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A COMPARATIVE STUDY ON THE PHYTOCHEMICALS, ANTIOXIDANTS AND ANTIMICROBIAL ACTIVITIES IN THE LEAVES OF *Aegle marmeloscorrea, Lawsonia inermis* AND *Tabernaemontana coronaria*

A PROJECT REPORT

Submitted by

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BONAFIDE CERTIFICATE

Certified that this project report **"A COMPARATIVE STUDY ON THE PHYTOCHEMICALS, ANTIOXIDANTS AND ANTIMICROBIAL ACTIVITIES IN THE LEAVES OF *Aegle marmeloscorrea, Lawsonia inermis* AND *Tabernaemontana coronaria*"** is the bonafide work of **"P.AMBIKA and K.BHAVANI"** who carried out the project work under my supervision.

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The report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Technology degree in Industrial Biotechnology of Anna University were evaluated and confirmed to be report of the work done by the above students. It was submitted for the viva voce examination submitted on 28/04/06.

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ABSTRACT

Medicinal plants play a key role in the development and advancement of modern studies on biological activities of substances. The present study was focussed on comparing three medicinal plants viz., *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria* for their phytochemical distribution and quantification, antioxidant and antimicrobial activities. The analysis revealed the presence of various phytochemicals in all the three plants. Out of the three plants, *Lawsonia inermis* (henna), a herb proved to possess a high concentration of flavonoids, tannins and non- enzymatic antioxidants like Vitamin C and Vitamin E. Studies also showed a high antibacterial and dye degradation activities for *Aegle marmeloscorrea* and *Tabernaemontana coronaria.*

Overall studies proved that the *Lawsonia inermis* possess a high antioxidant property and *Tabernaemontana coronaria* possess a high antifungal activity. Further, studies can be extended in isolating the bioactive principles and molecules that are responsible for the dye removal.

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1. INTRODUCTION

Since ancient times, man used various natural materials as a source of medicine, among them plants play a vital role (Abdolbaset Ghorbani, 2005). Recent reports of World Health Organisation (WHO) revealed that 80% of the people living in developing countries almost exclusively use traditional medicine for various diseases and in this forum, medicinal plants form a principle component (Eloff, 1998). The knowledge about the use of medicinal plants and their properties was acquired by means of trial and error methods. This has been transmitted from generation to generation (Lev and Amar, 2000; Abdolbaset Ghorbani, 2005) and adapted by all culture of people (Bacquar, 1995; Tsige Gebre - Marium *et al.*, 2005). The use of plant derived compounds for medication is believed to be less toxic and no side effects compared to synthetic chemical drugs. Since last decade, the importance of the traditional medicine has expanded globally (Tsige Gebre –Marium *et al.,* 2005). In view of that, 12,000 medicinal plants have been investigated based on the phytochemical or pharmacological point of view (Harborne *et al.*, 1999;Szaboles Nyriredy, 2004). In recent years, use of ethanobotanical information of medicinal plants have gained considerable attention towards the scientific community owing to their safety and efficacy usage as a traditional medicine (Fransworth, 1994; Eloff, 1998).

Basically, medicinal plants produce two types of constituents viz., primary metabolites (proteins, carbohydrates, nucleic acids etc.,) and secondary metabolites or phytochemicals [alkaloids, flavonoid, polyphenols, tannins, quinones, terpenoids, essential oils, saponins and cardiac glycosides] (Haensel *et al.,* 1999; Majorie Murphy Cowan, 1999; Szaboles Nyriredy, 2004). These phytochemicals possess antimicrobial (Rios *et al.*, 2005), antifungal (Duarte *et al.*, 2005), antidiarrhoeal (Majorie Murphy Cowan, 1999), toothache curing (Abdolbaset Ghorbani, 2005; Amachina Okechukwu Okeke, 2003) activities.

Alkaloids are the group of cyclic organic compounds containing nitrogen in a negative oxidation state. Alkaloids are divided into heterocyclic and non – heterocyclic alkaloids. These alkaloids play an important role in the plant protection and germination and

to be plant growth stimulants (Ameenah Gurib – Fakim, 2005). Alkaloids have been found to have microbicidal effects (Ghosal *et al.,* 1996).

Flavonoids are phenolic structures containing one carbonyl group. They are synthesized by plants in response to the microbial infection (Dixon *et al.,* 1983). They act as co – pigments. They protect plant from UV damaging effects. They possess pharmacological effects as anti inflammatory, anti infective, antioxidant, anti ulcerogic. Example: Quercetin is used for its antidiarrhoeal activity (Ameenah Gurib – Fakim, 2005).

Tannins are a group of polymeric phenolic substances capable of tanning leathers (Majorie Murphy Cowan, 1999). Tannins are used against diarrhoea and antidote in poisoning by heavy metals.Tannins have anticancer and anti HIV activities (Ameenah Gurib – Fakim, 2005).

Phenols are among largest group of secondary metabolites. They are used in dentistry due to its antibacterial and antiinflammatory activities (Ameenah Gurib – Fakim, 2005).

Aglycone part of cardiac glycosides is a tetracyclic steroid with an unsaturated lactone ring. They are used to treat cardiac insufficiency eg. Digitoxin (Padua *et al.,* 1999). Terpenoids are derived from isoprene units, which is biosynthesized from acetate. Iridoids are used for analgesic and anti-inflammatory activities (Ameenah Gurib – Fakim, 2005).

Even though medicinal plants have innumerable properties, the antimicrobial and antioxidant properties are considered to be significant because of their preservative effects conferred to the tissues (Djeridane *et al.*, 2005). Long before the existence of harmful microbes was discovered, the healing power of the plants as a remedy was also exploited. This property is due to certain antimicrobial principles present in the plants (Rios *et al.*, 2005). Antifungal activity was due to the lactones, terpenoids, alkaloids and saponins (Cowan, 1999).

Recently, interest has grown considerably to exploit naturally occurring low molecular antioxidants (Mittal, 2001;Djeridane *et al.,* 2005), which create some beneficial effects in the food and pharmaceutical industries [as an alternative for synthetic antioxidants] (Fulgenico Saura – Calixto *et al.*, 2004; Djeridane *et al.,* 2005). Scientific studies showed that the dietary antioxidants such as Vitamin C, Vitamin E, carotenoids, flavonoid and other phenolic compounds are able to neutralize free radicals (Fulgenico Saura-Calixto *et al.,* 2004).

Thus, the natural products research is an important part of the drug discovery process and the main advantage of these products are their tremendous molecular diversity with various functions (Robert P.Borris, 1996). Owing to an increased awareness about the natural products and phytomedicine among the public and scientific community, it was considered worthwhile to select and study extensively about three commonly available medicinal plants viz., *Aegle marmeloscorrea,Lawsonia inermis* and *Tabernaemontana coronaria* which has a limited documentation.

2.LITERATURE REVIEW

Plants have been utilized as medicines for thousands of years (Samuelsson, 2004). In more recent history, the use of plants as medicines has involved the isolation of active compounds in the early 19th century (Kinghorn, 2001).

2.1 *Aegle marmeloscorrea*

The major constituents of the leaf extracts of *Aegle marmeloscorrea* were identified to be tannins, skimmianins, essential oils (mainly cryophyllene, cineole, citral.citronellal, D-limonene and eugenol), sterols and /or triterpenoids, including lupeol, β- and γ-sitosterol, α – and β-amyrin, flavonoid (mainly rutin) and coumarins, including asgeline, marmesin and umbelliferone. The essential oils obtained from the leaves have shown a broad spectrum of antibacterial and antifungal activities (Pattnaik *et al.,* 1996;Rana *et al.,*). The hydroalcoholic extract of the leaves has been reported to have the chemoprotective potential, especially against chemical carcinogenesis (Singh *et al.,* 2000).

The ethanobotanical studies showed that leaves and ripe fruits of *Aegle marmeloscorrea* have been used in the treatment of diarrhoea and diabetes mellitus. The leaf extracts exhibits antidiabetic action in glucose induced hyperglycaemic rats (Sachdewa *et al.*, 2001).

The roots are astringent, bitter and febrifuge. They are useful in dyspepsia, stomachalgia (Shoba and Thomas, 2001), cardiopalmus, seminal weakness, vomiting, intermittent fever and swellings. The root and bark extract of this plant has been reported to be beneficial to cure intermittent fever, mental disease, pericarditis and angina pectoris. The fruit is used for the treatment of heart ailments.

Fresh aqueous and alcoholic extracts of the plant are reported to have a cardiotonic effects like digitalis and to decrease the need for circulatory stimulants.

Figure 2.1 ***Aegle marmeloscorrea***

The leaves of *Aegle marmeloscorrea* are useful as laxative, febrifuge, also in opthalmia, deafness, inflammations, diabetes, asthmatic and antifungal complaints.

2.2 *Lawsonia inermis*

Henna, an extract of the plant *Lawsonia inermis* has been used for centuries in many cultures, mainly as a dye for hair and nails as well as for decorative body painting.

Figure 2.2 ***Lawsonia inermis***

It is used worldwide not only as a cosmetic agent to stain the nail,hair and skin but also applied to the body as an ointment for the lesions caused by the fungal infections.

The plant leaves are used in the treatment of skin diseases, boils, burns and burning sensation in the feet. Far more than a simple or elaborate means of decorating the body, the henna leaves are said to contain medicinal compounds, which are good for allaying various skin problems. Henna is considered an anti irritant, a deodorant and an antiseptic. It is used by Ayurvedic physicians for skin irritations such as heat rashes and skin allergies and to cool the body during the intense heat of summer. Because of this cooling property, henna leaves and flowers are made into lotions and ointments to be used externally for boils, bruises and skin inflammations, including sores from leprosy.

The flowers are intellect promoting, cardiotonic, refridgerant, soporific, febrifuge and tonic. They are useful in curing cephalagia, burning sensation, cardiopathy, amentia, insomania and fever. Henna also contains mannite, tannic acid, mucilage, gallic acid and napthaquinone. Henna is used in hair shampoos, dyes, conditioners and rinses. Henna dye products are mixed with indigo or other plant material to obtain a greater color range. Extracts of henna are also used to stain wood and to dye fabrics and textiles.

As a medicinal plant, henna has been used for astringent, antihaemorrhagic, intestinal antineoplastic, cardio-inhibitory, hypotensive and sedative effects. It has also been used as a folk remedy against amoebiasis, headache, jaundice and leprosy. Henna extracts show antibacterial, antifungal and ultraviolet light screening activity. Henna has exhibited antifertility activity in animals and may induce menstruation. The dried leaf and petiole of henna are generally recognized as safe when used as a color additive.

There has been found in henna a brown substance of a resinoid fracture, having the chemical properties, which characterize the tannins and therefore named hennotannic acid. It has been employed both internally and locally in jaundice, leprosy, smallpox and affections of the skin. The fruit is thought to have emmenagogue properties.

2.3 *Tabernaemontana coronaria*

The ethanobotany of *Tabernaemontana coronaria* revealed that an infusion of the root is believed to have febrifugal properties. The roots of this plant were use to get relief from toothache (Bani Talapatra *et al.*, 1974).

An infusion of bark and rro is used against dysentery. The juices of the leaves are used as an antihypertensive, diuretic (Gupta *et al.*, 2004), for treating boils, ulcers, headache (Bani Talapatra *et al.*, 1974) and against convulsions. The flowers of this plant were used to treat the inflammation of cornea (Bani Talapatra *et al.*, 1974; Henriques *et al.*, 1995; Gupta *et al.*, 2004) and a remedy for toothache.

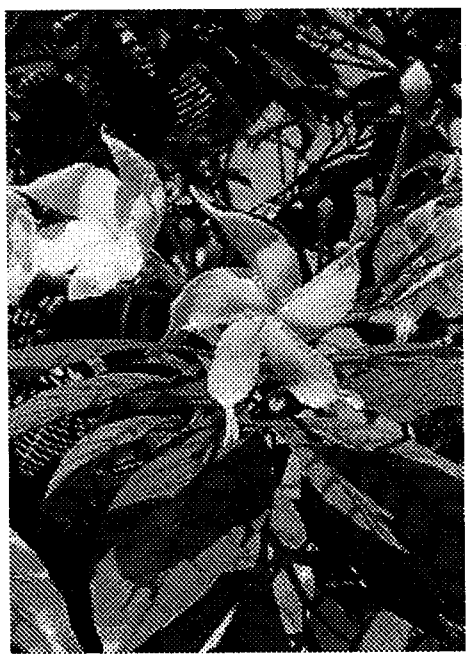


Figure 2.3 *Tabernaemontana coronaria*

The stem and bark of *Tabernaemontana coronaria* has been shown to contain atleast 15 alkaloids of complex indole type (Bani Talapatra *et al.*, 1975), which has antioxidant, antimicrobial(Rojas Hernandez, 1979), antibacterial, antifungal, antidiarrheal and anticancer activities.In addition, terpenoids, steroids, tannins, essential oils, glycosides and saponins produced by the stem, bark and leaves of this species also possesse antimicrobial, anticancer and antioxidant activities(Gupta *et al.*, 2004).

The plant also contain flavonoid or bioflavonoid, present in the leaves and flowers (Cook *et al.*, 2004) possessing various properties like antimicrobial (Gupta *et al.*, 2004),

antioxidant (Gupta *et al.*, 2004), anticancer (Hsu, 1967), hepatoprotective (Defeudis *et al.*, 2004), antiinflammatory, antiallergic and vasodilation effects (Wiseman, 1996).

Som worked on Malay Peninsula species and using HPLC and TLC recognized 16 different types of phenolic comounds including phenolic acids (protocatechuic acid and ellagic acid), 5 types of hydroxycinnamic acids, 4 types of coumarins, the flavonols: quercetin and kaempferol, the flavones: apigenin, the flavonones: narigenin and catechin.

In spite of these innumerable properties, no scientific studies have been validated on:

i) Quantification of the phytochemicals distributed in the three plants.
ii) Assessment of the non-enzymatic antioxidant in the plant samples.
iii) Antibacterial and antifungal activities against pathogenic microorganisms using various leaf extracts (aqueous and organic) of the three plants.

3. OBJECTIVES

1. To screen the presence of various phytochemicals in the leaf extracts of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria* using various solvents (water, methanol, ethanol, chloroform and heptane).
2. To quantify important phytochemicals like alkaloids, flavonoids, tannin and total phenolics in various solvent extracts.
3. Quantification of total proteins.
4. Assessment of non-enzymatic antioxidants (Vitamin C and Vitamin E).
5. To study the antibacterial (*Escherichia coli, Staphylococcus aureus, Salmonella typhii, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and antifungal (*Aspergillus niger, Pencillium chrysogenum, Giberella fujikori* and *Aspergillus terreus*) activities in different solvent extracts.

4.MATERIALS AND METHODS

4.1 PHYTOCHEMICAL SCREENING

4.1(a) ANALYSIS OF ALKALOIDS BY MARQUI'S TEST

SAMPLE PREPARATION:

Different solvents ranging from highly non-polar to polar (Heptane > chloroform > Ethanol > Methanol > Aqueous) were used to extract alkaloids from the leaf sample.

The extract of each sample was prepared by soaking 100g of dried powdered samples in 200mL of solvent for 12 h. The extracts were filtered using Whatman filter paper No 42 (125 mm).

PROCEDURE:

1. 1mL of leaf extract was mixed with 1mL of Marqui's reagent.
2. Observe the color change (dark orange/purple) for the positive result.

REAGENTS:

Marqui's reagent:

To 3mL of concentrated sulphuric acid, 2 drops of 40% formaldehyde was added and mixed well.

4.1(b) ANALYSIS OF FLAVONOID

SAMPLE PREPARATION:

Different solvents ranging from highly non-polar to polar (Heptane > chloroform > Ethanol > Methanol > Aqueous) were used to extract flavonoid from the leaf sample.

The extract of each sample was prepared by soaking 100g of dried powdered samples in 200mL of solvent for 12 h. The extracts were filtered using Whatman filter paper No 42 (125 mm).

PROCEDURE:

1. 5ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract.
2. Then concentrated H_2S0_4 was added.
3. Observe a yellow colouration for the positive result.

4.1(c) ANALYSIS OF TANNINS BY $FeCl_3$ TEST

PRINCIPLE:

Ferric chloride reacts with the (-OH) group attached to a aromatic ring to give a purple colored complex. The colored complex was due to the formation of a doubly coordinated bond to the Fe^{3+} given by the phenolic compounds.

$$3ArOH + FeCl_3 \xrightarrow{\text{pyridine}} \underset{\text{colored complex}}{Fe(OAr)_3}$$

PROCEDURE:

1. About 0.5g of the dried powdered samples was boiled in 20ml of water in a test tube
2. The mixture was then filtered.
3. A few drops of 0.1% ferric chloride were added to the filtrate.
4. Observe a brownish green or a blue-black colouration for the positive result.

4.1(d) ANALYSIS OF PHLOBATANNINS

PROCEDURE:

1. An aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid.
2. Check for the red precipitate for the positive result.

4.1(e) ANALYSIS OF SAPONIN BY FROTH TEST

PROCEDURE:

1. About 2g of the powdered sample was boiled in 20ml of distilled water in a water bath and filtered
2. 10ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth.
3. The frothing was mixed with 3 drops of olive oil and shaken vigorously.
4. Observe the formation of emulsion for the positive result.

4.1(f) ANALYSIS OF STERIODS BY LIBERMANN BURCHARD TEST

SAMPLE PREPARATION:

Different solvents ranging from highly non-polar to polar (Heptane > chloroform > Ethanol > Methanol > Aqueous) were used to extract steroids from the leaf sample.

The extract of each sample was prepared by soaking 100g of dried powdered samples in 200mL of solvent for 12 h. The extracts were filtered using Whatman filter paper No 42 (125 mm).

PROCEDURE:

1. 2ml of acetic anhydride was added to 0.5mL extract of each sample with 2ml H_2S0_4.
2. The colour changed from violet to blue or green in some samples indicating the presence of steroids.

4.1(g) ANALYSIS OF TERPENOIDS BY SALKOWSKI TEST

SAMPLE PREPARATION:

Different solvents ranging from highly non-polar to polar (Heptane > chloroform > Ethanol > Methanol > Aqueous) were used to extract terpenoids from the leaf sample.

The extract of each sample was prepared by soaking 100g of dried powdered samples in 200mL of solvent for 12 h. The extracts were filtered using Whatman filter paper No 42 (125 mm).

PROCEDURE:

1. 5ml of each extract was mixed in 2ml of chloroform, and concentrated H_2S0_4 (3ml) was carefully added to form a layer.
2. A reddish brown colouration of the interface was formed to show positive results for the presence of terpenoids.

4.1(h) ANALYSIS OF CARDIAC GLYCOSIDES BY KELLER-KILLANI TEST

SAMPLE PREPARATION:

Different solvents ranging from highly non-polar to polar (Heptane > chloroform > Ethanol > Methanol > Aqueous) were used to extract cardiac glycosides from the leaf sample.

The extract of each sample was prepared by soaking 100g of dried powdered samples in 200mL of solvent for 12 h. The extracts were filtered using Whatman filter paper No 42 (125 mm).

PROCEDURE:

1. 5ml of each extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution.
2. This was underlayed with 1ml of concentrated sulphuric acid.

3. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

4.1(i) ANALYSIS OF REDUCING SUGARS BY FEHLINGS TEST

PRINCIPLE:

Cupric ion complexed with tartrate ion is reduced to cuprous oxide. Cupric ion is present in the alkaline solution. Fehling's solution contains tartrate ion as a complexng agent to keep the copper ion in solution. So the reduction of Cu^{2+} to Cu^{+} by reducing sugars results in the formation of reddish brown precipitate of Cu_2O.

SAMPLE PREPARATION:

Different solvents ranging from highly non-polar to polar (Heptane > chloroform > Ethanol > Methanol > Aqueous) were used to extract reducing sugars from the leaf sample.

The extract of each sample was prepared by soaking 100g of dried powdered samples in 200mL of solvent for 12 h. The extracts were filtered using Whatman filter paper No 42 (125 mm).

PROCEDURE:

1. To 200µL of each extract, 200µL of Fehling's A and Fehling's B was added.
2. The tubes were heated in a boiling water bath for 1minute.
3. Observe a red precipitate for the positive result.

4.2 QUANTIFICATION OF PHYTOCHEMICALS

4.2.1 ESTIMATION OF TOTAL PROTEINS BY FOLIN-LOWRY'S METHOD

PRINCIPLE:

Proteins reacted with Folin Ciocalteau reagent give a blue coloured complex. The blue colour so formed was due to the reaction of alkaline copper with the protein and the reduction of phosphomolybdenate by tyrosine and tryptophan, present in the protein. The intensity of the colour depends on the amount of aromatic amino acids present and they vary for different proteins. The blue colour developed was spectrophotometrically measured at 750nm.

REAGENTS:

See Appendix 1

PROCEDURE:

1. Pipetted out 0.2, 0.4, 0.6, 0.8 and 1mL (concentration varying from 20 to 100µg) of the working standard solution into a series of test tubes.
2. Pipetted out 0.2mL of the sample into a test tube.
3. To all the tubes, including the blank, distilled water was added to make up to 1mL.
4. To all the tubes, added 2.1mL of Alkaline Copper reagent.
5. The tubes were incubated at room temperature for 10 minute.
6. Then added 0.2mL of folin's reagent in all the test tubes and kept at room temperature for 20 minute.
7. The absorbance was read spectrophotometerically at 750nm.
8. A graph was drawn by plotting the concentration of total protein along the X-axis and the optical density reading along Y-axis.
9. From the graph, the unknown sample concentration was calculated and the results were expressed as mg/g tissue.

CALCULATION

Graphical method:

Optical density reading b corresponds to a μg of proteins.

i.e.1 mL of unknown sample contains a μg of proteins.

Therefore, 100 mL of unknown sample contains (aX100) / (1X1000) mg/g of proteins.

Theoretical method:

$$\frac{\text{(OD of test) X Concentration of standard (μg)}}{\text{(OD of standard)}} \text{ X } \frac{\text{(made up volume) (mL)}}{\text{(sample volume) (mL)}}$$

4.2.2 ESTIMATION OF TOTAL PHENOLICS BY FOLIN-CIOCALTEAU METHOD

PRINCIPLE:

The hydroxyl (-OH) groups of phenolic compounds reduced the phosphomolybdic acid to molybdenum blue in the presence of an alkaline medium (present in Folin's reagent). The blue coloured complex was then spectrophotometrically measured at 650nm.

REAGENTS:

See Appendix 2

PROCEDURE:

1. Pipetted out 0.2, 0.4, 0.6, 0.8 and 1mL (concentration varying from 20 to 100μg) of the working standard solution into a series of test tubes.
2. Pipetted out 0.1mL of the sample into a test tube.
3. To all the tubes, including the blank, distilled water was added to make up to 4mL.
4. To all the tubes, added 0.5mL of folin's reagent.

5. The tubes were incubated at room temperature for 3 minute.
6. Then added 2mL of 20% Sodium carbonate in all the test tubes.
7. Kept at boiling water bath for 1 minute.
8. The absorbance was read spectrophotometerically at 650nm.
9. A graph was drawn by plotting the concentration of total phenolics along the X-axis and the optical density reading along Y-axis.
10. From the graph, the unknown sample concentration was calculated and the results were expressed as mg/g tissue.

CALCULATION

Graphical method:

Optical density reading b corresponds to a µg of proteins.

i.e.1 mL of unknown sample contains a µg of proteins.

Therefore, 100 mL of unknown sample contains (aX100) / (1X1000)mg/g of proteins.

Theoretical method:

$$\frac{\text{(OD of test) X Concentration of standard (µg)}}{\text{(OD of standard)}} \text{X} \frac{\text{(made up volume) (mL)}}{\text{(sample volume) (mL)}}$$

4.2.3 ESTIMATION OF ALKALOIDS BY HARBORNE METHOD

PRINCIPLE:

Alkaloid is extracted from plant material. It is precipitated by the addition of concentrated Ammonium hydroxide. After thoroughly washing with diluted Ammonium hydroxide, the precipitate is dried and weighed.

REAGENTS:

See Appendix 3

PROCEDURE:

1. The leaf sample was filtered and the extract was used for the experimental analysis.
2. Then,the extract was concentrated on a boiling water bath to one-quarter of the original volume.
3. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was completed.
4. Then, the whole solution was allowed to settle and the precipitate was collected.
5. Then the precipitate was washed with diluted ammonium hydroxide and then filtered with the Whatman filter paper. The filter paper was dried and weighed before and after filtration.
6. The difference in the weight gives the amount of alkaloid present in the extract.
7. The residue in the filter paper was scraped and qualitative test for alkaloids was performed and confirmed.

4.2.4 ESTIMATION OF FLAVONOID BY BOHM AND KOCIPAI-ABYAZAN METHOD

REAGENTS:

See Appendix 4

PROCEDURE:

1. 10g of the dried leaves was extracted repeatedly with 100mL of 80% Aqueous methanol at room temperature.
2. The whole solution was filtered through Whatman filter paper No 42 (125 mm).
3. The filtrate was later transferred into a beaker and evaporated into dryness over a water bath and weighed to a constant weight.
4. The difference in the weight gives the amount of flavonoid present in the extract.

5. The residue in the filter paper was scraped and qualitative test for flavonoid was performed and confirmed.

4.2.5 ESTIMATION OF TANNINS BY MODIFIED PRUSSIAN BLUE METHOD

PRINCIPLE:

Phenols oxidized Potassium ferricyanide to produce ferrous ions. These ferrous ions in turn reacted with ferric chloride in the presence of diluted HCl to form a Prussian blue colored complex, which can be measured spectrophotometricaly at 700nm.

REAGENTS:

See Appendix 5

PROCEDURE:

1. Pipetted out 0.2, 0.4, 0.6, 0.8 and 1mL (concentration varying from 20 to 100μg) of the working standard solution into a series of test tubes.
2. Pipetted out 0.1mL of the sample into a test tube.
3. To all the tubes, including the blank, distilled water was added to make up to 7mL.
4. To all the tubes, added 1mL of Potassium ferric cyanide and 1mL of $FeCl_3$. Mixed well.
5. The absorbance was read spectrophotometerically at 700nm.
6. A graph was drawn by plotting the concentration of tannin along the X-axis and the optical density reading along Y-axis.
7. From the graph, the unknown sample concentration was calculated and the results were expressed as mg/g tissue.

CALCULATION

Graphical method:

Optical density reading b corresponds to a μg of proteins.

i.e. 1 mL of unknown sample contains a μg of proteins.

Therefore, 100 mL of unknown sample contains (aX100) / (1X1000)mg/g of proteins.

Theoretical method:

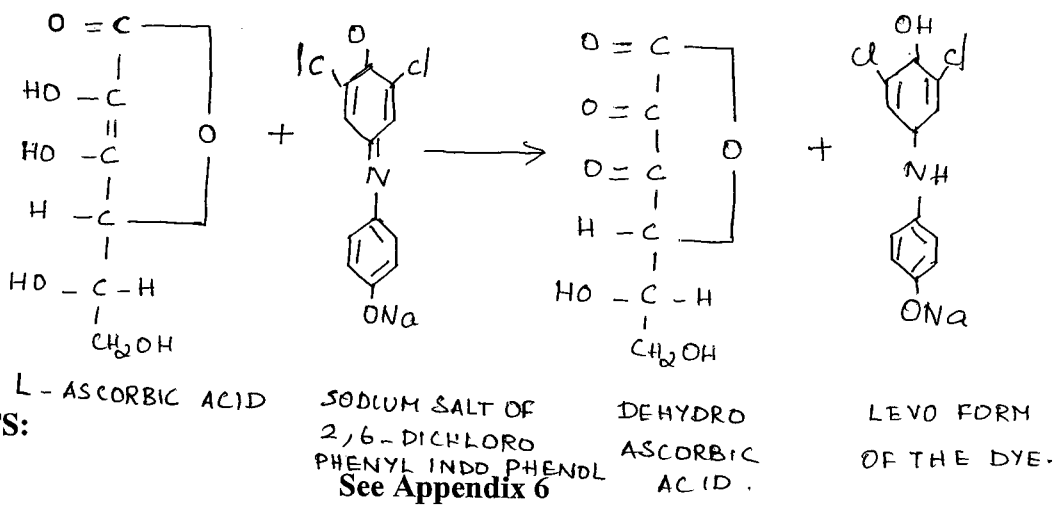
$$\frac{\text{(OD of test) X Concentration of standard (μg)}}{\text{(OD of standard)}} \text{ X } \frac{\text{(made up volume) (mL)}}{\text{(sample volume) (mL)}}$$

4.3 QUANTIFICATION OF NON-ENZYMATIC ANTIOXIDANTS

4.3.1 ESTIMATION OF ASCORBIC ACID BY 2,4-DNPH METHOD

PRINCIPLE:

Ascorbic acid was dehydrogenated in the presence of bromine water or activated charcoal (oxidizing agents) to form dehydroascorbic acid. Dehydroascorbic acid then reacted with 2,4,dinitrophenyl hydrazine to give an orange red colour osazone derivative, which was dissolved by 80% sulphuric acid. Thiourea acted as a preservative in the mechanism. The orange red colour was then spectrophotometrically measured at 540nm.



REAGENTS: See Appendix 6

PROCEDURE:

1. Pipetted out 0.2, 0.4, 0.6, 0.8 and 1mL (concentration varying from 20 to 100µg) of the working standard solution into a series of test tubes.
2. Pipetted out 0.6mL of the sample into a test tube.
3. To all the tubes, including the blank, distilled water was added to make up to 3mL.
4. To all the tubes, added 1mL of 2,4-DNPH.
5. The contents were mixed and incubated at 37°C for 3 hours.

6. After incubation, orange red osazone crystals were dissolved in 7mL of 80%H_2SO_4.
7. The absorbance was read spectrophotometerically at 540nm.
8. A graph was drawn by plotting the concentration of Vitamin C along the X-axis and the optical density reading along Y-axis.
9. From the graph, the unknown sample concentration was calculated and the results were expressed as mg/g tissue.

CALCULATION

Graphical method:

Optical density reading b corresponds to a µg of proteins.

i.e.1 mL of unknown sample contains a µg of proteins.

Therefore, 100 mL of unknown sample contains (aX100) / (1X1000)mg/g of proteins.

Theoretical method:

$$\frac{\text{(OD of test) X Concentration of standard (µg)}}{\text{(OD of standard)}} \text{X} \frac{\text{(made up volume) (mL)}}{\text{(sample volume) (mL)}}$$

4.3.2 ESTIMATION OF VITAMIN E BY BAKER AND FRANK METHOD

PRINCIPLE:

Vitamin E was oxidized in the presence of $FeCl_3$ (Fe^{3++}) to give an intermediate, which then reacted 2,2'-dipyridyl reagent to form a pink colored product. This was spectrophotometrically measured at 520 nm.

REAGENTS:

See Appendix 7

PROCEDURE:

1. Pipetted out 0.5, 1, 1.5, 2 and 2.5mL (concentration varying from 50 to 250µg) of the working standard solution into a series of test tubes.
2. To all the tubes, including the blank, distilled water was added to make up to 3mL.
3. Pipetted out 1.5mL of the sample and 1.5mL of ethanol into a test tube.
4. To all the tubes, added 1.5mL of Xylene.
5. The contents were centrifuged at 5000rpm for about half an hour.
6. Transferred 1.0mL of xylene layer into another stoppered tube, taking care not to include any ethanol or protein.
7. Added 1.0mL of 2,2' dipyridyl reagent to each tube.
8. Beginning with the blank, 0.33mL of ferric chloride solution was added to all the test tubes.
9. The absorbance was read spectrophotometerically at 520nm.
10. A graph was drawn by plotting the concentration of Vitamin E along the X-axis and the optical density reading along Y-axis.
11. From the graph, the unknown sample concentration was calculated and the results were expressed as mg/g tissue.

CALCULATION

Graphical method:

Optical density reading b corresponds to a µg of proteins.

i.e.1 mL of unknown sample contains a µg of proteins.

Therefore, 100 mL of unknown sample contains (aX100) / (1X1000)mg/g of proteins.

Theoretical method:

$$\frac{\text{(OD of test) X Concentration of standard (µg)}}{\text{(OD of standard)}} \text{X} \frac{\text{(made up volume) (mL)}}{\text{(sample volume) (mL)}}$$

4.4 ASSESSMENT OF ANTIBACTERIAL ACTIVITIES BY DISC-DIFFUSION METHOD (KIRBY-BAUER)

PRINCIPLE:

The spread plate technique requires a previously diluted mixture of micro-organism. During inoculation, the cells are spread over surface of the solid agar medium with a sterile L-shaped bent rod. The cells are pushed into separate areas on the surface so that they can form individual colonies.

MATERIALS:

Nutrient broth, petriplates, glasswares, paper discs and plant extracts.

TEST ORGANISMS:

The test organisms were selected on the basis that they cause a lot of infections in humans. Both Gram- positive and Gram- negative bacterial species were selected as test organisms. The following microorganisms were used for the study: *Escherichia coli, Staphylococcus aureus, Salmonella typhii, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.*

INOCULATION PROCEDURE:

50mL of the Nutrient broth was prepared for each of the test bacterial strain. 50mL of the broth was sterilized at 121°C for 15 minute. The broth was inoculated aseptically with the appropriate microorganisms. The inoculated broth was incubated at 37°C for 24 hour. The procedure was repeated for each bacterial species.

PROCEDURE:

1. 0.2mL of the bacterial suspension was pipetted into the appropriately labeled petri dish and spread uniformly on the Nutrient agar surface.
2. 5mm diameter paper discs were dipped in the plant extracts for 3 minute and placed on the agar surface.

3. Gentamycin was used as the positive control.
4. The plates were incubated at 37°C for 24 hours after which zones of inhibition was taken to be the diameter of the zone visibly showing the absence of growth including the diameter of the paper disc.
5. The zones of inhibition were measured and recorded.

4.5 ASSESSMENT OF ANTIFUNGAL ACTIVITIES BY DISC-DIFFUSION METHOD (KIRBY-BAUER)

PRINCIPLE:

The spread plate techniques require a previously diluted mixture of microorganism. During inoculation, the cells are spread over surface of the solid agar medium with a sterile L-shaped bent rod. The cells are pushed into separate areas on the surface so that they can form individual colonies.

MATERIALS:

Czapekdox broth (see Appendix 8), petriplates, glasswares, paper discs and plant extracts.

TEST ORGANISMS:

The test organisms were selected on the basis that they cause infections in humans. The following microorganisms were used for the study: *Aspergillus niger, Pencillium chrysogenum, Giberella fujikori* and *Aspergillus terreus*

INOCULATION PROCEDURE:

50mL of the Czapekdox broth was prepared for each of the test fungal strain. 50mL of the broth was sterilized at 121°C for 15 minute. The broth was inoculated aseptically with the appropriate microorganisms. The inoculated broth was incubated at room temperature for 4 days. The procedure was repeated for each fungal species.

PROCEDURE

1. 0.2mL of the fungal suspension was pipetted into the appropriately labeled petri dish and spread uniformly on the Czapekdox agar surface.
2. 5mm diameter paper discs were dipped in the plant extracts for 3 minute and placed on the agar surface.
3. Gentamycin was used as the positive control.
4. The plates were incubated at room temperature for 4 days after which zones of inhibition was taken to be the diameter of the zone visibly showing the absence of growth including the diameter of the paper disc.
5. The zones of inhibition were measured and recorded.

5. RESULTS AND DISCUSSIONS

This study was focused to compare the presence of various phytochemicals, quantification of certain important phytochemicals like total proteins, alkaloids, flavonoids, tannins, total phenolics and non- enzymatic antioxidants (Vitamin C and Vitamin E) using various solvents, in the leaf extracts of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria*. The study has been also concentrated on the antibacterial and antifungal activities of different solvent extracts. For the experimental analysis, the leaves of the above mentioned plants were collected from the medicinal garden located at Kumaraguru college of Technology campus, Coimbatore.

The leaf extracts screened for the presence of phytochemicals in the plant, *Aegle marmeloscorrea* showed the existence of alkaloids, flavonoids, tannins, phlobatannins, saponins, steroids, terpenoids, cardiac glycosides and reducing sugars. Significantly, the chloroform extract alone showed the presence of reducing sugars. The extract also showed to possess traces of alkaloids, flavonoids, steroids and terpenoids. It was proved that the aqueous extract lack terpenoids completely. **(See Table 5.1).** The study also revealed that traces of phlobatannins were present only in this plant. **(See Table 5.4).**

The leaf extract of *Lawsonia inermis* proved to have certain phytochemicals like alkaloids, flavonoids, tannins, saponins, steroids, terpenoids, cardiac glycosides and reducing sugars. Terpenoids were absent only in the aqueous extract. Heptane extract showed the absence of steroids and reducing sugars. The study also evidenced the presence of alkaloids, flavonoids and cardiac glycosides in all the solvent extracts. **(See Table 5.2).**

The phytochemical screening performed in the leaf extract of *Tabernaemontana coronaria* showed the presence of alkaloids, flavonoids, tannins, saponins, steroids, terpenoids, cardiac glycosides and reducing sugars. Terpenoids were absent only in the heptane extract. Steroids and terpenoids were present only in the aqueous and chloroform extract. Aqueous and heptane extract showed the absence of reducing sugars. The study significantly proved the presence of alkaloids, flavonoids and cardiac glycosides in all the solvent extracts. **(See Table 5.3).**

SOLVENT TYPE	ALKALOIDS	FLAVONOIDS	TANNINS	PHLOBA TANNINS	SAPONINS	STEROIDS	TERPENOIDS	CARDIAC GLYCOSIDES	REDUCING SUGARS
AQUEOUS	+	+	+	+ TRACES	+	+	–	+	–
METHANOLIC	+	+				+	+	+	–
ETHANOLIC	+	+				+	+	+	–
HEPTANE	+	+ TRACES				+	+	+	–
CHLOROFORM	+ TRACES	+ TRACES				+ TRACES	+ TRACES	+	+

Table 5.1 Analysis of Phytochemicals using various solvents for leaf extracts of *Aegle marmeloscorrea*

SOLVENT TYPE	ALKALOIDS	FLAVONOIDS	TANNINS	PHLOBA TANNINS	SAPONINS	STEROIDS	TERPENOIDS	CARDIAC GLYCOSIDES	REDUCING SUGARS
AQUEOUS	+	+	+	–	+	+	–	+	+ TRACES
METHANOLIC	+	+				+	+	+	+
ETHANOLIC	+	+				+	+	+	+
HEPTANE	+	+				–	+	+	–
CHLOROFORM	+	+				+	+	+	+

Table 5.2 Analysis of Phytochemicals using various solvents for leaf extracts of *Lawsonia inermis*

SOLVENT TYPE	ALKALOIDS	FLAVONOIDS	TANNINS	PHLOBA TANNINS	SAPONINS	STEROIDS	TERPENODS	CARDIAC GLYCOSIDES	REDUCING SUGARS
AQUEOUS	+	+	+	–	+	+ TRACES	+ TRACES	+	–
METHANOLIC	+	+				+	+	+	+
ETHANOLIC	+	+				+	+	+	+
HEPTANE	+ TRACES	+ TRACES				+	–	+	–
CHLOROFORM	+	+ TRACES				+ TRACES	+ TRACES	+	+

Table 5.3 Analysis of Phytochemicals using various solvents for leaf extracts of *Tabernaemontana coronaria*

LEAF SAMPLE	ALKALOIDS	FLAVONOIDS	TANNINS	PHLOBA TANNINS	SAPONINS	STEROIDS	TERPENOIDS	REDUCING SUGARS	CARDIAC GLYCOSIDES
Aegle marmeloscorrea	+	+	+	TRACES	+	+	+	+	+
Lawsonia inermis	+	+	+	–	+	+	+	+	+
Tabernaemontana coronaria	+	+	+	–	+	+	+	+	+

Table 5.4 Comparative phytochemical analysis of *Aegle marmeloscorrea*, *Lawsonia inermis* and *Tabernaemontana coronaria*

The comparative phytochemical analysis of the three plants showed that traces of phlobatannins were present only in the *Aegle marmeloscorrea,* but other phytochemicals like alkaloids, flavonoids, tannins, saponins, steroids, terpenoids, cardiac glycosides and reducing sugars were present in all the three plants. **(See Table 5.4).**

The quantification of total proteins analyzed by Folin – Lowry's method showed the aqueous extract to possess a higher amount than the other solvents i.e., more than 20mg of total proteins were present per gram tissue in all the 3 plants. It was observed that the two nonpolar solvents heptane and chloroform were too poor in extracting the proteins from all the plants. A moderate extraction was done by methanol and ethanol i.e., approximately **10mg/g tissue**. Of the three plants, *Aegle marmeloscorrea* was proved to possess a higher concentration of total proteins **(24.58mg/g tissue)**. **(See Figure 5.1 and Figure 5.2)**.

Analysis of total phenolics by Folin – Ciocalteau method proved that the solvent methanol has a higher extraction capacity than the other solvents (i.e., more than 8mg/g tissue). It was also observed that the nonpolar solvents like chloroform, heptane and the polar solvent water were too poor in extracting the phenolic compounds **(2mg/g tissue)**. Overall studies revealed that the plant *Tabernaemontana coronaria* possess *a* higher amount of phenolic compounds than the other 2 plants **(11.7mg/g tissue)**. The pie chart representation supported that *Tabernaemontana coronaria* possess *a* higher concentration of phenolic compounds (i.e., **41%**) when compared to other 2 plants. **(See Figure 5.3 and Figure 5.4)**.

Quantification of alkaloid content by using ammonium hydroxide precipitation method revealed that the aqueous leaf extract of *Tabernaemontana coronaria* possess a higher amount than the other two plants **(192mg/g tissue)**. The plant *Aegle marmeloscorrea* has found to contain least level of alkaloids **(120mg/g tissue)**. The Pie chart analysis revealed that the *Tabernaemontana coronaria* **(40%)** has about 1½ fold increase of alkaloid content than the *Aegle marmeloscorrea* **(25%)**. **(See Figure 5.5 and Figure 5.6).**

Gravimetric analysis was performed to quantify the flavonoid content in all the 3 plants. The studies have proved that the solvent methanol is an efficient one to extract significant amount of flavonoids **(140mg/g tissue).** It was also observed that the nonpolar solvents chloroform and heptane has extracted least amount of flavonoids **(20mg/g tissue).** Overall comparison of all the 3 plants revealed that *Lawsonia inermis* has the highest flavonoid content than the other 2 plants **(182mg/g tissue).** The Pie chart analysis showed that the *Lawsonia inermis* **(43%)** has about 1½ fold increase of flavonoid content than the *Aegle marmeloscorrea* **(25%). (See Figure 5.7 and Figure 5.8).**

Tannins were estimated by modified Prussian blue method. It was found to have a higher level of tannins in the aqueous leaf extract of *Lawsonia inermis* **(0.027mg/g tissue).** The study also revealed that *Tabernaemontana coronaria* possess *a* lower level of tannins than the other 2 plants **(0.013mg/g tissue).**.The Pie chart analysis showed that the *Lawsonia inermis* **(45%)** has about 2 fold increase of tannin content than the *Tabernaemontana coronaria* **(21%).** *(***See Figure 5.9 and Figure 5.10).**

Vitamin C (Ascorbic acid) was estimated using 2,4 - DNPH method. A significant increase in the vitamin content was noticed in the aqueous leaf extract of *Lawsonia inermis* than the other two plants **(2.55mg/g tissue).** An interesting observation was made with the help of Pie diagram. This diagram proved the presence of an efficient non enzymatic antioxidant system in the plant *Lawsonia inermis (56%)* than the other 2 plants **(20%, 24%). (See Figure 5.11 and Figure 5.12).**

Vitamin E (α-tocopherol) was analyzed by Baker and Frank method. It was found that the aqueous leaf extract of *Lawsonia inermis* to possess a higher concentration of vitamin E than the other two plants **(0.0016mg/g tissue).**
(See Figure 5.13 and Figure 5.14).

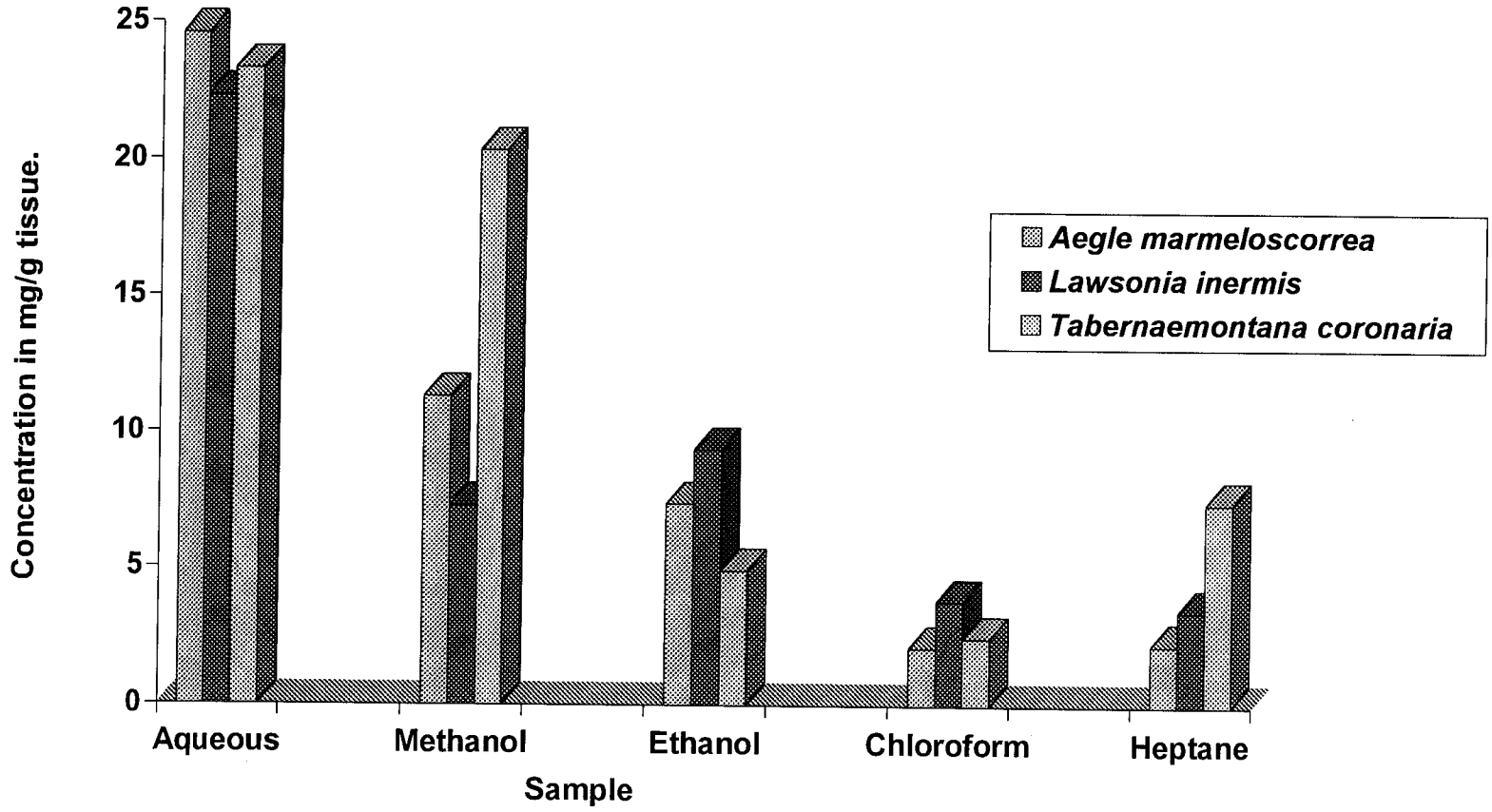


Figure 5.1 Comparison of total protein concentration using various solvents in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

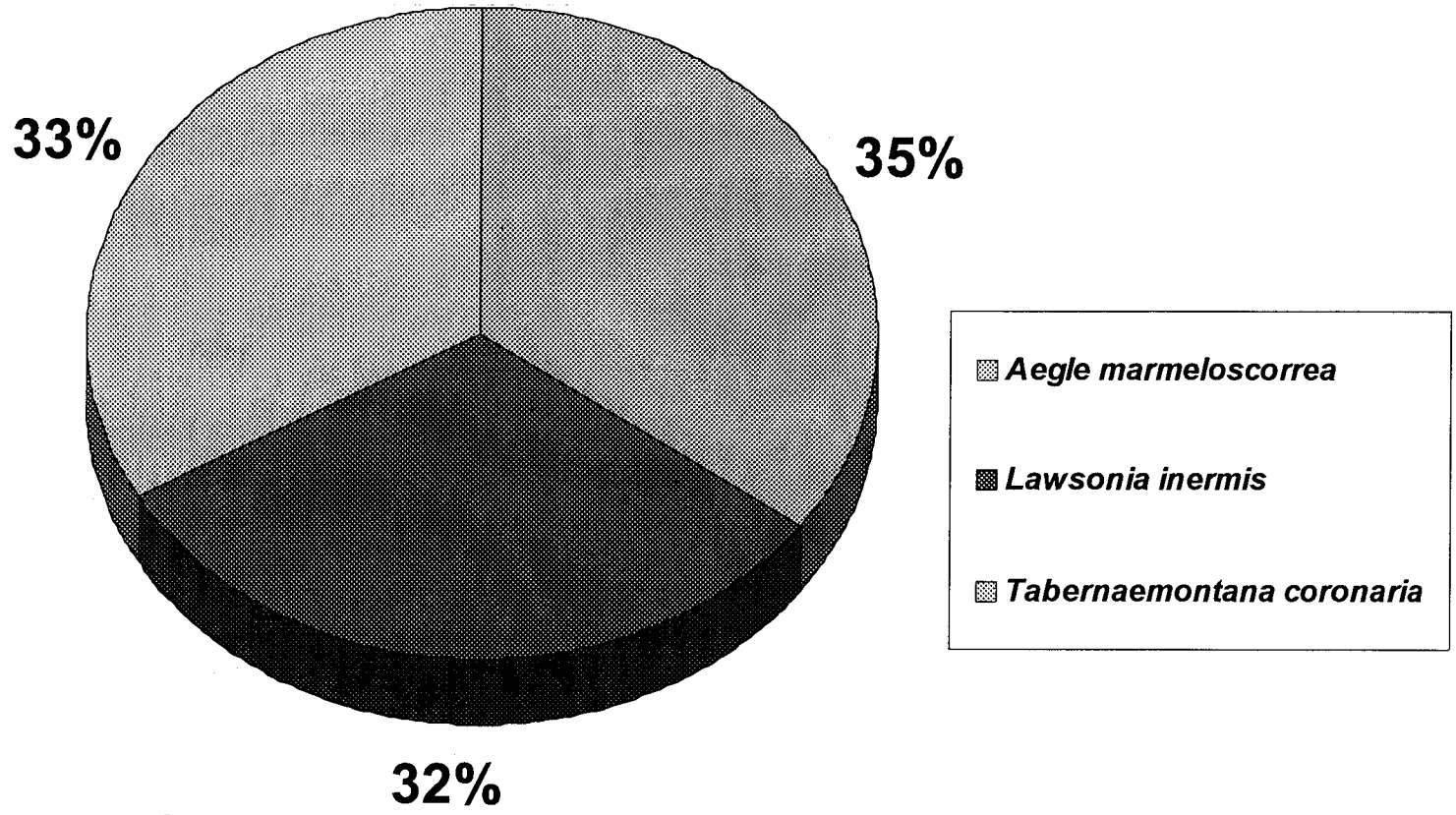


Figure 5.2 Comparison of total protein concentration in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

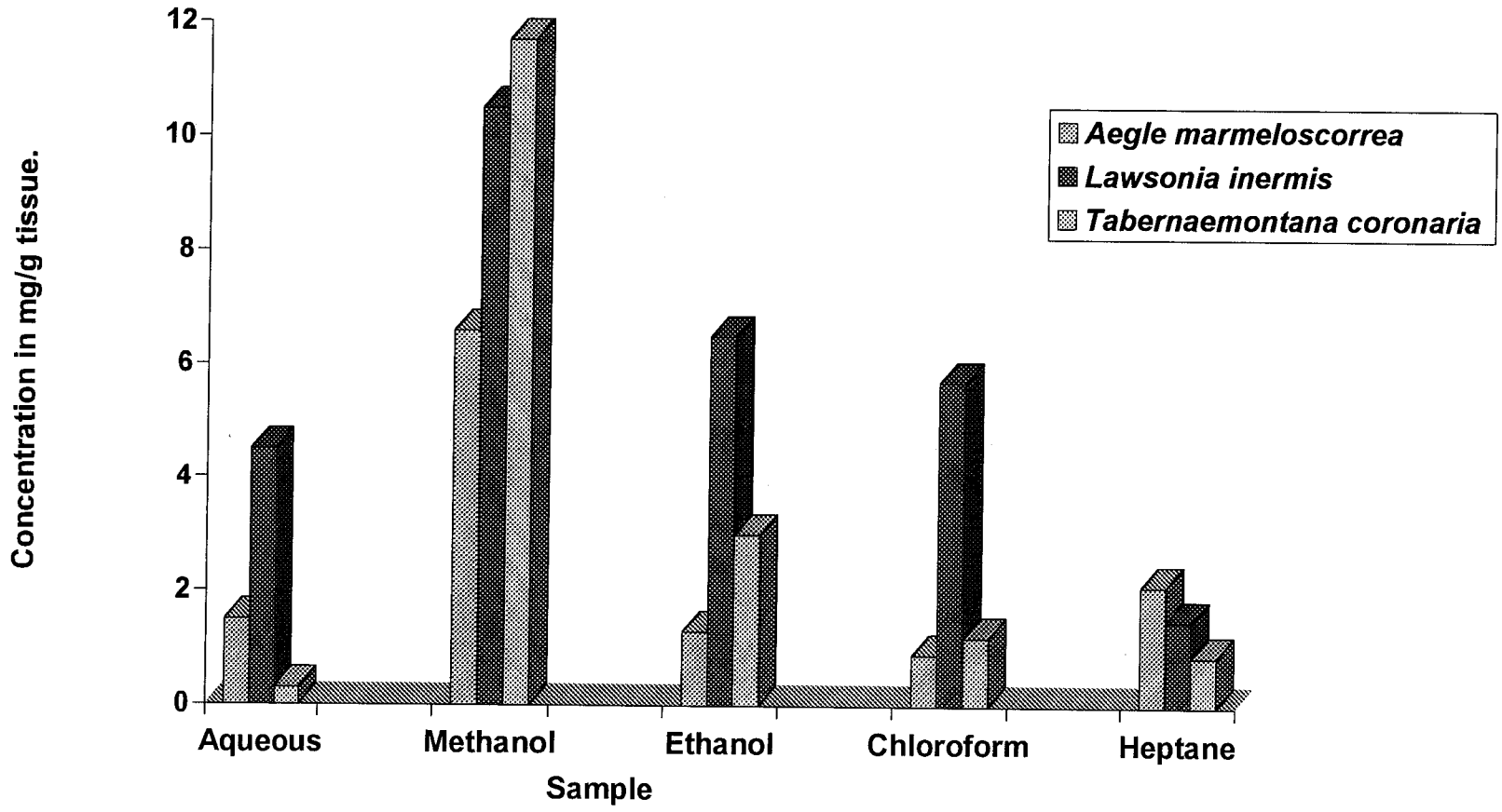


Figure 5.3 Comparison of total phenolics concentration using various solvents in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

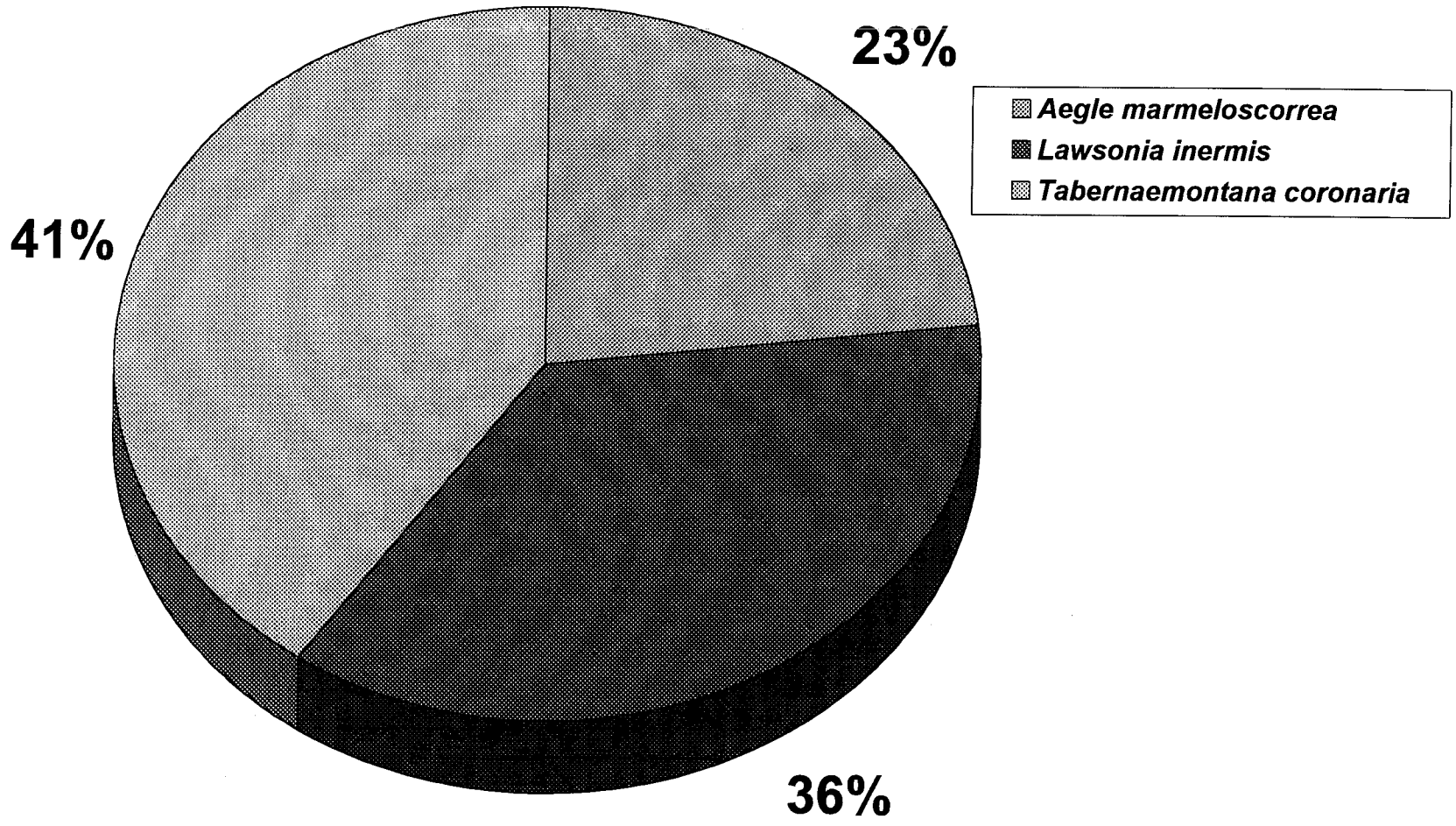


Figure 5.4 Comparison of total phenolics concentration in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

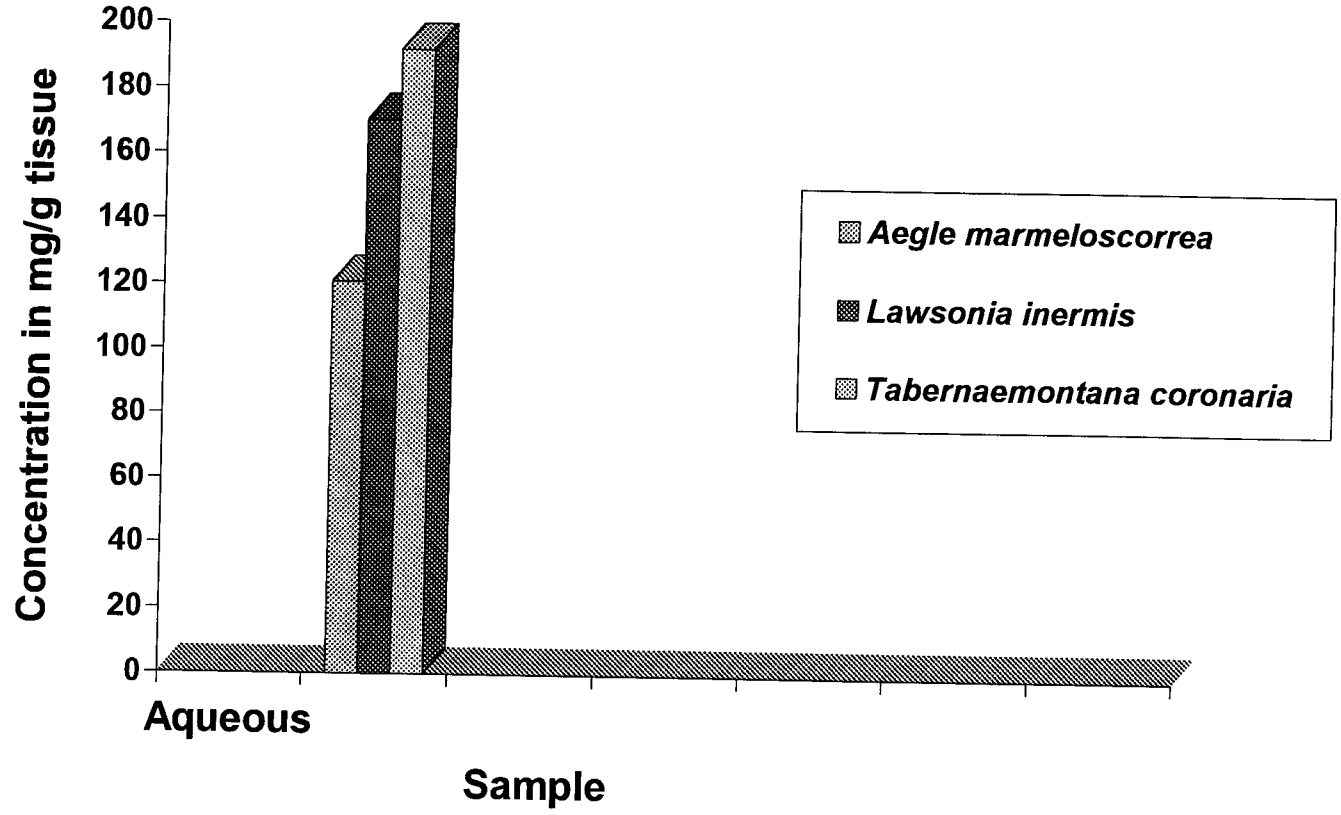


Figure 5.5 Comparison of alkaloids concentration in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

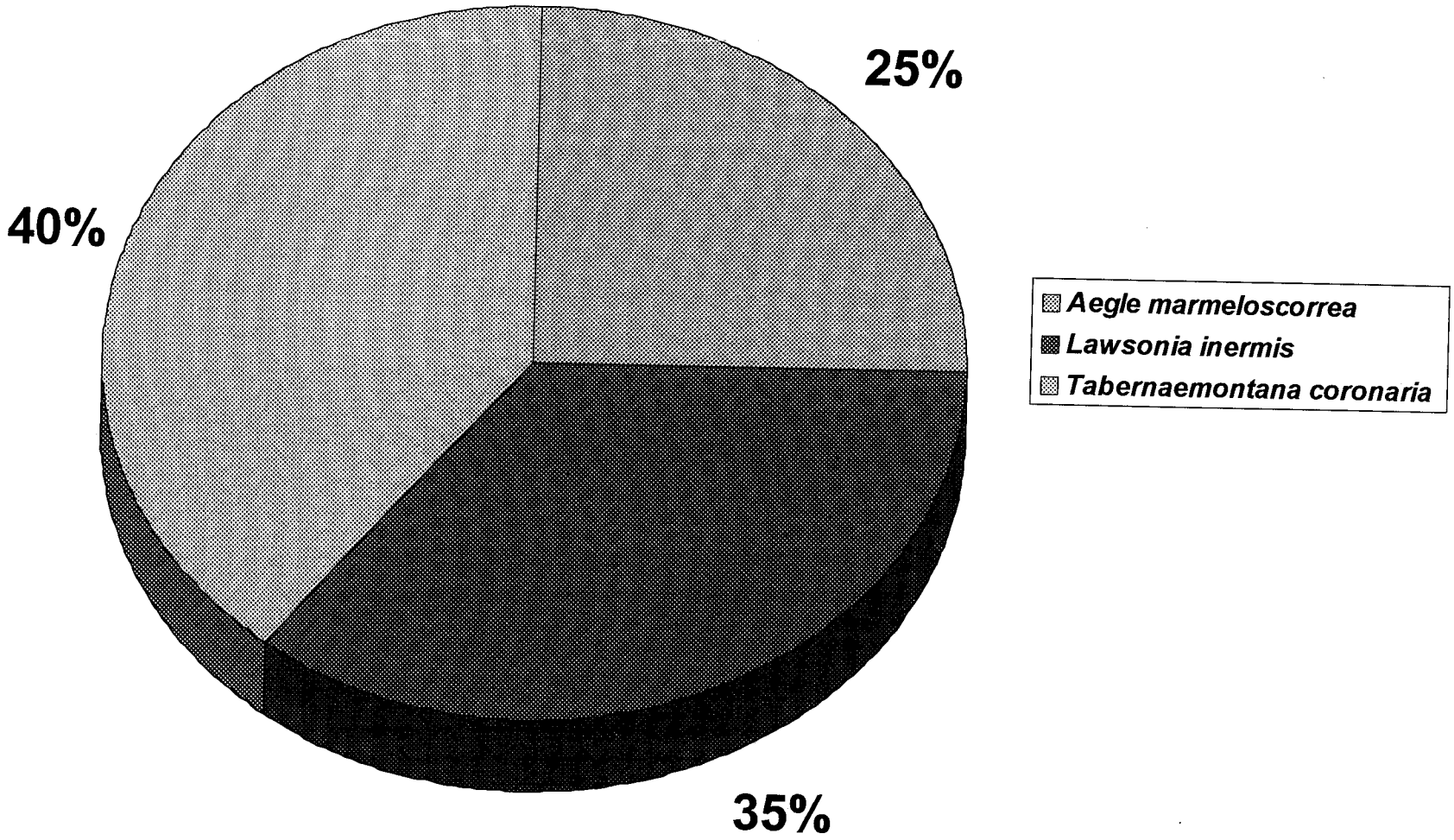


Figure 5.6 Comparison of alkaloids concentration in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

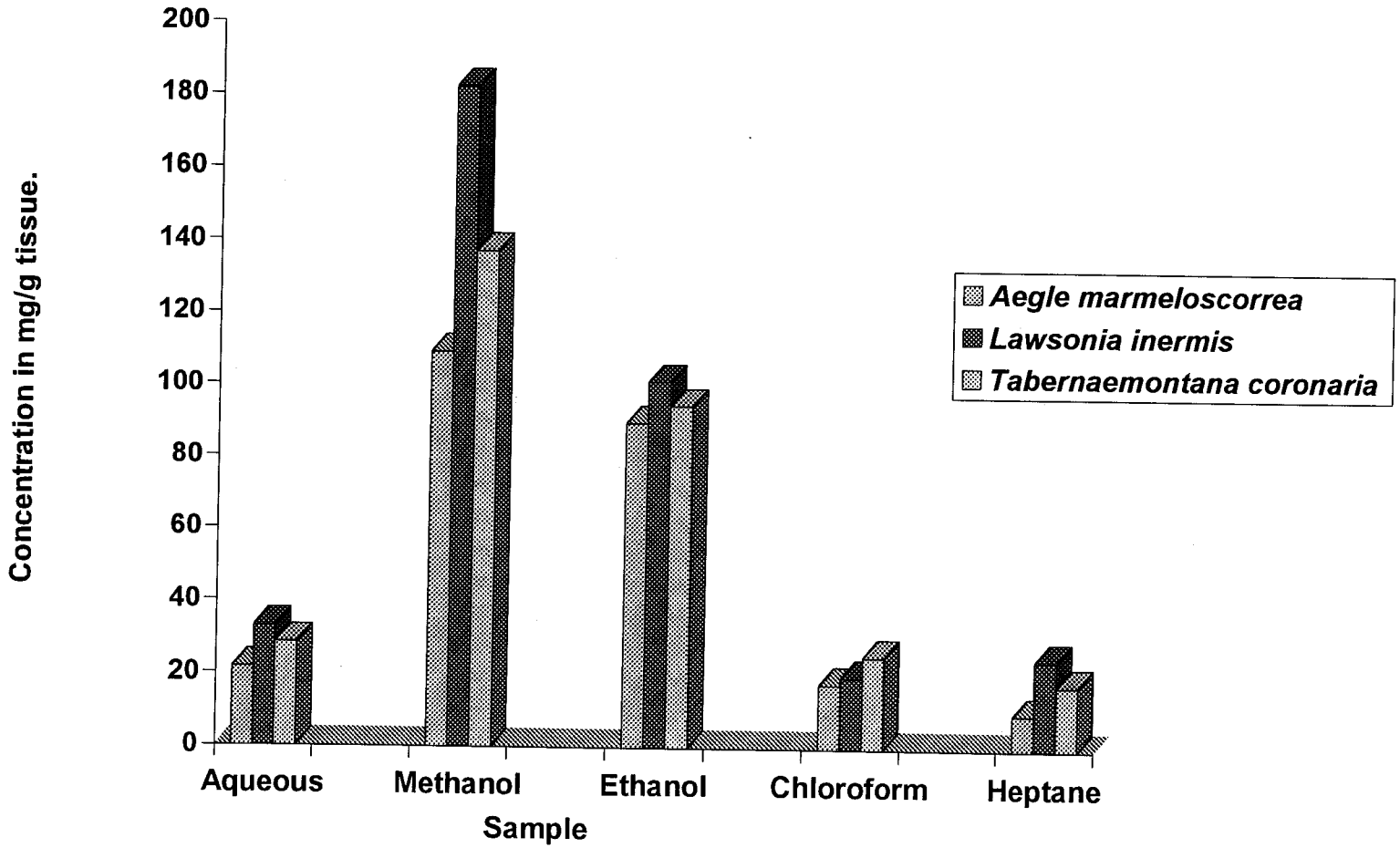


Figure 5.7 Comparison of flavonoids concentration using various solvents in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

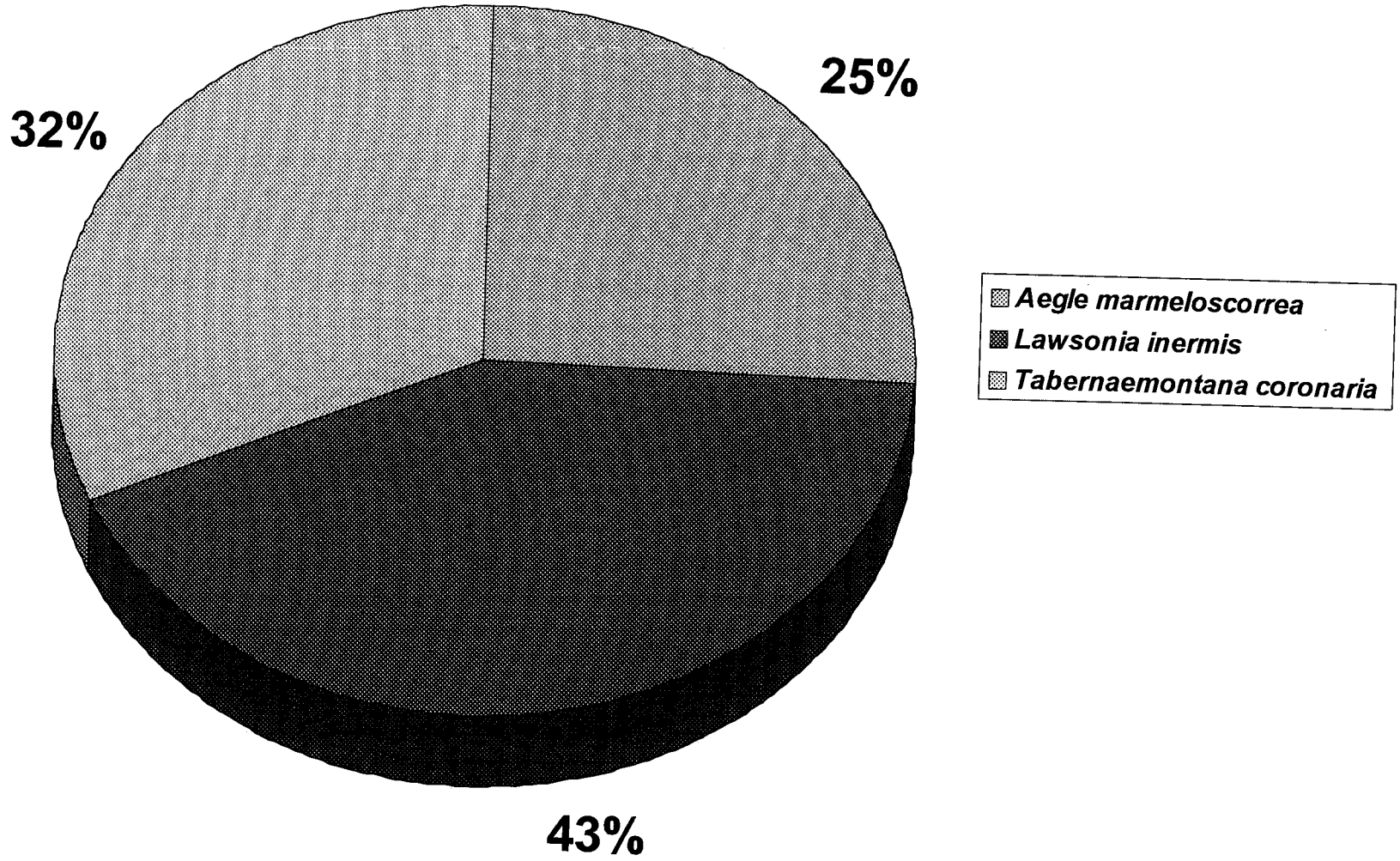


Figure 5.8 Comparison of flavonoids concentration in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

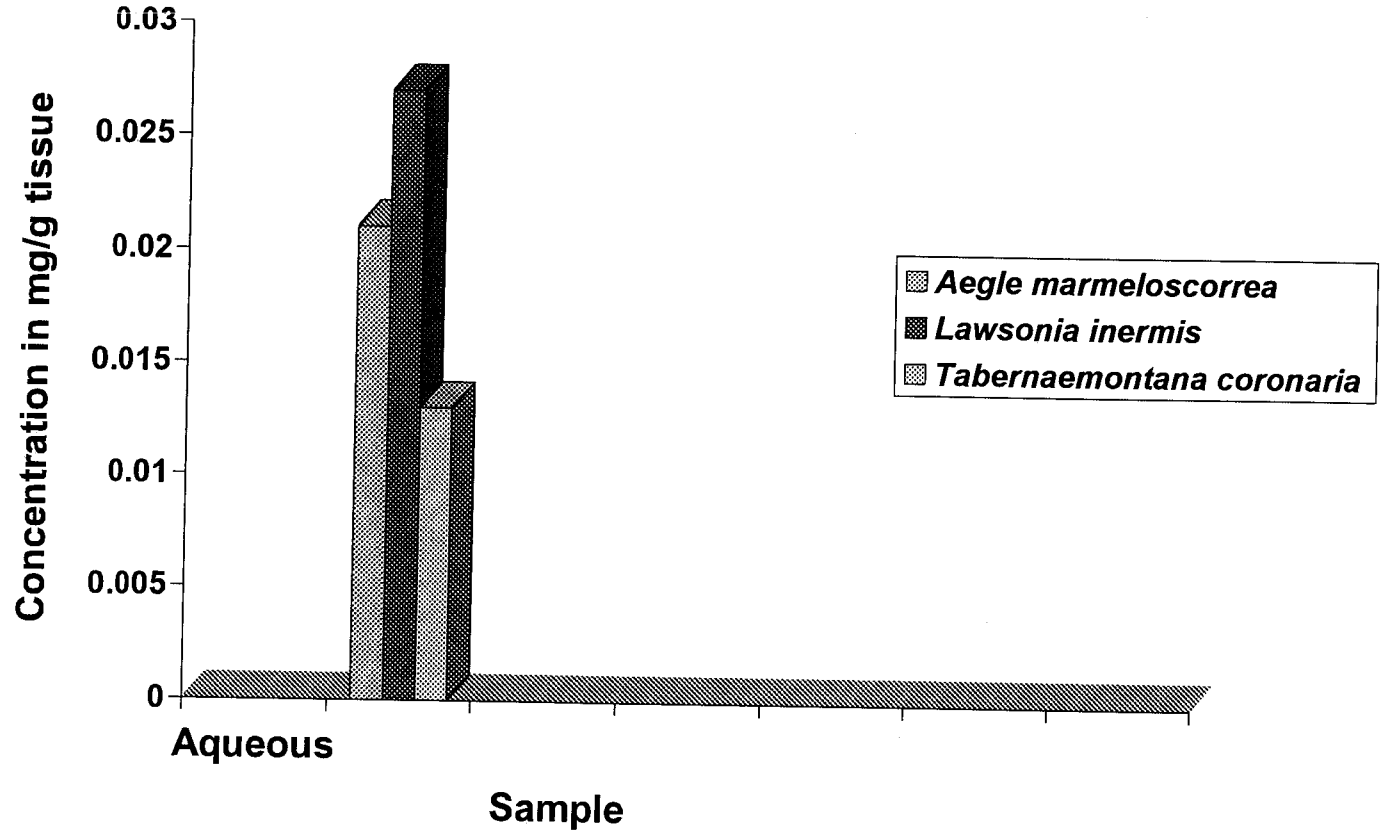


Figure 5.9 Comparison of tannins concentration in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

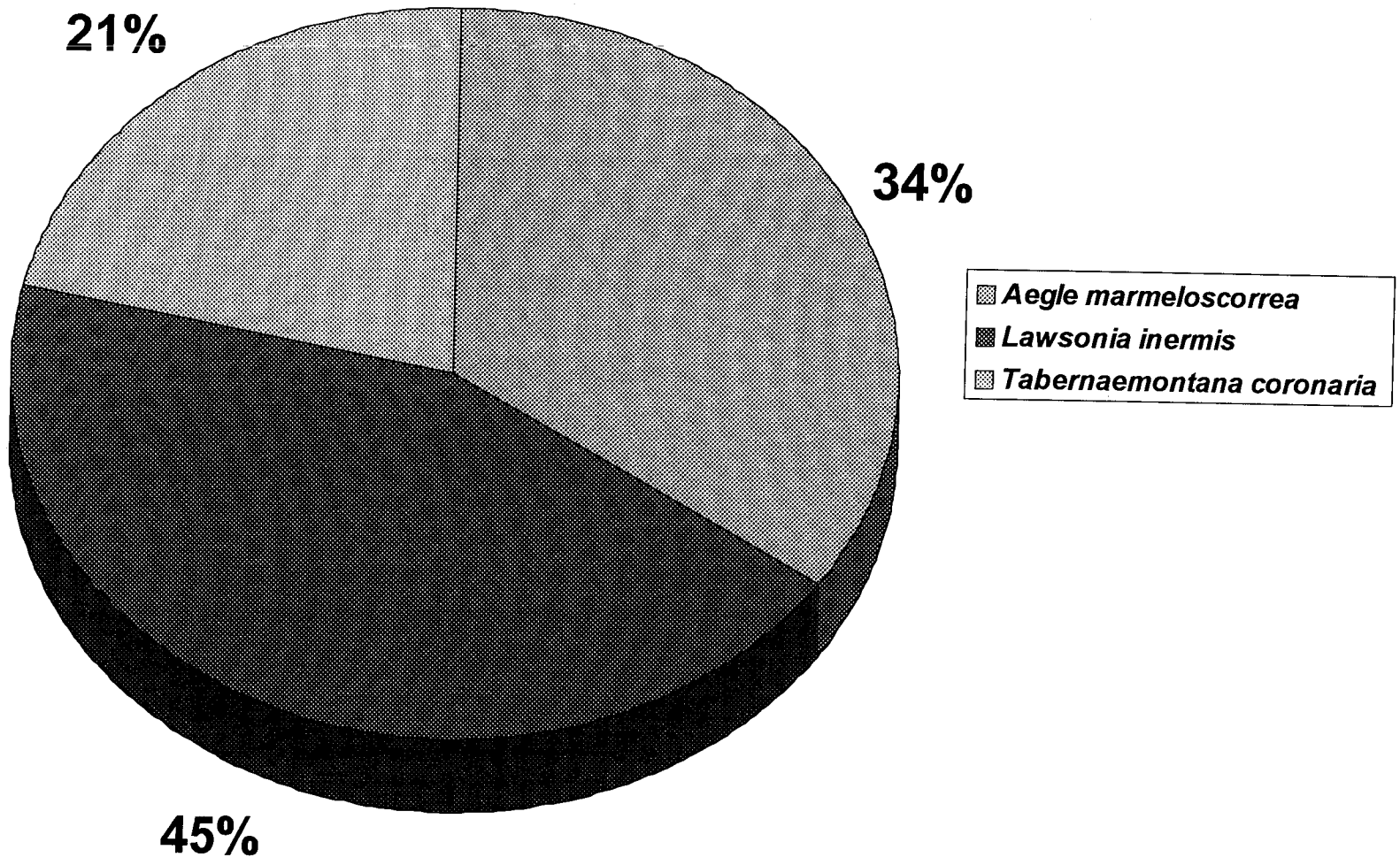


Figure 5.10 Comparison of tannins concentration in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

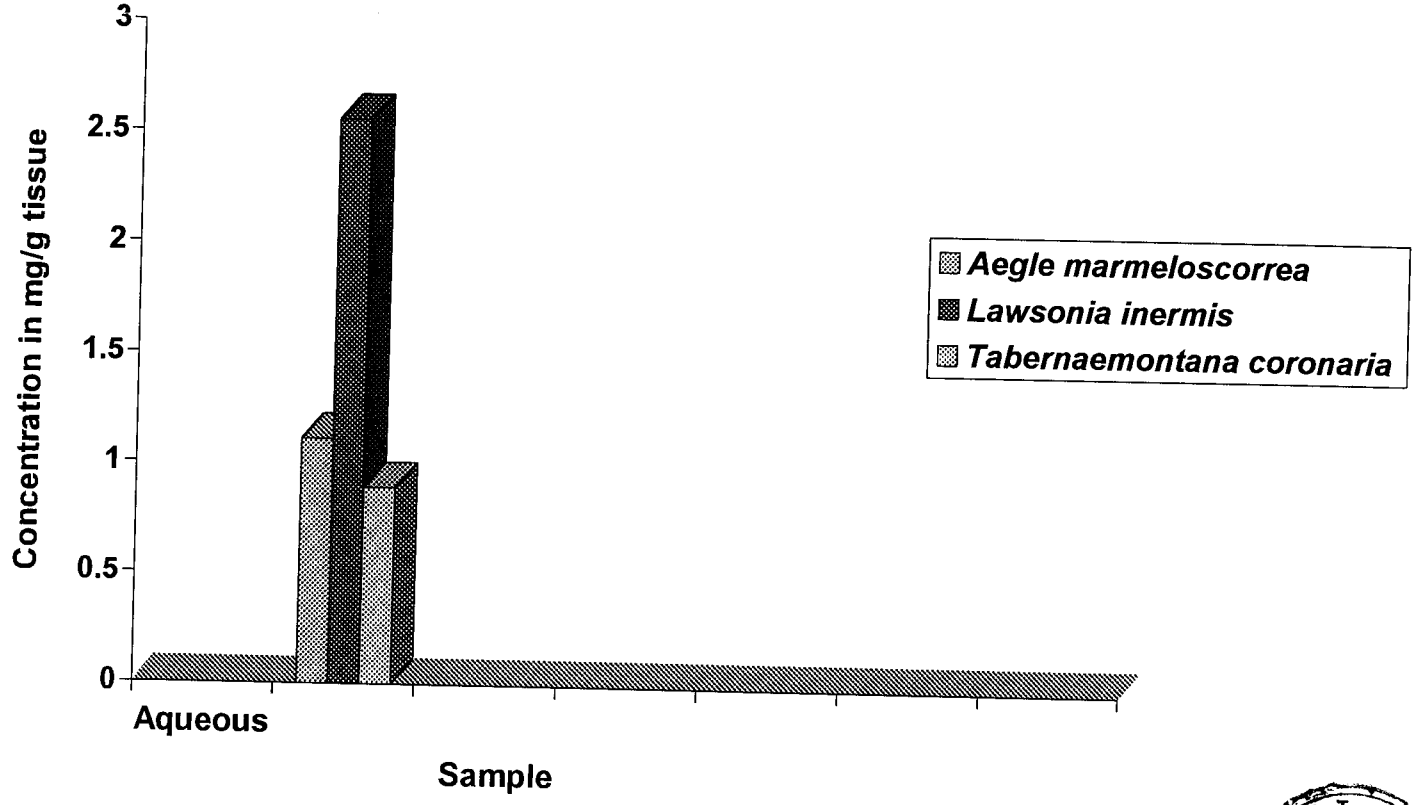


Figure 5.11 Comparison of Vitamin C concentration in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

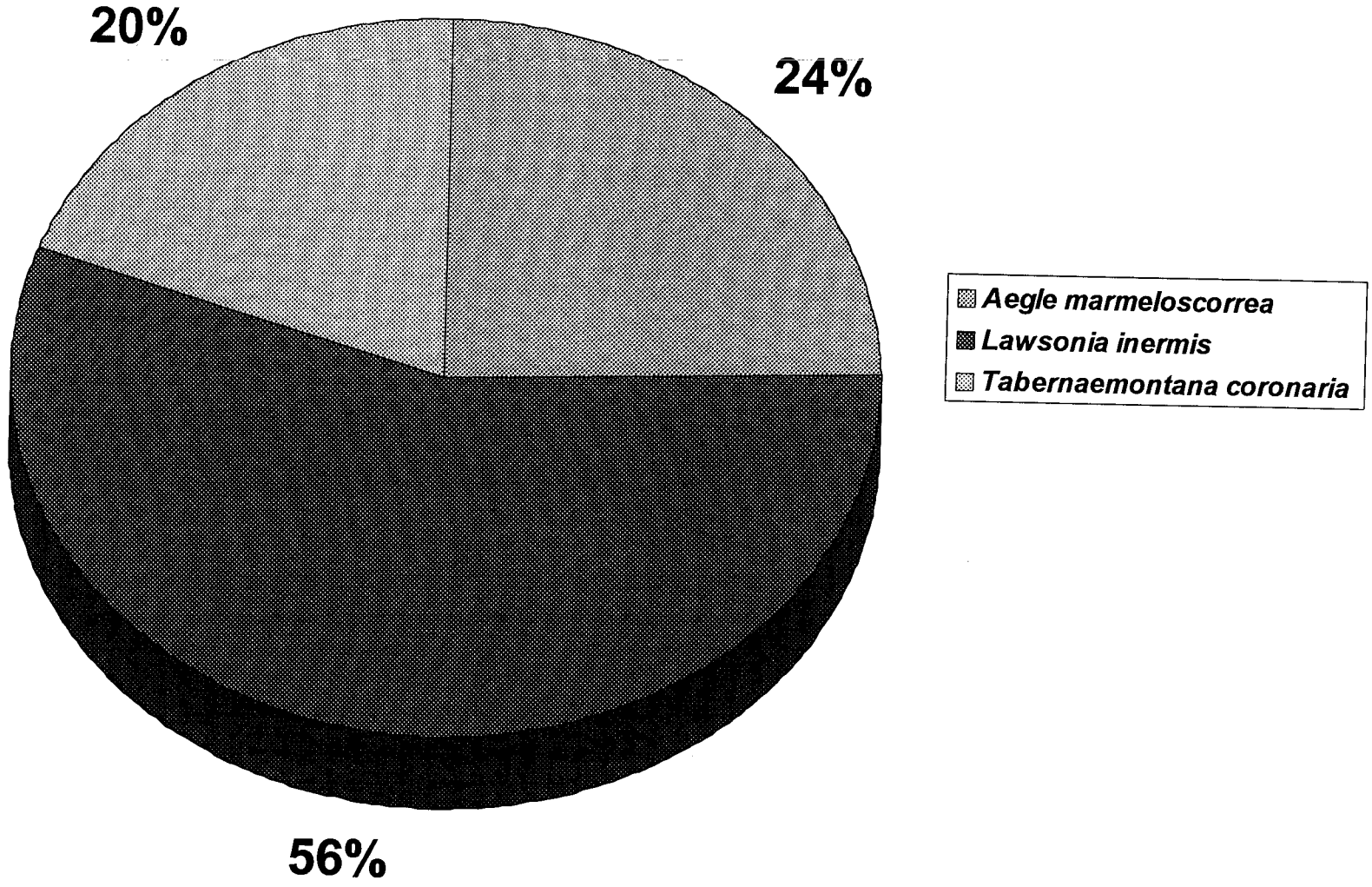


Figure 5.12 Comparison of Vitamin C concentration in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

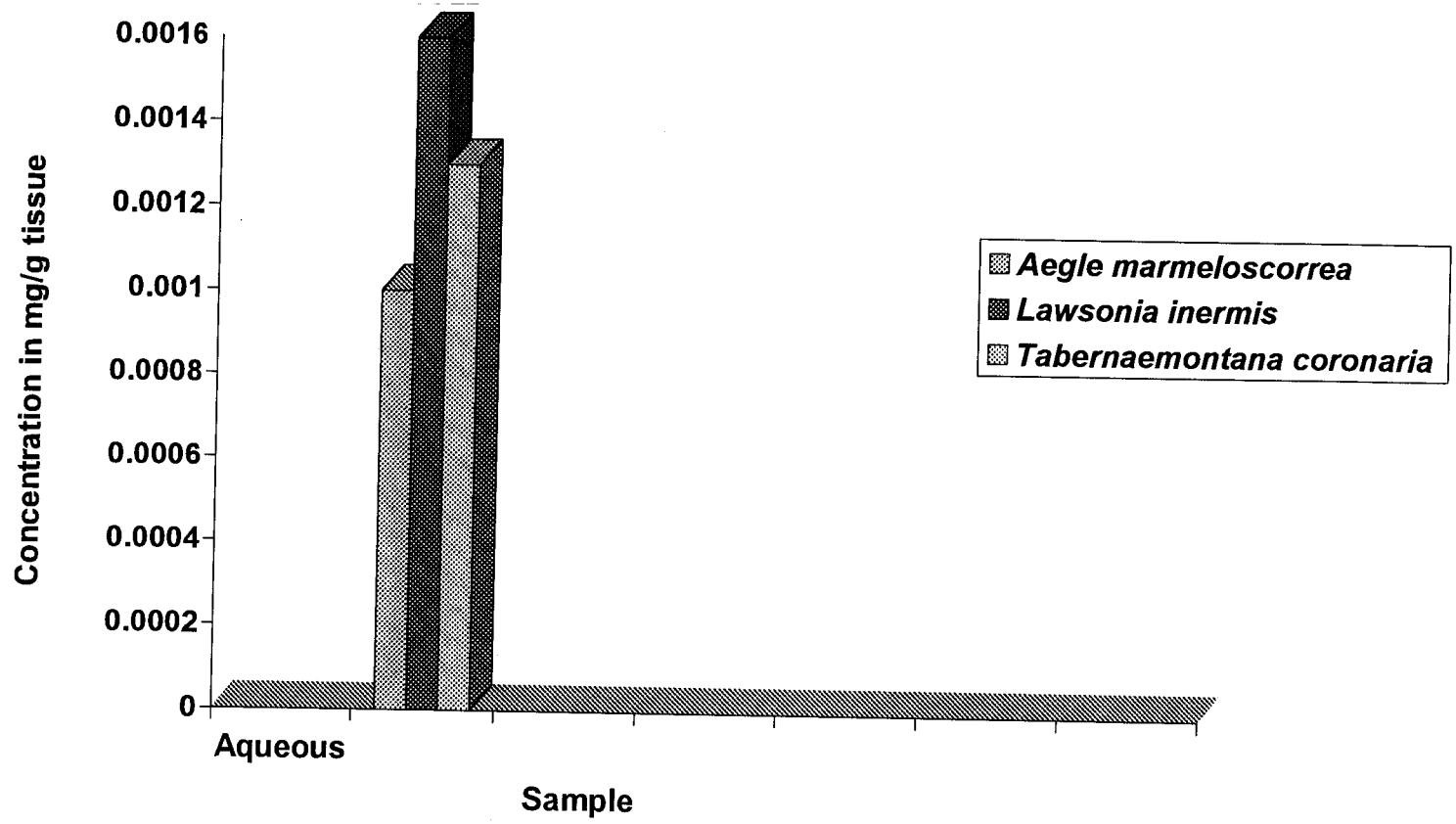


Figure 5.13 Comparison of Vitamin E concentration in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

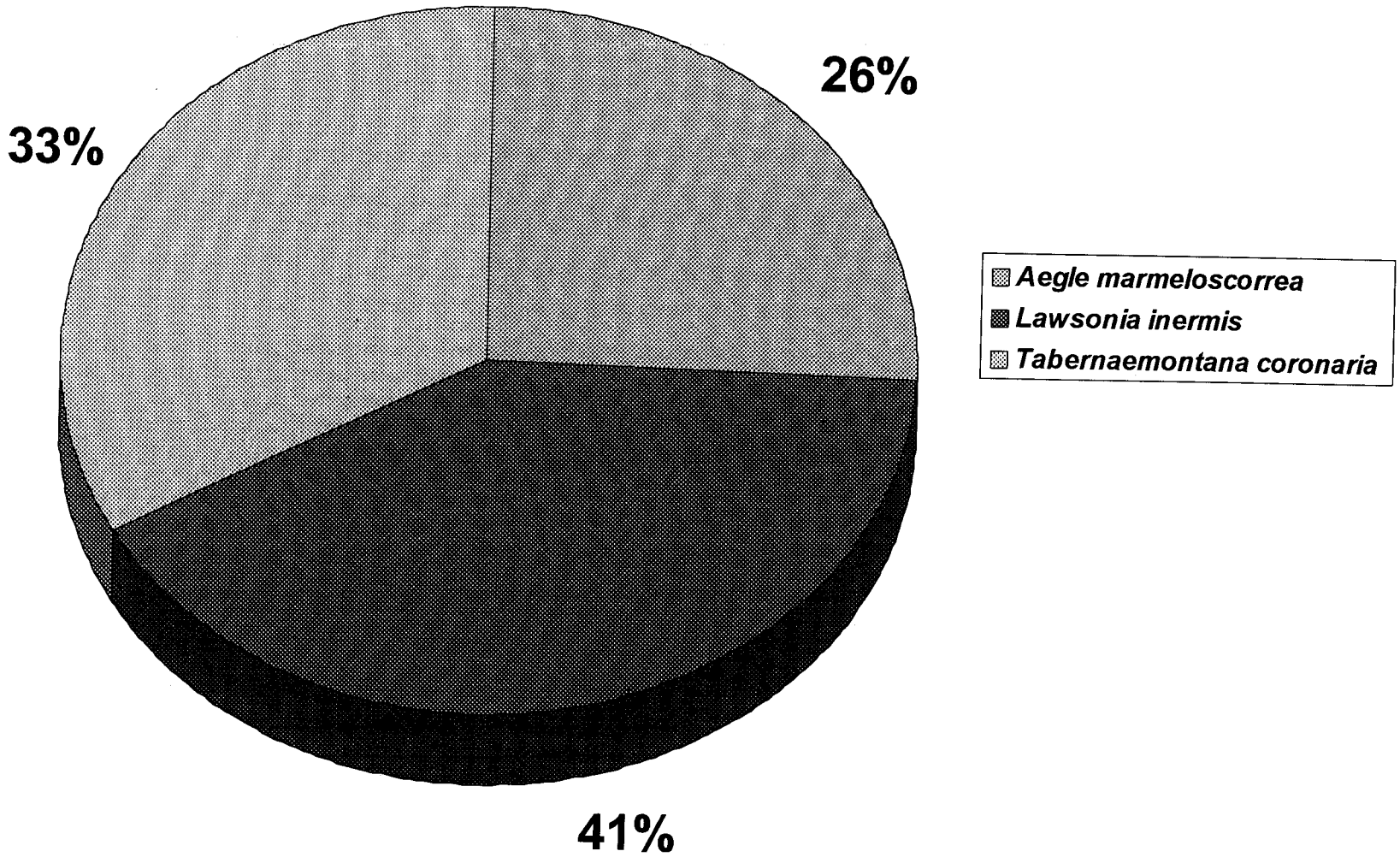


Figure 5.14 Comparison of Vitamin E concentration in the leaves of *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria.*

Antibacterial studies were performed by Disc diffusion method (Kirby – Bauer). In this study various solvents ranging from highly nonpolar to polar were used. Among these different solvents the aqueous solvent was proved to be the best one for inhibiting various bacterial species. It was proved that the aqueous extract of *Aegle marmeloscorrea* possessed the highest inhibition against *Pseudomonas aeruginosa* **(12mm)** and the lowest inhibition against *E.coli* **(10mm)** and *Salmonella typhii* **(10mm).** The ethanolic extract of this plant was proved to be poor in performing the antibacterial activties **(see table 5.5).**

Analysis of *Lawsonia inermis* against different bacterial species showed the highest inhibition against *Klebsiella pneumoniae* **(11mm)** and the lowest inhibition against *Pseudomonas aeruginosa* **(6mm).** The analysis also revealed that the heptane extract is poor and the aqueous extract is highly efficient in inhibiting the bacterial species **(see table 5.6).**

Studies of antibacterial activities in the aqueous leaf extract of *Tabernaemontana coronaria* showed to possess a good inhibition against *Pseudomonas aeruginosa* **(12mm)** and a poor inhibition against *Salmonella typhii* **(9mm).** The methanolic extract was found to be a poor one in inhibiting the bacterial species than the other solvents **(See table 5.7).**

Overall studies showed that *Aegle marmeloscorrea* have a good inhibitory property against all the five bacterial species than the other two plants **(See Figures 5.15, 5.16, 5.17, 5.18 and 5.19).**

The antifungal studies were performed by Disc diffusion method (Kirby – Bauer). Various plant and animal pathogens were selected for the studies. The analysis showed that all the three plants have good inhibition against *Aspergillus niger. Tabernaemontana coronaria* showed a significant inhibition against *Aspergillus terreus, Giberella fujikori* and *Pencillium chrysogenum.* In all the 3 plants the aqueous extract was proved to be poor in inhibiting the fungal species. **(See Table 5.8, Table 5.9, Table 5.10, Figure 5.20 and Figure 5.21).**

In extension, studies have been performed in the *Aegle marmeloscorrea* for dye degradation in the tannery effluents. This could be used as an alternative method for the chemical treatment of the tannery effluents. **(See Figure 5.22).**

Test organism	Zone of inhibition (in mm)				
	AQUEOUS	METHANOL	ETHANOL	CHLOROFORM	HEPTANE
E.coli	10	7	6	8	9
S.aureus	11	8	6	8	8
S.typhii	10	7	7	10	8
K.pneumoniae	11	7	7	10	7
P.aeruginosa	12	6	7	7	7

Table 5.5 Antibacterial activities of the leaf extracts of *Aegle marmeloscorrea*

Test organism	Zone of inhibition (in mm)				
	AQUEOUS	METHANOL	ETHANOL	CHLOROFORM	HEPTANE
E.coli	8	6	7	8	6
S.aureus	8	6	8	10	6
S.typhii	8	7	6	7	8
K.pneumoniae	11	6	9	8	7
P.aeruginosa	6	6	7	6	6

Table 5.6 Antibacterial activities of the leaf extracts of *Lawsonia inermis*

Test organism	Zone of inhibition (in mm)				
	AQUEOUS	METHANOL	ETHANOL	CHLOROFORM	HEPTANE
E.coli	11	8	8	8	6
S.aureus	10	6	7	7	9
S.typhii	9	6	6	9	8
K.pneumoniae	10	7	6	8	9
P.aeruginosa	12	7	8	6	8

Table 5.7 Antibacterial activities of the leaf extracts of *Tabernaemontana coronaria*

Test organism	Zone of inhibition (in mm)				
	AQUEOUS	**METHANOL**	**ETHANOL**	**CHLOROFORM**	**HEPTANE**
Aspergillus niger	9	8	7	7	10
Giberella fujikori	Non detectable	10	6	8	9
Pencillium chrysogenum	Non detectable	6	9	7	8
Aspergillus terreus	10	7	6	6	6

Table 5.8 Antifungal activities of the leaf extracts of *Aegle marmeloscorrea*

Test organism	Zone of inhibition (in mm)				
	AQUEOUS	**METHANOL**	**ETHANOL**	**CHLOROFORM**	**HEPTANE**
Aspergillus niger	10	7	9	8	7
Giberella fujikori	Non detectable	9	7	Non detectable	Non detectable
Pencillium chrysogenum	6	7	8	Non detectable	8
Aspergillus terreus	8	7	6	6	6

Table 5.9 Antifungal activities of the leaf extracts of *Lawsonia inermis*

Test organism	Zone of inhibition (in mm)				
	AQUEOUS	**METHANOL**	**ETHANOL**	**CHLOROFORM**	**HEPTANE**
Aspergillus niger	9	7	10	7	10
Giberella fujikori	8	9	7	8	9
Pencillium chrysogenum	9	8	7	8	8
Aspergillus terreus	9	10	6	8	6

Table 5.10 Antifungal activities of the leaf extracts of *Tabernaemontana coronaria*

FIG.5.1.5 ANTIBACTERIAL ACTIVITY OF THE THREE LEAF EXTRACTS ON *E.coli*.

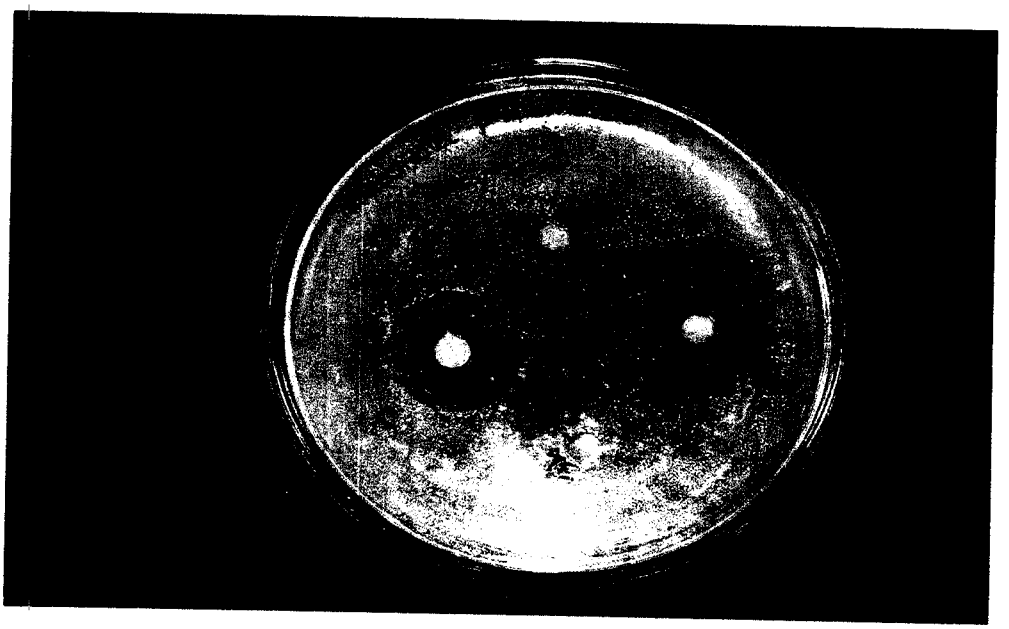


FIG.5.1.6. ANTIBACTERIAL ACTIVITY OF THE THREE LEAF EXTRACTS ON *Klebsiella pneumoniae*.

FIG.5.1.7. ANTIBACTERIAL ACTIVITY OF THE THREE LEAF EXTRACTS ON *Pseudomonas aeruginosa*.

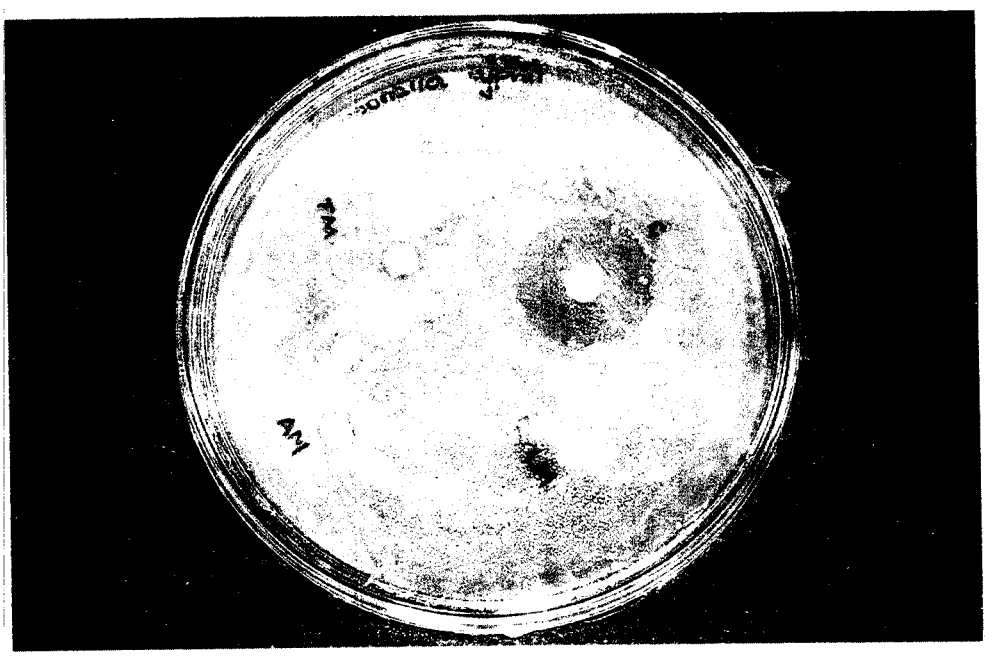


FIG.5.1.8. ANTIBACTERIAL ACTIVITY OF THE THREE LEAF EXTRACTS ON *Salmonella typhii*.

FIG. 5.1.9. ANTIBACTERIAL ACTIVITY OF THE THREE LEAF EXTRACTS ON *Staphylococcus aureus*.

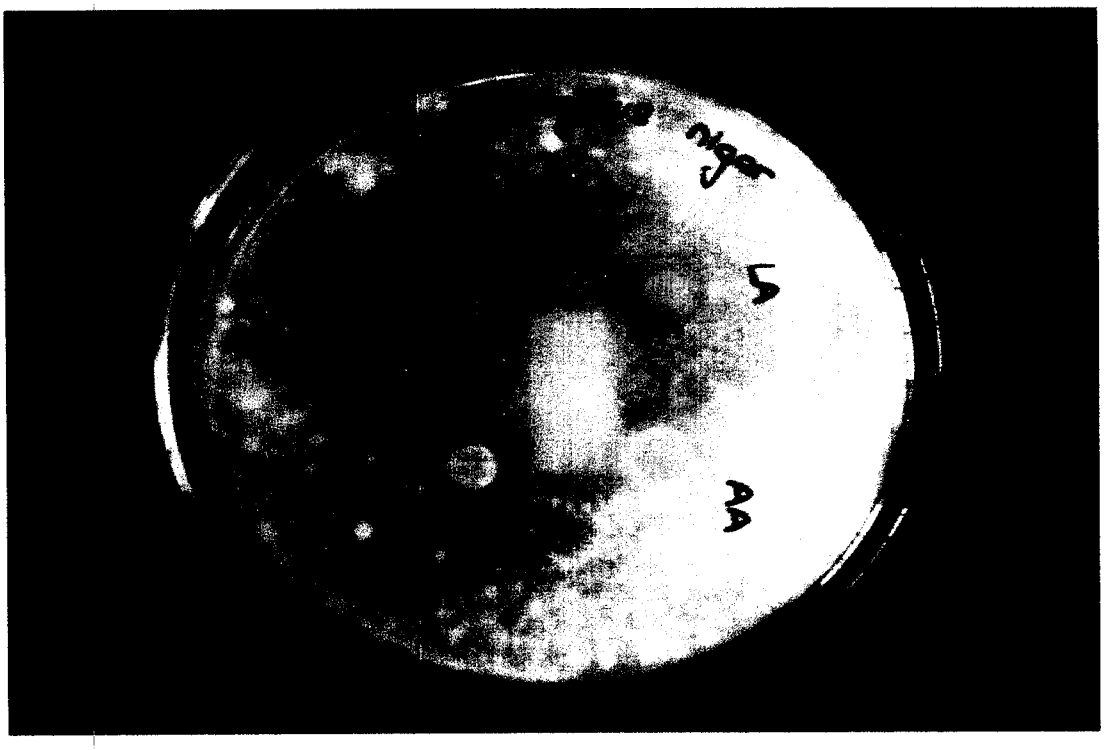


FIG.5.2.0. ANTIFUNGAL ACTIVITIES OF THE THREE LEAF EXTRACTS ON *Aspergillus niger*.

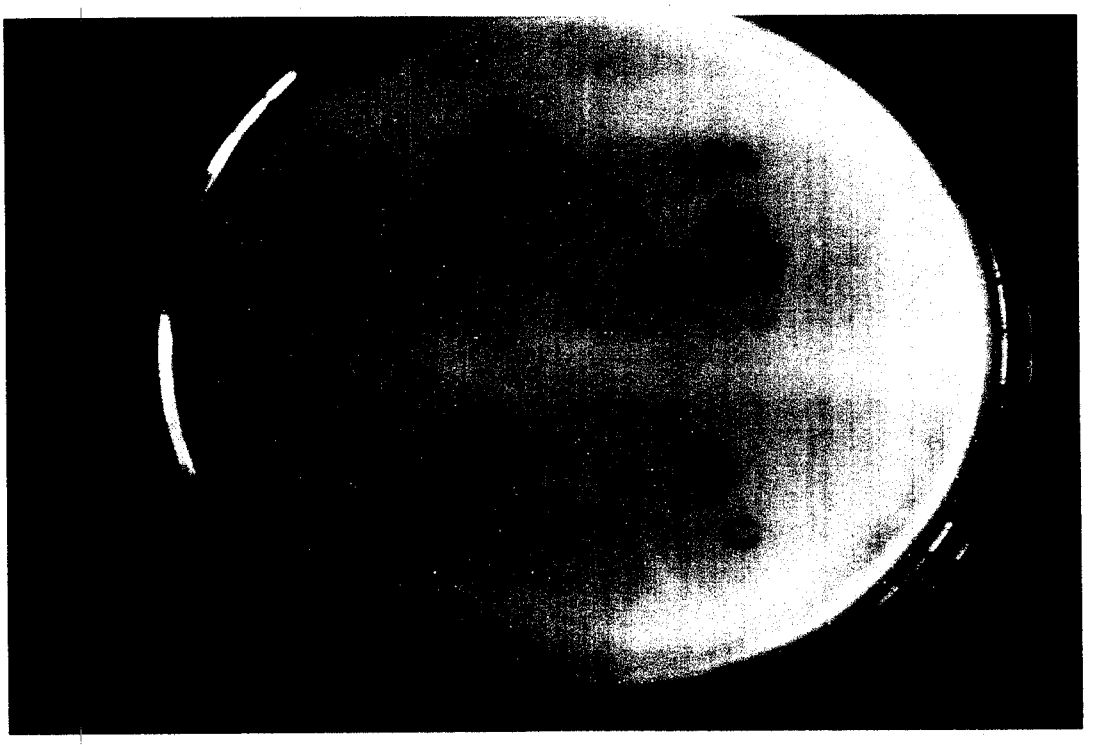


FIG.5.2.1. ANTIFUNGAL ACTIVITIES OF THE THREE LEAF EXTRACTS ON *Aspergillus terreus*.

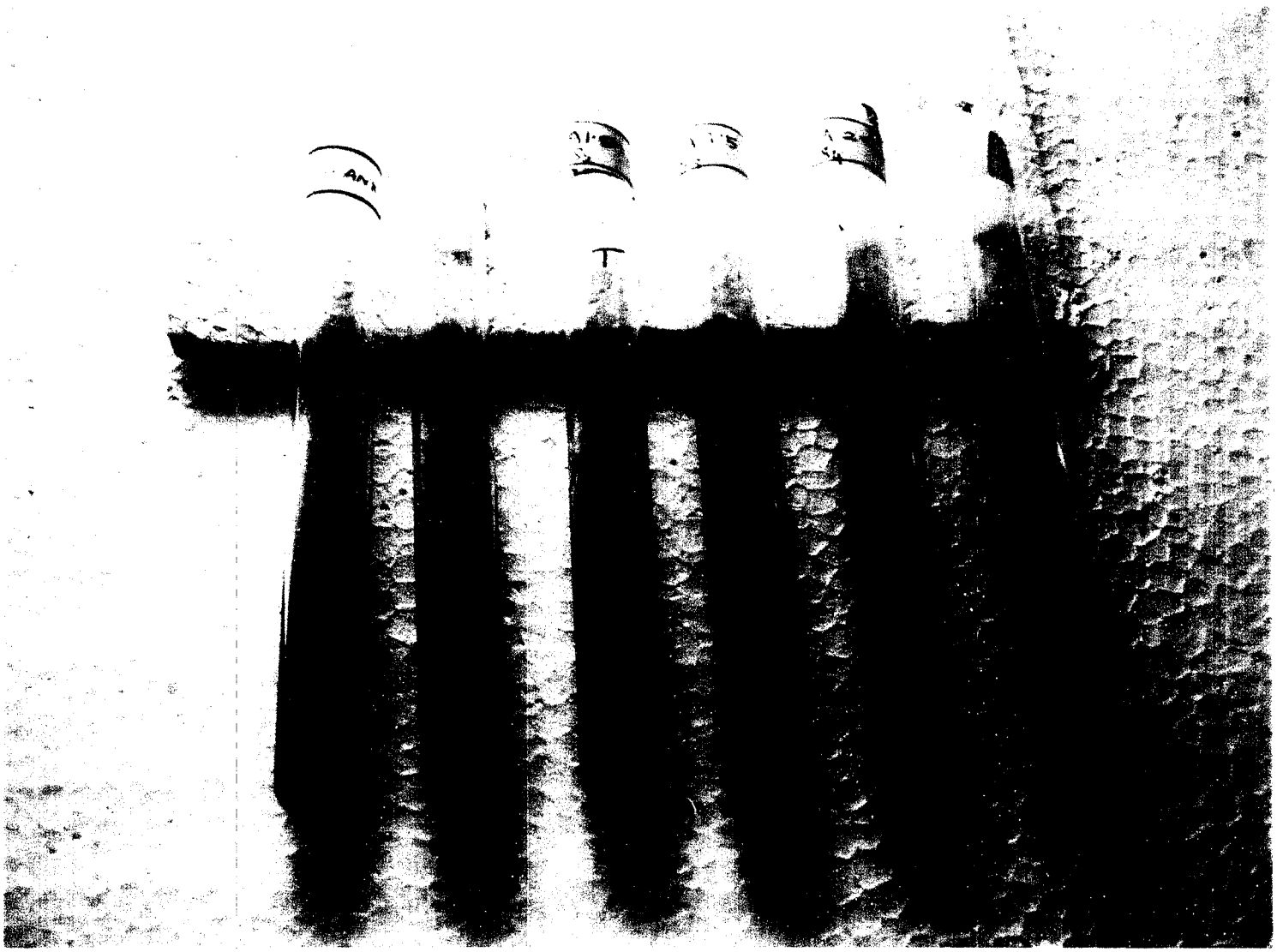


Fig. 5·22 DYE DEGRADATION BY THE LEAF EXTRACTS OF *Aegle marmelosmma*.

6. CONCLUSION

This study has been focused on the phytochemical screening and quantification, assessment of antioxidants and antimicrobial activities in the three commonly available plants viz., *Aegle marmeloscorrea, Lawsonia inermis* and *Tabernaemontana coronaria*. The leaves were collected from the medicinal garden located at Kumaraguru college of Technology campus, Coimbatore.

The phytochemical screening using various solvents showed that alkaloids, flavonoids, tannins, saponins, steroids, terpenoids, cardiac glycosides and reducing sugars were present in all the three plants. hlobatannins were present only in *Aegle marmeloscorrea*.

The quantification studies showed that the amount of total proteins in the aqueous leaf extract of *Aegle marmeloscorrea* is higher than the other two plant extracts. Studies for the quantification of total phenolics showed a higher level in *Tabernaemontana coronaria* than the other two plants. Alkaloids were found relatively higher in the leaf extracts of *Tabernaemontana coronaria* than the other two plants. Ethanolic extracts of all the three plants have a higher amount of flavonoids. Significant amounts of tannins, Vitamin C and Vitamin E were found in higher amounts in the leaf extracts of *Lawsonia inermis*.

Antibacterial studies proved that *Lawsonia inermis* possessed the highest inhibition against *Klebsiella pneumoniae*. *Aegle marmeloscorrea* showed inhibition against *E.coli, Staphylococcus aureus* and *Salmonella typhii*. *Tabernaemontana coronaria* and *Aegle marmeloscorrea* showed the highest inhibition against *Pseudomonas aeruginosa*. Overall studies showed that *Aegle marmeloscorrea and Tabernaemontana coronaria* relatively have a good inhibition against all the five bacterial species.

Antifungal studies showed that all the three plants have a good inhibition against *Aspergillus niger*. *Tabernaemontana coronaria* showed a significant inhibition against *Aspergillus terreus, Giberella fujikori* and *Pencillium chrysogenum*. In extension, studies have been performed in the *Aegle marmeloscorrea* for dye degradation in the tannery effluents. This could be used as an alternative method for the chemical treatment of the tannery effluents.

APPENDICES

APPENDIX 1

ESTIMATION OF TOTAL PROTEINS BY FOLIN-LOWRY'S METHOD

a) Sample preparation:

Different solvents ranging from highly non-polar to polar (Heptane > chloroform > Ethanol > Methanol > Aqueous) were used to extract total proteins from the leaf sample.

In a clean dry conical flask, weighed 0.5g of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) and added 50mL of distilled water. Kept this in an orbital shaker for an overnight. Filtered the extract. Took 0.01mL of the extract and made upto 1mL with corresponding solvent to get 1:100 dilution.

b) Stock solution for standard:

Dissolved 100mg of bovine serum albumin in 100mL of distilled water. (1mL = 1mg).

c) Working standard solution:

10mL of the stock was made up to 100mL with distilled water. (1mL=100μg)

d) Alkaline Copper reagent:

Solution A: 1% Copper reagent (Lowry's reagent)

Into a clean dry 100mL standard flask weighed 1g of Copper sulphate and made upto the mark with distilled water.

Solution B: 1% Sodium potassium tartrate

Into a clean dry 100mL standard flask weighed 1g of Sodium potassium tartrate and made upto the mark with distilled water.

Solution C: 2% Sodium carbonate in 0.1N NaOH

Into a clean dry 100mL standard flask weighed 2g of Sodium carbonate and made upto the mark with 0.1N NaOH.

1mL Solution A + 1mL Solution B + 98mL Solution C

e) Folin-Ciocalteau reagent

Folin's reagent was mixed with distilled water in the ratio of 1:1. This reagent should be prepared freshly.

APPENDIX 2

ESTIMATION OF TOTAL PHENOLICS BY FOLIN-CIOCALTEAU METHOD

a) Sample preparation:

Different solvents ranging from highly non-polar to polar (Heptane > chloroform > Ethanol > Methanol > Aqueous) were used to extract total phenolics from the leaf sample.

In a clean dry conical flask, weighed 0.5g of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) and added 15mL of solvent. Centrifuged the homogenate at 10000rpm for 20 minute. Saved the supernatant. Evaporated the supernatant to dryness.

a) Stock solution for standard:

Dissolved 100mg of phenol in 100mL of distilled water. (1mL = 1mg).

b) Working standard solution:

10mL of the stock was made up to 100mL with distilled water. (1mL=100μg)

c) 80% ethanol

d) Folin-Ciocalteau reagent

Folin's reagent was mixed with distilled water in the ratio of 1:1. This reagent should be prepared freshly.

e) 20% Sodium carbonate

APPENDIX 3

ESTIMATION OF ALKALOIDS BY HARBORNE METHOD

a) Sample preparation:

In a clean dry conical flask, weighed 1.25g of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) and added 50mL of 10% acetic acid in ethanol. Kept this in an orbital shaker for 4hours.

b) 10% Acetic acid in ethanol

5mL of Acetic acid was made upto 50mL with ethanol.

c) Concentrated Ammonium hydroxide (25% v/v)

d) Diluted Ammonium hydroxide

5mL of concentrated Ammonium hydroxide was made upto 25mL with distilled water.

APPENDIX 4

ESTIMATION OF FLAVONOID BY BOHM AND KOCIPAI-ABYAZAN METHOD

a) Sample preparation:

Different solvents ranging from highly non-polar to polar (Heptane > chloroform > Ethanol > Methanol > Aqueous) were used to extract flavonoid from the leaf sample.

In a clean dry conical flask, weighed 10g of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) and added 100mL of solvent. Kept this in an orbital shaker for an overnight.

b) 80% Aqueous methanol

80mL of methanol was made up to 100mL with distilled water.

APPENDIX 5

ESTIMATION OF TANNINS BY MODIFIED PRUSSIAN BLUE METHOD

a) Sample preparation:

In a clean dry conical flask, weighed 0.5g of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) and added 50mL of distilled water. Kept this in an orbital shaker for an overnight. Filtered the extract and the filtrate was made upto 50mL with distilled water. Took 5mL of the extract and made upto 50mL with distilled water to get 1:10 dilution.

b) Stock solution for standard:

Dissolved 50mg of tannic acid in 50mL of distilled water. (1mL = 1mg).

c) Working standard solution:

5mL of the stock was made up to 50mL with distilled water. (1mL=100μg)

d) 0.02M $FeCl_3$ in 0.1M HCl

Pipetted out 8.3mL of concentrated HCl in one litre standard flask and made upto the mark with distilled water. Then,added 3.24g of anhydrous $FeCl_3$ and mixed well.

e) 0.008M Potassium ferric cyanide

Dissolved 3.38g of Potassium ferric cyanide in one litre of distilled water.

APPENDIX 6

ESTIMATION OF ASCORBIC ACID BY 2,4-DNPH METHOD

a) Sample preparation

In a clean dry conical flask, weighed 1.5g of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) and added 15mL 4% Oxalic acid. This was centrifuged for half an hour. The contents were filtered with Whatman filter paper and filtrate was collected. To this added 2-3 drops of diluted bromine water or a pinch of activated charcoal. The solution was then subjected to aeration by repeated bubbling with pipette for facilitating the oxidation reaction. Filtered the extract and the filtrate was made upto 50mL with 4% Oxalic acid.

b) Stock solution

Dissolved 100mg of Vitamin C in 100mL of 4% Oxalic acid.(1mL = 1mg)

c) Working standard

5mL of stock was made upto 50mL with 4% Oxalic acid. To this added 2-3 drops of diluted bromine water or a pinch of activated charcoal. The solution was then subjected to aeration by repeated bubbling with pipette for facilitating the oxidation reaction. This converted the ascorbic acid to dehydroascorbic acid (1mL=100μg).

d) 0.5N Sulphuric acid

0.42mL of concentrated sulphuric acid was made up to 30mL with distilled water.

e) 2% 2,4-Dinitrophenyl hydrazine (DNPH) reagent

Dissolved 2g of 2,4-DNPH in 100mL 0.5N H_2SO_4 by gentle heating. The reagent was filtered and used.

f) 4% Oxalic acid solution

g) Bromine water

Dissolved 1-2 drops of liquid bromine in 100mL of distilled water.

Activated charcoal

h) 80% H_2SO_4

i) 10% Thiourea reagent

APPENDIX 7

ESTIMATION OF VITAMIN E BY BAKER AND FRANK METHOD

a) Sample preparation

In a clean dry conical flask, weighed 1g of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) and added 50mL of 0.1N sulphuric acid. Kept this in an orbital shaker for an overnight. The contents were filtered with Whatman filter paper and filtrate was collected.

b) Stock solution

Dissolved 50mg of Vitamin E in 50mL of ethyl alcohol.(1mL = 1mg)

c) Working standard

5mL of stock was made upto 50mL with ethyl alcohol. (1mL=100μg).

d) Absolute ethanol

e) Xylene

f) 0.2% 2,2' dipyridyl in n-propanol

g) 0.5% Ferric chloride

APPENDIX 8

ASSESSMENT OF ANTIFUNGAL ACTIVITIES BY DISC-DIFFUSION METHOD (KIRBY-BAUER)

Preparation of Czapekdox broth (for 100mL)

Sucrose	3g
Sodium nitrate	0.2g
Dipotassium phosphate	0.1g
Magnesium sulphate	0.05g
Potassium clrloride	0.05g
Ferrous sulphate	0.001g
Distilled water	100mL

pH must be adjusted to 7.3

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