# MICROWAVE – ASSISTED SOLVENT EXTRACTION OF BANABA CRUDE OIL AND ITS ANTIBACTERIAL ACTIVITY

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## APPROVAL SHEET

This graduate thesis is hereto entitled:

**"MICROWAVE – ASSISTED SOLVENT EXTRACTION OF BANABA CRUDE OIL AND ITS ANTIBACTERIAL ACTIVITY"**

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## ABSTRACT

Banaba (*Lagerstroemia speciosa*) is a deciduous tropical flowering tree found in India, the Philippines, and Vietnam. It is known worldwide especially for its antidiabetic and antiobesity abilities. The active ingredient responsible for these properties in humans is *corosolic acid*, a triterpenoid compound that stimulates glucose uptake by enhancing insulin receptor phosphorylation.

Although conventional methods have been used to extract banaba, they lead to low extraction efficiency, long extraction time, and even loss of some volatile compounds. Recently, microwave - assisted extraction with solvent (MASE) has been found to be widely used for herbal extraction because of its increased efficiency, reduced extraction time, and environment-friendliness.

Thus, MASE was investigated for banaba leaves and fruits in this study. Ethanol and ethyl acetate were used as solvents for extracting from banaba fresh leaves. The effect on yield of banaba extract was studied for the following parameters: 300W, 500W, and 800W microwave power; 2 minute and 6 minute extraction time; 6ml/g, 8ml/g, and 10ml/g solvent-to-material ratio; and, 50$^{o}$C, 60$^{o}$C, and 70$^{o}$C temperature.

The active compound in banaba extract, corosolic acid, was identified by high-performance liquid chromatography (HPLC) analysis. Furthermore, the antibacterial activity of banaba extract was tested by Kirby–Bauer method with two gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*).

Extraction of banaba by MASE obtained, on the one hand, the highest yield of 63.95% for ethanol, which made a good solvent for this process, compared to the highest yield of 7.9625% for ethyl acetate. The best conditions that gave the highest yields, on the other hand, were at 500W of power, 10:1(ml of ethanol/g of material) solvent-to-sample ratio, 6 minute, and 70$^{o}$C yielding 63.95% for fresh banaba leaves; and, at 500W power, 8:1(ml of ethanol/g of material) solvent-to-sample ratio, 6 minute, and 60$^{o}$C yielding 79.65% for fresh banaba fruits.

Corosolic acid was identified by HPLC analysis with the peak at around 8.5 minute, the yield of corosolic acid was 0.248% at the best conditions for MAE extraction with banaba fresh leaves extract. However, no presence of corosolic acid was observed in banaba fruits extract.

Measurement of five physico-chemical properties of the resulting banaba crude oil—i.e., pH, specific gravity, refractive index (RI), acid values, and ester values—gave the following characterization ranges: pH of 4–5, specific gravity of 0.927–1.012, and RI of 1.34–1.35. Banaba crude oil obtained a low range of acid values at 2.14–3.6 but obtained a high range of ester values at 12–15 for leaves, specifically giving 96.58 for fresh fruits and 117.67 for dried fruits.

Antibacterial testing of the extracts of banaba showed positive inhibition for antibacterial activity, which demonstrated the applicability of banaba extracts of leaves and fruits in the pharmaceutical and medical industries. The level of antibacterial ability varied with the extract product and with the bacteria used. All four microorganisms, however, exhibited activity at 100mg/ml with *E. coli* as the most sensitive and with the extract showing the lowest zone of inhibition diameter with *B. subtilis*. The best inhibition was exhibited by the extract of fresh banaba leaves while the worst inhabitation was displayed by the extract MASE with ethyl acetate as solvent.

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# CHAPTER I
# INTRODUCTION

## 1.1. BACKGROUND OF THE STUDY

Herbal medicine, also known as *herbalism* or *botanical medicine*, is the oldest form of healthcare in the world. The present recognition of its therapeutic and medicinal values has led people to resort more to herbal medicine as an alternative healthcare and this has resulted in the rapid growth of the herbal industry. The World Health Organization (WHO) estimated that approximately 4 billion people, about 80% of the world's population, utilize herbal medicine for their primary health care; and, thus, according to Ipsos Market & Opinion Research International (MORI), 77% of population agreed that herbal medicines need to regulated (MORI, 2008).

The WHO identified 119 plant-derived pharmaceutical medicines that include, among others, *Aloe Vera*, which is used for healing burns and wounds; *Agarius blazei*, which prevents specific kinds of cancer; and, *Artichoke*, which reduces cholesterol levels.

Another plant whose therapeutic properties show great promise is the *Lagerstroemia speciosa*, popularly known as *banaba* in the Philippines. *L. speciosa* belongs to the Lythracease family and is found abundantly in Southeast Asia. It is known as *Bungur* in India, *Jarul* in Bangladesh, and *BangLang* in Vietnam.

Quisumbing (1978) has established the importance of banaba as one of the Philippines' most important medicinal plants as a popular and common diuretic and hypoglycemic treatment. Filipinos use the banaba leaves to prepare a traditional medicinal beverage. Thus, Garcia (1940, 1941, 1957) studied the effects of orally administered *L. speciosa* decoction on hypoglycemia.

Although in some parts of Vietnam and of other Asian countries, the extract decoction from banaba bark is used to clean wounds or to make ointment for burns, *L. speciosa* is more popularly grown simply for decorative purposes.

During the 1990s, however, scientists started studying the bioactivity of banaba extract from leaves and fruits. Among the 23 medicinal plants that have been examined, banaba leaves showed a remarkable effect on glucose transport activity. The extract contained large amounts of corosolic acid, which indicated antidiabetic properties. (Marakumi et al., 1993; Kakuda et al., 1996).

Other studies noted that banaba extract can influence the glucose uptake of rat adipocytes, thereby reducing blood glucose levels (Hayashi et al., 2002) and countering diabetes and obesity (Klein et al., 2007). These studies proved that the activity of banaba leaves is not limited to corosolic acid but extends to other components of the extract that has similar activity, namely: lagerstroemin, flosin B, and reginin A (Hayashi et al., 2002) as well as valoneic acid, dilactone, and ellagice acid (Hattori K. et al., 2003). These compounds were isolated from the extract of banaba leaves by dissolving them with solvent and then determined by high performance liquid chromatography (HPLC) analysis.

The extraction of banaba oil from its leaves or fruits, however, did not focus on a single method alone. The methods differed depending on the component to be identified, isolated, or quantified.

In most of the previous researches, oil from banaba leaves was extracted by solvent extraction or by crude extraction (Kakuda et al., 1996; Unno et al., 2004; Deocaris et al., 2005). These studies differed only in the solvents used during extraction. The solvents used in these studies included methanol, ethanol, acetone, ethyl acetate, and even hot water.

Results, however, showed that the content of corosolic acid in the resulting banaba extract is only about 1%wt in powdered form. Furthermore, this conventional extraction method not only needed large amounts of solvent but also required a long extraction time of about 1.5 hours. (Judy, 2003)

Hence, to address these limitations, new technologies known as *green technologies*—such as microwave extraction, supercritical fluid, —had been tapped and are now being applied. (Camel, 2000)

The application of microwave technology seemed to be the most popular method in recent years. Several techniques had been devised to optimize the use of microwave in extraction, namely: Microwave-assisted solvent extraction (MASE), vacuum microwave hydrodistillation (VMHD), microwave hydrodistillation (MWHD), compressed air microwave distillation (CAMD), and solvent-free microwave extraction (SFME). (Loupy, André, 2006)

Among these methods, MASE and SFME were the two most popular plant extraction technologies by being able to decrease both time and amount of solvent used (Eskilsson & Bjorklun, 2000. E.Martino et al. (2006) supported these findings by proving that the use of microwave would be the most efficient method of extraction since it gave higher yields in relatively shorter time.

One such study, done by Huang et al. (2009) revealed that banaba extract by using SFME gave the higher yield of crude oil with shorter time than the conventional method. Other studies by Reniek (2007), extraction of Ginger oil and by Nguyen (2008), extraction of Garlic oil; however, showed that using MASE gave a higher yield of crude oil at an even shorter extraction time than using SFME. Therefore, MASE would be the preferred method in the extraction of oil from both banaba leaves and fruits.

Thus, the present study focused on the microwave-assisted solvent extraction (MASE) of *Lagerstroemia speciosa* (banaba) leaves and fruits as well as the testing of crude oil for antimicrobial activity.

## 1.2. STATEMENT OF THE PROBLEM

*Lagerstroemia speciosa* (L.) Pers. (Lythraceae) is considered a popular medicinal plant in Southeast Asia, especially in Philippines, because of its medicinal properties useful in the treatment of diabetes mellitus. (Kakuda et al., 1996; de Padua et al., 1997)

Although Judy et al. (1999) confirmed that *L. speciosa*, commonly called banaba in the Philippines, is high in corosolic acid that is used in many treatments for diabetes; it was found that the antidiabetic effect of banaba is not due to a single chemical but to several compounds that include ellagitannins, lagerstroemia, and flosin. (Murakami, 1993; Hayashi et al., 2002; Klein G. et al.., 2008)

In addition, banaba extract was reported to display potential antioxidant as well as anti-inflammatory and antihypertensive properties. Likewise, corosolic acid—the active component in banaba extract that improves glucose metabolism—also showed cytotoxic activities against certain human cancer cell lines (Yamaguchi et al., 2006)

Conventional methods, such as distillation and soxhlet extraction, are often utilized to extract essential oils from banaba. These methods, however, take larger amounts of solvent, longer extraction time, higher power consumption, and higher risks of negative effects on the environment.

Hence, efforts to overcome these obstacles had led to the exploration of new extraction methods, such as microwave-assisted solvent extraction. The simple procedure and relatively user-friendly installation involved in MASE had made it a widely used procedure for herbal extraction.

Despite this popularity, few studies had been conducted on corosolic acid in extracts from banaba fruits. It is, therefore, deemed necessary to research further both on the extraction from banaba fruits and on the characterization of the properties of the extract as well.

Thus, for this study the application of microwave solvent extraction method for both banaba leaves and fruits was investigated, the effect of the parameters of extraction on the oil yield was examined, and the antibacterial activity of banaba crude oil was explored.

## 1.3. OBJECTIVES OF THE STUDY

### 1.3.1. General Objective

The general objective of the study is to extract and to test the antibacterial activity of banaba leaves and fruits crude oil extracted by Microwave - Assisted Solvent Extraction (MASE).

### 1.3.2. Specific Objectives

In order to achieve the general objective, it is necessary to:

1. Determine the effects of the different parameters—such as extraction time, temperature, microwave power, and solvent to sample ratio—on the yield of banaba extract;
2. Determine the effects of solvents, such as ethanol and ethyl acetate, on the yield of banaba extract;
3. Identify the corosolic acid content in the banaba extract using high performance liquid chromatography (HPLC);
4. Determine the physico-chemical properties—namely, specific gravity, refractive index, acid value, ester value, and pH—of the crude oil produced; and,
5. Test the antimicrobial activity of crude banaba oil.

## 1.4. SIGNIFICANCE OF STUDY

Banaba is considered an extremely valuable plant because its extract can treat diabetes, has antioxidant effects, and, among other reasons, can aid in weight reduction. This plant is grown in many parts of Southeast Asia, specifically in the Philippines where banaba leaves are popularly used as source of local medication. It is well known in the Philippines that banaba leaves contain high levels of corosolic acid, which is the main component responsible for the plant’s various medicinal and therapeutic properties.

Banaba is available in the market both as leaves and as extract. The price depends on the product's corosolic acid content, which is especially true for banaba extract. Dried banaba leaves sell at US$3–5/kg (alibala.com), whereas banaba extract sell for over US$240/kg (bettyhan.en.made-in-china.com). Hence, there is a need for an effective method to increase both the high yields of banaba extract and the high concentration of corosolic acid in these yield extracts.

In recent times, the application of microwave energy in the extraction of oils has been the expected and preferred method for studies. The benefit of MASE over conventional methods, on the one hand, is that it reduces the time of extraction while it increases the recovery of oil. Furthermore, the mechanism of heat application employed in MASE preserves instead of destroys sensitive components in the oil (Mandal et al., 2007).

Recently, although pre-study has always focused on the anti-diabetic and other therapeutic activities of extract of banaba leaves (Ambujakshi et al. 2009); no report has been done on the antimicrobial activity of banaba extract by MASE.

Hence, the present study also investigated on the testing of the antibacterial activity of banaba crude oil, which would be another advantage of the banaba in the application of banaba oil on food and natural cosmetic products like the other essential oil i.e. Tea Tree Oil.

## 1.5. SCOPE AND LIMITATIONS

The present study focused on the yield of banaba oil as well as on the antimicrobial activity of the banaba oil extracted from banaba leaves and fruits by MASE.

It had the following limitations:

- The banaba leaves and fruits were purchased in Alabang, Metro Manila, Philippines, the same materials used by Huang et al., (2009) and De Jesus et al., (2011). They were bought between March and April. Banaba fruits were harvested in May.

- The banaba leaves and fruits were bought fresh and then air dried at room temperature.
- The microwave used for extraction was a domestic oven made by American Company with a frequency of 2445MHz and maximum delivered power of 1000W using 220V.
- The solvents used in the extraction were limited to ethanol and ethyl acetate.
- The solvent-to-material ratios used were 6:1 (ml/g); 8:1 (ml/g), and 10:1 (ml/g).
- The extraction temperatures were $50^{o}$C, $60^{o}$C, and $70^{o}$C to avoid reaching the boiling point of the solvent.
- MASE was carried out at extraction times of 2 minute and 6 minute.
- The corosolic acid in banaba crude oil was identified using high-performance liquid chromatography (HPLC).
- Antimicrobial activity was tested for gram-positive bacteria, such as *Staphylococcus aureus* and *Bacillus subtilis*, as well as gram-negative bacteria, such as *Eschrichia coli* and *Pseudomonas aeruginosa*, which were obtained from the Biology Department of De La Salle University, Manila.
- No economic feasibility was done.

# CHAPTER 2
# REVIEW OF RELATED LITERATURE

## 2.1. *Lagerstroemia speciosa* (Banaba)

*Lagerstroemia speciosa* of the Lythraceae family is commonly known as Queen's Crape Myrtle or Queen's Flower Pride of India and, locally, as *banaba* in the Philippines, *Banglang* in Vietnam, and *Jarul* in Bangladesh. It is grown in some parts of Southeast and South Asia, specifically in Bangladesh, India, Indonesia, Malaysia, Thailand, Vietnam, and the Philippines.

Banaba is a popular medicinal plant in the Philippines, where it is cultivated in most of the provinces in Northern Luzon as well as in the islands of Palawan, Mindanao, and the Sulu Archipelago.

**Figure 2.1.** ***Lagerstroemia speciosa***

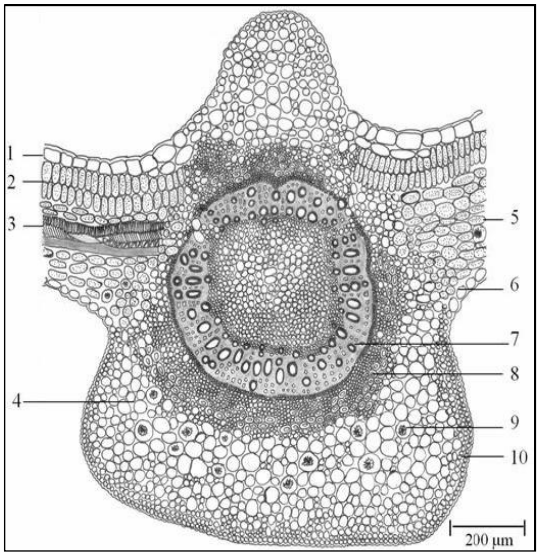
*L. speciosa* is a deciduous tropical plant that grows to about 18m in height and 9–12m in girth. The plant turns bright orange or red during the first month of the year as it begins to shed leaves. These leaves are large, oblate, or elliptical-ovate in shape; and, are 5.1–10.2cm wide and 12.7–20.3cm long. The petals of the banaba's pink, purple, or purplish pink flowers are 3.0–3.5cm long. The smooth bark, which is greyish to cream in color, peels off in irregular flakes. Banaba fruits have winged flaps, are ovate and small (only about 2.5cm long), and grow as much as six pieces as they mature.

Banaba grows best in rich deep alluvial loam and along the banks of rivers and streams as well as in swampy areas.

W.Thitikornpong et al. (2011) examined the microscopic characteristics of *L. speciosa* based on a transverse section of the plant and the plant in powder form.

The transverse section of an *L. speciosa midrib* in **Figure 2.2** shows the parenchyma, collenchymas, phloem, xylem, and parenchyma containing calcium oxalate crystals. It was found that epidermal cells were rectangular to round in shape; some epidermal cells contained spherical clusters of rosette aggregate calcium oxalate crystals, whereas some cells were enlarged and mucilaginous. Cells of the lower epidermis were about twice as small as those of the upper epidermis.

The leaf powder was olive-green in color and had a slightly bitter taste. Some of the microscopic characteristics of the powder include the stomata, rosette aggregate calcium oxalate crystals, fiber, and vessels.




1. Upper epidermis
2. Palisade parenchyma
3. Group of vascular bundles
4. Parenchyma
5. Spongy parenchyma
6. Lower epidermis
7. Xylem vessel
8. Phloem tissue
9. Rosette aggregate calcium oxalate crystal in a parenchyma cell
10. Collenchymas

**Figure 2.2. Microscopic Characteristics of Transverse Section of *L. speciosa* Midrib (Thitikornpong et al., 2011)**

### 2.1.1. History, Uses, and Importance of *L. speciosa*

*Lagerstroemia speciosa* is a famous medicinal plant in the Philippines where it is popularly known as *banaba*. The plant's decocted leaves are well-known as treatment for diabetes mellitus, as diuretic, and as purgative while the plant's root parts are used for stomach ailments.

As early as 1940, Garcia published the first study on banaba's insulin-like hypoglycemic ability and, with his group, continued up to 1957 to publish reports related to the plant's therapeutic activities. (Garcia, 1957) In 1978, Quisumbing affirmed again the antidiabetic properties of banaba decoction.

In 1993, researchers from the Hiroshima University School of Medicine examined the extract of 23 medicinal plants from the Philippines and 6 plants from Japan using Ehrlich ascites tumor cells to identify the anti-diabetes activity of these plants' components. The banaba turned out to be among the five plants that exhibited glucose transport activator effects. (Murakami et al., 1993*)* However, it was only in 1996 that interest in banaba extract grew after the study done by Kakuda et al., which considered the extract's hypoglycemic effects on diabetic KK-Ay mice. (Kakuda et al., 1996)

Today, scientists from Japan, Korea, the Philippines, and the United State are studying banaba. They recognized *L. speciosa* L. to contain not only corosolic acid but other ingredients that possess hypoglycemic ability. On the one hand, Unno et al. (2004), for instance, isolated valoneic acid dilactone and ellagic acid from the aqueous extracts of the *L. speciosa* leaves. The inhibited xanthine oxidase abilities of valoneic acid dilactone and ellagic acid were demonstrated to be stronger than that of *allopurinol*, a clinical drug used as a xanthine oxidase inhibitor. On another study, Okada (2003) was able to isolate trierpenoid, 3β, 23-dihydroxy-1-oxo-olean-12-en-28-oic acid from *L. speciosa* L. Pers extract.

In contrast, in the study done by Hayashi et al. in 2002, the three compounds—namely, lagerstroemin, flosin B, and reginin—that were purified from banaba extract were found to increase the glucose uptake activity on rat adipocytes.

### 2.1.2. Extract from *L. speciosa* Leaves

Research on *L. speciosa* was first reported by Garcia in 1940. From the 39 Philippine medicinal plants that were examined, only five were confirmed to be antidiabetic plants, namely: *Biophytum sensitivum, Catharantus roseus, Syzygum cumine, Ipomoea batata*s, and *Lagerstromia speciosa* L. This report and reports thereafter demonstrated that oral administration of the decoction from banaba produced hypoglycemic effect while oral administration of insulin did not. Oral administration of large doses of the decoction of banaba was never observed to produce convulsions or any toxic effect, unlike insulin which when injected in large doses resulted in convulsions or even in death. Testing of banaba extract on diabetes for humans in 1941 was implemented at the North General Hospital. The dose of 125g of dried young banaba leaves daily administered by mouth in the form of decoction as 4.9 units/100cc of 20% decoction, to be equal to 30.6 units produced practically a similar rate of reduction of blood sugar to the rate of blood sugar reduction obtained after daily infection of 30 units of protons of zinc insulin. (Garcia, 1940)

In 1993, the research of Murakami et al. used an Ehrlich ascites tumors cell line with a bioassay guided fractionation to screen compounds isolated by HPLC from 23 medicinal plants of Philippines for effects on glucose transport activity. Corosolic acid which was isolated from methanol extract faction of *banaba* showed a significant effect on a glucose uptakes assay at concentration 1μm. This result is shown in **Figure 2.3.**

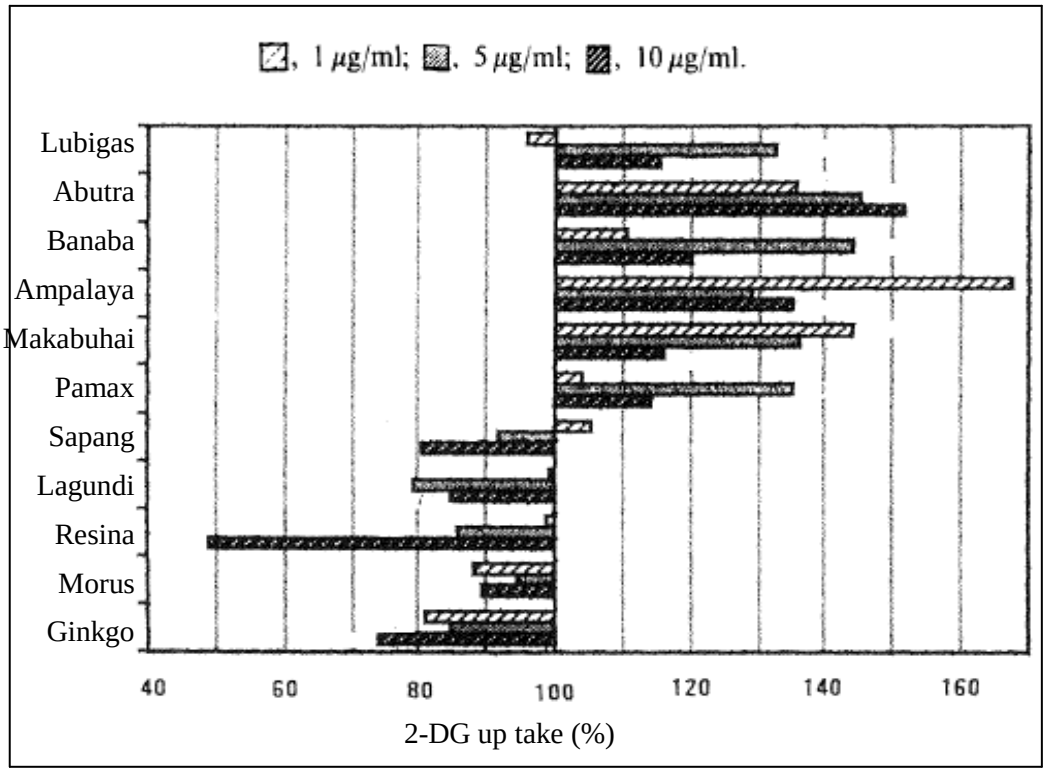



**Figure 2.3. Effects of MeOH Extract of Medicinal Plants on 2-DG Uptake Using Ehrlich Cells (Murakumi, 2003)**

The activity of *L. speciosa* L. Pers leaves on hypoglycemia had also been well studied. Deocaris et al. (2005) from Delhi University, for instance, identified that the banaba leaf extract dose which produced significant hypoglycemic effect, and changed blood levels, was at 250mg/100g ($p<0.05$). In addition, the works of Kakuda et al. (1996) showed that food containing 5% of banaba extract by hot water (HWE) was effective in decreasing blood glucose and insulin levels ($p<0.05$).

The hypoglycemic activity of *L. speciosa* L. was presented by Saha et al. (2009) based on experiments carried out on streptozotocin and focused on the underlying mechanism of the action. The group suggested that hot water extract of *L. speciosa* leaves attributed its prominent hypoglycemic activity on experimental diabetic mice through the suppression of gluconeogenesis and the stimulation of glucose oxidation using the pentose phosphate pathway. Recently more studies demonstrated the hypoglycemic activity of banaba carried out on alloxan–induced

diabetic mice (Tanquiluit et al., 2009), which proved further the hypoglycemic activity of irradiated banaba (Deocaris et al., 2005).

In most of the studies considered, isolated corosolic acid was evidently showed to be the active component. However, in the research of Klein et al. (2007) the whole herb was considered to have glucose-lowering effect and could be used to fight diabetes and obesity, especially Penta-O-glucopytanose (PGG). This conclusion was similar to that of a study done by Liu et al. (2005), whose results indicated that PGG had a significantly higher glucose transport stimulatory activity than lagerstroemin.

### 2.1.3. Components of *L. speciosa* Leaves

The study done by Josefina B. Manalo et al. (1993) on the photochemistry of *L. speciosa* L. Pers leaves found 16 amino acids in the extract of banaba leaves, which also contained tannins, corosolic acid, and other components (Moffett, 2006).

A phytochemical investigation of Banaba leaves was done by Josefina B. Manalo et al., (1993). TLC analysis using silica Gel GF254 adsorbent and Phenol: $H_2O$ (20:80 v/v) solvent system was used to identify components in banaba leaves. The result showed that there are sixteen amino acids, pyrogallol tannins and lipids, present in banaba leaves. **Table 2.1** indicates color and retention values of amino acids when comparing with their standard. The qualitative (TLC) and quantitative (HPLC) determinations were made to know the status or nature of the insulin-like principle in the leaves of banaba. With the sixteen amino acids present in both the crude and tannin free powder extracts, they consider that one can attribute the amino acids present in banaba leaves to the insulin- like principle stated in previous studies. (Manalo et al., 1993)

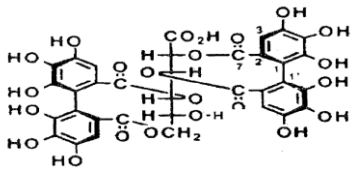
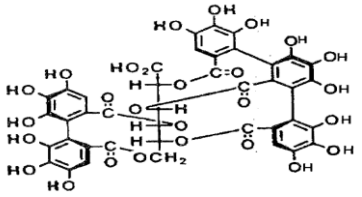
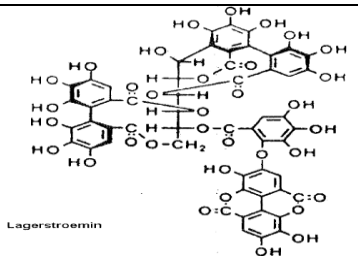
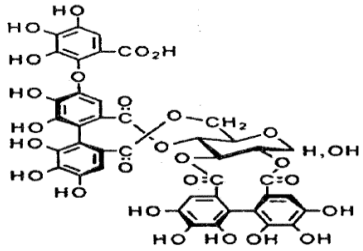
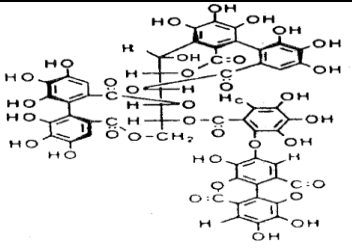
**Table 2.1. Retention Value (Rf) of Standard Amino Acids and Amino Acids in Banaba Leaves (Manalo et al.,1993)**

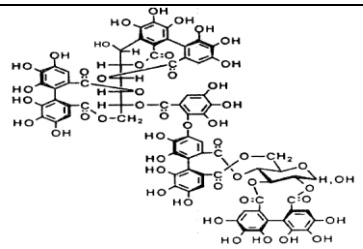
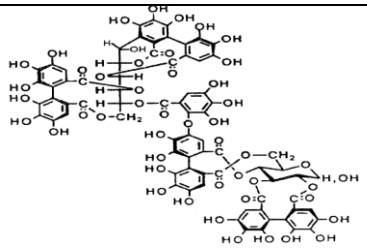
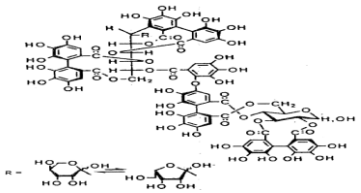
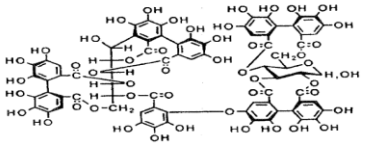
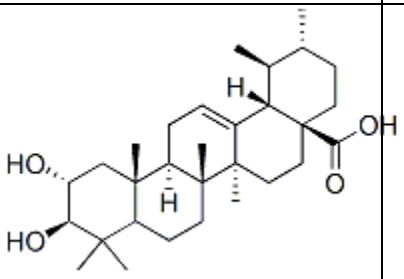
| Standard Amino Acid | Rf Value | Color | Amino Acid in Banaba | Rf Value | Color |
|---|---|---|---|---|---|
| Aspartic Acid | 0.065 | Violet | Aspartic Acid | 0.063 | Violet |
| Arginine | 0.192 | Purplish pink | Arginine | 0.196 | Purplish pink |
| Serine | 0.200 | Violet | Serine | 0.201 | Violet |
| Threonine | 0.203 | Purple violet | Threonine | 0.204 | Purple violet |
| Alanine | 0.276 | Dark purplish pink | Alanine | 0.280 | Dark purplish pink |
| Lysine | 0.297 | Dark purplish pink | Lysine | 0.296 | Dark purplish pink |
| Glutamic Acid | 0.386 | Light violet | Glutamic Acid | 0.350 | Light violet |
| Valine | 0.416 | Dark purplish pink | Valine | 0.405 | Dark purplish pink |
| Tyrosine | 0.420 | Dark purplish pink | Tyrosine | 0.423 | Dark purplish pink |
| Glycerin | 0.443 | Dark purplish pink | Glycerin | 0.446 | Dark purplish pink |
| Isoleucine | 0.456 | Dark purplish pink | Isoleucine | 0.466 | Dark purplish pink |
| Leucine | 0.480 | Light purplish pink | Leucine | 0.482 | Light purplish pink |
| Histidine | 0.530 | Dark gray pink | Histidine | 0.533 | Dark gray pink |
| Phenylalanine | 0.551 | Violet | Phenylalanine | 0.553 | Violet |
| Methionine | 0.664 | Purple violet | Methionine | 0.662 | Purple violet |
| Proline | 0.768 | Yellow | Proline | 0.766 | Yellow |

Furthermore, the other components of banaba leaves were isolated by Moffett et al. (2006). The study investigated relates to pharmaceutical, therapeutic, and dietary composition derived from Banaba leaves and novel extraction processes used to produce these controlled blends of varying composition with respect to corosolic acid, gallotannins, ellagitannins, and valoneic acid and dilactone. Some components isolated from the study are shown in **Table 2.2.**, which include the IUPAC name, formula, structure and molecule of active components in banaba extract. i.e. Lagerstannin A, Lagerstannin B, flosin and corosolic acid.

**Table 2.2. Components of Banaba Leaf Extract**

**(Moffett et al., 2006)**

| Name | IUPAC Name | Formula | Structure | Molar Weight |
|---|---|---|---|---|
| Lagerstannin A | 2,3;4,6-bis-O-(S)-hexahydroxydiphenoyl-D-gluconic acid | $C_{41}H_{27}O_{27}$ |  | 800 |
| Lagerstannin B | 2,3,5-O-(S,R)-flavogallynyl-4,6-O-(S)-hexahydroxydiphenoyl-D-gluconic acid | $C_{34}H_{24}O_{23}.H_2O$ |  | 950 |
| Lagerstroemin | | $C_{55}H_{32}O_{34}.H_2O$ |   | 1,236 |
| Flosin A | | $C_{41}H_{28}O_{27}$ |  | 952 |
| Flosin B | | $C_{55}H_{32}O_{34}$ |  | 1,236 |

| Name | IUPAC Name | Formula | Structure | Molar Weight |
|---|---|---|---|---|
| Reginin A | | $C_{75}H_{50}O_{48}$ |  | 1,718 |
| Reginin B | | $C_{55}H_{32}O_{34}$ |  | 1,718 |
| Reginin C | | $C_{80}H_{58}O_{52}$ |  | 1,850 |
| Reginin D | | $C_{75}H_{50}O_{58}.3H_2O$ |  | 1,718 |
| Corosolic Acid | (1S,2R,4aS,6aR,6aS,6bR,10R,11R,12aR,14bR)-10,11-Dihydroxy-1,2,6a,6b,9,9,12a-heptamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid | |  | 472.7 |

Samples of the study included an approximately 15.5% to about 98.5% mixture of a corosolic acid rich banaba leaf extract in a tannic acid enriched base for novel combinations for pharmaceutical, therapeutic and/or dietary compositions that

yield healthful benefits. Not all samples include corosolic acid, others at low concentration in combination particularly enriched in other compounds. Compounds without the presence of corosolic acid, were the product of extraction process that yield controllably increased ratios of gallotannins, ellagitannins, and valoneic acid dilactone. Both those compositions containing corosolic acid and those lacking corosolic acid are efficacious in effecting control of blood glucose levels, and can be further enhanced in their efficacy by post production and formulation strategies that utilize nanotechnological approaches, targeted deliver enteric coatings, and specialized microenacapsulations.

#### *2.1.3.1. Corosolic Acid (CA)*

*Corosolic acid* is a pentacylic triterpene acid. In recent years, it attracted much attention because of its biological activities—especially its antidiabetes, anti-inflammation, and antiproliferation activities—as well as its protein kinase C inhibition activities. **Table 2.3** shows the properties of corosolic acid.

**Table 2.3. Properties of Corosolic Acid**

| No. | Property | Property Value |
|---|---|---|
| 1 | Molecular Formula | $C_{30}H_{48}O_4$ |
| 2 | Formula Weight | 472.7g/mol |
| 3 | Index of Refraction | 1.566 |
| 4 | Molar Volume | 48.5dyne/cm |
| 5 | Density | 1.14g/cm$^3$ |
| 6 | Flash Point | 314.6°C |
| 7 | Enthalpy of Vaporization | 98.68kJ/mole |
| 8 | Boiling Point | 573.3°C at 760mmHg |
| 9 | Vapor Pressure | 1.56E-15mmHg at 25°C |
| 10 | Molar Refractivity | 135.03cm$^3$ |
| 11 | Surface Tension | 48.68kJ/mole |

In the 1990s, the popularity of this herbal medicine began to attract the attention of scientists worldwide. Since then, researchers have conducted numerous in vitro and in vivo studies that consistently confirmed the antidiabetic activity of banaba. Scientists were able to identify different components of banaba that were responsible for its therapeutic activity. Using tumor cells as a cell model, corosolic acid was isolated from the methanol extract of banaba and was shown to be active at a concentration of 1%. (Murakami, 1993)

In 2004, the group of Miura from the Suzuka University of Medical Science considered that corosolic acid-induced glucose transporter isoform 4 (GLUT4) in genetically type 2 diabetic mice did not change plasma insulin levels. Results proved that CA had significant effects on blood glucose in KK-Ay mice at a dose of over 2mg/kg after 4h ($p<0.05$) administration and decreased plasma glucose at level 4 after 7h. The team's series of continuous studies in 2006 demonstrated, too, the inhibitory effect of CA on the development of obesity and hepatic steatosis in KK-Ay mice as well as the hydrolysis of disaccharides (Miura et al., 2006).

In 2008, when Yamada et al. investigated the mechanism of action of CA on gluconeogenesis in rat liver the results indicated that corosolic acid inhibited fluconeogenesis by increasing the generation of hepatocyte fructose–2, 6–bisphosphate (F-2,6-BP) and inhibiting protein kinase activity in isolated hepatocytes. In addition, CA increases glucokinase activity in isolate hepatocytes without affecting gluco–6–phosphatase activity, suffering the promotion of glycolysis.

The corosolic acid content in *L. speciosa* leaf extract as determined by high-performance liquid chromatography (HPLC) and by high-performance thin-layer chromatography (HPTLC) methods are presented in **Figure 2.4** and **Figure 2.5**, respectively.

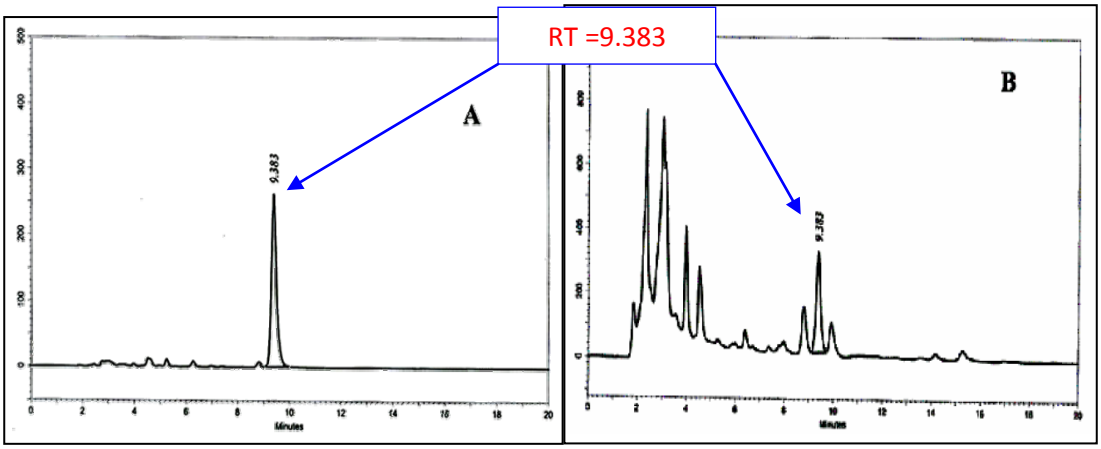



**Figure 2.4. Peak of Standard CA *vs* Peak of Banaba Leaf Extract by HPLC Analysis (Vijaykumar, 2006)**

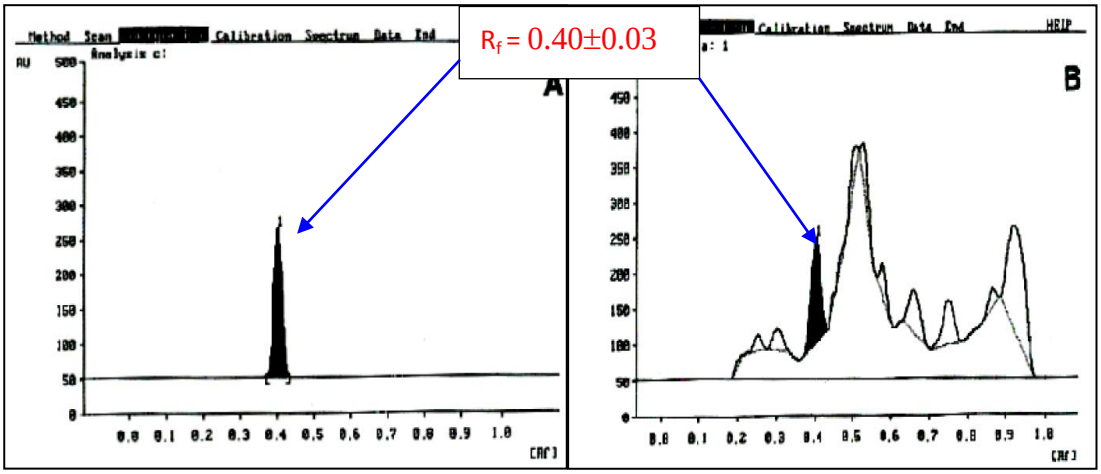



**Figure 2.5. Peak of Standard CA *vs* Peak of Banaba Leaf Extract CA by HPTLC Analysis (Vijaykumar, 2006)**

For HPLC, methanol was used as solvent to prepare the sample, mobile phase included acetonitrile and 0.1% (v/v) phosphoric acid in water (75:25, v/v). Corosolic acid was identified at 9.4±0.4min with 210nm. For HPTLC, at 9:1 chloroform: methanol composition of the mobile phase, the compound was identified as corosolic acid at the $R_f$ value of 0.40±0.03. (Vijaykumar, 2006)

### *2.1.3.2. Tannic Acid (Gallotannins)*

*Tannic acid* is a special commercial form of tannin, a polyphenolic compound. Tannic acid is present in the almost all plants in all climates, but most especially in the barks of trees like oak, walnut, pine, and mahogany as well as in nettles, seeds, and horse chestnuts. Tannic acid is based mainly on glucose esters of gallic acid. Tannic acid has been considered to possess antibacterial (Wakasa et al., 1998), antioxidant (Nelofer et al., 2000), and eicosanoid enzyme inhibition properties (Schubert et al., 1999).

According to report of Klein (2007), tannic acid was isolated as active compound from tannins fraction of *banaba* extract. The presence of tannic acid was identified by HPLC (Liu et al., 2005). **Figure 2.6** shows the structure of tannic acid (gallotannins).

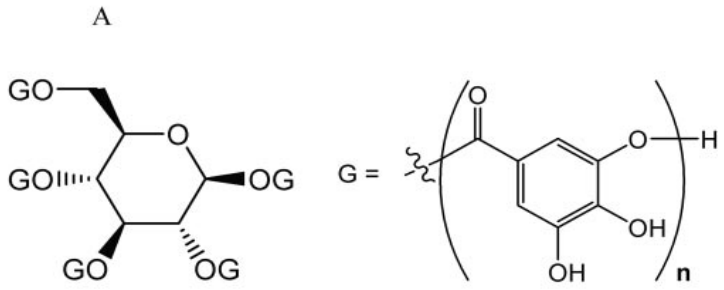



**Figure 2.6. Chemical Structure of Gallotannins (Tannic Acid) (Klein, 2007)**

In the study of Liu et al. (2005), it was noted that removing tannin from banaba extract did not exhibit glucose transport-stimulatory or adipocyte differentiation-inhibitory activity unlike when testing for activity in tannic acid and banaba extract tannin. Thus, it was suggested that tannic acid was a major component that possessed two of the aforementioned activities similar to that of insulin.

### *2.1.3.3. Ellagitannins*

*Ellagitannins* are esters of glucose with hexahydroxydiphenic acid that, when hydrolyzed, yield *ellagic acid*, the dilactone of hexahydroxydiphenic acid. Many forms of ellagitannins—including lagerstroemin; lagerstannin A, B, and C; reginin A, C, and D; flosin B; and, pterocarinin A—have been isolated in *L. speciosa*. Of these, the three most important components were identified as lagerstroemin, flosin B, and reginin A using nuclear magnetic resonance (NMR) spectroscopy and optical rotation. (Hayashi, 2002)

Hayashi et al. (2002) tested for the glucose transporter enhancement activity of banaba and insulin, a physiological activator of hexose transport. They found out that the ability to increase the glucose transporter did not only depend on the presence of corosolic acid, but also was supported by lagerstroemin, flosin B, and reginin A. Results of 2-DG uptake ability testing showed that insulin increased the rate by 0.48nmol/5minute; whereas, tannins increased the rate by 0.26 nmol/5minute. Although flosin B showed a similar activity to lagerstroemin, reginin A induced a marked increase in the hexose uptake. (Hayashi, 2001)

In 2003, Hattori et al. reported that ellagitannins played an important role in phosphorylation, hence, in the activation of insulin receptors. The team examined lagerstroemin for its biological activities in rat adipocytes, where the compound increased the rate of glucose uptake and decreased the isoproterenol-induced glycerol release. Results showed a conclusion similar to that of Hayashi's group, where the treatment of rat adipocytes with lagerstroemin increased the 2DG uptake, which at 150μM resulted in an uptake of 68±3.7%.

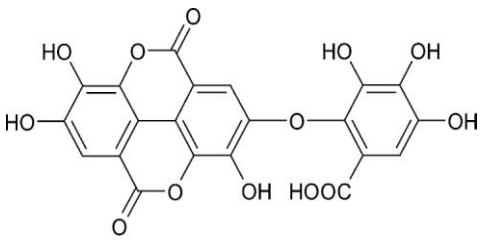
Furthermore, in Hattori et al. (2003) lagerstroemin was demonstrated to induce tyrosin phosphorylation of IRβ-receptors. They also examined whether trypsin treatment induced any change in the actions of lagerstroemin.

### *2.1.3.4. Other Components of L. speciosa and Their Activities*

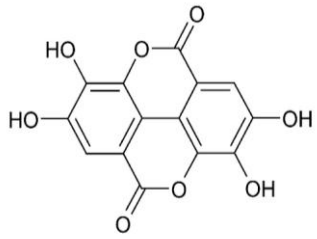
Up to the present time, most scientists had focused their attention to exploring the activities of *L. speciosa* leaves in relation to controlling blood glucose levels. However, research considered that banaba extract also played the important role of being a potent α-amylase inhibitor (Hosoyama et al., 2003).

This research carried out the α-amylase–inhibiting activities test of the *banaba* leaf decoctions. The whole valoneaic acid contents indicated the effect on the α-amylase–inhibiting activities.

In 2004, other scientist research intensively on the potency of *banaba* leaves extract. Several studies indicated that *banaba* leaves extract with valoneic acid as one of the component, was considered as action of xanthanine oxidase (XOD) inhibitors. XOD major function is to catalyze the oxidation of hypoxanthine to xanthine and of xanthine touric acid. XOD inhibitor can syntheze uric acid for the body (Unno et al., 2004). **Figure 2.7** shows the structure of valoneic acid dilactone (VAD) and ellagic acid (EA) while **Figure 2.8** shows the process to isolate and determine VAD and EA.




Valoneic acid dilactone (VAD)




Ellagic acid (EA)

**Figure 2.7. Structure of Valoneic Acid Dilactone (VAD) and Ellagic Acid (EA) (Unno, 2004)**

Comparison of the XOD – inhibitory effects with allopurinol, clinically used as drug for the XOD inhibitor, showed strongest XOD inhibitory (the concentration required to inhibitory effect 50% - IC50) was 2.5 μM whereas allopurinol and EA were 71.5μM as indicated in VAD (Hattori, 2003).

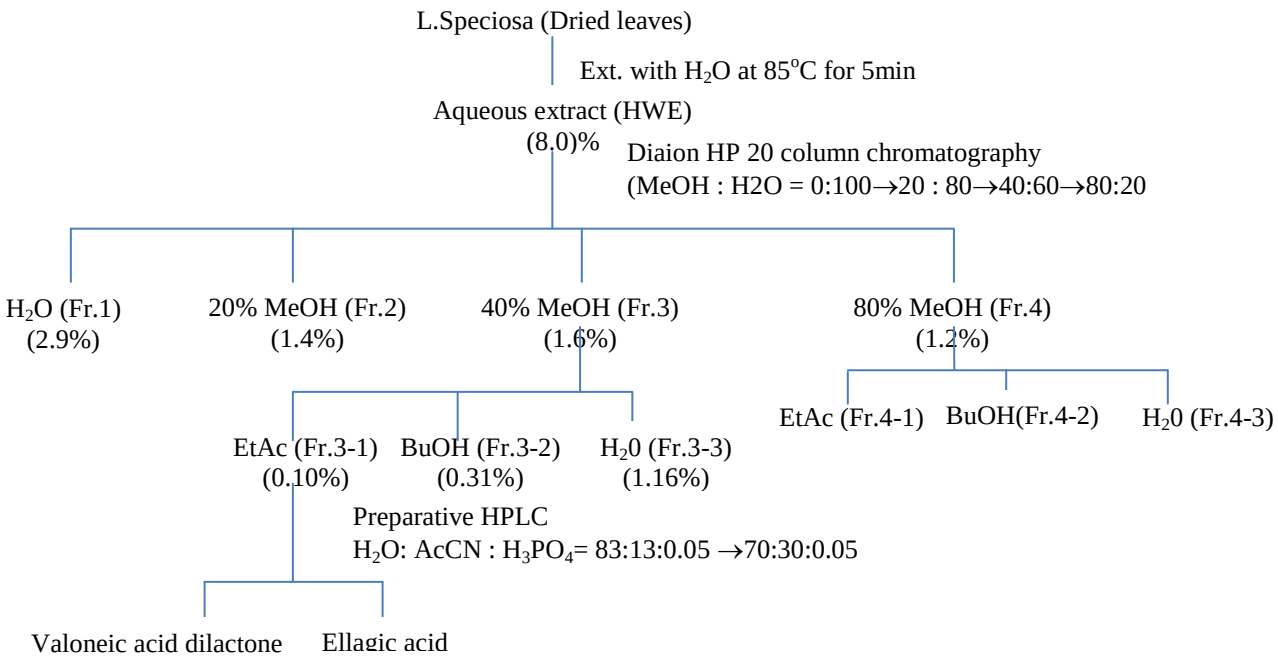



**Figure 2.8. The Process to Isolate and Determine VAD and EA (Unno; 2004)**

### 2.1.4. *L. speciosa* Fruits–Seeds

Banaba fruits have been used for beverage as well as for treatment and prevention of diabetes mellitus similar to banaba leaves. However, there are a few reports about *banaba* fruits (Jehan, 1990) that was used for DNP test for *Lagerstroemia speciosa* seed oil and indicated the presence of a keto group, the acid used as an ingredient of paints and varnishes. The article reported that there was 21.1% of a new keto fatty acid, known as 9-ketooctadec-cis-11-enoic acid, was found in banaba seed oil. The seed oil of *Lagerstroemia speciosa* also contained the other fatty acid such as palmitic acid, stearic acid, oleic acid, linoleic acid. All of fatty acid was measured by GC-MS method. MS were obtained by GC-MS at 70eV and GC was carried out on 15% DEGS on Chromosorb W with 240, 240 and 190 of temperature of inject, detector and oven at 30ml/min of $N_2$ flow rate.

Scientist from China in 2006 investigated the effect of pressure, temperature, flow rate, and particle size on extraction rate of *banaba* seed oil on supercritical $CO_2$.

The result showed the optimum conditions at 30Mpa, 40$^{o}$C, 120 min and 12kg/h of extraction pressure, temperature, time and $CO_2$ flow. Furthermore, GC-MS was used to determine the components of seed oil, 12 fatty acids were found with linoleic acid the highest (74.57%), (**Table 2.4)**.

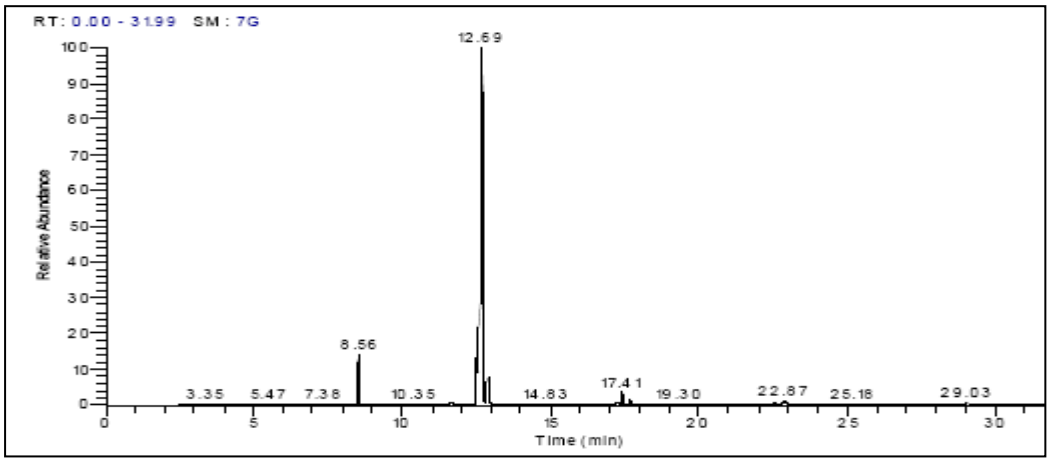



**Figure 2.9. GC-MS Profile of SCF Extracted Oil of Banaba Seed (Zong Wei et al., 2006)**

**Table 2.4. Components of Banaba Fruit Extract (Zong Wei et al., 2006)**

| **Peak** | **RT/min** | **Compounds** | **Area** *(%)* |
|---|---|---|---|
| 1 | 8.56 | Palmitic Acid | 6.68 |
| 2 | 10.61 | Hexadecanoic Acid | 0.25 |
| 3 | 11.68 | Behenic Acid | 0.88 |
| 4 | 12.54 | Oleic Acid | 8.38 |
| 5 | 12.70 | Linoleic Acid | 74.57 |
| 6 | 12.88 | Stearic Acid | 4.26 |
| 7 | 17.28 | Eicosenoic Acid | 0.37 |
| 8 | 17.41 | 11,13-1cesadienoic | 2.15 |
| 9 | 17.66 | Arachic Acid | 0.89 |
| 10 | 18.28 | Tricosanic Acid | 0.26 |
| 11 | 22.53 | Erucic Acid | 0.29 |
| 12 | 22.87 | Lignoceric Acid | 1.03 |

## 2.2. EXTRACTION TECHNOLOGY

Herbs such as aloe vera and ginseng are the valued medical plants. The major compounds in medicinal plants are alkaloids, saponins, flavonoids, anthraquinones, terpenoids, coumarins, lignans, polysaccharides, polypeptides and proteins. Extraction is the method to produce essential oil from herbs, it is also one of the steps needed to identify phytochemical, chemical assay of active components. There are several methods to extract medicinal herbs which include sonication, heating under reflux, and Soxhlet extraction. The selection of extraction method depends on active compounds of the naturally significant analyte made in the interaction matrix. Extraction for botanical can be done by both included two group conventional method and green technologies. **Figure 2.10** shows a summary of the tree diagram showing the wide branching specializations of essential oil field.

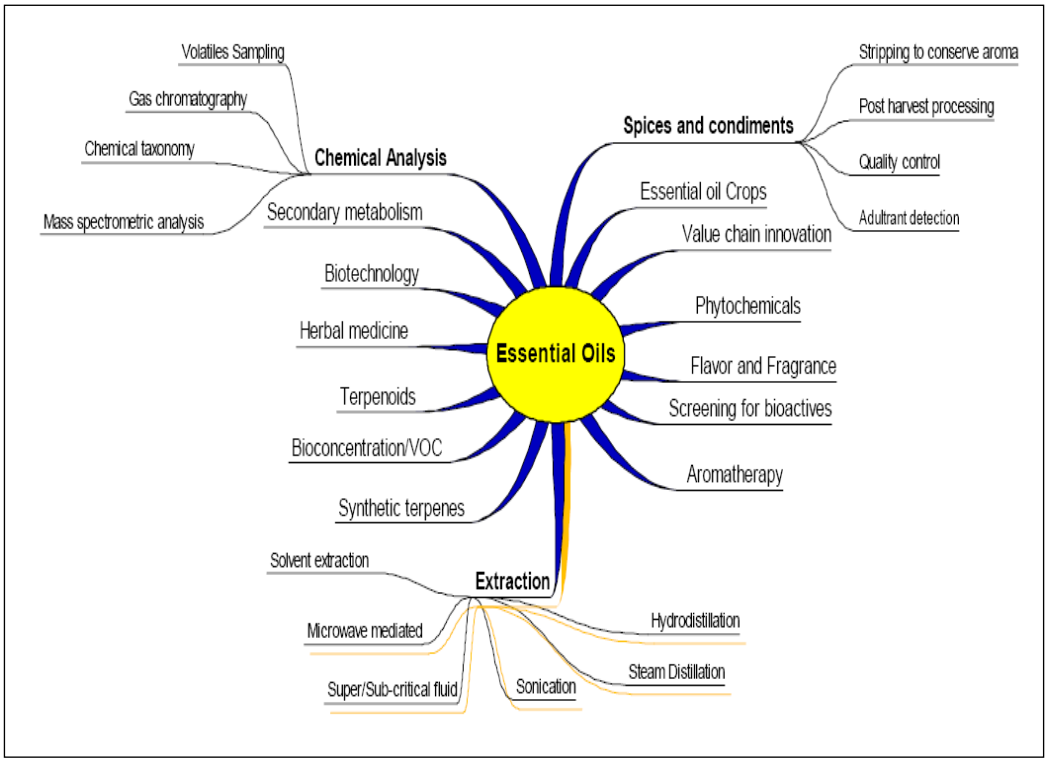



**Figure 2.10 Tree diagram showing the wide branching specializations in the field of essential oil (Ramanadhan, 2005)**

### 2.2.1. Conventional Methods

Conventional solid-liquid extraction techniques permit comparison across technology. Conventional methods were known as liquid-liquid extraction. The principle of liquid-liquid extraction is that the sample is distributed or partitioned between two immiscible solvents in which the analyte and matrix have different solubility. The main advantages of this approach are the wide availability of pure, solvents and the use of low-cost apparatus.

The theory of this technology was based on the use of discontinuous, continuous and hybrid approaches. Therefore conventional can be classify as solvent extraction for discontinuous distillation, continuous distillation, and conventional hybrid (continuous–discontinuous).

#### *2.2.1.1. Distillation*

Distillation is a method of separating mixtures based on differences in their volatilities in a boiling liquid mixture. Distillation is a unit operation, or a physical separation process, and not a chemical reaction. Steam distillation is a method for distilling compounds which are heat-sensitive. This process involves using bubbling steam through a heated mixture of the raw material. Today, steam distillation is a common method for extracting essential oils, such as lavender, peppermint, and eucalyptus.

However, this conventional technology has a major disadvantage like the risk of losses of thermolabile compounds and significant drawbacks such as infeasibility for automation and taking the long time for extraction. Thermolabile compound losses can be avoided by using vacuum distillation. This technology has required high firm material that resulted to be as one of its drawbacks also (Jame, 2002)

#### *2.2.1.2. Soxhlet Extraction*

Soxhlet extraction was introduced by Baron von Soxhlet in the mid-nineteenth century by Franz von Soxhlet. Condensed solvent would then drip into the sample, solubilizing extractable material and then siphon back into the boiling solvent, where this cycle would then repeat. After several cycles over many hours, the apparatus is disassembled and the solvent, now containing extract (fat), is evaporated off, leaving the residue for further analysis. The Soxhlet procedure remains the most exhaustive extraction technique, and today it is still widely used.

Soxhlet extractor is not limited to the extraction of lipids. Typically, a Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. However, over the years, there had been some improvements to the basic technique but still the procedure remained long, tedious, and prone to variability. (Luthria, 2004).

#### *2.2.1.3. Sonication Extraction*

Sonication is an extraction approach that used ultrasonic frequencies to detach to target analyte from the matrix. Horn-type sonic probes operate at pulse power 400-600W in the sample solvent container. Sonication is rapid and effective for certain situations because capitation raises the temperature at the particular surface creating localized superheating even though bulk heating is minimal.

Using organic solvent extraction has as its main shortcomings such as solvent residues in the extract, with the subsequent toxicological risk, and the long extraction time required in most case for achieving efficient extractions. Furthermore, organic solvents have a low selectivity. Therefore, apart from the desired components, high molecular weight, non–volatile components such as fatty oils, resins, waxes and coloring matters are co-extracted. The infeasibility of this technology is another important drawback to be taken into account. (John, 2009)

#### *2.2.1.4. Soxtec Extraction*

In the early 1970s, an improve extraction technique, based on the Soxhlet system, Edward Randall developed an accelerated extraction technique that cut the extraction time to as little as 30 min, is Soxtec. In the Randall method, the sample is lowered and totally immersed in the boiling solvent. The simple principle is that the material to be extracted, in this case, fats and waxes, is more soluble in hot solvent than in cold or room temperature solvent. The procedure included this new boiling step followed by a rinsing step to flush residual extract from the sample. The advantage of Soxtec over Soxhlet is reducing total time of extraction, however this methods has limited the time extract and solvent. (Luthria, 2004).

### 2.2.2. Green Technologies

Green technologies in extraction are the methods using the less solvent and energy. This group includes supercritical fluid, pressurized liquid, continuous subcritical water and microwave extraction.

#### *2.2.2.1. Supercritical fluid extraction (SFE)*

Supercritical fluid extraction (SFE) is becoming an important sample preparation method in the chemical analysis of food products, especially for fats and fatty oils. SFE has been used successfully for over a decade in analyses of food samples. The most popular SFE solvent is carbon dioxide ($CO_2$). There are several reasons for its popularity. First, $CO_2$ is inexpensive and commercially available even at high purity. Second, it is nonflammable, unlike many organic solvents, and is used in some fire extinguishers. Third, $CO_2$ is relatively nontoxic, especially in comparison to many organic solvents; it is actually present in air, foods, and drinks. Carbon dioxide does have a few disadvantages. First, it is practically the only solvent for SFE, as mentioned in the previous paragraph. Even though supercritical fluids offer

flexible solubility depending on pressure, $CO_2$ still has limited solvating power. As a rule of thumb, its solvent strength is comparable to that of hexanes. Because it is nonpolar, extracting polar analyses can be a challenge. (Luthria, 2004)

#### *2.2.2.2. Microwave–Assisted Solvent Extraction (MASE)*

Microwave-assisted extraction (MAE) utilizes electromagnetic radiation to desorb pollutants from their matrices. The microwave region is considered to exist at wavelengths from 0.3 mm to 1 m and frequencies of 100 GHz to 300 MHz. While the whole of this electromagnetic region is potentially available for use, this is not the case. All microwave ovens (domestic or scientific) operate at 2.45 GHz only.

Microwave–Assisted Extraction (MAE) is called Microwave Assisted Process and is a new extraction technology. It is a combined microwave and traditional solvent extraction (Hao et al., 2002).

The technology uses a microwave applicator as the energy source during solvent extraction leading to: faster processing time; improved yield and quality; direct extraction capability; lower energy consumption; reduced solvent levels; and lower capital investment, when compared to conventional extraction methods. The technology not only applies to liquid phase extraction but also to gas phase extraction.

### 2.2.3. Microwave-Assisted Solvent Extraction Related Studies

Today, speed and efficiency in the analytical chemistry laboratory is emphasized. Any technique that will improve speed and efficiency of solvent extraction is an important factor. Since 1985, applications of microwave heating for extraction of compounds from sample matrices have been in use. Granzlet et al. published the first exploration on the use of microwave energy in 1985 to partition various types of compounds from soil, seeds, foods and feeds as a sample preparation method prior to chromatographic analyses (Granzlet; 1985, 1986, 1987, 1991).

Another contribution to this appeared in a U.S patent by Paré et al. from the Ministry of Environment of Canada. In this patent, "Microwave Assisted Natural Products Extraction", the scientists demonstrated the use of microwave energy for the extraction of naturally produced compounds from plant tissues. The patent also proposes and provides evidence for the mechanism for the release of the compounds of interest from the tissues (Paré, 1991).

In another publication Nielson used microwave assisted solvent extraction to examine the extraction additives from polyolefin. They quantitatively extracted antioxidants and a UV-stabilizer with analyses by HPLC (Nielson, 1991).

In the pharmaceutical area, the researcher in Italy used methanol extraction in the closed system with controlled microwave irradiation. They consider again relative to comparisons with the classical extraction technique that means the microwave system with the internal temperature control provided completely analogous qualitative and quantitative composition of extracts (Beloarab, 1992). From the monograph in pharmacopecia, extraction method for botanicals and herbal such as sonication, heating under reflux, soxhlet extraction among others are commonly used. However, these methods take a long time and required the use of large amount of organic solvent and have lower extraction efficiencies. From the various botanicals studied in laboratory, it is considered that different methods of extraction and different conditions may often be required for the extraction of active compounds from different plant material (Ong, 2003). Microwave is the new method for the analysis of botanicals and herbal preparation. Using microwave assisted extraction, extraction time was reduced significantly, usually in less than 30 minute. Moreover, microwave assisted extraction also enables a significant reduction in the consumption of organic solvents. (Ong, 2003).

Besides that, the selection of the method would mainly depend on other advantages and disadvantages of the process, such as complexity, production cost, environmental friendliness and safety. In the case of two-step extraction, the same with case 1, microwave assisted is faster than others (Gringnois et al., 2005).

In the report published in 2002, Pan et al. used the extraction techniques such as microwave–assisted extraction, extraction at room temperature (ERT), heat reflux extraction, ultra-sonic extraction, and soxhlet extraction for tanshinoners from Salvia miltiorrhiza bunge. The results showed that microwave got the higher efficiency than conventional extraction methods. Furthermore, microwave need only 2 min, whereas ERT, heat reflux extraction, ultrasonic extraction and soxhlet extraction take 24 hours, 45, 75 and 90 min, respectively. Therefore, it was considered that microwave was the best selection method for saving of time and high extraction efficiency (Pan et al., 2002). **Table 2.5** shows brief summary of the experimental condition for various methods of extraction, such as sonication, soxhlet extraction, MAE. Pressurized Hot Water extraction (PHWE) and surfactant assistant assisted (PHWE) for medicinal plant (Song, 2004) while **Figure 2.11** shows comparison of microwave and conventional extraction techniques (experimental, conditions, solvent 95% (v/v) of 100ml ethanol, root of Salvia miltiorrhiza bunge 10.0 g L/S=10.0, MAE: 2min, ERT: 24h, heating reflux extraction 45 minute, ultrasonic extraction 75 minute, soxhlet extraction 90min.

**Table 2.5. Summary of Experimental Conditions for Some Extraction Methods for Medicinal Plants (Song, 2004)**

| | Sonication | Sohxhlet Extraction | Microwave assisted extraction (MAE) | Supercritical Fluid extraction | Accelerated Solvent extraction static | Pressurized Liquid Extraction dynamic (PLE) | Pressurized Hot Water extraction (PHWE) | Surfactant assisted (PHWE) |
|---|---|---|---|---|---|---|---|---|
| **Common Solvent Used** | Methanol, ethanol or mixture of alcohol and water | Methanol, ethanol or mixture of alcohol and water | Methanol, ethanol or mixture of alcohol and water | Carbon dioxide or carbon dioxide with modifiers, such as methanol 40-100 | Methanol | Methanol | Water or water with 10-30% ethanol | Water with surfactant, such as Trion X100 or SDS |
| **Temperature** *($^oC$)* | Can be heated | Depending on solvent used | 80-150 | 40-100 | 80-200 | 80-200 | 80-300 | 80-200 |
| **Pressure Applied** | NA | NA | Depending on if it is closed or opened vessel extraction | 250-450 | 100 bar | 10-20 bar | 10-50 bar | 10-20 bar |
| **Time Required** | 1H | 3-18h | 10-40min | 30-100min | 20-40min | 20-40min | 40-50min | 40-50min |
| **Volume of Solvent Required** | 50-100ml | 150-200ml | 20-50ml | NA | 20-40ml | 20-30 | 40-50 | 40-45 |

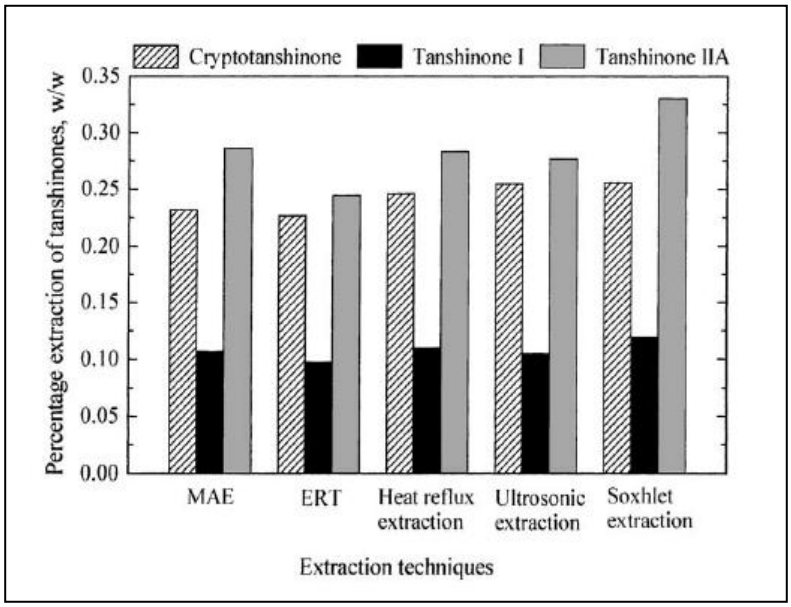



**Figure 2.11 Comparison of microwave and conventional extraction techniques** (experimental, conditions, solvent 95% (v/v) of 100ml ethanol, root of Salvia miltiorrhiza bunge 10.0 g L/S=10.0, MAE: 2min, ERT: 24h, heating reflux extraction 45min, ultrasonic extraction 75 minute, soxhlet extraction 90 minute).

Recently, Terigar's et al. (2010) had tried to design a continuous microwave extraction using temperature and residence time during and after microwave expose exposure. They used this method to extract isoflavones from soybeans with ethanol as solvent. This result showed that the optimum parameters for microwave-assisted extraction of isoflavones were 73$^{o}$C for 8 minute using 3:1 ethanol-to soy flour ratio and the total yield of isoflavones extracted doubled, the amount of oil extracted was 12.

## 2.3. ANTIMICROBIAL ACTIVITY STUDIES

Most microbiologists distinguish two groups of antimicrobial agent used in the treatment of infectious disease: antibiotics, which are natural substances produced by certain groups of microorganisms, and chemotherapeutic agents, which are chemically synthesized. The paper published by Guenther in 1952 considered that some of essential oils possess antimicrobial activities. After that, the studies of antimicrobial activities of essential oils have been investigated. In 1985, Naqvi et al. evaluated 163 plants of different families and found 30% of them active (Naqvi et al., 1985). Others studies demonstrated the antibacterial and antifungal activities of essential oils of different plant species against various microorganisms (Morris et al., 1979; Syed et al., 1991; Rahman et al., 2002). Some essential oils included in Pharmacopoeias as having antimicrobial activity are Sideritis of Greek Mountain Tea, Oregano oil, Tea tree Oil, Mint oil, Lavander oil, Lemon oil, Onion oil.

Tannins are the most popular component isolated from the leaves or bark of tree. There are many researches that examined the antimicrobial properties of tannins. In the review published in 1991, Scalbert considered that there are probably several mechanisms involved in tannin toxicity.

In 1999, the researcher from Université Catholique de Louvan, D. Djipa et al. tested antimicrobial activity of bark extracts of *Syzygium Jambos* (L.) Alston (Mytaceae). several flavonoids were isolated myricetin and quercetin 3-O-b-D-xylopyranosyl(1-2)a-L-rhamnopyranosides and ellagic acid derivatives: 3,3%, 4% - tri-O-methylellagic acid-4-O-b-D-glucopyranoside, 3,3%, 4% - tri- O - methylellagic acid orellagitannins (pedunculagin, casuarinin, tellimagrandin I, strictinin, casuarictin, 2,3-HHDP-glucose and traces of tellimagrandin II). Their aim was to determine the minimal inhibitory concentration (MIC: the minimal concentration completely inhibiting the growth of the micro-organism and determined the minimal active concentration (MAC: the minimal concentration reducing the growth of the microorganism as compared to controls) of *Syzygium Jambos* effect on both position and negative bacterial. The results showed *S. jambos* extract is generally more active on very sensitive strains (MIC<1000 mg/ml) compared with lower MIC value of H.

6irginiana on M. morganii 180 and the equal MIC values of H. 6irginiana on *S. aureus* 105 MC and S. Warnerii 215 and of A. 6ulgaris on M. morganii 180.

By measuring the mean diameter of zone of inhibition, Banso, et al. showed the antibacterial activity of tannin such as from *dichrostachys cinere*a. Furthermore, it was reported that the MIC of tannins isolated in this study against the test organisms ranged between 4.0 and 5.5 mg/ml while the MBC ranged between 4.5 and 6.0 mg/ml (Banso, 2007)

**Table 2.6: Antibacterial of tannin isolated from *dichrostachy cinerea* (Banso, 2007)**

| **Concentration** *(mg/ml)* | **Mean diameter of zone of inhition** *(mm±SD)* | | | | |
|---|---|---|---|---|---|
| | ***Staph.aureus*** | ***Sh.boydii*** | ***Sh.flexneri*** | ***E.Coli*** | ***P.aureuginosa*** |
| 0.5 | 0 | 0 | 0 | 0 | 0 |
| 1.0 | 0 | 0 | 0 | 0 | 0 |
| 1.5 | 7.5±0.1 | 9.5±0.01 | 12.0±0.1 | 8.5±0.1 | 6.5±0.1 |
| 2.0 | 10.0±0.02 | 11.0±0.05 | 15.0±0.2 | 10.5±0.1 | 8.0±0.02 |
| 2.5 | 13.5±0.5 | 14.0±0.2 | 19±0.01 | 14±0.1 | 11.0±0.01 |
| 3.0 | 15.0±0.08 | 17.0±0.1 | 22.0±0.02 | 16.5±0.2 | 13.5±0.2 |
| 3.5 | 18.0±0.02 | 21.0±0.05 | 24.0±0.05 | 18.5±0.3 | 16.0±0.1 |

**Table 2.7. Mininum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of tannins isolated from Dichrostachys cinerea (Banso, 2007)**

| Organism | MIC (mg/ml) | MBC (mg/ml) |
|---|---|---|
| *Staph.aureus* | 5.5 | 6.0 |
| *Sh.boydii* | 4.5 | 5.0 |
| *Sh.flexnero* | 4.0 | 4.5 |
| *E.coli* | 5.0 | 5.5 |
| *P.aeruginosa* | 5.5 | 5.5 |

The antibacterial activity of tannin constituent was studied in the different researches. Although tannin was extracted from variable plants, it demonstrated the effect on bacterial.

**Table 2.8. Antibacterial activity (MIC,μg/ml) of the *P. vulgaris* red bean (P), F.*esculentum buckwheat* (F), *C.avellana hazelnut* (C), *J nigra walnut tannin* (J) (Amarowicz et al.; 2007)**

| Baterial | Tannin | | | | Streptomycin |
|---|---|---|---|---|---|
| | P | F | C | J | |
| *Listeria monocytogenes* | 125 | 62.5 | 125 | 125 | 31.3 |
| *Staphytococcus aureas* | 250 | 250 | 250 | 500 | 31.4 |
| *Escherichia coli* | 250 | 500 | 125 | 125 | 15.6 |
| *Brochothrix thermosphacta* | 250 | 500 | 500 | 125 | 62.5 |
| *Pseudomonas fragi* | 250 | 250 | 250 | 250 | 31.3 |
| *Salmonella Typhimurium* | 125 | 125 | 500 | 250 | 7.8 |
| *Lactobacillus plantarum* | 125 | 250 | 125 | 125 | 7.8 |

In a recent study, Ambujakshi et al. (2009) carried out first test for the antibacterial activity of *Lagerstroemia speciosa (L.) pers*. The gram positive and gram negative bacterial were tested by cup-plate agar diffusion method. The result showed that water extract was more effective than ethanol extract.

**Table 2.9. Antibacterial Activity of *Lagerstroemia speciosa (L) Pers*. Leaves extract Ambujakshi et al. (2009)**

| **Zone of inhibition** *(diameter in mm)* | | | |
|---|---|---|---|
| **Microorganisms** | **Ethanol Extract** | **Water extract** | **Standard (Ampicillin)** |
| S. aureus | 14 | 15 | 22 |
| B. Subsisilis | 12 | 15 | 25 |
| P. aeruginosa | 14 | 17 | 23 |
| E. Coli | 16 | 17 | 25 |

Tannic acid, a form of tannins, was also considered to possess antibacterial activity (Wakasa et al., 1998; Akiyama et al., 2001; Zhao et al., 1997). 2009, Colak, tested antimicrobial efficacy of tannic acid used in the pickling stage by disc diffusion method with bacteria, moulds and yeasts and got the positive results for the three, tannic acid has antimicrobial activity, it against all bacteria, moulds and yeast. At 3%, tannic acid exhibited the most effective. **Table 2.10** shows the result of anti-microorganism of tannic acid.

**Table 2.10. Inhibition zones of bacteria, moulds and yeasts (mm)***

**(Wakasa et al., 1998)**

| | | **Experimental Group (% TA)** | | | | | **Control Group ***** |
|---|---|---|---|---|---|---|---|
| | | 0.1% | 0.5% | 1% | 2% | 3% | |
| **Bacteria** | *Bacillus cereus* | 12 | 13 | 14 | 14 | 14 | -*** |
| | *Pseudomonas aeruginosa* | 12 | 12 | 12 | 13 | 14 | - |
| | *Escherichia coli* | 11 | 12 | 12 | 12 | 13 | - |
| | *Staphylococcus aureus* | 14 | 14 | 14 | 15 | 15 | - |
| **Mould** | *Aspergillus niger* | 10 | 10 | 11 | 11 | 14 | - |
| | *Aspergillus flavus* | 10 | 10 | 10 | 11 | 12 | - |
| | *Penicillium granulatum* | 10 | 11 | 11 | 12 | 12 | - |
| **Yeast** | *Geotricum candiaum* | 10 | 10 | 10 | 11 | 12 | - |
| | *Rhodotorula rubra* | 11 | 11 | 12 | 12 | 13 | - |

*: value, including diameter of the chrome tanned leather sample (8.0mm), are means of three replicate.

**: No tannic acid was used.

***: No inhibition zone was observed.

# CHAPTER 3
# THEORETICAL CONSIDERATIONS

## 3.1. MICROWAVE

### 3.1.1. Microwave Radiation

*Microwaves* are electromagnetic waves with wavelengths of 1mm–1m, corresponding to frequencies between 0.3 and 300GH, and located between infrared radiation and radio waves **(Figure 3.1).** Microwave is used for transmission of information (telecommunication) and for transmission of energy. Microwave ovens and all dedicated microwave reactors for chemical syntheses that are commercially available today operate at a frequency of 2.45GHz (correlative wavelength of 12.25cm) in order to avoid interference with telecommunication, wireless networks, and cellular phone frequencies.

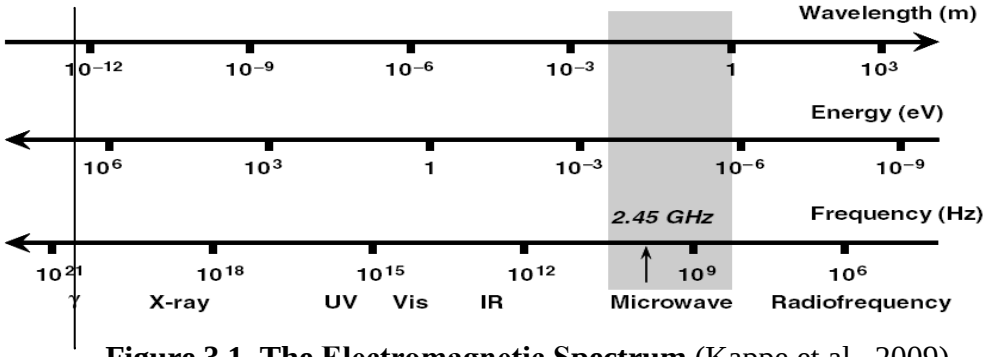



**Figure 3.1. The Electromagnetic Spectrum** (Kappe et al., 2009)

Chemical reactions under microwave radiation cannot be aborted directly with electromagnetic energy in contrast to ultraviolet radiation and visible radiation (Kappe et al., 2009), since variable radiation of this type gives higher Brownian motion than the energy of microwave photon at 2.45GHz (0.016eV) as can be seen in **Table 3.1**.

**Table 3.1. Microwave Photon, Brownian Motion, and Chemical Bond Energies (Loupy et al. 2006)**

| | Microwave Photon | Brownian Motion | Hydrogen Bonds | Covalent Bonds | Ionic Bonds |
|---|---|---|---|---|---|
| **Energy** *(eV)* | $1.2 \times 10^{-6}$ to $1 \times 10^{-5}$ | 0.017 | 0.04–0.44 | ~4.51 (C-H)<br>~3.82 (C-C) | 7.6 |

### 3.1.2. Microwave Heating

Microwave dielectric heating, which is a basic theory in microwave chemistry, is dependent on the ability of a specific material (a solvent or a reagent) to absorb microwave energy and to convert it into heat. Since microwaves are electromagnetic waves they consist of an electric component and a magnetic field component (Kappe et al., 2009), which are shown in **Figure 3.2.**

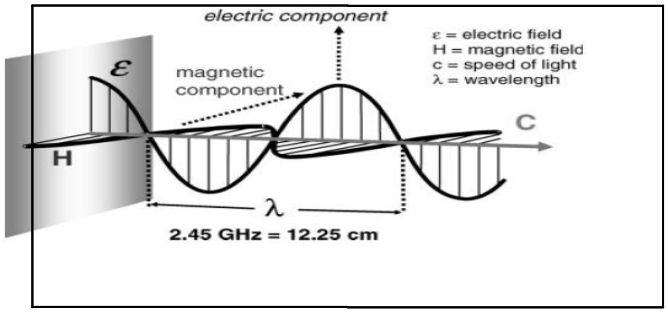



**Figure 3.2 Electric and Magnetic Field Components in Microwaves**

Heating was caused by the electric component of an electromagnetic field through two mains mechanisms: dipolar polarization and ionic conduction. *Dipolar polarization* mechanism is the interaction of the electric field component with the matrix (Baghurst, 1991; Gabriel, 1998) and the substance must possess a dipole moment; whereas, during the ionic conduction, under the influence of the microwave, particles in a sample (ions) oscillate back and forth and in colliding with neighboring molecules or atoms lead to agitation and motion, thus, creating heat. The heat-

generating capacity of the conductivity principle has a much stronger effect than its dipolar rotation mechanism (Kappe et al., 2009).

### 3.1.3. Loss Tangent

The dielectric properties of the material affect the heating characteristics of a particular material under microwave irradiation conditions. As microwave absorption occurs owing to the reorientation of the permanent dipoles by the electric field, the amount of energy absorbed is proportional to the dielectric constant (ϵ') of the solvent. In practice, the absorption is also proportional to the solvent polarity. Apart from absorbing the energy, the solvent must be able to convert the energy into the heat, so the efficiency of the conversion process is dependent on the dielectric factor loss (ϵ''). The overall efficiency of heating is then expressed by the loss tangent or dissipation factor.

$$\tan \delta = \frac{\varepsilon''}{\varepsilon'} \tag{3.1}$$

The dielectric constant plays an important role in heating generation of the sample in the microwave field. The greater the dielectric constant, the more thermal energy is eliminated and the heating system increases rapidly. Therefore, the effects of microwave depend on the nature of both the solvent and the matrix (Camel, 2000).

The loss tangent is a measure of reactance of a molecule. For instance, a material that has tan $\delta = 0$ is completely transparent to microwave irradiation, and incident irradiation passed through with its path unchanged ($\delta = 0$). A perfectly absorbing material has tan $\delta = \infty$, $\delta = \pi/2$ radians. Here, the material under irradiation shows complete resistance to the incident irradiant. On the other hand, material with tan δ achieving 1 are very strong microwave absorbers, i.e. ethanol (tan $\delta = 0.935$) is excellent absorber of microwave irradiation at 2.45 GHz. Based on the tangent δ value, there are three levels of solvent, namely: high (tan $\delta > 0.5$), medium (tan δ: from 0.1 to 0.5), and low microwave absorbing (tan $\delta < 0.1$). Loss tangent values depend on both frequency and temperature.

The loss tangent values depend on both frequency and temperature. For instant, **Figure 3.3** shows the relative of dielectric properties, dielectric loss and frequency. It is apparent that appreciable values of the dielectric loss ϵ" goes through a maximum as the dielectric constant falls. The heating, as indicated by ϵ", approaching its maximum about 18 GHz, however, the domestic microwave ovens operate at a much lower frequency, 2.45 GHz. If the frequency is optimal for a maximum heating rate, the microwaves are absorbed in the outer regions of the food, and penetrate only a short distance.

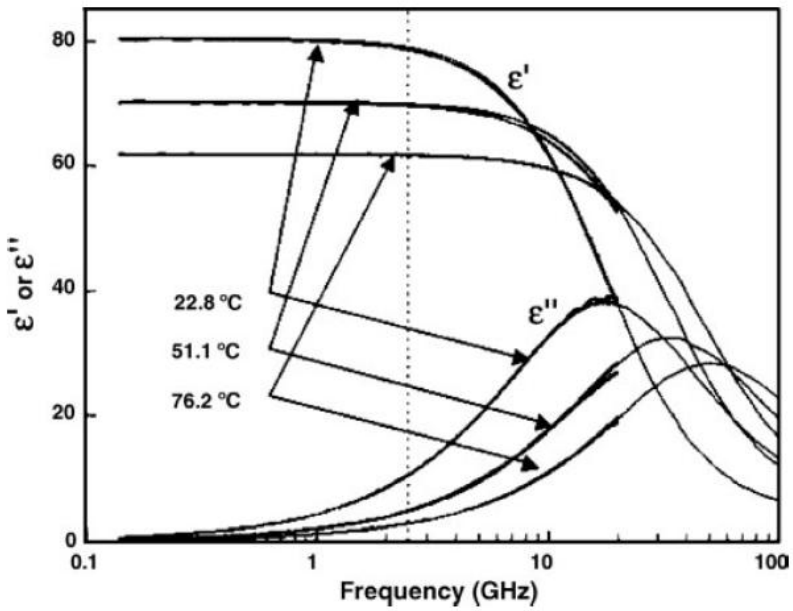



**Figure 3.3: Dielectric properties of water as a function of frequency at 25$^{o}$C (Kappe, et al., 2009).**

The dielectric loss and loss tangent of most organic solvent decreased with increasing temperature. Therefore absorption of microwave radiation in them decreases at higher temperature. From the practical point of view this may be somewhat inconvenient, since microwave heating at higher temperatures may often be compromised. On the other hand, from the standpoint of safety, it should be

stressed that the opposite situation may lead to a scenario where a material will become a stronger microwave absorber with increasing temperature. This is the case will that lead to the danger of a thermal runaway during microwave heating.

If the material surface reflects microwave radiation, there is no or very small energy provided for the system. The main heat of microwave makes increasing the material only marginally, especially for metals with high conductivity (Kappe, et al., 2009).

**Table 3.2. Loss Tangents (tan δ) of Low-Absorbing Materials at 2.45GHz, 25°C (Bogdal & Prociak, 2007)**

| Material | tan δ ($\times 10^{-4}$) | Material | tan δ ($\times 10^{-4}$) |
|---|---|---|---|
| Quartz | 0.6 | Plexiglas | 57 |
| Ceramic | 5.5 | Polyester | 28 |
| Phosphate glass | 46 | Polyethylene | 31 |

### 3.1.4. Dielectric Properties of Materials

The attenuation of electromagnetic wave at the microwave frequency range is caused by energy absorption at various other frequencies. The electromagnetic characteristics of materials are controlled by the macro-molecular, granular, as well as atomic scale behavior of the components constituting them. The interaction of electromagnetic radiation with matter depends on the frequency as well as the material properties (Mauritz, 2005) as shown in **Figure 3.4**

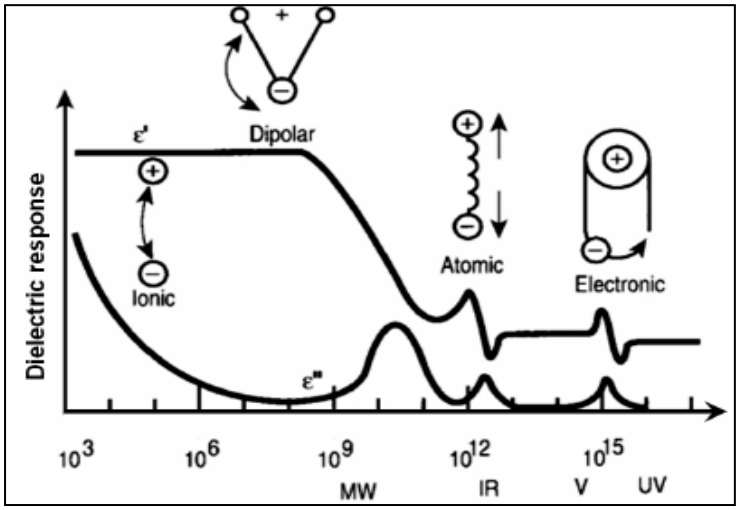



**Figure 3.4: Interaction of electromagnetic radiation with matter (Mauritz; 2005)**

Various effects occur at different frequency creating the characteristic stepwise development of dielectric properties with respect to frequency. The dielectric constant effectively remains constant at high and low frequencies. The relaxation phenomenon portrayed relates to the disturbance of polar molecules by an impressed electric field. There is an orienting effect experienced, associated with corresponding energy absorption in the quantum rotational band. If a polar molecule such as water is present in the medium, these molecules experience a force that acts to orient the permanent dipole moment in the direction of the electromagnetic field. This force is opposed by equilibrium state of the molecules. If an alternating field continuously propagates, the individual molecules will be induced to rotate in an oscillatory manner about an axis through their centers. Inertia however prevents them from spontaneous response. Similar effects are felt in the translational degree of freedom too.

The polarizing effect of propagating waves is related to the thermal mobility and therefore, sensitive to the temperature. The temperature, activation energy and the nature frequency of oscillation of polarized particles determine the relaxation time, often expressed as relaxation frequency (Lew, 2002).

### 3.1.5. Microwave Heating *vs* Conventional Thermal Heating

Microwave irradiation produces efficient internal heating (in core volumetric heating) by direct coupling of microwave energy with the molecules (solvents, reagents, catalysts) that are present in the reaction mixture. Thus, microwave irradiation raises the temperature of the whole volume simultaneously.

In contrast, conventional heating is carried out with an external heat source, such as oil-bath or heating mantle. Hence, transferring energy into the system occurs slowly and inefficiently. In addition, since conventional heating depends on convection currents, the thermal conductivity of the various materials must be penetrated since the temperature of the reaction vessel is higher than that of the reaction mixture. The very efficient internal heat transfer results in minimized wall effects (no hot vessel surface) which may lead to the observation of the so-called *microwave effect.*

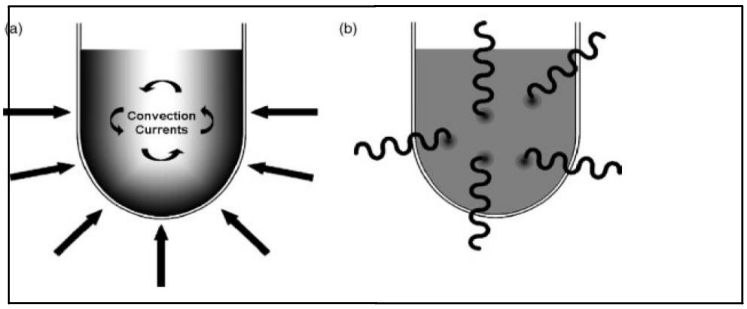



**Figure 3.5. Comparison of (a) Conventional and (b) Microwave Heating**

### 3.1.6. Microwave Effects

Microwave effects are the subject of considerable current debates and controversy and it is evident then that extensive research efforts are necessary to truly understand these and related phenomena. Essentially, three different possibilities for rationalizing rate enhancements have been observed in a microwave-assisted chemical reaction (Kappe et al., 2009).

Many scientists agree that in majority of the cases the reason for the observed rate enhancements in microwave chemistry is a *pure thermal/kinetic effect*; that is, a consequence of the high reaction temperatures that can rapidly be attained when irradiating polar materials in a microwave field. It needs to be noted that the rapid

heating and cooling typical of small-scale microwave-assisted transformations may lead to altered product distributions compared to conventional oil-bath reflux experiments. In many cases, microwave-assisted reactions have been found to be cleaner, leading to less by products compared to conventionally heated processes.

*Specific Microwave Effects* has been defined as the acceleration of chemical transformations in a microwave field that cannot be achieved or duplicated by conventional heating but essentially have thermal effects.

The first of these is the *superheating effect* of solvent at atmospheric pressure. (Baghurst, 1992; Saillard, 1995) It has been established that the enthalpy of vaporization is the same under both microwave and conventional heating (Abtal, 1985). Studies have also shown that the rate of vaporization, as well as the temperature of both vapor and liquid at the interface, depends largely on experimental conditions. Moreover, it has been considered that microwave-heated liquids boil at temperatures above the equilibrium boiling point at atmospheric pressure. (Baghurst, 1992)

For several solvents, the superheating temperature can be up to 40$^{o}$C above the classical boiling point (Chemat et al., 2001); therefore, in a microwave-heated reactor, the average temperature of the solvent can be significantly higher than the atmospheric boiling point as whole volume of the solvent is dissipated by microwave power. However, this will occur only at the existing liquid–gas interfaces in contrast to that in a thermally heated solvent where boiling typically occurs at nucleation point (cavities, pits, and scratches) on the glass reactor surface. (Baghurst, 1992)

The second of these microwave effects are the *wall effects*. Because the wall surface is not heated, the temperature at the inner surface of the reactor wall is lower than that of the bulk liquid. Hence, there is limited decomposition at the reactor surface compared to conventional heating. However, no dedicated study of the reduction of wall effects in microwave chemistry has been published to date.

The third of these microwave effects is the phenomenon characteristic of microwave dielectric heating called *mass heating*, which refers to the rapid, and even heating of the whole reaction mixture by microwave, or *volumetric heating*.

A fourth set of effects known as *nonthermal (althermal) microwave effects* has been classified and described as the acceleration of chemical transformations in a microwave field that cannot be rationalized by either purely thermal/kinetic or specific microwave effects. (Kappe, Dallinger, & Murphree, 2009) Essentially, nonthermal effects result from a proposed direct interaction of the electric field with specific molecules in the reaction medium. The presence of an electric field leads to orientation effects of dipolar molecules and changes the pre-exponential factor A (Jacob, 1995) or the activation energy (Berlan, 1991) in the Arrhenius equation.

## 3.2. MICROWAVE-ASSISTED SOLVENT EXTRACTION (MASE)

### 3.2.1. Principles of MASE

The use of microwave energy as a heating source in analytical laboratories began in the 1970s when it was applied to the acid digestion process. Later the development of microwave assisted to extract of compounds from soil, seeds, foods and feeds was first reported by Ganzler and co-workers, and, in 1995, Zlotorzynski reviewed the fundamental principles of microwave energy for digestion, extraction, and desorption. Microwave-assisted solvent extraction is based on the property which absorbs and transfers energy (Fattorusso & Tagalialatela–Scafati, 2008).

When using microwave for extraction, the moisture inside the plant is heated up due to microwave effects, evaporates, and generates high pressure on the cell wall, which causes plant cells to break up. The cell wall is pushed from inside by the pressure and stretched until it finally ruptures. These processes throws out the active constituents in the cell into the bulk solvent; thereby, improving the extraction's yield by microwave. More so, the plant matrix is sunk in the solvent with higher efficiency under microwave, at a higher tan $\delta$ value which can be enhanced by this phenomenon. Since higher temperatures increase the hydrolyzability of ether linkages of cellulose the constituent is converted into soluble fractions within 1–2 min. (Mandal V. et al., 2007)

The microwave energy's ability depends on the dielectric susceptibility of both the solvent and the solid plant matrix (Neas & Collins, 1988). As temperature increases penetration of the solvent into the matrix constituents are released into the surrounding hot solvent.

Here, there are two popular technologies involved: first is controlled pressure and temperature, which is called *pressurized microwave-assisted extraction* or *closed vessels*; and, second is *focused microwave-assisted extraction* (FMAE) or *open vessels*.

1. Closed-Vessel. In closed vessel, the heating temperature of the solvent can be above boiling point at atmospheric pressure. The main advantage of closed-vessel conditions is that solvents may be heated way above their boiling points because of pressure is contained and controlled. The higher the temperature, the shorter the extraction time. In addition, there is no evaporation; thus, loss solvent is required. However, the drawback of closed-vessel is the partitioning of the more volatile solutes into headspace when temperature rises rapidly, causing loss of these components. Moreover, since the system will need time for cooling to room temperature before opening the vessels to avoid loss of volatile solutes, overall extraction time is increased. (Camel, 2000)

2. Open-Vessel. Microwave systems work under atmospheric pressure. Consequently, the highest temperature is possible at boiling point of solvent. Using the open-vessel system can even be more effective with such increased safety, ease in adding the reagent at any time during extraction, and fully automated operations. Therefore, open-vessel can be applied easily to scale-up microwave-assisted reactions. Moreover, it allows more rapid heating compared to conventional techniques provided that the reaction mixture is highly microwave absorbing. (Mandal et al., 2007).

### 3.2.2. Influence of Different Parameters on the Extraction Process

#### 3.2.2.1. Choice of Solvent

Choosing the solvent is the most important step for obtaining an optimal extraction process even in MASE. The choice of solvent for MASE is influenced by the solubility of the target analyte, by the interaction between solvent and plant matrix, and the microwave-absorbing properties of the solvent. Firstly, the solvent should have a high selectivity towards the analyte of interest excluding unwanted matrix component and have the compatibility of the extracting solvent with further chromatographic analytical step. Secondly, the interaction between the solvent and the plant matrix is influenced by the solvent's dielectric properties.

Solvents interact differently with microwaves, according to their dielectric properties. The higher the loss tangent (tan δ), the better is the conversion of microwave energy into heat, and it will be more effective in microwave heating. Generally, the more polar the solvent, the greater is its ability to couple with microwave energy and temperature will rise rapidly. **Table 3.3** shows the dielectric properties and tan δ of five solvents.

**Table 3.3. Loss Tangents (tan δ) of Different Solvents at 2.45GHz, 20$^{o}$C**

(Kappe, et al., 2009)

| **Solvent** | **Dielectric Constant,** $\epsilon'$ | **Dipole Moment** | **Tan δ** | **Boiling Point** *($^{o}C$)* |
|---|---|---|---|---|
| Ethanol | 24.3 | 1.96 | 0.941 | 78 |
| Methanol | 32.6 | 2.87 | 0.659 | 65 |
| Water | 80.4 | 1.85 | 0.123 | 100 |
| Ethyl Acetate | 6.02 | 0.78 | 0.059 | 77 |
| Hexane | 1.89 | 0.00 | 0.020 | 69 |

Although the dielectric constants of water and methanol are higher than ethanol, their tan δ are less than tan δ of ethanol; therefore, ethanol has been considered as solvent of choice. Contrary, hexane and ethyl acetate have lower tan δ, which means they heat up lower under microwave. However, they may also be processed with microwaves if mixed with the solvent that has high tan δ.

Therefore, the dielectric properties of solvents are an important factor in MASE. For specific purposes, MASE may use solvents with low dielectric constants as in the case of the extraction of solutes by a cold solvent to avoid degradation of thermolabile components.

When using microwave for extraction with solvent, it must be ensured that the material is immersed in the solvent throughout the irradiation time. (Mandal .et al., 2007)

#### 3.2.2.2. Extraction Time

Extraction time is another parameter that is of interest and importance because it affects extraction efficiency. Generally, the extraction yield is higher with increasing time although there is the risk of degradation occurring. In extraction 15–20 min is often sufficient, but some reported that even 40s gave excellent recovery (Li et al., 2004; Wang et al., 2007).

The study by Pan, Niu, and Liu (2003) found that the extraction yields of polyphenols and caffeine were highest up to 4min and later decreased when extraction time was increased. However, in the extraction of Artemisia, Hao et al. (2002) found that the overall high of 92% extraction yield was achieved within 12min after which extract yield dropped.

Nevertheless, some extraction reports revealed that varying the extraction time did not significantly improve recovery like when Barbero, Palma, and Barroso (2006) varied extraction time for pepper at 5, 10, 15, and 20 minute. Results showed that increased extraction time failed to increase the recovery of capsaicinoids.

In conclusion, it may be said that extraction time may vary with the different plant parts used. Likewise, irradiation time is also influenced by the dielectric properties of solvents. For instance, solvents like water, ethanol, and methanol may heat up tremendously on longer exposure, thus risking the structure of thermolabile constituents.

#### *3.2.2.3. Microwave Power*

Microwave power is the third factor that influences the MASE process to a great extent. Microwave has an effect on the mechanism of interaction between the microwave and the sample in the system: Generally, the higher the power the higher the extraction efficiency. The study of Shu, Ko, and Chang (2003) showed that the extraction efficiency of ginsenosides between 30W and 150W improved with increased microwave power.

Similarly, microwave power also affects extraction time. Although high power can effect shorter extraction time, high power with prolonged exposure always involves the risk of thermal degradation. (Shu, Ko, & Chang, 2003)

Contrary, however, increasing power from 400W to 1,200W had no significant effect on the extraction yield of flavonoids although it was observed that extraction time was shortened by 45min at 1,200W. In closed-vessel systems, choosing power settings will depend on the number of samples to be extracted during a single extraction run, as up to 12 vessels can be treated in a single run. The power must be chosen correctly to avoid excessive temperatures, which can lead to solute degradation and to overpressure inside the vessel. (Letellier, 1999).

#### *3.2.2.4. Matrix Characteristics*

Water molecules have high dipole moment and strongly absorb microwave energy; therefore, the water content of the matrix is of great importance. In the extraction of chlorinated pesticides from sediments, Onuska (1993) considered matrix

moisture as a critical parameter with the best recoveries obtained at a 15% water level, which was also the sediment saturation level.

The plant particle size of the extracted materials often has a profound effect on the recovery of compounds. Generally, the particles are in the range of 100$\mu$m–2mm. Small-sized particles increase extraction yield by providing a larger surface area, therefore, enhancing contact between the plant matrix and the solvent. However, although finer particles improve the penetrability of the material in the solvent they are quite difficult to separate after microwave extraction. Hence, centrifugation or filtration is often used as a remedy.

In the extraction of ginseng saponins, Kwon et al. (2003) proved that extraction yield increased with reduction in particle size, but it was also seen that particles less than 60 mesh in size were no longer suitable for the filter.

#### 3.2.2.5. *Temperature*

Temperature is the most important factor with the biggest impact on extraction efficiency. Generally, high temperatures enhance extraction as a result of increased diffusivity of the solvent unto the internal parts of the matrix.

In the closed-vessel system, microwave power and temperature are varyingly interrelated to each other as when temperature gain a higher boiling point for the solvent under pressurized extraction. Additionally, solvents have a higher capacity to solubilize analytes at higher temperatures, while surface tension and solvent viscosity decrease with temperature. Therefore, the sample both easily wets and penetrates the matrix in the solvent. Care must be taken, however, when extracting at high temperatures because increased temperatures is associated with increased pressure and, hence, safety problems, in closed systems. It is interesting to note, too, that temperature was found to be a significant factor in the extraction of paclitaxel, used in cancer therapy. (Mandal et al., 2007; Talebi et al., 2004)

In the open-vessel system, temperature can be effectively controlled by proper combinations of extracting solvents that heat up differently. For example, in the microwave-assisted extraction of organotin compounds from marine tissues, high temperatures were required to reach complete tissue hydrolysis, even though they could induce degradation of compounds like monophenyl- and diphenyltin above 90$^{o}$C. (Mandal et al., 2007)

In using MAE to extract pesticides from several crops, Pylypiw (1997) found out that the optimum temperature depended on the matrix to be extracted; for instance, 100$^{o}$C was suitable for lettuces and 120$^{o}$C was the optimum temperature for tomatoes.

## 3.3. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Essential oils have widespread applications—from perfumery to pharmaceutical products to food flavorings—and must, therefore, be of high quality and purity.

The various techniques consistently used in analyzing oils give two types of information: first is *qualitative information* or the presence of components in a substance; and, second is *quantitative information*, or the amount of components in a substance. Chromatography is one of these many techniques most often used in analyzing essential oils.

*Chromatography* is a separation technique based on differences in structure of molecules or composition. The mixture dissolved in mobile phase is passed through a stationary phase. According to different interactions with the stationary support, the molecules in the mixture lead to the separation of molecules. The tighter the interactions in stationary phase, the more slowly the mixture is passed through the column. In this way, different types of molecules can be separated from the mixture when they pass the support material.

If the mobile phase is a gas the process is referred to as *gas chromatography* and if it is a liquid, it is called *liquid chromatography.*

HPLC is a form of liquid chromatography, which has been improved by allowing solvent to drip through a column under gravity or, for faster results, forced through under high pressures of up to 400atm. Nowadays, HPLC enjoys widespread use in pharmaceutics, biology, synthetic and natural polymers, and environment residues. In addition, detectors used in HPLC are nondestructive, hence, facilitating sample recovery and providing the opportunity for subsequent spectroanalytical studies.

Generally, depending on the relative polarity of the solvent and the stationary phase, there are two variations in using HPLC: the normal phase HPLC and the reversed phase HPLC.

*Normal phase HPLC,* similar to *thin-layer chromatography* or *column chromatography*, is based on the adsorption to a stationary surface chemistry and by polarity. The use of more polar solvents of the mobile phase will reduce the retention time of analytes, while more hydropholic solvents will increase the retention time. Polar solvents in a mixture being passed through the column will stick longer to polar silica than nonpolar compounds will.

In the case of *reversed phase HPLC*, the column size is the same but the silica is modified to make it nonpolar by attaching to its surface a long hydrocarbon chain $RMe_2SiCl$, where $R$ is a straight chain alkyl group, such as $C_{18}H_{37}$ or $C_8H_{17}$, referred to as $C_{18}$ *column* or $C_8$ *column*. Therefore, the polar solvent and polar molecules in the mixture being passed through the column will have strong attraction, the polar compound will spend a long time to move with the solvent, and the polar molecules will pass through the column quickly. On the other hand, nonpolar compounds in the mixture will tend to form an attraction with the hydrocarbon group by Van der Waals dispersion force. These are less soluble in solvent because they have to break hydrogen bonds as they squeeze in between water or alcohol. Hence, they spend less time in solution as the solvent slows them on their way down through the column.

## 3.4. PHYSICO-CHEMICAL PROPERTIES OF ESSENTIAL OIL

### 3.4.1. Specific Gravity

The ratio of the mass of a substance to that of an equal volume of some standard substance, which is water in the case of solids and liquids and air or hydrogen in the case of gases, is called *Specific Gravity*. It is a dimensionless quantity. At a given temperature, every essential oil has a specified specific gravity because of its unique constituents. It is quite a narrow range and any deviation from these limits will indicate impurity.

$$SG = \frac{\text{Mass of given volume of a substance}}{\text{Mass of equal volume of pure water}} \tag{3.2}$$

### 3.4.2. Refractive Index

*Refractive index* is the property of a substance that changes with the speed of light. It is calculated as the ratio of the speed of light in a vacuum to the speed of light through the substance. When light moves at an angle between two changed materials, the refractive indices identify the angle of transmission of the light beam. Generally, the refractive index varies based on the frequency of the light as well, hence, different colors of light at different speeds. The slower the speed of light can travels a medium, the higher is the index. The refractive index is always more than 1 for light passing from a less dense medium like air into a denser medium like oil.

Currently, a *refractometer* is used to measure the refractive index.

Essential oils, just like specific gravity, have a refractive index with a fairly narrow range of limits. For example, 1.457–1.464 for *Lavedula angustifolia* oil and 1.476–1.478 for *Citrus reticulate* oil.

An essential oil that deviates from these values indicates adulteration, which only means two things: that oil is of poor quality or that the oil is a different substance altogether.

### 3.4.3. Acid Value

Acid value is measured by the number of free fatty acids (FFAs) in an essential oil. It is expressed in milligrams of potassium hydroxide, which is required to neutralize the acidic constituents, in 1g of essential oil. Acid value is calculated as

$$AN = (V_{eq} - b_{eq})N\frac{56.1}{W_{oil}} \tag{3.3}$$

where: $V_{eq}$ is the amount of titrant (ml) consumed by the sample and 1ml spiking at the equivalent point, $B_{eq}$ is the amount of titrant (ml) consumed by 1ml spiking solution at the equivalent point, and *56.1* is the molecular weight of KOH.

### 3.4.4. Ester Value

*Ester value* is the measure of milligrams of potassium hydroxide required to saponify the neutral oil in a 1g sample, exclusive of that required to neutralize any free acid. The equation is given as

$$EV = 28.05\frac{(B - V)}{m} \tag{3.4}$$

Where: *B* is the volume of 1.5M hydrochloric acid required for the blank (ml), *V* is the volume of 1.5M hydrochloric acid needed to neutralize the excess alkali after hydrolysis (ml), and *m* is the mass of oil taken (g).

Ester value is important index because it is relative to physical firmness and it is an index of a substance's capacity to produce odor, with shorter-chain or lower molecular-weight fatty acids being more odorous. Hence, a high ester value means firmer essential oil. (Murano, 2003)

## 3.5. ANTIMICROBIAL SUSCEPTIBILITY TEST

Antimicrobial susceptibility test is the method to measure the ability of an antibiotic or an antimicrobial agent to inhibit bacterial growth in vitro. Antimicrobial susceptibility testing can be classified on the endpoint of the test as inhibitory or lethal. The inhibitory activity of an antimicrobial agent is determined either by dilution testing or disk diffusion testing and, recently, by E-test.

### 3.5.1. Methods of Antimicrobial Susceptibility Testing

#### *3.5.1.1. Dilution Testing*

*Dilution susceptibility testing* determines the minimal concentration of an antimicrobial agent. In the dilution testing method, antimicrobial agents are tested at $\log_2$ serial dilution using a range dependent on the bacterial agent. There are two methods of dilution: first is by the agar-based agar dilution method, and second is by broth-based broth dilution method.

On the one hand, agar dilutions, which are carried out in petri dishes, have the advantage of making possible the testing of several organisms on one dish. The dilutions are made in a small volume of water, added to agar and then melted and cooled to not more than $60^{o}$C. It is convenient to use 90mm-diameter petri dishes and to add 1ml of the desired drug dilutions to 19ml of broth with agar. On the other hand, broth dilutions are simple procedures for testing small numbers of isolates or even a single isolate. Through broth dilution the Minimum Bactericidal Concentration (MBC) can be measured.

One of the advantages of dilution testing is flexibility, since this test can be used with rapidly growing aerobic, facultative, fastidious, and anaerobic bacteria that are available from many vendors. Furthermore, dilution systems are adaptable to automation, resulting in the increased efficiency of technical personnel and in the feasibility of data generation.

#### *3.5.1.2. Disk Diffusion*

In this test method, an antibiotic-impregnated wafer and an antibiotic are placed onto an agar plate to which bacteria have been swabbed and the setup incubated overnight. The antibiotic diffuses from the disk into the agar in lessening amounts the further it goes away from the disk. On the disk's surface will be an area that goes around the disk and where bacteria cannot grow. This area is called the *zone of inhibition.*

Disk diffusion, or *Kirby–Bauer Test*, is a popular anti-microorganism testing method used in clinical laboratories worldwide. There are many advantages to disk diffusion: First, it allows accurate and reproducible testing of most bacterial pathogens; second, it shows acceptable batch-to-batch reproducibility for susceptibility testing; third, it is versatile, easy to perform, and inexpensive; and, lastly, it makes use of media, reagents, equipment, and supplies that are accessible and readily available from most clinical laboratories.

#### *3.5.1.3. E–Test*

The *E-test* or *AB Biodisk* is a quantitative method for antimicrobial susceptibility testing that applies both to methods of dilution and diffusion into the medium. E-test, also known as the *epsilometer test*, is an "exponential gradient" testing methodology, where *E* stands for *epsilon*.

In this test, a predefined stable antimicrobial gradient is present on a thin inert carrier strip. When this E-test strip is applied onto an inoculated agar plate, there is an immediate release of the drug. Following incubation, a symmetrical inhibition ellipse is produced. The intersection of the inhibitory zone edge and the calibrated carrier strip indicates the MIC value over a wide concentration range (>10 dilutions) with inherent precision and accuracy.

E-test can be used to determine the MIC for fastidious organisms, such as *S. pneumoniae*, *N. gonorrhoeae*, *Haemophilus* sp., and anaerobes. (Lalitha, 2004)

### 3.5.2. Microbial Agents Used to Test Microbial Susceptibility

#### *3.5.2.1. Staphylococcus*

In 1884, Rosembach discovered two types of staphylococci, *Staphylococcus aureus* (yellow) and *Staphylococcus albus* (white). At present, more than 20 species of staphylococcus were described in *Bergey's Manual of Systemic Bacteriology* (2001). *Staphylococcus* is found in most anatomical locales, including the skin, oral cavity, and gastrointestinal tract.

*Staphylococcus aureus* is a gram-positive *coccus*, which appears in grape-like clusters when viewed through a microscope. When they grow in the agar plate, their appear round and golden yellow. *S. aureus* frequently lives on the skin or in the nose of a healthy person and can cause illnesses ranging from minor skin infections like pimples, boils, and abscesses to life-threatening diseases like pneumonia, meningitis, and septicemia.

Some strains of *S. aureus* produce the exotoxin TSST-1, which causes toxic shock syndrome. Other strains produce an enterotoxin that is the causative agent of gastroenteritis, whose symptoms include nausea, vomiting, diarrhea, and abdominal pain.

Penicillin is the choice of treatment for *S. aureus* infection. However, penicillin-resistance is extremely common in most countries. In 2007, a group of researchers from Italy identified a bacteriophage active against *S. aureus*, including methicillin-resistant strains (MRSA), in mice and possibly humans. (Capparelli, 2007)

#### *3.5.2.2. Bacillus subtilis*

*Bacillus subtilis* was discovered by Christian Gottfried Ehrenberg. Originally named *Vibrio subtilis* in 1835, it was renamed *Bacillus subtilis* by Ferdinand Cohn in 1972. *B. subtilis Bacullius*, or *grass bacillus* is a gram- and catalase-positive bacterium of the genus Bacillus. It is shaped like a rod and has the ability to form a tough protective endospore, allowing the organism to tolerate extreme environmental conditions.

*B. subtilis* can be found in the food but it rarely causes food poisoning. Furthermore, *B. subtilis* can survive the extreme heat during cooking. *B. subtilis* also has the ability to divide symmetrically to make two daughter cells (binary fission) or to divide asymmetrically, generating a single endospore that can be resistant to environmental factors like heat, acid, and salt.

Two of the many uses of *B. subtilis* are its applications in bacterial chromosome replication and in horticulture and agriculture as soil inoculant. The enzymes produced by *B. subtilis* and *B. licheniformis* are widely used as additives in laundry detergents. *B. subtilis* also plays a role in the conversion of explosives into harmless compounds of nitrogen, carbon dioxide, and water; in the safe waste disposal of radionuclide with proton binding; and, in the production of amylase enzyme.

#### *3.5.2.3. Escherichia coli*

*Escherichia coli* is a gram-negative rod-shaped bacterium that is found in the lower intestines of humans and animals. Most of *E. coli* strains are harmless, except for setotype O157: H7, which can cause serious food poisoning in humans. *E. coli* was first identified as a cause of illness in 1982 during an outbreak of severe bloody diarrhea traced to contaminated hamburgers. *E. coli* usually infect children, older people, and those who are ill.

*E. coli* is facultative anaerobic and nonsporulating becterium. It uses mixed-acid fermentation in anaerobic conditions and produces lactate, succinate, ethanol, acetate, and carbon dioxide. *E. coli* grows at $37^{o}$C but it can multiply at temperatures of up to $49^{o}$C and can drive by aerobic or anaerobic means.

The nonpathogenic *E. coli* strain Nessle (Mutaflor) has been in use in medicine since 1917. This strain can treat various gastroenterological diseases, including inflammatory bowel disease. *E. coli* is treated with antibiotics; however, it is resistant to may antibiotics that are effective against gram-positive organisms.

Antibiotics that can effectively treat *E. coli* infection include amoxicillins, semisynthetic penicillina, many cephalosporins, and carbapenems.

#### *3.5.2.4. Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a gram-negative, aerobic rod-shaped bacterium of the family Pseudomonadaceae. It can be found in soil, water, skin flora, coastal marine habitats as well as on the cells of plants and animals.

*P. aeruginosa* causes diseases in both animals and humans. Among the diseases which caused by *P. aeruginosa* are urinary tract infections, respiratory system infections, dermatitis, and soft tissue infections, especially in victims of severe burns and in cancer and AIDS patients.

*P. aeruginosa* is identified by its pearlescent appearance and grape-like or tortilla-like odor in vitro. It can grow at 42$^{o}$C in diesel and jet fuel where it is known as a hydrocarbon-utilizing microorganism. Although *P. aeruginosa* is an aerobic organism, it is considered a facultative anaerobe as it is well adapted to proliferate in conditions of partial or total oxygen depletion. Moreover, *P. aeruginosa* can achieve anaerobic growth with nitrate as a terminal electron acceptor and can ferment arginine by substrate-level phosphorylation.

*P. aeruginosa* is isolated from nonsterile sites, such as mouth swabs and sputum; therefore, it often represents colonization and not infection under these environments. *P. aeruginosa*, which is resistant to many antibiotics, demonstrates additional resistance after unsuccessful treatment, particularly through modification of a porin.

# CHAPTER 4
# METHODOLOGY

## 4.1. FLOWCHART

Based on the objectives of the study, the experiments were carried out as presented in **Figure 4.1.** The study used Banaba leaves and fruits, both leaves and fruits used were fresh and one week air dried. Microwave - Assisted Solvent Extraction with ethanol and ethyl acetate as solvents were employed. However, ethyl acetate was used for fresh leaves only; because no oil was collected when dried leaves and fruits were extracted using the same solvent. Banaba extract was analyzed by High Performance Liquid Choromatography (HPLC) was used to identify corosolic acid. The physico - chemical properties of the banaba crude oil were determined. In addition, Kirby-Bauer diffusion method was used to test antibacterial activity of banaba crude oil.

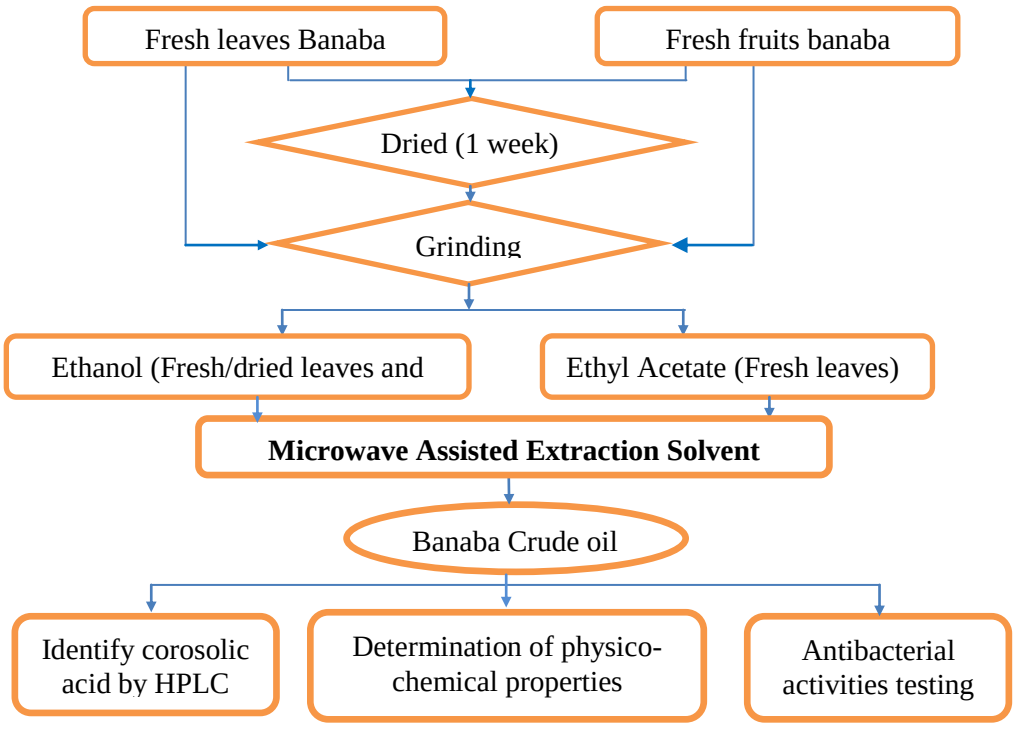



**Figure 4.1. Experimental Flowchart**

## 4.2. MATERIALS

### 4.2.1. Plant Material

This study used leaves and fruits of *Banaba* (*Lagerstroemia speciosa)* which were purchased from Filinvest compound, Alabang, Philippines. Banaba fresh leaves were oblong and about 30cm long, with green color and become brown after drying, while the fresh fruits of banaba is round or oval nutlike with diameter around 2 - 3cm, they became black or dark brown when dried.

### 4.2.2. Reagent and Chemicals

The study used analytical grade ethanol and ethyl acetate as solvents in MASE.

Hydrochloric acid, potassium hydroxide, and phenolphthalein, all of analytical grade, were used to determine the physico-chemical properties of the essential oil produced.

Mueller–Hinton agar, barium chloride dihydrate, ethanol 95%, and sodium chloride were used for the antimicrobial susceptibility test.

## 4.3. EXPERIMENTAL SETUP

In the present study, the microwave-assisted solvent extraction system used a domestic microwave oven, a reactor, a condenser and cooling system, a thermocouple, and a digital controller terminal **(Figure 4.4).**

### 4.3.1. Microwave Oven

A domestic microwave oven used made by American Company had the following specifications:

- Frequency of 2455MHz,
- Maximum Delivered Power of 1,000W using 220V with five levels, namely from 100W (10%), 300W (30%), 500W (50%), and 800W (80%) up to 1000W (100%) and,
- Cavity Dimension of 368×348×340mm.

The top cavity had a hole that connects the reactor inside to the temperature controller and the condensing system.

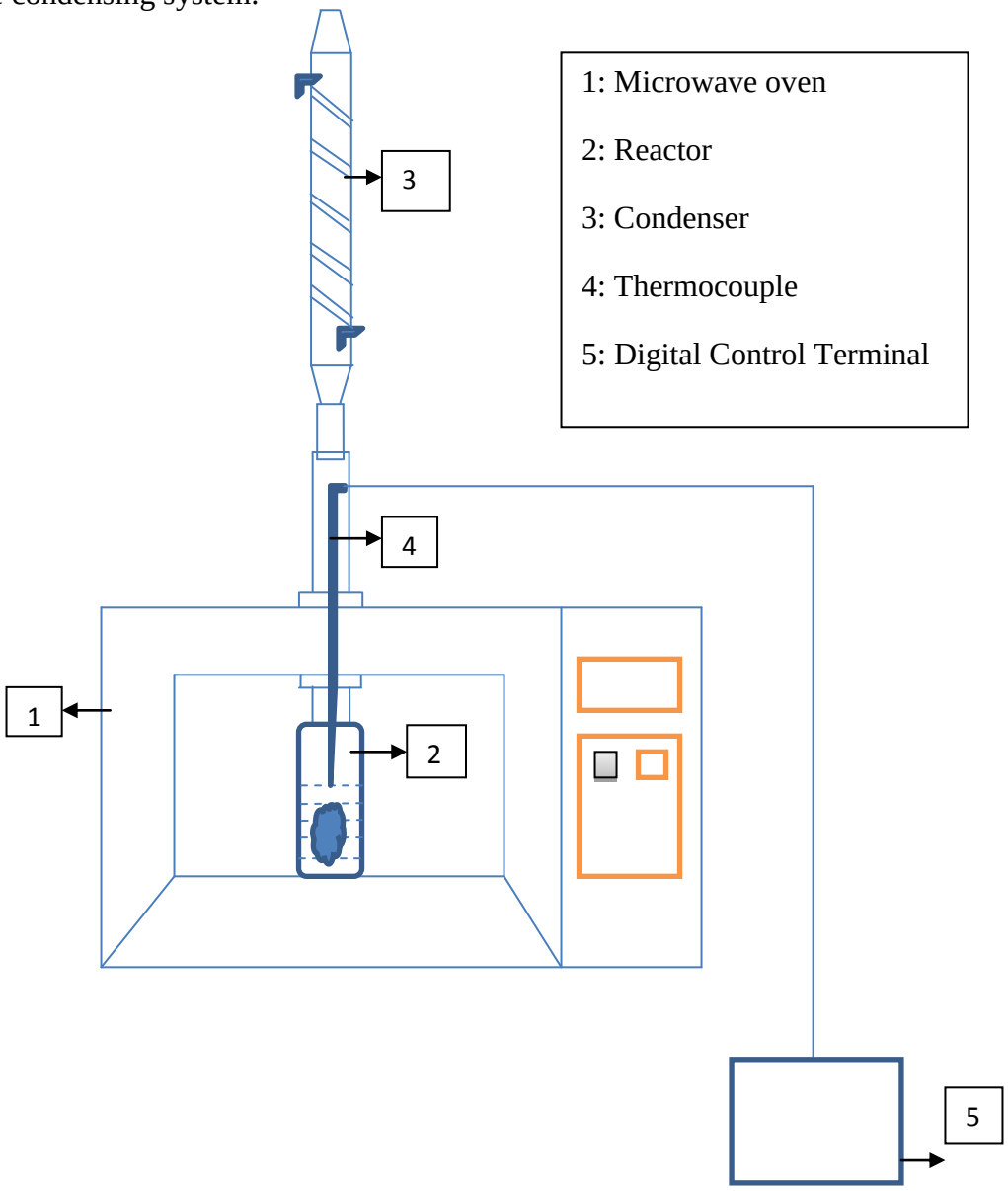



**Figure 4.2. Schematic Diagram of Experimental Set up**

### 4.3.2. Reactor

A 330ml cylinder glass reactor with a 100cm-diameter neck was used for the process.

### 4.3.3. Condenser and Cooling System

The Pyrex condenser had an outside diameter of 2.584cm and a length of 31cm. The banaba extract produced was released from the reactor inside the microwave oven, passed through the vertical condenser, and then cooled by the cooling water system.

### 4.3.4. Thermocouple

A type K thermocouple was used in the study to measure temperature inside the reactor (in solvent). The thermocouple, which was 2m long and 0.55mm in diameter, had a detecting temperature range of $-25^{o}C$ to $899^{o}C$.

### 4.3.5. Digital Control Terminal

The temperature inside the reactor was controlled by a Universal Digital Controller Model UDC 700. Temperature was kept at the desired set point and was controlled by feedback controller to the microwave power regulator.

## 4.4. EXPERIMENTAL PROCEDURE

Fresh and one week air dried banaba leaves and fruits were grinded (Supper Chopper of John Mills Limited House), and then it was placed in a 330ml extraction cylinder-reactor. Ethanol or ethyl acetate was added. The reactor was inserted inside the microwave cavity and fitted with a condenser. The sample then was irradiated with microwave (2445 MHz). The extraction conditions of temperature, time, solvent – to- material ratio, microwave powers and solvents, were described in later section.

After the microwave extraction, the solution was cooled in water for 5 minutes and it was filtered by using solvent filtration assembly with Whatman No.1. The residual banaba in reactor was washed with a premeasured amount of fresh solvent and the solvent was filtered and added to the extract. Then all of filtrates were concentrated in vacuum rotary evaporator (Heidolph VV 2000 series installed with Sibata WJ-20 Series), at $45^{o}C$ to remove solvent. The extracts were stored in vials which were kept in refrigerator.

The banaba crude oil was analyzed by HPLC (Aligent Technology 1200 series with Ascentis C18 column); to identify the presence of corosolic acid. The mobile phase was a mixture of acetonitrile in water containing 0.5% phosphoric acid (75/25 v/v) with a flow rate of 1ml/min. The peaks of corosolic acid were detected at a wavelength of 210nm. Finally, the physico-chemical and antibacterial activity of banaba crude were determined.

## 4.5. EXPERIMENTAL METHODOLOGY

### 4.5.1. Sample Preparation of Banaba Leaves and Fruits

Fresh banaba leaves and fruits were cleaned and washed with water and then air-dried at room temperatures of 25$^{o}$C to 30$^{o}$C for one week. The samples of the leaves and fruits were ground until the desired size of approximately 2mm$^{2}$ was achieved. The samples were then weighed before and after drying.

This weight was used to measure the percentage moisture content calculated using the following formula (Pordesimo, 2004):

$$\%\ \text{Moisture of banaba leaves} = \frac{\left[\begin{matrix}\text{weight of sample}\\ \text{before drying}\end{matrix}\right] - \left[\begin{matrix}\text{weight of sample}\\ \text{after drying}\end{matrix}\right]}{\left[\begin{matrix}\text{weight of sample}\\ \text{before drying}\end{matrix}\right]} \times 100\% \quad (4.1)$$

### 4.5.2. Determination of Residual Moisture of Banaba Leaves and Fruits

Approximately 10g of banaba leaves and fruits were placed in an oven at 100$^{o}$C for residual moisture determination. The samples were then weighed at 30 minute intervals for a period of 20 hours until the ratio weight/weight (%) reached less than 10%. When the constant weight was achieved the samples were stored in desiccators assuming that negligible amount of moisture in banaba after oven drying. At this point, the dried banaba leaves and fruits were considered *bone-dry* banaba leaves and fruits, whose residual moisture was calculated as the total water lost using the formula:

$$\%\text{Residual moisture} = \frac{\text{weight before oven drying-weight after oven drying}}{\text{weight before oven drying}} \times 100 \quad (4.2)$$

### 4.5.3. Microwave-Assisted Solvent Extraction (MASE)

#### 4.5.3.1. Experimental Design for MASE

Experimental design for banaba leaves and fruit extracted by MASE are shown in Table 4.1., its details are indicated in **Appendix H**.

**Table 4.1. Experimental Parameters for Banaba MAE with Solvent**

| **Variables** | | |
|---|---|---|
| Sample | Banaba Leaves | Banaba Fruits |
| Kind of Leaves | Fresh Leaves<br>1 week air-dried | Fresh Fruits<br>1 week air-dried |
| Kind of Solvent | Ethanol, Ethyl Acetate | Ethanol |
| Solvent/Solid Ratio *(ml/gr)* | 6:1, 8:1, and 10:1 | 6:1 and 8:1 |
| Power of Microwave *(% of maximum power)* | 30, 50, and 80 | 50 and 80 |
| Extraction Time*(min)* | 2 and 6 | 2 and 6 |
| Temperature of Reactor *($^oC$)* | 50, 60, and 70 | 60 and 70 |
| **Constants** | | |
| Size of Particles *(mm)* | 2 | 2 |
| Number of Replication | 2 | 2 |
| Mass of Fresh Banaba Leaves and Fruits | 30g | 30g |
| Pressure *(atm)* | 1 | 1 |

The solvents used in MASE were ethanol and ethyl acetate which had the following properties:

**Table 4.2. Properties of Ethanol and Ethyl Acetate Solvents**

| | Dielectric Constant, $\epsilon'$ | Dipole Moment, *debyte* | Loss Tangents, *tan* $\delta$ | Boiling point ($^{o}$C) |
|---|---|---|---|---|
| Ethanol | 24.3 | 1.96 | 0.941 | 78 |
| Ethyl acetate | 6.02 | 0.78 | 0.059 | 77 |

According to Huang et al. (2009), the use of microwave-assisted extraction without solvent would give higher concentrations of corosolic acid in banaba fruits extract than in banaba leaf extract. However, the source of banaba leaves is abundant and more stable than banaba fruits. Thus, given these considerations, both banaba leaves and banaba fruits were used in the present study.

Ethanol, on the one hand, was chosen because aside from being a popular solvent for MASE (Somenath Mitra, 2003), ethanol was also high loss tangent solvent. Therefore ethanol extremely absorbed microwave energy; it can be heated rapidly with microwave.

Okada (2003) used conventional method with three kinds of solvent, i.e. ethyl acetate, acetone and hot water to extract banaba leaves. The result shows that the crude oil extract with ethyl acetate contained not only corosolic acid but also contained the other active components, i.e. virgatic acid, β-sitosterol glucoside, urosolic acid which had antibacterial activity. Therefore, present study also used ethyl acetate as a solvent for MAE. However, ethyl acetate was used only for fresh leaves, because no oil was collected when dried leaves and fruits were extracted using the same solvent.

Plant particle size affects the recovery of compounds such that if the particle size is large, the efficiency of MASE will be low and if the particle size is small after extraction filtration process will be more difficult. Since the particle sizes of extracted materials generally fall within the range of 0.1–2mm (Kormin et al., 2002), banaba leaves and fruits cut to the size of $2mm^2$ were used in the experiment.

The experiment was carried out in an open-vessel MASE to keep temperatures below the boiling point of the solvents used. Thus, temperatures of 50$^{o}$C, 60$^{o}$C, and 70$^{o}$C were examined since the boiling points of the solvents were 77.1$^{o}$C for ethyl acetate and 78.3$^{o}$C for ethanol.

Longer extraction time was not necessary because it would lead to the decomposition of thermolabile analytes. Moreover, extraction time depended on the irradiation power. Therefore, for the present study, microwave power had been set to 30%, 50%, and 80% of 1,000W, which were compatible with extraction times of 2 minute and 6 minute. (Mandal et al., 2007)

#### *4.5.3.2. Determination of Extractable Oil*

The extractable oil was determined by repeated microwave extraction of banaba leaves and fruits until oil could no longer be detected. For this procedure, 30g of fresh banaba leaves and fruits had been extracted with appropriated solvent at 70$^{o}$C temperature and 500W microwave power.

After concentrating the filtrate in the rotary evaporator, the crude oil was collected, the reactor was removed from the rotary evaporator setup, and the mixture was filtered using Whatman No. 1 Filter Paper with 90mm diameter and 0.011mm pore size. The solid mixture that remained on the filter paper was returned to the reactor and was dissolved again with the same amount of solvent.

This filtration process was repeated until all traces of oil had been collected. After each step, the weight of oil was measured.

The total amount of oil extracted is the *extractable oil*.

After the extraction process, the oil (crude extract) product was weighed to determine the yield and the efficiency of extraction.

*Yield* is defined as:

$$\%Yield = \frac{\text{Mass of oil}}{\text{Mass of bone dried banaba}} \times 100 \qquad (4.3)$$

$$\text{Bone dried} = \frac{\text{Mass of banaba} \times (100 - \%\text{Residual moisture})}{100} \quad (4.4)$$

while the *Efficiency of Extraction* is defined as:

$$\%\text{Efficiency of extraction} = \frac{\text{Mass of crude oil extracted}}{\text{mass of extractable oil in raw material}} \times 100 \quad (4.5)$$

### 4.5.4. Statistical Analysis

The collected data of banaba leaves extracted by MASE were analyzed by Analysis of Variance (ANOVA) using MINITAB software. For the fresh banaba leaves, five major parameters—namely, kind of solvent, microwave power, extraction time, solvent – to- material ratio, and extraction temperature—were studied whether these factors significantly influenced the yield of banaba oil. For the banaba leaves and fruit, five major parameters – kind of material, microwave power, extraction time, solvent – to - material ratio, and extraction temperature- were used to study these factors significant effect on the yield.

### 4.5.5. Characterization of Essential Oil Properties

#### *4.5.5.1. Specific Gravity*

Using a micropipette, an accurate volume of 10$\mu$L of banaba oil was transferred to a previously weighed vial with cover and then weighed at 20$^{o}$C. The density of banaba oil was computed thus:

$$\%\ \rho_{banabaoil} = \frac{\text{the weight of } 10\mu l}{10\mu l} \quad (4.6)$$

The ratio between the density of banaba oil and the density of water at 20$^{o}$C is *specific gravity*, which is stated thus:

$$S = \frac{\rho_{\text{banaba oil}}}{\rho_{water}} \quad (4.7)$$

#### *4.5.5.2. Refractive Index (RI)*

The *refractive index* of a substance is the measure of the speed of light in that substance. It is expressed as the ratio of the speed of light in vacuum relative to that in the considered medium.

#### *4.5.5.3. Acid Value*

*Acid value* is the number that expresses in milligrams the quantity of potassium hydroxide required to neutralize approximately 1g of substance.

For the present study, approximately 1.5–2 g of the essential oil was measured into a 250ml Erlenmeyer flask to which was added 5ml of ethanol and 2ml of phenolphthalein indicator solution. The free acid in the solution was titrated with 0.1M ethanolic potassium hydroxide. Upon reaching the end point, the volume of titrant used was measured.

The acid value would then be calculated using the formula:

$$AV = 5.61\frac{V}{m} \tag{4.8}$$

where,

*V* is the volume of 0.1M potassium hydroxide required to neutralize the acid, *ml* and

*M* is the mass of the oil taken, *g*.

The content of the 250ml Erlenmeyer flask would be set aside for use in the subsequent determination of the ester value.

#### *4.5.5.4. Ester Value (EV)*

The *ester value* is the number that expresses in milligrams the quantity of potassium hydroxide required to saponify the esters present in 1g of the substance.

In determining the ester value of banaba oil, two mixtures were prepared and used: the blank solution and the neutralized solution.

The *blank solution* or *blank*, on the one hand, was prepared by boiling a mixture of 5ml of ethanol, 25ml of 0.5M ethanolic potassium hydroxide, and 0.2ml of phenolphthalein indicator solution under reflux system for 1h. After an hour, the solution was cooled and 20ml of distilled water was added to it. Then, the volume of titrant used was measured.

The *neutralized solution*, on the other hand, was the solution used earlier to determine acid value and to which was added 25ml of 0.5M ethanolic potassium hydroxide. This resulting mixture was boiled under reflux for 1h. After an hour, the solution was cooled then mixed with 20ml of distilled water and 0.5ml of phenolphthalein indicator solution. The solution was then titrated with 0.5M hydrochloric acid. And, similar to what was done for the blank solution, the volume of the titrant used was measured after the end point was reached.

The volume of the titrant obtained would be used to calculate the ester value of corosolic acid according to the following:

$$EV = \frac{28.05(B - V)}{m} \tag{4.9}$$

where, *B* is the volume of 0.5M hydrochloric acid required for the blank, *ml; V* is the volume of 0.5M hydrochloric acid required to neutralize the excess alkali after hydrolysis, *ml*; and, *M* is mass of the oil taken, *g*.

#### *4.5.5.5. pH*

The pH value was measured using Merck pH indicator paper. The pH indicator determined pH value according to the change in color of the paper which would be read against a range of 14 values that corresponded to five standard colors.

### 4.5.6. Antimicrobial Activity

#### 4.5.6.1. *Preparation of Filter Paper Disk*

Filter paper disks were used to contain the banaba crude oil in the disk diffusion assay. A puncher was used to produce these paper disks. A Petri dish with the filter paper was placed in the oven for 4–6 h. Then, the filter paper disk was impregnated with the banaba crude oil solution.

#### *4.5.6.2. Culture Medium of Bacteria*

*Nutrient agar*. which consisted of beef extract (0.3%), peptone (0.5%), and agar (1.5%) in water, was the medium used to culture bacteria prior to antimicrobial testing. The bacteria were inoculated employing the clock streak method using a sterile loop. After streaking had been finished, the inoculated plates were incubated at $37^{o}$C for 48 hours. (Crabtree et al., 1974)

#### *4.5.6.3. Kirby–Bauer Diffusion Method*

An antimicrobial activity test was performed on *Staphylococcus aureus, Bacillus subtilis, Escherichia coli,* and *Pseudomonas aeruginosa*, which were readily available in the laboratory. These microbes were chosen because they are known common causes of human diseases and infections.

Several studies (Syed, 1991; Rahman, 2002) identified disk diffusion method as a method frequently used in determining the *zone of inhibition*, which is the area around the microbial agent where there is no bacterial growth. For the present study, where the antimicrobial agent in the banaba extract was suspected to be due to total tannins and corosolic acid compounds, to determine the zone of inhibition would be to identify, too, the potency of the banaba extract.

The stock cultures of *S. aureus, B. subtilis, E. coli,* and *P. aeruginosa* were obtained from the DLSU–Manila Microbiology Laboratory. These cultures were subcultured by inoculating in Nutrient agar. In using Kirby–Bauer antimicrobial susceptibility test, Mueller–Hinton agar had been identified to be compatible with these four microorganisms and was, therefore, recommended as the culture medium.

After incubating the culture at 37.5$^{o}$C overnight a sterile cotton swab was utilized to obtain bacteria from the culture, which was transferred to a tube of Mueller–Hinton broth that had been thoroughly mixed. Furthermore, the turbidity was compared to the 0.5 McFarland standards. When the microbial suspension did not appear to have the same density as the standard, the turbidity was reduced by adding broth or was increased by adding more microbial growth.

A sterile cotton swab was dipped into suspension and the swab streaked over the entire surface of the medium three times. After each application, the plates were rotated at about 60 degrees to ensure even distribution of the inoculums.

The filter disks were prepared no longer than 15 minute after inoculation. The disks were placed individually using sterile forceps and pressed down gently onto the agar. The plates were incubated at 37.5$^{o}$C for 18–24 hours. After incubation, the plates were checked for zones of inhibition.

#### *4.5.4.1. Zone of Inhibition*

*Zone of inhibition* is the area on an agar plate where no organism grows, as indicated in **Figure 4.3**. The size of a zone of inhibition would indicate how effective a particular type of antibiotic would be against a specific type of microorganism. The larger the zone of inhibition the more effective that antibiotic is against that particular type of bacteria.

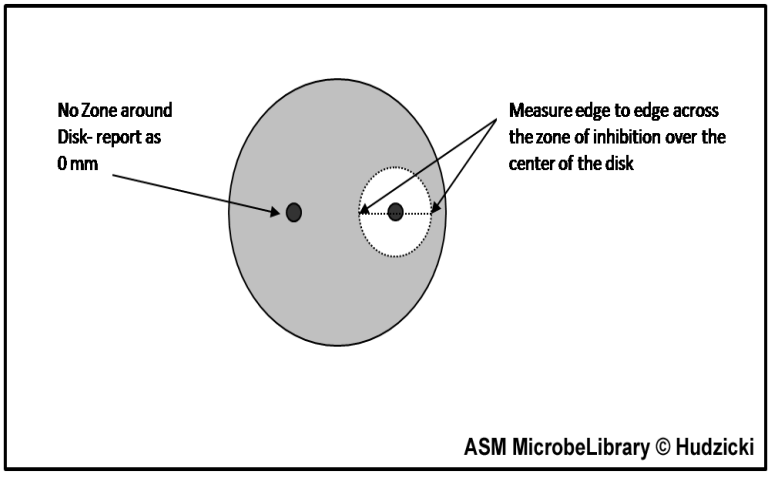



**Figure 4.3. Zone of Inhibition**

Each zone of inhibition was to be measured by a ruler or caliper, as shown in **Figure 4.4.** If the zones of inhibition adjacent to the antibiotic disks overlap, the zone diameter would be determined by measuring the radius of the zone.

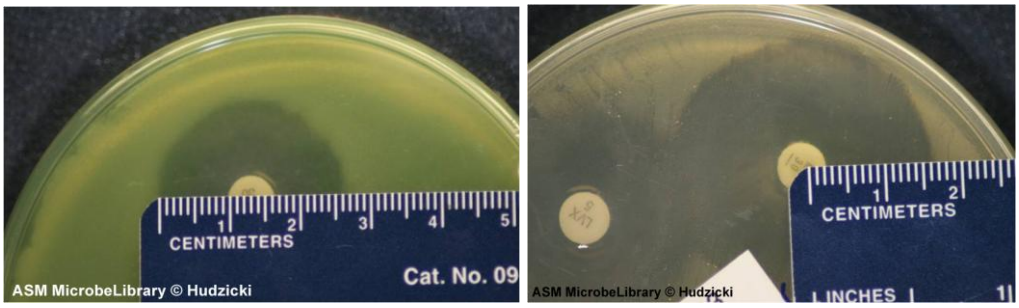



**Figure 4.4. Measuring the Zone of Inhibition**

In summary, the experimental design of the present study is presented in **Table 4.3.**

**Table 4.3. Experimental Design for Testing Antibacterial Activity of Banaba Crude Oil**

| **Variables** | | | |
|---|---|---|---|
| **Sample** | | **Concentration** *(µg/ml)* | **Microorganism** |
| MASE | Banaba Leaves (fresh; dried)<br>Banaba Fruits (fresh; dried) | 1000<br>500<br>200<br>100<br>50<br>10 | *Staphylococcus aureus*<br>*Bacillus subtilis*<br>*Escherichia coli*<br>*Pseudomonas aeruginosa* |
| Conventional Method | Hot Water<br>Ethanol | | |

# CHAPTER 5

# RESULTS AND DISCUSSIONS

## 5.1. MOISTURE OF BANABA

### 5.1.1. Moisture Content

Extraction process of essential oil was affected by the moisture content of raw material and, thereby, also greatly influenced the yield and quality of essential oil. In this study, the moisture content of banaba leaves and fruits was measured by the amount of water evaporated from the collected banaba to the storage environment.

The results presented in **Appendix E** show the average moisture at 72.89% for fruits and at 71.504% for leaves. Although the result for leaves agreed with the 71.79% moisture content obtained by Huang et al. (2009), the result for fruits was 39.5% less than the value reported by same authors. In their study, banaba fruits were naturally dried, therefore, only a little water was obtained. Furthermore, moisture content varies with the plant's type, variety, and growth conditions as well as the fruit's preservation method and storage conditions (Rajal, 2005).

Furthermore, the residual moisture content of one-week air-dried banaba leaves was 53.701 % whereas it was 63.484 % for banaba fruits.

### 5.1.2. Rate of Drying

The graph of moisture content versus time and the graph of drying rate versus moisture content of dried banaba leaves and fruits are shown in **Figure 5.1** and **Figure 5.2**, respectively. The drying curves shown in **Figure 5.1** and **Figure 5.2** contain two well-defined main zones.

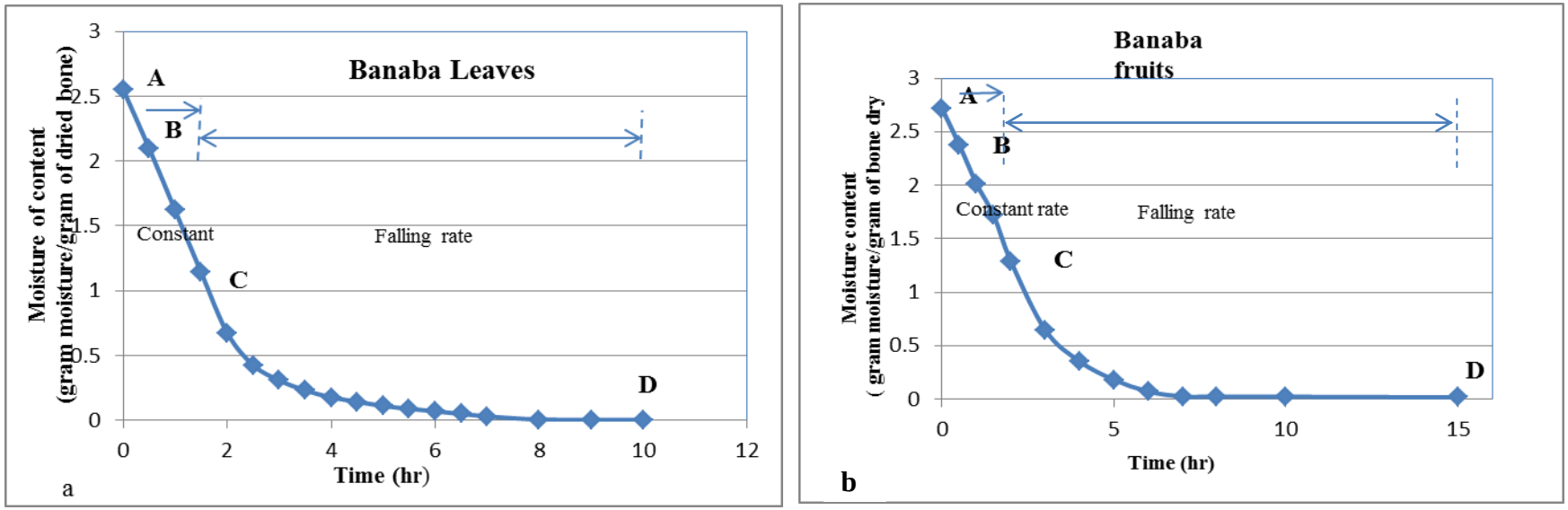



**Figure 5.1. Residual Moisture of Banaba Leaves (a) and Fruits (b)**

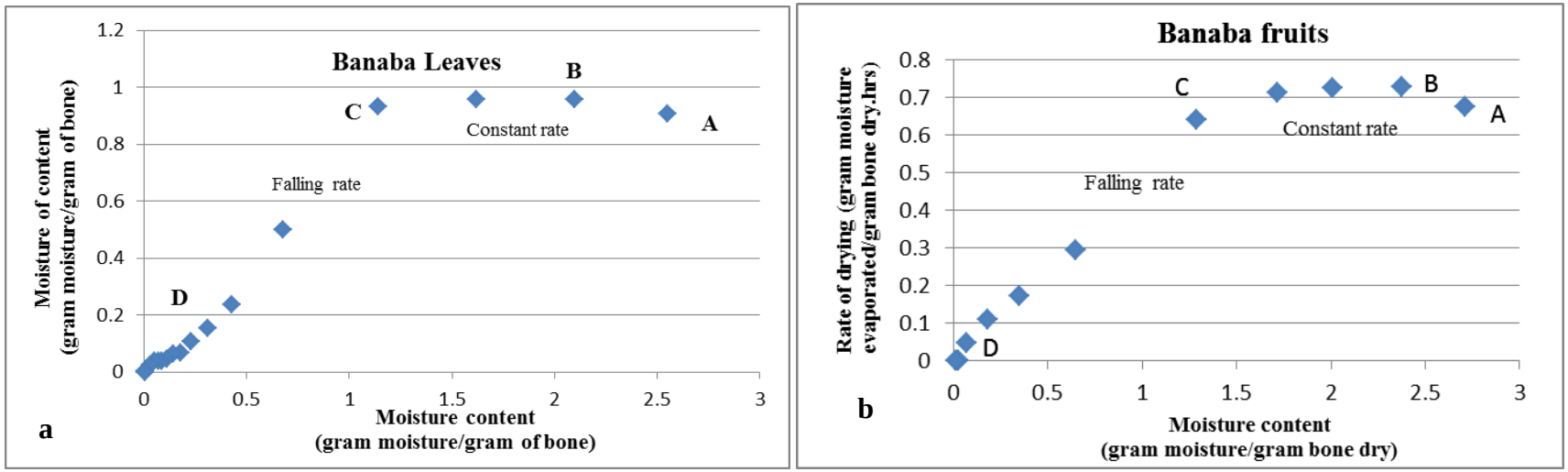



**Figure 5.2: Rate of Drying Curve of Banaba Leaves (a) and Banaba fruits (b)**

The first zone presents the constant rate period as *BC* where the rate of drying is constant while the second zone presents the falling rate period where the amount of moisture content steadily goes down. Point C, known as *critical moisture content*, gives moisture content at the end of the constant rate period a value of 1.14 (gram moisture content/gram bone dry) for banaba leaves and 1.4 (gram moisture content/gram bone dry) for banaba fruits. Moreover, point D, at the end of the curve, presents the moisture content of the dried banaba leaves and fruits in equilibrium condition with the drying condition.

In the curve, section *BC* corresponds to conditions at which the surface is no longer capable of supplying sufficient free moisture to saturate the air in contact with it. At these conditions, the mechanism by which the moisture from inside the material is transferred to the surface affects the rate of drying very much.

## 5.2. MICROWAVE-ASSISTED SOLVENT EXTRACTION (MASE)

### 5.2.1. Qualitative Analysis

**Figure 5.3** shows crude oils of banaba leaves and fruits extracted by MAE using ethanol and ethyl acetate as solvents, by solvent-free microwave extraction or SFME (Huang et al., 2009), and by conventional method using ethanol and hot water.

The extract of MAE without solvent using SFME is yellowish in color. In contrast, the crude oil extracted with ethanol as solvent is dark brown and with a mild characteristic odor. This is suspected to be due to the presence of oleoresin in the extract. In the case of dried leaves, the extract is observed to be darker than the others. This is entirely reasonable because the extract by MAE includes *oleoresin*, which consists of both volatile substance (volatile oil) and nonvolatile compounds that are characterized by their brown color and high viscosity.


**Figure 5.3**: **Extracts of Banaba Leaves and Fruits**

Fresh leaves (MAE + ethanol); B. Dried leaves (MAE + ethanol); C. Fresh fruits (MAE + ethanol); D. Dried fruits (MAE + ethanol); E. Fresh leaves (MAE + ethyl acetate); F: Fresh leaves (conventional method + ethanol); G: Fresh leaves (conventional method + hot water). $N_1$: Fresh leaves (MAE without solvent).

### 5.2.2. Yield and Best Extraction Conditions

Banaba leaves and fruits were run by MAE following the different conditions given in the experimental design. The study also extracted banaba fresh leaves by conventional method with hot water and ethanol as solvents for comparison. The run that obtained the highest yield conditions was analyzed by HPLC to identify corosolic acid.

The best combination of conditions for microwave power, extraction time, solvent-to-sample ratio, kind of solvent, and extraction temperature to obtain the highest yields are presented in **Table 5.1**.

**Table 5.1: The highest yields of banaba leaves and fruits extracted by MASE**

| Method | Material | Condition | Yield *(%)* | % Efficiency |
|---|---|---|---|---|
| MAE with ethanol as solvent | Fresh banaba leaves | 500W,70$^{o}$C, 6min, 10ml/g cylinder-bottomed | 63.950 | 74.525 |
| MAE with ethanol as solvent | Dried banaba leaves | 800W; 60$^{o}$C, 6min, 8ml/g cylinder-bottomed | 51.590 | 92.289 |
| MAE with ethyl acetate as solvent | Fresh banaba leaves | 800W, 70$^{o}$C, 6min, 6ml/g cylinder-bottomed | 7.963 | 40.625 |
| MAE with ethanol as solvent | Fresh banaba fruits | 500W, 60$^{o}$C, 6min, 8ml/g cylinder-bottomed | 79.650 | 90.800 |
| MAE with ethanol as solvent | Dried banaba fruits | 800W, 6min, 60$^{o}$C, 6ml/g, cylinder-bottomed | 47.110 | 79.530 |
| Conventional method with hot water | Fresh banaba leaves | 2h, 90$^{o}$C in shaking water bath, 10ml/g | 33.756 | 39.338 |
| Conventional method with ethanol | Fresh banaba leaves | 6 days, room temperature, 10ml/g | 26.023 | 30.326 |

Different optimal yields of banaba crude oil were obtained at different sets of conditions as shown in **Table 5.1**. Generally, the yield as well as the extraction efficiency of MAE with solvent is attributed to its heating effect, which is caused by the dipole rotation of the solvent in the microwave field. This causes the solvent temperature to rise, which then increases the solubility of the compound of interest. Specifically, solvent heating by the microwave occurs, when molecules of the polar solvent could not align themselves quickly enough to the high frequency electric field of microwave. (Kappe et al., 2009)

Both fresh leaves and fresh fruits gave the yields higher than those of dried leaves and dried fruits. This was due to the presence of moisture in the sample. After microwave irradiation, the matrix moisture improved the extraction recovery in most cases. As proven by Lopez-Avilla et al., (1994) the extraction of polar pollutants is

improved in the presence of moisture. This was confirmed by Nguyen (2008) with a study that showed fresh garlic with 74.38% moisture content had higher product recovery than dried garlic.

In the present study, fresh banaba leaves were extracted with ethanol and ethyl acetate as solvents. Ethanol gave more yield than ethyl acetate. This had been due to the nature of the solvents, where ethanol possessed a higher loss tangent (tan $\delta$ = 0.935) than that of ethyl acetate.

Furthermore, MAE was compared with the conventional method, using hot water and ethanol as solvents to extract banaba leaves. Basing on the data in **Table 5.1**, yield extracts by conventional method were much lower compared to those by MASE despite having a longer extraction time.

The yield of present study was compared to that of Huang et al. (2009) for MAE without solvent and De Jesus et al. (2011) for MASE with ethanol as solvent **Table 5.2** shows their results.

**Table 5.2: The yield of banaba extraction**

| **Method** | **Material** | **Condition** | **Yield** *(%)* |
|---|---|---|---|
| MAE without solvent [(1)] | Fresh banaba leaves | 600W, 60min, 170$^{o}$C | 47.610 |
| MAE without solvent [(1)] | Dried banaba leaves | 600W, 60min, 170$^{o}$C | 39.330 |
| MAE without solvent[(1)] | Dried banaba fruits | 400W, 30min, 85$^{o}$C | 29.170 |
| MAE with ethanol as solvent [(2)] | Dried banaba leaves | 800W, 6min, 8ml/g round-bottomed | 64.690 |

[(1)] Huang et al. (2009) [(2)] De Jesus et al. (2011)

In the case of oil extract without solvent the yield was smaller with longer time than that of an extract with solvent that contained both oil and resin.

The highest yield (79.65%) was for fresh banaba fruits extracted by MAE with ethanol as a solvent at 500W of microwave power, 60$^{o}$C of temperature, 6 minute of time extraction, and 8 (ml ethanol/g of fresh fruit). This had been due to the moisture

of contain in babana fruits (72.29 %) higher than of the others. The presence of water in the cell leads to higher heating rates for the whole mixture, therefore the cells was broken easily and releasing more the oil.

### 5.2.3. Extractable Oil and Microwave Extraction Efficiency

*Efficiency of extraction* is calculated by the amount of extractable oil, which was determined from MASE with ethanol as solvent at best conditions for both fresh and dried leaves and fruits of banaba.

The total amount of crude oil, which was obtained by repeated extraction of banaba leaves and fruits by MASE with ethanol until oil could no longer be detected, is considered *extractable oil*. The detailed calculation for extractable oil is shown in **Appendix G**.

The yields of extractable oil were 6.1129g and 5.8184g corresponding to 85.81% of fresh leaves and 87.72% of fresh fruits. The corresponding values were much lower for dried leaves 4.9641g (55.90%) and for dried fruits 3.929g (59.2341%). The big difference had been due to the moisture content in fresh leaves (72.168%) and dried leaves (53.70131%) since the amount of water in raw material greatly affected the efficiency of MASE.

During heating water inside the raw material create internal pressure which is higher than the external pressure; thus, the cells of leaves break easily and released oil. In addition, water has a high dielectric constant and, under the influence of microwave, temperature inside the leaves rise quickly.

Furthermore, the extraction efficiency at a best condition was calculated by the ratio between the yields in grams to the total extractable oil. The MAE extraction efficiency with ethanol as solvent gave the highest efficiency, specifically for the efficiencies of MAE of dried banaba leaves and fresh banaba fruits which were over 90%. This is the advantage of using the MASE method.

The plot of the oil content follows the extraction time of leaves and fruits as shown in **Figure 5.4.**

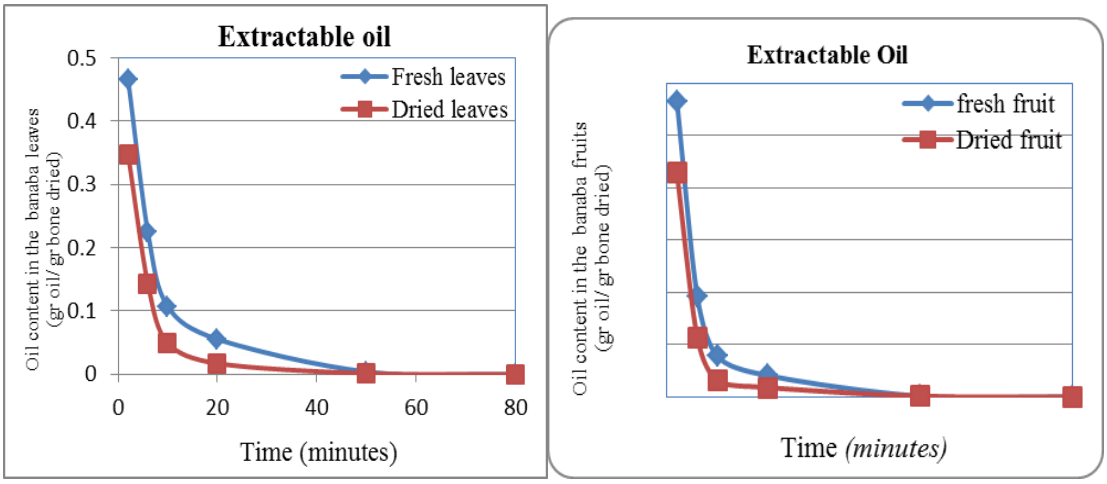



**Figure 5.4. Extractable Oil of Fresh and Dried Banaba Leaves**

Similar to moisture content, oil content also reduce with increasing extraction time. During the first 20 minutes, crude oil decreased with extraction time; but, after that, very little oil was obtained until the time reached 50 minutes and no more oil could be extracted.

## 5.3. STATISTICAL ANALYSIS

### 5.3.1. Statistical analysis of Parameters for Fresh Banaba leaves extracted by MASE with ethanol and ethyl acetate

Data of experiment of Fresh Banaba leaves used the ANOVA Five Factors with replication at the level of significance (alpha) of 0.05 that was displayed in this study. Results shown in **Appendix I** present the five factors individually as significantly affecting the yields of banaba crude oil extracted by MAE with solvent.

Likewise, **Figure 5.5** shows the variations on the main factor parameters—that is, temperature, power, time, material-to-solvent ratio, and kind of solvent—that generated p-values of less than 0.05 (alpha) for the influence of solvent (A), ratio (E),

interaction of solvent and time (AB), interaction of time and ratio, and interaction of solvent, time, and ratio (ACE).

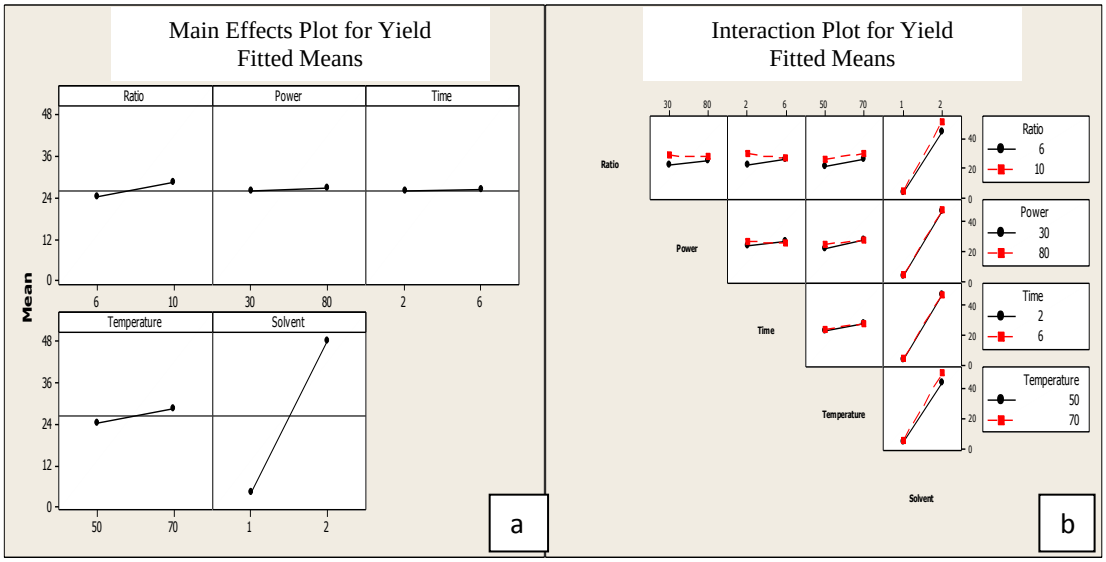



**Figure 5.5. Main Effect Plot and Interaction Plots for Yield**

The results illustrated that, the kind of solvent used played an important role in the MASE. High yield in banaba oil was obtained when ethanol was used. It was also evident in the interaction of solvent and temperature (AB), and the interaction of solvent, ratio and time (ACE), (**Figure 5.5b**). There was no significant increase in the yield when ethyl acetate was used. However, with ethanol as a solvent higher yield was obtained.

Solvent-to material ratio also is another important parameter to consider in the fresh banaba extraction MASE, the yield of crude oil was increased when solvent – to material increase in range 6-10ml/g. Similar to ratio, temperature had positive effect on the yield in the range of 50$^{o}$C-70$^{o}$C.

On the other hand, power and time were not that significant in the experiment range. However, the interaction of temperature and solvent had affected the yield in a way that was similar to the effect on yield of the interaction of time, solvent, and ratio as may be seen from **Figure 5.5**

### 5.3.2. Statistical analysis for Banaba leaves

An analysis of data of Banaba leaves using ANOVA-five factor; i.e. fresh banaba leaves and dried banaba leaves; solvent – to material ratio; microwave power; time extraction and temperature; with also at the levels of significance (alpha) of 0.05 was performed as shown in **Appendix I**

**Figure 5.6** indicates the main effect plot and interaction of parameters on the yield. Three significant factors was kind of material, ratio and time; whereas interaction of kind of material and ratio, interaction of ratio and microwave power, and interaction of microwave power and temperature affected on the yield of banaba leaves extracted by MASE.

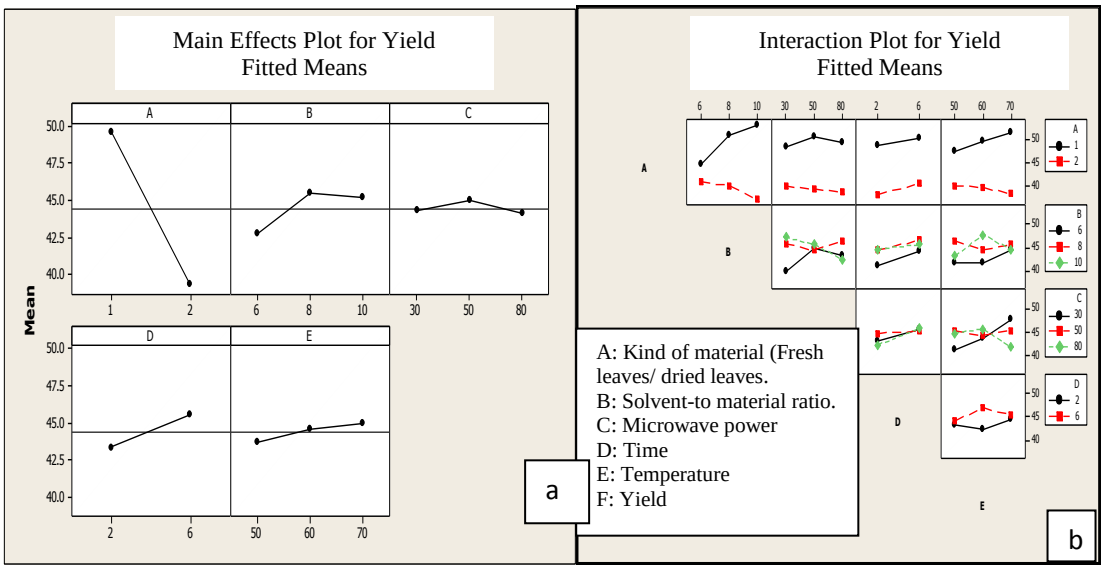



**Figure 5.6: Main Effects Plot and Interaction Plot on Yield of banaba leaves**

The kind of banaba leaves extremely affected on the yield, fresh leaves gave the higher yield than dried leaves. In the range of 6 - 10 ml/g, the yield was influenced by solvent-to material, however, the yield increased from 6 - 8 ml/g quickly, whereas no difference in range of 8 - 10 ml/g. In addition, time was important factor in using MASE to extract banaba leaves, the yield at 6 minute higher than at 2 minute.

### 5.3.3. Statistical analysis for Banaba fruit

ANOVA-five factor; i.e. kind of material fresh banaba fruits and dried banaba fruits; solvent – to material ratio; microwave power; time extraction and temperature; was used to analysis the data of banaba fruits. The level of at the levels of significance (alpha) was 0.05. The result was shown in **Appendix I** and the **Figure 5.7.**

**Appendix I.3** shown almost the main factors and their interactions had effect on the yield of banaba fruit crude oil, expect influence of solvent-to material ratio, influence of some interactions such as interaction of status of material and ratio, interaction of power and ratio or interaction of time and ratio.

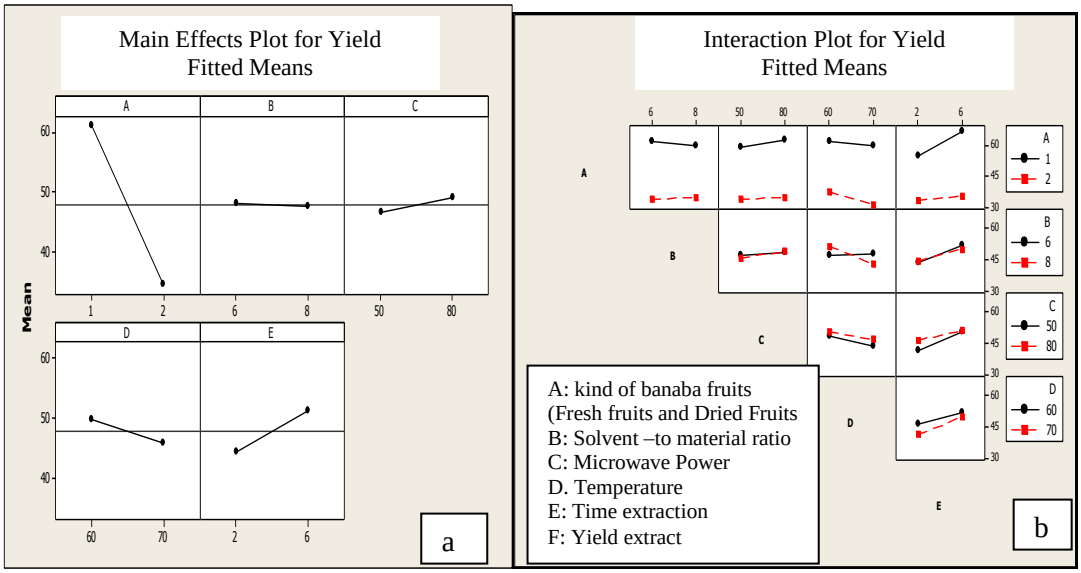



**Figure 5.7: Main Effects Plot and Interaction Plot on Yield of banaba Fruits**

Similar to banaba leaves, fresh banaba fruit also gave higher yield than dried leaves. In MASE, higher temperature, higher yield was given, however in case of banaba fruits, when increasing the time from 60$^{o}$C-70$^{o}$C, the yield of crude was slightly decrease. Contrast to in case of time and power extraction the yield increase in range of 2-6 minute, and of 500 - 800W of microwave oven.

Ratio has no significant effect on the yield; however its interaction with temperature, its interaction with status of material and power, or with temperature and time affected the yield. The result was shown in **Figure 5.7b.**

## 5.4. EFFECTS OF PARAMETERS ON YIELD

The present study investigated the influence of the nature of solvent, extraction time, power extraction, and solvent-to-solid ratio on the yield of oil extract from the fresh and dried leaves and fruits of banaba.

In the case of banaba fruits, the data where only ethanol was used as a solvent for MAE extraction at 2 minute and 6 minute, 500W and 800W power, 6ml/g and 8ml/g, and 60$^{o}$C and 70$^{o}$C are presented in **Appendix J**

### 5.4.1. Influence of Solvents on the Extraction Efficiency of MASE

Two solvents, ethanol and ethyl acetate, were selected to test the influence of solvents on the extraction efficiency of banaba leaves.

The results for fresh banaba leaves are presented in **Appendix H.**

Ethanol is both a good absorber to microwave energy, and a good solvent to dissolve leaf components, such as tannins; whereas, ethyl acetate is neither a good absorber to microwave energy nor a good solvent to dissolve components of banaba oil like tannins.

The effects of ethanol and ethyl acetate as solvents on fresh banaba leaves, which were extracted by MAE at the same conditions was illustrated in **Appendix J.**

The results proved the important role played by solvents in the MAE process. The yields of banaba leaf oil, which were extracted by ethanol as solvent, were higher than that by ethyl acetate in almost all of the conditions studied. This may be due to the difference in dielectric constants or tan $\delta$ of ethanol and ethyl acetate.

According to the studies of Pare, Lapointe, and Segouin (1991) and Hao et al. (2002), the polarity of the solvent had a significant effect on microwave extraction, because a solvent with higher polarity more easily absorbed microwave irradiation. Thus, rapidly increasing the heating would cause cell membranes to rupture and,

therefore, to release higher yields of essential oils of banaba. These results were similar to those when MAE was used to extract garlic (Nguyen, 2009).

Furthermore, ethyl acetate is a microwave transparent medium (Pare, Lapointe, & Segouin, 1991), thereby its ability to absorb microwave energy is less, therefore it is material i.e. the banaba leaves may have absorbed microwave energy. Moreover, the presence of water in banaba leaves may have contributed too in increasing the heat because water is a high polarity solvent with a dielectric constant of $\epsilon' = 80.4$ and $\tan \delta = 0.123$.

### 5.4.2. Influence of Nature of Material (Dried and Fresh Banaba Leaves and Fruits) on Extraction Efficiency of MASE

The kind of the plant matrix was significant factor in MASE (section **5.3**) can have a profound effect on the recovery of compounds. The results of the present study, which carried out MASE on both fresh and one-week dried leaves and fruits of the banaba, are presented on **Appendix J**

In almost all of the conditions of extraction, using fresh banaba gave higher yields than using dried banaba leaves and fruits. This had been due to the nature of the raw materials, which had been illustrated similarly in the studies of Lay–Keow (2003) with cigar tobacco and of Nguyen (2008) with garlic.

This effect on microwave extraction occurred in the presence in water in the cells, which had a high dielectric constant and loss tangent ($\tan \delta = 0.123$). Therefore, the water in these cells had been selectively heated and rapidly distilled off from the mixture, releasing the cells' essential oils and giving higher yields. As the moisture level increased, the number of water-containing plant cells increased and more cells ruptured.

However, at 6 ml/g, 800W power, $70^{o}$C, and 6 minute the yield for dried leaves of 47.51882% was higher than for fresh leaves of 44.3914%.

Actually, this difference had not been that significant, it is due to the loss of some oil during the course of the experiment. This result may also be explained by the

use of similarly strong conditions i.e. high power for dried leaves (800W) caused the cells in it to rupture as much as those in fresh leaves.

### 5.4.3. Influence of Microwave Power on Extraction Efficiency of MASE

Different microwave power levels were used; namely: at 300W, 500W, and 800W for leaves and at 500W, 800W for fresh fruits to investigate their influence on the yield (**Appendix G).**

In general, the extraction efficiency was improved by raising microwave power.

**Figure 5.6 and 5.7,** main plot effect on the yield of banaba leaves fruits; show that the yield extraction was greatly influenced by both the microwave power and time extraction. However in range of 500W to 800W, no change of yield occurred when increasing the microwave power (**Figure 5.6**). Similar to the result was report by Song (2011), when microwave power was lower than 350W, the extraction increased with the increase in microwave; however, extraction decreased with any further increase of power.

At higher power level setting the extraction pattern was same whereas purity reduced substantially. Rapid rupture of cell wall takes place at higher temperature when kept at higher power, as a result together with the desire analytes impurities are also leached out into solvent. However, at lower power levels the cell wall rupture might take place gradually this enables selective MAE.

### 5.4.4. Influence of Time extraction on Extraction Efficiency of MASE

The present study examined experiment at 2 minute and 6 minute. The result was shown in **Appendix G.**

As shown in the **Figure 5.6** and **Figure 5.7**, time extraction is significant factors in case both of banaba leaves and fruit extracted by MAE with ethanol or ethyl acetate as solvent. When increasing the time extraction, the yield of extract increased. The result was similar to Ginger extraction (Reniek, 2007); Garlic extraction by MAE

(Nguyen, 2008); or the study of Pan et al. (2002). Therefore, almost all the yields at 6 minute were higher than those at 2 minute.

However, some conditions gave the yield of oil at 2 minute higher than 6 minute, almost corresponding with high microwave power of 500W and 800W. The results were similar to the study of Zhou et el., (2007) which showed the yield of oil increase from 3min up to 6min, above which no increase had been observed.

On the one hand, in Kong et al. (2009), the application of MAE on cajaninstilbene acid (CSA) and pinostrobin from pigeon pea leaves showed that extraction yield was not significantly higher when extraction time was increased to 30 minute. On the other hand, in Song (2011), the recovery of total phenolics delivered from *Ipomoea batatas* leaves increased with increased MAE time within 90 second and decreased when it exceeded 90 seconds.

### 5.4.5. Influence of Solvent –to material ratio on Extraction Efficiency of MASE

The solvent volume must ensure that the material remains immersed throughout the extraction process. Therefor; solvent-to-material ratios (6:1 ml /g; 8:1 ml/g; and 10:1 ml/g) on extraction yield are shown in **Appendix J**.

These results of statistical analysis indicated that when the ratio was no significant on the yield extraction of banaba fruits, whereas in case of banaba leaves, the yield increased in range of 6-8ml/g, no difference in range of 8 - 10ml/g, i.e. highest yield was 10:1 (ml of solvent/g of dried leaves) at 70$^{o}$C temperature and 500W power.

In general, a higher ratio of solvent volume to solid matrix may be effective in conventional extraction method. However, in MAE a higher ratio may yield lower recoveries, which may be due to inadequate stirring of the solvent by microwave. For instance, the recovery of total phenolics also had no significant difference between the recoveries at 25–30 ml/g (Song, 2011). On the other hand, too much of extracting solvent will mean more energy and time to condense the extraction solution in the later step and purification process. The heating efficiency of the solvent under

microwave should also be given due consideration as the evaporation of the solvent will depend how rapidly it heats up under microwave.

### 5.4.6. Influence of Temperature on Extraction Efficiency of MASE.

Because the microwave extraction system of study is open vessel, the temperature must be low than boiling point. 50, 60, and 70$^{o}$C were the temperature level of banaba leaves extraction and 60 and 70$^{o}$C of banaba fruits extraction.

Base on the result of ANOVA analysis, temperature was also significance factor effect on the yield. Generally, when elevating temperature, enhancing the extraction. However, the yield of banaba leaves increased in range of 50 - 60$^{o}$C, slightly decreasing in range of 60 - 70$^{o}$C, similar to banaba fruits in range of 60 - 70$^{o}$C. It proved that the extraction yields of CSA significantly increased when the temperature was raised from 35$^{o}$C to 55$^{o}$C but had no significant influence with the increase in temperature from 55$^{o}$C to 75$^{o}$C. The reason was that higher temperatures led to reducing intermolecular interactions with the solvent and caused both higher molecular motion and increased solubility.

This elevated temperature does indeed result in improved the yield since when desorption of analyte from active sites in the matrix will increase. In addition, solvents have higher capacity to tension and solvent viscosity decrease with temperature, which will improve sample wetting and matrix penetration respectively. (Mandal, 2007)

## 5.5. PHYSICO-CHEMICAL PROPERTIES OF BANABA CRUDE OIL

Essential oils are characterized by their physico-chemical properties and sensory properties, which determine the intended uses and purities of essential oils. The present study identified some important physico-chemical properties of banaba oil extract, such as its pH, specific gravity, refractive index, acid value, and ester values.

### 5.5.1. pH

**Table 5.3** shows the pH values of banaba crude oil from leaves and fruits extracted by MAE with solvents (ethyl acetate and ethanol) and by conventional method (hot water and ethanol). The pH values were determined after removing solvent by evaporation.

**Table 5.3. pH of Banaba Crude Oil**

| Name | pH |
|---|---|
| MAE with solvent (ethyl acetate, fresh banaba leaves) | 4.0 |
| MAE with solvent (ethanol, fresh banaba leaves) | 4.0 |
| MAE with solvent (ethanol, dried banaba leaves) | 4.0 |
| Conventional method (hot water, fresh leaves) | 4.5 |
| Conventional method (ethanol, fresh leaves) | 4.0 |
| MAE with solvent (ethanol, fresh fruits) | 4.0 |
| MAE with solvent (ethanol, dried fruits) | 4.5 |

The pH value of the oil varies due to the presence of different components in the crude oil, especially, the percentage of the acid and the alcohol in the extracts. In **Table 5.3**, pH of banaba extract is slightly acidic.

### 5.5.2. Specific Gravity

Specific gravity is an important property that helps to identify the quality and purity of an essential oil. In addition, it is a property that is reported most frequently in the literature. Generally, the specific gravity of essential oils is less than one.

**Table 5.4** gives the specific gravity of banaba crude oil by the extraction method used. A specific gravity of less than one was achieved, although specific gravities of 1 were achieved by MAE with solvent for dried leaves as well as dried fruits—and by conventional method of hot water on fresh leaves—because the extracts contained oleoresin, which consisted of both resin and essential oil.

**Table 5.4: Specific Gravity of Banaba Crude Oil**

| Name | Specific Gravity |
|---|---|
| MAE with solvent (ethyl acetate, fresh banaba leaves) | 0.950755 |
| MAE with solvent (ethanol, fresh banaba leaves) | 0.927321 |
| MAE with solvent (ethanol, dried banaba leaves) | 1.081316 |
| Conventional method (hot water, fresh leaves) | 1.004319 |
| Conventional method (ethanol, fresh leaves) | 0.977537 |
| MAE with solvent (ethanol, fresh fruits) | 0.927321 |
| MAE with solvent (ethanol, dried fruits) | 1.011014 |

### 5.5.3. Refractive Index (RI)

Refractive index presents the purity of crude oil. The RI values of banaba crude oil from MAE with and without solvent were determined by using an Abbe Refractometer. Similar to specific gravity, there has been no standard yet for banaba extract; however, an RI of 1.300–1.7000 for essential oils will be practical.

**Table 5.5. Refractive Index of Banaba Crude Oil**

| Name | Refractive index |
|---|---|
| MAE with solvent (ethyl acetate, fresh banaba leaves) | 1.3512 |
| MAE with solvent (ethanol, fresh banaba leaves) | 1.3570 |
| MAE with solvent (ethanol, dried banaba leaves) | 1.3579 |
| Conventional method (hot water, fresh leaves) | 1.3491 |
| Conventional method (ethanol, fresh leaves) | 1.3545 |
| MAE with solvent (ethanol, fresh fruits) | 1.3557 |
| MAE with solvent (ethanol, dried fruits) | 1.3414 |

Refractive index of banaba crude oil was shown in **Table 5.5**. The range of refractive index was 1.341-1.36 which satisfied the requirement of the standard of essential oil.

### 5.5.4. Acid Value

Essential oils are often referred to as neutralizers; therefore, they have low acidity. It is the acid value that determines if there an abundance of the hydrolytic of the free fatty acids in the oil has occurred (Murano, 2003). Thus, the oil is considered better if it possessed low acid value.

The acid values of banaba crude oils, which were extracted by MAE with ethanol and ethyl acetate as solvents and by conventional method, are presented in **Table 5.6.**

**Table 5.6: Acid Value of Banaba Crude Oil**

| Name | Acid Value |
|---|---|
| MAE with solvent (ethyl acetate, fresh banaba leaves) | 2.145 |
| MAE with solvent (ethanol, fresh banaba leaves) | 2.344 |
| MAE with solvent (ethanol, dried banaba leaves) | 2.6136 |
| Conventional method (hot water, fresh leaves) | 2.8513 |
| Conventional method (ethanol, fresh leaves) | 3.535 |
| MAE with solvent (ethanol, fresh fruits) | 3.2436 |
| MAE with solvent (ethanol, dried fruits) | 2.2117 |

Banaba crude oils extracted by conventional method had acid values higher than those by microwave. However, the acid value of banaba crude oil extracted with solvent using ethanol and ethyl acetate as solvents were both small. For the crude oils of banaba fruits, those from fresh fruits (3.2436) presented higher acid values than those from fresh fruits (2.2117). However, because a standard acid value for banaba extract has not been issued yet, data cannot be compared.

Nevertheless, the acid values of banaba extracts by MAE with solvent were less than the acid values of banaba extracted by MAE without solvent at 44.384 of leaves and 30.644 of fruits (Huang et al., 2009) but were comparable the acid value of garlic extract at 13.778 (Nguyen, 2009).

Banaba crude oil extract both by MAE without solvent and by conventional method obtained high acid values due perhaps to the presence of more free acids in these oils as well as the long extraction time or could even be due to the hydrolysis of esters by the catalytic action of light (Bandares et al., 1987).

### 5.5.5. Ester Value

Ester value, which is also directly involved in the quality of essential oils, is frequently determined by saponification with a measure quantity of standard base. The ester value is important for it gives the average molecular weight of the fatty acids in a fat. Likewise, ester value affects the physical firmness of essential oils; that is, the higher the ester value the more firm is the oil.

**Table 5.7: Ester Value of Banaba Crude Oil**

| Name | Ester Value |
|---|---|
| MAE with solvent (ethyl acetate, fresh banaba leaves) | 12.46297 |
| MAE with solvent (ethanol, fresh banaba leaves) | 13.9206 |
| MAE with solvent (ethanol, dried banaba leaves) | 14.72625 |
| Conventional method (hot water, fresh leaves) | 12.59291 |
| Conventional method (ethanol, fresh leaves) | 12.46297 |
| MAE with solvent (ethanol, fresh fruits) | 96.5766 |
| MAE with solvent (ethanol, dried fruits) | 117.6717 |

**Table 5.7** shows the ester values of banaba extract by MAE and by conventional method. The ester value of banaba leaf extract obtained by MAE at 13.9206 is a little bit higher than the ester value obtained by conventional method. Noteworthy, especially, are the extracts of banaba fruits that showed high ester values at 96.5766 for fresh fruits and at 117.6717 for dried fruits.

## 5.6. HPLC ANALYSIS AND COROSOLIC ACID

*Corosolic acid* is the important component in banaba crude oil that is responsible for the plant's medicinal properties, especially of banaba's antidiabetic activity. With the aid of High- Pressure Liquid Chromatography quantification technique, the corosolic acid content in banaba extract was identified.

The chromatogram of standard corosolic acid at 89.9% purity from ChromaDex gives a distinctive peak at 8.051min as shown on **Figure 5.8**. This is best seen against **Figure 5.10** with the chromatogram of purified banaba extracts and note how the peak corresponding to corosolic acid separates well from the adjacent peaks. This condition for HPLC can, therefore, be used for quantification.

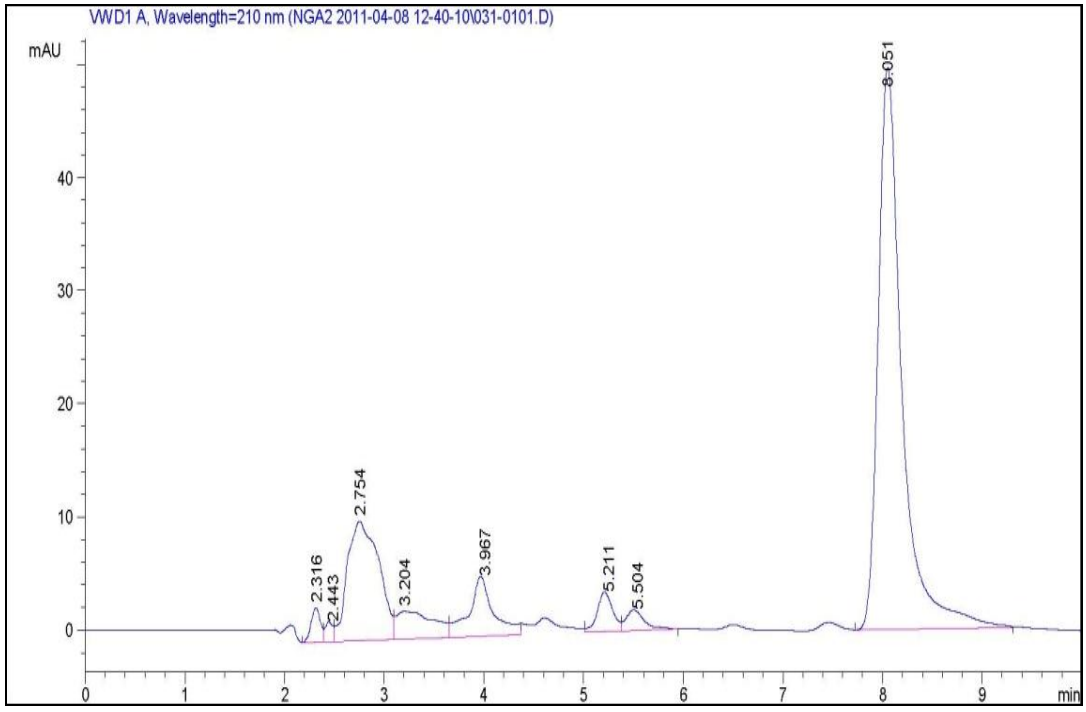



**Figure 5.8. HPLC of Standard Corosolic Acid**

**Figure 5.9** shows the calibration curve of the peak area versus concentration with commercial corosolic acid as standard at concentrations ranging from 3.125 ppm to 1,200 ppm. The linear regression shows an $R^2$ of 0.9936 and a peak area–concentration relationship of $y = 10.117x + 115.48$.

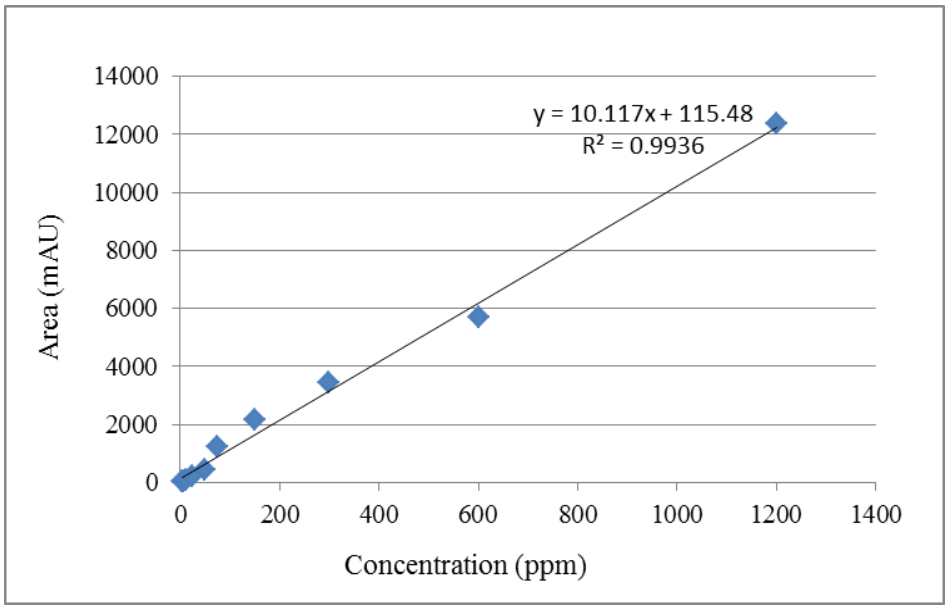



**Figure 5.9. Calibration Curve for HPLC Quantification with Commercial Standard Corosolic Acid**

The corosolic acid content of banaba leaf crude oil extracted by MAE with ethanol and ethyl acetate as solvents was identified by HPLC analysis and the results shown in **Figure 5.10** and the results in **Figure 5.11** for banaba crude oil extracted by conventional method. Meanwhile, the chromatogram of banaba fruit extract by MAE appears in **Figure 5.12**

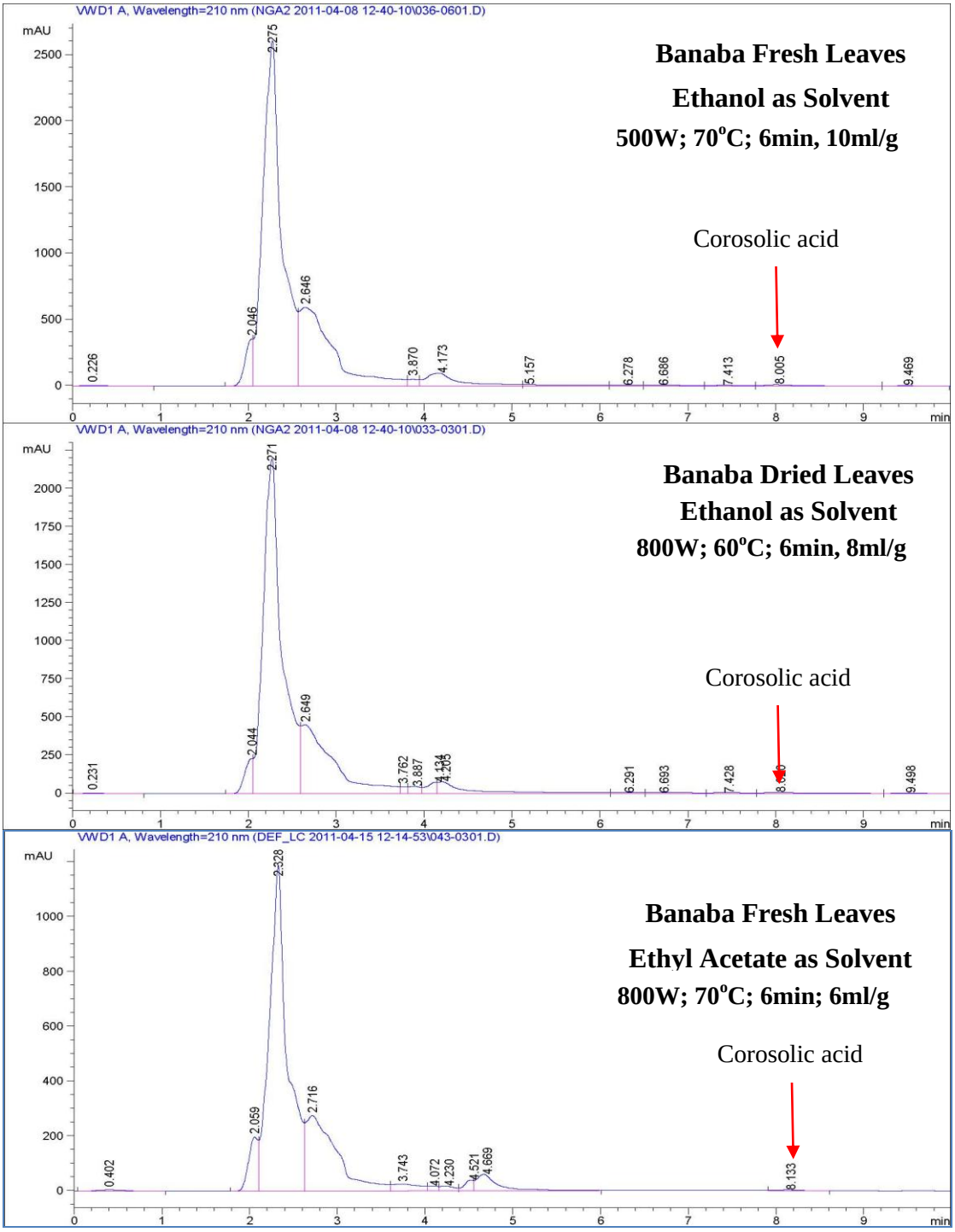



**Figure 5.10. HPLC of Banaba Extract by MAE**

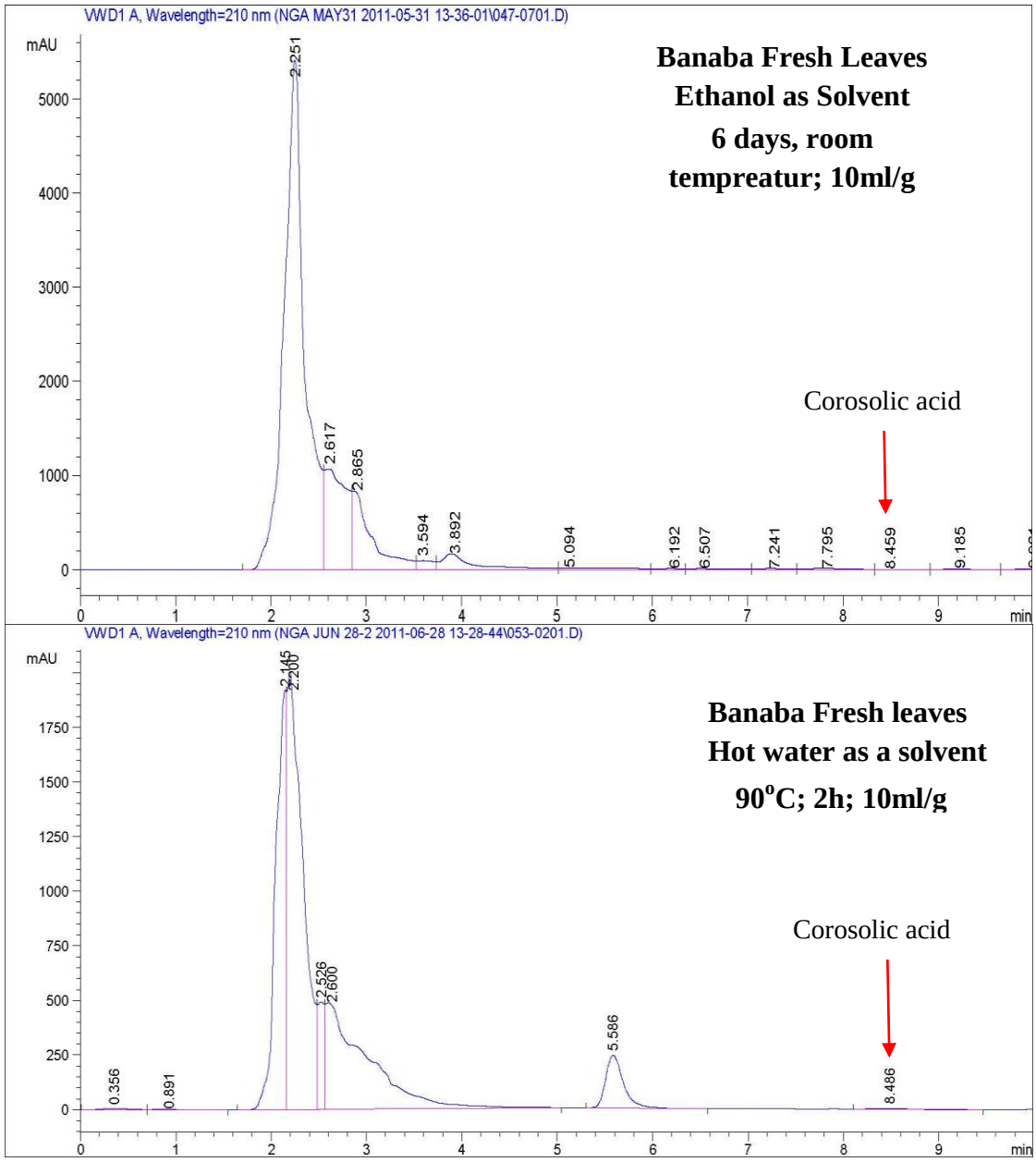



**Figure 5.11. HPLC of Banaba Extracted by Conventional Method**

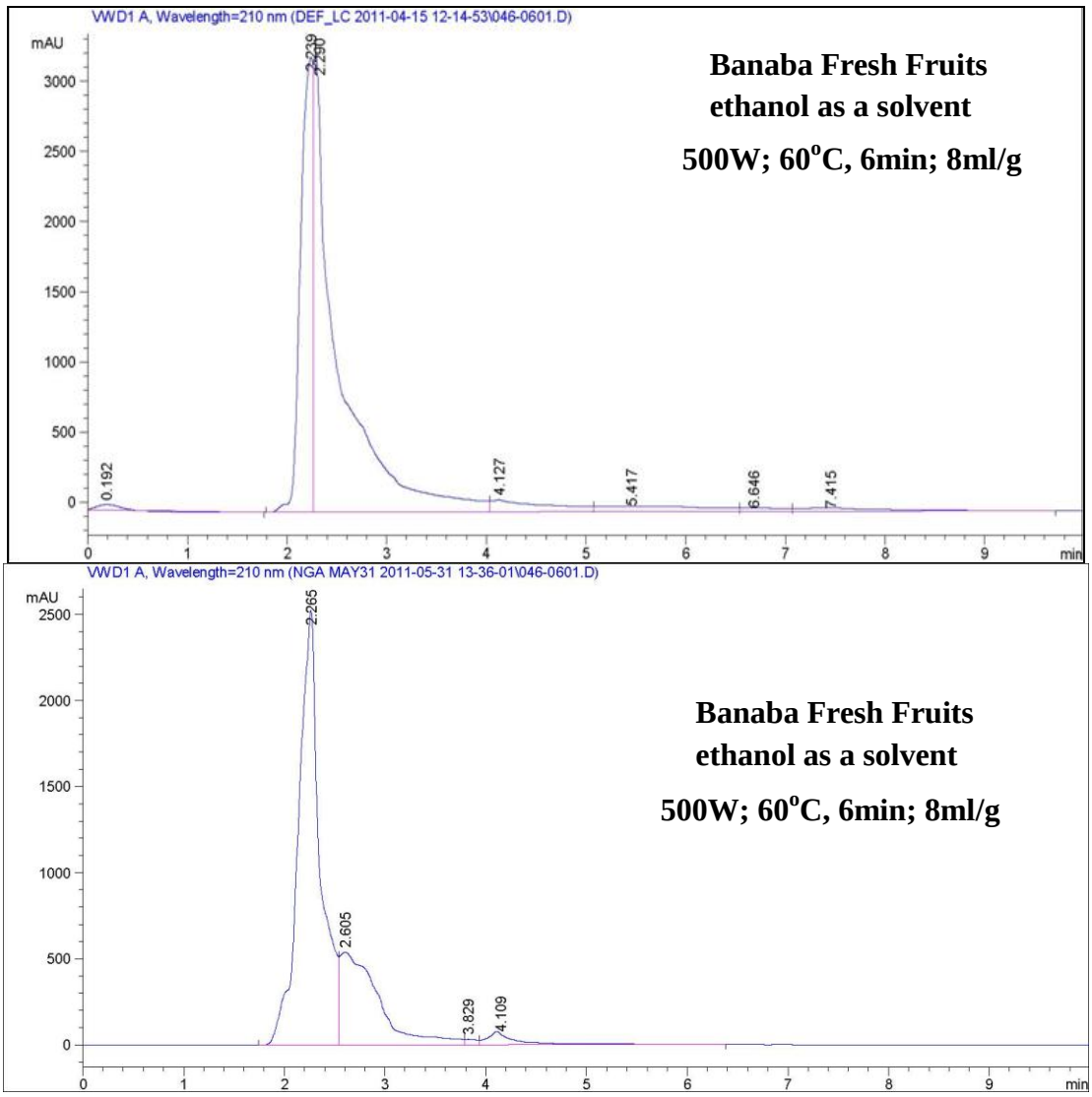



**Figure 5.12. HPLC of Banaba Fruit by MASE**

In addition, the yield of corosolic acid and the % yield of the corosolic acid peak at best conditions are presented in **Table 5.8**. As can be seen, different rates of extraction were given varying with corosolic acid concentration. The highest concentrations of corosolic acid obtained by MAE were for fresh leaves followed by dried leaves. However, the peaks of corosolic acid for fresh and dried fruits of banaba were not present in the HPLC chromatograms.

**Table 5.8. Yield of Corosolic Acid**

| **Method** | **Material** | **Yield of Oil** *(%)* | **Yield of Corosolic Acid** |
|---|---|---|---|
| MAE with ethanol as solvent | Fresh banaba leaves | 63.950 | 0.2488 |
| MAE with ethanol as solvent | Dried banaba leaves | 51.590 | 0.1798 17 |
| MAE with ethyl acetate as solvent | Fresh banaba leaves | 34.860 | 0.0228 |
| MAE with ethanol as solvent | Fresh banaba fruits | 79.650 | --- |
| MAE with ethanol as solvent | Dried banaba fruits | 47.110 | --- |
| Conventional method with hot water | Fresh banaba leaves | 33.756 | 0.0184 |
| Conventional method with ethanol | Fresh banaba leaves | 26.023 | 0.0385 |

The chromatogram of banaba extracted by MAE with ethyl acetate as solvent displayed the peak of corosolic acid at 8.113min, with the yield of corosolic acid in its extract lowest at 0.0284. Although this suggests that the choice of solvent for MAE is very important, this yield is still higher than when using conventional method, which corresponded only to 0.018% with hot water and 0.039 % with ethanol as solvents.

Results were compared with those found by Huang et al. (2009) using MAE without solvent to extract banaba leaves and fruits and by De Jesus et al. (2011) using MAE to extract banaba dried leaves. On the one hand, in Huang et al. (2009), yield of corosolic acid was 0.071% which corresponds to the highest yield of oil; however the highest yield of corosolic acid was 0.203% for fresh leaves and 0.225% for dried leaves. Whereas, in De Jesus et al. (2011), the highest yield of corosolic acid was 0.552%.

From these results, it may be expected that MAE is better than conventional method. Furthermore, MAE with solvent is also showed more advantage compared to MAE without solvent, which took a long time and gave lower yields.

However, when analyzing banaba fruit's crude oil by HPLC, the peak of corosolic acid was not presented. Although, in Huang et al. (2009), the yield of corosolic acid in banaba fruit extract at 2.645% was higher than in banaba leaves at 0.225 %. Several reasons may be able to explain these differences. At the time that Huang et al. (2009) carried out their experiment, which was around August, the banaba fruits available were already old and nature dried; whereas, when the present study was done, the banaba fruits available had just grown. The banaba flowers around March and bear fruits sometime in May; so, perhaps, the use of young fruits for this present study had effect on the presence of corosolic acid.

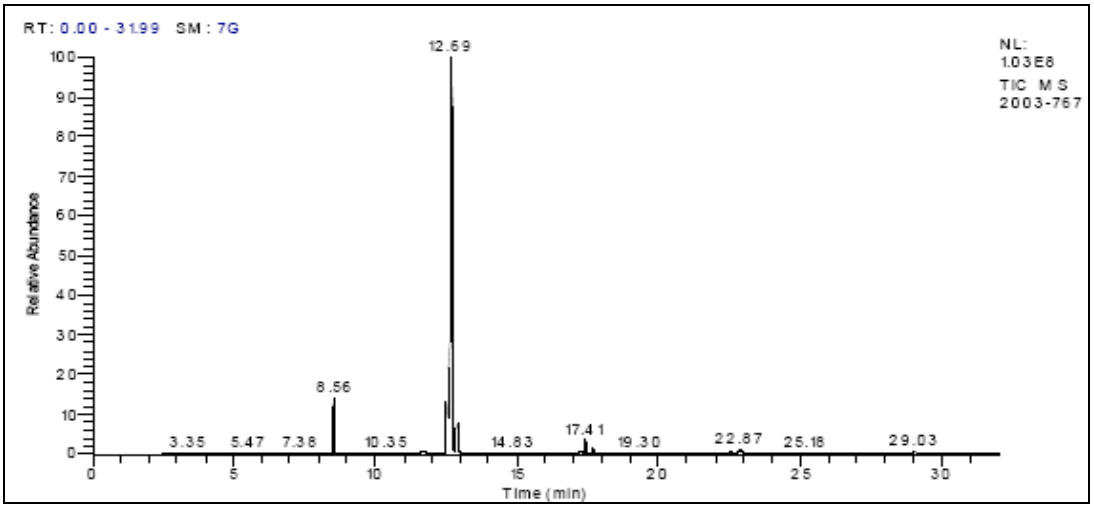



**Figure 5.13. GC–MS Profile of Banaba Seed Extracted Oil (Zong Wei et al., 2006)**

Furthermore, all of the chromatograms presented the other peaks beside that of the appearance of corosolic acid.

Based on literature, the many components contained in banaba leaf oil include not only tannic acid (Klein, 2007; Liu et al., 2005), with the tannins at about 14–17 % in banaba fruits and about 13% in banaba leaves, but also:

- ellagitannins, including: lagerstroemin; lagerstannin A, B, and C; reginin A, C, and D; flosin B; and, pterocarinin A (Hayashi, 2002);

- valoneaic acid and even ellagic acid (Hosoyama, 2003);
- ursolic acid, alphitolic acid (3), and, asiatic acid (4); and,
- fatty acids, such as 9-ketooctadec-cis-11-enoic acid. (Jehan, Daulatabad, & Nirajkar, 1990)

In addition, when Zongwei and Hanjun (2006) used GC–MS for the analysis of banaba crude oil extracted by supercritical $CO_2$ they found fatty acids—of which linoleic acid was highest at 74.57% in crude oil of banaba fruits—including palmitic, stearic, and oleic acids (**Figure 5.13**).

Moreover, banaba extract contained antibacterial components as a mixture of nonanedioic acid, 12-acetyloxy-9-octadecenoic acid, and 16-methyl-heptadecanoic acid. (Sinhababu et al., 1996)

## 5.7. ANTIBACTERIAL ACTIVITY OF BANABA CRUDE OIL

The antibacterial susceptibility test was done using microorganisms from De La Salle University's Microbiology Laboratory. Banaba extracts, both by MAE and by conventional method, were tested for their inhibition zones as shown in **Table 4.3** by Kirby–Bauer method, which was also used to determine the minimum inhibition concentrations or MICs of the oil extracts.

### 5.7.1. Effects of Banaba Crude Oil on Microorganisms

Banaba extract was diluted in saline solution at different concentrations to test for antibacterial activity, the results of which are shown in **Table 5.9**.

The banaba leaves and fruits at the conditions which gave highest yields were used to test for antibacterial activity using four microorganisms, namely: two gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*; and, two gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*.

The extract made a clear zone with all the microorganisms tested. The clear zones confirmed the effect of banaba crude oil on these four microorganisms. In the present study, ethanol was used like a control agent. Again, the data on **Table 5.9** will illustrate that all extracts of banaba leaves and fruits inhibit the tested microorganisms, with the inhibition of depending on both the concentration of extract and the type of microorganism.

#### *5.7.1.1. Banaba Extracts Against Gram-Negative Bacteria*

Banaba fresh leaves extracted by MAE had the sensitivity at minimum concentration of 10mg/ml for *E. coli,* which causes diarrhea especially in infants and newborns, as against the other extracts showing sensitivity at 100mg/ml.

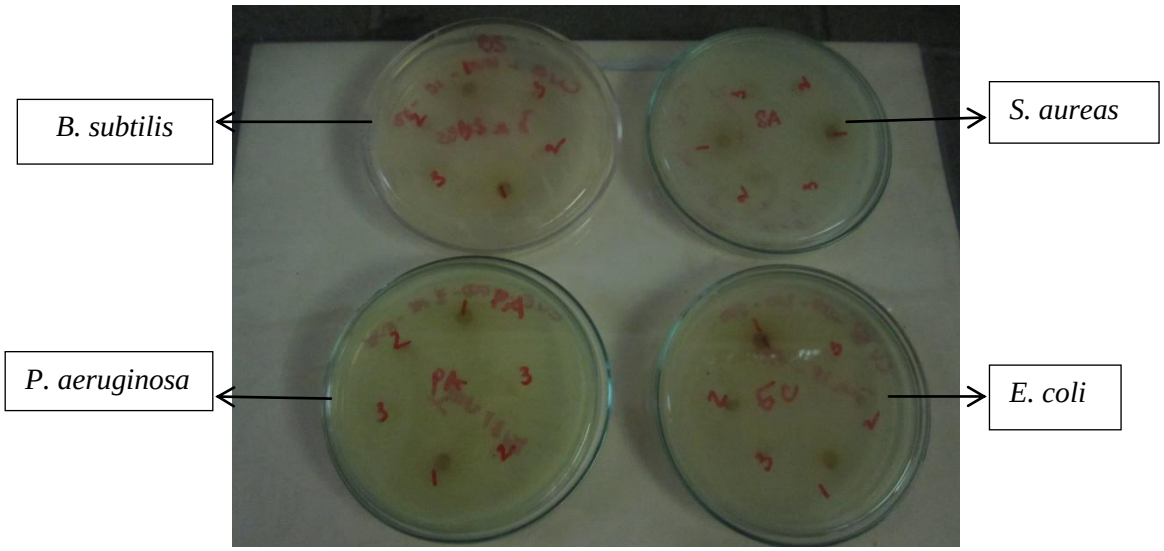



**Figure 5.14. Zone of Inhibition, by Microorganism**

**Table 5.9. Effects of Banaba Crude Oil on antibacterial activity by Zone of Inhibition**

| Concentration *(mg/ml)* | Microwave-Assisted Solvent Extraction | | | | | Conventional Method | |
|---|---|---|---|---|---|---|---|
| | **FL** | **DL** | **FF** | **DF** | **FLA** | **FLW** | **FLE** |
| ***Escherichia coli*** | | | | | | | |
| 1000 | + | + | + | + | + | + | + |
| 500 | + | + | + | + | + | + | + |
| 200 | + | + | + | + | + | + | + |
| 100 | + | + | + | + | + | + | + |
| 50 | + | - | - | - | - | - | - |
| 10 | + | - | - | - | - | - | - |
| ***Pseudomonas aeruginosa*** | | | | | | | |
| 1000 | + | + | + | + | + | + | + |
| 500 | + | + | + | + | + | + | + |
| 200 | + | + | + | + | + | + | + |
| 100 | + | + | + | + | - | + | + |
| 50 | + | + | + | - | - | - | - |
| 10 | - | - | - | - | - | - | - |
| ***Staphylococcus aureus*** | | | | | | | |
| 1000 | + | + | + | + | + | + | + |
| 500 | + | + | + | + | + | + | + |
| 200 | + | + | + | + | + | + | + |
| 100 | + | + | + | + | + | + | + |
| 50 | - | - | + | - | - | - | - |
| 10 | - | - | + | - | - | - | - |
| ***Bacillus subtilis*** | | | | | | | |
| 1000 | + | + | + | + | + | + | + |
| 500 | + | + | + | + | + | + | + |
| 200 | + | + | + | + | + | + | + |
| 100 | + | + | + | + | + | + | + |
| 50 | - | - | - | - | - | - | - |
| 10 | - | - | - | - | - | - | - |

Legend: -FL: Banaba fresh leaves extracted by MAE with ethanol as solvent.
-DL: Banaba dried leaves extracted by MAE with ethanol as solvent.
-FF: Banaba fresh fruits extracted by MAE with ethanol as solvent.
-DF: Banaba dried fruits extracted by MAE with ethanol as solvent
-FLA: Banaba fresh leaves extracted by MAE with ethyl acetate as solvent
-FLW: Banaba fresh leaves extracted by conventional method with hot water
-FLE: Banaba fresh leaves extracted by conventional method with ethanol.

*P. aeruginosa*—that which causes dermatitis as well as causes infectons of the urinary tract, respiratory system, and soft tissues—was the hardest microorganism to be inhibited. Although most of the banaba extracts exhibited an inhibition zone diameter of around 100mg/ml, especially that of crude oil of banaba leaves and fresh fruits, the *P. aeruginosa* was inhibited at 50mg/ml of concentration.

#### *5.7.1.2. Banaba Extracts Against Gram-Positive Bacteria*

*B. subtilis*—which is a known cause of an array of infections from ear infection to meningitis and urinary tract infection to septicemia—was inhibited by banaba extract at 100mg/ml of concentration.

In contrast, *S. aureas*—which is one of the root causes of pneumonia and septicemia as well as boils as well as kidney and wound infections—was inhibited by almost all of the tested banaba extracts.

The extract of banaba fresh fruits by MAE resulted in the highest sensitivity among the extracts with a minimum concentration at 10mg/ml compared to 50mg/ml minimum concentration for of the other extracts.

### 5.7.2. Effects of Concentration on Inhibition Zone Diameter

All the microorganisms that were tested at different levels of concentration of banaba extract by MAE were illustrated in **Table 5.9**. The concentrations were carried out at 1000mg/ml, 500mg/ml, 200mg/ml, 100mg/ml, 50mg/ml, and 10mg/ml. In addition, the results were compared to those obtained in banaba extracted by conventional method.

The diameter of inhibition zone increased when the concentration of banaba extract increased as shown in **Figure 5.15**.

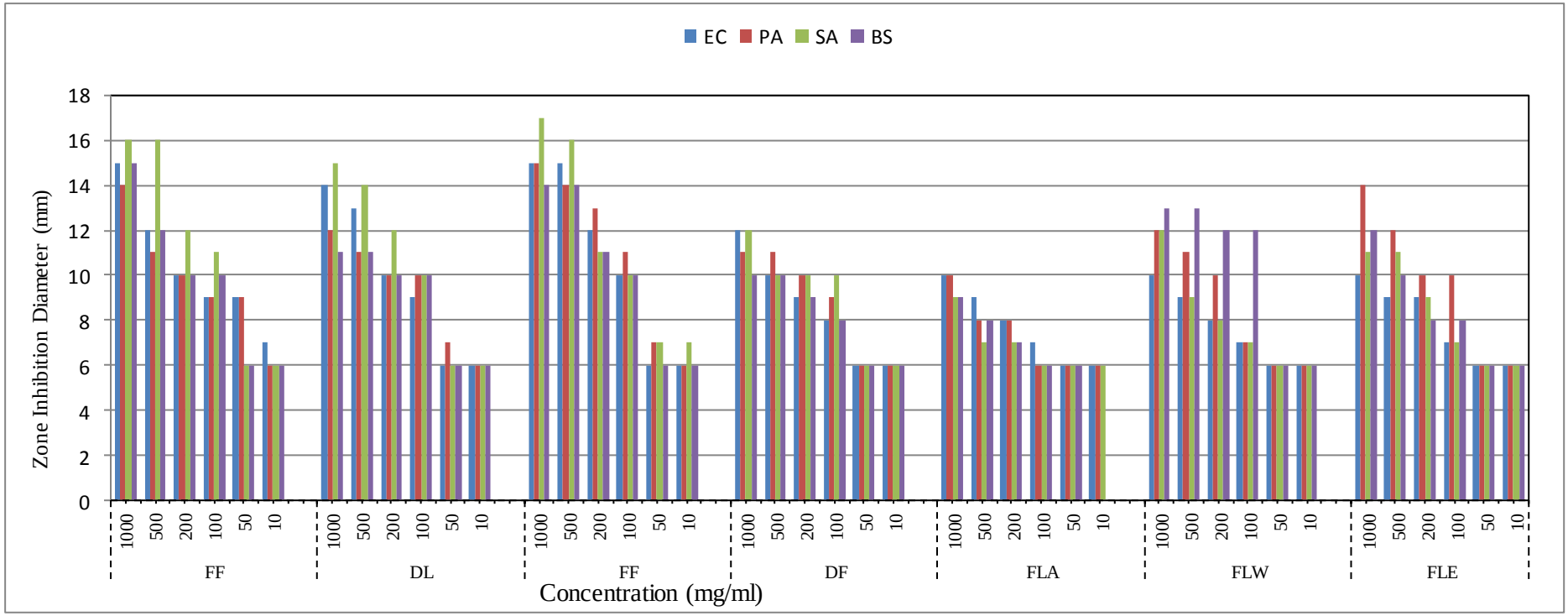



**Figure 5.15. Sensitivity of Microorganisms to Banaba Extract**

Notes: -FL: Banaba fresh leaves extracted by MAE with ethanol as a solvent.
-DL: Banaba dried leaves extracted by MAE with ethanol as a solvent.
-FF: Banaba fresh fruits extracted by MAE with ethanol as a solvent.
-DF: Banaba dried fruits extracted by MAE with ethanol as a solvent
-FLA: Banaba fresh leaves extracted by MAE with ethyl acetate as a solvent
-FLW: Banaba fresh leaves extracted by conventional method with hot water
-FLE: Banaba fresh leaves extracted by conventional method with ethanol.
-6mm: is the diameter of disc paper.

Microorganisms: EC *E. coli* , BS *B. subtilis* , SA *S. aureus*, PA *P. aeruginosa*

At the lowest concentration of 10mg/ml, the extract of banaba fresh leaves inhibited *E.coli* and the extract of banaba fresh fruits inhibited *S. aureus*; hence, the other extracts could have inhibited almost all at 100mg/ml of concentration.

At the highest test of 1,000mg/ml concentration of banaba extract by MAE and conventional method, all of the microorganisms exhibited sensitivity. *S. aureus* was the most sensitive (17mm)—followed by *E. coli* and *P. aeruginosa*—and *B. subtilis* had the worst sensitivity.

According to Ambujakshi (2009), banaba extracted by water and ethanol as solvents showed good sensitivity to *E. coli* (17mm) and lower for *B. subtilis* (15mm).

The extract of banaba fresh fruits exhibited higher inhibition zone diameters than the other extracts. Banaba fresh fruits were followed by banaba fresh leaves, banaba dried leaves, and banaba dried fruits.

The lowest performance, however, was by banaba crude oil extracted by MAE with ethyl acetate as solvent (see **Figure 5.15**). This is especially because, as previously discussed, the crude oil of banaba fruits did not exhibit the peak of corosolic acid during HPLC analysis. Nevertheless, extract of banaba fruits displayed better inhibition with microorganism than the other extracts.

It may then be said that the antibacterial activity of banaba extract was not dependent only on the presence of corosolic acid in the extract and was also affected by the other components.

Based on the findings of Sinhababu et al. (1996), the extract of seeds (fruits) from banaba contained a mixture of antibacterial components, such as nonanedioic acid, 12-acetyloxy-9-octadecenoic acid, and 16-methyl-heptadecanoic acid. In determining the minimum inhibitory concentration (MIC) of the fraction they showed values of up to <400µg/ ml. In addition, according to literature, banaba extracts included tannins, which like garlic acid exhibited antibacterial activity. This explains why banaba fruits extracted by MAE and even banaba leaves extracted by conventional method performed good antibacterial activity.

# CHAPTER 6

# CONCLUSIONS AND RECOMMENDATIONS

## 6.1. CONCLUSIONS

This study tested the antibacterial activity of banaba crude oil by Microwave Assisted Solvent Extraction (MASE) method. Fresh and dried leaves and fruits were the materials used in the MASE experiment. The varied conditions of time, temperature, power, and ratio of solvent to material was investigated to study the effects of these parameters on the yield of banaba extract.

Furthermore, the solvents ethyl acetate and ethanol were used for extraction by MASE to compare the influence of the matrix of solvent to the yield. After that, HPLC analysis was used to identify the presence of corosolic acid in the extract at the best conditions of extraction and the yield of corosolic acid was compared at the best conditions for each material matrix and solvents.

The physico-chemical properties of pH, specific gravity, acid value, and ester value were likewise investigated in the study. Finally, testing for antibacterial activity was carried out with four microorganisms, namely: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa.*

The best conditions for microwave power, temperature, and extraction time for the extraction of banaba leaves and fruits were realized at the following parameter combinations:

1. 500W power, $70^{o}$C temperature, and 6 minute for 10ml/g (ethanol/material) for fresh leaves with 63.949% yield;

2. 800W power, 60$^{o}$C temperature, and 6min for 8ml/g (ethanol/material) for dried leaves with 51.59% yield;
3. 500W power, 60$^{o}$C temperature, and 6 minute for 8ml/g (ethanol/material) for fresh fruits; with 79.65% yield and;
4. 800W power, 60$^{o}$C temperature, and 6 minute for 6ml/g (ethanol/material) for dried fruits with 47.11% yield.

The effect of solvent on extraction by MASE was that ethanol as a solvent gave higher yield than ethyl acetate. The highest yield of 63.9498% was obtained using ethanol as solvent compared to the highest yield of 7.963% using ethyl acetate as solvent.

The physico-chemical properties of banaba crude oil—such as refractive index, specific gravity, acid value, pH, and ester value—were measured as well. The results gave the following values:

1. A pH value of 4 was obtained for MASE extraction with ethyl acetate and ethanol as solvents of banaba leaves and a pH value of 4.5 for MAE extraction with ethanol as solvent of dried banaba fruits;
2. The range of specific gravity for banaba extract was 0.927–1.012. The range of refractive index was around 1.34–1.35;
3. The acid values of banaba extracts were found to be in the range of 2.14–3.6, which would be low; and,
4. The ester values of banaba extract, however, turned out relatively high at the range of 12–15 for leaves, specifically at 96.58 for fresh fruits and 117.67 for dried fruits.

The peak of corosolic acid was identified by HPLC analysis at around 8.5min. The yield of corosolic acid at best conditions for MASE extraction was realized for banaba leaf extract at 0.248%. However, the presence of corosolic acid in banaba fruits extract was not realized after HPLC analysis.

Nevertheless, free acids and fatty acids were both found to exist in banaba leaves and fruits, whose extracts possessed positive inhibition for antibacterial activity.

The antibacterial activity of banaba extracts demonstrated its applicability for use in the pharmaceutical and medical industries. The level of antibacterial ability, however, varied with the kind of extract and with the bacteria used. Nevertheless, all of tested bacteria exhibited sensitivity to the extracts at 100mg/ml.

On the one hand, *E. coli* exhibited the highest sensitivity among the four bacteria tested and *B. subtilis* had the lowest zone of inhibition diameter. On the other hand, banaba extract from fresh leaves by MASE with ethanol as a solvent displayed the best inhibition and the worse inhibition came from the extract by MASE with ethyl acetate.

The ANOVA test of five factors for fresh banaba leaves using MASE showed that temperature, the kind of solvent and the ratio had the most significant effects on the yield. In addition, the interaction of solvent and temperature, interaction of time and ratio as well as the interaction of solvent, time, and ratio turned out as the most significant terms in this investigated model.

## 6.2. RECOMMENDATIONS

1. The banaba fruits used may have been young fruits, such that the peak of corosolic acid was not present in the HPLC chromatogram. It is recommended that old banaba fruits be used.
2. The analysis of banaba extract by HPLC revealed the existence of other components besides corosolic acid. Hence, banaba extract should be analysis also by GC–MS.
3. The components of banaba crude oil should be isolated in order to determine exactly which of its many components possessed antibacterial activity.

4. The possibility of comparing MAE with both traditional extraction methods, such as by steam distillation and by Soxhlet, and green techniques, such as by supercritical $CO_2$, should also be considered.
5. Likewise, the application of banaba fruit extracts on biodiesel need to be studied as well.
6. In addition, the storage of essential oils needed to be considered and studied as well because essential oils are easily affected by the presence of oxygen, heat, and moisture. Moreover, essential oils are easily catalyzed by exposure to light and, possibly also, exposure to metals to bring about oxidation, polymerization, resinification, and hydrolysis of esters. It is best for essential oils to be first dried before storing them in refrigeration units and free from air and light.

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# APPENDIX A

## APPLICATION OF REFRACTOMETER

1. To introduce the sample unlock the prism, lift the top prism, spread a few drops of the sample on the bottom prism, close the prism slowly, and lock the prism again.
2. Turn the instrument on (on/off switch)
3. Focus the eyepiece on the scale by rotating it.
4. Turn the scale adjustment so that the critical ray boundary is visible in the top part of the viewer (a dividing line between light and dark halves is visible).
5. Turn the Amici prism adjustment so as to achromatize the boundary.
6. Turn the scale adjustment so that the boundary between light and dark coincides with the center of the cross hair.
7. Read and record the refractive index on the top scale in the lower part of the viewer (the bottom scale is for the concentration of sugar in water; ignore it). Three decimal places can be read, the fourth place is estimated. The below imagines shows a reading of 1.3433 (notice the smallest division is 0.0005).
8. If the specific dispersion is required, read and record the Amici prism adjustment knob.
9. Read and record the temperature on the thermometer.
10. Clean the prism by opening them and wiping them (top and bottom). Use water to remove water soluble compounds, toluene or petroleum ether for water insoluble compounds. Be sure not to scratch the prism.
11. Leave the prim in the open position so they can air dry.
12. To compare the refractive index with a reported literature value determines at a different temperature, the temperature correction factor may be used.

## APPENDIX B

## INSTRUCTIONS FOR OPERATING THE ROTARY EVAPORATOR

### Set up

1. Be sure that both the aspirator pump and the recalculating water bath (5 gallon bucket) are filled with ice.
2. Check that the power strip is turned on and plugged in.
3. Verify that the pump trap is clean and dry.
4. Attach the round-bottom flask to the ground glass joint of the bump trap as shown at the right. (Note: some assemblies will have an adapter between the bump trap and round-bottom flask.)
5. Ensure that all glassware is held securely in place with a place Keck clip and/or ring cap.
6. Turn on the rotary evaporator motor (green switch).
7. Adjust the dial to rotate the flask at medium speed.
8. Turn the aspirator pump.
9. Seal the vacuum by closing the valve at the top of the diagonal rotary evaporator condenser.
10. If necessary, carefully lover the round-bottom flask into the water heating bath.

### Shutdown

1. Lift the flask out of the water bath.
2. Break the vacuum by opening the top valve at the top of the rotary evaporator condenser.
3. Turn off the aspirator pump.
4. Turn the flask rotation dial down to zero.
5. Carefully remove the round-bottom flask.

**Tips and general considerations**

1. Rotary evaporator evaporates solvent, which is very different from drying a solvent/
2. Understand the factors that contribute to the rapid evaporation of solvent in rotary evaporator.
3. The round-bottom flask should be no more than half full. Large amouts of solvent can be removed either by transferring the solution to a larger flask or evaporating sequentially in smaller portions.
4. It may be advantageous to know the mass of the clean, empty round-bottom flask prior to rotary evaporation.
5. There is no need to turn off the rotary evaporator unless it is the end of the lab period.

# APPENDIX C

## DETAILED PROCEDURE

### 1. Extractable oil

Fresh/dried banaba which is equivalent to 30grams of fresh banaba was placed in a reactor and added with certain amount of solvent. The reactor was inserted inside the microwave cavity and fitted with a condenser. The irradiation was carried out according to one of the predetermined sequences. Upon the completion of radiation, the extract was filtered by using solvent filtration assembly. The residue (in reactor) was washed with a pre-measure amount of fresh solvent and the solvent was filtered and added to the extract. Then the residue of banaba was placed in a reactor and added with new solvent then inserted again inside the microwave cavity and fitted with a condenser. It was repeated until the oil was extracted.

### 2. MAE with solvent

Banaba was placed in a 330ml extraction cylinder-reactor, and then solvent was added. The reactor was inserted inside the microwave cavity and fitted with a condenser. The sample then was irradiated with microwave (2445 MHz). After the extraction, the solution was filtered by using solvent filtration assembly with Whatman No.1. The residue banaba in reactor was washed with a premeasure amount of fresh solvent and the solvent was filtered and added to the extract. Then all of filtrates were concentrated in vacuum rotary evaporator at $45^{o}$C.

### 3. Conventional method

Hot water: Fresh leaves Babana was place in glass and put in water bath at temperature 90oC for 2 hours.

Ethanol: The samples were macerated in 95% ethanol and to stand for 6days at room temperatures. After incubation, the samples were filtered and remove water like MAE.

# APPENDIX D

## 1. MULLER HINTON AGAR

**Formula in g/l of**

| | |
|---|---|
| Beef infusion: | 2.0 |
| Corn starch: | 1.5 |
| Acid casein peptone (H) | 17.5 |
| Bacteriological agar | 17.0 |

**Preparation**

Suspend 38 grams of medium in one liter of distilled water. Mix well. Heat agitating frequently and boil for about 1 minute. Dispense and sterilize in autoclave at 116$^{o}$C-121$^{o}$C for 15 minutes. Cool to 45$^{o}$C or 50$^{o}$C and add defibrinated blood if desired. The blood mixture should be chocolate by heating to 80$^{o}$C for 10 minutes if Neisseria development is desired. DO NOT OVERHEAT. To remelt the cold medium, heat as briefly as possible.

Follow manufacturer's instruction to prepare medium. After autoclaving, cool medium to 50$^{o}$C. Measure 25-30 ml per plate into 100-mm diameter plates. Agar should be poured into flat bottom glass Petri dishes on a level pouring surface to a uniform depth 4 mm. using more or less agar will affect the susceptibility.

Freshly prepared plates may be used the same day or stored in a refrigerator (2$^{o}$C to 8$^{o}$C) for up to 2 weeks. If plates are not used within 7 days of preparation they should be wrapped in plastics to minimize evaporation. Just before use, if excess moisture is on the surfaces, plates should be placed in an incubator (35$^{o}$C to 37$^{o}$C) until the moisture evaporates (usually 10 to 30 minutes). Do not leave lids agar because the medium is easily contaminated.

**2. Turbidity standards (McFarland)**

McFarland 0.5 turbidity standard are available from various manufactures. Alternatively, the 0.5 McFarland may be prepared by adding 0.5 ml of 1.175% (wt/vol) Barium chloride dehydrate ($BaCl_2.2H_2O$) solution to 99.5ml of 1% (vol/vol) sulfuric acid. The turbidity standard is then aliquot into test tube identical to those used to prepare the inoculums suspension. Seal the McFarland standard tube with wax, parafilm or some others means to prevent evaporation. McFarland standard may be stored for up to 6 months or sooner if any volume it lost. Before each use, shake well, mixing the fine white precipitate of barium sulfate in the tube.

The accuracy of the density of a prepared McFarland standard should be checked by using a spectrophotometer with a 1cm light path, for 0.5 McFarland standards, the absorbance at a wavelength of 625mnm should be 0.08 to 0.1

# APPENDIX E

## MOISTURE OF BANABA LEAVES AND FRUITS

**1. Moisture content in fresh Banaba leaves**

| Weight of Banaba leaves before drying (gram) | Weight of Banaba leaves after drying (gram) | Moisture content (%) |
|---|---|---|
| 30.0437 | 8.468592 | 71.812 |
| 30.057 | 8.657666 | 71.19584 |
| **Average** | | **71.50413** |

**2. Moisture of one-week air dried Banaba leaves**

| Weight of Banaba leaves before drying (gram) | Weight of Banaba leaves after drying (gram) | Moisture content (%) |
|---|---|---|
| 100.13 | 59.9847 | 40.09318 |
| 100.0578 | 61.234 | 38.80137 |
| **Average** | | **39.44729** |

**3. Residual moisture of one-week air dried Banaba leaves**

| **Weight of Banaba leaves before drying (gram)** | **Weight of Banaba leaves after drying (gram)** | **Moisture (%)** |
|---|---|---|
| 18.158 | 8.581 | 52.19187 |
| 18.174 | 8.14 | 55.21074 |
| **Average** | | **53.70131** |

4. **Moisture content of fresh Banaba fruit**

| **Weight of Banaba leaves before drying (gram)** | **Weight of Banaba leaves after drying (gram)** | **Moisture content (%)** |
|---|---|---|
| 30.0832 | 8.139 | 72.94503 |
| 30.0872 | 8.1681 | 72.85245 |
| **Average** | | **72.89874** |

5. **Residual moisture content of one - week air dried Banaba fruit**

| **Weight of Banaba fruits drying (gram)** | **Weight of Banaba leaves after drying (gram)** | **Moisture content (%)** |
|---|---|---|
| 18.165 | 6.655 | 63.36361 |
| 18.173 | 6.614 | 63.60535 |
| **Average** | | **63.4847** |

# APPENDIX F

## TEMPERATURE PROFILE

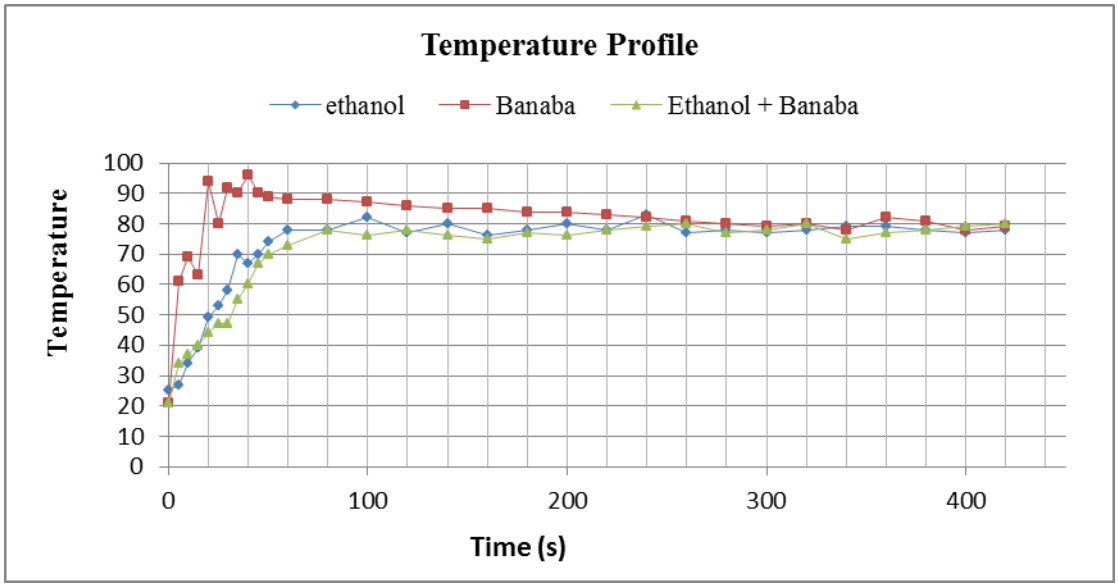



**Figure E.1:** Temperature profile of ethanol as solvent

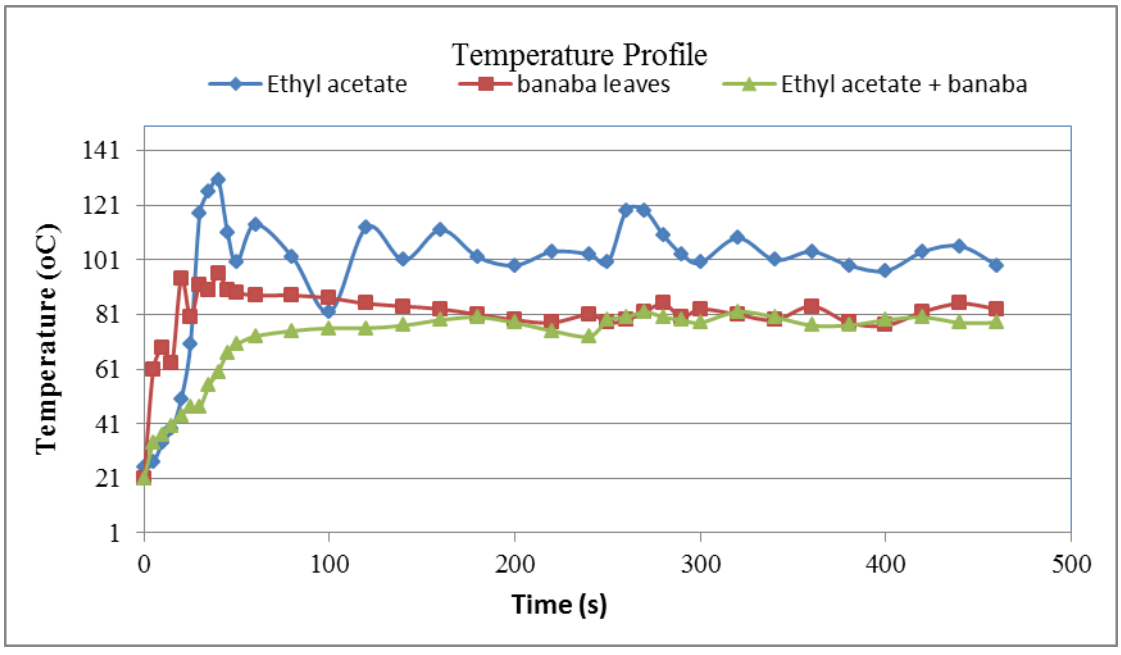



**Figure E.2:** Temperature profile of ethyl acetate as solvent

# APPENDIX G

## EXTRACTABLE OIL

### 1. Extractable oil of banaba leaves

| | Fresh leaves | | Dried leaves | |
|---|---|---|---|---|
| **Time** | **Weight of oil** | **Yield** | **Weight of oil** | **Yield** |
| 2 | 4.0575 | 0.466379 | 3.0274 | 0.347977 |
| 6 | 1.9572 | 0.224966 | 1.2441 | 0.143 |
| 10 | 0.932 | 0.107126 | 0.4306 | 0.049494 |
| 20 | 0.4834 | 0.055563 | 0.15 | 0.017241 |
| 50 | 0.035 | 0.004023 | 0.012 | 0.001379 |
| 80 | 0 | 0 | 0 | 0 |
| Total | 6.1129 | 85.80575 | 4.9641 | 55.9092 |

### 2. Extractable of banaba fruits

| | Fresh fruit | | Dried Fruit | |
|---|---|---|---|---|
| **Time** | **Weight of oil** | **Yields** | **Weight of oil** | **Yields** |
| 2 | 3.7434 | 0.56436 | 2.835 | 0.427408 |
| 6 | 1.279 | 0.192824 | 0.757 | 0.114126 |
| 10 | 0.516 | 0.077793 | 0.208 | 0.031358 |
| 20 | 0.27 | 0.040706 | 0.119 | 0.017941 |
| 50 | 0.01 | 0.001508 | 0.01 | 0.001508 |
| 80 | 0 | 0 | 0 | 0 |
| | 5.8184 | 0.87719 | 3.929 | 0.592341 |

# APPENDIX H

## 1. Fresh Banaba leaves extracted by MAE with ethanol as a solvent

| Run | Ratio | Power | Time | Tempt | W.Sample | | W.oil | | Yield | | Avg |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Trial 1 | Trial 2 | Trial 1 | Trial 2 | Trial 1 | Trial 2 | |
| 1 | 6 | 30 | 2 | 50 | 30.0572 | 30.0604 | 2.8632 | 3.1011 | 33.42884 | 36.20254 | 34.81569 |
| 2 | | 30 | 6 | 50 | 30.0451 | 30.0483 | 4.4968 | 2.8387 | 52.52282 | 33.15261 | 42.83771 |
| 3 | | 50 | 2 | 50 | 30.0275 | 30.077 | 4.281 | 3.4693 | 50.03157 | 40.47859 | 45.25508 |
| 4 | | 50 | 6 | 50 | 30.0667 | 30.0552 | 3.723 | 4.005 | 43.45356 | 46.76285 | 45.1082 |
| 5 | | 80 | 2 | 50 | 30.0148 | 30.0435 | 3.8264 | 3.2701 | 44.73763 | 38.19694 | 41.46729 |
| 6 | | 80 | 6 | 50 | 30.0336 | 30.0674 | 4.1664 | 3.2542 | 48.68236 | 37.981 | 43.33168 |
| 7 | | 30 | 2 | 60 | 30.0732 | 30.0448 | 4.2717 | 2.8641 | 49.84702 | 33.45314 | 41.65008 |
| 8 | | 30 | 6 | 60 | 30.0287 | 30.0698 | 4.3218 | 2.3273 | 50.50638 | 27.16063 | 38.83351 |
| 9 | | 50 | 2 | 60 | 30.0282 | 30.0586 | 3.68 | 3.3676 | 43.00675 | 39.31605 | 41.1614 |
| 10 | | 50 | 6 | 60 | 30.0169 | 30.0553 | 3.6904 | 4.5582 | 43.14452 | 53.2219 | 48.18321 |
| 11 | | 80 | 2 | 60 | 30.0315 | 30.0511 | 3.572 | 3.3986 | 41.74001 | 39.68787 | 40.71394 |
| 12 | | 80 | 6 | 60 | 30.0102 | 30.0065 | 2.6469 | 4.9449 | 30.95186 | 57.83094 | 44.3914 |
| 13 | | 30 | 2 | 70 | 30.0812 | 30.0808 | 2.897 | 3.8371 | 33.79648 | 44.7643 | 39.28039 |
| 14 | | 30 | 6 | 70 | 30.0615 | 30.049 | 5.2189 | 4.2017 | 60.92372 | 49.06967 | 54.99669 |
| 15 | | 50 | 2 | 70 | 30.0343 | 30.088 | 5.0228 | 3.984 | 58.68761 | 46.46694 | 52.57728 |
| 16 | | 50 | 6 | 70 | 30.0279 | 30.0318 | 4.4104 | 3.864 | 51.54317 | 45.15167 | 48.34742 |
| 17 | | 80 | 2 | 70 | 30.0594 | 30.008 | 4.1917 | 4.3351 | 48.93594 | 50.69675 | 49.81635 |
| 18 | | 80 | 6 | 70 | 30.0385 | 30.0308 | 4.2506 | 4.1492 | 49.6581 | 48.48591 | 49.072 |
| 19 | 8 | 30 | 2 | 50 | 30.0323 | 30.0025 | 4.5914 | 3.6963 | 53.6506 | 43.23424 | 48.44242 |
| 20 | | 30 | 6 | 50 | 30.0335 | 30.0335 | 4.723 | 4.015 | 55.18615 | 46.91348 | 51.04982 |
| 21 | | 50 | 2 | 50 | 30.0055 | 30.0417 | 5.314 | 3.4039 | 62.14966 | 39.7622 | 50.95593 |
| 22 | | 50 | 6 | 50 | 30.0036 | 30.0485 | 4.1988 | 4.015 | 49.11 | 46.89006 | 48.00003 |
| 23 | | 80 | 2 | 50 | 30.0273 | 30.0313 | 6.0937 | 3.2 | 71.21687 | 37.39331 | 54.30509 |
| 24 | | 80 | 6 | 50 | 30.068 | 30.0223 | 5.1255 | 4.2617 | 59.82046 | 49.81464 | 54.81755 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | | 30 | 2 | 60 | 30.026 | 30.0059 | 4.4095 | 3.094 | 51.53591 | 36.18526 | 43.86059 |
| 26 | | 30 | 6 | 60 | 30.0055 | 30.0452 | 5.9408 | 3.377 | 69.48037 | 39.44337 | 54.46187 |
| 27 | | 50 | 2 | 60 | 30.0416 | 30.017 | 4.5366 | 3.7591 | 52.99385 | 43.94755 | 48.4707 |
| 28 | 8 | 50 | 6 | 60 | 30.0559 | 30 | 4.7301 | 3.6241 | 55.22792 | 42.39328 | 48.8106 |
| 29 | | 80 | 2 | 60 | 30.0808 | 30.0235 | 4.956 | 3.9422 | 57.81759 | 46.07819 | 51.94789 |
| 30 | | 80 | 6 | 60 | 30.0214 | 30.0499 | 5.6595 | 4.4907 | 66.15539 | 52.44319 | 59.29929 |
| 31 | | 30 | 2 | 70 | 30.0345 | 30.0127 | 4.6042 | 4.132 | 53.79623 | 48.31404 | 51.05513 |
| 32 | | 30 | 6 | 70 | 30.0455 | 30.0616 | 4.2748 | 4.7344 | 49.92918 | 55.26764 | 52.59841 |
| 33 | | 50 | 2 | 70 | 30.0689 | 30.0068 | 4.7713 | 5.3307 | 55.68488 | 62.34227 | 59.01358 |
| 34 | | 50 | 6 | 70 | 30.271 | 30.0581 | 4.5406 | 3.8174 | 52.63863 | 44.56811 | 48.60337 |
| 35 | | 80 | 2 | 70 | 30.0419 | 30.0781 | 3.7 | 3.1454 | 43.22076 | 36.6981 | 39.95943 |
| 36 | | 80 | 6 | 70 | 30.0021 | 30.0077 | 3.9951 | 4.8345 | 46.72982 | 56.53754 | 51.63368 |
| 37 | | 30 | 2 | 50 | 30.019 | 30.0537 | 4.3025 | 3.8072 | 50.29708 | 44.45553 | 47.3763 |
| 38 | | 30 | 6 | 50 | 30.0169 | 30.0748 | 3.997 | 3.5365 | 46.72899 | 41.26568 | 43.99733 |
| 39 | | 50 | 2 | 50 | 30.067 | 30.0124 | 4.3712 | 3.5611 | 51.01861 | 41.63912 | 46.32887 |
| 40 | | 50 | 6 | 50 | 30.0332 | 30.0533 | 4.4907 | 4.7157 | 52.47235 | 55.06455 | 53.76845 |
| 41 | | 80 | 2 | 50 | 30.0715 | 30.0189 | 5.553 | 3.1532 | 64.80234 | 36.86165 | 50.832 |
| 42 | | 80 | 6 | 50 | 30.0715 | 30.0988 | 4.865 | 3.8005 | 56.77352 | 44.3108 | 50.54216 |
| 43 | | 30 | 2 | 60 | 30.0291 | 30.0745 | 4.7168 | 4.1641 | 55.12178 | 48.58932 | 51.85555 |
| 44 | | 30 | 6 | 60 | 30.0069 | 30 | 4.985 | 4.9651 | 58.29913 | 58.07976 | 58.18945 |
| 45 | 10 | 50 | 2 | 60 | 30.0523 | 30.0407 | 4.8754 | 5.5478 | 56.93124 | 64.80803 | 60.86963 |
| 46 | | 50 | 6 | 60 | 30.0687 | 30.0324 | 5.5558 | 4.0213 | 64.84105 | 46.98882 | 55.91493 |
| 47 | | 80 | 2 | 60 | 30.0716 | 30.0744 | 4.5325 | 4.5374 | 52.89314 | 52.94539 | 52.91927 |
| 48 | | 80 | 6 | 60 | 30.0419 | 30.07056 | 4.6768 | 4.309 | 54.63104 | 50.28669 | 52.45887 |
| 49 | | 30 | 2 | 70 | 30.0569 | 30.0216 | 5.166 | 5.4741 | 60.31541 | 63.98777 | 62.15159 |
| 50 | | 30 | 6 | 70 | 30.0423 | 30.0438 | 5.1975 | 4.5388 | 60.71268 | 53.01567 | 56.86418 |
| 51 | | 50 | 2 | 70 | 30.025 | 30.0379 | 5.088 | 3.0847 | 59.46784 | 36.03806 | 47.75295 |
| 52 | | 50 | 6 | 70 | 30.0169 | 30.0256 | 4.7172 | 6.2246 | 55.14886 | 72.75081 | 63.94983 |
| 53 | | 80 | 2 | 70 | 30.0806 | 30.0135 | 5.5628 | 4.3588 | 64.89706 | 50.96457 | 57.93082 |
| 54 | | 80 | 6 | 70 | 30.035 | 30.0154 | 3.66126 | 3.66126 | 42.77805 | 42.80599 | 42.79202 |

## 2. Banaba dried leaves extracted by MAE with ethanol as a solvent

| Run | Ratio | Power | Time | Tempt | W.Sample | | W.oil | | Yield | | Yield |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Trial 1 | Trial 2 | Trial 1 | Trial 2 | Trial 1 | Trial 2 | |
| 1 | 6 | 30 | 2 | 50 | 18.1962 | 18.16 | 3.1287 | 2.8437 | 37.13766 | 33.822 | 35.47983 |
| 2 | | 30 | 6 | 50 | 18.1626 | 18.15 | 3.2583 | 2.828 | 38.74756 | 33.6538 | 36.20068 |
| 3 | | 30 | 2 | 60 | 18.1352 | 18.16 | 2.9885 | 3.1101 | 35.5928 | 36.99047 | 36.29164 |
| 4 | | 30 | 6 | 60 | 18.179 | 18.152 | 2.9984 | 3.7693 | 35.62467 | 44.85052 | 40.2376 |
| 5 | | 50 | 2 | 50 | 18.1832 | 18.157 | 3.8918 | 3.163 | 46.22868 | 37.62586 | 41.92727 |
| 6 | | 50 | 6 | 50 | 18.1723 | 18.16 | 4.0146 | 3.2693 | 47.71596 | 38.88394 | 43.29995 |
| 7 | | 80 | 2 | 50 | 18.1749 | 18.1604 | 4.0094 | 3.363 | 47.64734 | 39.99749 | 43.82242 |
| 8 | | 80 | 6 | 50 | 18.1647 | 18.1549 | 4.3106 | 3.5781 | 51.25554 | 42.56865 | 46.9121 |
| 9 | | 50 | 2 | 60 | 18.1945 | 18.1583 | 3.6745 | 3.4028 | 43.62038 | 40.47553 | 42.04795 |
| 10 | | 50 | 6 | 60 | 18.1763 | 18.1573 | 3.689 | 3.616 | 43.83636 | 43.01386 | 43.42511 |
| 11 | | 30 | 2 | 70 | 18.158 | 18.1584 | 3.5844 | 2.7207 | 42.63632 | 32.36193 | 37.49913 |
| 12 | | 30 | 6 | 70 | 18.1538 | 18.1582 | 3.2984 | 3.567 | 39.24344 | 42.42888 | 40.83616 |
| 13 | | 50 | 2 | 70 | 18.1737 | 18.159 | 3.4861 | 3.5825 | 41.43123 | 42.61138 | 42.0213 |
| 14 | | 50 | 6 | 70 | 18.1703 | 18.156 | 3.6757 | 3.7243 | 43.69274 | 44.30531 | 43.99902 |
| 15 | | 80 | 2 | 60 | 18.1609 | 18.64 | 3.1452 | 3.0357 | 37.40608 | 35.17582 | 36.29095 |
| 16 | | 80 | 6 | 60 | 18.1547 | 18.166 | 3.943 | 4.0478 | 46.91039 | 48.12725 | 47.51882 |
| 17 | | 80 | 2 | 70 | 18.17 | 18.1567 | 2.6408 | 3.4448 | 31.39149 | 40.97872 | 36.1851 |
| 18 | | 80 | 6 | 70 | 18.173 | 18.1519 | 3.4433 | 3.3948 | 40.92414 | 40.39461 | 40.65937 |
| 19 | 8 | 30 | 2 | 50 | 18.1614 | 18.1553 | 3.1049 | 2.8726 | 36.92577 | 34.17457 | 35.55017 |
| 20 | | 30 | 6 | 50 | 18.1702 | 18.1615 | 3.2361 | 3.277 | 38.46746 | 38.9723 | 38.71988 |
| 21 | | 30 | 2 | 60 | 18.1549 | 18.1601 | 3.3205 | 2.5386 | 39.50399 | 30.19307 | 34.84853 |
| 22 | | 30 | 6 | 60 | 18.1556 | 18.1608 | 3.3057 | 3.5548 | 39.32639 | 42.27771 | 40.80205 |
| 23 | | 30 | 2 | 70 | 18.1637 | 18.164 | 3.9056 | 4.0556 | 46.44241 | 48.2253 | 47.33385 |
| 24 | | 30 | 6 | 70 | 18.1681 | 18.157 | 4.1936 | 4.1781 | 49.85501 | 49.7011 | 49.77806 |
| 25 | | 50 | 2 | 50 | 18.1711 | 18.1557 | 3.1563 | 3.8825 | 37.51702 | 46.18806 | 41.85254 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 26 | | 50 | 6 | 50 | 18.1702 | 18.1699 | 3.7274 | 3.2622 | 44.30754 | 38.77835 | 41.54294 |
| 27 | | 50 | 2 | 60 | 18.1678 | 18.1602 | 2.5649 | 2.9313 | 30.49295 | 34.8635 | 32.67822 |
| 28 | | 50 | 6 | 60 | 18.1537 | 18.1608 | 2.9812 | 2.3622 | 35.46968 | 28.09396 | 31.78182 |
| 29 | | 50 | 2 | 70 | 18.1703 | 18.1528 | 3.2783 | 4.083 | 38.96888 | 48.58107 | 43.77497 |
| 30 | | 50 | 6 | 70 | 18.1592 | 18.155 | 2.9056 | 3.4137 | 34.55974 | 40.61256 | 37.58615 |
| 31 | 8 | 80 | 2 | 50 | 18.1576 | 18.158 | 3.8014 | 4.6751 | 45.21853 | 55.61017 | 50.41435 |
| 32 | | 80 | 6 | 50 | 18.1634 | 18.1557 | 3.375 | 3.4525 | 40.13358 | 41.07258 | 40.60308 |
| 33 | | 80 | 2 | 60 | 18.166 | 18.1541 | 2.5901 | 3.177 | 30.79559 | 37.79843 | 34.29701 |
| 34 | | 80 | 6 | 60 | 18.1535 | 18.1616 | 4.5075 | 4.1665 | 53.62986 | 49.55056 | 51.59021 |
| 35 | | 80 | 2 | 70 | 18.1604 | 18.15 | 2.8952 | 2.129 | 34.43376 | 25.33555 | 29.88466 |
| 36 | | 80 | 6 | 70 | 18.1604 | 18.15 | 3.0735 | 3.1693 | 36.55435 | 37.71534 | 37.13485 |
| 37 | | 30 | 2 | 50 | 18.1834 | 18.1565 | 3.1546 | 3.7494 | 37.47145 | 44.60268 | 41.03706 |
| 38 | | 30 | 6 | 50 | 18.1532 | 18.1623 | 3.2416 | 3.5405 | 38.56892 | 42.10416 | 40.33654 |
| 39 | | 30 | 2 | 60 | 18.528 | 18.1578 | 3.9507 | 3.4506 | 46.055 | 41.04523 | 43.55012 |
| 40 | | 30 | 6 | 60 | 18.5626 | 18.1604 | 3.7605 | 3.2301 | 43.75605 | 38.41686 | 41.08645 |
| 41 | | 50 | 2 | 50 | 18.1592 | 18.1653 | 3.1619 | 3.9107 | 37.60821 | 46.49896 | 42.05359 |
| 42 | | 50 | 6 | 50 | 18.178 | 18.1597 | 3.2291 | 3.789 | 38.36778 | 45.06582 | 41.7168 |
| 43 | | 80 | 2 | 50 | 18.15 | 18.15 | 2.5802 | 2.1367 | 30.70492 | 25.42718 | 28.06605 |
| 44 | | 80 | 6 | 50 | 18.1758 | 18.1678 | 2.8134 | 2.5272 | 33.43253 | 30.04475 | 31.73864 |
| 45 | | 50 | 2 | 60 | 18.6432 | 18.1546 | 3.0313 | 3.478 | 35.11881 | 41.37845 | 38.24863 |
| 46 | 10 | 50 | 6 | 60 | 18.1538 | 18.1543 | 2.9622 | 3.769 | 35.24343 | 44.84127 | 40.04235 |
| 47 | | 30 | 2 | 70 | 18.1556 | 18.1635 | 3.7362 | 3.4624 | 44.44785 | 41.17267 | 42.81026 |
| 48 | | 30 | 6 | 70 | 18.1646 | 18.1589 | 2.8036 | 3.4785 | 33.33662 | 41.3746 | 37.35561 |
| 49 | | 50 | 2 | 70 | 18.1906 | 18.1703 | 2.3445 | 2.4507 | 27.83777 | 29.13127 | 28.48452 |
| 50 | | 50 | 6 | 70 | 18.1906 | 18.1534 | 2.2788 | 2.6139 | 27.05768 | 31.10014 | 29.07891 |
| 51 | | 80 | 2 | 60 | 18.155 | 18.1702 | 2.577 | 2.43 | 30.6584 | 28.88537 | 29.77188 |
| 52 | | 80 | 6 | 60 | 18.5079 | 18.1639 | 4.1665 | 3.9167 | 48.62342 | 46.57389 | 47.59866 |
| 53 | | 80 | 2 | 70 | 18.1876 | 18.15 | 2.1123 | 3.2504 | 25.08485 | 38.68045 | 31.88265 |
| 54 | | 80 | 6 | 70 | 18.1545 | 18.1549 | 2.5617 | 3.0557 | 30.47721 | 36.35366 | 33.41544 |

**3. Banaba fresh leaves extracted by MAE with ethyl acetate as a solvent (mass of sample = 30g)**

| Run | Ratio | Power | Time | Tempt | W.Sample of oil | | Yield | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Trial 1 | Trial 2 | Yield 1 | Yield 2 | Yield |
| 1 | 6 | 30 | 2 | 50 | 0.2004 | 0.1471 | 2.344199 | 1.720717 | 2.032458 |
| 2 | 6 | 30 | 6 | 50 | 0.2417 | 0.1359 | 2.82731 | 1.589704 | 2.208507 |
| 3 | 6 | 80 | 2 | 50 | 0.3485 | 0.4055 | 4.076614 | 4.743377 | 4.409996 |
| 4 | 6 | 80 | 6 | 50 | 0.242 | 0.206 | 2.83082 | 2.409706 | 2.620263 |
| 5 | 6 | 30 | 2 | 70 | 0.3295 | 0.126 | 3.85436 | 1.473898 | 2.664129 |
| 6 | 6 | 30 | 6 | 70 | 0.3288 | 0.2699 | 3.846171 | 3.157183 | 3.501677 |
| 7 | 6 | 80 | 2 | 70 | 0.2805 | 0.2545 | 3.281177 | 2.97704 | 3.129108 |
| 8 | 6 | 80 | 6 | 70 | 0.7517 | 0.6097 | 8.793087 | 7.132028 | 7.962557 |
| 9 | 10 | 30 | 2 | 50 | 0.2399 | 0.2402 | 2.806255 | 2.809764 | 2.808009 |
| 10 | 10 | 30 | 6 | 50 | 0.618 | 0.5035 | 7.229118 | 5.889742 | 6.55943 |
| 11 | 10 | 80 | 2 | 50 | 0.30766 | 0.2721 | 3.598884 | 3.182917 | 3.390901 |
| 12 | 10 | 80 | 6 | 50 | 0.4455 | 0.5187 | 5.211281 | 6.067546 | 5.639414 |
| 13 | 10 | 30 | 2 | 70 | 0.4275 | 0.4942 | 5.000725 | 5.780955 | 5.39084 |
| 14 | 10 | 30 | 6 | 70 | 0.3793 | 0.4239 | 4.4369 | 4.958613 | 4.697757 |
| 15 | 10 | 80 | 2 | 70 | 0.5883 | 0.6831 | 6.881699 | 7.990632 | 7.436165 |
| 16 | 10 | 80 | 6 | 70 | 0.4279 | 0.3475 | 5.005404 | 4.064917 | 4.53516 |

**4. Banaba Fresh Fruits extracted by MAE with Ethanol as a solvent**

| Run | Ratio | Power | Tempt | Time | Weight of fruit | | weight of oil | | Yield | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Trial 1 | Trial 2 | Trial 1 | Trial 2 | Trial 1 | Trial 2 | Average |
| 1 | 6 | 50 | 60 | 2 | 24.069 | 24.08 | 4.4149 | 3.842 | 67.67868 | 58.86944 | 63.27406 |
| 2 | 6 | 50 | 60 | 6 | 24.061 | 24.0805 | 4.107 | 3.9325 | 62.97963 | 60.25489 | 61.61726 |
| 3 | 6 | 50 | 70 | 2 | 24.059 | 24.0617 | 3.2558 | 3.334 | 49.93088 | 51.12441 | 50.52764 |
| 4 | 6 | 50 | 70 | 6 | 24.0521 | 24.0428 | 4.8944 | 5.1787 | 75.08194 | 79.47394 | 77.27794 |
| 5 | 6 | 80 | 60 | 2 | 24.0463 | 24.0477 | 3.695 | 3.7781 | 56.69637 | 57.96808 | 57.33223 |
| 6 | 6 | 80 | 60 | 6 | 24.0585 | 24.0817 | 3.439 | 3.214 | 52.74152 | 49.24337 | 50.99245 |
| 7 | 6 | 80 | 70 | 2 | 24.0648 | 24.0727 | 3.679 | 3.996 | 56.40746 | 61.24769 | 58.82758 |
| 8 | 6 | 80 | 70 | 6 | 24.0467 | 24.0618 | 4.944 | 5.1296 | 75.85986 | 78.65828 | 77.25907 |
| 9 | 8 | 50 | 60 | 2 | 24.0577 | 24.0551 | 2.574 | 2.925 | 39.47694 | 44.86501 | 42.17097 |
| 10 | 8 | 50 | 60 | 6 | 24.0612 | 24.0591 | 4.9088 | 5.4796 | 75.27436 | 84.03467 | 79.65452 |
| 11 | 8 | 50 | 70 | 2 | 24.0703 | 24.0458 | 3.0372 | 2.789 | 46.55657 | 42.79552 | 44.67604 |
| 12 | 8 | 50 | 70 | 6 | 24.0801 | 24.0803 | 3.379 | 3.5432 | 51.77486 | 54.29037 | 53.03262 |
| 13 | 8 | 80 | 60 | 2 | 24.0501 | 24.0453 | 4.2194 | 4.738 | 64.73257 | 72.70326 | 68.71792 |
| 14 | 8 | 80 | 60 | 6 | 24.0882 | 24.0505 | 5.0339 | 4.354 | 77.1062 | 66.79645 | 71.95132 |
| 15 | 8 | 80 | 70 | 2 | 24.0746 | 24.0537 | 3.5133 | 3.7852 | 53.84498 | 58.06253 | 55.95376 |
| 16 | 8 | 80 | 70 | 6 | 24.0443 | 24.0555 | 4.1132 | 4.214 | 63.11851 | 64.63521 | 63.87686 |

**4. Banaba Dried Fruits extracted by MAE with Ethanol as a solvent**

| Run | Ratio | Power | Tempt | Time | Weight fruit | | Weight of oil | | Yield | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Trial 1 | Trial 2 | Trial 1 | Trial 2 | Trial 1 | Trial 2 | Average |
| 1 | 6 | 50 | 60 | 2 | 18.153 | 18.1645 | 1.7734 | 1.4067 | 26.752 | 21.20684 | 23.97942 |
| 2 | 6 | 50 | 60 | 6 | 18.1558 | 18.1557 | 2.9161 | 2.2252 | 43.98302 | 33.56248 | 38.77275 |
| 3 | 6 | 50 | 70 | 2 | 18.1565 | 18.1587 | 1.6007 | 2.2321 | 24.14214 | 33.66099 | 28.90157 |
| 4 | 6 | 50 | 70 | 6 | 18.1586 | 18.1723 | 2.0837 | 2.24 | 31.42323 | 33.75485 | 32.58904 |
| 5 | 6 | 80 | 60 | 2 | 18.1609 | 18.1736 | 2.9882 | 2.1091 | 45.05783 | 31.78002 | 38.41893 |
| 6 | 6 | 80 | 60 | 6 | 18.1579 | 18.153 | 2.975 | 3.2711 | 44.86621 | 49.34503 | 47.10562 |
| 7 | 6 | 80 | 70 | 2 | 18.2507 | 18.1543 | 1.7981 | 2.1091 | 26.9794 | 31.81381 | 29.39661 |
| 8 | 6 | 80 | 70 | 6 | 18.1804 | 18.1823 | 1.8903 | 2.41 | 28.47248 | 36.29662 | 32.38455 |
| 9 | 8 | 50 | 60 | 2 | 18.1583 | 18.16 | 3.1107 | 3.091 | 46.91168 | 46.61022 | 46.76095 |
| 10 | 8 | 50 | 60 | 6 | 18.1827 | 18.1605 | 2.0309 | 2.5603 | 30.58639 | 38.60656 | 34.59647 |
| 11 | 8 | 50 | 70 | 2 | 18.1774 | 18.1494 | 2.0309 | 2.5153 | 30.5953 | 37.9512 | 34.27325 |
| 12 | 8 | 50 | 70 | 6 | 18.1578 | 18.1532 | 2.2665 | 2.0506 | 34.18145 | 30.93327 | 32.55736 |
| 13 | 8 | 80 | 60 | 2 | 18.1548 | 18.16 | 2.3807 | 2.2765 | 35.90965 | 34.3281 | 35.11888 |
| 14 | 8 | 80 | 60 | 6 | 18.1672 | 18.19 | 2.4784 | 2.298 | 37.35781 | 34.59516 | 35.97648 |
| 15 | 8 | 80 | 70 | 2 | 18.1645 | 18.1603 | 2.2936 | 1.8506 | 34.57739 | 27.90535 | 31.24137 |
| 16 | 8 | 80 | 70 | 6 | 18.1593 | 18.1712 | 2.0054 | 2.107 | 30.24126 | 31.75257 | 30.99692 |

## APPENDIX I

### 1. Analysis of Variance (ANOVA) for Fresh leaves extracted by MAE with ethyl acetate and ethanol as a solvent

**General Linear Model: Yield versus Solvent, Temperature,**

| Factor | Levels | Values |
|---|---|---|
| Solvent : | 2 | 1(ethyl acetate), 2(ethanol) |
| Temperature | 2 | 50, 70 |
| Time | 2 | 2, 6 |
| Power | 2 | 30, 80 |
| Ratio | 2 | 6, 10 |

| Source | DF | SS | MS | F value | P values | Decision |
|---|---|---|---|---|---|---|
| A-Solvent | 1 | 30522.3 | 30522.3 | 922.76 | 0.000 | **Significant** |
| B-Temperature | 1 | 281.0 | 281.0 | 8.50 | 0.006 | **Significant** |
| C- Time | 1 | 3.0 | 3.0 | 0.09 | 0.765 | Insignificant |
| D-Power | 1 | 10.6 | 10.6 | 0.32 | 0.576 | Insignificant |
| E-Ratio | 1 | 298.4 | 298.4 | 9.02 | 0.005 | **Significant** |
| A*B | 1 | 146.1 | 146.1 | 4.42 | 0.044 | **Significant** |
| A*C | 1 | 1.8 | 1.8 | 0.06 | 0.816 | Insignificant |
| A*E | 1 | 124.6 | 124.6 | 3.77 | 0.061 | Insignificant |
| A*D | 1 | 2.3 | 2.3 | 0.07 | 0.793 | Insignificant |
| BC | 1 | 11.7 | 11.7 | 0.35 | 0.556 | Insignificant |
| BD | 1 | 44.8 | 44.8 | 1.35 | 0.253 | Insignificant |
| BE | 1 | 2.5 | 2.5 | 0.08 | 0.785 | Insignificant |
| CD | 1 | 61.5 | 61.5 | 1.86 | 0.182 | Insignificant |
| CE | 1 | 161.9 | 161.9 | 4.89 | 0.034 | **Significant** |
| ED | 1 | 42.0 | 76.1 | 76.1 | 0.268 | Insignificant |
| ABC | 1 | 0 | 0.1 | 0.0 | 0.678 | Insignificant |
| ABD | 1 | 76.1 | 76.1 | 2.30 | 0.139 | Insignificant |
| ABE | 1 | 0.1 | 0.1 | 0.00 | 0.962 | Insignificant |
| ACE | 1 | 138.1 | 138.1 | 4.18 | 0.049 | **Significant** |
| ACD | 1 | 46.9 | 46.9 | 1.42 | 0.242 | Insignificant |
| ADE | 1 | 12.6 | 12.6 | 0.38 | 0.542 | Insignificant |
| BCD | 1 | 19.6 | 19.6 | 0.59 | 0.447 | Insignificant |
| BCE | 1 | 92.3 | 92.3 | 2.79 | 0.105 | Insignificant |
| BDE | 1 | 6.0 | 6.0 | 0.18 | 0.672 | Insignificant |
| CDE | 1 | 42.1 | 42.1 | 1.27 | 0.267 | Insignificant |
| ABCD | 1 | 51.8 | 51.8 | 1.57 | 0.220 | Insignificant |
| ABCE | 1 | 1.7 | 1.7 | 0.05 | 0.821 | Insignificant |
| ABDE | 1 | 40.8 | 40.8 | 1.23 | 0.275 | Insignificant |
| ACDE | 1 | 5.1 | 5.1 | 0.15 | 0.698 | Insignificant |
| BCDE | 1 | 29.9 | 29.9 | 0.90 | 0.349 | Insignificant |
| ABCDE | 1 | 0.9 | 0.9 | 0.03 | 0.872 | Insignificant |
| Error | 32 | 1058.5 | 33.1 | | | |
| Total | 63 | 33342.9 | | | | |

## 2. Analysis of Variance (ANOVA) for Banaba leaves extracted by MAE with ethanol

**General Linear Model: Yield versus Solvent, Temperature,**

| Factor | Levels | Values |
|---|---|---|
| Status | 2 | 1(fresh), 2(Dried) |
| Ratio | 3 | 6; 8; 10 (ml/gr) |
| Power | 3 | 30; 50. 80% of maximum power |
| Time | 3 | 2; 6 minutes |
| Temperature | 3 | 50; 60; 70 |

| Source | DF | SS | MS | F value | P values | Decision |
|---|---|---|---|---|---|---|
| A-Status | 1 | 5653.24 | 5653.24 | 107.09 | 0.000 | **Significant** |
| B-Ratio | 2 | 336.06 | 168.03 | 3.18 | 0.045 | **Significant** |
| C- Power | 1 | 32.07 | 16.04 | 0.30 | 0.739 | Insignificant |
| D-Time | 1 | 253.42 | 253.42 | 4.80 | 0.031 | **Significant** |
| E-Temperature | 2 | 56.38 | 28.19 | 0.53 | 0.588 | Insignificant |
| A*B | 2 | 1371.08 | 685.54 | 12.99 | 0.000 | **Significant** |
| A*C | 2 | 81.15 | 40.57 | 0.77 | 0.466 | Insignificant |
| A*E | 2 | 316.79 | 158.39 | 3.00 | 0.054 | Insignificant |
| A*D | 1 | 5.23 | 5.23 | 0.10 | 0.754 | Insignificant |
| BC | 4 | 591.83 | 147.96 | 2.80 | 0.029 | Significant |
| BD | 2 | 51.39 | 25.69 | 0.49 | 0.616 | Insignificant |
| BE | 4 | 386.71 | 96.68 | 1.83 | 0.128 | Insignificant |
| CD | 2 | 93.60 | 46.80 | 0.89 | 0.415 | Insignificant |
| CE | 4 | 670.95 | 167.74 | 3.18 | 0.016 | **Significant** |
| ED | 4 | 163.12 | 81.56 | 1.55 | 0.218 | Insignificant |
| ABC | 4 | 81.05 | 20.26 | 0.38 | 0.820 | Insignificant |
| ABD | 2 | 9.86 | 4.93 | 0.09 | 0.911 | Insignificant |
| ABE | 4 | 284.10 | 71.02 | 1.35 | 0.258 | Insignificant |
| ACE | 4 | 95.33 | 23.83 | 0.45 | 0.771 | Insignificant |
| ACD | 2 | 155.62 | 77.81 | 1.47 | 0.234 | Insignificant |
| ADE | 2 | 50.51 | 25.26 | 0.48 | 0.621 | Insignificant |
| BCD | 4 | 323.68 | 80.92 | 1.53 | 0.198 | Insignificant |
| BCE | 8 | 562.59 | 70.32 | 1.33 | 0.235 | Insignificant |
| BDE | 4 | 99.38 | 24.85 | 0.47 | 0.757 | Insignificant |
| CDE | 4 | 173.01 | 43.25 | 0.82 | 0.516 | Insignificant |
| ABCD | 4 | 206.44 | 51.61 | 0.98 | 0.423 | Insignificant |
| ABCE | 8 | 402.24 | 50.28 | 0.95 | 0.477 | Insignificant |
| ABDE | 4 | 6.09 | 1.52 | 0.03 | 0.998 | Insignificant |
| ACDE | 4 | 96.67 | 24.17 | 0.46 | 0.766 | Insignificant |
| BCDE | 8 | 556.32 | 69.54 | 1.32 | 0.243 | Insignificant |
| ABCDE | 8 | 343.03 | 42.88 | 0.81 | 0.593 | Insignificant |
| Error | 108 | 5701.04 | 52.79 | | | |
| Total | 215 | 19209.99 | | | | |

## 3. Analysis of Variance (ANOVA) for Banaba Fruit extracted by MAE with ethanol

**General Linear Model: Yield versus Solvent, Temperature,**

| Factor | Levels | Values |
|---|---|---|
| Status | 2 | 1(fresh), 2(Dried) |
| Temperature | 2 | 60, 70 |
| Time | 2 | 2, 6 |
| Power | 2 | 50, 80 |
| Ratio | 2 | 6, 8 |

| Source | DF | SS | MS | F value | P values | Decision |
|---|---|---|---|---|---|---|
| A-Solvent | 1 | 11239.82 | 11239.82 | 615.17 | 0.000 | **Significant** |
| B-Temperature | 1 | 3.15 | 3.15 | 0.17 | 0.681 | **Insignificant** |
| C- Time | 1 | 104.49 | 104.49 | 5.72 | 0.023 | **Significant** |
| D-Power | 1 | 245.46 | 245.46 | 13.43 | 0.001 | **Significant** |
| E-Ratio | 1 | 771.03 | 771.03 | 42.20 | 0.000 | **Significant** |
| A*B | 1 | 45.72 | 45.72 | 2.50 | 0.124 | Significant |
| A*C | 1 | 37.43 | 37.43 | 2.05 | 0.162 | Insignificant |
| A*E | 1 | 373.39 | 373.39 | 20.44 | 0.000 | **Significant** |
| A*D | 1 | 72.72 | 72.72 | 3.98 | 0.055 | Insignificant |
| BC | 1 | 8.03 | 8.03 | 0.44 | 0.512 | Insignificant |
| BD | 1 | 342.35 | 342.35 | 18.74 | 0.000 | **Significant** |
| BE | 1 | 34.84 | 34.84 | 1.91 | 0.177 | Insignificant |
| CD | 1 | 8.00 | 8.00 | 0.44 | 0.513 | Insignificant |
| CE | 1 | 99.99 | 99.99 | 5.47 | 0.026 | **Significant** |
| ED | 1 | 28.31 | 28.31 | 1.55 | 0.222 | Insignificant |
| ABC | 1 | 474.90 | 474.90 | 25.99 | 0.000 | **Significant** |
| ABD | 1 | 373.00 | 373.00 | 20.41 | 0.000 | **Significant** |
| ABE | 1 | 249.91 | 249.91 | 13.68 | 0.001 | **Significant** |
| ACE | 1 | 191.63 | 191.63 | 10.49 | 0.003 | **Significant** |
| ACD | 1 | 126.21 | 126.21 | 6.91 | 0.013 | **Significant** |
| ADE | 1 | 81.90 | 81.90 | 4.48 | 0.042 | **Significant** |
| BCD | 1 | 2.71 | 2.71 | 2.71 | 0.703 | Insignificant |
| BCE | 1 | 0.01 | 0.01 | 0.00 | 0.982 | Insignificant |
| BDE | 1 | 165.54 | 165.54 | 9.06 | 0.005 | **Significant** |
| CDE | 1 | 36.11 | 36.11 | 1.98 | 0.169 | Insignificant |
| ABCD | 1 | 154.51 | 154.51 | 8.46 | 0.007 | **Significant** |
| ABCE | 1 | 115.46 | 115.46 | 6.32 | 0.017 | **Significant** |
| ABDE | 1 | 672.97 | 672.97 | 36.83 | 0.000 | **Significant** |
| ACDE | 1 | 82.47 | 82.47 | 4.51 | 0.041 | **Significant** |
| BCDE | 1 | 26.25 | 26.25 | 1.44 | 0.239 | Insignificant |
| ABCDE | 1 | 185.03 | 185.03 | 10.13 | 0.003 | **Significant** |
| Error | 32 | 584.68 | 18.27 | | | |
| Total | 63 | 16938.02 | | | | |

*DF: Degree of Freedom. SS= Sum of square, MS: Mean Square.

**Note**:

- Degree of freedom (DF) = the number level -1 For example test at 2 level of time therefore DF=2-1=1.

- Sum of square:

$$SS_E = \sum_{i=1}^{n} e_i^2 = \sum_{i=1}^{n} (y_i - \hat{y}_i)^2$$

- **Mean of Square**

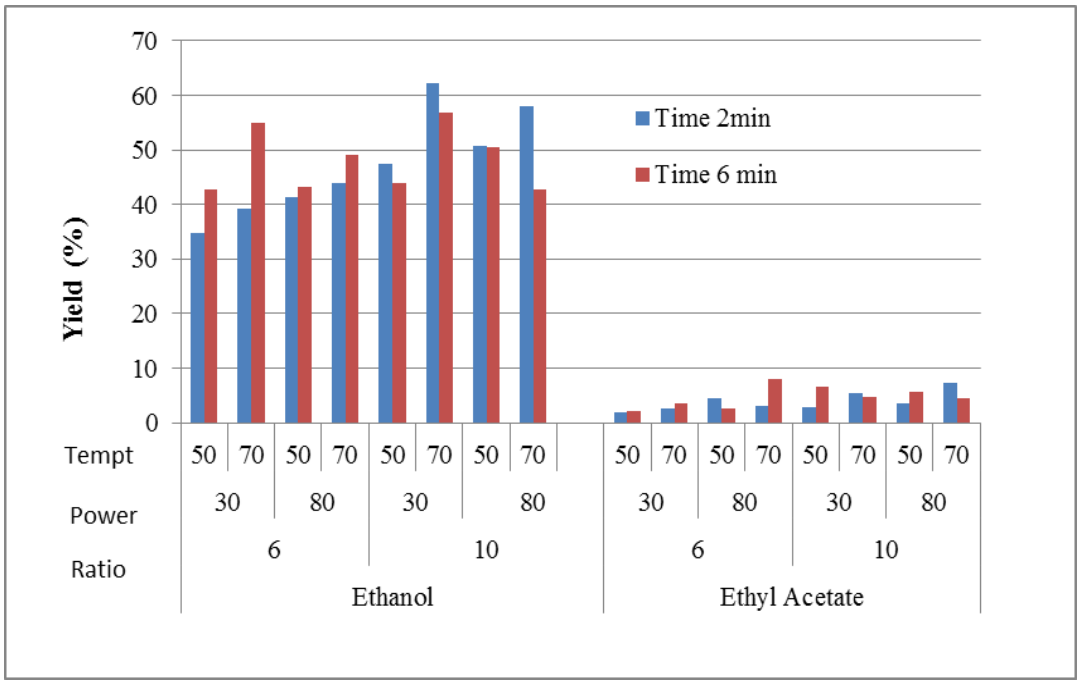
$$MS_E = \frac{SS_E}{dof(SS_E)}$$

**DF=dof(SSE)**

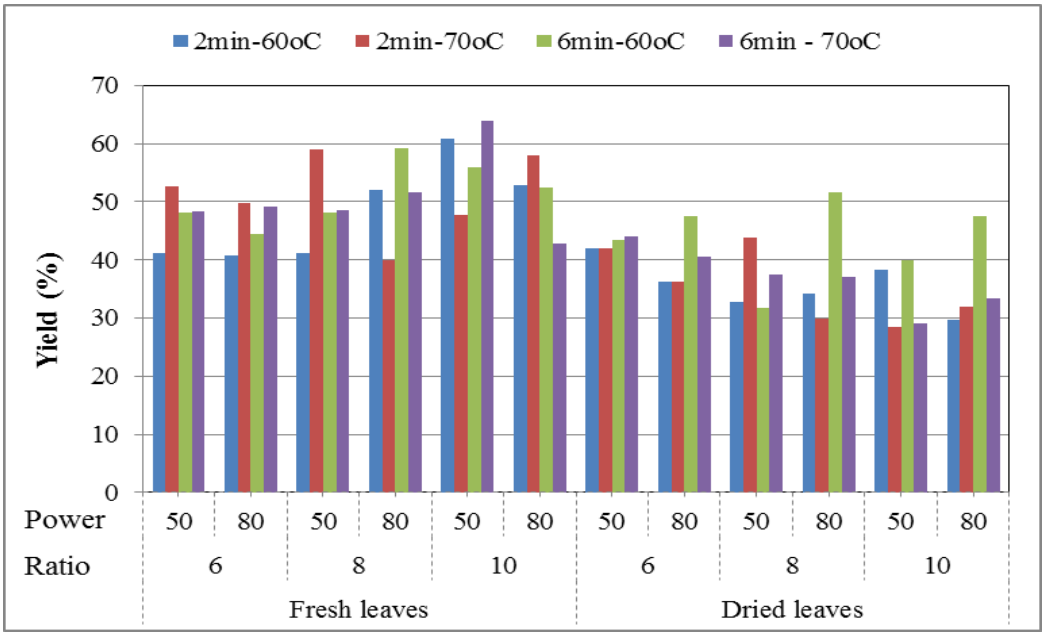
Because DF=1 therefore, SS=MS (Table J.1)

# APPENDIX J

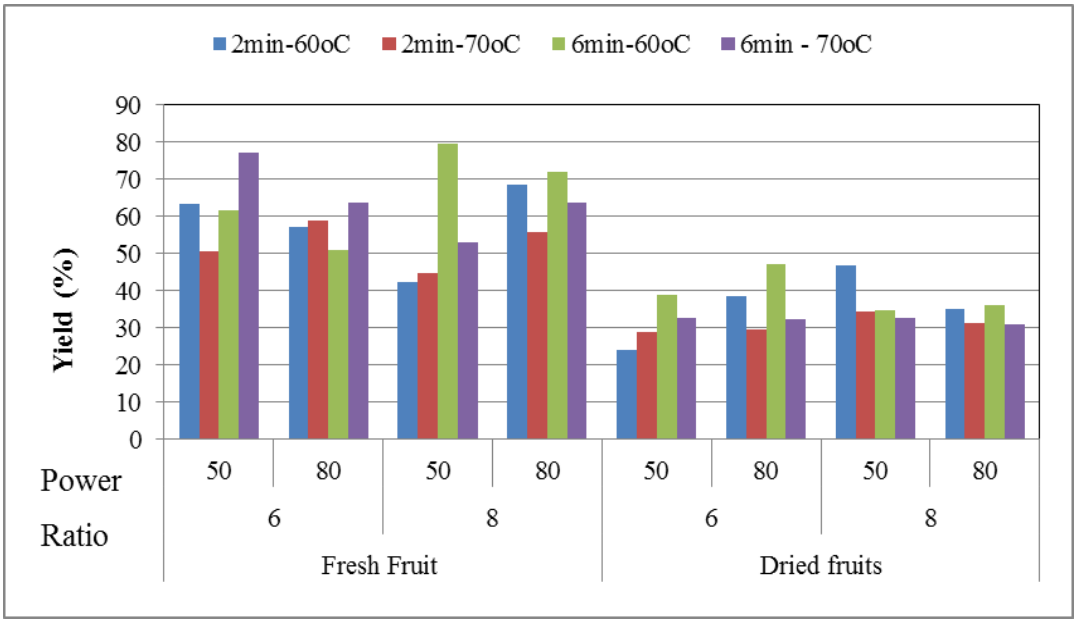
## 1. Influence of Solvent on Fresh Banaba Leaves



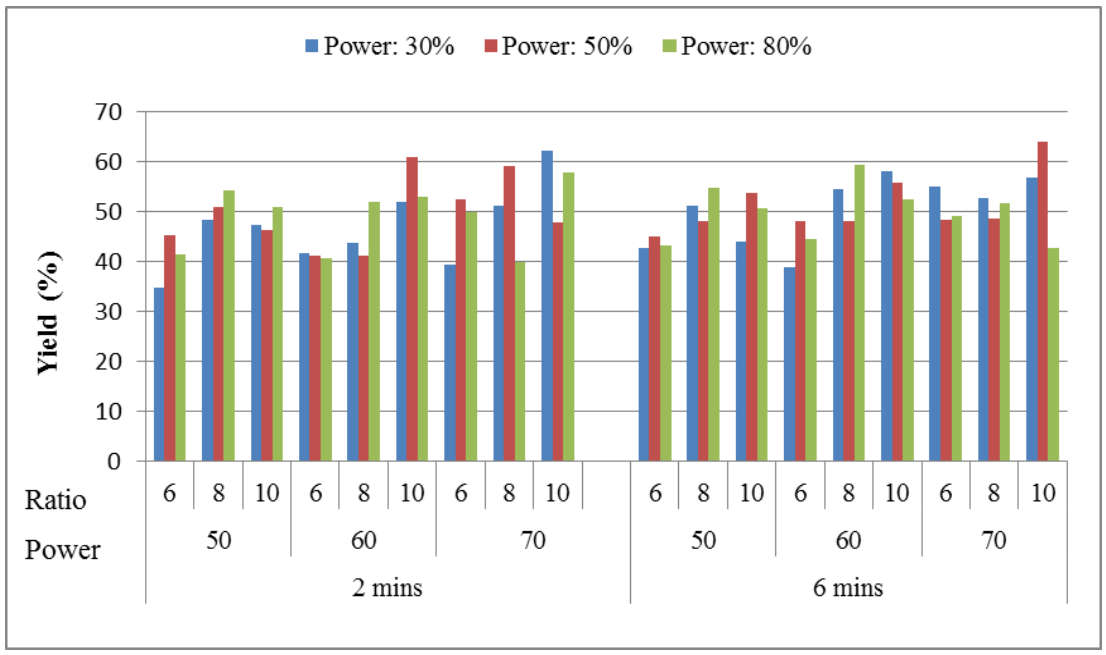

## 2. Effect of Nature of Banaba Leaves on Yield



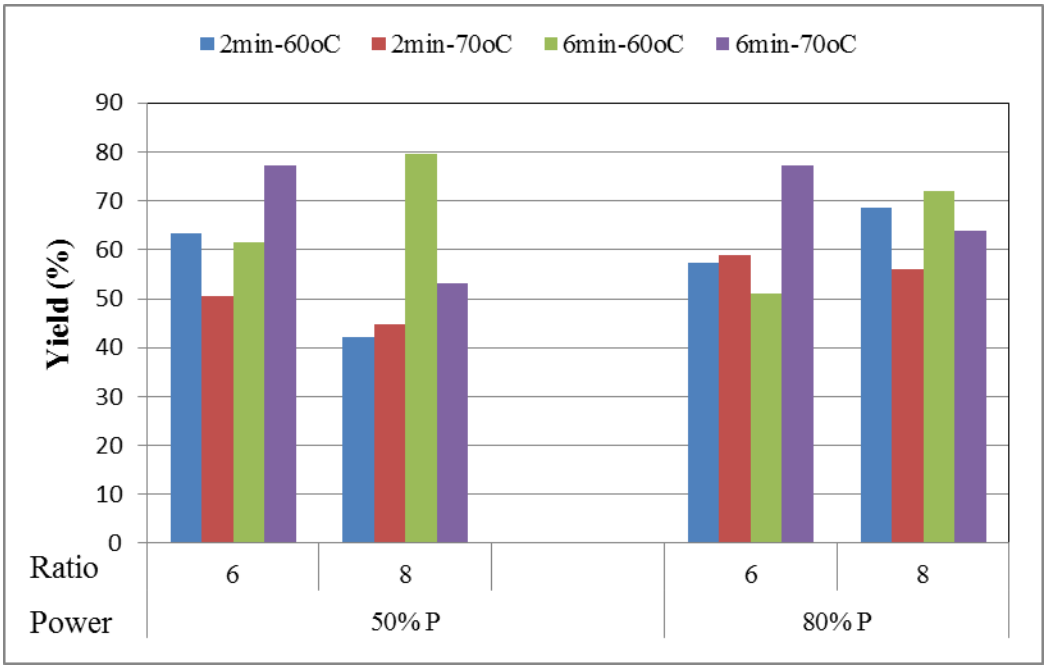
### 3. Effect of Nature of Banaba Fruits on Yield



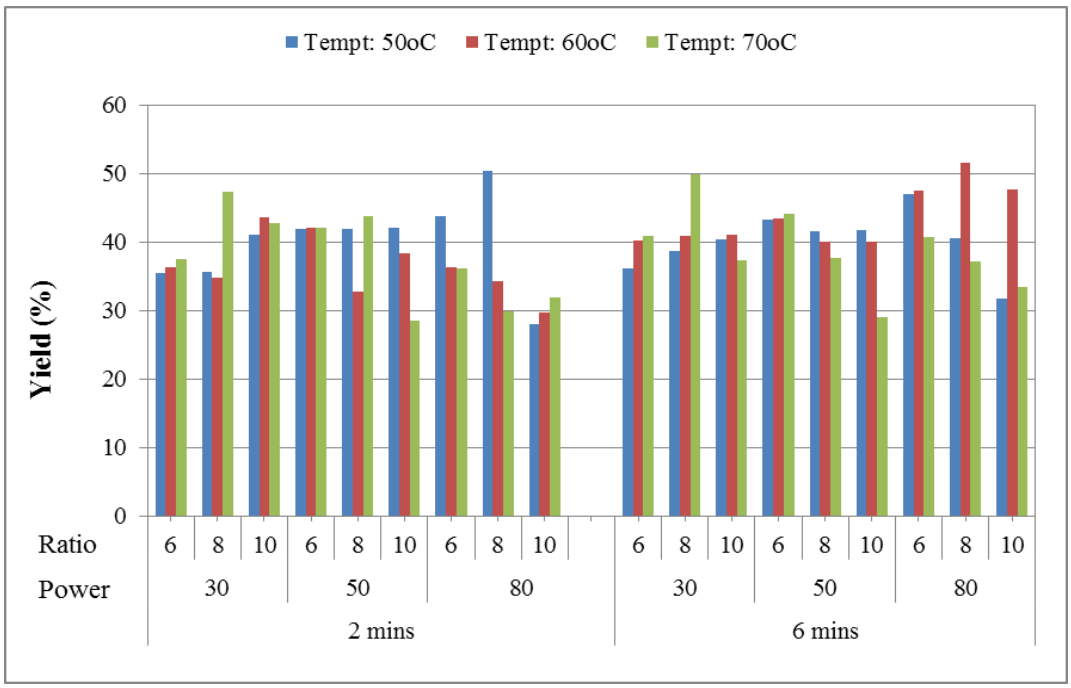

### 4. Effect of Power and Time on Yield of Fresh Banaba Leaves



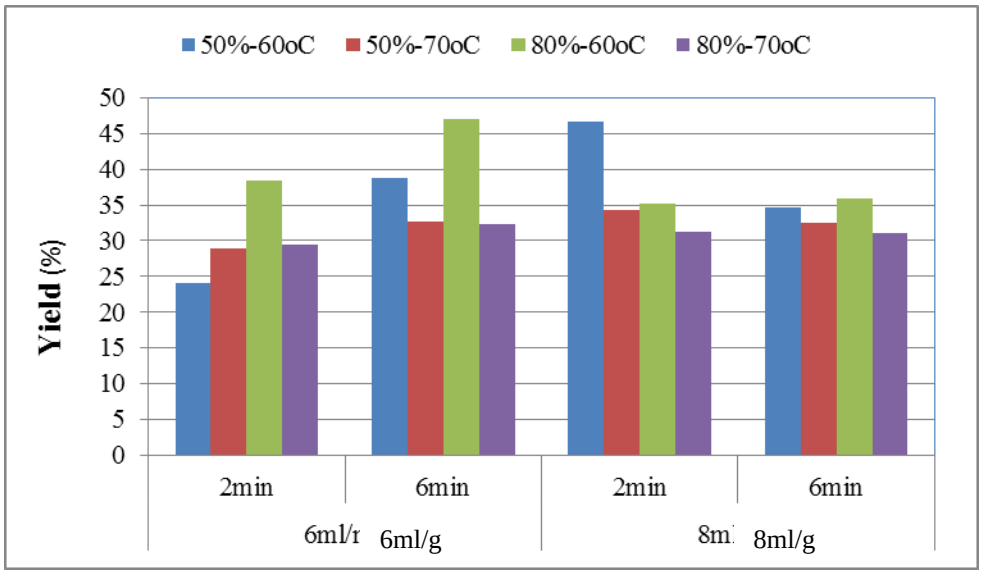
## 5. Effect of Parameters on Yield of Fresh Banaba Fruits




## 6. Effect of Parameters on Yield of Dried Banaba Leaves



## 7. Effect of Parameters on Yield of Dried Banaba Fruits




**Note: Microwave power: 30% of maximum power oven (300W)**
**50% of maximum power oven (500W)**
**80% of maximum power oven (800W)**

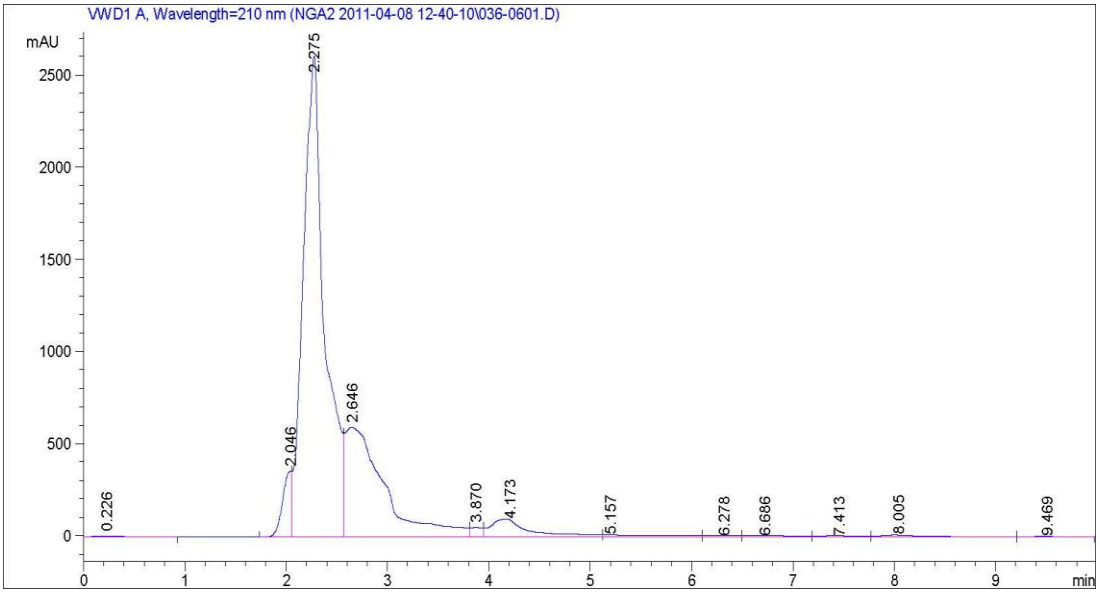
# Appendix K
# HPLC of some conditions

## 1. Banaba Dried leaves - MASE with ethanol as a solvent Power: 800W; Temperature: 60°C; Time: 6 minutes, Ratio: 1:8. Trial 1.

Data File C:\CHEM32\1\DATA\NGA2 2011-04-08 12-40-10\036-0601.D Sample Name: Sample 5

==========

========== Acq. Operator : Francis Seq. Line : 6 Acq. Instrument : Instrument 1 Location : Vial 36 Injection Date : 4/8/2011 1:38:20 PM Inj : 1Inj Volume : 25.0 µlAcq. Method : C:\CHEM32\1\DATA\NGA2 2011-04-08 12-40-10\KAREN.M Last changed : 3/4/2011 10:18:29 AM by BingAnalysis Method : C:\CHEM32\1\DATA\DEF_LC 2011-04-15 12-14-53\KAREN.MLast changed : 3/4/2011 10:18:29 AM by Bing

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Area Percent Report

==========

Sorted By : SignalMultiplier: : 1.0000 Dilution: : 1.0000 Use Multiplier & Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=210 nm

Peak RetTime Type Width Area Height Area
  # [min] [min] mAU *s [mAU ] % ----|-------|----|-------
|----------|----------|--------|
      1 0.226 BB 0.2860 82.80219 4.50065 0.1351
       2 2.046 BV 0.0936 2135.30103 357.06326 3.4846
       3 2.275 VV 0.1935 3.86933e4 2606.89575 63.1431
       4 2.646 VV 0.3680 1.60392e4 595.74896 26.1741
       5 3.870 VV 0.1123 423.53381 52.08036 0.6912
       6 4.173 VB 0.3350 2581.31934 98.46072 4.2124

        Instrument 1 4/15/2011 2:25:39 PM Francis Page 1 of 2
Data File C:\CHEM32\1\DATA\NGA2 2011-04-08 12-40-10\036-0601.D Sample
Name: Sample 5

Peak RetTime Type Width Area Height Area # [min] [min] mAU *s
  [mAU ] % ----|-------|----|-------|----------|----------|------
  --|

       7   5.157 BV   0.5464   496.05127   11.44672   0.8095
       8   6.278 VV   0.3324   138.98213    6.24530   0.2268
       9   6.686 VV   0.4771   216.23660    6.37771   0.3529
      10   7.413 VV   0.3679   147.88136    5.46936   0.2413
      11   8.005 VV   0.3665   280.80588   10.43055   0.4582
      12  9.469 VBA   0.3074   43.35938     2.06658   0.0708

           Totals :      6.12788e4      3756.78593

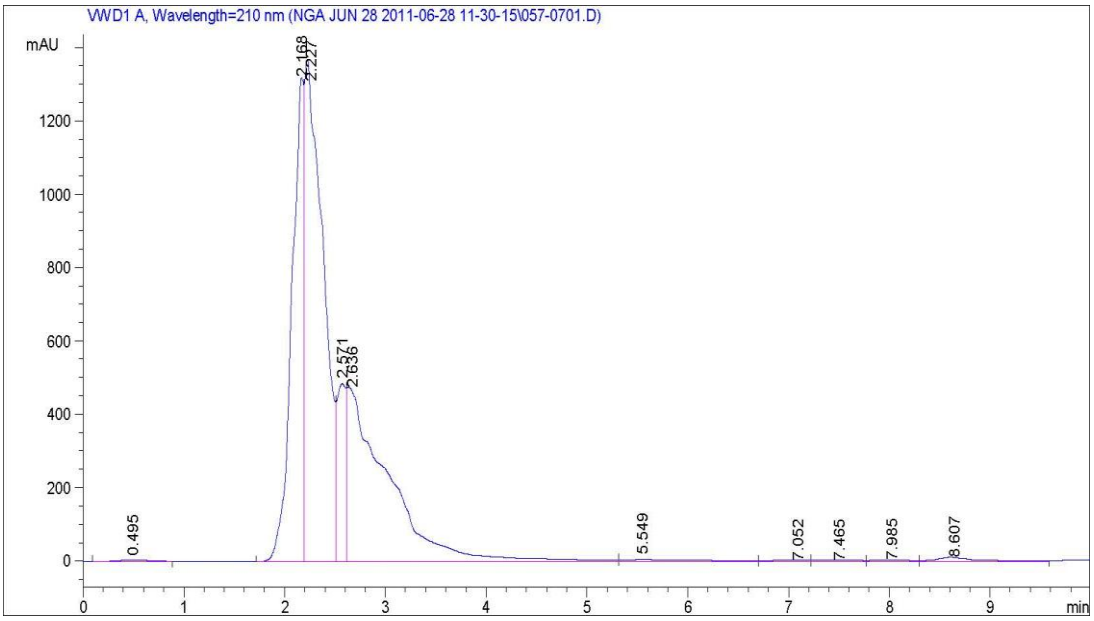
============================================================
============ *** End of Report *** Instrument 1 4/15/2011
2:25:39 PM Francis Page 2 of 2

2. **Banaba Dried leaves: -MASE with ethanol as a solvent Power: 800W; Temperature: 60°C; Time: 6 minutes, Ratio: 1:8. Trial 2.**

Data File C:\CHEM32\1\DATA\NGA JUN 28 2011-06-28 11-30-15\057-0701.D Sample Name: Sample 6

==========

========= Acq. Operator : Francis Seq. Line : 7 Acq. Instrument : Instrument 1 Location : Vial 57 Injection Date : 6/28/2011 12:39:41 PM Inj : 1 Inj Volume : 25.0 µlAcq. Method : C:\CHEM32\1\DATA\NGA JUN 28 2011-06-28 11-30-15\KAREN.M Last changed : 3/4/2011 10:18:29 AM by BingAnalysis Method : C:\CHEM32\1\METHODS\KAREN.MLast changed : 3/4/2011 10:18:29 AM by Bing

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Area Percent Report

==========

Sorted By : SignalMultiplier: : 1.0000 Dilution: : 1.0000 Use Multiplier & Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=210 nm

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|

|----------|----------|--------|
1 0.495 BV 0.3757 80.58770 3.24401 0.1770
2 2.168 BV 0.1050 1.03995e4 1320.74390 22.8429
3 2.227 VV 0.1645 1.77623e4 1369.06567 39.0155
4 2.571 VV 0.0864 2930.39014 485.94073 6.4367
5 2.636 VB 0.3641 1.34770e4 475.57733 29.6028
6 5.549 BV 0.6279 292.64374 5.82958 0.6428


Data File C:\CHEM32\1\DATA\NGA JUN 28 2011-06-28 11-30-15\057-0701.D
Sample Name: Sample 6

| Peak | RetTime | Type | Width | Area | Height | Area |
|---|---|---|---|---|---|---|
| # | [min] | | [min] | mAU *s | [mAU ] | % |
| 7 | 7.052 | VV | 0.3648 | 79.94364 | 2.93097 | 0.1756 |
| 8 | 7.465 | VV | 0.3778 | 101.24651 | 3.56648 | 0.2224 |
| 9 | 7.985 | VV | 0.3302 | 92.25398 | 3.97838 | 0.2026 |
| 10 | 8.607 | VB | 0.3972 | 310.27350 | 10.86733 | 0.6815 |
| Totals : | | | | 4.55261e4 | 3681.74437 | |

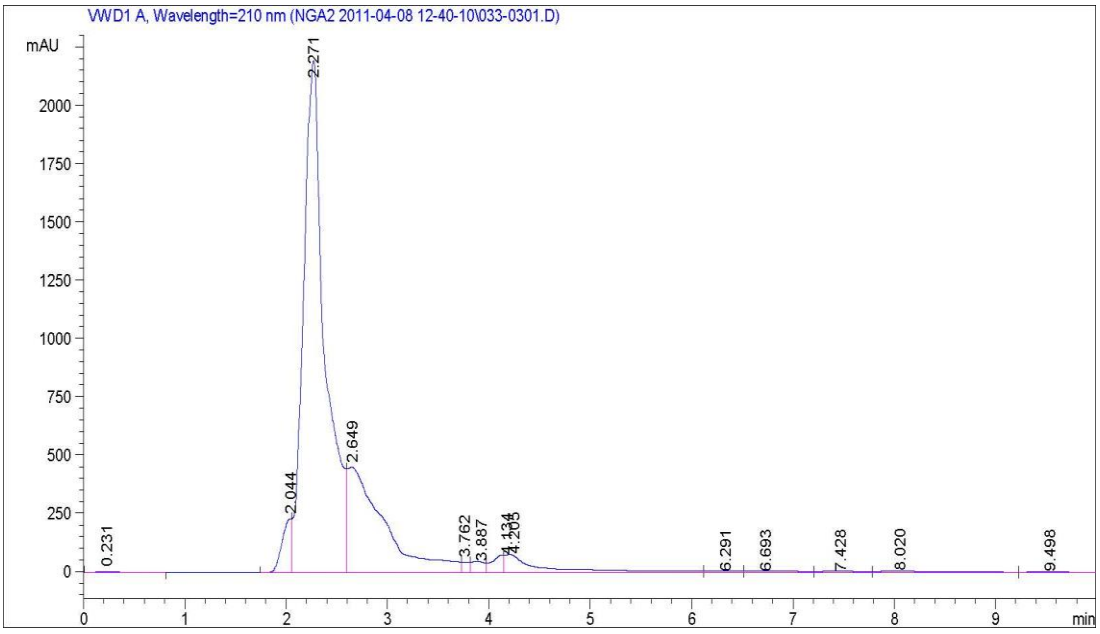
==========
========== *** End of Report *** 

**3. Banaba Fresh leaves: - MASE with ethanol as a solvent Power: 50)W; Temperature: 70°C; Time: 6 minutes, Ratio: 1:10. Trial 1.**

Data File C:\CHEM32\1\DATA\NGA2 2011-04-08 12-40-10\033-0301.D
Sample Name: sample 2

==========

= Acq. Operator : Francis Seq. Line : 3 Acq. Instrument : Instrument 1 Location : Vial 33 Injection Date : 4/8/2011 1:03:55 PM Inj : 1 Inj Volume : 25.0 µlAcq. Method : C:\CHEM32\1\DATA\NGA2 2011-04-08 12-40-10\KAREN.M Last changed : 3/4/2011 10:18:29 AM by BingAnalysis Method : C:\CHEM32\1\DATA\DEF_LC 2011-04-15 12-14-53\KAREN.MLast changed : 3/4/2011 10:18:29 AM by Bing

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Area Percent Report

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Sorted By : SignalMultiplier: : 1.0000
Dilution: : 1.0000 Use Multiplier &
Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=210 nm

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 1 | 0.231 | BB | 0.2923 | 54.47455 | 2.91413 | 0.1088 |
| 2 | 2.044 | BV | 0.1066 | 1464.86267 | 231.39110 | 2.9263 |
| 3 | 2.271 | VV | 0.1950 | 3.25784e4 | 2195.47583 | 65.0807 |
| 4 | 2.649 | VV | 0.3369 | 1.15220e4 | 451.66858 | 23.0171 |
| 5 | 3.762 | VV | 0.0710 | 225.02185 | 45.37809 | 0.4495 |
| 6 | 3.887 | VV | 0.1270 | 442.00522 | 49.02579 | 0.8830 |



Data File C:\CHEM32\1\DATA\NGA2 2011-04-08 12-40-10\033-0301.D Sample Name: sample 2

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 7 | 4.134 | VV | 0.1147 | 597.64374 | 75.06497 | 1.1939 |
| 8 | 4.205 | VB | 0.3464 | 2094.13892 | 77.71166 | 4.1834 |
| 9 | 6.291 | BV | 0.3228 | 159.35651 | 6.91387 | 0.3183 |
| 10 | 6.693 | VV | 0.5061 | 240.70241 | 6.61480 | 0.4808 |
| 11 | 7.428 | VV | 0.3971 | 180.33199 | 6.05344 | 0.3602 |
| 12 | 8.020 | VV | 0.5478 | 364.13800 | 8.55016 | 0.7274 |
| 13 | 9.498 | VBA | 0.4603 | 135.38060 | 3.98639 | 0.2704 |
| Totals : | | | | 5.00584e4 | 3160.74881 | |

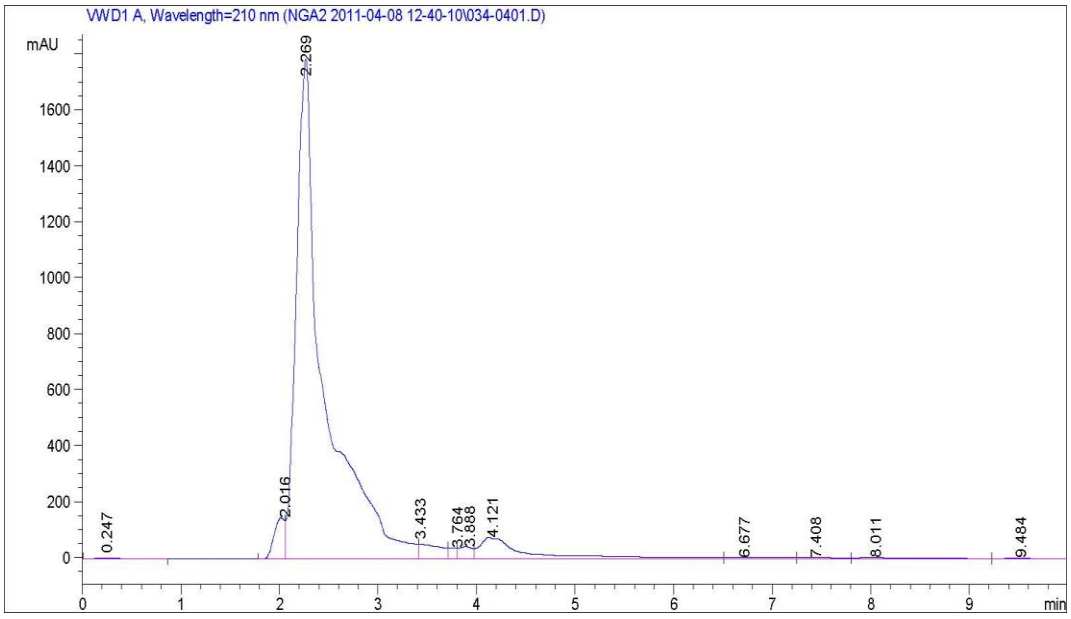
*** End of Report *** Instrument 1 4/15/2011 2:26:24 PM Francis Page 2 of 2

**4. Banaba Fresh leaves: - MASE with ethanol as a solvent Power: 500W; Temperature: 70°C; Time: 6 minutes, Ratio: 1:10. Trial 2.**

Data File C:\CHEM32\1\DATA\NGA2 2011-04-08 12-40-10\034-0401.D
Sample Name: Sample 3

==========

= Acq. Operator : Francis Seq. Line : 4 Acq. Instrument : Instrument 1 Location : Vial 34 Injection Date : 4/8/2011 1:15:24 PM Inj : 1 Inj Volume : 25.0 µlAcq. Method : C:\CHEM32\1\DATA\NGA2 2011-04-08 12-40-10\KAREN.M Last changed : 3/4/2011 10:18:29 AM by BingAnalysis Method : C:\CHEM32\1\DATA\DEF_LC 2011-04-15 12-14-53\KAREN.MLast changed : 3/4/2011 10:18:29 AM by Bing

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Area Percent Report

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Sorted By : SignalMultiplier: : 1.0000
Dilution: : 1.0000 Use Multiplier &
Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=210 nm

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 1 | 0.247 | BB | 0.3002 | 46.91645 | 2.42233 | 0.1141 |
| 2 | 2.016 | BV | 0.1177 | 1064.24829 | 147.37685 | 2.5881 |
| 3 | 2.269 | VV | 0.2514 | 3.51197e4 | 1785.07117 | 85.4054 |
| 4 | 3.433 | VV | 0.2138 | 818.95905 | 52.13876 | 1.9916 |
| 5 | 3.764 | VV | 0.0807 | 224.95418 | 39.68613 | 0.5471 |
| 6 | 3.888 | VV | 0.1306 | 407.00650 | 43.67360 | 0.9898 |



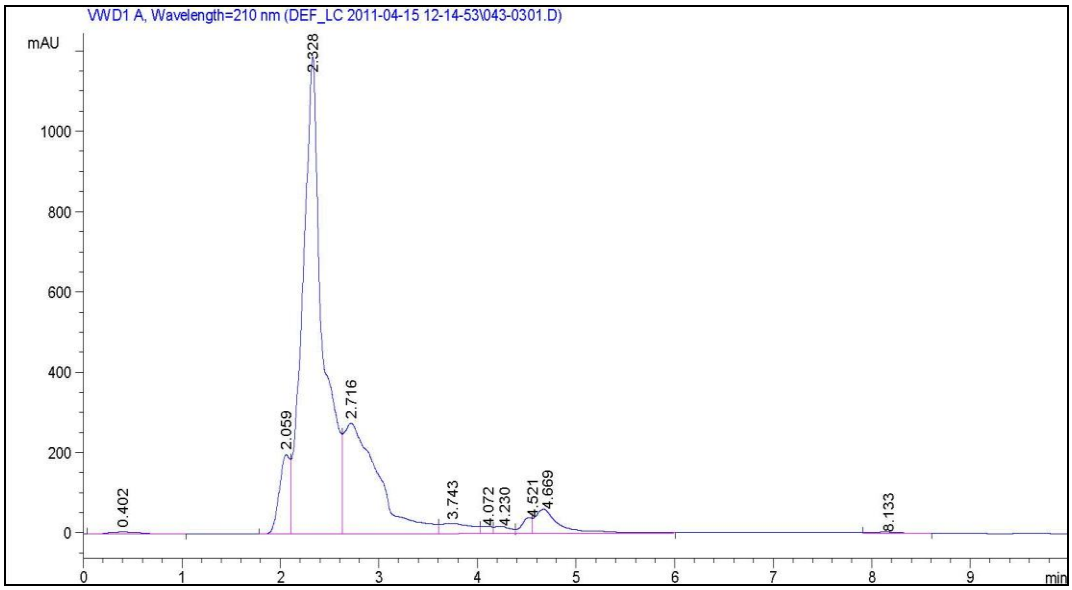
Data File C:\CHEM32\1\DATA\NGA2 2011-04-08 12-40-10\034-0401.D Sample Name: Sample 3

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 7 | 4.121 | VB | 0.4394 | 2711.55396 | 76.29250 | 6.5941 |
| 8 | 6.677 | BV | 0.5515 | 224.86765 | 5.59903 | 0.5468 |
| 9 | 7.408 | VV | 0.4180 | 140.94846 | 4.57047 | 0.3428 |
| 10 | 8.011 | VV | 0.6727 | 264.49670 | 4.98613 | 0.6432 |
| 11 | 9.484 | VBA | 0.4789 | 97.53752 | 2.65934 | 0.2372 |
| | | Totals : | | 4.11212e4 | 2164.47630 | |

==========

5. **Banaba Fresh leaves: - MASE with Ethyl Acetate as a solvent Power: 800W; Temperature: 70$^{o}$C; Time: 6 minutes, Ratio: 1:6. Trial 1.**

Data File C:\CHEM32\1\DATA\DEF_LC 2011-04-15 12-14-53\043-0301.D
Sample Name: DE30
==========
=
Acq. Operator : Francis Seq. Line : 3
Acq. Instrument : Instrument 1 Location : Vial 43
Injection Date : 4/15/2011 12:38:38 PM Inj : 1 Inj Volume : 25.0
µlAcq. Method : C:\CHEM32\1\DATA\DEF_LC 2011-04-15 12-14-53\KAREN.M
Last changed : 3/4/2011 10:18:29 AM by BingAnalysis Method :
C:\CHEM32\1\DATA\DEF_LC 2011-04-15 12-14-53\043-0301.D\DA.M
(KAREN.M, FromData File)
Last changed : 4/15/2011 2:13:32 PM by Francis

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Area Percent Report
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Sorted By : SignalMultiplier: : 1.0000
Dilution: : 1.0000 Use Multiplier &
Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=210 nm

Peak RetTime Type Width Area Height Area
  # [min] [min] mAU *s [mAU ] % ----|-------|----|-------
|----------|----------|--------|
      1 0.402 BB 0.3145 106.36209 5.10285 0.3873
       2 2.059 BV 0.1083 1471.69641 198.50417 5.3596
       3 2.328 VV 0.1848 1.69071e4 1189.20349 61.5720
       4 2.716 VV 0.3108 6768.99268 276.03256 24.6512
       5 3.743 VV 0.3186 542.13074 24.29587 1.9743


Data File C:\CHEM32\1\DATA\DEF_LC 2011-04-15 12-14-53\043-0301.D
Sample Name: DE30

Peak RetTime Type Width Area Height Area # [min] [min] mAU *s
  [mAU ] % ----|-------|----|-------|----------|----------|------
  --|

| | | | | | | |
|---|---|---|---|---|---|---|
| 6 | 4.072 | VV | 0.1164 | 135.37227 | 16.95794 | 0.4930 |
| 7 | 4.230 | VV | 0.1518 | 192.64107 | 17.88928 | 0.7016 |
| 8 | 4.521 | VV | 0.1071 | 273.04147 | 38.68761 | 0.9944 |
| 9 | 4.669 | VB | 0.2367 | 1007.84949 | 59.55995 | 3.6704 |
| 10 | 8.133 | VB | 0.2308 | 53.88861 | 3.49628 | 0.1963 |

Totals : 2.74591e4 1829.73000

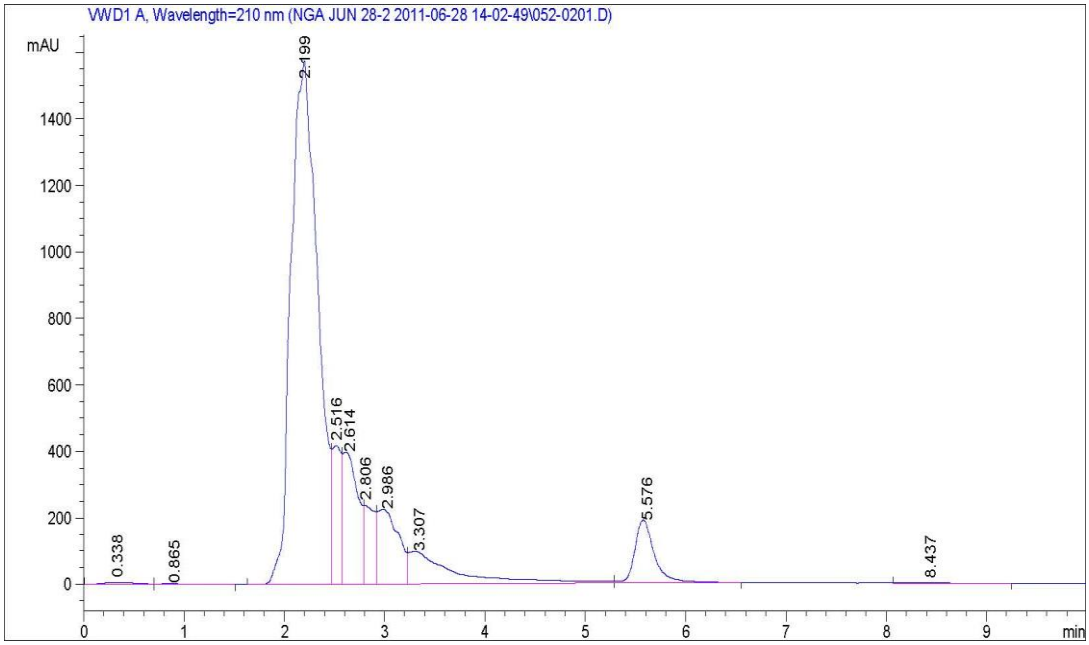
==========
========== *** End of Report *** Instrument 1 4/15/2011
2:13:34 PM Francis Page 2 of 2

6. **Banaba Fresh leaves: -MASE with Ethyl Acetate as a solvent Power: 800W; Temperature: 70°C; Time: 6 minutes, Ratio: 1:6. Trial 2**

Data File C:\CHEM32\1\DATA\NGA JUN 28-2 2011-06-28 14-02-49\052-0201.D Sample Name: fresh leaves 1

==========

= Acq. Operator : Francis Seq. Line : 2 Acq. Instrument : Instrument 1 Location : Vial 52 Injection Date : 6/28/2011 2:17:44 PM Inj : 1 Inj Volume : 25.0 µlAcq. Method : C:\CHEM32\1\DATA\NGA JUN 28-2 2011-06-28 14-02-49\KAREN.M Last changed : 3/4/2011 10:18:29 AM by BingAnalysis Method : C:\CHEM32\1\METHODS\KAREN.MLast changed : 3/4/2011 10:18:29 AM by Bing

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Area Percent Report

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Sorted By : SignalMultiplier: : 1.0000
Dilution: : 1.0000 Use Multiplier &
Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=210 nm

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 1 | 0.338 | BV | 0.3595 | 113.48009 | 4.86576 | 0.2431 |
| 2 | 0.865 | VB | 0.3584 | 46.43718 | 1.90643 | 0.0995 |
| 3 | 2.199 | BV | 0.2295 | 2.90040e4 | 1574.47632 | 62.1379 |
| 4 | 2.516 | VV | 0.0889 | 2605.16528 | 417.14465 | 5.5813 |
| 5 | 2.614 | VV | 0.1522 | 4185.11084 | 396.68753 | 8.9661 |
| 6 | 2.806 | VV | 0.0957 | 1704.21863 | 236.53397 | 3.6511 |


Data File C:\CHEM32\1\DATA\NGA JUN 28-2 2011-06-28 14-02-49\052-0201.D Sample Name: fresh leaves 1

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 7 | 2.986 | VV | 0.2040 | 3281.12134 | 224.51241 | 7.0294 |
| 8 | 3.307 | VV | 0.4048 | 3128.02588 | 98.27602 | 6.7015 |
| 9 | 5.576 | VB | 0.2044 | 2560.05151 | 189.21376 | 5.4846 |
| 10 | 8.437 | VB | 0.3780 | 49.20586 | 1.79551 | 0.1054 |
| Totals : | | | | 4.66768e4 | 3145.41236 | |

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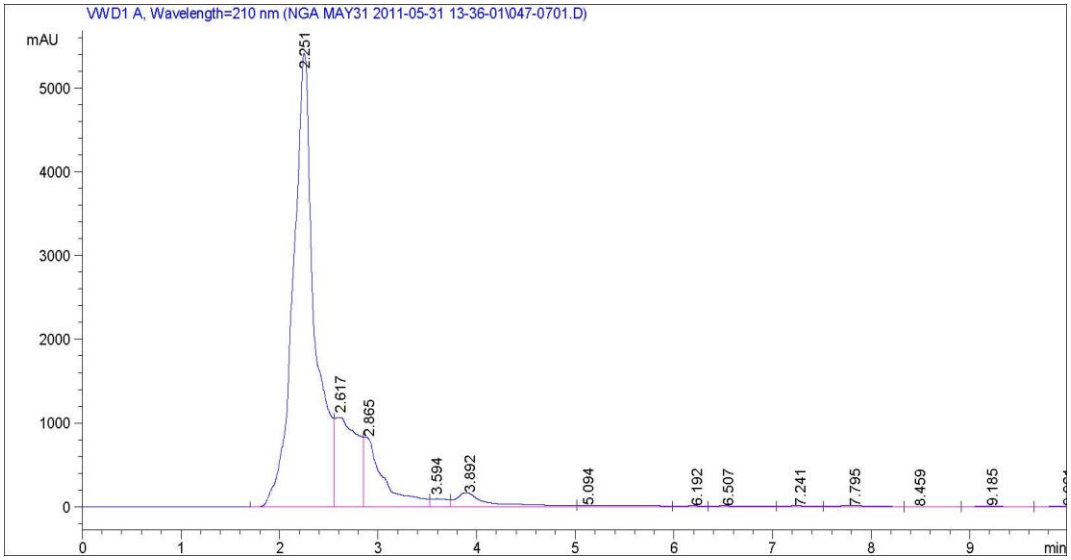
## 7. Banaba Fresh leaves: - Conventional method with Ethanol as a solvent – Trial 1

Data File C:\CHEM32\1\DATA\NGA MAY31 2011-05-31 13-36-01\047-0701.D Sample Name: Sample 6

==========

Acq. Operator : Francis Seq. Line : 7
Acq. Instrument : Instrument 1 Location : Vial 47
Injection Date : 5/31/2011 2:45:46 PM Inj : 1 Inj Volume : 25.0 µlAcq. Method : C:\CHEM32\1\DATA\NGA MAY31 2011-05-31 13-36-01\KAREN.M Last changed : 3/4/2011 10:18:29 AM by BingAnalysis Method : C:\CHEM32\1\DATA\DEF_LC 2011-06-06 09-41-57\GRONA-ROSBERO1.MLast changed : 6/3/2011 10:31:20 AM by Francis

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Area Percent Report

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Sorted By : SignalMultiplier: : 1.0000
Dilution: : 1.0000 Use Multiplier &
Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=210 nm

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 1 | 2.251 | BV | 0.2012 | 8.19074e4 | 5416.50732 | 68.7758 |
| 2 | 2.617 | VV | 0.2006 | 1.68164e4 | 1071.13574 | 14.1204 |
| 3 | 2.865 | VV | 0.1934 | 1.20017e4 | 837.55774 | 10.0775 |
| 4 | 3.594 | VV | 0.1858 | 1189.93665 | 95.73737 | 0.9992 |
| 5 | 3.892 | VV | 0.3295 | 4101.30078 | 168.25931 | 3.4438 |
| 6 | 5.094 | VV | 0.5689 | 889.31635 | 19.86401 | 0.7467 |


Data File C:\CHEM32\1\DATA\NGA MAY31 2011-05-31 13-36-01\047-0701.D
Sample Name: Sample 6

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 7 | 6.192 | VV | 0.2873 | 261.63205 | 12.52708 | 0.2197 |
| 8 | 6.507 | VV | 0.5140 | 460.29037 | 12.54339 | 0.3865 |
| 9 | 7.241 | VV | 0.3180 | 303.92975 | 13.13578 | 0.2552 |
| 10 | 7.795 | VV | 0.3893 | 499.59869 | 17.15106 | 0.4195 |
| 11 | 8.459 | VV | 0.4129 | 211.51675 | 6.60522 | 0.1776 |
| 12 | 9.185 | VV | 0.4238 | 292.43393 | 9.37144 | 0.2455 |
| 13 | 9.961 | VBA | 0.2151 | 157.96512 | 12.24229 | 0.1326 |
| Totals : | | | | 1.19093e5 | 7692.63775 | |

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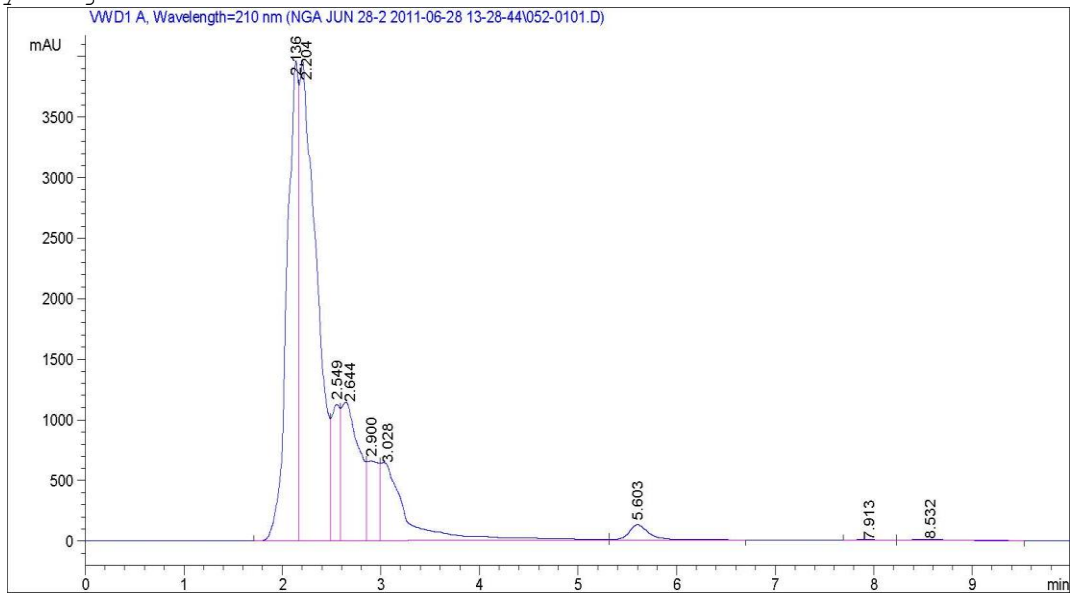
## 8. Banaba Fresh leaves: - Conventional method with Ethanol as a solvent – Trial 2

Data File C:\CHEM32\1\DATA\NGA JUN 28-2 2011-06-28 13-28-44\052-0101.D Sample Name: ethanol

==========

====== Acq. Operator : Francis Seq. Line : 1 Acq. Instrument : Instrument 1 Location : Vial 52 Injection Date : 6/28/2011 1:32:49 PM Inj : 1 Inj Volume : 25.0 µlAcq. Method : C:\CHEM32\1\DATA\NGA JUN 28-2 2011-06-28 13-28-44\KAREN.M Last changed : 3/4/2011 10:18:29 AM by BingAnalysis Method : C:\CHEM32\1\METHODS\KAREN.MLast changed : 3/4/2011 10:18:29 AM by Bing

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Area Percent Report

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Sorted By : SignalMultiplier: : 1.0000
Dilution: : 1.0000 Use Multiplier &
Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=210 nm

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 1 | 2.136 | VV | 0.1068 | 3.18242e4 | 3962.49683 | 26.5949 |
| 2 | 2.204 | VV | 0.1520 | 4.73026e4 | 3973.86621 | 39.5299 |
| 3 | 2.549 | VV | 0.0792 | 6391.96582 | 1127.55359 | 5.3417 |
| 4 | 2.644 | VV | 0.1744 | 1.46507e4 | 1142.45813 | 12.2433 |
| 5 | 2.900 | VV | 0.1197 | 5417.85742 | 655.80042 | 4.5276 |
| 6 | 3.028 | VV | 0.2386 | 1.19451e4 | 642.99225 | 9.9823 |



Data File C:\CHEM32\1\DATA\NGA JUN 28-2 2011-06-28 13-28-44\052-0101.D Sample Name: ethanol

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 7 | 5.603 | VB | 0.2236 | 1914.98511 | 126.25108 | 1.6003 |
| 8 | 7.913 | VV | 0.2414 | 55.20024 | 3.54519 | 0.0461 |
| 9 | 8.532 | VB | 0.2845 | 160.13713 | 8.53139 | 0.1338 |
| | | Totals : | | 1.19663e5 | 1.16435e4 | |

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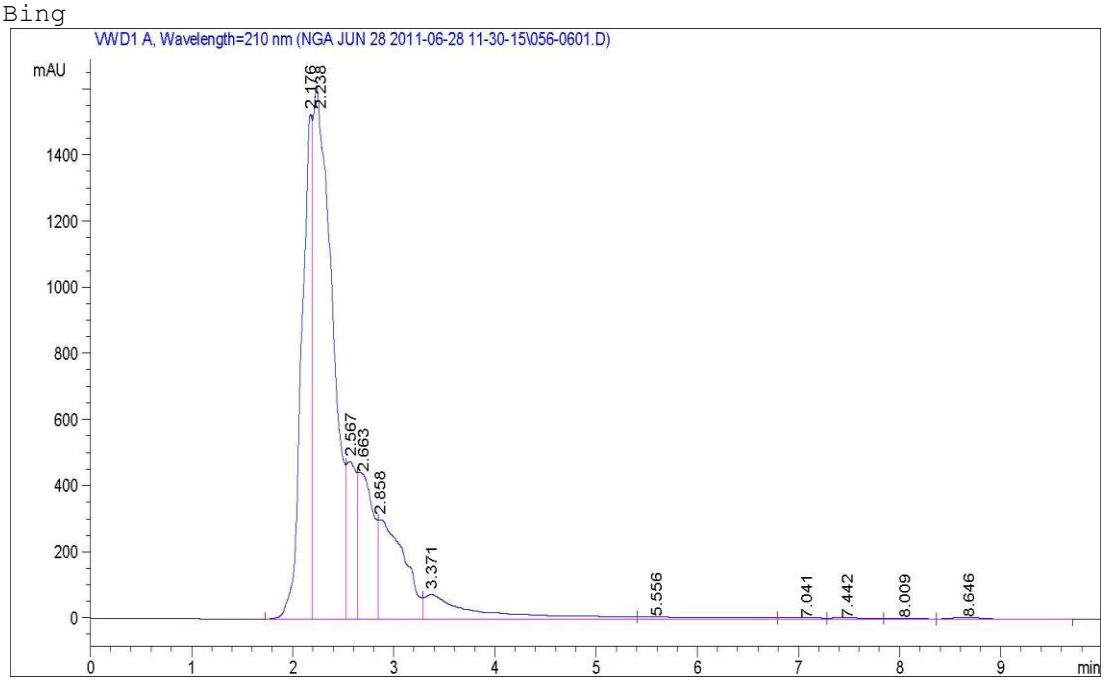
========== *** End of Report *** Instrument 1 6/28/2011 2:09:11 PM Francis Page 2 of 2

## 9. Banaba Fresh leaves: - Conventional method with Hot water as a solvent –Trial 1

Data File C:\CHEM32\1\DATA\NGA JUN 28 2011-06-28 11-30-15\056-0601.D Sample Name: sample 5

==========

==== Acq. Operator : Francis Seq. Line : 6 Acq. Instrument : Instrument 1 Location : Vial 56 Injection Date : 6/28/2011 12:28:13 PM Inj : 1 Inj Volume : 25.0 µlAcq. Method : C:\CHEM32\1\DATA\NGA JUN 28 2011-06-28 11-30-15\KAREN.M Last changed : 3/4/2011 10:18:29 AM by BingAnalysis Method : C:\CHEM32\1\METHODS\KAREN.MLast changed : 3/4/2011 10:18:29 AM by Bing

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Area Percent Report

==========

Sorted By : SignalMultiplier: : 1.0000
Dilution: : 1.0000 Use Multiplier &
Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=210 nm

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 1 | 2.176 | BV | 0.0960 | 1.08503e4 | 1526.31482 | 22.3653 |
| 2 | 2.238 | VV | 0.1666 | 2.11891e4 | 1610.86328 | 43.6765 |
| 3 | 2.567 | VV | 0.0929 | 3136.32642 | 475.90707 | 6.4648 |
| 4 | 2.663 | VV | 0.1584 | 4747.98730 | 443.32156 | 9.7869 |
| 5 | 2.858 | VV | 0.2250 | 5210.16650 | 299.14716 | 10.7395 |
| 6 | 3.371 | VB | 0.4513 | 2572.92847 | 73.63395 | 5.3035 |


Data File C:\CHEM32\1\DATA\NGA JUN 28 2011-06-28 11-30-15\056-0601.D
Sample Name: sample 5

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 7 | 5.556 | BV | 0.6738 | 410.70609 | 7.62232 | 0.8466 |
| 8 | 7.041 | VV | 0.3737 | 98.28123 | 3.61749 | 0.2026 |
| 9 | 7.442 | VV | 0.3928 | 97.26517 | 3.29146 | 0.2005 |
| 10 | 8.009 | VV | 0.3542 | 66.18832 | 2.62162 | 0.1364 |
| 11 | 8.646 | VB | 0.3695 | 134.60655 | 5.19092 | 0.2775 |
| Totals : | | | | 4.85138e4 | 4451.53166 | |

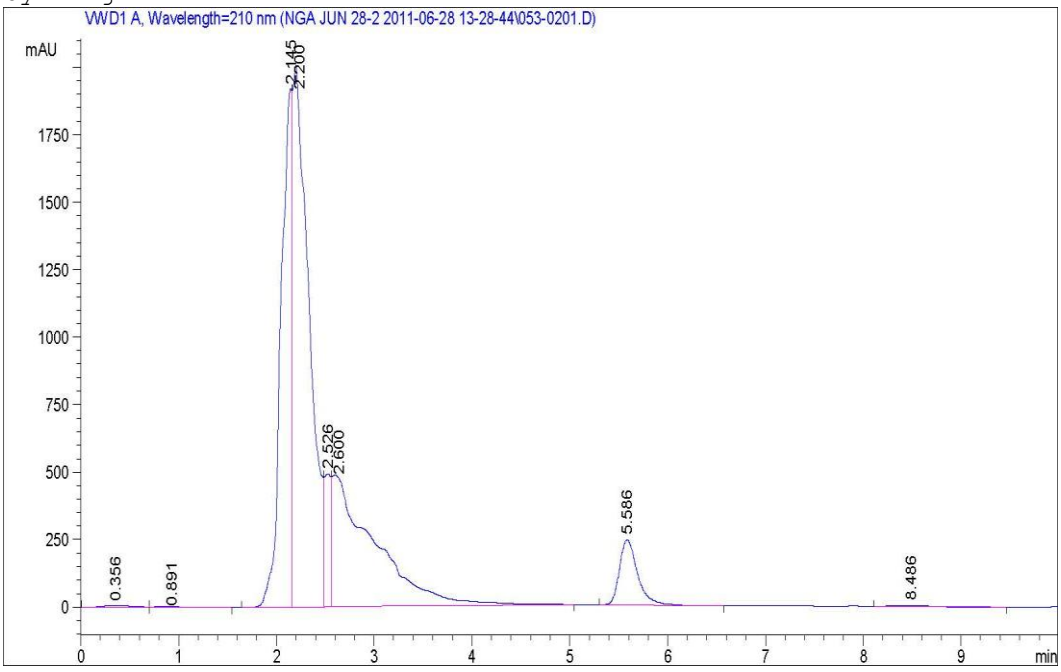
==========

## 10. Banaba Fresh leaves: - Conventional method with Hot water as a solvent –Trial 2

Data File C:\CHEM32\1\DATA\NGA JUN 28-2 2011-06-28 13-28-44\053-0201.D Sample Name: fresh leaves

==========

====== Acq. Operator : Francis Seq. Line : 2 Acq. Instrument : Instrument 1 Location : Vial 53 Injection Date : 6/28/2011 1:44:16 PM Inj : 1 Inj Volume : 25.0 µlAcq. Method : C:\CHEM32\1\DATA\NGA JUN 28-2 2011-06-28 13-28-44\KAREN.M Last changed : 3/4/2011 10:18:29 AM by BingAnalysis Method : C:\CHEM32\1\METHODS\KAREN.MLast changed : 3/4/2011 10:18:29 AM by Bing

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Area Percent Report

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Sorted By : SignalMultiplier: : 1.0000 Dilution: : 1.0000 Use Multiplier & Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=210 nm

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 1 | 0.356 | BV | 0.3526 | 130.48671 | 5.64671 | 0.2237 |
| 2 | 0.891 | VB | 0.3689 | 66.84293 | 2.69964 | 0.1146 |
| 3 | 2.145 | BV | 0.1018 | 1.43545e4 | 1920.45410 | 24.6100 |
| 4 | 2.200 | VV | 0.1510 | 2.34467e4 | 2004.99121 | 40.1982 |
| 5 | 2.526 | VV | 0.0674 | 2354.00952 | 492.48697 | 4.0358 |
| 6 | 2.600 | VB | 0.3876 | 1.47692e4 | 488.60159 | 25.3211 |

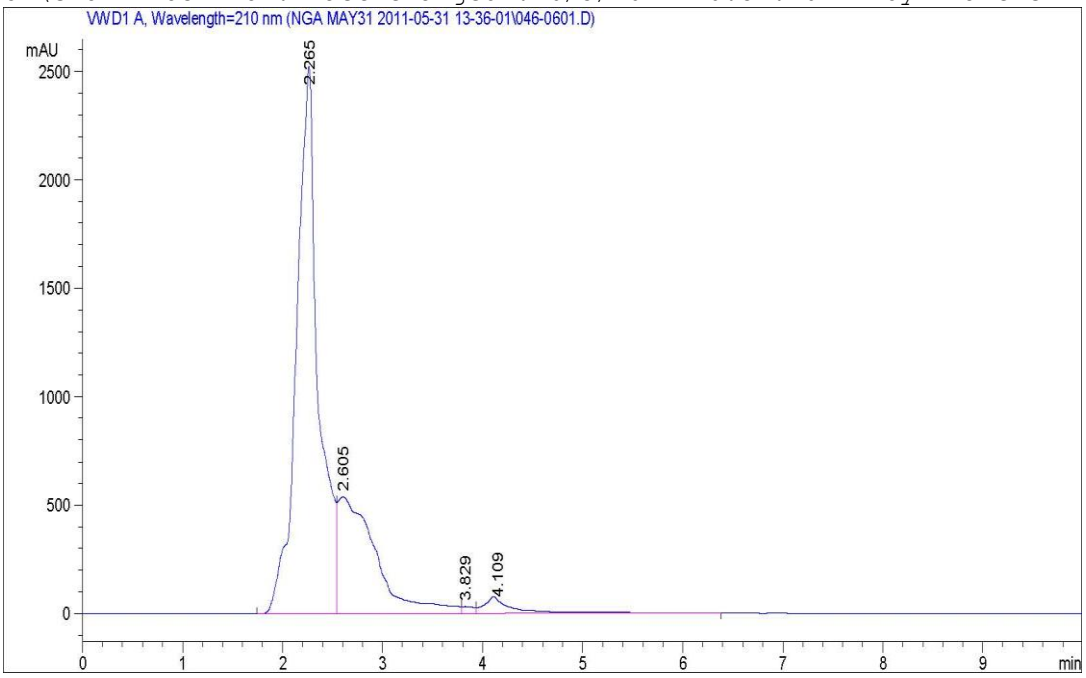

Data File C:\CHEM32\1\DATA\NGA JUN 28-2 2011-06-28 13-28-44\053-0201.D Sample Name: fresh leaves

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 7 | 5.586 | BB | 0.1952 | 3104.63306 | 241.39165 | 5.3227 |
| 8 | 8.486 | VV | 0.3101 | 101.36078 | 4.75038 | 0.1738 |
| Totals : | | | | 5.83277e4 | 5161.02225 | |

========== *** End of Report *** Instrument 1 6/28/2011 2:09:25 PM Francis Page 2 of 2

**11. Banaba Dried fruits : - MASE with ethanol as a solvent – Power; 500W, Temperature: 60$^{o}$C; time: 6min; Ratio: 6 (ml/gr)**

```
Data File C:\CHEM32\1\DATA\NGA MAY31 2011-05-31 13-36-
01\046-0601.D Sample Name: Sample 5
==================================================================
Acq. Operator : Francis Seq. Line : 6
Acq. Instrument : Instrument 1 Location : Vial 46
Injection Date : 5/31/2011 2:34:17 PM Inj : 1
 Inj Volume : 25.0 µlAcq. Method : C:\CHEM32\1\DATA\NGA MAY31 2011-
05-31 13-36-01\KAREN.M Last changed : 3/4/2011 10:18:29 AM by
BingAnalysis Method : C:\CHEM32\1\DATA\DEF_LC 2011-06-06 09-41-
57\GRONA-ROSBERO1.MLast changed : 6/3/2011 10:31:20 AM by Francis
```




```
==================================================================
Area Percent Report
==================================================================
Sorted By : SignalMultiplier: : 1.0000
Dilution: : 1.0000 Use Multiplier &
Dilution Factor with ISTDs
```

Signal 1: VWD1 A, Wavelength=210 nm

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 1 | 2.265 | BV | 0.1950 | 3.81094e4 | 2524.92896 | 70.1667 |
| 2 | 2.605 | VV | 0.3408 | 1.43196e4 | 538.58453 | 26.3651 |
| 3 | 3.829 | VV | 0.1069 | 254.71367 | 32.70899 | 0.4690 |
| 4 | 4.109 | VB | 0.2735 | 1628.92065 | 75.96590 | 2.9992 |

Totals : 5.43126e4 3172.18839



Data File C:\CHEM32\1\DATA\NGA MAY31 2011-05-31 13-36-01\046-0601.D Sample Name: Sample 5

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**12. Banaba Dried fruits : - MASE with ethanol as a solvent –Power; 500W, Temperature: 60°C; time: 6min; Ratio: 8 (ml/gr)**

Data File C:\CHEM32\1\DATA\DEF_LC 2011-04-15 12-14-53\046-0601.D
Sample Name: FRE01

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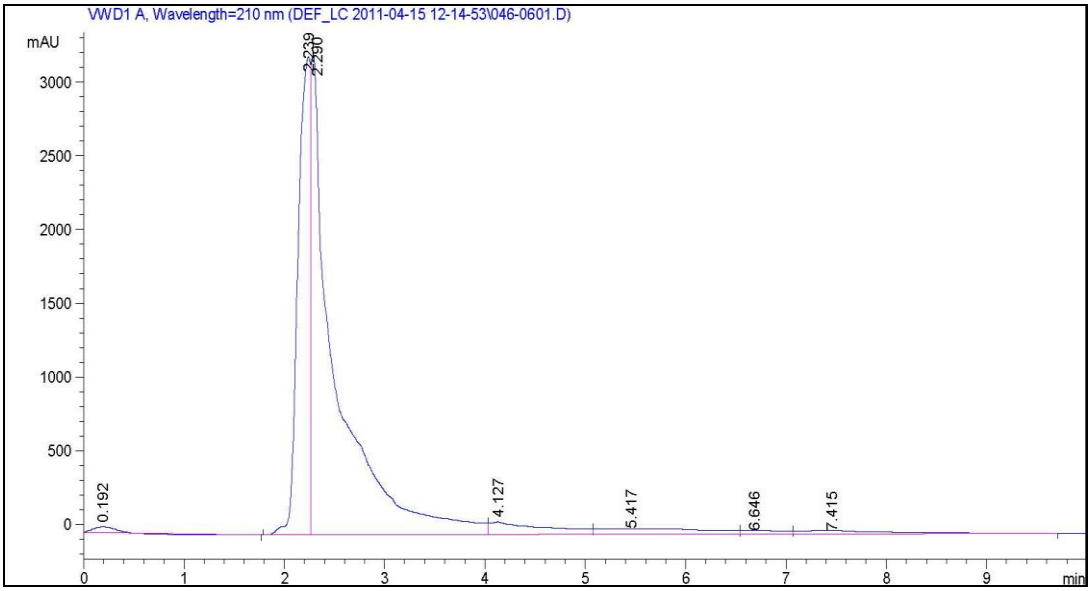
Acq. Operator : Francis Seq. Line : 6
Acq. Instrument : Instrument 1 Location : Vial 46
Injection Date : 4/15/2011 1:13:03 PM Inj : 1 Inj Volume : 25.0 µlAcq. Method : C:\CHEM32\1\DATA\DEF_LC 2011-04-15 12-14-53\KAREN.M
Last changed : 3/4/2011 10:18:29 AM by BingAnalysis Method : C:\CHEM32\1\DATA\DEF_LC 2011-04-15 12-14-53\046-0601.D\DA.M (KAREN.M, FromData File)
Last changed : 4/15/2011 2:14:31 PM by Francis

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Area Percent Report

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Sorted By : SignalMultiplier: : 1.0000
Dilution: : 1.0000 Use Multiplier &
Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=210 nm

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 1 | 0.192 | BB | 0.1633 | 318.56076 | 39.16137 | 0.3537 |
| 2 | 2.239 | BV | 0.1409 | 2.74105e4 | 3241.22827 | 30.4327 |
| 3 | 2.290 | VV | 0.2108 | 5.42086e4 | 3246.10938 | 60.1854 |
| 4 | 4.127 | VV | 0.4673 | 3258.98657 | 84.53172 | 3.6183 |
| 5 | 5.417 | VV | 1.0830 | 2844.59766 | 38.84029 | 3.1582 |


Data File C:\CHEM32\1\DATA\DEF_LC 2011-04-15 12-14-53\046-0601.D
Sample Name: FRE01

| Peak # | RetTime [min] | Type | Width [min] | Area mAU *s | Height [mAU ] | Area % |
|---|---|---|---|---|---|---|
| 6 | 6.646 | VV | 0.4325 | 657.61053 | 22.04120 | 0.7301 |
| 7 | 7.415 | VB | 0.8698 | 1370.44885 | 21.55714 | 1.5215 |
| Totals : | | | | 9.00693e4 | 6693.46936 | |

========== *** End of Report *** Instrument 1 4/15/2011 2:14:34 PM Francis Page 2 of 2

# APPENDIX L

## SAMPLE CALCULATION

### 1. Moisture content

$$\%\ \text{moisture of banaba leaves} = \frac{\left[\begin{matrix}\text{weight of sample}\\ \text{before drying}\end{matrix}\right] - \left[\begin{matrix}\text{weight of sample}\\ \text{after drying}\end{matrix}\right]}{\left[\begin{matrix}\text{weight of sample}\\ \text{before drying}\end{matrix}\right]} \times 100\%$$

$$\%\ \text{moisture of banaba leaves} = \frac{[30.0437] - [8.4689]}{[30.0437]} \times 100\% = 71.812$$

The rest of calculations were done using the same formula as above

### 2. Residual Moisture Content

$$\%\text{Residual moisture} = \frac{\text{weight before oven drying-weight after oven drying}}{\text{weight before oven drying}} \times 100$$

$$\%\text{Residual moisture} = \frac{18.158\text{-}8.581}{8.14} \times 100 = 52.1919$$

### 3. % Yield and % Extraction Efficiency

$$\%\text{yield} = \frac{\text{Mass of oil}}{\text{mass of bone dried banaba}} \times 100\%$$

$$\%\text{yield} = \frac{3.0374}{8.15} \times 100\% = 34.79\%$$

$$\%\text{efficiency of extraction} = \frac{\text{Mass of crude oil extracted}}{\text{mass of extractable oil in raw material}} \times 100\%$$

$$\%\text{efficiency of extraction} = \frac{3.0274}{6.1129} \times 100\% = 49.525\%$$

The rest of calculations were done using the same way above

Yield of corosolic acid

$$\%\text{yield of corosolic acid} = \%\text{yield of crude oil} \times \frac{\text{Area of corosolic acid (sample)}}{\text{Area of corosolic acid(std)}} \times \frac{\text{weight of corosolic acid std}}{\text{weight of sample}}$$

Where: 5mg was the gram of corosolic acid standard

$$\%\text{yield of corosolic acid} = 63.95 \times \frac{364.138}{822.7096} \times \frac{5mg}{500.2mg} 100\% = 0.2839\%$$

# APPENDIX M

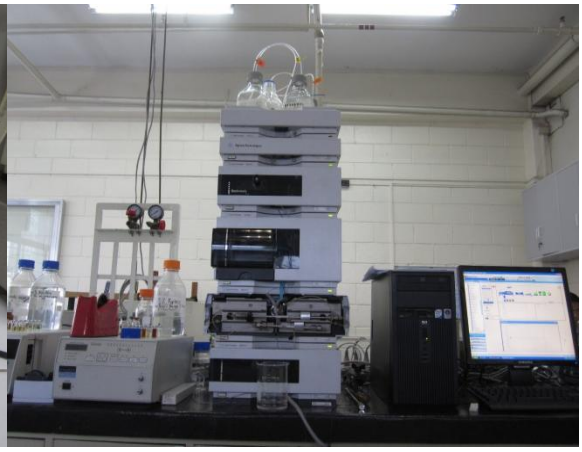
## EXPERIMENTAL SET UP

# APPENDIX N
## MATERIAL

**Banaba leaves and Fruits.**

**Grounded banaba fruit**

**Filtration**



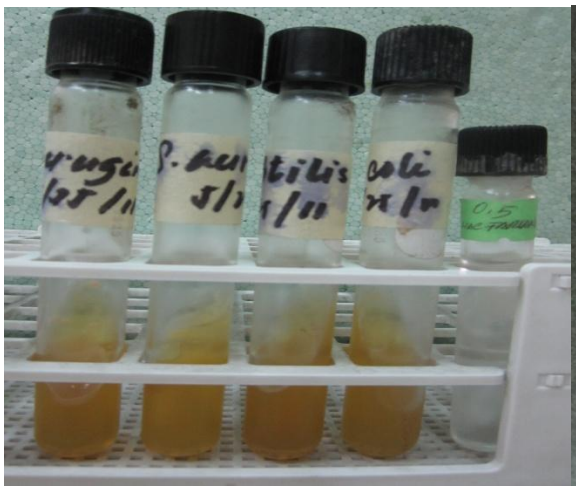
**HPLC system**

**Rotary Evaporator**

# APPENDIX O

## ANTIBACTERIAL ACTIVITY TEST


**Autoclave**



**Microorganisms and MCFarland standard**

**Streaked petri disk**