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Bioactive Metabolites from *Phaseolus Lunatus*

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ABSTRACT

The study involves the extraction, bioactivity testing and partial characterization of the bioactive metabolites from the dried leaves of lima beans (*Phaseolus lunatus*).

The dried leaves were extracted with ethanol and subjected to solvent partitioning technique using n-hexane, dichloromethane and ethyl acetate solvents.

The bioactivity potential of each fraction was tested using Brine-Shrimp Lethality Assay. The ethanol-water fraction showed the lowest LC_{50} value and was considered the most bioactive fraction. The metabolites were separated using Gravity Column Chromatography whereby gradient elution technique was employed. The separation yields two isolates. The two isolates were tested for its anti-bacterial activity using *Escherichia coli* and *Bacillus subtilis*. The isolates were characterized by its melting point and infra-red spectra.

The two isolates are compounds that have the same functional groups. The isolates exhibited different melting point ranges. A spectrum of a saponin was retrieved from the Advance Institute for Science and Technology (AIST) and showed that similar functional groups were present in both isolates and the saponin spectrum. The isolates are possibly saponins.

The two isolates showed anti-bacterial activity to *E. coli* and *B. subtilis* and is a possible anti-bacterial agent.

INTRODUCTION

Man has been battling with different diseases since time immemorial, with each generation producing a breakthrough in the treatment and prevention of diseases. Although the methods of diagnosing and treating the disease have improved greatly since 1930's, the World Health Organization (WHO) has estimated 2-3 million deaths worldwide are caused by new strains of multi-drug-resistant bacteria. *(WHO, 2023)*

Approximately 80% of the world's inhabitants rely mainly on traditional medicine to cure certain diseases. *(Fokunang et al., 2011)*

A large number of human, animal and plant diseases are caused by pathogenic microbes (fungi, bacteria and algae). Infections due to fungi and bacteria have been the major cause of death in higher organisms. The discovery of antibiotic penicillin by Fleming is therefore considered to be one of the most important discoveries in the world. Historically, many of the new antibiotics were isolated from natural sources (soil microbes, plants, etc). Many more were later synthesized and introduced in clinical practices. Unfortunately, human struggle against pathogenic microbes is far overdue because microbes evolve from generation to generation. The discovery and development of new antimicrobial agents is therefore an on-going process. The remarkable diversity of chemicals present in biological samples has tremendous potential in the search for new antimicrobial agents. *(Thomsen et al., 2001)*

A recent study of the leguminous plant *Maesa balansae* was examined and found to have strong anti-leishmanial potential. The polar extract of the leaves exhibited potent and selective *in vitro* and *in vivo* activity

against intracellular *Leishmania infantum* amastigotes. Bioassay-guided fractionation led to the isolation and characterization of six anti-leishmanial triterpene saponins. *(De Kimpe et al., 2005)*

This study aims to isolate and characterize the bioactive metabolites in *Phaseolus lunatus* and test its antibacterial property, this is in hope to increase the economic role of legumes in the Philippines according to its feasibility for pharmaceutical use specifically on its antibacterial activity.

Statement of the Problem

This study aims to determine the bioactive metabolite from the dried leaves of *Phaseolus lunatus*.

Explicitly, the study aims to:

1. extract the bioactive metabolites through solvent extraction using the solvents: ethanol, hexane, dichloromethane and ethyl acetate;
2. determine the bioactivity potential of each extract using the Brine-Shrimp *(Artemia salina)* Lethality Assay; *(Waghulde, et al, 2019)*
3. isolate the active metabolite from the most bioactive fraction by gravity column chromatography;
4. determine the antibacterial activity of the most active isolate against gram- negative bacterium, *Escherichia coli* and gram-positive bacterium, *Bacillus subtilis;*
5. characterize the melting point and infrared spectra of the most active isolate

Significance of the Study

The results of this study are important in evaluating the potentials of local legumes like genus *Phaseolus lunatus* that are possible alternative constituents for some pharmaceutical products. The study provides information that serves as a starting point for phytochemical and pharmacological researchers whose interests are in the development of medicinal preparations using local materials like legumes, which are abundant in the locality. Moreover, the information that will be obtained with regards to the bioactive metabolites isolated from the plant will serve as a foundation for future researchers about Phaseolus lunatus.

The bioactivity assay will be determined by the Brine-Shrimp *(Artemia salina)* Lethality Method and the antibacterial activity of the isolate from the most active fraction will be studied with the hope that it has potential as an antibacterial agent against gram- negative bacterium, *Escherichia coli* and gram-positive bacterium, *Bacillus subtilis.* The antibacterial property of the most active isolate will be compared to a potent antibacterial drug, Chloroamphenicol ®.

Scope and Limitations

The leaf samples were collected from Argao, Cebu last June 2006. Only mature and healthy leaves were gathered because the recent study of the leguminous plant *Maesa balansae* used mature and healthy leaves as a possible source for the bioactive metabolite.

The leaves were air-dried for two days and then oven-dried at 35-40 □C and then powdered using a blender. Sufficient leaves were collected to obtain about 250-g of the dried powdered leaves. The scope of this study was to isolate the most active metabolite from the leaf extract of *Phaseolus lunatus* through solvent extraction using the solvents: ethanol, hexane, dichloromethane and ethyl acetate. The bioactivity potential utilized the Brine-Shrimp *(Artemia salina)* Lethality Assay. The gram-negative bacterium strain that was used, *Escherichia coli* and gram-positive bacterium, *Bacillus subtilis*, was obtained from the College of Pharmacy. Characterization was limited to Infrared Spectra and melting point determination used the Fisher-John's Melting Point Apparatus. The above procedure was carried out at the Chemistry Research Laboratory of the Department of Chemistry, University of San Carlos, Cebu City.

INTERNATIONAL JOURNAL OF RESEARCH AND SCIENTIFIC INNOVATION (IJRSI)
ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue X October 2024

The study did not include characterization using the Mass Spectroscopy, Proton Nuclear Magnetic Resonance (^{1}H-NMR) and 2D Nuclear Magnetic Resonance.

REVIEW OF RELATED LITERATURE AND RELATED STUDIES

Phaseolus lunatus is locally known as *patani* in Filipino and *lima beans* in English. *Phaseolus lunatus* is thoroughly naturalized and common in thickets at low and medium altitudes, ascending to 2,000 meters. It was introduced from the tropical America, and is now pantropic in distribution. *(Quisumbing, 1951)*

This is a climbing, slender, annual, smooth or sparingly hairy, herbaceous vine reaching a length of 4 meters or more. The leaves are thin, compound, with three leaflets which are ovate, 6 to 12 centimeters long and somewhat rounded at the base and pointed at the tip. The flowers are greenish or pale yellow, about 13 millimeters long, and borne on auxiliary, solitary racemes, 8 to 20 centimeters long, and about 2 centimeters wide and contain from 1 to 4 large variously colored or white seeds. *(Quisumbing, 1951:421)*

Analyses of lima beans show that they are very high in carbohydrates and fairly high in protein, but only fair in iron, and deficient in calcium. *(Quisumbing, 1951:422)*

Dunstan and Henry isolated from the seeds phaseolunatin ($C_{10}H_{17}O_6N$), a cyanogenetic glucoside, which yields about tenth of 1% of hydrocyanic acid (HCN) calculated on dry beans. They state that the dark purple beans are most toxic. Guerin reports that the leaves also contain phaseolunatin. Many cases of animal poisoning have been reported. *(Quisumbing, 1951:422)*

Studies proved that plants that have toxic effects on animals and human are said to have active biological metabolites. Some of these toxic but biologically active components are saponins, flavonoids, steroids and alkaloids.

Saponins are glycosides, which, even at low concentrations, produce a frothing in aqueous solution, because they have surfactant and soap-like properties. The name comes from the Latin *sapo*, soap, and the plant materials containing saponins were originally for cleansing clothes, e.g. soapwort (*Saponaria officinalis*; Caryophyllaceae) and quillaia or soapbark (*Quillaja saponaria*; Rosaceae). These materials also cause haemolysis, lysing red blood cells by increasing the permeability of the plasma membrane, and thus they are highly toxic when injected into the blood stream. Some saponin-containing plant extracts have been used as arrow poisons. However, saponins are relatively harmless when taken orally, and some of our valuable materials, *e.g. beans, lentils, soybeans, spinach and oats*, contain significant amounts of saponins. Toxicity is minimized during ingestion by low absorption, and by hydrolysis. Acid-catalyzed hydrolysis of saponin liberates sugar(s) and an aglycone (sapogenin), which can be either triterpenoid or steriodal in nature. Some plants may contain exceptionally high amounts of saponins, e.g. about 10% in quillaia bark. *(Dewick, 2002)*

Triterpenoid saponins are rare in monocotyledons, but abundant in many dicotyledonous families. Medicinally useful examples are mainly based on the □-amyrin subgroup and many of these possess the carboxylic acid groups derived by oxidation of methyl groups, those at positions 4 (C-23), 17 (C-28), and 20 (C-30) on the aglycone ring system being subject to such oxidation. □-amyrin is a pentacyclic triterpenoid that is oleanane substituted at the 3 □-position by a hydroxy group and containing a double bond between positions 12 and 13. It is one of the most commonly occurring triterpenoids in higher plants. It has a role as a plant metabolite and an Aspergillus metabolite. *(Dewick, 2002)*

Sugar residues are usually attached to the 3-hydroxyl, with one to six monosaccharide units, the most being glucose, galactose, rhamnose and arabinose, with uronic acid units (glucuronic acid and galacturonic acid). *(Dewick, 2002)*

The triterpenoid skeletons may be subjected to a variety of structural modifications. Pre-eminent amongst such degraded triterpenoids are the steroids, and these are so important that they are considered separately. Other degraded triterpenoids include the limonoids (tetranortritepenoids), in which four terminal carbons from the side-chain are removed, and the quassinoids, which have lost ten carbons, including one of the C-4 methyls.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue X October 2024

(Dewick, 2002)

Triterpenoids have many biological activities such as inhibition of tumor promoting action, anti-inflammatory activities, hepatoprotective effects, anti-tumor activity and NO inhibitory activity. *(Fu et al., 2005)*

A study of the plant *Maesa balansae* was examined and found to have strong anti-leishmanial potential. *Maesa balansae* Mez (Myrsinaceae), a shrub growing in the northern part of Vietnam, is a traditional medicine for the treatment of allergies, sprains, worm infections, skin ulcers, drunkenness and headache. The polar extract of the leaves exhibited potent and selective *in vitro* and *in vivo* activity against intracellular *Leishmania infantum* amastigotes. Bioassay-guided fractionation led to the isolation and characterization of six anti-leishmanial triterpene saponins. *(De Kimpe et al., 2005) (Mishra et al., 2009)*

A survey of the literature reveals that saponins are also found in most common fish poisons. Native fishermen have identified most fish poisons from plants in use. For example, the Chiriguano Indians of South America use the bark of *Myrsine pellucida*, a tree that grows wild in Bolivia. The Australian aborigines often use the seeds of *Barringtonia asiatica*. Fishermen in parts of the Philippines use *Heritiera littoralis* as fish poisons. *(Cannon et al., 2004)*

Anecdotal evidence from the Aboriginal people living in Kimberly districts of North Australia showed that the bark of *Barringtonia acutangula* has potent analgesic properties. The bark is chewed to form a paste, which is applied, externally to wounds, after which the pain is said to be relieved quickly and wound to heal rapidly. According to the study, saponins were also present in the plant that would act as the primary active factor to its analgesic properties. *(Quinn et al., 2005)* In Ethiopia, treatment of tapeworms includes the use of the seeds of *Glinus lotoides*

L. (Mollunginaceae), locally known as "Mettere". Tenicidal activities have been shown in vitro and in vivo against *Tenia saginata* and *Hymenolopis nana* worms. The cestocidal and pharmacological activities of the seeds of *Glinus loitodes* are probably due to its saponins. This study also proves that saponins have anti-helminthic activity against worm infections. *(Merfort et al., 2005)*

The saponins of fruits of *Mimusops laurifolia* were reported also to poses anti- inflammatory activity and that those from stem bark of *Mimusops elengi* exhibited anti-ulcer activity. *(Long et al., 2005)*

The genus *Asparagus*, which is widely distributed in eastern Asia including China, Korea, and Japan, has over 300 species. Steriodal saponins have been isolated from many of these species. The rhizomes of *Asparagus oligoclonos* Maxim (Liliaceae) have been used as cough and asthma remedies in Far Eastern countries. They isolated three steriodal saponins and found out that they were cytotoxic against five human tumor cell lines with IC_{50} values of 2.05 – 2.84 μg/mL. *(Kim et al., 2005)*

Albizia grandibracteata leaves contain high levels of saponins. In Kibale National Park in Uganda, red colobus monkeys *(Procolobus badius)* consume most of the leaves of the specie frequently. These primates have polygastric digestive system that allows efficient detoxification. By contrast, they are consumed so rarely and briefly by chimpanzees that they may be ingested for self-medication rather than nutrient maximization. *Albizia grandibracteata* is also sometimes used as traditional medicine. The terminal bud decoction is used to treat wounds, while the bark is used as treatment for parasitism and lumbago. It was also reported that the methanolic extract of the leaves of *Albizia grandibracteata* exhibited a significant anti-helminthic activity against *Rhabditis* worms. The crude extract and the pure compound showed significant inhibitory activity against the KB and MCF7 tumoral cell lines. They found out that triterpenoid saponins were the active component of the crude extract and the pure compound. *(Lavaud et al., 2005)*

A preparation made from the roots of *Pulsatilla koreana*, *Panax ginseng* and *Glycyrrhiza glabra* has been used in traditional medicine practice to treat cancer. Currently, this preparation, which is called SB31, is under going phase II clinical studies in Korea. It was previously reported that SB31 exhibited cytotoxic activity against some human cancer cell lines and potent anti-tumor activity on a mouse tumor model. The study

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue X October 2024

found out that an active component was found to be deoxy podophyllotoxin (DPT), which also exhibited anti-angiogenic activity and good anti-tumor activity against mice bearing Lewis lung carcinoma (LLC). However, they doubt that DPT was responsible for the anti-tumor property of the specie since it is found to be in very low concentrations. Another study of the specie was done and found out that the main active component of the extract was triterpenoid saponins. In their study they found out 17 triterpenoid saponins, among which 11 of them has already been isolated. The cytotoxic activity of the new isolated compounds was also studied and evaluated against A-529 human lung carcinoma. *(Ahn et al., 2005)*

The roots of the herbaceous plants *Panax ginseng* (Araliaceae) from China, Korea, and Russia, and *Panax* related species, e.g. *P. quinquefolium* (American ginseng) from the USA and Canada, and *P. notoginseng* (Sanchi-ginseng) from China, have been widely used in China and Russia for the treatment of a number of diseases including anaemia, diabetes, gastritis, insomnia, sexual impotence, and as a general restorative. Interest in the drug has considerably increased in recent years. Ginseng is classified as an 'adaptogen', helping the body to adapt to stress, improving stamina and concentration, and providing a normalizing and restorative effect. It is also promoted as an aphrodisiac. Long term use of ginseng can lead to symptoms similar to those of corticosteriod poisoning, including hypertension, nervousness and sleeplessness in some people, yet hypotension and tranquilizing effect in others. *(Dewick, 2002)*

Many of the secondary metabolites present in the root have now been identified. It contains a large number of triterpenoid saponins based on the dammarane subgroup, saponins that have been termed as ginsenosides by Japanese investigators; or panaxosides by Russian researchers. *(Dewick, 2002*

These are derivatives of two main aglycones, protopanaxadiol and protopanaxatriol respectively. Acid-catalyzed cyclization in the side-chain produces an ether ring. Sugars that is present as substituents in the saponins on the 3- and 20- hydroxyls in the diol series and 6- and 20- hydroxyls in the triol series. *(Dewick, 2002)*

An activity-guided isolation of antifungal, antibacterial, and antioxidant compounds from a dichloromethane extract of the plant *Eriophorum scheuchzeri* led to the isolation of eight flavanoids. Three isoflavones and one flavone are new natural compounds. The pure compound was tested for antifungal activity by bioautography on thin layer chromatograms and by agar overlay method. The isolate exhibited an activity against the fungi *Candida albicans* and *Candida cucumerinum*. *(Hostettmann et al., 2005)*

Bioactive compounds are often toxic to brine-shrimp *(Artemia salina)* larvae. Hence, *in vivo* lethality to brine-shrimp larvae can be used as a rapid and simple preliminary monitor for bioactive compounds during the isolation of natural products. The eggs of the brine- shrimp *Artemia salina* (Leach) are readily available as fish foods in pet shops. When placed in filtered seawater, the eggs hatch within 48 hours, providing a large number of larvae. These tiny brine-shrimp larvae have been extensively used as a tool to monitor the bioactivity of samples under study. This is a rapid, inexpensive, in-house, general bioassay, which has been developed for screening, fractionation and monitoring of physiological active natural products. *(Meyer et al., 1982)*

Chromatography is widely used for the separation, identification and determination of the chemical components in a complex mixture. It could readily separate non-volatile, non- isomeric compounds using gradient elution methods, where, different solvent eluents are widely introduced. Normal chromatography is often used in natural products especially when dealing with non-polar interests to which polar stationary phase (silica gel) is commonly used. *(McMurry, 1984)*

METHODS

Plant Material

Mature and healthy leaves of *Phaseolus lunatus* were collected from Argao, Cebu, Philippines. The leaves were washed with distilled water to remove foreign materials and dusts, and then air-dried for two days and then followed by oven-drying at 35-40 □C. The dried leaves were powdered using a blender.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue X October 2024

Extraction

About 250-g of the powdered leaves were subsequently soaked in 500-mL ethanol. It was filtered after 24 hours, and the residue was re-soaked in 500-mL ethanol. The soaked residue was filtered and the second filtrate was added to the first filtrate. The collected filtrate was concentrated to about 100-mL of its original volume in a rotary evaporator at 40 C.

Solvent Partitioning

The concentrated filtrate was added with equal amount of water to make a 50% ethanol-water solution. A mixture of 100-mL of ethanol-water solution and 50-mL of *n*- hexane was added to the concentrated filtrates in a separatory funnel. The layers were then separated and the ethanol layer was extracted again with 50-mL of *n*-hexane. The second *n*-hexane layer was added to the first *n*-hexane extract. The same procedure was employed for ethyl acetate and then with dichloromethane (DCM). This will create four solvent fractions of the ethanol extract of the sample: hexane, ethyl acetate, dichloromethane (DCM) and 50% ethanol in water. Each solvent fraction was labeled as Fraction A, B C and D respectively. Each solvent fraction was concentrated in the rotary evaporator and kept in a clean container.

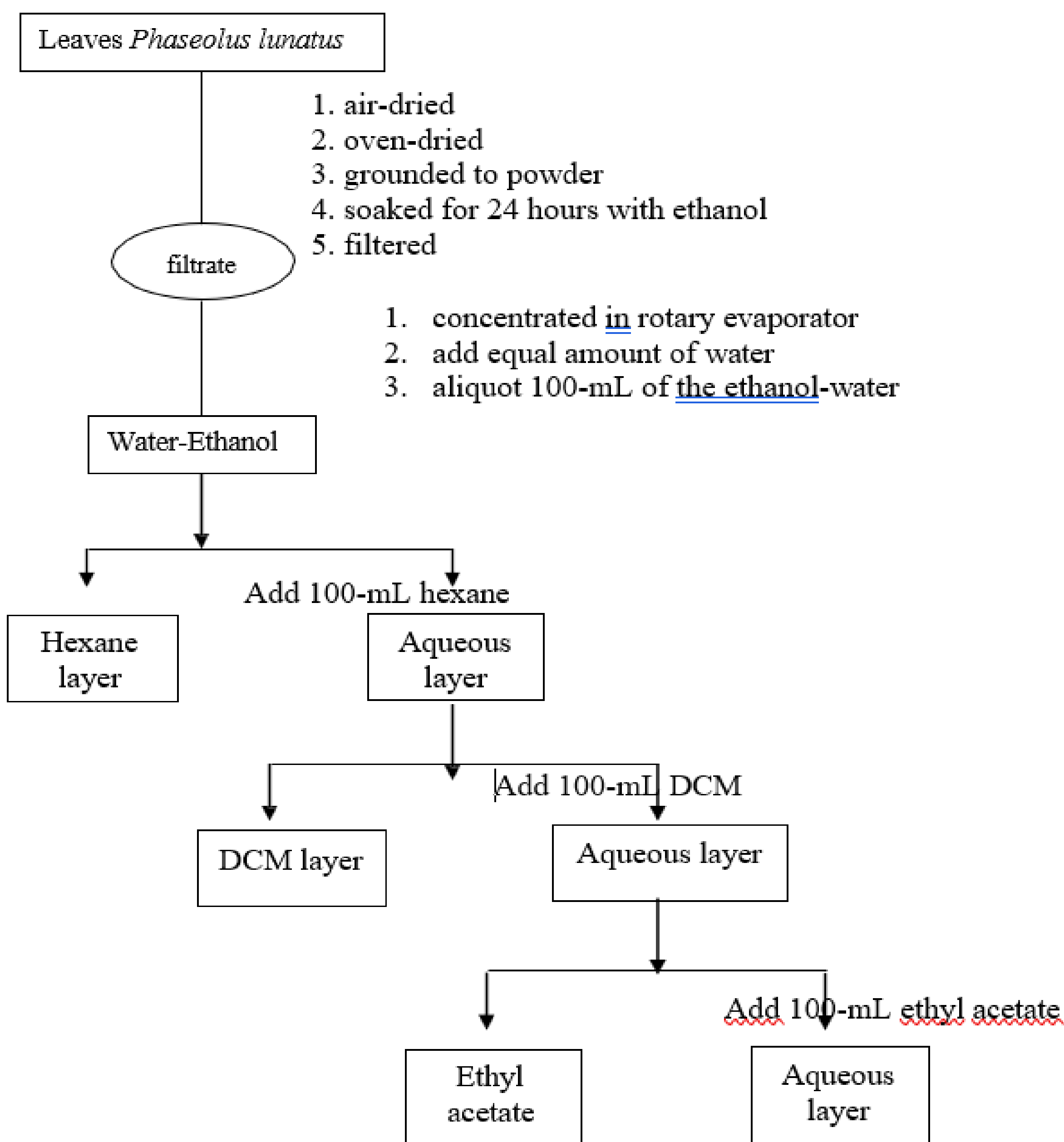

Figure 1. Extraction and Solvent Partitioning of *Phaseolus lunatus*

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue X October 2024

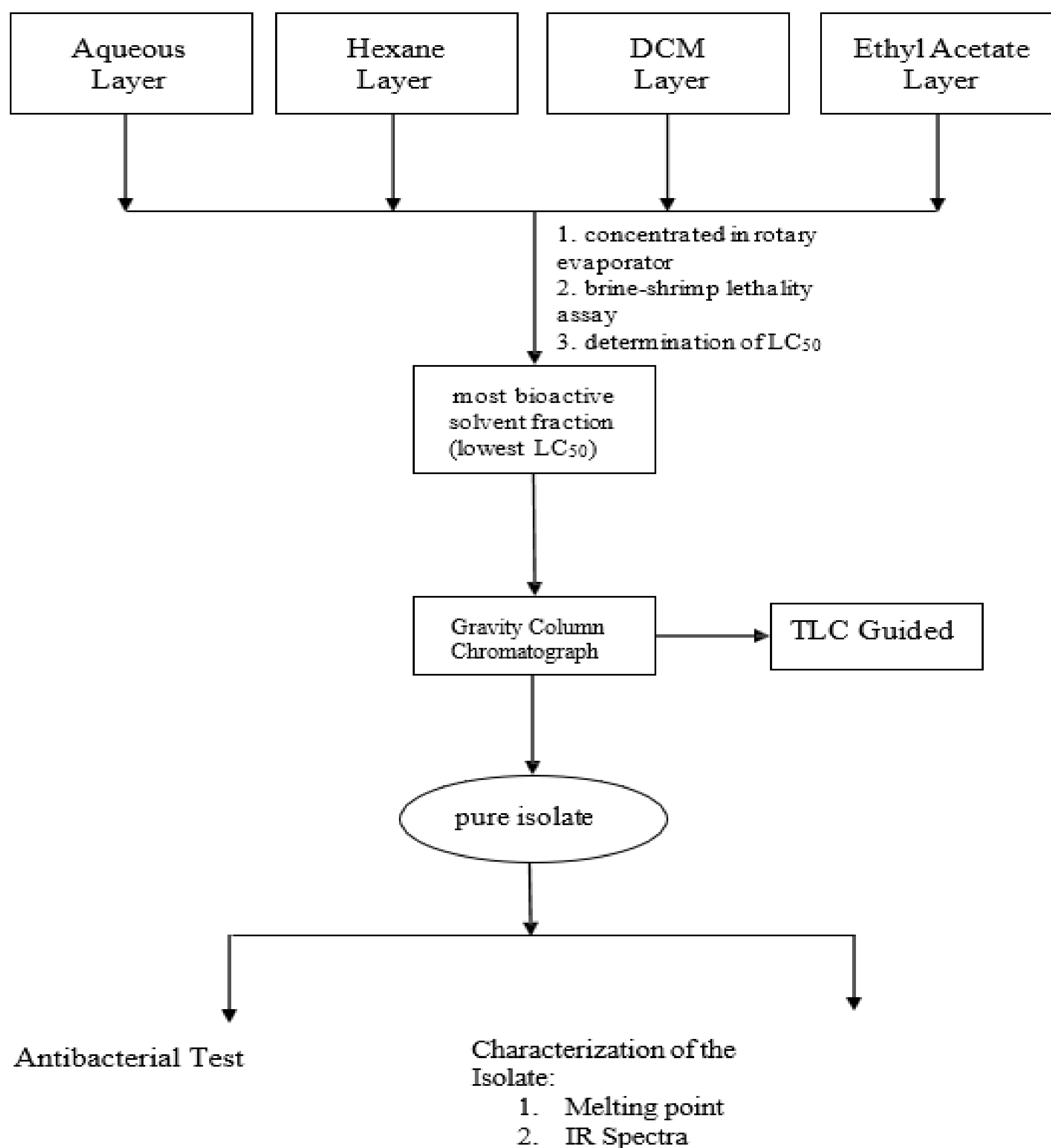

Figure 2. Schematic Diagram of the Bioactivity Testing, Isolation and Characterization of the Isolate

Preparation of Brine-Shrimp Larvae

Seawater was filtered and then boiled. It was allowed to cool to room temperature. The seawater was placed in a small unequally divided tank. The shrimp eggs were placed in the larger compartment of the tank, which was darkened by covering it with aluminum foil. The smaller compartment was illuminated with a table lamp. The illuminated compartment attracted the hatched shrimp larvae (nauplii) through the perforations in the dam. The eggs were allowed to hatch and mature in 48-hours at a temperature of 22-29 C. The brine-shrimp larvae were separated from the eggs by pipetting them 2-3 times into small beakers containing seawater.

Preparation of Test Solutions for Range Finding Test

The test samples were prepared by dissolving 25-mg of crude extract in 20-mL of 1% ethanol solution and 5 drops of Tween 20 {Polyoxyethylene (20) sorbitan monolaurate} to make up a stock concentration of 1000 g/mL in seawater. Serial dilutions were made by pippeting 10-mL and 1-mL of the stock solution. Three concentrations were made: 1000, 100, 10 g/mL respectively.

Brine-Shrimp Lethality Assay

Culture tubes were prepared for brine-shrimp lethality assay. A maximum of four culture tubes were made for each concentration. The culture tubes were added with 25- mL of the test solution. Ten (10) nauplii were added to each culture tubes. The time was noted after the addition of the nauplii. A control sample was made using seawater and the solvent.

Determination of the LC_{50} Value

After 24-hours, the culture tubes were examined using a hand lens. The number of dead (non-mobile) nauplii was counted in each culture tube. MS Excel calculated the LC_{50} using the Linear Regression.

Preparation of the Test Solution for the Definitive Test

The test samples were prepared by dissolving 25-mg of the most active crude extract in 1% ethanol solution and 5 drops of Tween 20 {Polyoxyethylene (20) sorbitan monolaurate} to make up a stock concentration of 1000 g/mL in seawater. Serial dilutions were made according to the LC_{50} value. Two concentrations above and below the LC_{50} value were made.

Isolation of the Bioactive Metabolite

The bioactive metabolite was isolated from the most active solvent fraction (lowest LC_{50} value).

A column, using an ordinary burette was prepared. The column was plugged with glass wool. Slurry of ethyl acetate solvent and silica gel (70-230 mesh) was prepared in a column in which the tip was opened to remove some of the solvent.

The most bioactive fraction was subjected to gravity column chromatography using gradient elution technique whereby it was eluted with 20-mL of the solvent systems (100% ethyl acetate, 5, 10, 20, 30, 40, 50 hexane in ethyl acetate and then 100% hexane). Approximately 2-mL fractions were collected into vials, which was pre-weighed. The vials was evaporated of the solvent and re-weighed. The difference of mass of vials with the isolate and mass of previously weighed vials was used to determine the mass of isolate. The vials with abundant isolates were chosen and were weighed. The 2-mL fractions were also subjected to TLC and the R_f values were calculated. The TLC assay was done simultaneously before evaporating the solvent from the 2-mL fractions. The isolate fractions with the same R_f values were pooled together. The combined solvent fractions were evaporated to dryness in an evaporating dish. The solvent-free extract was kept in covered containers and then desiccated.

Preparation of Test Solutions for Anti-Bacterial Test

About 30-g of the test extract was dissolved in appropriate solvent shaken to obtain homogenous solutions. The test solutions were labeled as Test Solution E1. The prepared test solution was used for antibacterial testing. A control of 30-g Chlorampenicol discs.

Preparation of Filter Paper Discs

Ashless filter paper was prepared using a 6-mm diameter puncher. The paper discs were wrapped in aluminum foil and were placed in a Petri dish, wrapped in paper, ready for sterilization.

Preparation of Culture Medium for Sterilization

About 5.6-gram of nutrient agar was suspended and dissolved in 200-mL distilled water. The mixture was boiled with constant stirring until the solution was clear. It was filtered while hot with a muslin cloth. The pH of the nutrient agar was adjusted to meet the required pH for bacterial growth. This was done by addition of 10 drops of Bromthymol blue to 10-mL of the nutrient agar solution. Bromthymol blue exhibits a yellow, color at pH 6.0, a blue color at pH 7.6 and a grass green color at pH 7.0, the pH needed for bacterial growth. If the

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue X October 2024

indicator added would produce a yellow color, a sufficient amount of dilute alkali (0.1 N NaOH) was added to the solution drop by drop to produce a grass green color. If a blue color was produced, sufficient amount of dilute acid (0.1 N HCl) was added to produce a grass green color. The number of drops was noted down. The volume of the acid or alkali that was added will calculate the necessary adjustment of the remaining portion of the medium. A maximum of 12 culture tubes, each with half-full of nutrient agar was made. All culture tubes were wrapped together in a non-absorbent paper ready for sterilization.

Sterilization of Test Materials

Petri dishes were washed and wiped dry with a clean cloth and wrapped in paper before sterilization. The culture tubes with medium, prepared filter paper discs and Petri dishes were sterilized using the standard sterilization procedures.

The inoculating loop was sterilized before and after use by flaming it to red-hot using an alcohol lamp.

Sources of Strain

Pure cultures of microorganism namely: *Escherichia coli* (Code number: 5622646171) and *Bacillus subtilis* was obtained from the University of San Carlos, College of Pharmacy, Cebu City, Philippines.

Preparation of Fresh Agar Slant Cultures

The preparation of fresh agar slant cultures was done at the Pharmacy laboratory. Fresh cultures of microorganism were made as often as necessary. This was done by streaking each culture of microorganism on the surface of the freshly prepared sterile agar slant with the use of a sterile inoculating loop while observing aseptic technique. The culture tubes were labeled accordingly. The agar slants was incubated at 37 □C for 24 hours.

Anti-Bacterial Activity Test

The test tubes of the nutrient agar were melted over a boiling water bath. The melted nutrient agar was allowed to cool to 45 □C in a constant temperature bath. A small amount of the *B. subtilis* was fished out from the fresh agar solution by an inoculating loop. The bacteria were dipped into the culture tube containing the sterile melted nutrient agar. The culture tube was rolled rapidly between the palms of the hand and the mouth of the tube was flamed. One side cover of the Petri dish was raised sufficient enough to insert the mouth of the tube. The seeded nutrient agar was poured into a sterile Petri dish aseptically. The Petri dish was rotated to distribute the nutrient agar evenly. The nutrient agar was allowed to solidify. The same process was done for *E. coli* culture.

A sterile filter paper disk was removed from the Petri dish using sterile forceps previously flamed to red-hot. Then it was dipped halfway into the Test Solution E1, the liquid was allowed to diffuse into the disk by capillary action. Allowing the disk to touch the side of the test tube will drain off the excess solution. The sterile filter paper disk was placed in a Petri dish containing the inoculated agar at its designated location observing proper aseptic technique. The same procedure was done to all bacterial strains. Each microorganism was placed on plates. The plates were labeled accordingly for their identification and were incubated in an inverted position at 37 □C for 24 hours.

Determination of % Antibacterial Activity

The zone of inhibition (ZOI) was measured using a millimeter-calibrated ruler. The zone diameter was recorded. The antibacterial activity was determined by comparing zones of inhibition that was produced by the test solution with that of the positive control, Chlorampenicol, using the formula:

$$\%\ \text{Antibacterial Activity} = \frac{\text{Zone of Inhibition produced by test extracts}}{\text{Zone of Inhibition produced by positive control (Chlorampenicol)}} \times 100$$

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue X October 2024

Characterization of the Isolate

Characterization included determination of the melting point and Infrared spectra.

Melting point was done using the Fisher-John melting point apparatus against standard pure benzoic acid.

Spectroscopic analysis was done with the Fourier-Transform Infrared Spectrophotometer (FTIR).

RESULTS AND DISCUSSIONS

Extraction

Ethanol Extraction. The extraction was done as stated in the methodology. The ethanol extract was a dark-green solution. The solution was kept in a fridge to prevent oxidation of the metabolites.

Solvent Partitioning. This was also done as stated in the methodology. The n- hexane layer was a dark green solution. The dichloromethane extract was a brownish- yellow solution. The ethyl acetate extract was a yellow solution. The ethanol-water layer after extraction was an orange-brown solution. Upon evaporation of the solvent, amorphous solids were obtained. These were kept in desiccators to avoid oxidation.

Brine-Shrimp Lethality Assay

Range Finding Test. The four solvent-free fractions were subjected to Brine- Shrimp Lethality Assay. The amorphous solids were weighed. The n-Hexane extract was weighed at 0.2569-g, ethanol-water extract at 0.2567-g, dichloromethane extract at 0.2359-g and ethyl acetate extract at 0.2359-g. Each of the solids were dissolved in 20- mL of 1% ethanol in distilled water and five (5) drops of Tween 20, was added before finally diluting each to 250-mL with boiled and filtered seawater. These stock solutions had a final concentration of 1000 □g/mL. Concentrations of 100 and 10 □g/mL were then prepared.

Results of the *Range Finding Test* (RFT) of the Brine-Shrimp Lethality Assay are shown in Table 1. This measured the activity of the four solvent fractions in variable concentration, monitored at 8, 12 and 24 hours.

Table 1. *Range Finding Test* of Brine-Shrimp Lethality Assay of the Four Solvent-Free Fractions.

Solvent Fraction	Concentration □g/mL	Average % Mortality of the Nauplii *(Artemia salina)*		
		8 hours	12 hours	24 hours
Ethanol-Water	0	0.00	0.00	0.00
	10	20.0	22.5	22.5
	100	7.50	15.0	20.0
	1000	22.5	27.5	90.0
Ethyl Acetate	0	0.00	0.00	0.00
	10	15.0	15.0	17.5
	100	10.0	12.5	32.5
	1000	15.0	15.0	15.0
Dichloromethane	0	0.00	0.00	0.00
	10	7.50	7.50	10.0
	100	5.00	15.0	22.5

	1000	7.50	25.0	85.0
n-Hexane	0	0.00	0.00	0.00
	10	0.00	0.00	0.25
	100	0.00	0.00	0.25
	1000	0.00	0.25	0.25

The results showed that as the number of hours of exposure increased, the number of deaths increased significantly. This indicated that the extracts showed bioactivity except for the n-hexane extract.

The LC_{50}, after 24 hours of monitoring the individual solvent-free extracts are shown in Table 2. Using MS Microsoft Excel, the LC_{50} was calculated using Linear Regression. The concentration was graphed against per cent (%) mortality. Upon obtaining the equation of the line, the LC_{50} was computed by substituting the y-value with 50%. Pearson's R gives the linearity of the values plotted. This assessed the reliability of the method being used. Ideally, calibration curves must have values of 1.000-0.9800, but in biological testing an R value of 0.8000 is tolerated due to unpredictable phenomena like mortality.

Table 2. LC_{50} of the Four Solvent-Free Fractions of *Phaseolus lunatus*.

Solvent Fraction	Time (Hours)	*LC_{50}	Pearson's R
Ethanol-Water	24	450.76	0.9871
Ethyl Acetate	24	2046.3	0.9546
Dichloromethane	24	517.99	0.9946
n-Hexane	24	29183	0.8176

Results of the lethality test of the four solvent-free extracts showed significant bioactivity after 24 hours of monitoring. The ethanol-water fraction gave an LC_{50} of 450.76 □g/mL. The ethyl acetate fraction gave an LC_{50} of 2046.3 □g/mL. The dichloromethane fraction gave an LC_{50} of 517.99 □g/mL. The hexane fraction gave an LC_{50} of 29183 □g/mL. The National Cancer Institute of the United States of America has established that an extract with LC_{50} that is less than or equal to 30 □g/mL is considered a potential bioactive extract. *(Meyer and Ferrigni, 1982)*

Since these are crude samples it is not necessary to obtain an LC_{50} that is less than or equal to 30 □g/mL. The lethality that was shown by the Brine-Shrimp Lethality Assay is necessary to explain that the extracts have bioactive properties.

Definitive Test. The ethanol-water and dichloromethane fraction were again subjected to Brine-Shrimp Lethality Assay. The stock solution concentration was 1000 □g/mL. Concentrations of 500, 450 and 400 □g/mL were prepared and the solutions were diluted to volume using boiled, filtered seawater.

The two fractions were the only ones that were considered bioactive based on the results of the *Range Finding Test*. The other fractions have shown a very large value of LC_{50} which tells us that there is relatively lesser bioactivity.

The result of the *Definitive Test* of the Brine-Shrimp Lethality Assay is shown in Table 3. This measured the activity of the ethanol-water and dichloromethane fraction in variable concentration after 8, 12 and 24 hours of monitoring. The *Definitive Test* reassesses the LC_{50} of the individual extracts since the concentrations are in close range within each other.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue X October 2024

Table 3. *Definitive Test* of Brine-Shrimp Lethality Assay of the Ethanol-Water and Dichloromethane Solvent Fraction.

Solvent Fraction	Concentration g/mL	Average % Mortality of the Nauplii *(Artemia salina)*		
		8 hours	12 hours	24 hours
Ethanol-Water	0	0.00	0.00	0.00
	400	80.0	82.5	82.5
	450	90.0	90.0	90.0
	500	90.5	90.5	95.0
Dichloromethane	0	0.00	0.00	0.00
	400	0.00	5.00	10.0
	450	10.0	15.0	22.0
	500	15.0	85.0	85.0

Table 4 shows the LC_{50} of the ethanol-water and dichloromethane extracts after 24 hours of monitoring. Based on the result, it showed that the ethanol-water fraction is the most bioactive fraction.

Table 4. LC_{50} of the Ethanol-Water and Dichloromethane Extracts of *Phaseolus lunatus*.

Solvent Fraction	Time (Hours)	*LC_{50}	Pearson's R
Ethanol-Water	24	136.66	0.9868
Dichloromethane	24	464.44	0.8710

Isolation of the Bioactive Metabolite from the Most Bioactive Fraction

Thin-Layer Chromatography (TLC) and Spot Identification. Table 5 shows a data set for R_f values from the chromatograms derived from the eluted isolate extracts of the ethanol-water extract, their corresponding color reactions in UV light and Acetic Anhydride-Sulfuric Acid.

Fractions after 2.10 were colorless fractions and are mainly composed of the solvent used for elution. They showed no spot on the TLC plates and upon viewing under UV light gave no color. Fraction 1.1 has no active isolate also, is mainly composed of the solvent itself. The fractions with the same and almost the same R_f values were pooled together.

Table 5. Results of the Thin-Layer Chromatography of the Eluted Isolate Extracts of the Ethanol-Water Fraction.

Fraction	Spot Number	R_f Value	Color Reaction in UV	Color Reaction in Acetic Anhydride- H_2SO_4
1.1	None	-	-	-
1.2	1	0.644	Dark spot	Colorless
1.3	1	0.531	Dark spot	Colorless
	2	0.691	Dark spot	Colorless
1.10	1	0.523	Dark spot	Colorless

	2	0.636	Dark spot	Colorless
2.1	1	0.609	Dark spot	Colorless
	2	0.738	Dark spot	Colorless
2.3	1	0.636	Dark spot	Colorless
	2	0.673	Dark spot	Colorless
2.4	1	0.636	Dark spot	Colorless
	2	0.673	Dark spot	Colorless
2.5	1	0.636	Dark spot	Colorless
	2	0.673	Dark spot	Colorless
2.6	1	0.689	Dark spot	Colorless
2.7	1	0.672	Dark spot	Colorless
2.8	1	0.655	Dark spot	Colorless
2.9	1	0.585	Dark spot	Colorless
2.10	None	-	-	-

The results showed that fractions 1.3 to 1.10 and 2.1 to 2.5 have two spots. This indicated that the solvent eluted two compounds. This also shows that fraction 1.2 has one spot and has a single compound eluted. This fraction was named isolate 1. Fractions from 2.6 to 2.9 also and one spot and also mean that it has a single compound eluted. This fraction was named isolate 2.

Anti-Bacterial Assay of the Isolates

In the Anti-Bacterial Assay, isolate 1 and isolate 2 were used. A 30 μg/mL aqueous solution was made. Chlorampenicol was used as positive control, with a concentration of 30 μg/mL. Distilled water was used as the negative control. Concentrations of both isolates and control were made equal so that comparisons of the anti-bacterial activity later will be easy.

The bioactivity of the isolates of *Phaseolus lunatus* against *E. coli* and *B. subtilis* are shown in Table 6.

Table 6. Zone of Inhibitions of the Isolates against *E. coli* and *B. subtilis*.

Isolate	Average Zone of Inhibition *(mm)**	
(at 30 μg/mL)	*E. coli*	*B. subtilis*
Isolate 1	4.21	4.18
Isolate 2	3.45	3.55
Chlorampenicol	15.95	15.95

* Average of three trials

The first isolate showed a slightly larger zone of inhibition compared to the second isolate. There is quite a significant difference in their percent anti-bacterial activity compared to the positive control, Chlorampenicol. The results of the assay are shown in Table 7.

Table 7. Anti-Bacterial Activity of the Isolates against *E. coli* and *B. subtilis*.

Isolate *(at 30 □g/mL)*	Average % Anti-Bacterial Property	
	E. coli	*B. subtilis*
Isolate 1	26.39	26.21
Isolate 2	21.63	22.26

The first isolate gave 26.39% and 26.21% activity against *E. coli* and *B. subtilis* respectively. The second isolate gave 21.63% and 22.26% activity against *E. coli* and *B. subtilis* respectively.

Infra-Red Spectroscopy

In the infra-red spectroscopy analysis, the KBr pellet method was used. The fractions with the same R_f values were pooled together in a 50-mL beaker and were allowed to dry in desiccators. After drying, the isolates were subjected to infra-red spectroscopy. To ensure that there would be no contamination of the solvent, the fractions were re-dissolved in absolute methanol and diethyl ether. All isolates were treated this way and then allowed to dry in the desiccators. (See Table 8)

Table 8. Infra-Red Spectroscopy Data of Isolates from the Ethanol Extract.

Isolate	Wave number (cm^{-1})	Interpretation
1	3388.13	O-H stretch
	2934.19	$-CH_3$ stretch
	2358.90	C-H stretch
	1721.76	C=O stretch
	1652.30, 1512.97	C=C aromatic stretch
	1383.71	$-CH_2$-O stretch
	1208.01	O-H, aromatic substituent
	1075.87	C-O stretching
	621.55	HC=CH bending
2	3425.07	O-H stretch
	2917.52	$-CH_3$ stretch
	2849.34, 2363.25	C-H stretch
	1711.00	C=O stretch
	1650.71, 1514.73	C=C aromatic stretch
	1076.52	C-O stretching

The prominent peaks observed in all fractions substantiate that each fraction contains fairly almost the same functional groups, specifically, the O-H stretch, C=O stretch, C-O stretch and the C=C aromatic stretch. Isolate 2 may have a different substituent because it showed no peak in the 1400-1300 regions compared to isolate 1. Figures 3 and 4 shows the infra-red spectra of isolate 1 and isolate 2 respectively.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue X October 2024

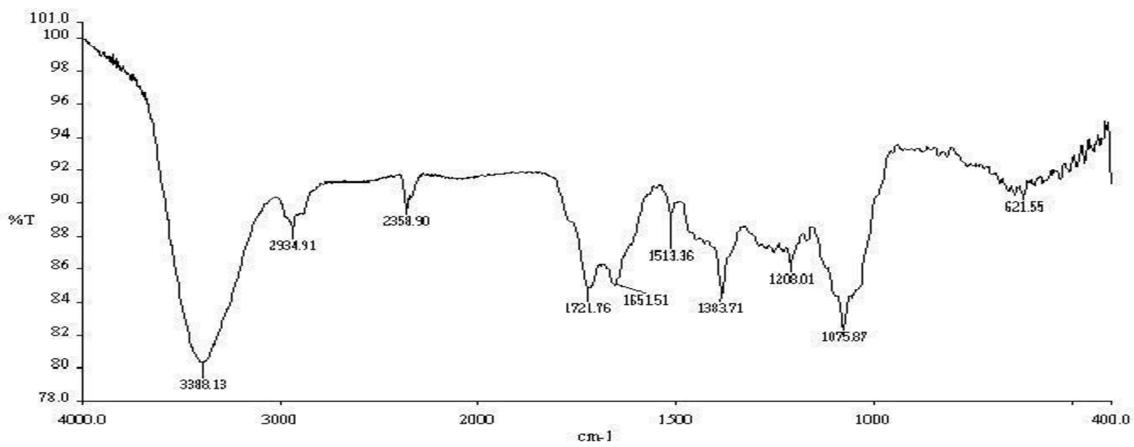

Figure 3. Infra-Red Spectrum of the Isolate 1 as KBr Disk.

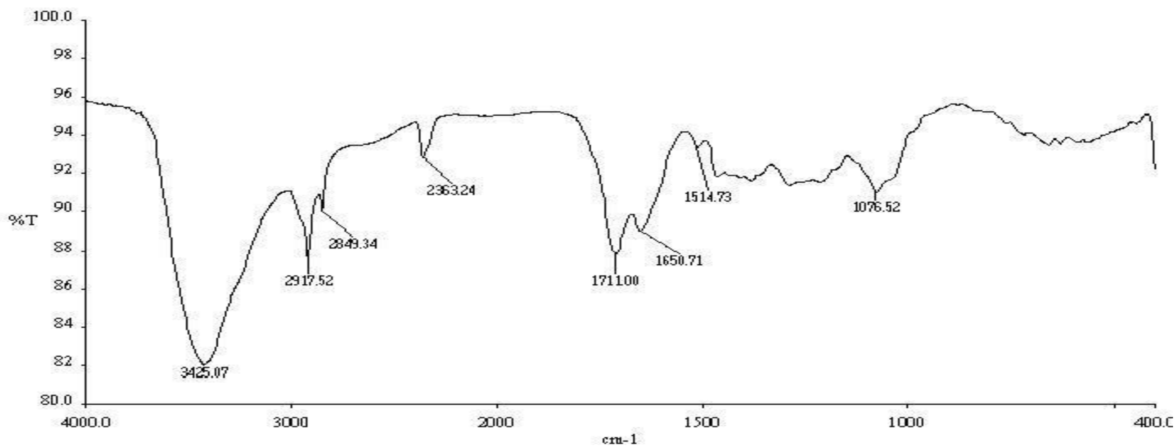

Figure 4. Infra-Red Spectra of Isolate 2 as KBr Disk.

The Advance Institute for Science and Technology (AIST) has the general infra- red spectra of a saponin, shown in Figure 5. The significant peaks have also been listed in Table 9. The spectra show that the functional groups that are present in both isolates 1 and 2 have similarities to the saponin spectra by AIST.

Based from this data, it can be inferred that the Isolate 1 and Isolate 2 are possible types of saponin. It showed that the significant peaks of the isolates are similar to the peaks of the saponin.

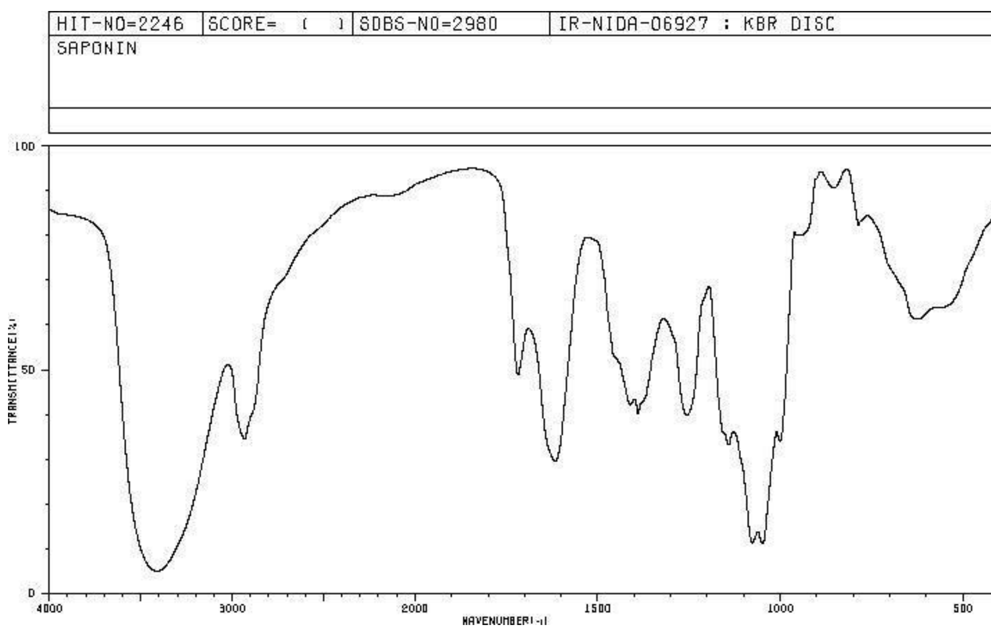

Figure 5. Infra-Red Spectrum of a Saponin

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XI Issue X October 2024

Table 9. Infra-Red Spectroscopic Data of a Saponin.

Wave number (cm^{-1})	Interpretation
3407	O-H stretch
2931	$-CH_3$ stretch
1716	C=O stretch
1616	C=C aromatic stretch
1411	$-CH_2$-O stretch
1390	O-H, aromatic substituent
1266	C-O stretching
622	HC=CH bending

Melting Point of the Isolated Metabolites

Isolate 1 was an amorphous yellowish to colorless solid which melts at 102-104

°C. Isolate 2 was an amorphous yellow-brown solid which melts at 92-94 °C.

CONCLUSIONS

This study determines the bioactivity of the crude extracts from the leaves of the legume, *Phaseolus lunatus*. It also does partial characterization of the isolates from the most bioactive fraction.

The ethanol extracts of the dried leaves were subjected to solvent partitioning. These created four solvent fractions: n-hexane, dichloromethane, ethyl acetate and ethanol-water fractions. These were tested for its bioactivity using the Brine-Shrimp *(Artemia salina)* Lethality Assay. The ethanol extract gave the lowest LC_{50} and was considered the most bioactive solvent fraction.

The bioactive metabolites of the ethanol-water extract were separated by gravity column chromatography employing a gradient elution technique. Two abundant metabolites were isolated. An amorphous yellowish to colorless solid and the other was an amorphous yellow-brown solid. The two isolates were tested for its anti-bacterial property using a gram-negative bacterium, *Escherichia coli* and gram-positive bacterium, *Bacillus subtilis*. They exhibited an anti-bacterial property towards the test organism. The first isolate gave 26.39% and 26.21% activity against *E. coli* and *B. subtilis* respectively. The second isolate gave 21.63% and 22.26% activity against *E. coli* and *B. subtilis* respectively.

The two isolates were subjected to Infra-Red (IR) Spectroscopy, and both gave spectra with the same significant peaks. Isolate 1 has a melting point of 102-104 °C and Isolate 2 has a melting point of 92-94 °C. It could be inferred that both the isolates are compounds that have the same functional group.

The bioactive metabolite from *Phaseolus lunatus* are possibly saponins. The ethanol-water fraction obtained from solvent partitioning of the ethanol extract of the *Phaseolus lunatus* yielded metabolites with anti-bacterial activity. The isolates have different melting point ranges. They have similar peaks of the IR region and are also similar to the peaks of the saponin spectra from AIST. It can be inferred that the isolates are possibly saponins. These possible saponins that were isolated can be the already isolated saponins or another new type of saponin.

The following conclusions may be formulated: (1) The two isolates are probably compounds with the same functional groups. (2) The two isolates are possible saponins. (3) The activities of the isolates showed that they have an anti-bacterial property against *E. coli* and *B. subtilis*.

RECOMMENDATION

1. Future studies should increase the amount of sample to increase the metabolite yield.

2. Different species of gram-positive and gram-negative bacteria should be included as a test organism including *Mycobacterium tuberculosis*.

3. The result of the study proved that ethanol can be used as a solvent to extract the bioactive metabolites but other more polar solvents, such as methanol, can also be used and studied.

4. The isolates should also be tested for other types of bioactivity such as anti- fungal tests, anti-cancer tests, etc.

5. Further characterization may also be done, such as Mass Spectroscopy, Proton Nuclear Magnetic Resonance (^{1}H-NMR) and 2D Nuclear Magnetic Resonance, to further elucidate the structure of the isolates.

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