Evaluation of In-Vitro Antimicrobial activity of *Cissampelos pareira*

A thesis report is submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy.

Submitted to

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Declaration by the research candidate

I, Khadija Begum, here by declare that the dissertation entitled "**A study on Antimicrobial activity of *Cissampelos pareira***" submitted by me to the Department of Pharmacy, in the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Honors) is a record of original research work carried out by me during Fall-2011 to spring-2012 under the supervision and guidance of Ms. Nazia Hoque, Lecturer, Department of Pharmacy, East West University, Dhaka.

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Thesis Certificate by the Invigilator

This is to certify that, the research work on **"A study on Antimicrobial activity of *Cissampelos pareira*"** submitted to the Department of Pharmacy, East West University, Dhaka, in the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy carried by Khadija Begum (ID: 2008-1-70-030) under our guidance, supervision & that no part of the thesis has been submitted for any other degree. We further certify that all the sources of information of this connection duty acknowledged.

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Endorsement by the Chairperson

This is to certify that, the research work on **"A study on Antimicrobial activity of *Cissampelos pareira***" submitted to the Department of Pharmacy, East West University, Dhaka, in the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy carried by Khadija Begum (ID: 2008-1-70-030) under our guidance, supervision & that no part of the thesis has been submitted for any other degree. We further certify that all the sources of information of this connection duty acknowledged.

Dr. Sufia Islam

Chairperson and Associate Professor

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East West University, Dhaka.

Dedication

This thesis paper is dedicated to

My Parents & Brothers.

Acknowledgement

At first thanks to almighty Allah who enabled me to pursue my study in Pharmacy as well as to submit me to the script for the degree of Bachelor of Pharmacy, Department of Pharmacy, East West University, Dhaka.

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Abstract

Cissampelos pareira (Menispermaceae family) is widely distributed in tropical regions of Asia, and used in the treatment of many diseases. The traditional use of this plant suggests its possible antibacterial properties. The present investigations evaluate the antimicrobial activity of *Cissampelos pareira* against selected gram positive, gram negative bacteria and fungal strains. In this study, the crude ethyl acetate, ethanol & water extract of *Cissampelos pareira* (stem, and leaves) was assed for their antimicrobial activity using disc diffusion method. The activity was performed against common pathogenic bacterial (*Bacillus sereus, Bacillus subtilis, Staphylococcus aureus, Sarcina lutea, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi, Shigella dysenteriae, Shigella boydii, Vibrio mimicus, Candida albicans, Candida albicans, Saccharomyces cerevisiae,Vibrio parahemolyticus, & Bacillius megateri).* The antimicrobial activity of the extract for the concentration of 400µg/disc and 800µg/disc were observed against the standard antimicrobial agent Cephradine. The activities were understood by observing and measuring the zone of inhibition for each concentration. Maximum activity was revealed by ethyl acetate than ethanol extracts but less active was revealed by water. This plant effective for treatment of different pathogenic diseases. The other parts of the plant like root, seed showed many activities that confirms the significance of the plant for medicinal compounds.

Keywords: Menispermaceae family, *Cissampelos pareira*, Cephradine, disc diffusion, zone of inhibition.

Chapter One

Introduction

Plant Family

1.1. Menispermaceae: Facts

The Menispermaceae family of flowering plants is a medium-sized family comprising of 70 genera and about 420 species. Most species in this family are climbing plants and found in the tropics. Although the number of species in this family is not large compared to some other plant families, a number of plants belonging to this family are important plants, being used in the traditional medicines of a number of countries. Seven species belonging to the Menispermaceae family were observed to be in use by the Kavirajes. These seven species were *Cocculus hirsutus*, *Stephania glabra*, *Stephania japonica*, *Tinospora cordifolia*, *Tinospora crispa*, *Cissampelos pareira* and *Tinospora sinensis*. Of the six species, *Stephania japonica* and *Tinospora cordifolia* were most frequently used for treatment of ailments like diabetes, edema, pain, bone fracture, debility, gastrointestinal disorders, respiratory tract disorders, helminthiasis, malaria, hepatic disorders, tuberculosis, measles, urinary tract disorders, and hypertension. The folk medicinal use of several of these plants has been validated through scientific studies. [2]

Menispermaceae family Plants of the order Ranunculales, subclass Magnoliidae, class of Magnoliopsida. Members are mostly vines and shrubs and they contain isoquinoline alkaloids, some of which have been used as arrow poisons. [1]

Three acetyl cholinesterase inhibitors have been isolated from tubers of the medicinal plant belonging to the Menispermaceae family, namely *Stephania venosa*. They have been identified as quaternary protoberberine alkaloids--stepharanine, cyclanoline, and N-methyl stepholidine. Anti-bacterial, antifungal, anti-plasmodial, and cytotoxic activities have been reported for the root bark alkaloidal extract of the plant *Albertisia villosa* and an isolated bisbenzylisoquinoline--cycleanine, which validates its traditional use in Congolese medicine for treatment of malaria and other infectious diseases. The methanolic leaf extract of *Cissampelos mucronata* reportedly demonstrated protective action against indomethacin-induced ulcer in rats. Antinociceptive and anti-arthritic activity has been reported of *Cissampelos pareira*. [2]

1.2. Menispermaceae: Chief Genera and species

***Table 1. 1*:** Chief Genera and species of Menispermaceae family: [3, 15]

Genus	Species
Albertisia	*A. delagoensis*
Antizoma	*A. angustifolia*
Cissampelos	*C. capensis*
Cissampelos	*C. pareira*
Cocculus	*C. hirsutus*
Tiliacora	*T. funifera*
Tinospora	*T. caffra*

1.3. Scientific classification

***Table 1.2*:** Scientific classification of *Cissampelos pareira:* [3]

Domain	*Eukaryota*	Whittaker & Margulis,1978 - eukaryotes
Kingdom	*Plantae*	Haeckel, 1866 – Plants.
Subkingdom	*Viridaeplantae*	Cavalier-Smith, 1981.
Phylum	*Tracheophyta*	Sinnott, 1935 ex Cavalier-Smith, 1998 - Vascular Plants.
Subphylum	*Euphyllophytina*	
Infraphylum	*Radiatopses*	Kenrick & Crane, 1997
Class	*Magnoliopsida*	Brongniart, 1843 - Dicotyledons
Subclass	*Ranunculidae*	Takhtajan Ex Reveal, 1992
Order	*Ranunculanae*	Dumortier, 1829
Family	*Menispermaceae*	A.L. de Jussieu, 1789, nom. cons. - moonseeds
Genus	*Cissampelos*	C. Linnaeus, 1753
Specific epithet	*pareira* - L.	
Botanical name	*Cissampelos pareira* L	

1.4. Classical Names

Table 1.3: Classical or traditional names of Names of *Cissampelos pareira* plants includes: [13]

Language Name	Classical or traditional names
Bengali	Akanadi, kijri.
English	False pareira root.
Hindi	Patha, Pardhi, Akanadi, Dakhnirdissi, Harjeuri, nirbisi, Pahre, Pharha, Advibanka teega, Pahan, Bhatindu.
Kannada	Padaval, Padvali, Gutte, Neemukha, Ambashtha, Hondikiballi, Maneballi.
Khasi	Jyrmi salla.
Malayalam	Kattuvalli, patakkilannu, patuvalli, pata, cattvalli.
Marathi	Pahad-mul, pahadamoola, padavel, paharmul, pahaad mool.
Nepali	Sulara, barel-panrhe, Batulay paat..
Oriya	Ghodakur, ambashtha, ambashthai-patha, amboostha, devi, laghupatha, malati, papanalil.
Sankrit	Pratanini, rasa, shishira, sthapini, vallika, vara, venivel, vrittaparni.
Tamil	Appatta, tuvigaba, ponmucuttai, ampattai, appakam, matarapanni, varititta.
Telugu	Adivibankatige, pateru tivva, esaboddi, paata, chiruboodi.
Tibetan	Batha, pa-tha.
Urdu	Patha.

1.5. Botanical Names: Synonyms of *Cissampelos pareira*

Other scientific names of *Cissampelos pareira:* [5]

1. *Cissempelos acuminate*
2. *Cissempelos argenta,*
3. *Cissempelos auriculata,*
4. *Cissempelos benthamiana,*
5. *Cissempelos boivinii,*
6. *Cissempelos bojeriana*
7. *Cissempelos canescens,*
8. *Cissempelos cocculus,*
9. *Cissempelos convolvulacea*
10. *Cissempelos cordifolia,*
11. *Cissempelos delicatula,*
12. *Cissempelos diffusa,*
13. *Cissempelos discolor,*
14. *Cissempelos gracilis,*
15. *Cissempelos grallatoria,*
16. *Cissempelos guayaquilensis,*
17. *Cissempelos hernandifolia,*
18. *Cissempelos hirsuta,*
19. *Cissempelos pannosa,*
20. *Cissempelos piolanei,*
21. *Cissempelos subreniformis*
22. *Cissempelos tamoides,*
23. *Cissempelos testudinum,*
24. *Cissempelos tomentocarpa*.

1.6. Parts used

1. Leaves,
2. roots,
3. seeds and
4. Barks.

1.6. Plant Description:

Cissampelos pareira linn (family-Manisperaceae) is perennial climbing hurbs/shrubs with small greenish yellow flowers, peltate or orbicular-reniform, ovate-sub-reiform leaves with truncate cordata base, glabrous or hairy above up to 3-12 cm long. It produces inedible, dark, grape-sized barriers. It belongs to the genus *Cissampelos*, of which 30 to 40 species are distributed in the topical and subtropical world. One species occur in India.

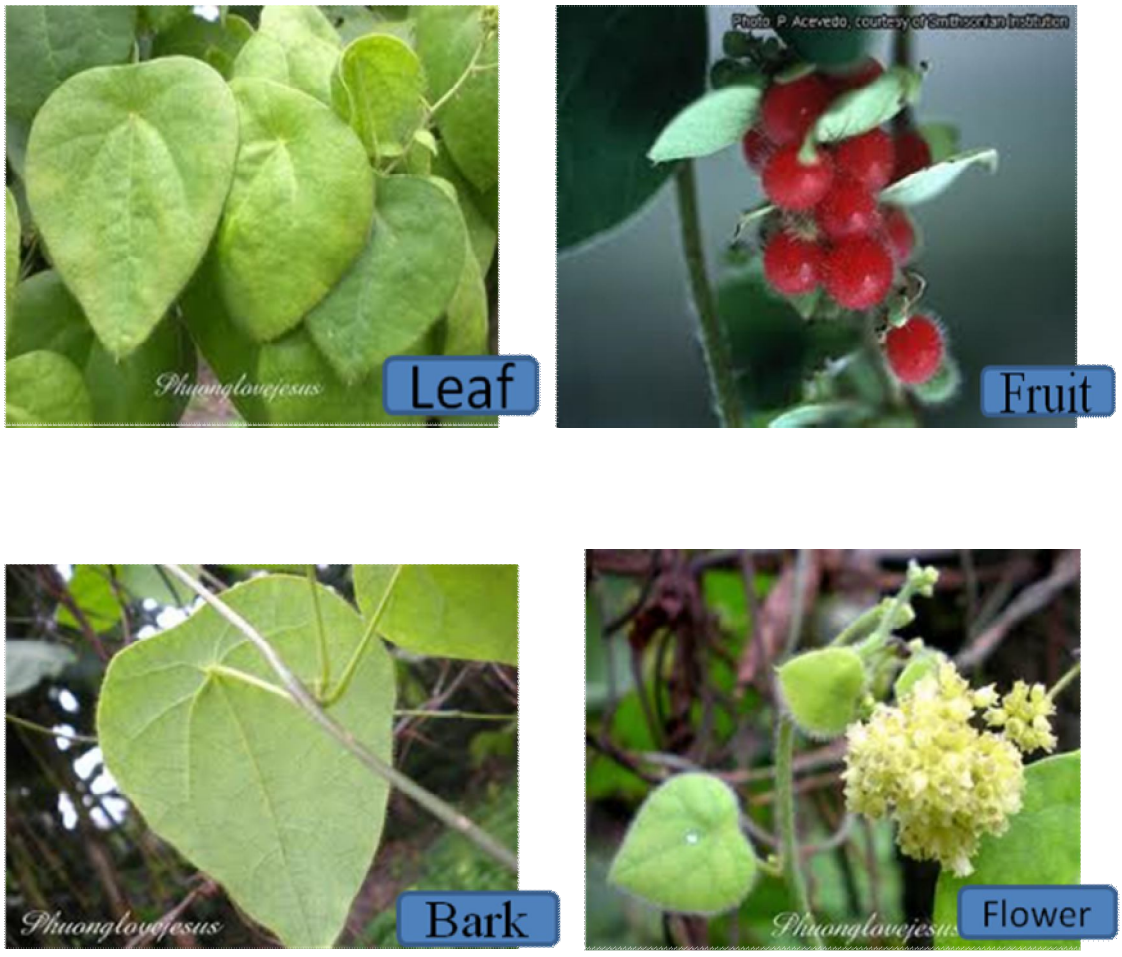


Figure1.1*: *Cissampelos pareira plant parts.

Flowers unisexual; pedicel up to 2mm long; male flowers with 4 sepals, ovate to obviate, c.1.5 mm × c.0.5 mm, keeled, hairy outside, greenish or yellowish, corolla cup-shaped, c.1 mm long, filaments of stamens completely fused; female flowers with 1 sepal c. 1.5 mm long, 1 obtriangular to kidney- shaped petal c. 1.5 mm × 2 mm, ovary superior, hairy, 1-celled, style thick with spreading, 3-lobed stigma. Fruit a short-hairy, orange to red drupe c. 5 mm

long, curved with style-scar near base; stone with 2 rows of very prominent transverse ridges, 1-seeded. Seed horseshoe-shaped; embryo elongate, narrow, embedded in endosperm, cotyledons flattened. The flowers of *Cissampelos pareira* are probably pollinated by small insects. The plant is common in orchards, hedges, park and gardens of moist soils, ether creeping or twining around other plants, also common on the hilly tracts along water courses. It can also be propagated from root cuttings, planted at the beginning of monsoon. Sometimes it dies back in hot water. *Cissampelos pareira* is mostly collected from the wild. *Cissampelos pareira* is very widespread and locally common. The quantity and composition of the alkaloids found in the leaves and roots seem to differ between plants from different regions. This may be a result of its great genetic diversity. [4]

1.8. Geographical Abundance:

Cissampelos pareira was first described from Latin America, but actually occurs throughout the tropics; in some countries it has been introduced for its ornamental value. In Africa it has been recorded from Sierra Leone east to eastern DR Congo, Rwanda and Tanzania and south to northern Angola and Zambia. It also occurs in the Comoros, Madagascar and Mauritius, and formerly on Assumption Island (Seychelles). Its presence in Benin is uncertain. [16]

Figure 1.2: Geographical distribution of *Cissampelos pareira.*

1.9. Chemical composition:

Cissampelos pareira contains a number of alkaloids, especially bisbenzylisoquinoline alkaloids. The rhizome contains hayatine, hayatidine, hayatinine, d-4”-O-methylbebeerine, l-bebeerines, isochondrodendrine, dicentrine, dehydrodicentrine, insularine; the rhizome and leaves contain cycleanine, while cissampareine has been isolated from the whole plant and the chalcone-flavone dimer cissampeloflavone from the aerial parts. The rhizomes have also been found to be a rich source of tropoloisoquinoline alkaloids. Pareirubrine A , pareirubrine B, grandirubrine, isoimerubrine and pareitropone have been isolated, all of which showed potent antileukaemic activity. Furthermore, two cytotoxic azafluoranthene alkaloids, structurally strongly related to tropoloisoquinoline alkaloids, have been isolated from the same extract, as has cissamine chloride. Several experiments on rhizome extracts of *Cissampelos pareira* have been done in recent years. A water-ethanol extract of the rhizomes reduced the growth and multiplication rate of benzoic pyrene-induced fore stomach tumours in mice in a dose-dependent manner. [11]

In another series of tests with rat models for acute, subacute and chronic inflammation, a similar extract showed significant anti-inflammatory activity without carcinogenic effects or causing gastric lesions. Mice administered the extract also showed reduced reactions against several pain stimuli. Ethanolic rhizome extracts have shown antihistaminic, hypotensive, antispasmodic and anticonvulsant properties. In a test to confirm the antifertility use of the plant, a methanol extract of the leaves administered to rats caused a significant increase in the duration of the dioestrus and a reduction in the number of litters. Altered gonadotropine and oestradiol secretion were involved. *Cissampelos pareira* exhibits curare-like activity, depressing the central nervous system and relaxing smooth muscles, and has hypertensive and hypoglycaemic actions. The compound hayatinine is structurally similar to tubocurarine from *Chondrodendron tomentosum*, the active compound in curare. It shows comparable neuro-muscular blocking activities. Cycle nine has shown significant inhibition of nitric oxide production in macrophages. Cycle nine and bebeerines suppressed hepatic injury and reduced the level of tumour necrosis factor in mice treated with lip polysaccharide and BCG, a model for the study of fulminant hepatitis. [11]

1.10. Medicinal uses of *Cissampelos pareira:*

1.*Cissampelos pareira* possesses antibacterial, anti-inflammatory, antihistamine, antioxidant, antispasmodic, diuretic, hypotensive, muscle relaxant, uterine relaxant, antiseptic, aphrodisiac, analgesic, ant hemorrhagic, cardio tonic, diaphoretic, expectorant, febrifuge, hepatoprotective stimulant and tonic activities.[11]

2. *Cissampelos pareira* mainly used for treating women's diseases. It has been used since the ancient times as a cure for menstrual problems, hormonal imbalance, and to ease childbirth, postpartum pain, prevent miscarriage, and control uterine hemorrhages, hormonal acne and premenstrual syndrome (PMS). A decoction of the whole vine is taken by women for 2 months before and throughout pregnancy and again three months after delivery. It is then used to bah infants as a tonic and prevention for skin diseases and taken internally for convulsions, ulcers, indigestion, skin irritations, cough, fever, intestinal worms, and wounds and also used in treatment of heart disorders, kidney stones, asthma, arthritis, muscle cramps, stomachaches, and malaria.[6]

3. *Cissampelos pareira* mainly uses stops bleeding, balances menstruation, relieves pain, reduces spasms, relaxes muscles, stops inflammation, , increases urination, prevents convulsions, reduces fever, balances hormones, menopausal libido loss, hormonal acne, premenstrual syndrome, childbirth and protects liver.

4. *Cissampelos pareira* also uses for heart problems (irregular heartbeat, high blood pressure, heart tonic).and also uses for cramps, erysipelas, fever, menstrual disorders, rheumatism, snakebite, water retention, and to increase perspiration.

5. Medicinaly *Cissampelos pareira* uses for Tonic, diuretic, aperients; acts as an antiseptic to the bladder, chiefly employed for the relief of chronic inflammation of the urinary passages, also recommended for calculus affections, leucorrhoea, rheumatism, jaundice, dropsy, and gonorrhoea.

6 *Cissampelos pareira* is a very useful herb for women's affections. Its antispasmodic action makes it influential in treating cramps, painful menstruation and pre and post-natal pain. *Cissampelos Pareira* roots are used in tropical countries to prevent a threatened miscarriage. The herb is also used to stop uterine haemorrhages. [6]

Table 1.4: worldwide ethnomedical uses:

Worldwide ethnomedical uses	
Amazonian	for childbirth, colic, fever, muscle spasms and pain, nervous children, pinta, snakebite
India	Ache(Stomach), Bite(Dog), Boil, Bronchitis, Burn, Chill, Cholera, Cold, Convulsion, Delirium, Diarrhea, Dysentery, Epilepsy, Eye, Gravel, Hematuria, Madness, Pimples, Rabies.
Argentina	for diarrhea, menstrual disorders, respiratory tract infections, urinary tract infections
Brazil	for abortions, anaemia, asthma, bladder problems, colic, congestion, constipation, contusions, cramps, cystitis, digestive problems, detoxification (by inducing sweating), dysentery, dyspepsia, drowsiness, edema, excessive phlegm and mucous, fever, gallbladder problems (to stimulate bile), hepatitis, inflammation, kidney stones, menstrual disorders, muscle aches, pains and spasms, testicular inflammation, threatened miscarriage, pre-and postnatal pain, rheumatism, snakebite, stomach problems, urinary tract disorders, uterine haemorrhages, water retention
Guatemala	for cramps, erysipelas, fever, menstrual disorders, rheumatism, snakebite, water retention, and to increase perspiration
Mexico	for bladder problems, dermatitis, diarrhoea, dysentery, edema, excessive phlegm and mucous, fever, insect bites, jaundice, menstrual disorders, muscle inflammation, nephritis, pain, pimples, rheumatism, snakebite, urogenital problems, vaginal discharge, water retention, and as a female balancing aid
Nicaragua	for bites, fever, skin rash, sores, stings, venereal disease
United States	for haemorrhages and excessive bleeding, constipation, kidney stones, menstrual disorders, muscle spasms, premenstrual syndrome (PMS), testicular inflammation, urinary tract irritation, water retention
Panama	Bite(Snake)
Trinidad	Boil, Diabetes, Hypertension, Palpitation

Elsewhere	for abortions, anaemia, arrow poisoning, asthma, boil, childbirth, constipation, cough, cystitis, diabetes, diarrhoea, dyspepsia, excessive phlegm and mucous, edema, eye problems, fetal growth problems, fever, haemorrhages, hypertension, indigestion, itch, kidney stones, malaria, menstrual disorders, pain, post-menstrual haemorrhages, rheumatism, snakebite, sores, sterility, threatened miscarriage, urogenital inflammation, uterine hemorrhage, venereal disease, water retention, wounds and as a female balancing aid.

1.11. Cephradine: Overview

Cephradine is the antimicrobial agent which was used in this research as control antimicrobial substance for the antimicrobial susceptibility test.

Cephradine is a first generation cephalosporin. It is bactericidal inhibiting bacterial septum and cell wall synthesis. Cephradine is effective against Gram positive and Gram negative bacteria such as Staphylococci including beta - lactamase - producing Staphylococcus aurous, beta-haemolytic Streptococci, Streptococcus pneumoniae; E.coli, Haemophilus influenzae, and Proteus mirabilis. Cephradine is widely distributed to body tissues and fluids. It is excreted unchanged in urine with 60-80% of an intramuscular dose being recovered within 6 hours. Cephradine is excreted into human milk in small amounts. Adverse effects in the nursing infant are unlikely. [32]

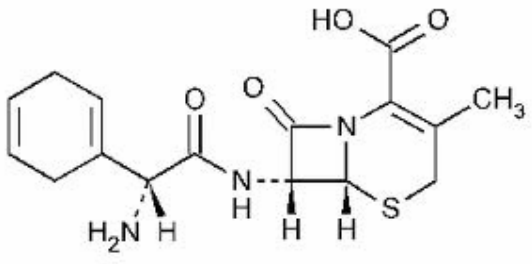


Figure 1.3: Chemical structure of Cephradine.

Cephradine stops the bacteria from making their protein cell wall, so the bacteria die. Cephradine is used to treat bacterial infections of the respiratory tract, urinary tract, skin, and bone and joint. Cephradine is a broad-spectrum bacterial antibiotic active against both Gram-positive and Gram-negative bacteria. It is also highly active against most strains of penicillin's -producing staphylococci. [31] Cephradine is used to treat infections caused by bacteria, including upper respiratory infections, ear infections, skin infections, and urinary tract infections. Do not use this medication if you are allergic to Cephradine, or to similar antibiotics such as Ceftin, Cefzil, Keflex, Omnicef, and others. [31]

1.12. Disc diffusion method

Principle:

Paper discs impregnated with specific antibiotics or the test substances are placed on the surface of the Muller Hinton agar medium inoculated with the target organisms, which is recommended for the diffusion of antimicrobial agents as described in NCCLS approved standard. The plates are incubated and the zones of inhibition around each disc are measured. [42]

Procedure:

Muller Hinton Agar plates were prepared and the test microorganisms were inoculated by the spread plate method. Filter paper discs approximately 6mm in diameter were soaked with 15µl of the plant extract and placed in the previously prepared agar plates. Each disc was pressed down to ensure complete contact with the agar surface and distributed evenly so that they are no closer than 24 mm from each other, center to center. The agar plates were 45 then incubated at 37ºC. After 16 to 18 hours of incubation, each plate was examined. The resulting zones of inhibition were uniformly circular with a confluent lawn of growth. [42]

1.13. Solvent system: ethyl acetate

Physical and chemical properties:

***Table1. 5*:** Physical and chemical properties of ethyl acetate [34]

Molecular formula	$C_4H_8O_2$
Molar mass	88.105 g/mol
Appearance	colourless liquid
Density	0.897 g/cm³, liquid
Melting point	−83.6 °C, 190 K, -118 °F
Boiling point	77.1 °C, 350 K, 171 °F
Solubility in water	8.3 g/100 mL (20 °C)
Solubility in ethanol, acetone, diethyl ether, benzene	Miscible
Refractive index (n_D)	1.3720
Viscosity	0.426 cP at 25 °C

Chemical structure:

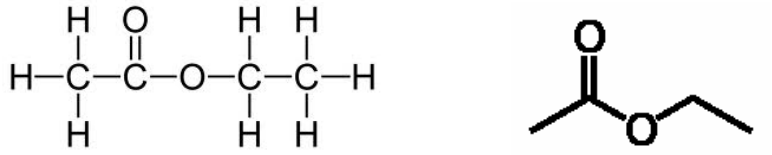


Figure 1.4: Chemical structure of Ethyl acetate.

Uses:

Ethyl acetate is used primarily as a solvent and diluents, being favoured because of its low cost, low toxicity, and agreeable odour. For example, it is commonly used to clean circuit boards and in some nail varnish removers. Coffee beans and tea leaves are decaffeinated with this solvent. Ethyl acetate is the most familiar ester to many chemistry students and possibly the ester with the widest range of uses. Esters are structurally derived from carboxylic acids by replacing the acidic hydrogen by an alkyl or aryl group. Ethyl acetate itself is a colourless liquid at room temperature with a pleasant "fruity" smell, 77°C. It is also used in paints as an activator or hardener. Ethyl acetate is present in confectionery, perfumes, and fruits. In perfumes, it evaporates quickly, leaving only the scent of the perfume on the skin. [33]

1.14. Solvent system: ethanol

Physical and chemical properties:

***Table 1. 6*:** Physical and chemical properties of ethanol [35]

Molecular formula	C_2H_6O
Molar mass	46.07 g/mol
Exact mass	46.041864814 g mol−1
Appearance	colourless liquid
Density	0.789 g/cm³, liquid
Melting point	−114 °C, 159 K, - Density 173 °F
Boiling point	78 °C, 351 K, 172 °F
log P	-0.18
Vapor pressure	5.95 kPa (at 20 °C)
Acidity (pK_a)	15.9
Basicity (pK_b)	-1.9
Refractive index (n_D)	1.36
Viscosity	0.0012 Pa s (at 20 °C)
Dipole moment	1.69 D

Chemical structure:

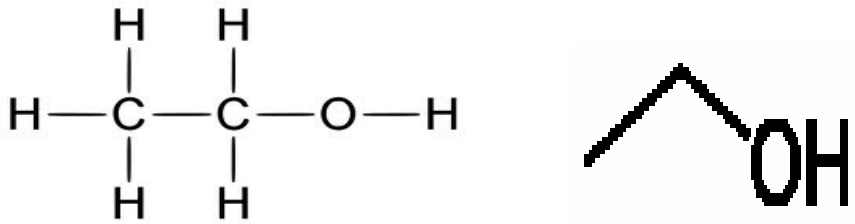


Figure1.5: Chemical structure of Ethanol.

Uses: Ethanol is a colourless, flammable liquid which is produced from the fermentation of sugar. Ethanol is used extensively as a solvent in the manufacture of varnishes and perfumes; as a preservative for biological specimens; in the preparation of essences and flavourings; in many medicines and drugs; as a disinfectant and in tinctures (e.g., tincture of iodine); and as a fuel and gasoline additive. [36] Ethanol is used in antiseptic and some antibacterial soaps and wipes. Ethanol is effective against viruses, fungi and most bacteria but is ineffective against bacterial spores. Ethanol can be used as a fuel for motor vehicles. Ethanol makes a good fuel for cars because it reduces the emission of harmful gases such as carbon monoxide. As ethanol is soluble in water, it can be used in a variety of different products. These include paint, permanent markers, perfumes and deodorants. Ethanol may also be used as a solvent in cooking, such as vodka sauce. [37]

1.15. Solvent system: water

Physical and chemical properties:

***Table 1.7*:** Physical and chemical properties of water [38]

Molecular formula	H_2O
Molar mass	18.01528(33) g/mol
Appearance	white solid or almost colorless, transparent, with a slight hint of blue, crystalline solid or liquid
Density	1000 kg/m^3, liquid (4 °C) (62.4 lb/cu. ft). 917 kg/m^3, solid
Melting point	0 °C, 32 °F, (273.15 K).
Boiling point	99.98 °C, 211.97 °F (373.13 K)
Acidity (pK_a)	15.74 ~35–36
Basicity (pK_b)	15.74
Refractive index (n_D)	1.3330
Viscosity	0.001 Pa□s at 20 °C

Chemical structure:

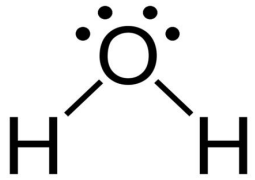


Figure1.6: Chemical structure of water.

Uses:

Water is estimated that 22% of worldwide water use is industrial. Major industrial users include hydroelectric dams, thermoelectric power plants, which use water for cooling, ore and oil refineries, which use water in chemical processes, and manufacturing plants, which use water as a solvent. Uses of fresh water can be categorized as consumptive and non-consumptive Losses to sub-surface seepage and evaporation are considered consumptive, as is water incorporated into a product. Water that can be treated and returned as surface water, such as sewage, is generally considered non-consumptive if that water can be put to additional use. Water use in power generation and industry is generally described using an alternate terminology, focusing on separate measurements of the withdrawal and consumption. [39]

Chapter Two

Literature Review

2.1. Phytochemical studies on *Cissampelos Pareira*:

An amorphous, white alkaloid, *pelosine* was studied in association with an indifferent body, *deyamittin*. Cissampelosine was reported from *Cissampelos Pariera* which was later on shortened as pelosine (Wiggers, 1838). [23]

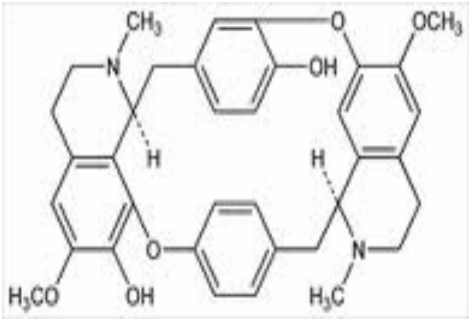


Figure.2.1*:* Structure of Pelosine.

A comparative analysis of *Cissampelos pareira* demonstrated presence of starch, gum, tannin, phlobaphene, and an alkaloid (Ringer and Brooke, 1982). Cissampareine was reported from *Cissampelos pareira* growing in Peru. Cissampareine was found to show significant and reproducible inhibitory activity against human carcinoma of the nasopharynx carried in cell culture. (Kupchan, 1964). [24]

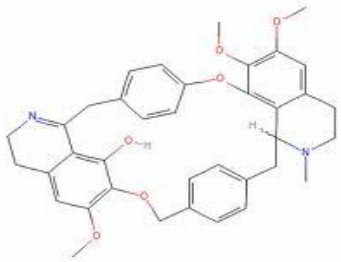


Figure.2.2: Structure of Cissampareine.

(++)-4 -*O*-methylcurine, *l*-curine *d*-isochondrodendrine, and hayatine were isolated from the roots and vines of *Cissampelos pareira* from Madras (Kupchan *et al*,1966). Preliminary pharmacological study of the methanol-extractable alkaloids, of the methiodide prepared

from the latter mixture, and of the quaternary alkaloids, showed that all had curare-like activity. (Mukerji and Bhandari, 1959). [25]

Figure2.3*:* Structure of *d*-iso-chondrodendrine.

Chemical investigation on the roots from Kashmir, reported 0.33 % of alkaloids, mainly hayatine and bebeerines 0.2 % essential oils, 3.4 % fixed oils and a sterol. In the same year, stereochemistry of hayatidine and hayatinine was reported. (Kirtikar and Basu, 2001). [26]

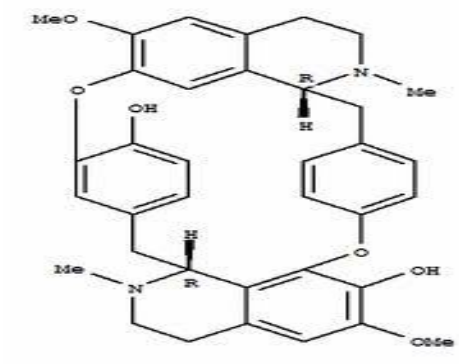


Figure 2.4: Structure of Hayatine.

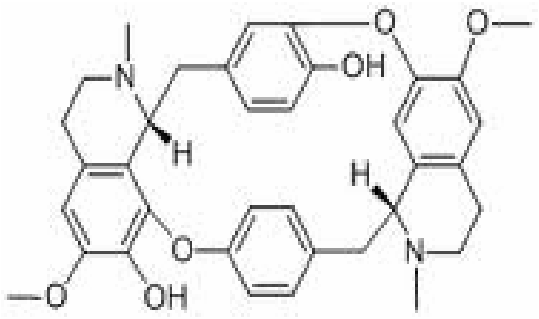


Figure2 .5*:* Structure of Bebeerine.

Cissamine and cycleanine have been reported from the roots. Root is reported to contain *l*-curine. Root bark is reported to contain menismine, pareirine and hayatinine. (Anwer et al. & Bhattacharji et al. 1968.).[27]

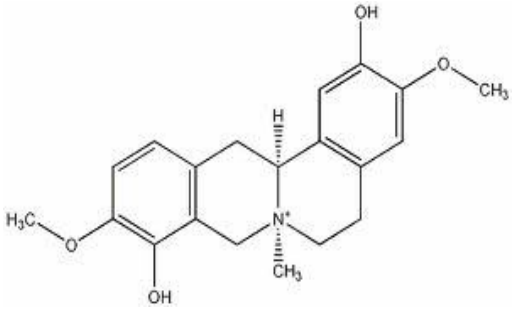


Figure 2.6: Structure of Cissamine.

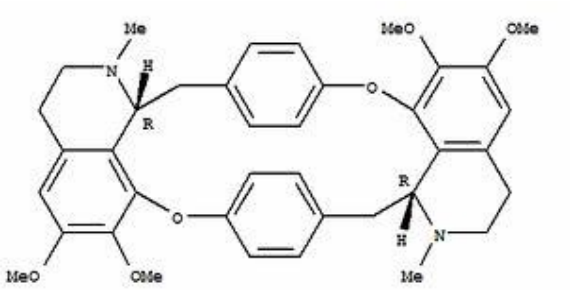


Figure 2.7: Structure of Cycleanine.

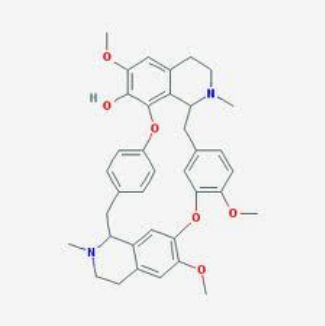


Figure 2 .8*:* Structure of Hayatinine.

Tetrandrine has been reported from the roots of *Cissampelos pareira* growing in Thailand. Dicentrine, dihydrodicentrine, cycleanine, insularine and isochondrodendrine have been reported from roots of the plant growing in Ghana (30). Isolation of pareirubrine A and B, novel tropoloisoquinoline alkaloids with antileukemic activity has been reported (Morita *et al*., 1993). [28]

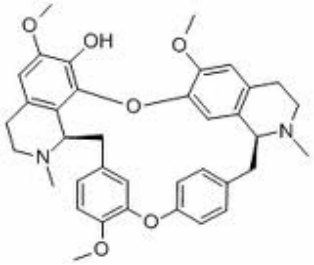


Figure2. 9*:* Structure of Tetrandrine.

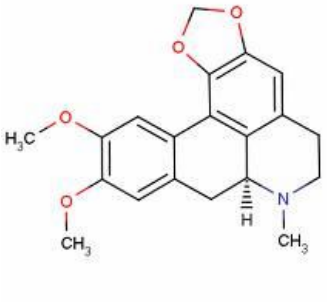


Figure 2.10. Structure of Dicentrine.

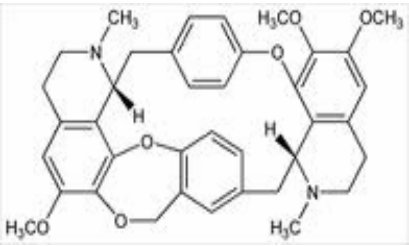


Figure2.11. Structure of Insularine.

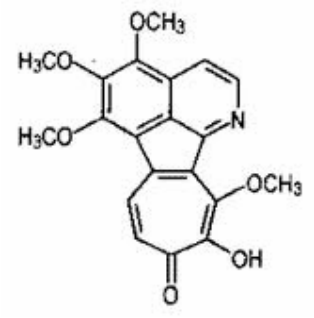


Figure 2.12*:* Structure of Pareirubrine A.

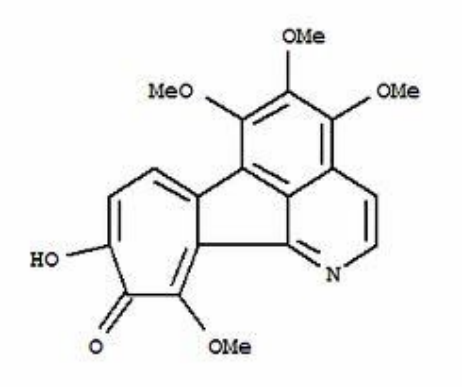


Figure 2.13: Structure of Pareirubrine B.

Tropoloisoquinoline alkaloid pareitropone has been reporte. A novel azafluoranthene alkaloid, norimeluteine, has been isolated as a cytotoxic substance from *Cissampelos pareira* together with an alkaloid having the same skeleton, norruffscine (Morita *et al*.,2005)[29]

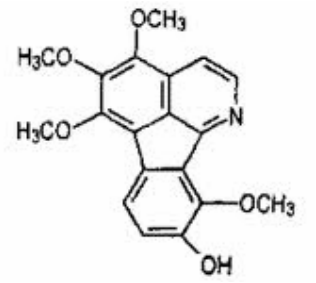


Figure2.14. Structure of Norimeluteine.

An antiprotozoal chalcone-flavone dimer, cissampeloflavone has been isolated from the aerial parts of *Cissampelos pareira*. It has good activity against *Trypanosoma cruzi* and *T. brucei rhodesiense* and has a low toxicity to the human KB cell line. DQurecitol and grandirubrine have been reported. (Carabot *et al.*, 2007)[30]

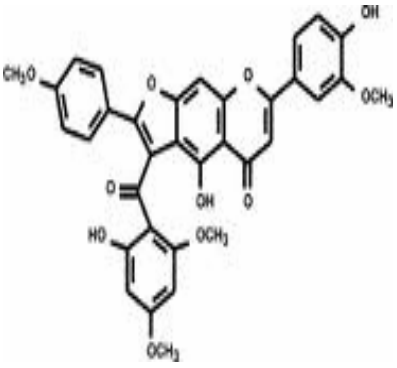


***Figure 2.15**:* Structure of Cissampeloflavone.

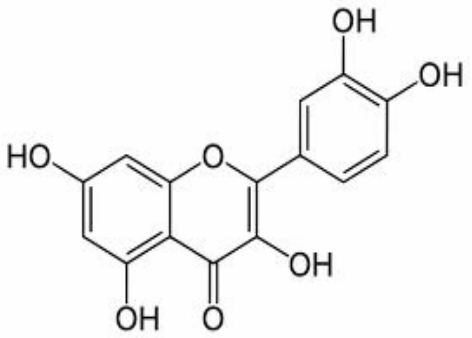


***Figure 2.16**:* Structure of D-Qurecitol.

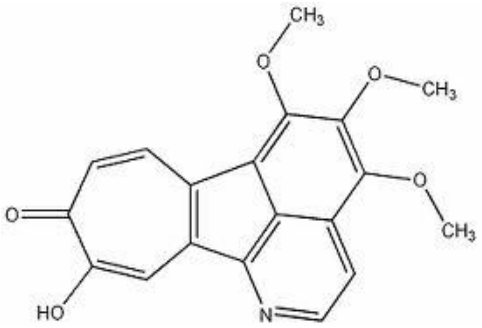


***Figure 2.17**:* Structure of grandirubrine.

A preliminary study of *Cissampelos pareira* Linn. From Peru yielded a new alkaloid, Cissampareine. Evidence is presented for assignment to Cissampareine of the empirical formula, C37H38N2O6. Cissampareine and four other bisbenzylisoquinoline alkaloids isolated from menispermaceous plants were found to show significant and reproducible inhibitory activity against human carcinoma of the nasopharynx carried in cell culture (KB).[41]

2.2. Summary of the Phytochemical studies on *Cissampelos Pareira*.

Table 2.1: Summary of the Phytochemical studies on *Cissampelos Pareira:*

Time/Chronicles	Names of Researchers	Plant of the part	Findings
1838	Wiggers *et al*	All parts	Pelosine
1964	Kupchan *et al*	All parts	Cissampareine
1966	Kupchan *et al*.	roots	(++)-4–*O*methylcurine, *l*-curine, hayatine, and *d*-isochondrodendrine.
1968	Anwer *et al.*	Bark & roots	Cissamine ,cycleanine, pareirine and hayatinine
1982	Ringer and Brooke.	Leaves	starch, gum, tannin, phlobaphene, and an alkaloid
1993	Morita *et al*.	roots	Tetrandrine, Dicentrine, Pareirubrine A, Pareirubrine B.
2001	Kirtikar and Basu,	roots	Hayatine and bebeerines.
2005	Morita *et al*.	All parts	Pareitropone, Norimeluteine.
2007	Carabot *et al*.	Leaves	Cissampeloflavone, D-Qurecitol, grandirubrine
2010	S.Morris Kupchan *et al*.	All parts	Bisbenzylisoquinoline.

2.3. Pharmacological studies on *Cissampelos Pareira*:

Cissampelos pareira Linn. Var. hirsuta is a very variable, lofty, slender, dioecious, perennial, climber commonly distributed throughout topical and sub topical India, ascending up to an altitude of *c* 2,000m, traditionally known as Laghupatha in Ayurveda, an Indian traditional system of medicine. It is thought to be an excellent remedy to alleviate and help with symptoms associated with menstruation and balances hormones in women. Members of the Palikir tribe in Guyana use a poultice of *Cissampelos pareira* as a topical pain-reliever, and the Wayapi Indians use a decoction of the leaf and stem as an oral analgesic.(Kirtikar *et al.*, 2001).[17]

Antinociceptive and antarthritic activity in the present study, 50% aqueous ethanolic extract of roots of *C. pareira* at the dose levels of 100–400 mg/kg, once daily for 3days exhibited significant ($P < 0.001$) resistance against mechanical pain after 30 min in analgesymeter induced pain in mice. In acetic acid (0.6%; i.p.) inducing writhing, *C. Pareira* significantly ($P < 0.05$) decreased the writhing episodes; the degree of percent protection at 200 and 400 mg/kg was 22.73 and 51.63. The hot plate reaction time was increased by 2.07 ($P < 0.05$) and 2.70 ($P < 0.001$) folds, respectively. Further *C. pareira* showed the dose dependent significant protective effect against complete Freund's adjuvant induced arthritis (Amresh *et al*., 2001). [20]

Bisbenzylisoquinoline alkaloids are the main active components of abuta and consist of grisabine and grisabutine, panurensine and norpanurensine, krukovine and limacine1, peinamine, 7-O-demethylpeinamine, N-methyl, 7-O-demethylpeinamine, macolidine and macoline. Protoberberine alkaloids have been found in the roots of the Cissampelos pareira Linn. Another important alkaloid of the C. pareira is hayatinin methochloride which possesses curariform activity. The cytotoxic alkaloid, cissampareine, has been reported to possess anti-tumor activity. The tropoloisoquinoline alkaloids, pareirubrines A and B, from C. pareira have been reported to possess the anti-leukemic action. Cissampeloflavone, a chalcone-flavone dimer from the aerial parts of the C. pareira L. (Menispermaceae), has been reported to have activity against Trypanosoma cruzi and T. brucei rhodesiense and to have low toxicity to the human KB cell line. (Bhatnagar *et al*.2003) [40]

Antifertility activity in *C. pareira* leaf extract, when administered orally, altered the estrous cycle pattern in female mice, prolonged the length of estrous cycle with significant increase in the duration of diestrus stage and reduced significantly the number of litters in albino mice. The analysis of the principal hormones involved in estrous cycle regulation showed that the plant extract altered gonadotropin release (LH, FSH and prolactin) and estradiol secretion. The oral LD50 of the extract was found to be 7.3 g/kg in mice. (Ganguly *et al*, 2007). [21]

To establish the antihemorrhagic activity of aqueous extract from leaves of *C. pareira*, the skin of mice was injected with a mixture of extract and venom, and it as found that extract produced a total inhibition of this activity. On the other hand, experiments regarding the anti-proteolyti activity were conducted observing the effect on casein in a test tube or on biotinylated casein in a microplate. None of the two procedures was able to show any inhibitory activity (Badilla *et al*., 2008). [22]

In the 2009 some South India scientists found that the *Cissampelos pareira* is Antimicrobial Activity of Laehiums Prepared by Herbal Venders. The results on the antimicrobial activity of medicinal formulations showed that all the formulations were effective against tested microorganisms with different zone of inhibition. The hexane extracts and water extracts of laehiums and podimarundhugal showed least antimicrobial activity when compared ethanol and petroleum-ether extracts. [18]

In the 2010 fourth scientists found that the *Cissampelos pareira* is significant medicinal plant of herbal arterial medica. It is used in the treatment of wide range of diseases in Traditional Chinese Medicine, Ayurevda and western herbalism. The plant abounds in isoquinoline alkaloids; the chemicals that received a great deal of attention. Antitumour potential of cissampareine and neuromuscular blocking effects of hayatine are of special interest. The review summarizes ethno pharmacological investigations carried out on the plant with special reference to isoquinoline alkaloids. It appeared to be about one-third as potent as tubocurarine. The neuromuscular block produced by this drug could be completely reversed by neostigmine. It was relatively free from serious side-effects and appears to be a promising muscle relaxant. [19]

In the 2010 fourth scientists study Anti-inflammatory activity of the ethanolic extract of the *Cissampelos pariera* Linn leaves was studied in albino wistar rats using the carrageenan induced rat paw edema model. The ethanolic extract of *Cissampelos pariera* (400 mg/kg) inhibited carrageenan induced rat paw edema. The extract was also studied for its preliminary phytochemical screening and acute toxicity studies. The result indicated that the extract produced significant ($P < 0.05$) anti-inflammatory activity when compared with the standard drug indomethacin (10 mg/kg) and untreated control. [14]

Cissampelos pareira L. inhibited the propagation of rodent parasite Plasmodium berghei in vivo. In a typical four day experiment, the BALB/c mice were administered with ethanol extracts of *Cissampelos pareira* L. The parasitaemian in untreated control group ranged between 17.31% and 30.02% whereas the root extracts of *Cissampelos pareira* L. resulted in inhibition of *Plasmodium berghei* significantly. The inhibitory properties of extracts of two plants require further studies so that the antimalarial activity is elucidated. [9]

In the 2011 two Indian scientists said that Birth control becomes essential part of our life. Synthetic antifertiliity agents have severe side effect like breast cancer, cervical cancer etc c*issampelos pareira Linn.* Is a perennial twining shrub with small yellow flower commonly is one of the folk medicinal plant used as an agert birth control among rural people. *Cissampelos pareira* was fist described from Latin America, but actually occurs throughout the tropic which implies that it is widely available. [13]

The alkaloidal fraction of roots of *Cissampelos pareira* Linn Was screened for *in-vitro* antioxidant activity and immunomodulatory activity in mice. The HPTLC finger print profile was also established for the identification of alkaloid fraction which was found to contain 0.176 % of barbering. Alkaloid fraction possess strong Antioxidant activity which was revealed by its ability to scavenge the stable free Radical DPPH, super oxide ion and to inhibit lipid per oxidation in rat liver Homogenate induced by iron/ADP/Acerbate complex. Alkaloid fraction was found to have Significant immunosuppressive activity at lower doses (25 and 50 mg/kg) while No activity was observed at higher doses (75 and 100 mg/kg). Humoral antibody Titre was significantly ($p<0.01$) lowered by AFCP at the doses of 25 and 50 Mg/kg. Delayed type hypersensitivity response was also significantly ($p<0.01$) Suppressed by the alkaloidal fraction at the dose of 75 mg/kg. Thus the present study revealed the

immunosuppressive and antioxidant activities of the alkaloid fraction of *Cissampelos pareira* roots. [7]

The extract of *C. pareira* roots of 25 mg kg–1 had no effect, 50 and 100 mg kg–1 doses inhibited defecation by 100% in the initial 2 h compared to normal defecation in mice. The activity was reduced to 40.0% and 73.0%, respectively, at the higher doses in the third hour. In the present investigation, the ethanolic extract of *C. pareira* roots showed dose dependent antidiarrhoeal activity in various validated models in rats. Castor oil produced characteristic semisolid diarrhoea droppings in all animals of the control group. The effect of the *C. pareira* root extract at the dose of 25–100 mg kg–1 caused a dose dependent decrease in the total faecal matter (29.2 and 60.0%). Diphenoxylate HCl, a standard antidiarrhoeal drug, inhibited the diarrhoea by 70.8%. The action of castor oil as diarrhoea inductors has been largely studied and it is known that its most active component is the ricinoleic acid, which produces an irritating activity in the small intestine The results of the present study justify the traditional claims of *C. pareira* extract being an antidiarrhoeal drug.[8]

The objective of the present study was to investigate the protective effect of ethanolic extract of *Cissampelos pareira* (CPE) against gastric cancer and its mode of action. The bioassay-guided fractionation of the CPE yielded a compound, identified as 2-(3,4-dihydroxyphenyl)-3,5,7- trihydroxy-4H-chromen-4-one (Quercetin) with the help of NMR and various spectrophotometer methods as U.V., I.R. and other chemical tests. The protective effects of *C. pareira* were studied against Benzo (a) pyrene induced gastric cancer, tumor multiplicity, micronucleus polychromatic erythrocytes (MnPCEs) in mice. The effect of CPE on SOD, CAT, LPO and GST, GPx, GSH was also studied. [10]

2.4. Summary of the Pharmacological studies on *Cissampelos Pareira*.

Table 2.2: Summary of the Pharmacological studies on *Cissampelos Pareira:*

Time/Chronicles	Names of Researchers	Solvent system	Plant of the part	Assertions
1999	Vikram *et al.*	ethanol	root	Anti malarial activity
2001.	Kirtikar *et al.*	Ethanol	leaf and stem	oral analgesic & menstruation and balances hormones in women
2001	Amresh, G *et al.*	ethanolic extract	root	Antinociceptive and antiarthritic activity
2007	Ganguly *et al.*	Ethanol	leaf extract	Antifertility activity.
2008	Badilla, *et al.*	water	leaves	Anti-hemorrhagic effects
2009	C.Chitravadivu, *et al.*	water	Leaf	Dandruff and Externally applied on hair for healthy hair
2010	Amritpal S, *et al.*	water	Full plant	Anti-inflammatory activity, isoquinoline alkaloid, sneuromuscular blocking effects of hayatine are of special interest.
2010	B. Gopalakrishna, et al.	Ethanol	Leaves	Anti-inflammatory activity, analgesic activity Toxic properties.
2011	S, Jhuma, S Bhattacharya.	Ethanol	Full plant	Folk medicinal plant, Antifertility &fever reducing properties.

Chapter Three

Materials & Methods

3.1. Materials Reagents:

1. Closed test tube.
2. Beaker.
3. Electric balance (ELH 3000, Shimadzu, Japan).
4. Aluminium foil paper.
5. Spatula.
6. Micropipette.
7. Pipette.
8. Refrigerator.
9. Appendrof micropipette (Eppendrof, Germany).
10. Appendrof micropipette tip.
11. Appendrof tube/ micro centrifuge tubes.
12. Petri dishes.
13. Nutrient agar (Himedia Laboratories, India).
14. Sodium Chloride.
15. Distilled water.
16. Cotton Buds.
17. Sample micro organisms.
18. Gas burner.
19. Forceps.
20. Filter paper (Whatman 40).
21. Hole puncher.
22. Permanent marking pen.
23. Plant extracts.
24. 50ml beakers.
25. Inoculating Loop.
26. Vortex mixer.
27. Glass container.
28. Autoclave (HIRAYAMA, Japan).
29. Laminar air flow (ESCO, Singapore).
30. Hot air oven (FN-500, Niive).
31. Incubator (BK 4266).

32. Ruler.
33. Disc (Prepared by filter paper).

3.2. Solvent:

1. Water
2. Ethanol.
3. Ethyl acetate.

3.3. Methods:

3.3.1. Preparation of sample and dosage form:

These plant samples were collected from the microbiology lab, Department of pharmacy, East West University. Crude extracts were measured in analytical balance, and dissolved in Water, Ethanol, Ethyl Acetate in a specific concentration 20 mg/ mL and 40 mg/ mL. The liquid form of sample was made for optimal concentration of dose in each paper disk. The disk concentrations used were 400 µg and 800 µg.

3.3.2. Collection of Micro organism samples:

Bacillus sereus, Bacillus subtilis, Staphylococcus aureus, Sarcina lutea, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi, Shigella dysenteriae, Shigella boydii, Vibrio mimicus, Candida albicans, Candida albicans, Saccharomyces cerevisiae, Vibrio parahemolyticus, Bacillius megaterium. These fifteen microorganism samples were collected from the microbiology lab, Department of pharmacy, East West University. All the strains were cultured from the mother strains as a stock culture in vials, and then they were re-cultured in Petri dishes.

3.3.3. Description of tested microorganisms:

I had performed the antimicrobial test against thirteen micro organisms. A brief description is given below about these micro organisms.

1. ***Bacillus cereus:***

Bacillus cereus is an endemic, soil-dwelling, Gram-positive, rod-shaped, beta haemolytic bacterium. Some stains are harmful to humans and cause food borne illness. *Bacillus cereus* causes two types of food poisoning in humans including diarrhoeal syndrome and emetic

syndrome. *Bacillus cereus* causes gangrene, bovine mastitis, gynogenic infections, infant death, septic meningitis, periodontal disease, lung abscesses and endocarditic. *Bacillus cereus* can cause ocular infections such as keratits, endophthalmitis and panophthalmitis.

2. **Bacillus *subtilis:***

Bacillus subtilis, known also as the hay bacillus or grass bacillus, is a Gram-positive, catalyse-positive bacterium commonly found in soil. It may contaminate food but rarely causes food poisoning. *Bacillus subtilis* produces the proteolytic enzyme subtilisin. It has associated with outbreaks of food poisoning by lechithinase enzyme activity which disrupts membranes of mammalian cells.

3. ***.Staphylococcus aureus:***

Staphylococcus aureus is a facultatively anaerobic, Gram-positive coccus and is the most common cause of staph infections. *Staphylococcus aureus* can cause a range of illnesses from minor skin infections such as pimples, impetigo, boils (furuncles), cellulites folliculate, carbuncles, scalded skin syndrome, and to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditic, toxic shock syndrome (TSS), chest pain food poisoning, bacteraemia, and sepsis.

4. ***Sarcina lutea:***

Sarcina lutea is an older name Micrococcus luteus. *Sarcina lutea* is a Gram-positive bacterium in the Fimicutes phylum. It is found in soil and air. *Sarcina lutea* can also live on human skin and in the mouth.

5. ***Escherichia coli (E.coli):***

Escherichia coli are a Gram-negative rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. Virulent strains of *Escherichia coli* can cause gastroenteritis, urinary tract infections, and neonatal meningitis. In rare case, virulent strains are also responsible for haemolytic-uremic syndrome, peritonitis, mastitis, septicaemia and Gram-negative pneumonia etc.

6. *Pseudomonas aeruginosa:*

Pseudomonas aeruginosa is a Gram-negative, aerobic, rod-shaped bacterium with unipolar motility. *Pseudomonas aeruginosa* is a common bacterium that can cause disease in animals, including humans. It is found in soil, water, skin flora, and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also in hypoxic atmospheres, and has, thus, colonized many natural and artificial environments. It uses a wide range of organic material for food; in animals, the versatility enables the organism to infect damaged tissues or those with reduced immunity.

7. *Salmonella typhi:*

Salmonella typhi is a serotype of the species Salmonella enteric. Salmona survives well in foods and on surfaces, 190 days on chocolate biscuit, 230 days on sweets, for 4 days in shellfish at 10 to 13□C and in excess of 90 days in ice. Infection of *Salmonella typhi* leads to the development of typhoid or enteric fever.

8. *Salmonella paratyphi:*

Salmonella paratyphi is part of the Enterobacteriaceae family; it is a Gram-negative motile, aerobic rod which is facultativelly anaerobic. *Salmonella paratyphi* can cause a disease known as paratyphoid fever. *Salmonella paratyphi* cause bacterial enteric fever which is characterised by an abrupt onset, continued fever, malaise, headache, anorexia, enlargement of spleen, bradycardia, complications include perforation/ulceration of the intestines, less frequently psychosis, hepatitis, cholecystitis, pneumonitis, endocarditis and pericarditis.

9. *Shigella dysenteriae:*

Shigella dysenteriae are Gram-negative bacteria, very closely related to *Escherichia coli. Shigella dysenteriae* can cause shigellosis (bacillary dysentery). *Shigella dysenteriae*, spread by contaminated water and food, causes the most severe dysentery because of its potent and deadly Shiga toxin, but other species may also be dysentery agents.

10. *Shigella boydii:*

Shigella boydii is a Gram-negative bacterium of the genus Sghigella. Like other member of the genus, *Shigella boydii* is a non-motile. Non-spore forming, rod-shaped bacteria which can cause dysentery in humans through fecal- oral contamination.

11. *Vibrio mimicus:*

Vibrio mimicus are largely classified into two distinct groups: Vibrio cholera infections and no cholera Vibrio infections. *Vibrio mimicus* gastroenteritis, septicaemia and wound infection include fever, blood diarrhea, diaeehea, vomiting and dehydration. *Vibrio mimicus* is most common organism responsible for food poisoning.

12. *Candida albicans:*

Candida albicans is a diploid fungus (a form of yeast) and a causal agent of opportunistic oral and genital infections in humans. Under normal circumstances, *Candida albicans* lives in 80% of the human population with no harmful effects, although overgrowth results in candidasis. Candidasis is often observed in immunocompromised individuals such as HIV-positive patients.

13. *Saccharomyces cerevisiae:*

Saccharomyces cerevisiae is one of the most important fungi in the history of the world. *Saccharomyces cerevisiae* has both asexual and sexual reproduction. They are found in the wild growing on the skins of grapes and other fruits. The yeast *Saccharomyces cerevisiae* has traditionally found useful applications in the production of food and alcoholic beverages. The completed sequence of its genome has revolutionized the use of yeast as a model system for the investigation of eukaryotic cell processes at a whole-genome level. The production of enzymes and recombinant proteins and the development of drug screening assays are commercial applications of yeast cells.

14. *Vibrio parahemolyticus:*

Vibrio parahemolyticus is a gram negative enteric bacterium, from the same family that causes cholera found abundantly along the coastal waters all over the world. It lives in

brackish saltwater and causes gastrointestinal illness in humans. *Vibrio parahemolyticus* is a curved rod-shaped, Gram-negative bacterium found in saltwater.

15. *Bacillius megaterium:*

Bacillus megaterium is a gram positive, endospore forming, rod shaped bacteria. It is considered aerobic. It is found in soil and considered a saprophyte. *Bacillus megaterium* is one of the first bacteria's genome that has been fully coded. *Bacillus megaterium* has often been used in the laboratory, and is used as an industrial organism that is able to produce a variety of proteins and sources of bioremediation. *Bacillus megaterium* is a good source of industrial proteins because it is both a desirable cloning host and produces a large variation of enzymes. It is considered as non-pathogenic to human.

3.3.4. Sterilization process:

Before starting the antimicrobial assay, sterilization of all the materials like Petri dishes were wrapped with paper and placed inside an autoclaved in the autoclave machine (HIRAYAMA, Japan) at 121℃ for 15 minutes. After the autoclaving, all the apparatus were washed by using detergents and then left for drying in the Hot air oven (FN-500, Niive) for 50-60 minutes. All the apparatus that were autoclaved and dried were placed into laminar air flow (ESCO, Singapore) to prevent contamination.

Figure 3.1: Hot air oven (FN-500, Niive).

Figure 3.2: Laminar air flow (ESCO, Singapore).

3.3.5. Preparation of Agar solution/Medium:

A standard rule is, 28 gram Nutrient Agar is require for 1000ml distilled water. As a result 5.6 gram Nutrient agar was weighed and then 200ml distilled water was added to prepare 200ml Agar solution. Same as 8.4gram Nutrient agar was weighed and then 300ml distilled water was added to prepare 300ml Agar solution. This preparation was kept in a 400ml glass container. To eliminate the chance of contamination from the agar solution, the prepared agar solution was autoclaved in the autoclave machine at 121℃ for 15 minutes and after that, as no drying was required here; the glass container containing the nutrient agar medium was placed inside the laminar air flow to prevent contamination.

Figure 3.3: Glass container.

Figure 3.4: Autoclave machine (HIRAYAMA, Japan).

3.3.6. Streaking and inoculation:

After the sterilization by autoclave & hot air oven, sterile Petri dishes and Agar solution glass container were kept under laminar air flow to prevent contamination. The prepared Agar solution was poured into each of the fifteen Petri dishes in a way so that each Petri dish gets 15-20 ml agar medium. Agar medium was dispensed into each Petri dish to gets 3-4mm depth of agar media in each Petri dish. After pouring the agar medium, all Petri dishes were kept in room temperature, so that agar medium can become properly solidified.

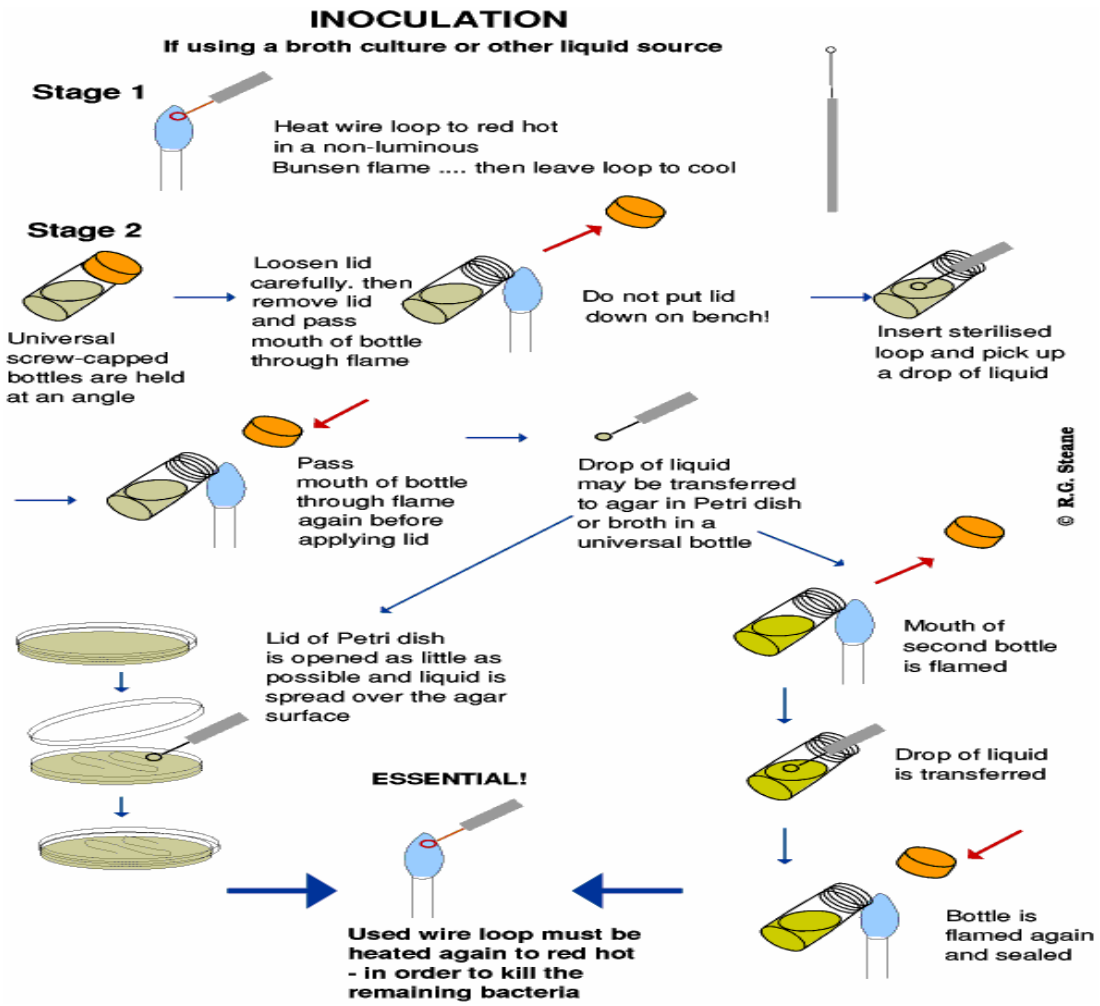


Figure 3.5: Inoculation process.

The Inoculating loop was soaked in ethanol and also placed under laminar air flow. All the sample micro organism was contained in separate closed test tubes. To inoculate the sample

bacteria to the agar medium of Petri dish, the bacterial sample test tube cap was opened and the top was exposed to the flame of the gas burner. Before inoculation, the inoculating loop was exposed to the flame of burner to ignite the ethanol in it. The sterile inoculating loop was touched inside the bacterial sample test tube slightly, then the inoculators with the micro organism on it, was streaked across the surface of agar medium of plate leaving he micro organism on the agar medium and a "zigzag" pattern was drawn. In this manner, one by one, all the agar plates were inoculated with micro organism according to their respective labels.

Figure 3.5: Inoculating loop.

3.3.7. Incubation:

Then all the prepared agar plates with respective micro organisms were placed inside a bacteriological incubator for 18 hours to allow the growth of pure fresh culture of micro organism in each of the Petri dishes.

Figure 3.6: Incubator.

3.3.8. Preservation of cultured micro organisms:

After 18 hours incubation, all the Petri dishes with respective micro organism cultures were removed from Incubator and then were kept in a refrigerator for further use in in-vitro antimicrobial test.

3.3.9. Preparation of dried filter paper discs:

Whatman filter paper no. 1 is used to prepare discs approximately 6 mm in diameter by a Hole puncher. The loop is used for delivering the antibiotics is made. These discs were also sterilized by autoclave.

Figure 3.7: Hole puncher.

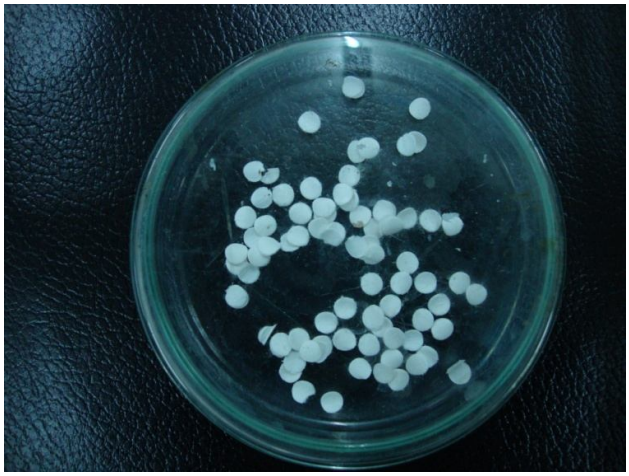


Figure 3.8: Filter paper discs.

3.3.10. Inoculums' preparation:

According to the method, the prepared 0.9% Sodium Chloride saline preparation was poured into 3 Appendrof tubes in a way that each may be able to contain 1ml of saline. These Appendrof tubes were labelled into the 3 respective sample micro organism names. Then, a micro organism culture plate that was stored in refrigerator was brought. With a sterile loop micro organism colonies from the bacterial culture plate was isolated and dipped into tube containing saline suspension. Then the tube was closed and the micro organism inoculums were mixed properly by a vortex mixer. The suspension was adjusted to match the 0.5

McFarland turbidity standards, using saline and a vortex mixer. This process was done for each of the 3 test microorganism.

Figure 3.9: Appendrof tube/ micro centrifuge tube.

Figure 3.10: vortex mixer.

3.3.11. Inoculation of Test plates:

1. Optimally, within 15 minutes after adjusting the turbidity of the inoculums suspension (tube containing suspension of micro organism), a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This helped to remove excess inoculums from the swab.
2. The dried surface of the agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60□ each time to ensure an even distribution of inoculums. As a final step, the rim of the agar was swabbed. This process was performed for each of the fifteen micro organisms. All the plates were labelled with name of micro organisms, dose of the plant disc, standard antibiotic dose at backside.
3. The lid may be left agar for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the plant impregnated disked. An extreme in inoculums density was avoided.

Figure 3.11: Cotton Buds.

3.3.12. Application of plant extracts to the prepared filter paper Discs:

A 20-200µL micropipette was adjusted to 20µL, so that a single press on the micropipette will deliver a concentration 400µg of plant extract of filter paper discs. In same way adjusted to 20µL so that a single press on the micropipette will deliver a concentration 800µg of plant extract of filter paper discs. By this process, 15 discs were treated with 400µg dose of leaf plant extract and 800µg dose of leaf plant extract.

Figure 3.12: Appendrof micropipette (Eppendrof, Germany) & Appendrof micropipette tip.

3.3.13. Procedures:

1. 800ml media (Agar) was prepared with Distilled Water. Where Nutrient Agar needed 22.4gm.
2. Media and other equipments were sterilized in the Autoclave for 1 hour. Where temperature range is 60°C To 121°C and Pressure is 1 atp.

3. Petri dish cleaned and kept in the Hot Air Woven to dry and sterilize.
4. Bacterial subculture was made before experiment at least 1 day.
5. There were fourteen types of Bacteria solution prepared with 1ml Normal Saline (0.9% NaCl) and micro organisms each.
6. Labelling was done before pouring the media into Petri dish.
7. Media was poured into fourteen Petridis equally and wait for being solid the media.
8. Speeded the Bacterial solution on solid media with very careful.
9. Then placed the Antibiotic disc after drying the disc to each Petri dish very carefully.
10. The plates should be incubated soon after placing the disc.
11. The temperature range of 35°c ±2°c is normally required for incubation and the incubation time was 24 hours which were considered as standard for this test.

Figure 3.13: Forceps.

3.3.14. Measuring zone sizes:

1. Following incubation, the zone sizes were measured to the nearest millimetre using a ruler. The diameter of the disc was included in the measurement.
2. When measuring zone diameter, it was round up to the next millimetre.
3. The plate was held a few inches above a black, non-reflecting surface illuminated with reflected light.
4. The plate was viewed using a direct, vertical line of sight to avoid any parallax that might result in misreading.
5. The zone sixed was recorded on the recording sheet.
6. If the placement of the disc or the size of the zone did not allow to read the diameter of the zone, then it was measured from the centre of the disc to a point on the circumference of the zone where a distinct edge was present(the radius) and the measurement was multiplied by 2 to determine the diameter.

Chapter Four

Result & Discussion

Table 4.1: **Antimicrobial activity of different solvent extracts of *Cissampelos pareira* leaves at a concentration of 800µg/disc.**

Name of Microorganism	Zone (mm) of Inhibition by Water Extract	Zone (mm) of Inhibition by Ethanol Extract	Zone (mm) of Inhibition by Ethyl Acetate Extract	Zone (mm) of Inhibition by Cephradine
Bacillus sereus	7	11	16	20
Bacillus subtilis	0	7	13	26
Staphylococcus aureus	10	8	8	26
Sarcina lutea	0	10	13	22
Escherichia coli	0	12	18	23
Pseudomonas aeruginosa	0	10	11	25
Salmonella typhi	0	9	0	20
Salmonella paratyphi	0	12	7	24
Shigella dysenteriae	0	0	0	22
Shigella boydii	15	10	18	30
Vibrio mimicus	0	15	12	20
Candida albicans	0	0	12	20
Saccharomyces cerevisiae	0	13	12	24
Vibrio parahemolyticus	0	13	9	20
Bacillius megaterium	0	0	10	22

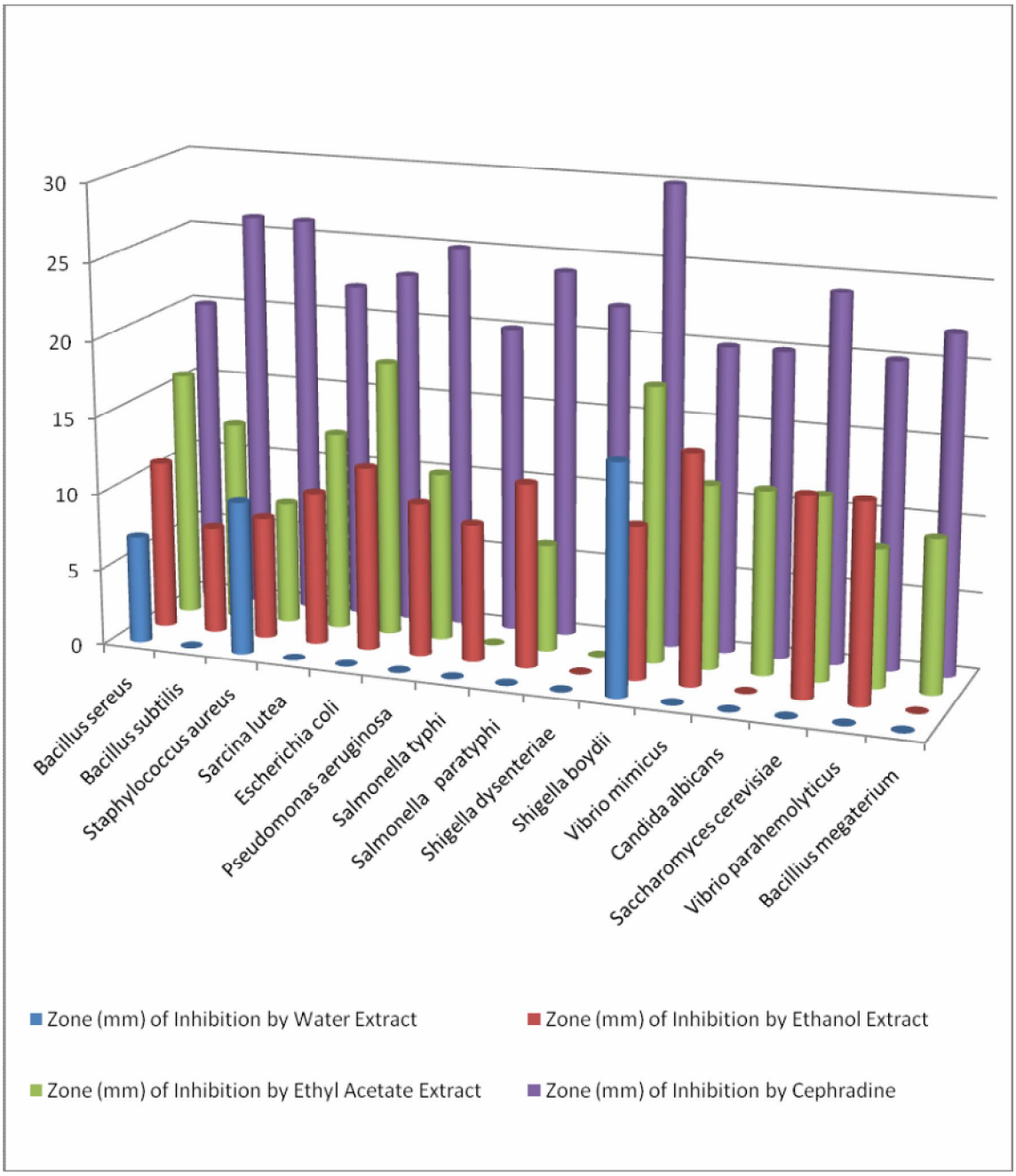


***Figure 4.1*:** Antimicrobial activity of *Cissampelos pareira* leaves extract against fifteen different microorganisms at a concentration of 800µg/disc.

Table 4.2: **Antimicrobial activity of different solvent extracts of *Cissampelos pareira* leaves at a concentration of 400µg/disc.**

Name of Microorganism	Zone (mm) of Inhibition by Water Extract	Zone (mm) of Inhibition by Ethanol Extract	Zone (mm) of Inhibition by Ethyl Acetate Extract	Zone (mm) of Inhibition by Cephradine
Bacillus sereus	0	0	12	20
Bacillus subtilis	0	0	7	26
Staphylococcus aureus	0	7	7	26
Sarcina lutea	0	6	10	22
Escherichia coli	0	11	14	23
Pseudomonas aeruginosa	0	12	7	25
Salmonella typhi	0	8	0	20
Salmonella paratyphi	0	7	9	24
Shigella dysenteriae	0	0	0	22
Shigella boydii	7	12	12	30
Vibrio mimicus	0	8	7	20
Candida albicans	0	0	0	20
Saccharomyces cerevisiae	0	12	11	24
Vibrio parahemolyticus	0	11	8	20
Bacillius megaterium	0	7	8	22

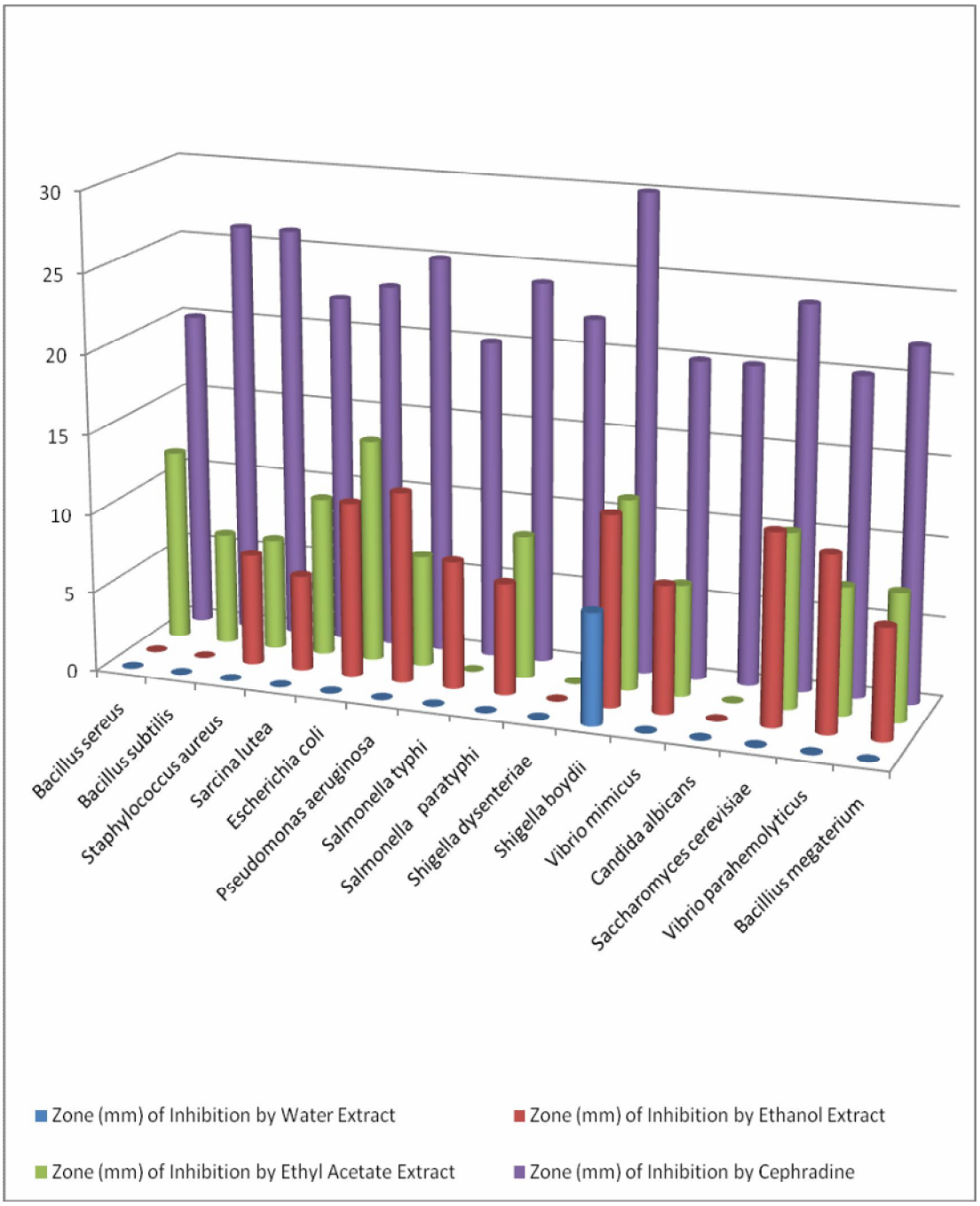


***Figure 4.2*:** Antimicrobial activity of *Cissampelos pareira* leaves extract against fifteen different microorganisms at a concentration of 400µg/disc.

Table 4.3: **Antimicrobial activity of different solvent extracts of *Cissampelos pareira* Bark at a concentration of 800µg/disc.**

Name of Microorganism	Zone (mm) of Inhibition by Water Extract	Zone (mm) of Inhibition by Ethanol Extract	Zone (mm) of Inhibition by Ethyl Acetate Extract	Zone (mm) of Inhibition by Cephradine
Bacillus sereus	0	7	8	20
Bacillus subtilis	0	10	9	26
Staphylococcus aureus	0	8	17	26
Sarcina lutea	0	7	8	22
Escherichia coli	0	12	17	23
Pseudomonas aeruginosa	0	8	12	25
Salmonella typhi	0	9	0	20
Salmonella paratyphi	0	7	7	24
Shigella dysenteriae	0	0	0	22
Shigella boydii	7	11	10	30
Vibrio mimicus	0	7	9	20
Candida albicans	0	6	8	20
Saccharomyces cerevisiae	0	7	11	24
Vibrio parahemolyticus	0	0	10	20
Bacillius megaterium	0	9	8	22

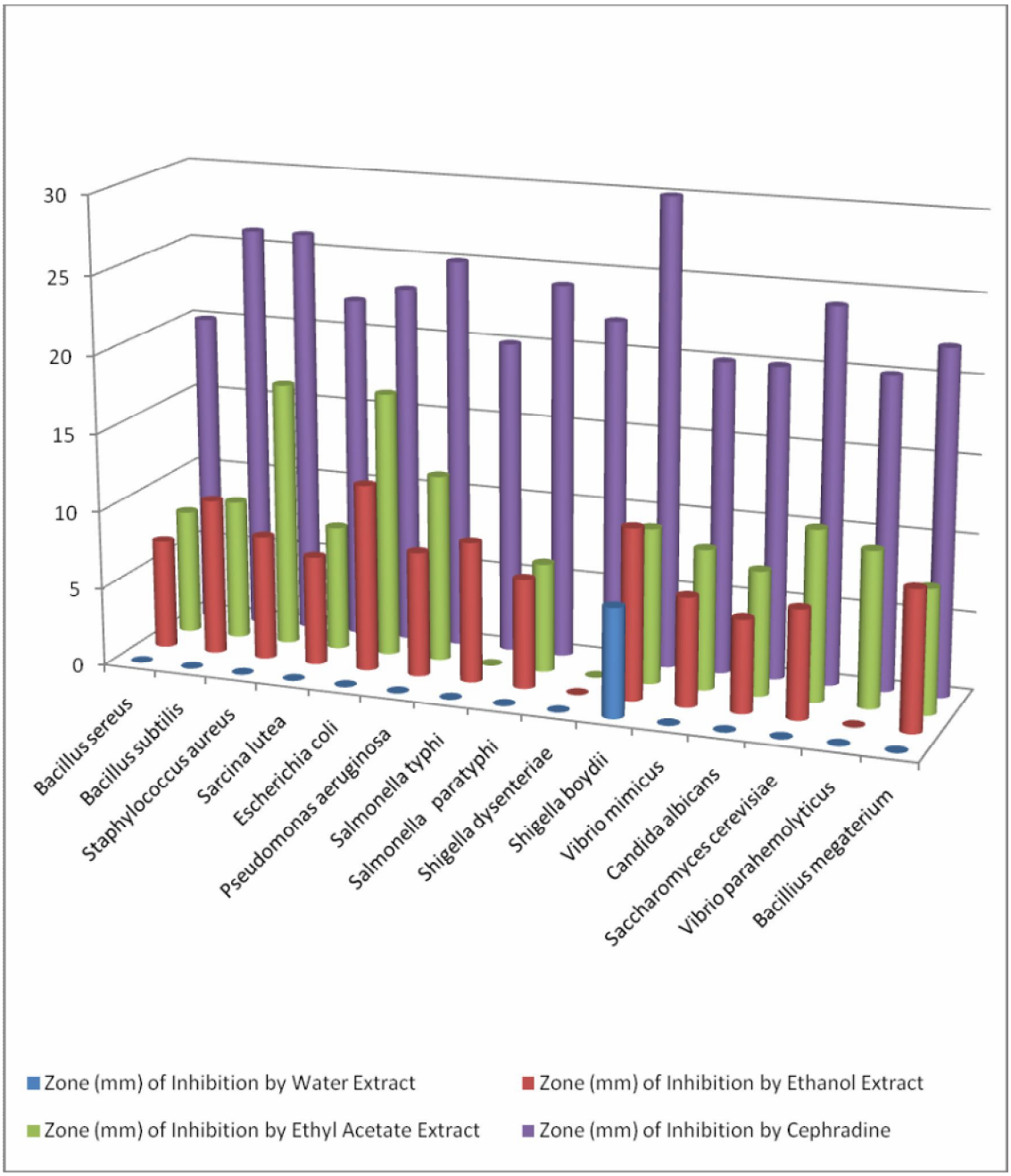


Figure 4.3: Antimicrobial activity of *Cissampelos pareira* bark extract against fifteen different microorganisms at a concentration of 800µg/disc.

Table 4.4: **Antimicrobial activity of different solvent extracts of *Cissampelos pareira* Bark at a concentration of 400µg/disc.**

Name of Microorganism	Zone (mm) of Inhibition by Water Extract	Zone (mm) of Inhibition by Ethanol Extract	Zone (mm) of Inhibition by Ethyl Acetate Extract	Zone (mm) of Inhibition by Cephradine
Bacillus sereus	0	0	7	20
Bacillus subtilis	0	8	7	26
Staphylococcus aureus	0	7	16	26
Sarcina lutea	0	0	0	22
Escherichia coli	0	14	11	23
Pseudomonas aeruginosa	0	9	10	25
Salmonella typhi	0	0	0	20
Salmonella paratyphi	0	10	7	24
Shigella dysenteriae	0	0	0	22
Shigella boydii	0	8	8	30
Vibrio mimicus	0	9	8	20
Candida albicans	0	0	0	20
Saccharomyces cerevisiae	0	7	8	24
Vibrio parahemolyticus	0	0	12	20
Bacillius megaterium	0	7	8	22

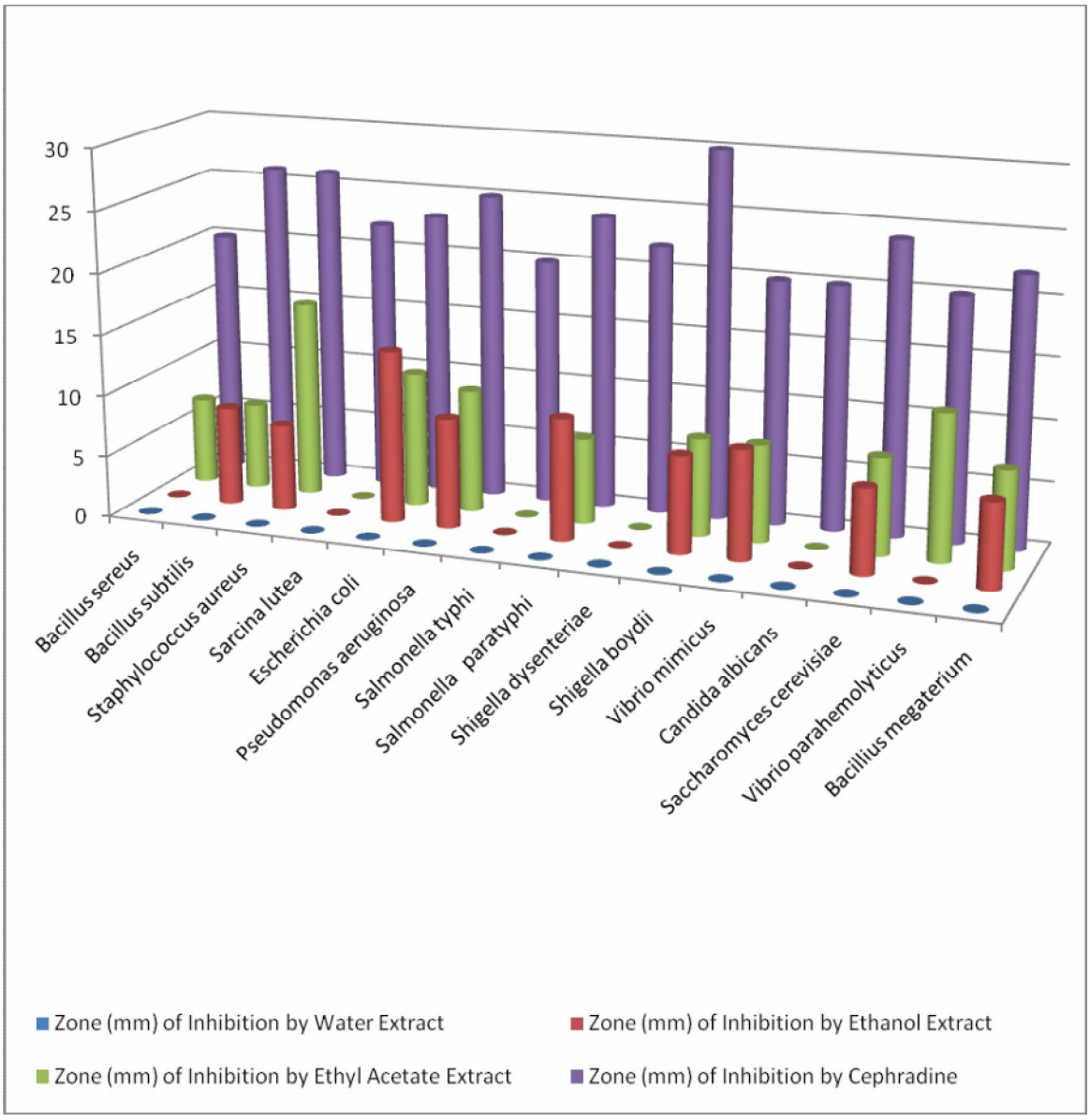
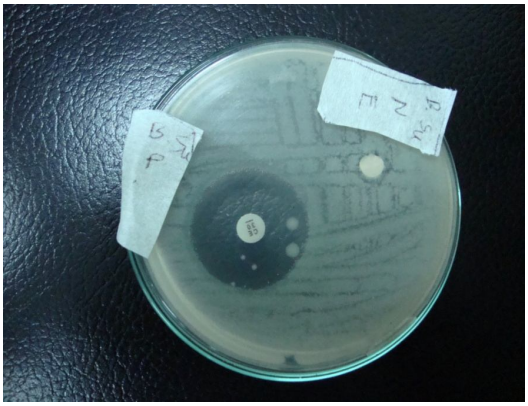
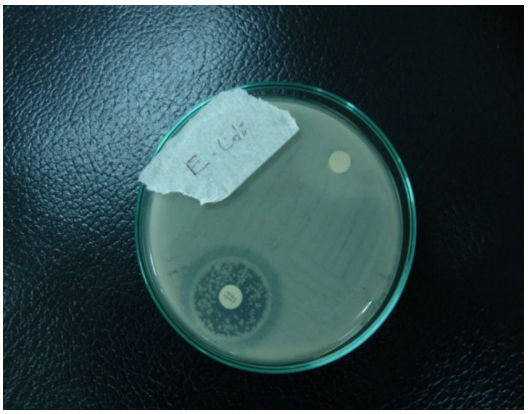


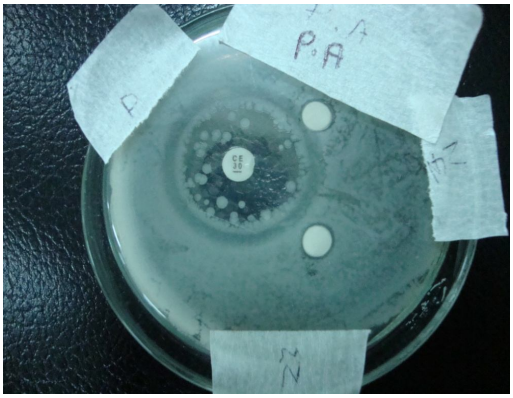
***Figure 4.4*:** Antimicrobial activity of *Cissampelos pareira* Bark extract against fifteen different microorganisms at a concentration of 400µg/disc.



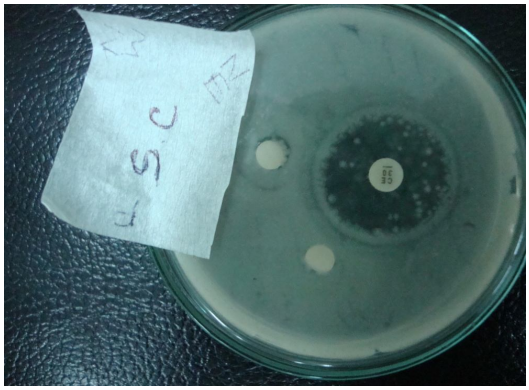
Positive control of *Bacillus subtilis.*



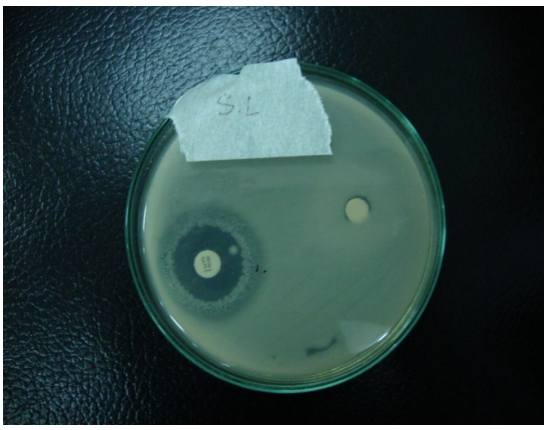
Positive control of *Escherichia coli.*



Positive control of *Pseudomonas aeruginosa*.

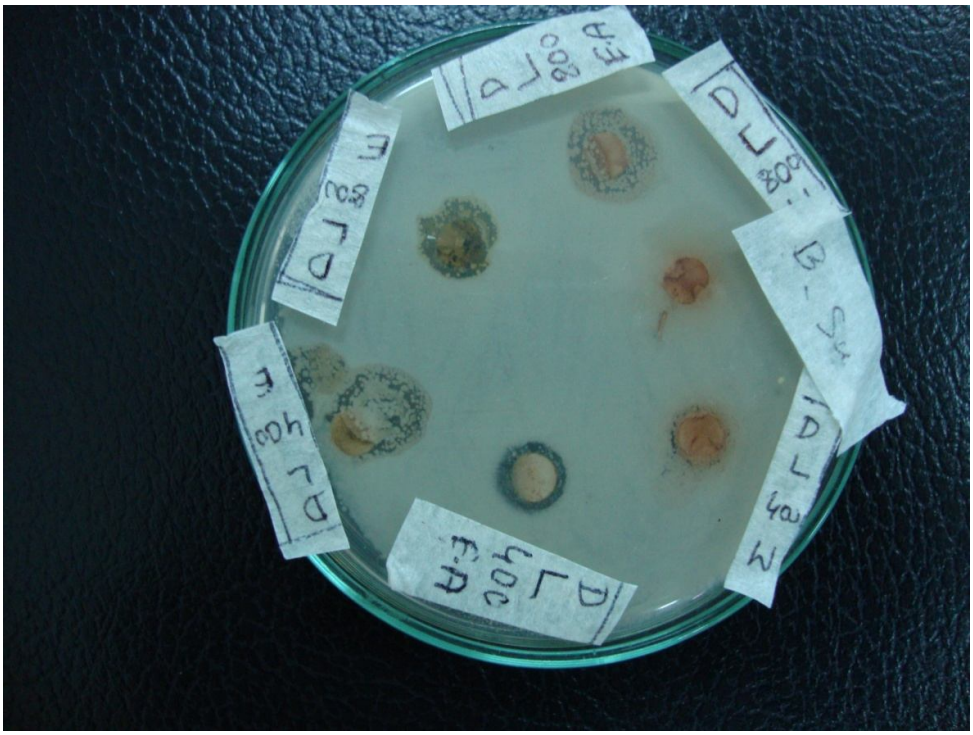


Positive control of *Saccharomyces cerevisiae*

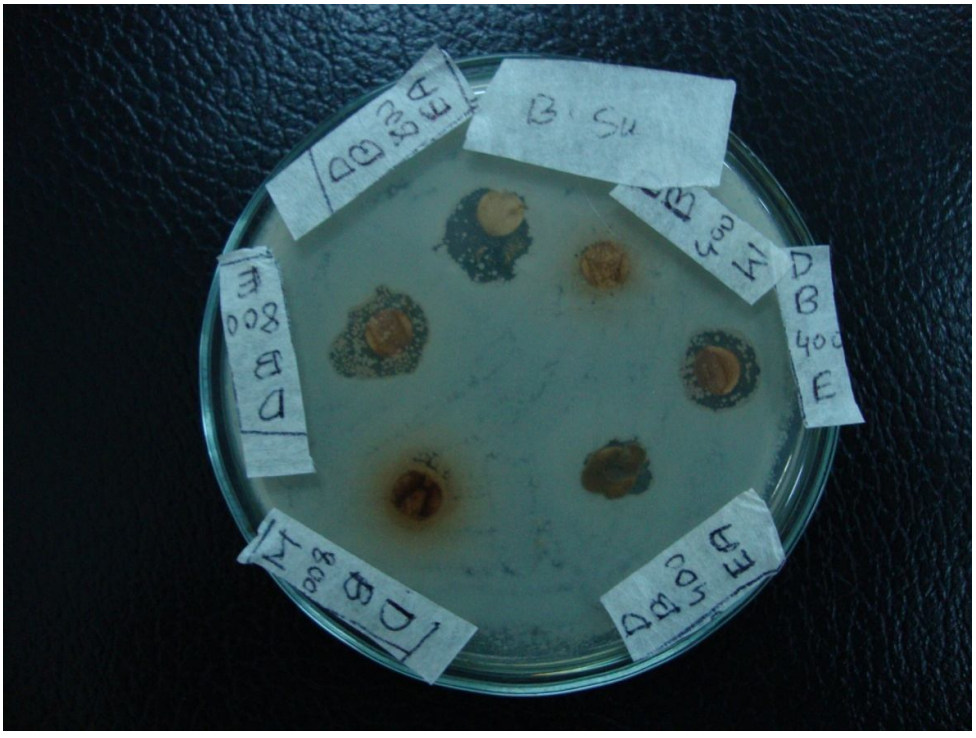


Positive control of *Sarcina lutea*.

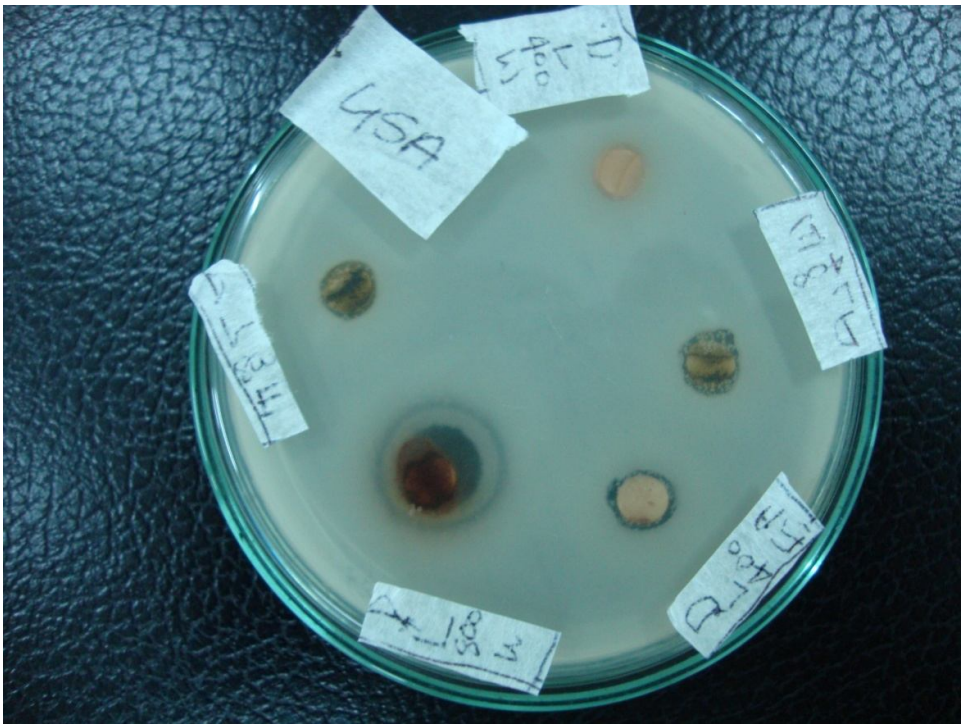
Positive control of *Staphylococcus aureus.*



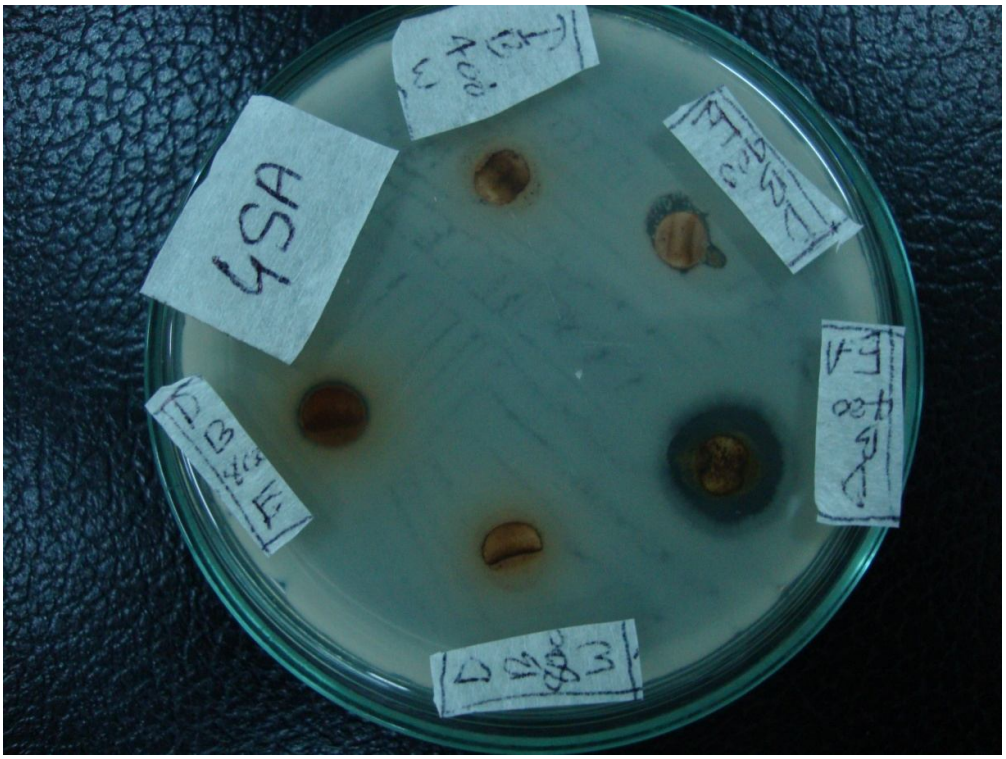
Antimicrobial activity against *Bacillus subtilis* of *Cissampelos pareira* leaves (400µg & 800µg) extract.



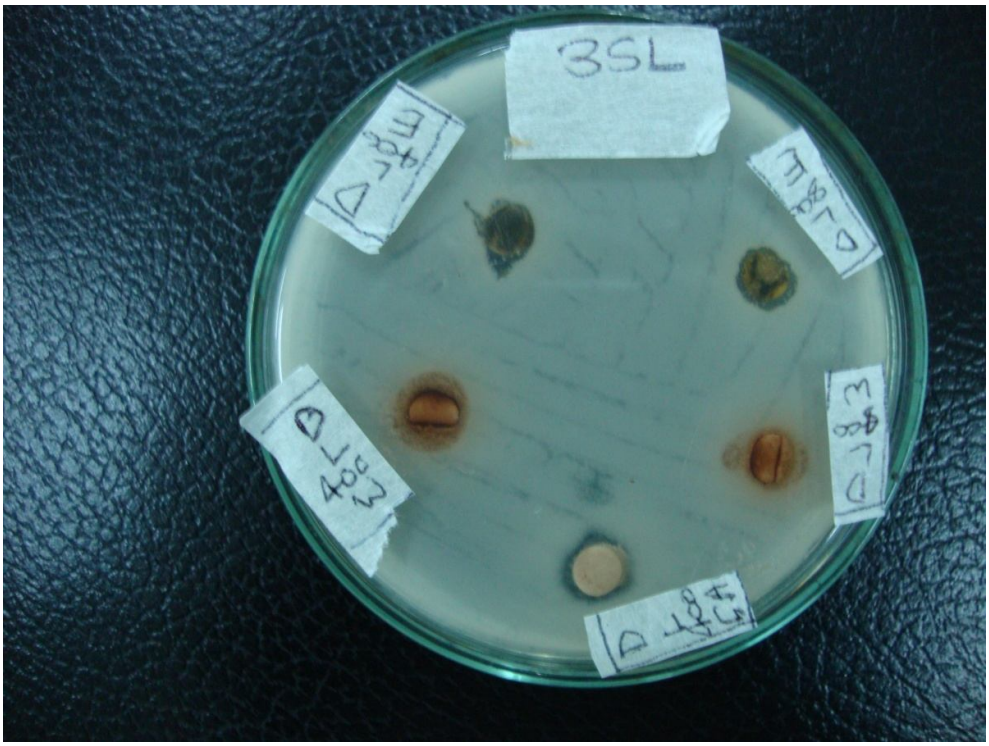
Antimicrobial activity against *Bacillus subtilis* of *Cissampelos pareira* Bark (400µg & 800µg) extract.



Antimicrobial activity against *Staphylococcus aureus* of *Cissampelos pareira* leaves(400µg & 800µg) extract.

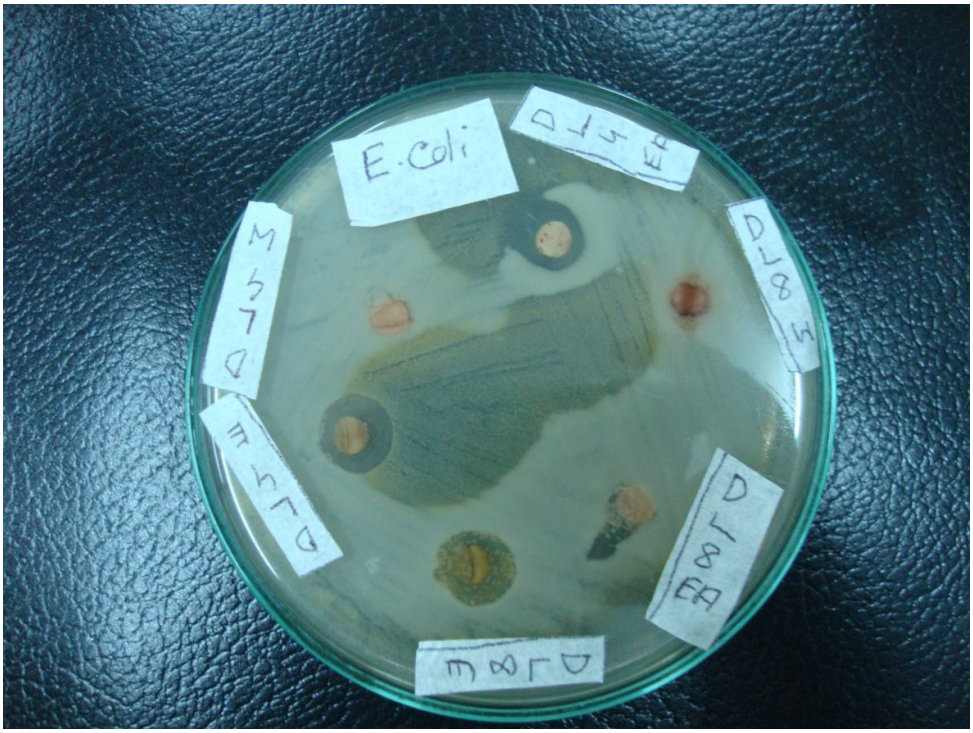


Antimicrobial activity against *Staphylococcus aureus* of *Cissampelos pareira* Bark (400µg & 800µg) extract.

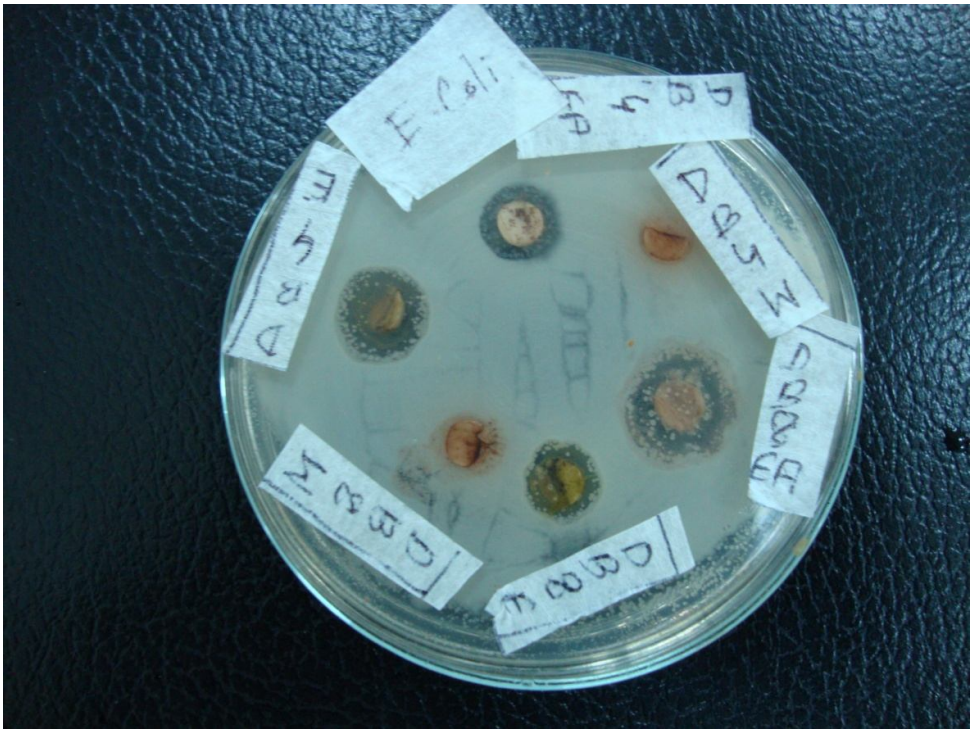


Antimicrobial activity against *Sarcina lutea* of *Cissampelos pareira* leaves(400µg & 800µg) extract.

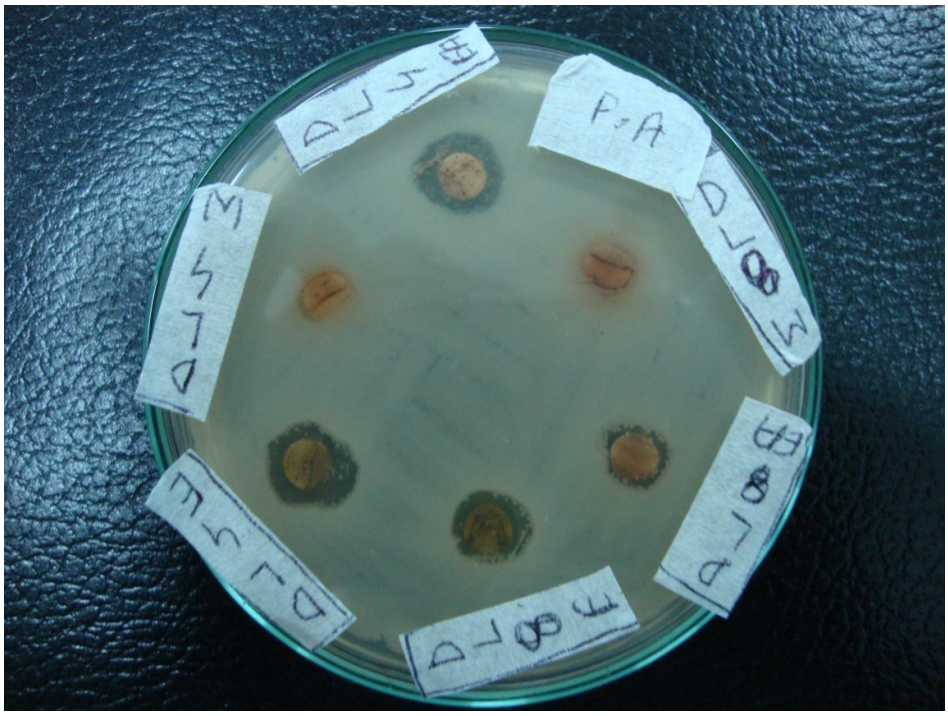
Antimicrobial activity against *Sarcina lutea* of *Cissampelos pareira* Bark (800µg) extract.



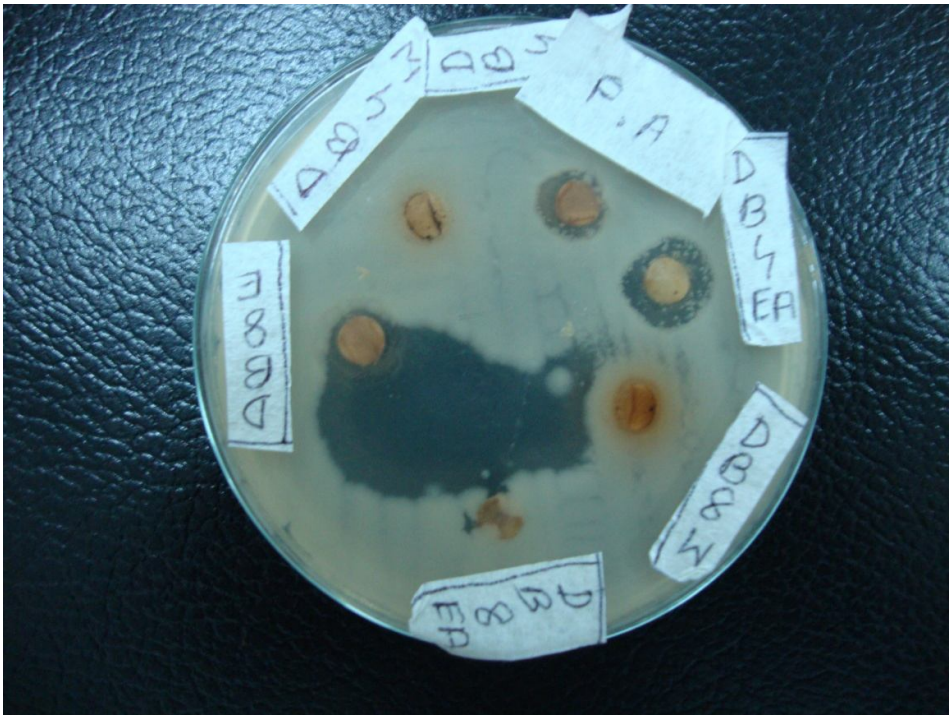
Antimicrobial activity against *Escherichia coli* of *Cissampelos pareira* leaves(400µg & 800µg) extract.



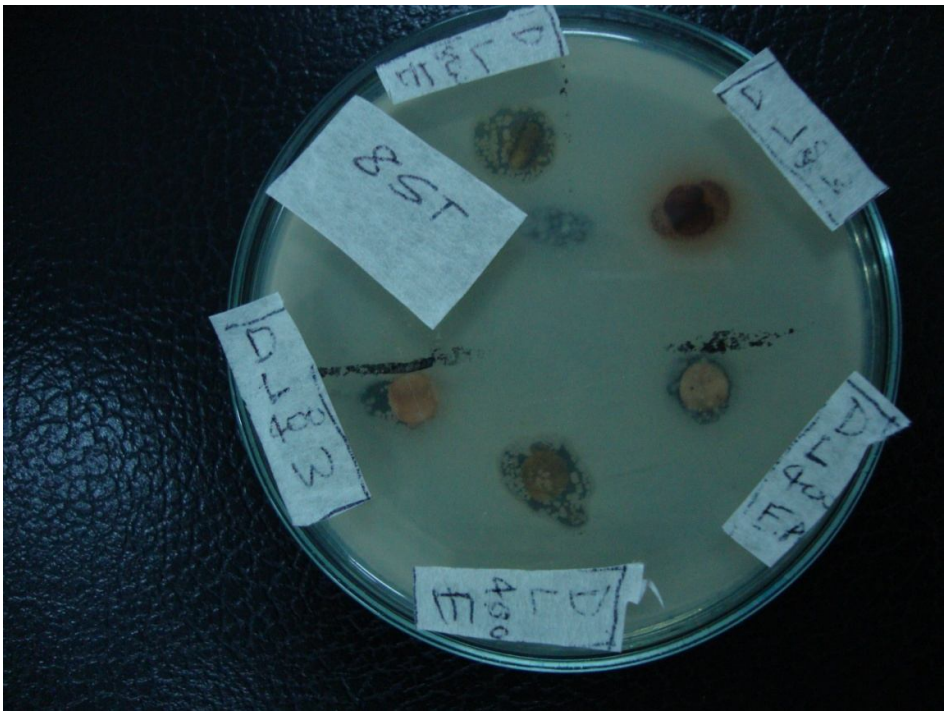
Antimicrobial activity against *Escherichia coli* of *Cissampelos pareira* Berk (400µg & 800µg) extract.



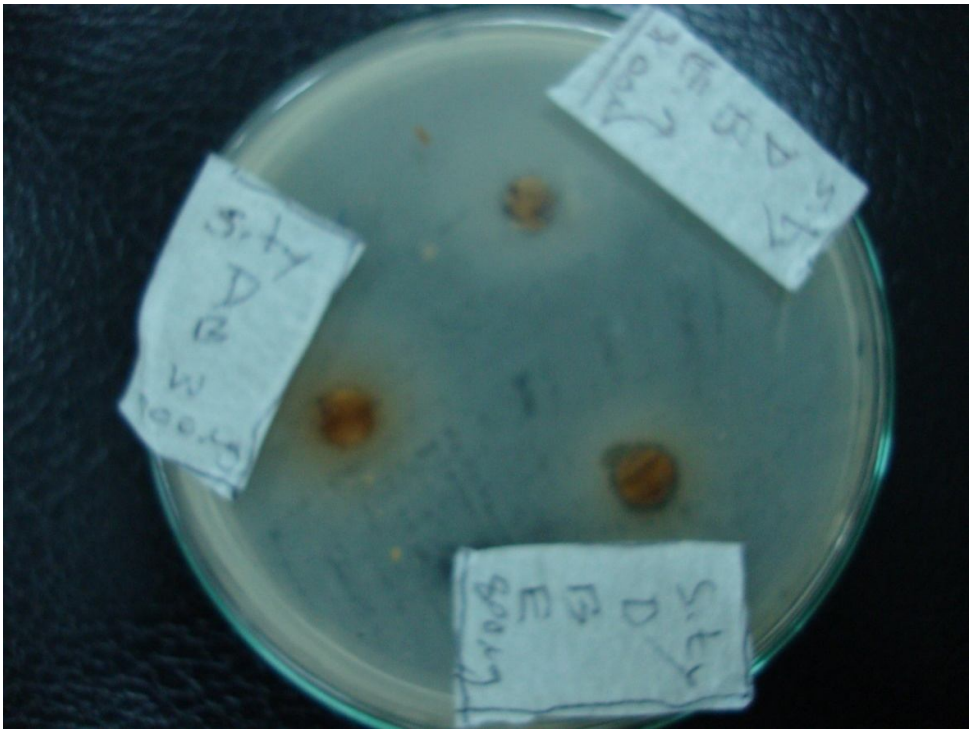
Antimicrobial activity against *Pseudomonas aeruginosa* of *Cissampelos pareira* leaves (400µg & 800µg) extract.



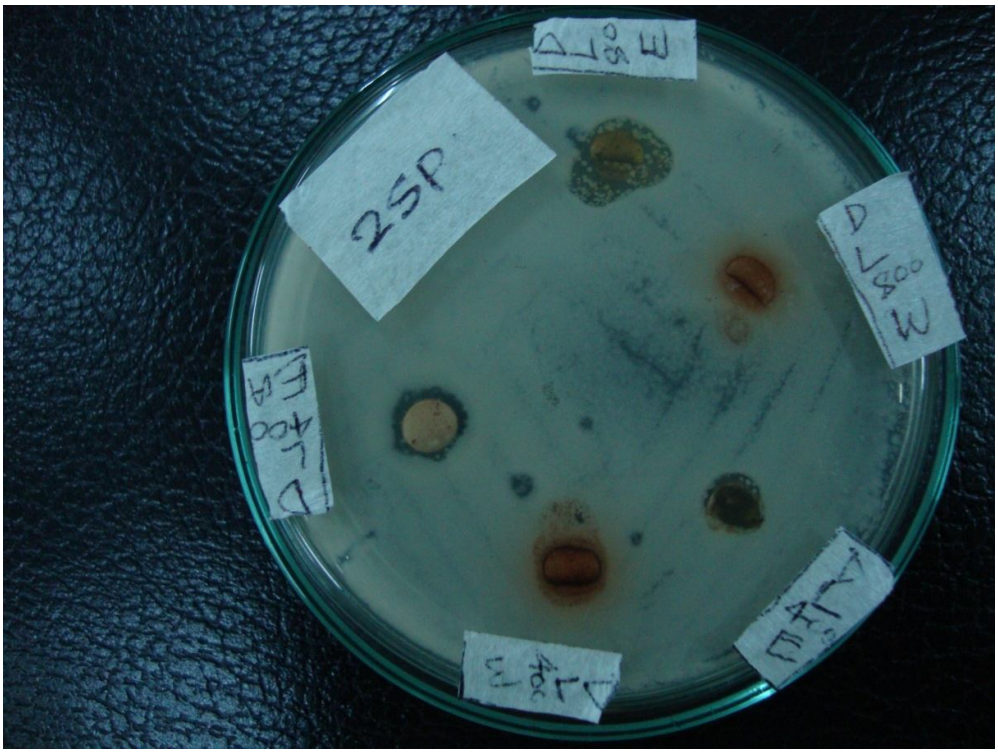
Antimicrobial activity against *Pseudomonas aeruginosa* of *Cissampelos pareira* Bark (400µg & 800µg) extract.



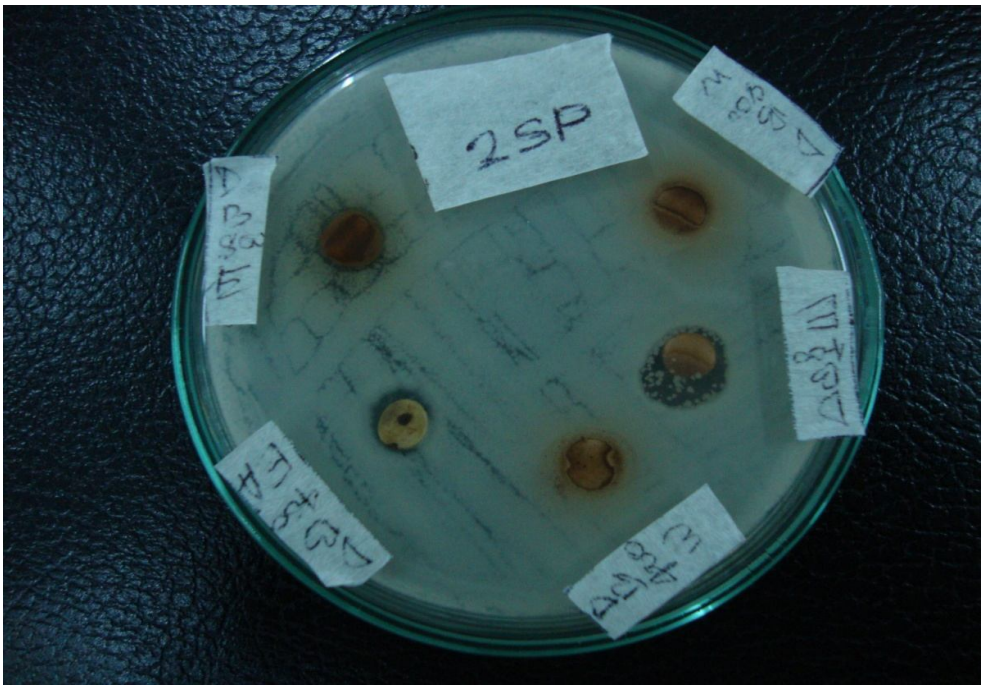
Antimicrobial activity against *Salmonella typhi* of *Cissampelos pareira* leaves (400µg & 800µg) extract.



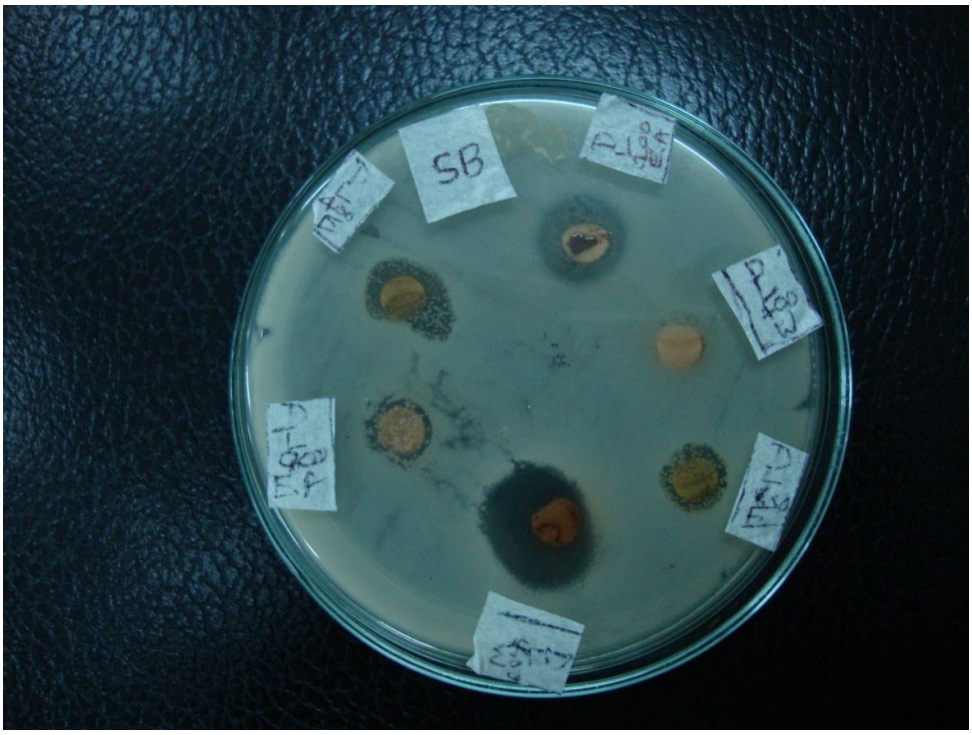
Antimicrobial activity against *Salmonella typhi* of *Cissampelos pareira* Bark (800µg) extract.



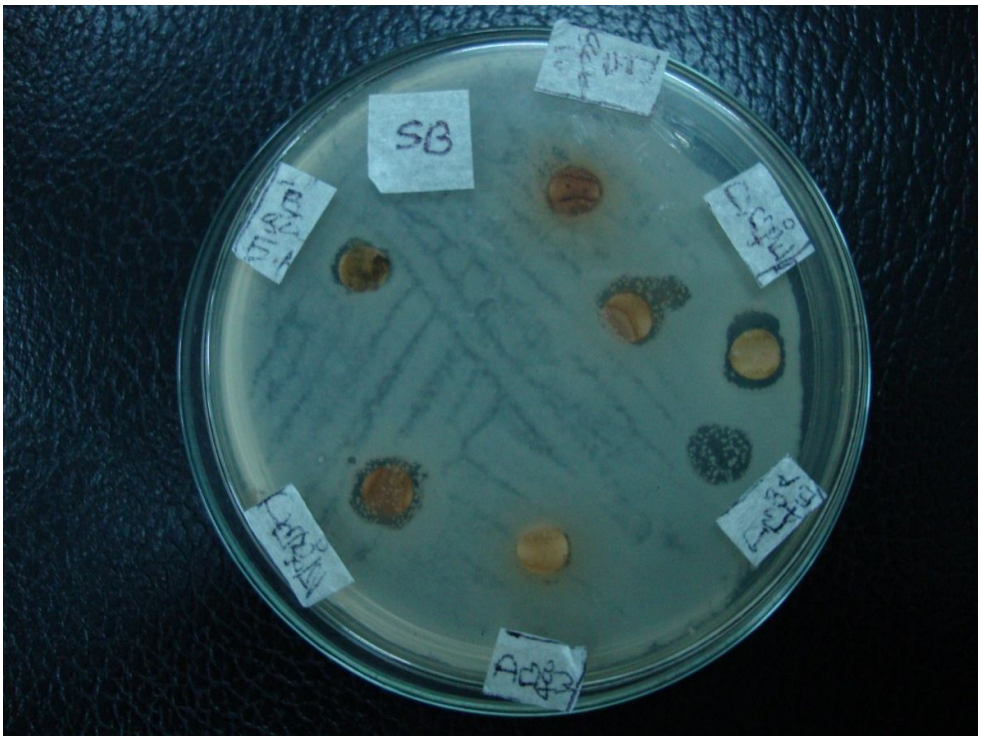
Antimicrobial activity against *Salmonella paratyphi* of *Cissampelos pareira* leaves (400µg & 800µg) extract.



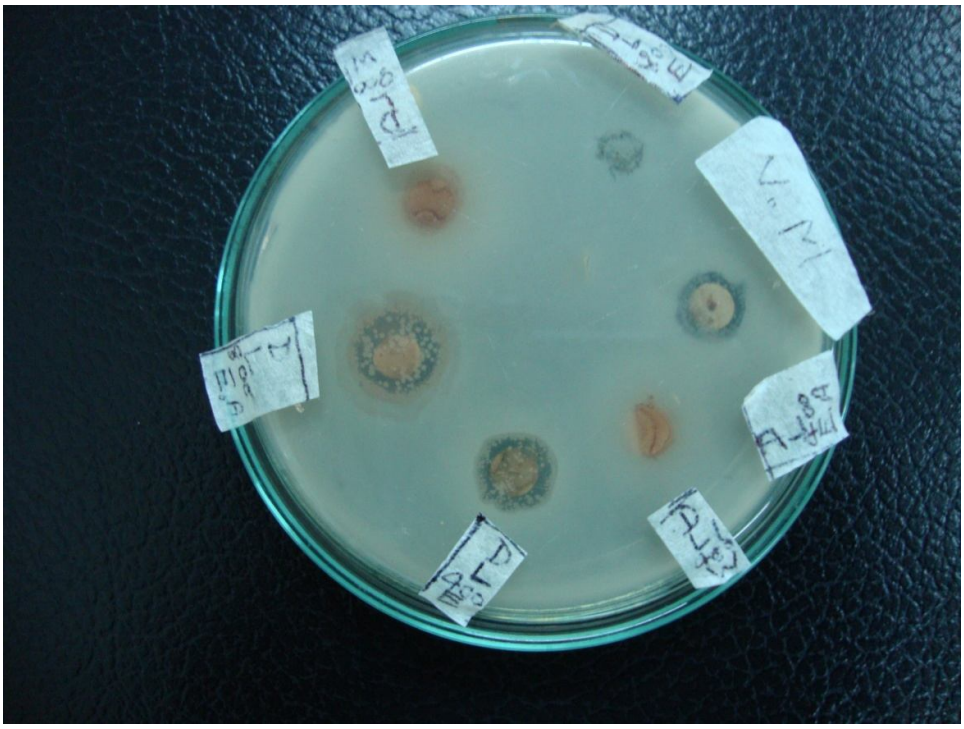
Antimicrobial activity against *Salmonella paratyphi* of *Cissampelos pareira* Bark (400µg & 800µg) extract.



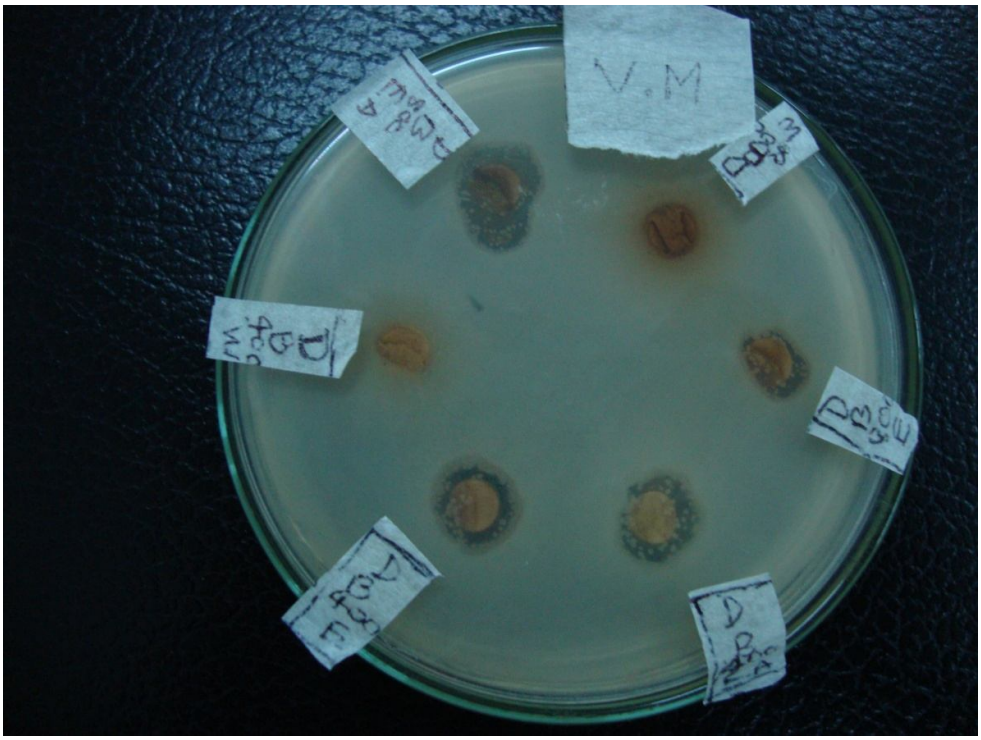
Antimicrobial activity against *Shigella boydii* of *Cissampelos pareira* leaves(400µg & 800µg) extract.



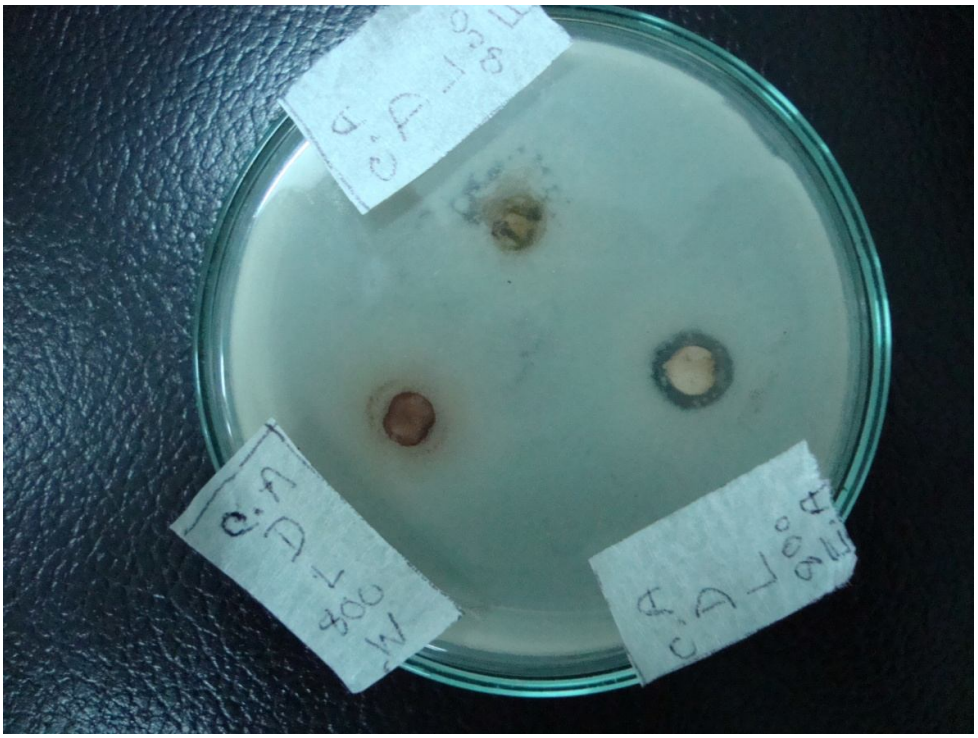
Antimicrobial activity against *Shigella boydii* of *Cissampelos pareira* Bark (400µg & 800µg) extract.



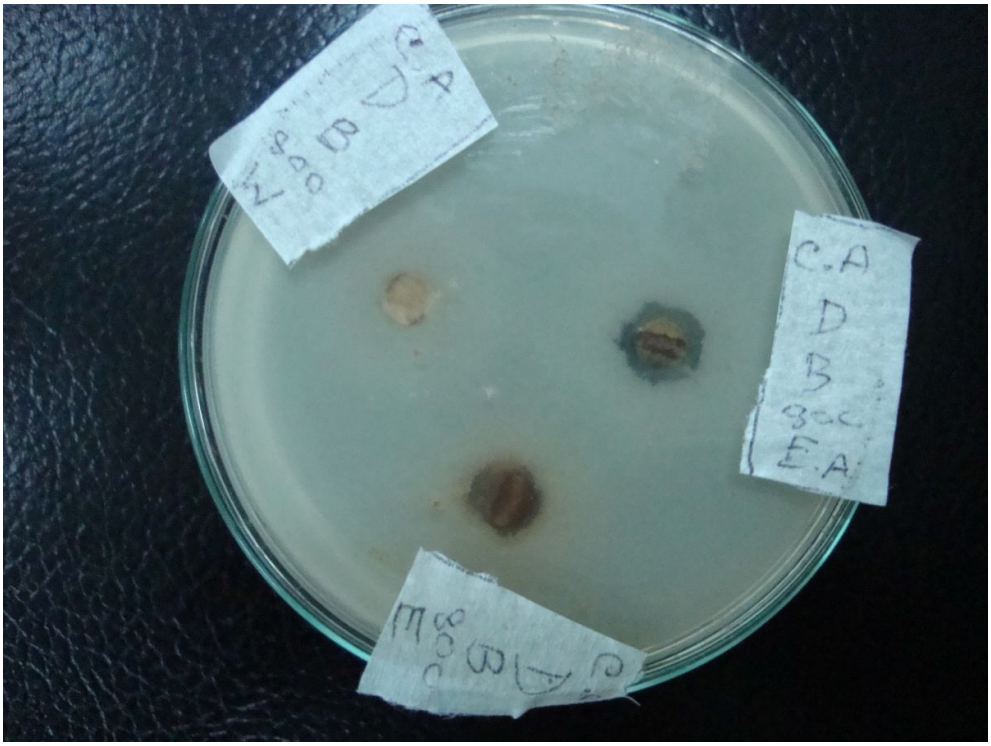
Antimicrobial activity against *Vibrio mimicus* of *Cissampelos pareira* leaves(400µg & 800µg) extract.



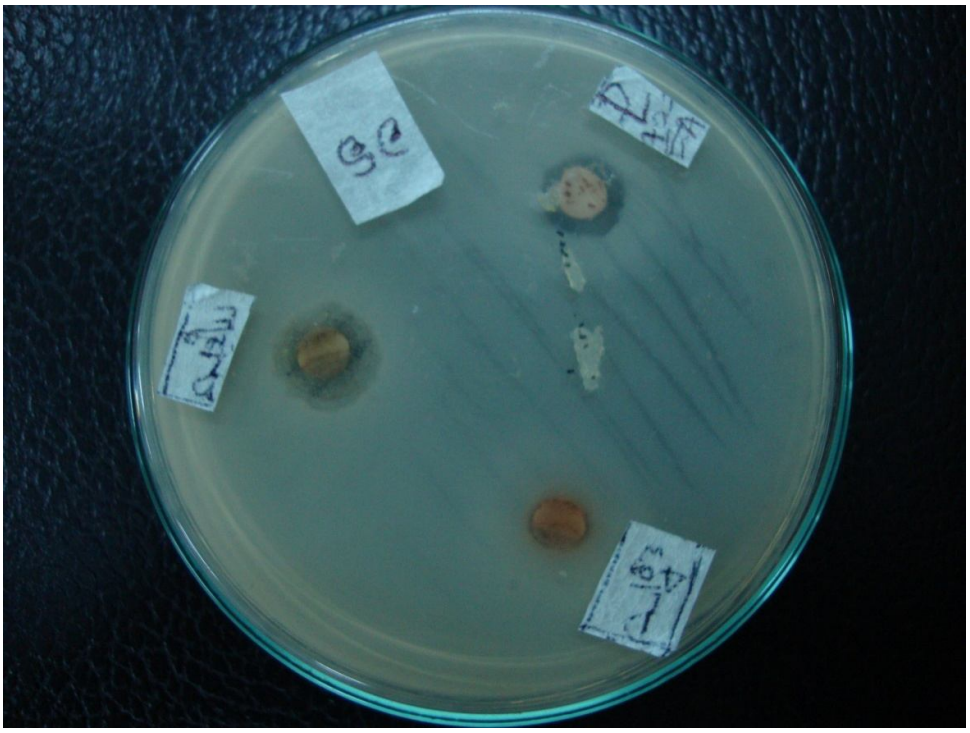
Antimicrobial activity against *Vibrio mimicus* of *Cissampelos pareira* Bark (400µg & 800µg) extract.



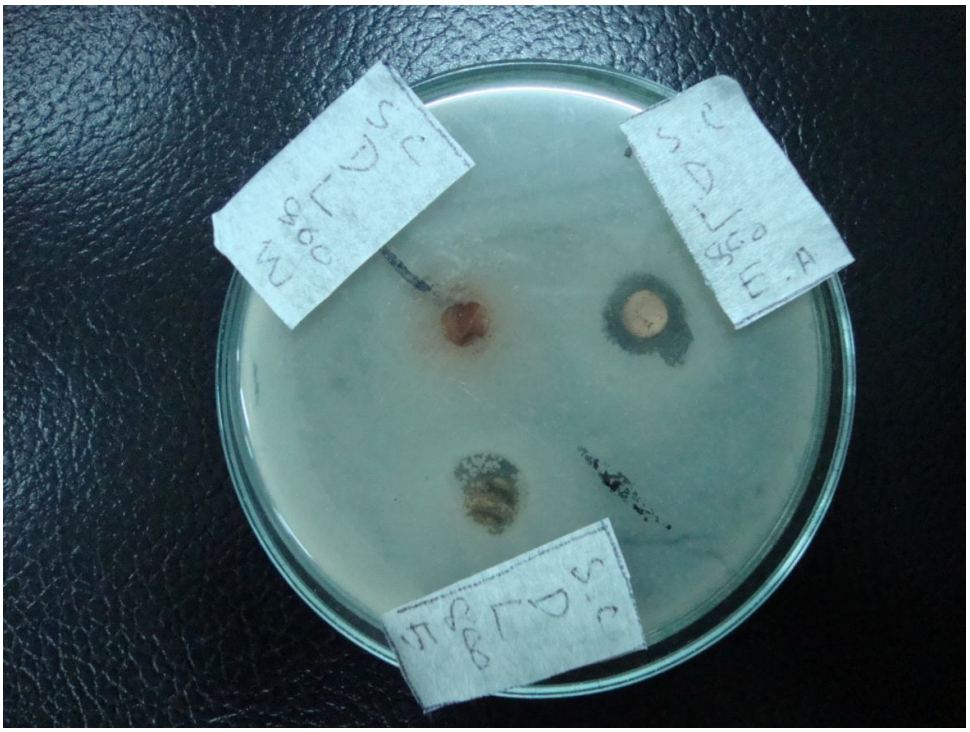
Antimicrobial activity against *Candida albicans* of *Cissampelos pareira* leaves(800µg) extract.



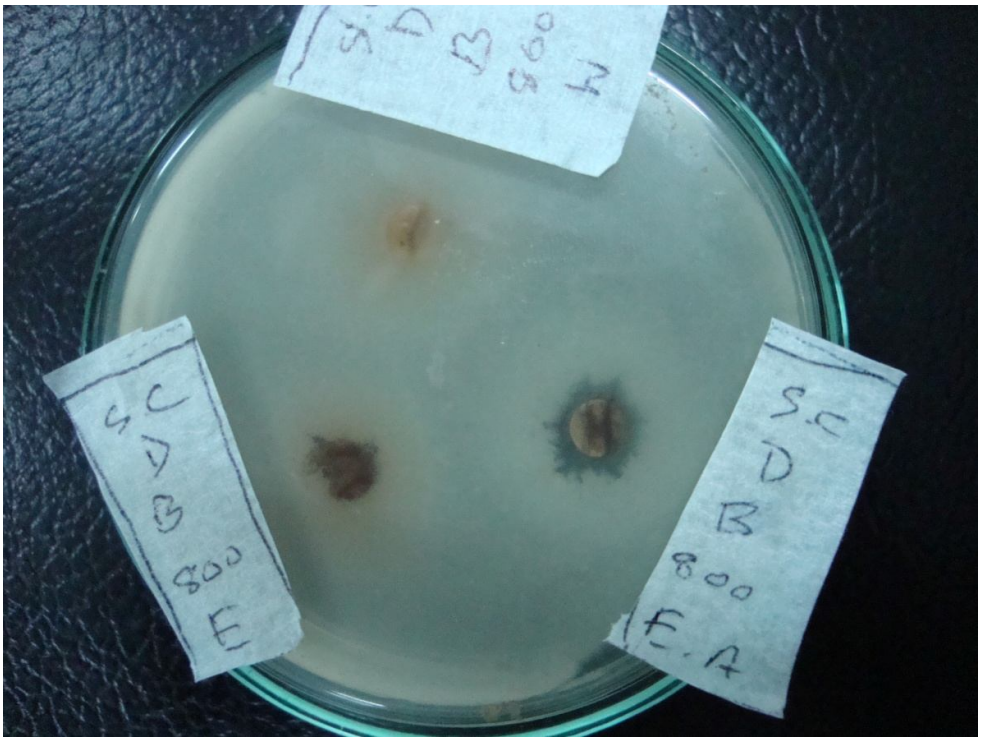
Antimicrobial activity against *Candida albicans* of *Cissampelos pareira* Bark (800µg) extract.



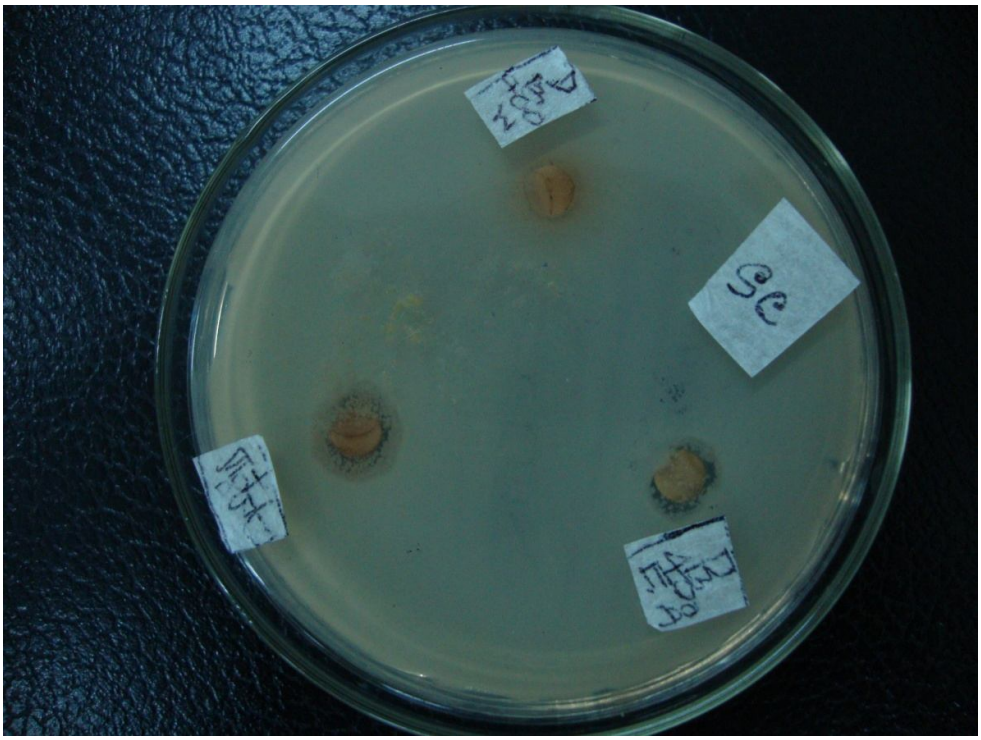
Antimicrobial activity against *Saccharomyces cerevisiae* of *Cissampelos pareira* leaves (400µg) extract.



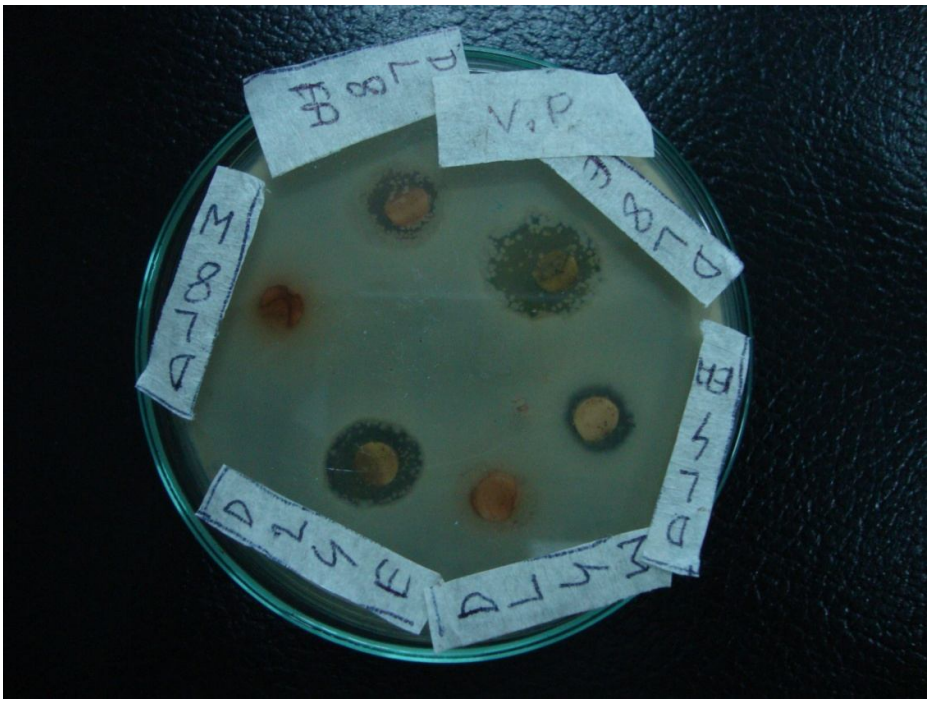
Antimicrobial activity against *Saccharomyces cerevisiae* of *Cissampelos pareira* leaves (800µg) extract.



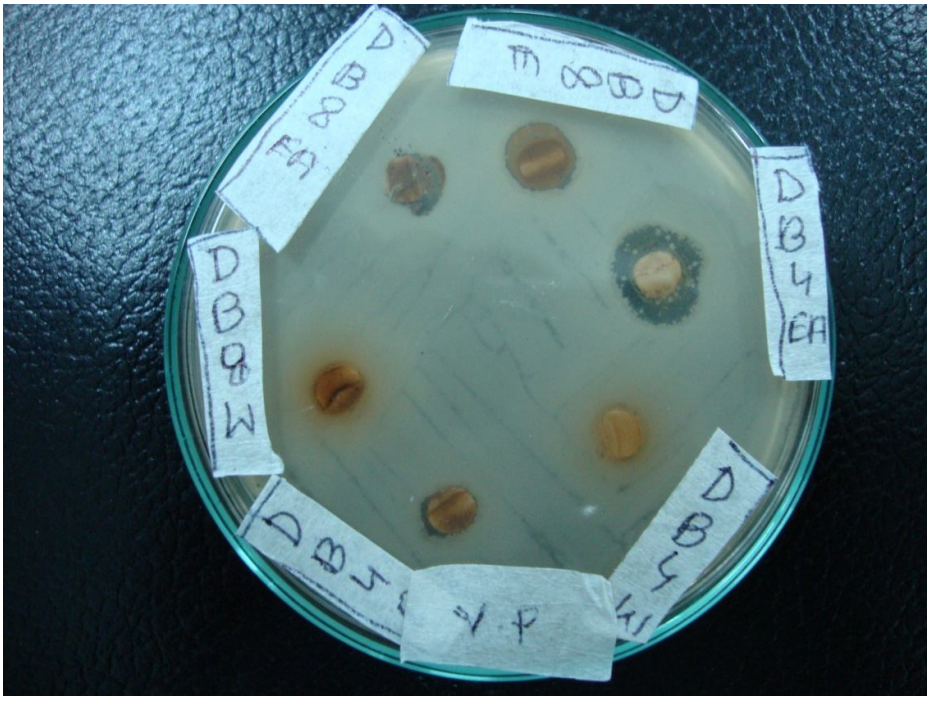
Antimicrobial activity against *Saccharomyces cerevisiae* of *Cissampelos pareira* Bark (800µg) extract.



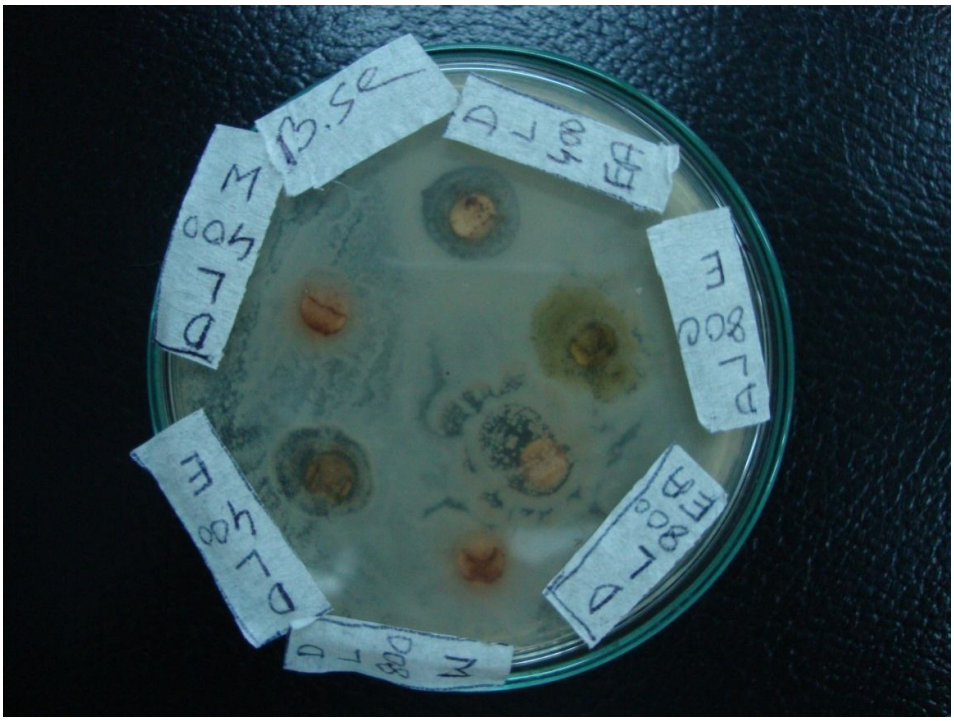
Antimicrobial activity against *Saccharomyces cerevisiae* of *Cissampelos pareira* Bark (400µg) extract.



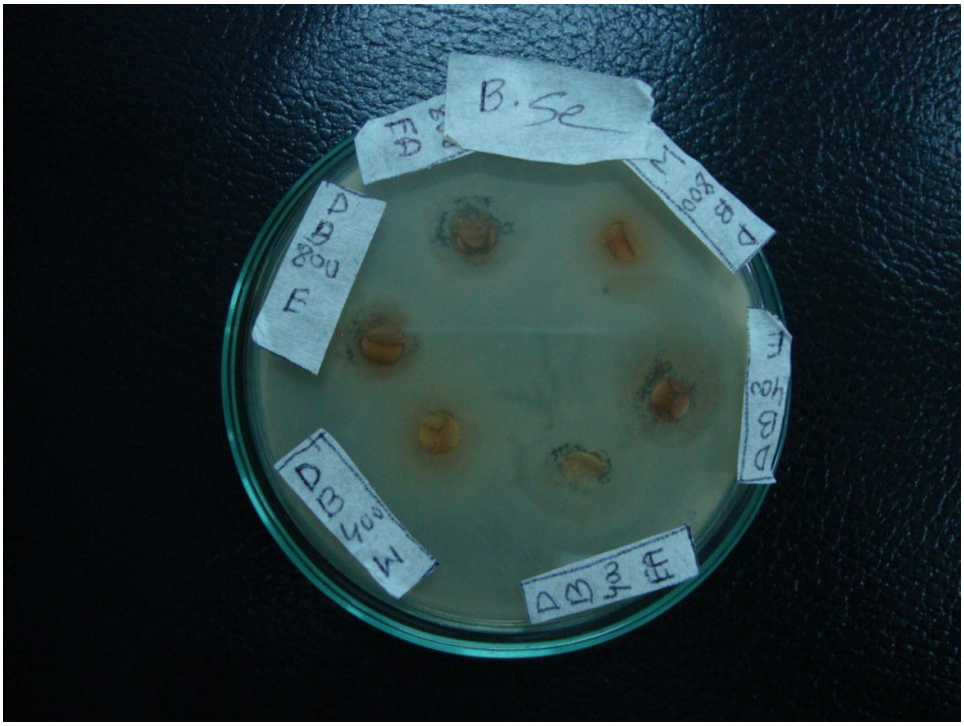
Antimicrobial activity against *Vibrio parahemolyticus* of *Cissampelos pareira* leaves(400µg & 800µg) extract.



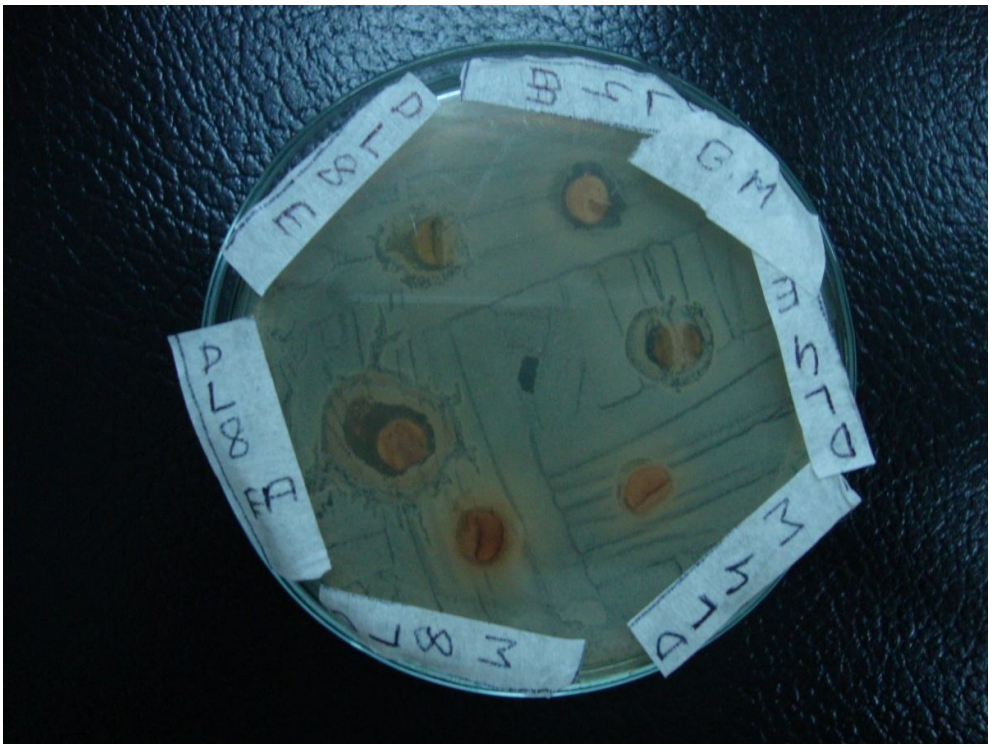
Antimicrobial activity against *Vibrio parahemolyticus* of *Cissampelos pareira* Bark (400µg & 800µg) extract.



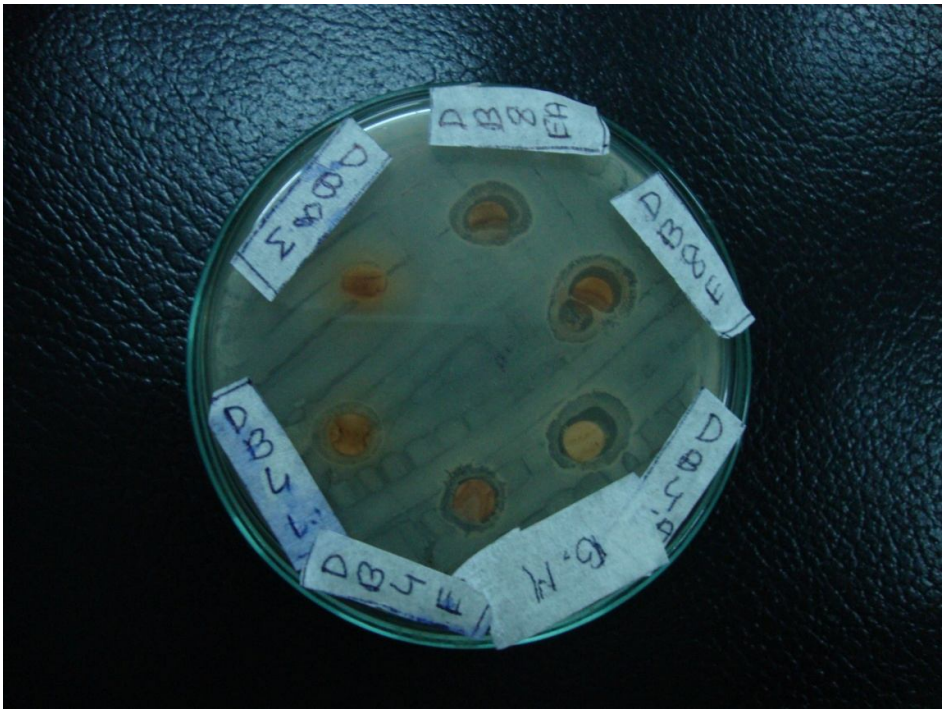
Antimicrobial activity against *Bacillius sereus* of *Cissampelos pareira* leaves (400µg & 800µg) extract.



Antimicrobial activity against *Bacillius sereus* of *Cissampelos pareira* Bark (400µg & 800µg) extract.



Antimicrobial activity against *Bacillius megaterium* of *Cissampelos pareira* leaves (400µg & 800µg) extract.



Antimicrobial activity against *Bacillius megaterium* of *Cissampelos pareira* Bark (400µg & 800µg) extract.

Discussion

The antimicrobial efficacy of *Cissampelos pareira* plant extracts (Bark & leaf) against bacterial and fungal strains was evaluated by the disk diffusion method via determination of the surrounding zones of inhibition (Table 4.1-4.4). The inhibition zone by the plant extracts were ranged from 6mm-18mm. the leaf extract of *Cissampelos pareira* was found to be more or less active against all pathogen tested. Among the 3 solvent extracts (water, ethanol, ethyl acetate), the ethyl acetate extract showed a higher activity than other extracts. This may be due to the solvent extract containing different constituents having antimicrobial activity.

Almost all the gram-positive strains showed sensitivity to ethyl acetate extract, but promising activity was found against *Bacillus cereus* and *Staphylococcus aureus* at a concentration of 800µg/disk. The ethanol extract demonstrated mild sensitivity against the gram-positive strains. The water extract showed very little sensitivity only against *Bacillus cereus* and *Staphylococcus aureus.*

Among the gram-negative organisms, Escherichia coli and *Shigella boydii* exhibited promising sensitivity towards ethyl acetate extract of leaf and bark respectfully. The ethanol extracts again showed mild sensitivity against the gram-negative organisms. The water extract showed activity only against *Shigella boydii*.

Studies on the antifungal activities showed that ethyl acetate extract has shown medium zone of inhibition against the fungi *Candida albicans* and *Saccheromyces cerevisiae*. Water extract had no activity.

The results of present study supports that *Cissampelos pareira* plants extracts containing compounds with antibacterial properties can be used antibacterial agents in new drugs for the therapy with infectious diseases caused by pathogens and further work may be carried out for photochemical evaluation.

Conclusion

Extracts of *Cissampelos pareira* in this study demonstrated a broad-spectrum of activity against both gram-positive and gram-negative bacteria. The broad-spectrum antibacterial activities of the plant extract, possibly due to the identified alkaloids. Bioactive substances from this plant can therefore be employed in the formulation of antimicrobial agents for the treatment of many diseases. Isolation, identification and purification of these phytoconstituents and determination of their respective antimicrobial potencies and toxicological evaluation with the view to formulating novel chemotherapeutic agents should be the future direction for investigation.

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