to form the ten carbon geranyl pyrophosphate. This comprises of the first class: monoterpenoids. To this, another molecule of IPP may be added to form the fifteen carbon molecule farnesyl pyrophosphate. This comprises of the second class: sesquiterpenoids.

Another molecule of IPP is added to this molecule to form geranyl-geranyl pyrophosphate, a twenty carbon molecule. This comprises of the third class: diterpenoids. The process then starts over with the dimerization of farnesyl pyrophosphate to form a thirty carbon molecule. This comprises of the fourth class: triterpenoids. Next, geranyl pyrophosphate is dimerized to form a forty carbon molecule. This comprises of the fifth class: tetraterpenoids. After this, any number of IPP molecules may be added forming the sixth class: polyterpernoids. (Langeheim 455)

Undoubtedly, each class of compounds carries it own unique set of characteristics and functions. Table 1.A briefly delineates what these are:

Name	# of Carbons	Product
Monoterpenes	C_{10}	essential oils
Sesquiterpenes	C_{15}	essential oils
Diterpenes	C_{20}	gibberellins
Triterpenes	C_{30}	saponins
Tetraterpenes	C_{40}	carotenoids
Polyterpenes	C_n	rubber
mono+sesqui+di+triterpenes	C_n	resin

Table 1.A Terpenoid Derivatives and Their Products

Both monoterpenes and sesquiterpenes form essential oils. Some examples of well known monoterpenes are citrus, menthol and camphor. (Langeheim 457) Together, monoterpenoids and sesquiterpenoids enhance a plants odor and flavor, help to attract pollinating insects and repel predatory ones, also they are able to inhibit the growth of parasitic organisms. (Langeheim 457) As essential oils they also are able to reduce water loss for plants growing in arid environments and can deter grazing animals. (Martin 69) Seperately, monoterpenes are most often used by the plant as a substrate for energy metabolism, where sesquiterpenoids as abscisic acid and xanthoxin are used for regulation in the plants physiological systems. (Langeheim 457)

Diterpenoids can come in the form of phytol which is a constituent of the chlorophyll molecule. Also, in the form of gibberellins they are able to stimulate growth within the plant. Triterpenoids can come in the form of sterols and can be incorporated by animals as a part of the structure of their membranes. They also aid in the vegetative and reproductive activity of fungi. Tetraterpenoids take the form of carotenoids, a yellow to

orange pigment that associates with chlorophyll. It is used by the plant to cue pollinators, which subsequently disperse the ripe seeds of the plants. Polyterpenoids, like their isoprenoid parents, take the form of long, unbranched chains. In addition to forming rubber, they are a component of latex, and act as insecticides, fungicides and bactericides. Combinations within the plant of mono, sesqui, di and sometimes triterpenoids produce a thick sap known as resin. This was used by ancient Egyptians to preserve mummies, and in the plant is used to defend against attacks from insects and other microorganisms. (Langeheim 457) Humans derive most of the terpenes they use from a plant's leaves. These are then incorporated into food flavorings, perfumes, medicines, germicides and fungicides. (Langeheim 458)

Steroids are four fused carbon rings which contain no nitrogen therefore, excluding them from the family of alkaloids. (Langeheim 436) Some familiar steroids are the sex hormones androgen and estrogen. In the case of a feeble heart beat they may be used medicinally as heart stimulants because they "stimulate the heart muscles, prolonging the period of relaxation in each heartbeat, and does not affect the period of contraction. Therefore the heart gets a better blood supply." (Langeheim 436) In plants there is a unique link between terpenes and steroids. The triterpenoids undergo a reaction to produce sterols. Sterols are "a subgroup of steroids with a hydroxyl group in the 3-position of the A-ring." (Wikipedia-sterols) They are important in food additives, cosmetics and medicine (they are known to reduce cholesterol in humans). As a result of this special relationship, terpenes and steroids are screened for together with the result being the presence of one compound over the other.

Saponins are related to both steroids and triterpenes as they are the structures of each with an added sugar (glycoside). (Ramarisololalao) They are characterized by the persistent foam or mousse they create when agitated with water, and by their ability to lyse red blood cells. This property makes many of the compounds in this family toxic. It is found most often in leaves, stems, roots, bulbs, blossom, and fruit. Its medicinal uses include treatments for hypercholesterolemia, hyperglycemia, free radicals, cancer, inflammation, obesity and to gently cleanse the blood. (Wikipedia-saponins)

Two pharmacological screens (or assays) were performed with varying doses of the extract: anti-inflammatory and toxicity. Inflammation is natural response by the body to infections and pathogens. It is characterized by redness, swelling and extreme tenderness in the immediate area surrounding the infection. In pharmacology, any substance which reduces the inflammation, that is to say the swelling, is known as having anti-inflammatory properties. In pharmacology, it is just as important to know how much of a treatment is beneficial, as is to know what the specific treatment of a physiological ailment is to be. For this reasons toxicity screens are performed with large doses of a treatment to understand at what point the medicine ceases to be helpful and becomes deadly. Also, it is important to note in toxicity screens the bad effects observed before death from the product occurs. From this you learn how the chemicals react as they spread through the body.

Methods

Hydroalcoholic Extraction

The process, known as hydro-alcoholic extraction, begins by gathering the plant from its natural environment and carefully drying it to arrest enzymatic reactions which might destroy the compounds before they may be detected. Afterwards, the plant was chopped and ground into a powder in the laboratory. One hundred grams of the powder was placed in a jar with enough of a solution made of two parts methanol and one parts water to cover the surface. The jar is sealed and allowed to sit for 24-48 hours. The liquid is drained off of the powder and placed in hot water to distill the water/methanol solution from the extract.

Flavanoids/Flavonoids

There are two separate tests for the flavanoid/flavonoid screening. For the first of two tests a little of the extract is poured into a test tube. Next, a sliver of Magnesium metal and hydrochloric acid is added. After shaking the mixture lightly the color is regarded. For test two, again a little extract is poured into a test tube. Afterwards, a potassium pellet is dropped in. After slight agitation, the color is regarded. Table 1.B prorates the color-chemical associations:

Flavone	yellow-orange
Flavane	pink
Flavonol	yellow-red
Flavanol	red-purple

Table 1.B Color-Chemical Associations in Flavanoid/Flavonoid Screening

Alkaloids

Before any of the alkaloid screens are performed, it is necessary to dry the extract out. For the first test it is necessary to prepare some Mayer reagent. This is composed of 1.36 g HgCl2, 1.60 g IK and 40 ml of distilled water. After making the reagent, mix it and a little of the extract in a test tube and observe the color change. For the second test it is necessary to make a potassium iodine reagent. This is comprised of 16.00 g of IK and 40 ml of distilled water. After making the reagent, mix it and a little of the extract in a test tube and observe the color change. For the third and final test requires Dragendorff reagent. This is comprised of 4.5948 g of bismuth nitrate, acetic acid and distilled water. After making the reagent, mix it and a little of the extract in a test tube and observe the color change. Table 2.B prorates the color-chemical associations:

Mayer reagent	white
IK reagent	orange-yellow
Dragendorff reagent	brown-yellow

Table 2.B Color-Correspondence by Reagent for Positive Result in Alkaloid Screening

Tannins

To screen for tannins place a small amount of the coarse powder into a test tube and agitate it with hot water. Drain the liquid into three test tubes (one is to be used as a control for color comparison). In the first test tube place a small amount of NaCl. In the second tube, place NaCl and gelatin. If the gel precipitates in the second test tube assume that there are tannins. To verify this, pour some of the liquid from the second test tube into a third. Place a few drops of $FeCl_3$ into the third tube and use table 3.B to determine the class of tannins present:

Ellagic-Gallic Tannins	blue-black
Tannins Catéchétique	green-black

Table 3.B Color-Chemical Associations for Tannin Screening

Terpenes/Steroids

To screen for terpenes and steroids place a miniscule amount of the coarse powder into test tube. Add a small amount of acetic anhydride, chloroform and H_2SO_4 while the test tube is submerged in cold water. Agitate the mixture a bit and regard the color. Table 4.B delineates the color-compound associations:

Steroids	green
Terpenes	blue

Table 4.B Color-Chemical Associations for Steroid/Terpene Screening

Saponins

To screen for saponins place some hot water into a test tube with some of the coarse powder and let stand for a while. Drain the liquid off into a separate test tube and shake vigorously. Look to see if there is a mousse on top of the liquid that persists. If a persistent mousse is observed it is necessary to perform a hemolytic test to confirm the presence of saponins. Prick the finger with a sharp needle and place a drop of blood into a well. Pour some of the hot water solution into the well. Table 5.B gives the color-result correlation:

Blood stays red	Negative result
Blood turns white + presence of mousse	Positive result

Table 5.B Color Association of +/- Results in a Saponine Screening

Anti-Inflammatory Assay

To perform an anti-inflammatory assay one needs a plethysmometer. This is an instrument designed to measure changes in the volume of a mouse's paw. The paw is dipped into a vial containing an isotonic solution. At the bottom of this vial is a sensor, which is connected to an adjacent vial filled the same amount of the solution as the other. The second vial contains a cell that is able to detect slight changes in the amount of fluid. When the mouse's paw is dipped into the vial, it displaces the liquid casing a change of equal dimensions in the liquid of the second vial. The sensor and cell calculate this change and it is read out on a connected device, which has been previously calibrated, as a value in m^3. In addition to this instrument, five lots of three to ten mice are needed (this experiment contained three per lot). Two are used as controls while the other three are injected with

varying doses of the extract. Two syringes, carragènène (an inflammatory agent) and enough extract to give the desired doses are also needed.

To begin, the control mice with no injections have the value of their right paw measured at a time (0). Next, the second lot of control mice is injected with 10ml/kg (0.25ml) of carragènène in the right paw and the paw is immediately measured at a time (0). For this experiment, the first set of test mice were injected with 10ml/kg (0.25ml) of carragènène in the right paw and 10ml/kg (0.25ml) of the extract in the abdominal region. The right paw was then immediately measured at a time (0). This step was repeated for the remaining two experimental lots at 10ml/kg (0.25ml) of carragènène in the right paw each and 1ml/kg (0.025ml) and 5ml/kg (0.125ml) of extract in the abdominal region respectively. The measurements were repeated at forty five minutes, one hour and two hours from the time of injection.

After the data is collected, an average is calculated for the mice in each group at each time interval. Afterwards, it is necessary to use these numbers to calculate a percentage of inflammation inhibition by the extract. The formula for this is as follows:

$$[1- X/Y] \times 100 = \% \text{ of inhibition}$$

In the formula, (X) represents the value of the volume of a treated mouse's paw as read from the plethysmometer at a given time. (Y) represents the value of the volume of a control mouse's paw as read from the plethysmometer at a given time, which is the same as the treated mouse.

Toxicity Assay

To perform a toxicity assay four lots of three to ten mice are needed (this experiment used three). In addition, two syringes, a physiological serum (salt water at 9g/L) and enough extract to administer the desired doses are also needed. The control lot of mice was injected with physiological serum at 10ml/kg (0.25ml). The first lot of experimental mice was injected with 7.5ml/kg (0.18ml) of extract. The second lot of experimental mice was injected with 10ml/kg (0.25ml) of extract. The third lot of experimental mice was injected with 15ml/kg (0.37ml) of extracted. The mice were observed and any immediate changes in behavior were noted. The behaviors were recorded again at thirty minutes, one hour, two hours, four hours and twenty four hours from injection.

Results

Chemical Screens

Table 1.C shows the result of all the chemical screens:

Class of Compound	+/-	Specific Type
Flavanoids	+	Flavane, Flavanol
Alkaloids	-	N/A
Tannins	+	Catéchétique
Terpenoids	-	N/A
Steroids	+	Unknown
Saponins	-	N/A

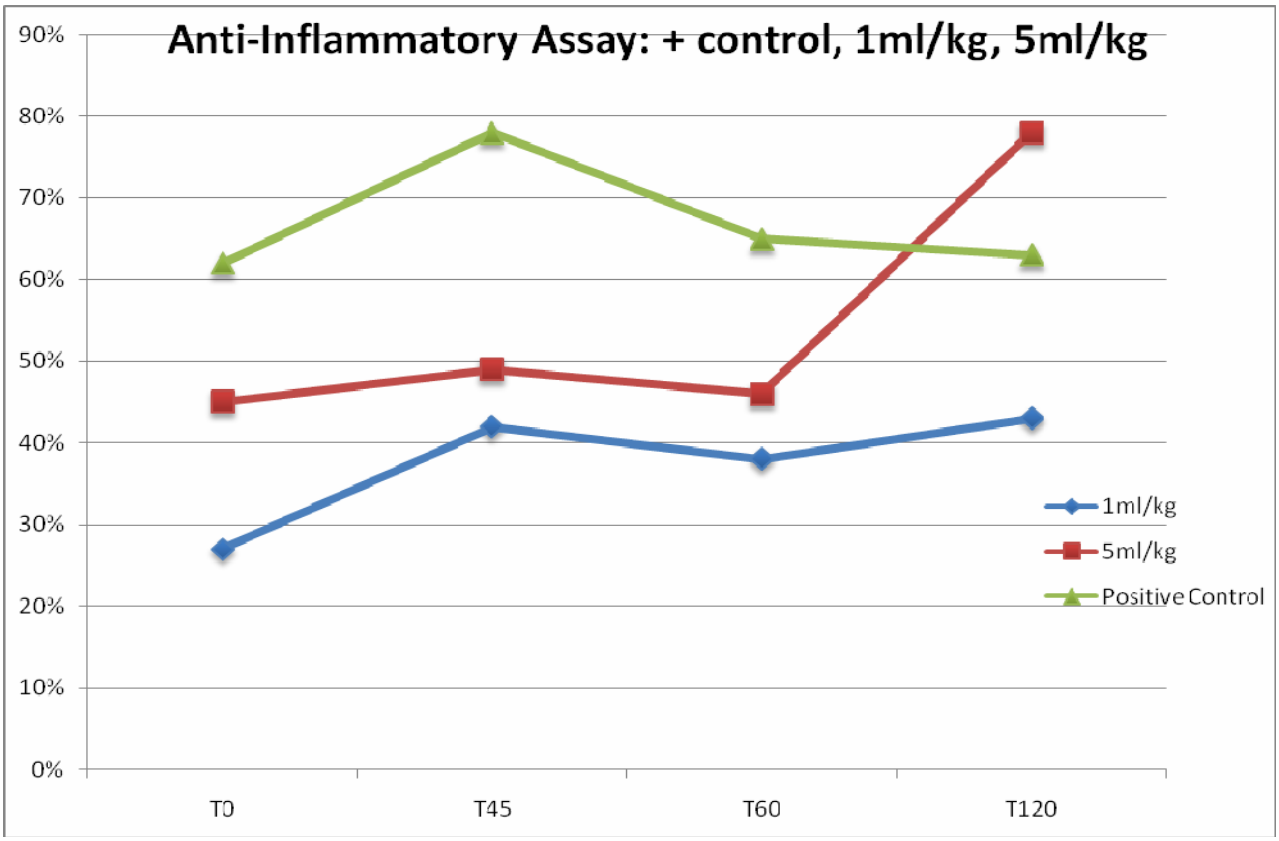
Table 1.C Results of All the Chemical Screens

Anti-Inflammatory Assay

In the anti-inflammatory assay, the positive control mice (injected only with the inflammatory agent carragènène) showed a slight decrease in inflammation as a function of time. Mice injected with a dose of Madravasarotra extract at 10ml/kg showed immediate paralysis of the right paw and were all dead within an hour and a half. Thus this does was deemed toxic and the two subsequent doses were diminished significantly. At a dose of 1ml/kg the extract showed a noticeable percentage of inhibition. At each time interval this

remained consistently in the low forties. At a dose of 5ml/kg the extract showed an extreme percentage of inhibition. In the time interval between one and two hours the inflammation in the mice was inhibited by 78% returning their paw's volume level to "normal" (as compared to the negative control mice that were injected with nothing).

Graph 1.C below represents an inverse relationship between the treated control mice and those injected with the extract. The control (green line) shows decrease in inflammation over time. The red and blue lines show the increase of the extract's inhibition of inflammation over time. This too is expressed physiologically by a decrease in inflammation of the mouse's paw:



Graph 1.C Results of the Anti-Inflammatory Assay

Toxicity Assay

The results of the toxicity assay showed some very interesting results. The control mice maintained their normal activity throughout the testing period. However, nearly all mice showed paralysis in varying degrees immediately after injection. While all showed paralysis in the right paw, those receiving higher doses became almost completely paralyzed over time; either losing ability to move altogether as those receiving 15ml/kg, or the desire as those receiving 10ml/kg and 7.5ml/kg. Another universal effect was that rate of respiration was increased greatly for each mouse, with the blood vessels in the ears becoming highly inflated.

The mice receiving 15 and 10ml/kg kept their eyes closed at least halfway, while for those 7.5ml/kg were able to open them fully towards the end of the experiment. One mouse who receive 10ml/kg began to defecate, but could not finish the bowel movement. Towards the end of the experiment it became evident that the extract is toxic at higher doses, as the mice receiving the two higher doses hardly moved, and the one receiving the lower does were able to move (and did) even though slightly paralyzed. At 24 hours just one mouse had died at 7.5ml/kg, all three mice had died at 10ml/kg, and two mice had died at 15ml/kg. Within two days, the surviving mice had overcome their paralysis and were behaving normally once again.

There was one mouse who received the highest dose (15ml/kg) that after receiving the injection continued to behave normally. Only after an hour did he begin to exhibit behaviors characteristic of toxicity. He remained able to move (though slowly and infrequently), while the other mice in the lot had loss all control of movement. At twenty four hours he was in the same shape as the surviving mice who had received the lower dose

and after two days was completely recovered. Perhaps there was a problem with his injection, or he had mutated some natural defense to the toxic effect of the extract.

Altogether, the results of this assay suggest that Madravasarotra is assuredly lethal to mice at 10ml/kg (the same result occurred inadvertently with the mice in the anti-inflammatory assay), and is highly likely to cause death in mice at doses higher than this. As for the immediate paralysis it shows the extract is quickly absorbed into the body, especially the nervous system. However, the surviving mice show that in doses lowed than 10ml/kg, the extract, in general, is processed and passed through the body within forty eight hours.

After the experiment was complete one test mouse and a mouse injected with 15ml/kg of the extract were dissected to observe the internal effect of the extract. The intestines of the treated mouse had been constricted greatly. Also the heart of the treated mouse was much larger that that of the control mouse. The lungs of the test mouse had diminished greatly and thus had retreated and were very hard to find. Lastly, the color of the organs and blood were much darker than that of the test mouse. This was especially noticeable in the liver. Though it was the same size as the test mouse's liver, it was very dull and dark while the test mouse's liver was glossy and had a high sheen.

Discussion

There are several interesting connections between the known classes of secondary metabolites found in Mandravasarotra and the physical manifestations observed in the pharmacological screens. It is important to note that the mice already have some natural ability to reduce inflammation, as was demonstrated by the positive control mice in the assay. However, some component within the extract was able to work with this natural system to enhance the effect. Mandravasarotra has been shown to have the presence of flavane and flavanol, which are effective anti-oxidants and have been show, in previous studies, to have an anti-inflammatory effect. Perhaps it is these molecules that were able to inhibit the effects of carragènène. However, it is necessary to mark the high toxicity of the extract in the mice in both assays. This could possibly also be due the presence of a high concentration of flavanoids, which as mentioned in the introduction has shown to be toxic to laboratory animals. Also, in nature, flavanoids are known to inhibit proteases and digestive enzymes in the digestive systems of insects. Perhaps this same inhibition working in the mice is why the liver and intestines appeared as unhealthy as they did.

In the chemical screening it is interesting to note the presence of steroids over terpenes in the plant. Though it is not sure, this could be because many of these terpenes had been previously converted into sterols. In any case, steroids are known to work on the heart and cause the period of relaxation to be accentuated without affecting the period of contraction. Perhaps these and other unknown chemicals stimulated the brain of the mice to cause this effect evidenced by the rapid breathing. Also, this may be evidence into how the chemicals from the extract enter the central nervous system to cause the instantaneous paralysis.

In the preparation of the powder used to make the extract, as well as in the preparation of traditional medicines, the bark of Mandravasarotra is a major component. The bark is rich in tannins, which has high astringent properties, or the ability to induce constrictions in the tissues. Perhaps it is this class of compounds that stimulates the contraction of the uterine walls in humans. It is also highly likely that this class of compounds is the reason for the highly constricted walls of the intestines, and the almost complete dissolution of the lungs in the treated mouse.

Conclusions

As in any scientific study, the end of this story only leads to more questions. Certainly, many interesting and research worthy hypotheses may be drawn from the striking results of these various screenings. I have quickly learned that my recent research in phytochemistry involves only the beginning steps of a path that leads into a wild world of discovery. Certainly it is rewarding to verify that the "ordinary plants" an outsider might regard as they pass through the street markets of Madagascar, indeed do have medicinal properties, via secondary metabolites.

I also feel fortunate to be able to appreciate both the chemical and physical ramifications of the compounds contained within Mandravasarotra. If time had permitted, I feel that isolation of specific compounds from the extract should be made and further tests should be run with them. In fact, "screening is of little scientific merit, standing alone. It only confirms the presence of compounds not the functions." (Martin 78) The reason this is true is because in the alcoholic extract I used, though I was able to identify three major classes of secondary metabolites, there could be several more present that possibly play a more integral part in the results observed that the classes identified. Also, from a pharmacological viewpoint, the assays are helpful but further along, strict, carefully controlled clinical trials must be run so that all the physiological effects of a substance can be known before a subsequent drug is developed and released to the market.

I feel privileged to have been able to work with a plant, which, for centuries, has been used to heal, those who might not be able to access the catalogue of pills, liquids and powders known as "western medicine". Mandravasarotra indeed has veritable effects, and

allows for an economically sound way for an individual to maintain his or her health. It was a pleasure, to hopefully add to the cannon of married traditional and scientific knowledge generally known as ethnobotany, because it has truly ameliorated the lives of millions.

. “Evaluating the Medicinal Properties of Mandravasarotra (*Cinnamosma sida*) Through Chemical and Pharmacological Screening”

Appendix A

. “Evaluating the Medicinal Properties of Mandravasarotra (*Cinnamosma sida*) Through Chemical and Pharmacological Screening”

Appendix B

Bibliography

1. American Chemical Society. "High Doses Of Phytochemicals, Including Flavanoids, In Teas And Supplements Could Be Unhealthy." ScienceDaily 1 May 2007. 6 December 2007 <http://www.sciencedaily.com /releases/2007/04/070430224756.htm>.

2. Langeheim, Jean H. and Kenneth V. Thiman. Botany: Plant Biology and Its Relation to Human Affairs. New York: John Wiley & Sons, 1982.

3. Martin, Gary J. Ethnobotany: A Methods Manual. London: Chapman & Hall, 1995.

4. Quansah, Nat. Personal Interviaw. 11 November 2007.

5. Quansah, Patricia. Personal Interview 11 November 2007.

6. Ramarisololalao, Amélie, Personal Interview 14 November 2007.

7. Randimbivolona, Fanantenanirainy. Personal Interview 5 December 2007.

8. Randrianasolonjanahary, Henri. "Mandravasarotra." 1 December 2007 http://www.antsanita.com/mandravasarotra/presentation.html

9. Ruud, Jorgen. Taboo: A Study of Malagasy Customs and Beliefs. Olso: University Press, Norway, 1960.

10. "Saponin." Wikipedia, The Free Encyclopedia. 2 Dec 2007, 00:41 UTC. Wikimedia Foundation, Inc. 6 Dec 2007 <http://en.wikipedia.org/w/index.php?title=Saponin&oldid=175164486>.

11. "Sterol." Wikipedia, The Free Encyclopedia. 26 Nov 2007, 20:52 UTC. Wikimedia Foundation, Inc. 6 Dec 2007 <http://en.wikipedia.org/w/index.php?title=Sterol&oldid=173970530>.

12. 1 December 2007. <http://www.jayateas.com/about_tea/health_benefits_of_tea.html>.

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