BIBLIOGRAPHY

MACARAEG– DIZA, MARIA THERESA C. November 2006. Extraction and Characterization of the Essential Oil from the Leaves of Karimbuaya (*Euphorbia neriifolia Linn).* Benguet State University, La Trinidad, Benguet.

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ABSTRACT

The fresh Karimbuaya (*Euphorbia neriifolia Linn.)* leaf extract was determined to be green, with leafy like odor, sweet with an after taste and contains 4.2% mass sucrose. Phytochemical analysis showed that the fresh leaf extract contains saponin and tannin, but it is found non-toxic. The LD_{50} of the fresh leaf extract using the brine shrimp toxicity assay was 58.73 ppm.

The essential oil from the leaves of Karimbuaya (*Euphorbia neriifolia Linn.)* was extracted using a combination of Soxhlet extraction and chromatographic separation techniques. The chromatogram of the product obtained showed 4 spots with Rf values 0.20, 0.52, 0.8, and 0.95. The fatty like, odorless, and orange colored essential oil has a melting point range of 36-40^{o}C and is found soluble in all organic solvents used in the solubility test. One component isolated from the essential oil is a white crystalline solid found to have a high affinity with chloroform and melts at 142^{o}C. The percentage yield of the white crystal was 0.142%

The consumer panel type evaluation conducted showed that Karimbuaya mixture as flavor enhancer for grilled chicken is favored over the use of salt or lemongrass mixture.

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INTRODUCTION

Background of the Study

Flavor enhancers are used to intensify or improve food flavor. Essential oil from plants and culinary herbs include a broad range of plant species that are used for flavor enhancement in food and beverages, as well as fragrances in pharmaceutical and industrial products. Essential oil are derived from aromatic plants of many genera which are distributed worldwide. These oils are not only used for flavor enhancement, but they are also used in aromatherapy, a form of herbal medicine in which healing effects are ascribed to the aromatic compounds in essential oils and other plant extract.

Culinary herbs refer to herbaceous aromatic plants grown and marketed fresh or dried and include aromatic plants which are grown for their extractable essential oils. Significant quantities of dried culinary herbs are imported into the Philippines. A large amount of selected herbs are domestically produced for the dried spice or condiments market.

Many herbs are commercially produced, albeit in small quantities, often in relatively small farms. Herbs which show promise for the fresh market and as source of essential oil are coriander (*Coriandrum sativumL)*, oregano (*Origanum*spp), celery (*Apium graveolens* L), lemon (*citrus limon* L), anise *(Pimpinella anisum* L.), lemongrass (*Cymbopogon citrates)*, and parsley (*Petroselinum crispum* Mill.).

Herbs and essential oils are used in a variety of meat and sausage products, salads, stews, sauces and soups.

Filipinos love to eat food's mixed with flavor enhancers. They used mostly artificial flavoring and only few use fresh herbs. Lechon, *grilled whole pig*, or *chicken* is one among the favorites of Filipinos. This Filipino delicacy is always present in the table during Filipino occasions. The taste of lechon is enhanced by putting herbs inside the pig or chicken before it is grilled. Lemongrass commonly known as tanglad is favored by Cebuanos as a flavor enhancer, Ilocanos on the other hand use Karimbuaya.

Karimbuaya (*Euphorbia neriifolia Linn*) as locally known by the Ilocanos is popular for enhancing the flavor of lechon. The leaves of this plant is used for lechon baboy (*grilled pig),*and lechon manok (*grilled chicken).* Hearsay claims that the plant gives a distinct aroma and sweet taste to the meat. The introduction of the use of Karimbuaya as a food flavor enhancer would contribute to the growth of the fresh herb market.

The extraction and characterization of the essential oil from the leaves of Karimbuaya (*Euphorbia neriifolia Linn.)* using physico-chemical test, phytochemical screening and toxicity testing was conducted at the chemistry laboratory of the Department of Chemistry, Benguet State University, La Trinidad, Benguet. The study was done from June to October 2006.

This study aims to:

1. Characterize the crude extract from the fresh leaves of Karimbuaya.
2. Determine the toxicity of the leaves of Karimbuaya.

3. Extract and characterize the essential oil from the dry leaves of Karimbuaya.
4. Determine the acceptability of Karimbuaya leaves as a flavor enhancer.

Importance of the Study.

This study finds importance for providing the scientific basis for the utilization of Karimbuaya as a food flavor enhancer. The result of the study would encourage local farmers to cultivate the plant and thus provide them additional source of income for local farmers

REVIEW OF LITERATURE

Karimbuaya (*Euphorbia neriifolia Linn.)*

Botanical Description

The plant sample *Euphorbia neriifolia Linn.* have several scientific designation which include *Euphorbia ligualaria Roxb., Euphobia pentagona Blanco, Euphorbia trigona Merr.* It is commonly known as *Bait* (pamp), *Karimbuaya* (ilk), *soro-soro (*tag), and *common milk hedge (*eng). Karimbuaya is cultivated in gardens and is nowhere spontaneous. As shown in Plate 1a, this plant is a shrubby, erect, branched, fleshy, cactus like plant, 2 to 4 meters high, the trunk and older branches being grayish and cylindric: the medium branches being slightly twisted, stout, fleshy, and 4 or 5 angled or winged; the younger ones usually 3-winged, the wings

(a)

(b)

Plate 1. Karimbuaya (*Euphorbia neriifolia Linn.)* (a) Matured Karimbuaya tree
(b) Matured fresh leaves

lobulate, with a pair of stout, sharp, 2-4mm long spines rising from the thickened bases at each leaf or petiole-scar. The cymes are short, solitary in the sinuses and usually of 3 involucres. The involucres are green or pale yellow and about 6 millimeters in diameter with the lobes fimbriate (Plate 1b). This plant is cultivated in garden as a hedge plant and it is propagated by stem cuttings (Anon., 2006).

Bioactivity

According to Nadkarni, Euphorbon, resin gum caoutchouc, malate of calcium are its constituents (Anon, 2006). A fluid extracted from the roasted leaves is used for earache. It is also similarly used in Malaya, as reported by Burkill and Haniff. Nadkarni states that the milky juice of this tree is used as a drastic cathartic. The expressed juice of the leaves is reported as very effective in relieving the paroxysm of spasmodic asthma. The leaves are considered diuretic. The root mixed with black

pepper is employed in snake bites both internally and externally (Quisumbing, 1979). The root is considered diuretic. According to folklore, the roots have been used for snake bites. The milky juice is regarded as a purgative internally and a rubefacient externally. Burkill quotes Dongen, who states that the latex maybe used as purgative, diuretic and vermifuge, and for asthma. Applied to glandular swellings, it prevents suppuration. Mixed with margosa oil, it is applied to limbs with contracted rheumatism. Turmeric powder mixed with the juice of Euphorbia neriifolia is recommended as an application to piles (Anon, 2006).

Euphorbia neriifolia Linn. shows wound healing activity in different pharmacological models and patients (Rasik, 1996). The resin obtained from the plant incorporated in the manufacture of a keratolytic ointment was proven effective in removing warts (Banaybay, 1980).

Flavor Enhancers

These are substances used to enhance the flavor of foods or to modify the flavor without contributing any significant flavor of their own. Controlled quantities of these plants are safe but if ingested in large quantities, they may lead to overdose. Generating an overall flavor experience requires more than the basic taste. Aromas released in the mouth stimulate olfactory receptors and transmit odors to the brain. The total flavor experience is a combination of taste, aroma and chemical feeling. Cations produce physiological response recognized as saltiness in sodium cations. It is believed that the cations travel through special channels in the outer membrane of

taste cells and enter receptor cells. This changes the voltage across the receptor cell membrane, electrically exciting the cell and causing the release of neurotransmitters that stimulate nerve cells to signal saltiness to the brain. In addition, anions saccharin have been found to contribute to the sweet response in sodium saccharine (Hegenbart, 1996).

Plant Flavor Enhancer

Flavor enhancers are derived from plants and laboratory synthesis. The plant source can be used directly in fresh, dried, or powdered form.

Lemongrass is used mainly in cheap fragrance work. Its applications include aerosol deodorants, floor polishes, household detergents, and soaps (Robbins, 1983). Basil is used in perfumery for its clear, sweet and mildly spicy aroma. Citronella oil, obtained from a relative of lemongrass, is used as an insect repellant and in perfumery. In food, several plant herbs are used to enhance the taste (Arctander, 1960).

In fish, meat and stir-fry dishes the following herbs are used as flavor enhancers: mustard (very potent), turmeric (very potent), garlic, thyme, rosemary, sage. Cayenne, cinnamon, cloves, ginger are not only used as flavor enhancers they also aid in digestion. For pasta dishes flavor enhancers are cayenne, turmeric, garlic, thyme, rosemary, ginger, mustard. Turmeric, garlic, sage, fennel, cinnamon powder, ginger, cloves are herbs used in baking. As flavor enhancers they also aid in digestion (Anon, 2006).

Synthetic Flavor Enhancer

An example of synthetic food enhancer is the monosodium glutamate (MSG). MSG is the sodium salt of the amino acid glutamic acid. Its chemical formula is $C_5H_5NO_4Na$. It is sold as a fine white crystal substance similar in appearance to salt or sugar. MSG is added as an ingredient to many snack foods, frozen dinners and instant meals like instant noodles. MSG stimulates specific receptors located in taste buds, like amino acid receptor (T1R1/T1R3), or metabotropic receptor (mGluR4 and mGluR1) which induce the taste known as *umami*. *Umami* is a Japanese loanword, referring to savoury or "more-ish". Sensitivities to MSG like migraines, nausea, heart palpitations, asthma, and anaphylactic shock are attributed to the free glutamic acid component (Wikipedia, 2006). Chinese restaurant syndrome is often used as an example of MSG symptom complex, a condition characterized by one or more of the following symptoms: facial pressure, chest pain, violent dream, bronchospasm, burning sensation in the back of the neck, forearms and chest (FDA, 1993).

Glutamate is found naturally in our bodies and in protein-containing foods such as cheese, milk, meat, peas and mushrooms. Bound and unbound glutamates are found naturally in nearly every protein-rich food. Some of the glutamate in foods is in a "free form and this free form of glutamate enhances food flavor. Certain cheese or fermented protein products are due to the presence of free glutamate. Studies showed that glutamate in the body plays an important role in normal functioning of the nervous system raising questions about whether glutamate in food could affect the nervous system (Meadows, 2003).

Glutamic acid is an amino acid commonly found in foods. It belongs to the class of chemicals known as excitotoxins. Abnormally high levels of excitotoxins have been shown in hundreds of animal studies to cause damage to areas of the brain unprotected by the blood brain barrier and that a variety of chronic disease can arise out of this neurotoxicity (FDA, 1993).

Sodium chloride, table salt, is commonly used as a flavor enhancer for food and has been identified as one of the basic tastes (Sizer, 1997).

Essential oil

The oil derived from plants is called essential oil. Essential oils extracted from plants are concentrated hydrophobic liquid containing volatile aromatic compounds. It is also known as volatile oil and ethereal oil. It may also be referred as "oil of the raw plant material" from which it was extracted, such as oil of clove or lemongrass oil. The term essential is intended to indicate that the oil is the fragrant essence of the plant from which it is extracted and not in the more common sense of being indispensable (Wikipedia, 2006). Essential oils are found in plant oil cells, glandular trichomes and oil or resin ducts (Schnaubelt, 1999).

The formation and accumulation of essential oils in plants have been reviewed by Croteau (1986), Guenther (1972) and Runeckles and Mabry (1973). Chemically, the essential oils are primarily composed of mono-and sesquiterpenes and aromatic polypropanoids synthesized via the mevalonic acid pathway for terpenes and the shikimic acid pathway for aromatic propanoids.

The essential oils from aromatic plants are for the most part volatile and thus, lend themselves to several methods of extraction such as hydrodistillation, steam distillation and solvent extraction (Guenther, 1972). The specific extraction method employed is dependent upon the plant material and the desired end-product. Essential oil components arise via the secondary metabolism of plants.

Citrus oils, produced by the mechanical pressing of citrus peels, are also called dry-distilled oils according to the International Standard Organization (ISO) (Simon, 1990).

Basil oil extracted from sweet basil *Ocimum basilicum* from the leaves and flowering tops are used as flavor enhancers for meat, pasta and fishes (Simon, 1990). The European basil oils which are the highest quality contain methyl chavicol dlinalool (Guether 1985, Simon 1990).

In aromatherapy, to receive the healing effects of essential oils, they are used to massage into the skin, inhaled, or added to bath water (Burfield, 1990).

Toxicity Test

Brine Shrimp Assay

Toxicity means the ability of a substance to cause harmful effect. The brine shrimp assay is a rapid, reliable, reproducible, non-tedious and inexpensive general bioassay tool for active plant extract. The procedure allows determination of the LD_{50} values in microgram per milliliter of active constituents in the brine shrimp medium. This bioassay has been used in the analysis of natural products (Guevara, 2005). This is done by counting mortalities on artemia, adult brine shrimp.

Artemia salina, commonly known as brine shrimp are crustaceans that live in saline environment. Their eggs hatch quickly and the larvae are sensitive to small doses of biologically-active chemicals (Tarpley, 1958). Brine shrimp grow faster in slightly higher salt concentration but hatch more rapidly at lower salt concentration, 3.5% saline solution (Guevara, 2005).

Phytochemical Screening

The term "phytochemical" is derived from the Greek word *phyto* which means plant. This is defined as the nonnutrient compounds in plant-derived foods having biological activity in the body (Sizer, 1997). This include the compound that gives hot peppers their burning taste, the compound that gives garlic its pungent flavor, the pigments that give spinach and tomatoes their dark green and dark red color (Criag, 1996).

Phytochemicals have health enhancing abilities and possibly curative abilities. They number in the hundreds in most plant foods. They are found in fruits, vegetables, legumes and grains. Phytochemicals work together with nutrients to promote health and prevent diseases. Examples of phytochemicals include antioxidant, enzyme stimulators, estrogen, estrogen blockers, compounds that bind potential cancer-causing chemicals, suppressors of cancer cells (Malaspina, 1996).

Polk (1996) estimates that there may be more than 100 different phytochemicals in just one serving of vegetables. Table 1 shows the different kinds of food with their secondary metabolites.

According to Bloch (1995), phytochemicals are associated with the prevention and/or treatment of at least four of the leading causes of death in the United States -- cancer, diabetes, cardiovascular disease, and hypertension. They are involved in many processes including ones that help prevent cell damage, prevent cancer cell replication, and decrease cholesterol levels.

Table 1. Most common food analyzed with their Phytochemical constituent

FOOD	TYPE OF PHYTOCHEMICALS
Allium vegetables (garlic, onions, chives, leeks)	Allyl sulfides
Cruciferous vegetables (broccoli, cauliflower, cabbage, brussel sprouts, kale, turnips, bok choy, kohlrabi	Indoles/glucosinolates, sulfaforaphane, Isothiocyanates/ Thiocyanates, Thiols
Umbelliferous vegetables (carrots, celery, cilantro, parsley, parsnips)	Carotenoids, Phthalides, Polyacetylenes
Citrus fruits (oranges, lemons, grapefruit) Glucarates	Monoterpenes(limonene), Carotenoids
Beans, grains, seeds (soybeans, oats, barley, brown rice, whole wheat, flax seed) Protease inhibitors	Flavonoids (isoflavones), Phytic acid, Saponins
Herbs, spices (ginger, mint, rosemary, thyme, oregano, sage, basil, tumeric, caraway, fennel)	Gingerols, Flavonoids, Monoterpenes (limonene)

Alkaloids

Alkaloids include literally thousands of bitter, nitrogenous compounds found throughout the plant kingdom. They often contain one or more rings of carbon atom with a nitrogen atom in the ring. The position of the nitrogen atom in the carbon rich

varies with different alkaloids and with different plant families. In some alkaloids, such as mescaline, the nitrogen atom is not in a carbon ring. The following alkaloids include belladonna type (atropine and cocaine), LSD-type (ergine and psilocybin) and peyote type (mescaline). Some of these have remarkable structural similarities with neurotransmitters in the human central nervous system, including dopamine, serotonin, and acetylcholine (Armstrong, 2001).

Saponins

Saponins are steroid/triterpenoid glycosides which are characterized by their ability to froth when the aqueous solution is agitated. They usually exert a powerful hemolytic action on red blood cells and when injected into the blood streams are highly toxic. Saponins are extracted from the plant material by hot water or alcohol and after concentration of the extract in vacuo, may undergo precipitation with ether. A simple test for saponins is to shake an aqueous alcoholic plant extract in a test tube and note whether persistent honeycomb froth is formed above the liquid surface (Guevarra, 1979).

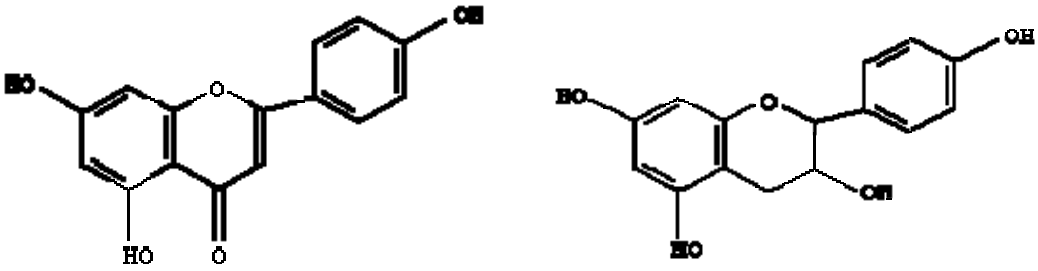
Saponins are natural surfactants or detergents extracted from plants which are commonly used as foaming agents for beverages (Clark, 2005). They have hemolytic, expectorative, anti-inflammatory and immune-stimulating activity. Saponin demonstrates antimicrobial properties against fungi, bacteria and protozoa. Saponins are found in number of herb including ginseng, paprika and red variety of onion (Sahelian, 2006).

Cardenolides and Bufadienolides

Cardenolides and bufadienolides are steroids containing deoxysugars and an unsaturated lactone ring. They are called “cardiac glycosides” as they act on the muscle of the heart. Plant extracts are usually evaluated for cardiotonic activity either through in-vitro or in-vivo biological tests. In phytochemical screening for cardiac glycosides, the test may be applied either to the crude alcoholic extract or the partially purified extract. Chemical test are designed to detect the presence of the deoxysugars, the steroid nucleus and the unsaturated lactone (Guevarra, 2005).

Flavonoids

The flavonoids are plant pigments based on C6-C3-C5 carbon skeleton and generally containing the γ-benzopyrone nucleus like the flavones (Figure 1a), isoflavones, flavonols and flavonones. Other flavonoids are the anthocyanins (Figure 1b), leucoanthocyanins, catechins, chalcones, and aurones. Flavonoid compounds usually occur in plants as glycosides wherein one or more of the phenolic hydroxyl groups are combined with sugar residue. Many flavonoids exhibit different biological activities like antiviral, antifungal, anti-inflammatory and cytotoxic activities (Guevara, 2005).



(a) (b)

Figure 1. Flavonoids (Wikipedia, 2006) (a) flavone are ketone derivatives of flavonoid; (b) anthocyanin that occur in tissues of plants that provides color in leaves, stem, roots, flowers and fruits

Tannins

The term "tannins" was first applied to plant constituents capable of forming raw animal skin into leather because of their ability to cross-link with protein. Tannins consist mainly of gallic acid residues that are linked to glucose via glycosidic bonds.

Tannins are located mainly in the vacuoles or surface wax of the plants. In these sites they do not interfere with plant metabolism. Only after cell breakdown and death can they act to have metabolic effects. In leaf tissues, tannins are most common in the upper epidermis. They serve to reduce palatability and, thus, protect against predators (Guevara, 1979). Recent studies shows tannins have potential value as cytotoxic or antineoplastic agents. In medicine tannins are used as an astringent and

for treatment of burns (Columbia Encyclopedia, 2006). In food, tannins are essential to the development of flavor in red wines (Klahorst, 2006).

Anthraquinones

Anthraquinones are naturally occurring quinine pigments. They occur in plants usually as hydroxylated, methylated, or carboxylated derivatives of anthraquinones, anthrones or dianthrone. They are commonly used as dyes and cathartics or purgatives (Guevara, 2005). Figure 2 shows the structural formula of anthraquinone

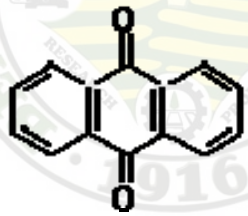


Figure 2. Chemical structure of Anthraquinone

Cyanogenic Glycosides

Cyanogenic glycosides are phytotoxins which occur in at least 2000 plant species of which a number of species are used as food in some areas of the world. Cassava and sorghum are especially important staple foods containing cyanogenic glycosides (Nartey, 1980). Cyanide, released from a cyanogenic glycoside in food by

ß-glucosidase either of plant or from gut microflora origin and when taken up, follows the known cyanide metabolic pathway and toxicokinetics both for animals and man. Cyanide is detoxified by the enzyme rhodanase, forming thiocyanate, which is excreted by urine (Conn, 1979 a, b; Oke, 1979). Due to several factors influencing hydrolysis of cyanogenic glycosides and the confounding influence of nutritional status (such as riboflavin, vit. B12, sodium, methionine intake) human case studies and epidemiological studies of the chronic toxicological effects have shown very variable results and were not conclusive. In several studies both in animals and man the toxicity of cyanogenic glycosides is often expressed as milligram releasable cyanide (Conn, 1979 a, b).

Extraction of Essential Oil

Extraction refers to separating by physical or chemical means the desired material from a plant with the aid of a solvent (Bossert, 1970). Extraction procedure includes soxhlet extraction and column chromatography.

Soxhlet Extraction

Soxhlet extractor (Plate 2) is used to extract solutes from solids, using any desired volatile solvents, which can be water-miscible or water-immiscible (Morrison and Freiser, 1957). The solvent is vaporized and when it condenses, drops on the solid substance contained in the extraction thimble and extracts the soluble compounds. When the liquid level fills the body of the extractor, it automatically

siphons into the flask. This process is continuously repeated as the solvent in the flask is vaporized and condensed. Soxhlet extractors are standard equipment in laboratories that analyze fats and oils in biologic samples. Separations can be achieved at low temperatures in inert atmospheres on a micro or macro scale by a discontinuous or continuous process (Lo et.al., 1983).

Plate 2. Soxhlet extractor

Chromatography

Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in a definite direction (IUPAC, 1993). Component molecules in a sample mixture are transported by a mobile phase over a stationary phase. Different components will have different affinities for the stationary phase with respect to the mobile phase and will therefore move at different rates. Thin-layer chromatography is a separation technique in which a stationary phase consisting of an appropriate material is spread in a uniform thin layer and fixed on a support of glass, metal or plastic. The separation is carried out by migration through the thin layer of solutes in a solvent or suitable mixture of solvents.

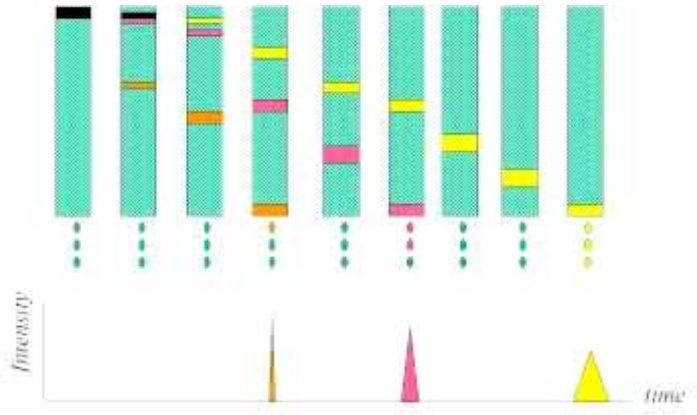


Plate 3. Chromatographic separation. A mixture of compounds is separated as it flows down a column packed with stationary phase particles. Separation is due to different degree of interaction between sample molecules and stationary phase. (IUPAC, 1993)

MATERIALS AND METHODS

The Plant Material

Collection of Plant Sample

Matured leaves of *Euphorbia neriifolia Linn* commonly known in ilocano as Karimbuaya, were collected from Pangasinan, and Vigan, Ilocos Sur, provinces north of Manila. The plant sample was identified at the Institute of Biology Herbarium, at U.P. Diliman, Quezon City.

Plant Preparation

The collected mature leaves of Karimbuaya (*Euphorbia neriifolia Linn.*) were washed thoroughly with tap water and air dried for 10 days. After which the dried leaves were cut into small pieces then powdered prior to analysis. Plate 4 shows samples ready for the extraction process.

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Plate 4. Cut and powdered Karimbuaya leaves

Characterization of the Fresh Karimbuaya Leaf Extract

Physico-Chemical Properties

The fresh leaves of Karimbuaya leaves were homogenized using a blender and the juice was extracted by the use of several layers of cheesecloth.

Color. The color of the extract was determined based on a color chart.

Taste. About 5 drops was used to taste the extract.

Odor. This process was done by smelling after the juices have been extracted from the leaves.

Refractive index. The amount of sugar on the sample was measured using Atago refractometer.

Phytochemical screening

The fresh Karimbuaya leaf extract was screened for the presence of phytochemicals using methods of Guevara (2005). The fresh extract was prepared by liquefying the thoroughly washed and drained fresh mature leaves of Karimbuaya (*Euphorbia neriifolia Linn*). Table 2 shows the different tests used for the phytochemical screening. A confirmatory test (Table 3) for the presence of secondary metabolites or phytochemicals using thin layer chromatography (TLC) was also performed using the methods from Guevara (2005).

Table 2. Phytochemical Analysis

TEST	COMPOUND TESTED
Culvenor-Fitzgerald, Dragendorff's and Mayer	Alkaloid
Froth and Liebermann Burchard	Saponins
Keller-Kiliani and Kedde	Cardenolide & bufadienolide
Bate-Smith&Metcalf and Wilstatter	Flavonoids
Gelatin and Ferric chloride	Tannins
Modified Borntrager's	Anthraquinones
Guignard	Cyanogenic glycoside

Table 3. Confirmatory Test for Phytochemicals

VISUALIZING AGENT	COMPOUND TESTED
Antimony (III) chloride	Flavonoids/Steriods
Potassium ferricyanide-ferric chloride	Phennol/tannin/flavonoid
Dragendorff's reagent	Alkaloid
Methanolic KOH	Phenols/Anthrone
Vanillin-sulfuric acid	Essential Oils
α-Napthol-sulfuric acid	Sugars

Toxicity Test

The toxicity of the Karimbuaya leaves was evaluated using the Brine Shrimp Assay.

Hatching of Brine Shrimp Eggs

A hatching medium was prepared by dissolving 3.8 grams of rock salt per 100 ml distilled water. This solution has a 3.8% NaCl concentration. About 250 mg of brine shrimp egg and 80 ml hatching medium were placed in the hatching dish. The hatching dish has two unequal compartment provided by a plastic divider punched with several 2 mm holes. The brine shrimp eggs were sprinkled into the larger compartment and then covered with cardboard to block the light. The smaller compartment was illuminated for 48 hours to allow the eggs to hatch. The nauplii were then harvested for the assay.

Preparation of Stock Solution

Fresh Karimbuaya leaves were washed thoroughly and oven dried at 60^{o}C for 24 hours. About 500 mg of the dried leaves were powdered using mortar and pestle. The powdered sample was transferred to a 250 ml beaker and 50 ml of artificial sea water (3.8% NaCl) was added. The extract was filtered using 4 layers of cheesecloth following one hour of incubation. The filtrate collected is the stock solution.

Dilution of the Stock Solution

Five test tubes of 20 ml capacity were prepared and labeled (1to 5) for the dilution of the stock solution. The dilution series was prepared as follows: To test tube 1, 10 ml of the stock solution was placed while 9ml of artificial sea water (3.8% NaCl) was placed to test tube # 2 to 5. The dilution was carried out by transferring 1.0 ml stock solution from test tube #1 to test tube #2. From the mix solution in test tube #2, 1.0 ml was transferred to test tube #3, 1.0ml from test tube #3 was transferred to test tube #4 and from this, 1.0 ml was pipetted and added to test tube #5. Table 4 presents the dilution ratio.

Table 4 Dilution of the stock solution used for the bioassay

TEST TUBE NO.	DILUTION RATIO
1	Stock solution
2	1: 10
3	1:100
4	1:1,000
5	1:10,000

Bioassay Procedure

The diluted stock solutions in the 5 test tubes were transferred to 5 previously cleaned petri dishes and labeled similarly as the test tubes. Using a Pasteur pipette, ten live brine shrimps (nauplii) were deposited in each Petri dish.

After 24 hours, the Petri dishes were examined for brine shrimp mortality with the aid of a magnifying lens.

Data Analysis

The toxicity of the plant extract was determined based on the value obtained for the LD_{50}, which refers to the amount (lethal dose) of a substance that kills half of the test organisms. The LD_{50} was determined from the equation of the line obtained by plotting the percent mortality against the log extract concentration.

Extraction of the Essential Oil

Soxhlet Extraction

Previously cut air dried Karimbuaya leaves were powdered using a blender. Each 15 gram batch of the powdered leaves was wrapped in filter paper and placed in the extracting thimble of a soxhlet extractor, where 250 mL of petroleum ether was added as the extracting solvent. The extraction process for each batch of leaves lasted for 5 hours, until the color of the solvent in the body of the soxhlet extractor became colorless. The petroleum ether extract was concentrated under reduced pressure at 40^{o}C. The concentrated plant extract was allowed to dry to determine the percentage yield.

Chromatographic Separation

A weighed amount of the concentrated petroleum ether extract was subjected to rapid column chromatography by vacuum elution, as in Plate 5. A column of 4.5 cm diameter was packed with 7 cm high of Silica Gel 60 Gf (Merck). Solvent used for elution was increasing percentage of hexane-ethyl acetate mixture starting from pure hexane to 10% ethyl acetate.

The chromatographic separation of the ether extract was monitored through thin layer chromatography using prepared TLC plates (Merck). The chromatograms were developed using different solvent system based on the concentration of the solvent used for elution. The spots were first developed using iodine vapors followed by vanillin-sulfuric acid spray and was then heated in the oven at 110^{o}C for color development. Components of the same Rf value were collected and grouped as one.

Plate 6 shows the over-all extraction process.

Purification of the Essential Oil Extract

Repeated column chromatography using different column sizes and isocratic elution yielded the oil extract. This is illustrated in Plate 7. Isocratic elution was carried out using pure hexane (A.R. grade). The fraction identified by TLC to contain the essential oil was further separated using a smaller column (2.0 cm in diameter). Solubility difference using pure ethyl acetate (A.R.) and filtration with the use of Whatman 42 separated the white crystals from the oil.

Plate 5. Column Chromatographic Separation of the Concentrated Petroleum ether extract from Karimbuaya leaves

Fresh leaves of Karimbuava

Dried Powderized Leaves

Soxhlet extraction

Ether extract

Concentrated under vacuo
(rotary evaporator)

Concentrated Petroleum
Ether Extract

Separation by Column
Chromatography

Fractions containing
Essential Oil

Essential Oil

Plate 6. Schematic diagram of the extraction of Essential oil from the leaves of Karimbuaya.

Plate 7. Chromatographic Separation of Essential Oil from Karimbuaya leaves

Characterization of the Essential Oil Extract

Physico-chemical Test

Solubility test. Each two drops of extracted oil was mixed with 2 ml of the different organic solvent in a test tube. Solubility of the oil extract was determined in different organic solvent which included chloroform, ethanol, ethyl acetate, hexane and petroleum ether.

Melting point determination. The melting point of the oil extract was analyzed as follows: About 0.1ml of the viscous oil was placed in a 50 ml beaker that was suspended in a water bath. This set-up is shown in Plate 8. The temperature of the water bath starting from 10^{o}C was gradually increased until the oil melted.

Plate 8. Melting point set-up for the essential oil

The melting point of the white crystals separated from the extracted oil was analyzed using the Thiele-Dennis tube. About 1 mm height of the white crystals was placed inside the sealed capillary tube. A thermometer was attached to the capillary tube containing the sample and was then placed inside the Thiele-Dennis tube filled with glycerol. The tube was heated until the white crystals melted. The set-up is presented in Plate 9.

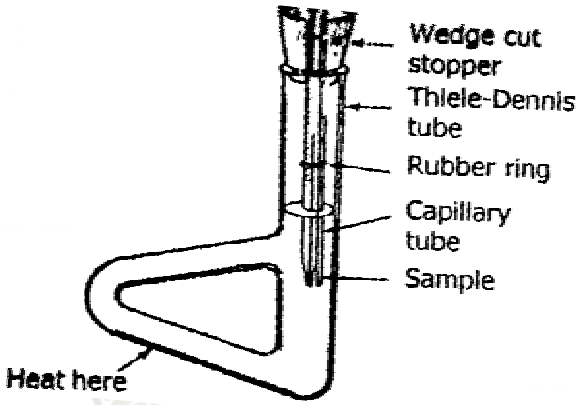


Plate 9 A Thiele-Dennis tube for melting point determination of the crystal (Visconde A.D. et al, 2003)

Acid Number. About 100 mg of oil was mixed with mixture containing 12.5 ml of ethanol (95%) and 12.5 ml ether. The mixture was titrated with 0.1M KOH until a red color remains after swirling. Phenolphthalein indicator was used to indicate the end point of the titration. Plate 10 presents the color of the sample solution before and after titration.

A. Before KOH Titration

B. After KOH titration

Plate 10. Acid number determination of the Essential Oil

Acceptability Test

A consumer type panel consisting of 48 members ranging in age from 20 to 60 years was asked to evaluate the acceptability of the aroma and taste of *lechon manok,* grilled with Karimbuaya leaves. Common spices for flavoring *lechon* sold in the market were the standard ingredients in the acceptability test conducted.

Preparation of Sample.

Dressed chickens previously washed and drained were used for the three treatments. The chickens were then stuffed with the respective treatment ingredients as shown in Table 5. The stuffed chickens were grilled at the same time.

Table 5. Treatments used in the chicken for the acceptability test

Treatment	Ingredients
T_1	Salt
T_2	Karimbuaya leaves, 5 pieces chopped onion bulbs, salt and pepper
T_3	Lemongrass, 5 pieces chopped onion bulbs, salt and pepper

Taste Test

Before the taste test, a sensory evaluation form (Appendix B) was given to each consumer to mark after tasting. The grilled chickens were cut into bite sizes and given to the members of the panel. The consumers were asked to rinse their mouth with water after tasting each of the treated samples.

Data Analysis

One way ANOVA test was used to determine if there was a significant difference between the three treatments. Computer software SPSS was used in the data analysis. This software computes using the P-value approach.

RESULTS AND DISCUSSION

Characterization of the Fresh Karimbuaya Leaf Extract

Physico-chemical Properties

The liquid squeezed from the homogenized fresh leaves of Karimbuaya was green in color, with a leafy like odor, and sweet with an after taste. The percentage (%) mass sucrose content of the fresh extract was 4.2%. This was obtained using a refractometer.

Phytochemical Screening

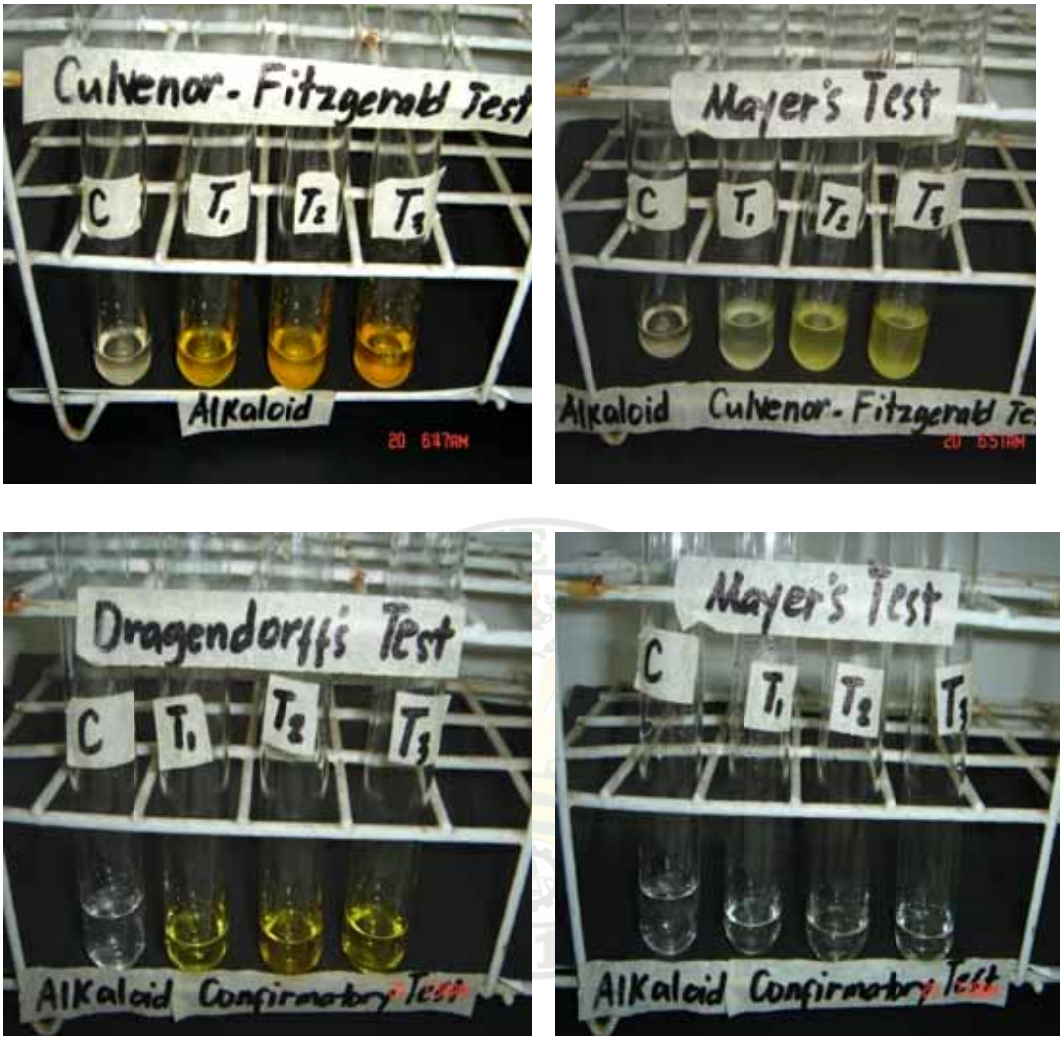
To identify the secondary metabolites present in the extract, phytochemical analysis was done. The results summarized in Table 6, showed that the fresh extract contains only the secondary metabolites, saponin and tannin. Although a slight turbidity was observed in the Culvenor-Fiztgerald test for alkaloid, this result is considered negative according to Guevara (2005). Presence of alkaloid in the sample is indicated by a heavy precipitation evaluated as (+++). The result of on the Laboratory test tube method, no formation of precipitates, confirms that alkaloid is not present in the sample.

Plate 11 represents the above results.

Table 6. Phytochemical Analyses of Fresh Karimbuaya Leaf Extract

TESTS	CLASS OF COMPOUNDS	RESULTS
Culvenor-Fitzgerald	Alkaloid	
Dragendorff		(+) slight turbidity yellow precipitate
Mayer		(+) slight turbidity yellow precipitate
Laboratory test tube		
Dragendorffs		(-) yellow liquid
Mayer's		(-) colorless liquid
Froth	Saponins	(+) frothing
Liebermann-Burchard		(+) green liquid
Guignard	Cyanogenic glycosides	(-) yellow color
Keller-Kiliani	Cardenolides and Bufadienolides	(-) orange ring at the interface
Kedde		(-) orange brown liquid
Bate-Smith&Metcalf	Flavonoids	(-) orange brown liquid
Wilstatter		(-) light orange liquid
"Cyanidine"		
Gelatin	Tannin	(+)jelly precipitate
Ferric chloride		(+) brownish green liquid
Modified Borntragers	Anthraquinones	(-) colorless in the ammoniacal layer

To confirm the above results, phytochemical analyses using thin-layer chromatography (TLC) was also performed. The tests confirmed the presence of saponin and tannin. The absence of alkaloids and other secondary metabolites

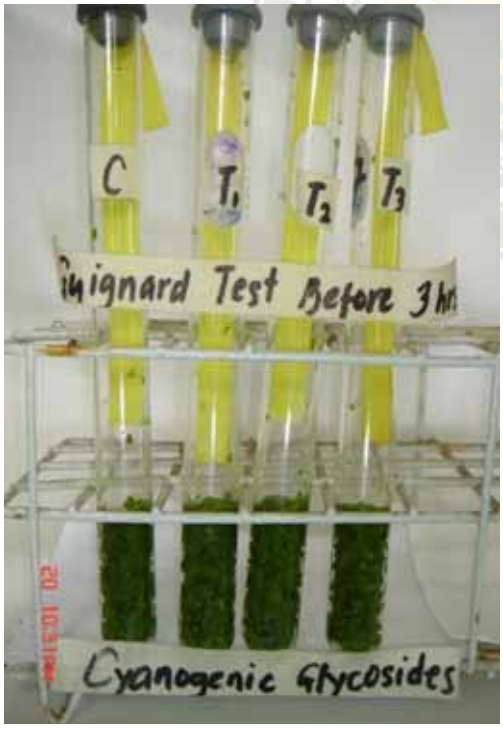


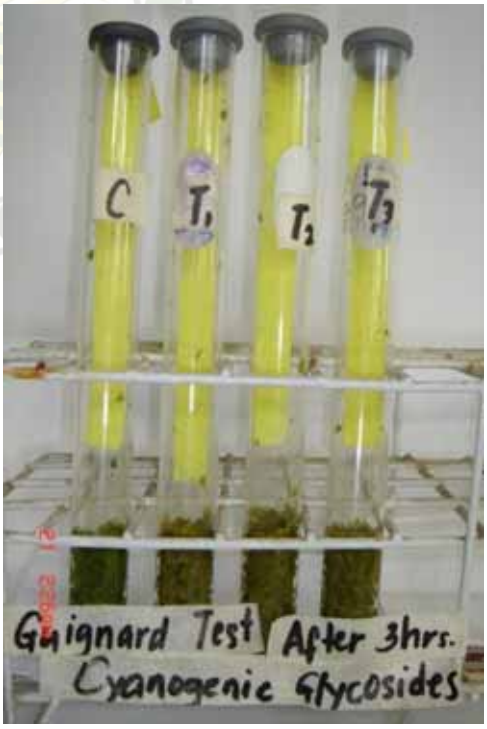
A. Alkaloid Test (negative result)

Plate 11. Phytochemical Screening Result

Plate 11. Continued…

B. Saponin (positive result)





C. Cyanogenic glycosides

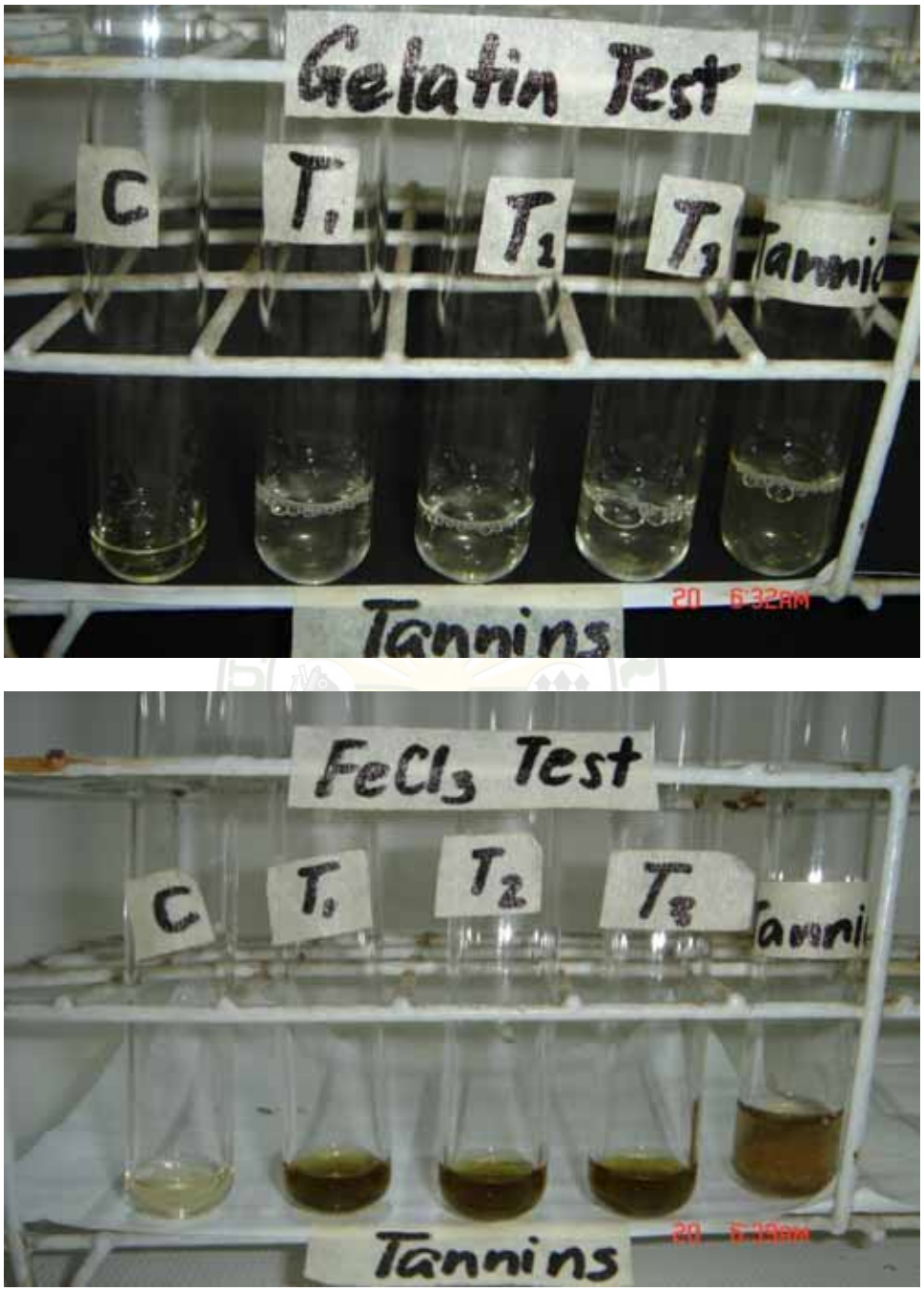
Plate 11. Continued…

D. Cardenolides and Bufadienolides (negative result)

Plate 11. Continued…

E. Flavonoids

Plate 11. Continued …



F. Tannins (positive result)

Plate 11. Continued….

G. Anthraquinone (negative result)

were also confirmed. The results are presented in Table 7. The same table also shows that essential oil and sugar are present in the fresh extract. The violet spots observed upon spraying the developed TLC plates with the vanillin-sulfuric acid visualizing agent, indicate that the extract contains essential oil. Using the α-Napthol-sulfuric spray reagent, a blue spot was observed which confirmed the presence of sugar in the Karimbuaya leaves. Results collaborates the finding using refractometer for the presence of sugar.

Table 7. Phytochemical Analyses using Thin-Layer Chromatography (TLC)

SPRAY REAGENTS	CONSTITUENT TESTED	RESULT
Antimony(III) chloride	Flavonoids/steroids	(-) no spot
Potassium ferricyanide-ferric chloride	Phenol/tannin/flavonoid	(-) yellow spot
Dragendorff's reagent	Alkaloid	(-) yellow spot
KOHmethanolic	Phenols/anthrone	(-) no spot
Vanillin-sulfuric acid	Essential oils	(+) wide range of color
α-Napthol-sulfuric acid	Sugars	(+) blue spot

Toxicity Test Using The Brine Shrimp Assay

Table 8 showed that the concentrated stock solution of Karimbuaya has an average of 13.3 % mortality after 24 hours. The diluted concentration of the stock solution showed 0% mortality on the nauplii.

Table 8. Average % mortality after 24 hours

CONC. OF EXTRACT (ppm)	AVERAGE % MORTALITY (24 hours)
11363.63	13.3
1136.36	0
113.63	0
11.36×10^{-3}	0
11.36×10^{-7}	0

From the graph (Figure 3) the LD_{50} of the plant solution was computed to be 58.73 ppm. Activities are considered significant if the LD_{50} value is less than 30 ppm (Saupe, 2006). The result indicates that the Karimbuaya extract is non-toxic.

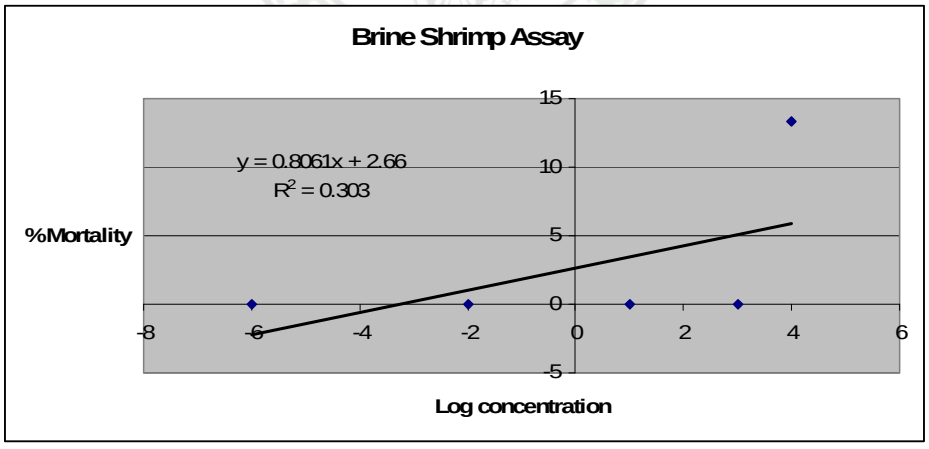


Figure 3. Determination of LD_{50}

Extraction of Essential Oil

Crude Fat Extraction

The petroleum ether extract obtained from the soxhlet extraction using 100 grams of powdered dried Karimbuaya leaves was dried under vacuo at 40^{o}C to yield 5.1664 gram of crude fat or concentrated petroleum ether extract. The recovered crude fat is solid and colored dark green. Plate 12 shows the sample of the crude fat

Plate 12. Extracted crude Fat from Karimbuaya

Separation of Essential Oil

The essential oil was obtained from the crude fat or petroleum ether extract after repeated chromatographic processes guided by thin layer chromatographic techniques. Isocratic elution using pure hexane was performed to elute the essential oil. Table 9 shows the chromatographic characteristics of the nine fractions obtained from the crude fat. These fractions are shown in Plate 13.

Essential oils, because they are non-polar are the first to be eluted from the column. Based on the TLC chromatogram, fractions I to III which contain the oil were pooled and concentrated under vacuo at 40^{o}C.

Table 9. Thin-Layer Chromatography of the Petroleum Ether Extract

FRACTION NUMBER	DEVELOPING SOLVENT	CHROMATOGRAPHIC CHARACTERISTIC	
		Rf values	Color of Spots (Vanillin-Sulfuric Acid Spray)
I	100% Hexane	0.73	Violet
		0.88	Violet
		0.93	Violet
II	0.2% E-H	0.7	Violet
III	0.2% E-H	0.43	Violet
		0.68	Violet
IV	0.4%E-H	0.13	Brown-violet
V	0.4%E-H	0.08	Brown-violet
VI	0.4%E-H	0.05	Brown-violet
VII	10%E-H	0.22	Brown
		0.3	Brown violet
		0.4	Violet
		0.48	Violet
		0.6	Violet
		0.82	Violet
VIII	10%E-H	0.13	Green-violet
		0.23	Violet
		0.30	Violet
IX	15%E-H	0.07	Brown-violet
		0.12	Brown-violet

* E-H Ethyl acetate in hexane

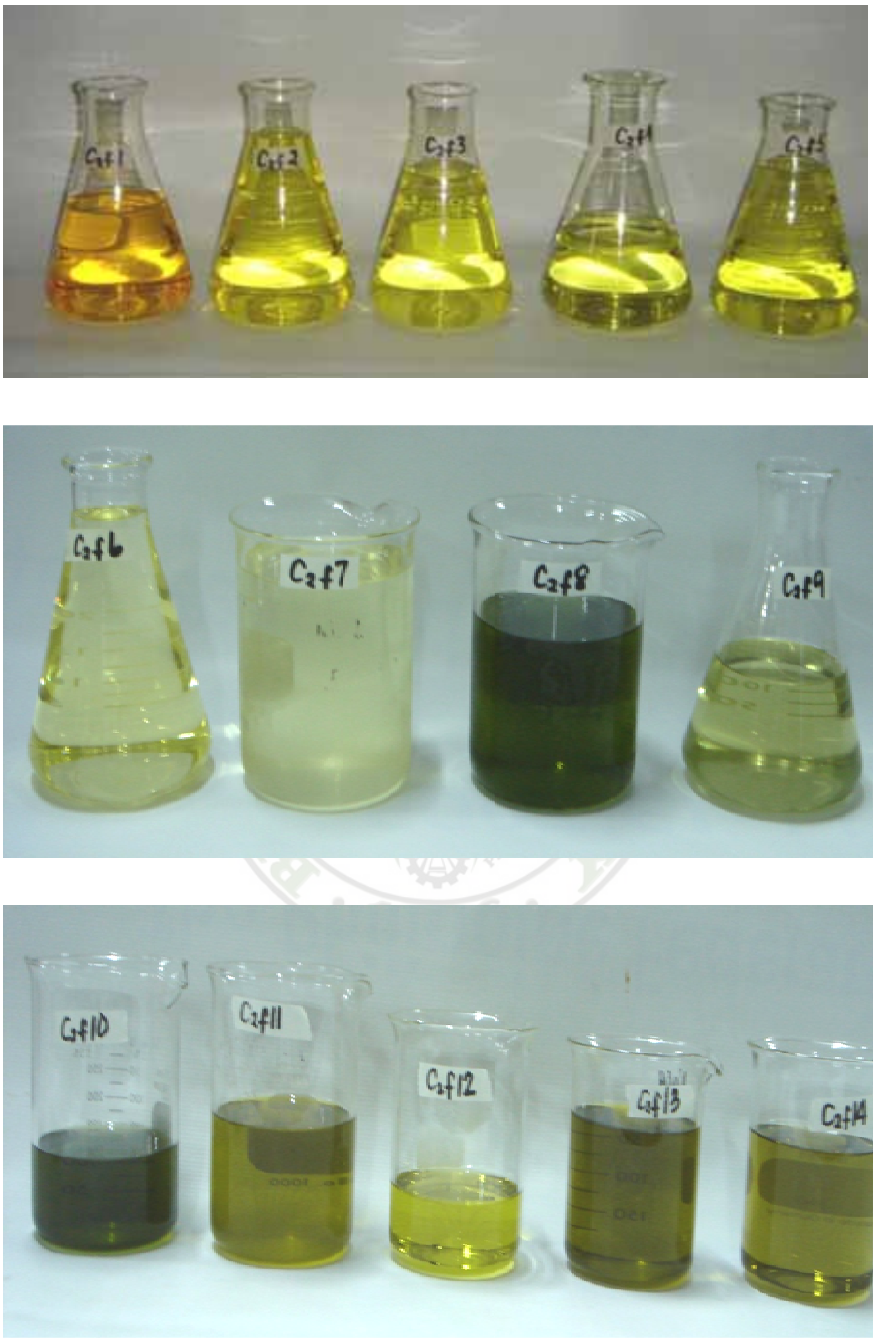


Plate 13. Eluates from the Column Chromatography from the concentrated petroleum extract of the Karimbuaya leaves

The pooled Fractions (I-III) was again subjected to rapid column chromatography to yield 9 fractions as shown in Figure 4. After several extraction using chromatographic techniques, an orange-colored fat-like substance was obtained. This is the essential oil.

The chromatogram (Plate 14) of the essential oil showed four spots, indicating the presence of four components. The over-all extraction process for the separation of the essential oil from the leaves of Karimbuaya is summarized in Figure 4.

Purification of Essential Oil

The essential oil extracted showed presence of white crystals. The large spot (Plate 14) located at the lower part of the chromatogram with Rf value of 0.20 corresponds to the white crystals.

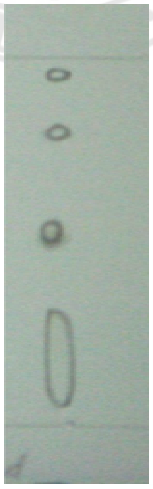


Plate 14. Chromatogram of the essential oil using Vanillin-sulfuric acid as visualizing agent

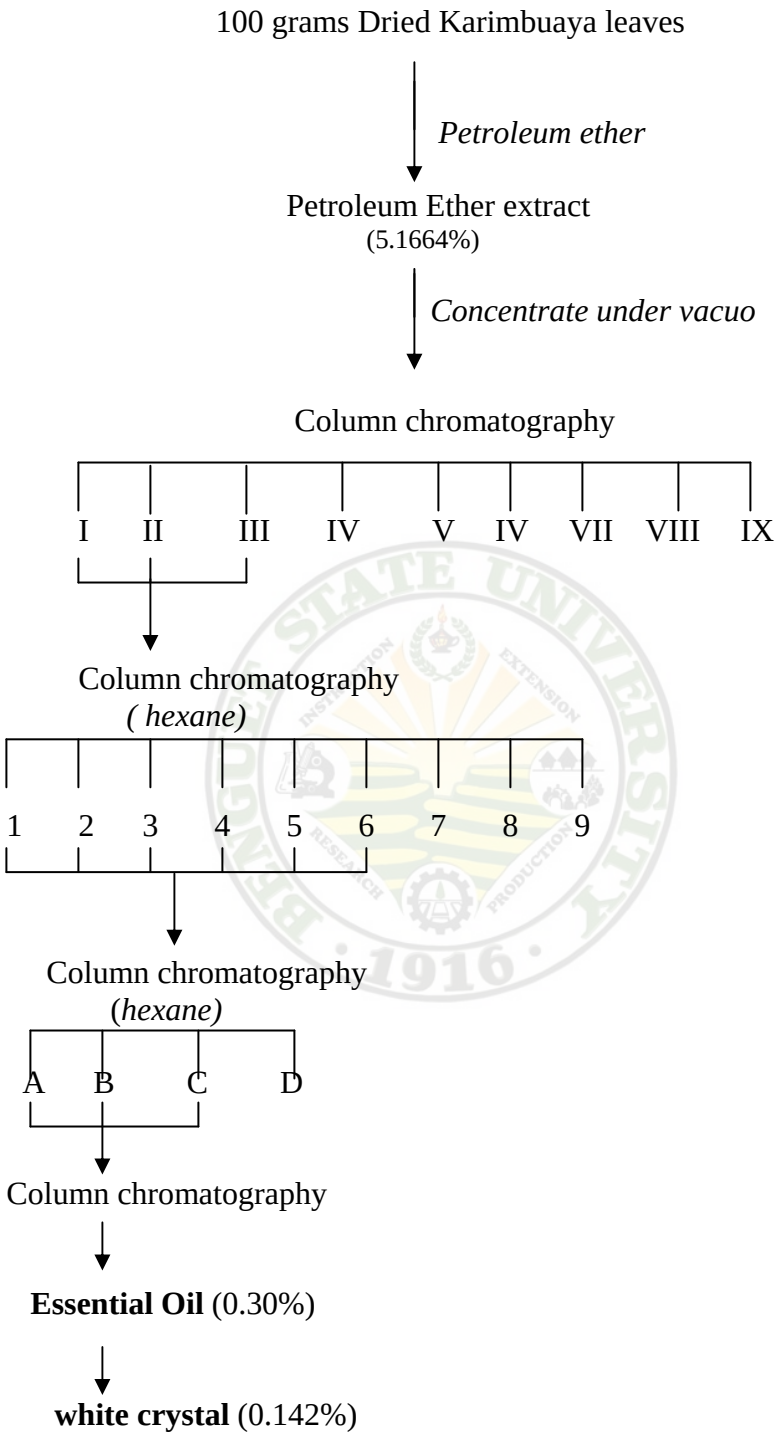


Figure 4. Schematic diagram of the Over- all Extraction of Essential oil

The presence of the crystals probably explains why the essential oil is solid at room temperature. Applying selective solubility difference followed by filtration, the white crystals were separated from the oil. This is shown in Plate 15. Pure ethyl acetate solution was used to recover the oil from the mixture. Percent recovery for the white crystals was 0.142%.

Plate 15. White Crystal Isolate from the leaves of Karimbuaya

Characterization of the Essential Oil

Physico-chemical Properties

Table 10 gave a summary of the physico-chemical properties of the essential oil and the white crystals isolated from the leaves of Karimbuaya.

Table 10. Physico-chemical Properties of the Essential Oil and Crystal

PARAMETERS	PHYSICO-CHEMICAL PROPERTIES	
	Essential Oil	Crystals
Color	Orange	White
Odor	Odorless	Odorless
Melting point	36°C – 40°C	142 °C
Solubility	Hexane Ethyl acetate Ethanol Petroleum ether Chloroform	Chloroform
Physical state	solid	solid
Acid value	2.9	

Essential Oil. The essential oil extracted was odorless and orange in color. This is shown in Plate 16. The melting point determined ranges from 36-40°C. The wide range of melting point denotes that the oil has other components, which is confirmed by the presence 4 spots observed in the chromatogram revealed in Plate 14. The essential oil is soluble in all of the organic solvents used in the solubility test. The oil has an acid value of 2.9. The acid value measures the amount of the fatty acids hydrolyzed. The higher acid number indicates the degree of degradation of the oil (Anon, 2006).

Crystals. The solid separated from the essential oil is white in color and is odorless. It has a melting point of 142°C. The solubility of the crystal was tested

A

B

C

Plate 16. A. Essential oil extracted from Karimbuaya leaves
B. top view of the oil in the beaker
C. The essential oil in a Florence flask after concentrating in vacuo

in several organic reagents and is found soluble in chloroform. as shown in Table 11.

Table 11. – Solubility of the White Crystal Isolate

REAGENTS	RESULT	REAGENTS	RESULT
70% ethanol	Insoluble	Ethyl acetate	Insoluble
80% ethanol	Insoluble	Hexane	Insoluble
85% ethanol	Insoluble	Diethyl ether	Insoluble
90% ethanol	Insoluble	Chloroform	Soluble
Petroleum ether	Insoluble	2%EtOAc-Hexame	Insoluble

Acceptability of Karimbuaya Leaves as a Flavor Enhancer

The sensory attributes of foods are of paramount importance as people take pleasure from a combination of their visual, olfactory, taste and tactile perception. Overall flavor requires not just the basic taste: sweet, salty, bitter, and sour. According to Hegenbart(1996) the total flavor experience is a combination of taste, aroma and the chemical feeling. Modifications on the flavors of food are achieved by addition of flavor enhancers.

The acceptability of fresh Karimbuaya leaves as a flavor enhancer in lechon preparation was compared to the common commercial ingredients. The aroma, taste and overall acceptability were evaluated.

Table 12. Multiple Comparison on Aroma

SAMPLE	MEAN DIFFERENCE	SIG.
Karimbuaya mixture vs. salt only	0.38	0.202
Karimbuaya mixture vs. Lemongrass mixture	0.52*	0.046
Salt only vs. Lemongrass mixture	0.15	0.784

* significant at 0.05

Aroma

Table 12 shows that the mean difference of 0.38 between Karimbuaya mixture and salt only is not significant at the 0.05 level. This indicates that there is no difference on the aroma of the grilled chicken stuffed with Karimbuaya mixture and salt only, respectively. On the other hand, between Karimbuaya mixture and lemongrass mixture, the mean difference (0.52) is significant. This implies that the aroma of grilled chicken as enhanced by Karimbuaya leaves is preferred by the consumer panel. Between the two standard ingredients, salt only and lemongrass mixture, the mean difference is not significant. This shows that the aroma of grilled chicken stuffed with lemongrass is the same as that of the salt.

Table 13. Multiple Comparison on Taste

SAMPLE	MEAN DIFFERENCE	SIG.
Karimbuaya mixture vs. salt only	0.15	0..820
Karimbuaya mixture vs. Lemongrass mixture	0.96*	0.000
Salt only vs. Lemongrass mixture	0.81*	0.002

* significant at 0.50

Taste

Comparing the taste of the grilled chicken treated with Karimbuaya mixture to the one treated with salt only, the mean difference (0.15) obtained is not significant. The result implies that the taste is the same. Karimbuaya mixture when compared with lemongrass mixture, the mean difference is significant. This means the consumers prefer the taste of chicken flavored with Karimbuaya mixture than the lemongrass mixture. The standard ingredients used in the analysis, salt only and lemongrass mixture, revealed a significant difference on the taste of the grilled chicken.

Overall Acceptability

The overall acceptability shown in Table 14 reveals a significant difference only between Karimbuaya mixture and lemongrass mixture. This implies that the Karimbuaya mixture as flavor enhancer on grilled chicken is accepted by consumers.

Table 14. Multiple Comparisons on Overall acceptability

SAMPLE	MEAN DIFFERENCE	SIG.
Karimbuaya mixture vs. salt only	0.15	0..820
Karimbuaya mixture vs. Lemongrass mixture	0.96*	0.000
Salt only vs. Lemongrass mixture	0.81*	0.002

* significant at 0.05

Table 15. Acceptability test of Karimbuaya mixture

PARAMETERS	$F_{computed}$	SIG.
Aroma	2.996*	0.05
Taste	9.020*	0.00
Overall acceptability	3.486*	0.03

* significant at 0.05

In table 15, statistical analysis using the F test (one-way ANOVA) showed that the computed F values for the aroma, taste and overall acceptability were higher than the F value (2.66) at the 0.05 level of significance. The results imply that addition of chopped Karimbuaya leaves to the usual spices for lechon preparation, is favored by the consumer panel. This denotes that the grilled meat with Karimbuaya leaves produces an aroma and taste that is much preferred than lemongrass and the salt.

SUMMARY, CONCLUSION and RECOMMENDATION

Summary

This study aims to characterize the extracts (fresh leaf and the essential oil) from the leaves of Karimbuaya (*Euphorbia neriifolia Linn).* The toxicity and acceptability as a flavor enhancer of the fresh leaves of Karimbuaya was also determined.

The characterization of the fresh Karimbuaya leaf extract was found to be green with leafy like odor and sweet with an after taste. The sweetness was confirmed with the presence of 4.2% mass sucrose using a refractometer. Results of the phytochemical analysis of the leaf extract showed the presence of saponin and tannin. The same metabolites were confirmed present in the sample from the results of the thin-layer chromatography (TLC). The presence of the essential oil in the sample was also revealed in the chromatogram which was developed using a vanillin-sulfuric spray reagent.

The LD_{50} equivalent to 58.73 ppm obtained from the brine shrimp assay indicates that the Karimbuaya leaves are non-toxic. This indicates that the concentration of the saponin and tannin present is below lethal dose (30 ppm).

The percentage yield of the recovered essential oil of the Karimbuaya leaves obtained from repeated rapid column chromatography was 0.30%. The chromatogram of the orange colored essential oil indicates the presence of four (4) components with Rf values 0.20, 0.52, 0.8, and 0.95. The essential oil

extracted is solid at room temperature with a melting point range of 36^{o} – 40^{o}C and is soluble to all the organic solvents (ethanol, ethyl acetate, hexane, petroleum ether, chloroform) used in the test.

The percentage yield of the white crystal isolate was 0.142%. The chromatogram shows the crystals to have an Rf value of 0.20. It has high chloroform affinity and a melting point of 142^{o}C.

As a flavor enhancer, the fresh Karimbuaya leaves were compared with the standard ingredients, salt and lemongrass. Results indicated that the Karimbuaya leaves were preferred by the consumer panel as a flavor enhancer on grilled chicken.

Conclusion

Based on the results, the following conclusions were drawn:

1. The Karimbuaya leaf extract is a green solution with leafy like odor, sweet with an after taste and is non-toxic as indicated by the LD_{50} value of 58.73 ppm;
2. The orange colored essential oil extracted from the leaves of Karimbuaya was fatty like with a wide melting point range of 36-40^{o}C. The chromatogram of the oil shows 4 spots indicating that the oil is a mixture. One component is a white crystalline mixture with a melting point of 142^{o}C;

3. The Karimbuaya leaves as a flavor enhancer for grilled chicken was preferred over the standard ingredients, salt and lemongrass.

Recommendation

The use of fresh Karimbuaya (*Euphorbia neriifolia Linn.)* leaves as a flavor enhancer on grilled chicken is recommended.

Further study should be done to determine the following:

1. effect of the fresh Karimbuaya leaves as a flavor enhancer on other meat and fish products;
2. the constituents responsible for the other properties of the plant not included in the study;
3. isolate and identify other constituents of the essential oil;
4. and to analyze the bioactivity of the plant.

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Appendix A-1

Analysis of Variance (ANOVA) for AROMA

	Sum of Squares	df	Mean Square	F	Sig
Between Groups	6.931	2	3.465	2.996	.05*
Within Groups	163.063	141	1.156		
Total	169.993	143			

Multiple Comparison

Dependent Variable : AROMA
Tukey HSD

(I) TREAT	(J) TREAT	Mean Difference (I-J)	Std. Error	Sig.
1	2	.38	.22	.202
	3	.15	.22	.784
2	1	.38	.22	.202
	3	.52*	.22	.046
3	1	.15	.22	.784
	2	.52*	.22	.046

* mean difference is significant at 0.5