PHARMACOGNOSTIC, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF *Clausena dentata* Willd.(RUTACEAE)

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CERTIFICATE

This is to certify that the dissertation entitled **"PHARMACOGNOSTIC, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF THE LEAVES OF *Clausena dentata* (Willd.) (RUTACEAE)"** submitted by **Mr. K. KALAIVANAN (Reg. No. 26108663)** in partial fulfilment of the requirement for the award of the degree of **MASTER OF PHARMACY** in **PHARMACOGNOSY** by The Tamil Nadu Dr. M.G.R. Medical University is a bonafied work done by him during the academic year 2011-2012 under my guidance at the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai-625 020.

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Project Supervisor

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CONTENTS

INTRODUCTION

An infection is the colonization of a host organism by parasite species. Infecting parasites seek to use the host's resources to reproduce, often resulting in disease. Colloquially infections are usually considered to be caused by "microorganism" or micro parasites like viruses, bacteria, viroids, though larger organism like "macro parasites" and fungi can also infect. [1]

Hosts normally fight infections themselves via their immune system. Mammalian hosts react to infections with an innate response, often involving inflammations followed by an adaptive response. Pharmaceuticals can also help to fight infections.

Infections may be divided into following

- Bacterial infections
- Fungal infections
- Parasitic infections
- Protozoan infections
- Viral infections
- Worms infestation

Bacterial infections

Pathogenic bacteria are bacteria which causes bacterial infections. Although the vast majority of bacteria are harmless or beneficial, quite a few bacteria are pathogenic. The highly pathogenic bacteria are classified in to two types "gram positive bacteria" and "gram negative bacteria". The pathogenic bacteria contribute to globally important diseases such as pneumonia, which are caused by bacteria such as *Streptococcus* and

Pseudomonas and food borne illness such as tetanus, typhoid fever, diphtheria, syphilis, and leprosy. [2]

The following bacteria are highly pathogenic in clinical characteristics

Gram positive bacteria	Gram negative bacteria
Staphylococcus aureus *Basillus substilis* *Streptococcus viridians,S. pyogens*	*Esherichia coli* *Klebsiella* *Proteus albus* *Salmonella typhi* *Proteus mirabilis* *Pseudomonas aeruginosa*

Staphylococcus aureus can cause a range of illness ranging from minor skin infections, such as pimples, boils, cellulitis, folliculitis, scaled skin syndrome to life threatening diseases such as pneumonia, meningitis, endocarditis, osteomyelitis, toxic shock syndrome (TSS), bacteremia and sepsis. It affects the skin; soft tissue, respiratory tract, bones, joints etc. It is one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections.

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 and thrives not only in normal atmospheres, but also in hypoxic atmospheres, and hence has colonized many natural and artificial environments. The organism generally infects damaged tissues or those with reduced immunity. The symptoms of infections are generalized inflammation and sepsis. If such colonization occurs in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal. Because it thrives on most surfaces, this bacterium is also found on and in medical equipment, including catheters, causing cross-

infections in hospitals and clinics. It is implicated in hot-tub rash. It is also able to decompose hydrocarbons and has been used to break down tar balls and oil from oil spills. [3]

Fungal infections

Pathogenic fungi are those that cause disease in humans or other organisms. The study of pathogenic fungi is referred to as medical mycology. Although fungi are eukaryotic organisms many pathogenic fungi are also microorganisms. A **mycosis** is a fungal infection of animals, including humans. Mycoses are common, and a variety of environmental and physiological conditions can contribute to the development of fungal diseases. Inhalation of fungal spores or localized colonization of the skin may initiate persistent infections; therefore, mycoses often start in the lungs or on the skin.

People are at risk of fungal infections when they are taking strong antibiotics for a long period of time because antibiotics kill not only damaging bacteria, but healthy bacteria as well. This alters the balance of microorganisms in the mouth, vagina, intestines and other places in the body, and results in an overgrowth of fungus. Individuals with weakened immune systems are also at risk of developing fungal infections. This is the case of people with HIV/AIDS, people under steroid treatments, and people taking chemotherapy. People with diabetes also tend to develop fungal infections. Very young and very old people, also, are groups at risk.

Mycoses are classified according to the tissue levels initially colonized

- Superficial mycoses
- Cutaneous mycoses
- Subcutaneous mycoses

- Systemic mycoses due to primary pathogens
- Systemic mycoses due to opportunistic pathogens

Candida species are important human pathogens that are best known for causing opportunist infections in immune compromised hosts (e.g. transplant patients, AIDS sufferers and cancer patients).

The most common pathogenic species are *Aspergillus fumigatus* and *Aspergillus flavus*. *Aspergillus flavus* produces aflatoxin which is both a toxin and a carcinogen and which can potentially contaminate foods such as nuts etc. *Aspergillus fumigatus* and *Aspergillus clavatus* can cause allergic diseases. Some *Aspergillus* species cause disease on the grain crops, especially maize, and syntheise mycotoxins including aflatoxin. Aspergillosis is the group of diseases caused by *Aspergillus*. The symptoms include fever, cough, chest pain or breathlessness. Usually, only patients with weakened immune systems or with other lung conditions are susceptible.[4]

Cryptococcus neoformans is another major human and animal pathogen. *Cryptococcus laurentii* and *Cryptococcus albidus* have been known to occasionally cause moderate-to-severe disease in human patients with compromised immunity.

Viral Infections

Viruses are tiny organisms that are made up of the genetic material known as DNA or RNA, which the virus uses to replicate. It invades and attaches itself to a living cell in order to survive and then multiply and produce more virus particles. Viruses can be transmitted in numerous ways, such as through contact with an infected person,

swallowing, inhalation, or unsafe sex. Poor hygiene and eating habits increases the risk of contracting a viral infection.[5]

Types of Viral Infections

Viruses affect any part of the body and cause infections such as the common cold, flu, gastroenteritis, chicken pox or herpes. The most common type of viral infections involves the respiratory tract.

The common cold is a frequently occurring viral infection and symptoms are sneezing, stuffy nose, sore throat and coughing. A cold can last from two days to two weeks though it is a minor infection of the nose and throat. They are highly contagious and are spread by fluids from sneezing or coughing, which contain the infection.

Influenza, also known as the "flu", is a respiratory infection that is caused by viruses. The flu differs in several ways from the common cold. Symptoms of the flu include body chills, fever, headache, muscle ache and sore throat. Unlike many other viral respiratory infections, the flu can cause severe illness and life-threatening complications in many people. The flu is contracted in the same airborne manner as the common cold. The viruses are easily transmitted especially in highly populated areas.

Diseases such as herpes are caused by the herpes simplex virus (HSV). This infection can infect the mouth, genitals and anus. Oral herpes causes sores around the mouth and face, while genital herpes affects the genitals, buttocks and anus. Genital herpes is known as a sexually transmitted disease (STD) and it is transmitted through sexual contact through the mouth and genitals. This virus can be spread even when sores are not present. Like chickenpox, this virus will remain in the body forever; however, a person with herpes may continue to deal with reoccurrences or "outbreaks" for life. The

symptoms include fever, muscle aches, coughing, sneezing, runny nose, headache, chills, diarrhea, vomiting, rashes and weakness. More severe symptoms include personality changes, neck stiffness, dehydration, seizures, paralysis of the limbs, confusion, back pain, loss of sensation, impaired bladder and bowel function, sleepiness that can progress into a coma or death.

Protozoan infections

Protozoan infections are parasitic diseases organisms formerly classified in the Kingdom Protozoa. They include organisms classified in Amoebozoa, Excavata, and Chromalveolata. Examples include *Entamoeba histolytica*, *Plasmodium* (some of which cause malaria), and *Giardia lamblia*. *Trypanosoma brucei*, transmitted by thetsetse fly and the cause of African sleeping sickness, is another example.

The species traditionally collectively termed "protozoa" are not closely related to each other, and have only superficial similarities (eukaryotic, unicellular, motile, though with exceptions.) The terms "protozoa" (and protist) are usually discouraged in the modern biosciences. However, this terminology is still encountered in medicine. This is partially because of the conservative character of medical classification, and partially due to the necessity of making identifications of organisms based upon appearances and not upon DNA.

Malaria is a mosquito-borne infectious disease of humans and other animals caused by eukaryotic protists of the genus *Plasmodium*. The disease results from the multiplication of *Plasmodium* parasites within red blood cells, causing symptoms that typically include fever and headache, in severe cases progressing to coma or death. It is

widespread in tropical and subtropical regions. Five species of malarial parasites can infect and be transmitted by humans. Severe disease is largely caused by *Plasmodium falciparum* while the disease caused by *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* is generally a milder disease that is rarely fatal. *Plasmodium knowlesi* is a zoonosis that causes malaria in macaques but can also infect humans. The symptoms of malaria include fever, shivering, arthralgia (joint pain), vomiting, anemia (caused by hemolysis), jaundice, hemoglobinuria, retinal damage and convulsions.[6]

Malaria transmission can be reduced by preventing mosquito bites by distribution of mosquito nets and insect repellants or by mozquiot control measures such as spraying insecticides and draining standing water.

Tropical diseases

Tropical diseases are infectious diseases that either occurs uniquely in tropical and subtropical regions (which is rare) or, more commonly, are either more widespread in the tropics or more difficult to prevent or control. Since the advent of air travel, people more frequently visit these regions and contract many of these diseases, most notably malaria and hepatitis.

In 1975 the Special Programme for Research and Training in Tropical Diseases (TDR) was established to focus on neglected infectious diseases which disproportionately affect poor and marginalized populations in developing regions of Africa, Asia, Central America and South America. It was established at the World Health Organization, which is the executing agency, and is co-sponsored by the United Nations Children's

Fund, United Nations Development Programme, the World Bank and the World Health Organization to study and carry out research on tropical and subtropical diseases.[7]

In the present study, an attempt is made to find out whether the volatile oil and ethanolic extract of the leaves of the plant *Clausena dentata* will be useful for the treatment of some of the infections commonly occurring in India.

AIM AND SCOPE

Plants have been used for healing purposes and form the origin of much of the modern medicine. Many medicinally important drugs originate from plant sources: a century ago, most of the few effective drugs were plant-based. The production of drugs from plants continues with many pharmaceutical companies engaged in large-scale pharmacologic screening of herbs.

The present study has been planned to carry out the pharmacognostic, phytochemical and pharmacological evaluation of the extract and volatile oil of the leaves *C. dentata.*

Pharmacognostic evaluation

1. Plant description based upon its macro – morphological and cyto-morphological evaluation.
2. Microscopical evaluation of the leaves and stem
3. Development of standardization parameters as per the WHO 1996 guidelines as a preliminary step for the inclusion of this plant to Ayurvedic Pharmacopoeia.

Phytochemical evaluation

1. Preliminary phytochemical screening on its powder form and crude extracts.
2. Estimation of some secondary metabolities present in the plant.
3. Standardization parameters including GC-MS analysis of volatile oil

4. TLC and HPTLC analysis of ethanolic extract as a preliminary step for authentication of *Clausena dentata.*

Pharmacological evaluation

1. To study *in-vitro* antioxidant activity of the ethanolic extract by various methods.
2. To study *in-vitro* antimicrobial and antifungal screening for both ethanolic extract and volatile oil of *C. dentata*.
3. To study *in-vitro* larvicidal screening for both the ethanolic extract and volatile oil of *C. dentata*.

REVIEW OF LITERATURE

The chapter provides retrieval of papers both from primary and secondary sources. The review of literature encompasses information on pharmacognosy, biological studies and phytochemistry of the various species of *Clausena*. It was found that very little information was available on *Clausena dentata* and hence the rationale for the present study.

Clausena dentata (Willd) Romer

Govindachari TR *et al.*, (1968) have isolated the coumarins imperatorin and two new coumarins dentain, nordentain from the root bark of *Clausena dentata* [8].

Subba Rao GSR *et al.*, (1981) have isolated 3-(1,1-dimethyl allyl) xanthyletin from the hexane extract of the root bark of *Clausena willdenovii* and its structure was elucidated [9].

Kamaraj RJ and Vasantha (2003) have isolated the 3-(1, 1 dimethyl allyl) xantheletin from *Clausena dentata* [10].

Rajesh SV *et al., (*2009) have demonstrated the hepatoprotective activity of the ethanol extract (250mg/kg po) of *Clausena dentata* against acetaminophen-induced hepatotoxicity in rats. The activity of the extract was comparable to the standard drug silymarin (50mg/kg po) [11].

Sivakumar M *et al.*, (2009) have evaluated the anti-inflammatory and analgesic effect of chloroform, hexane, and methanol extract of the root bark of *Clausena dentata.* The analgesic activity was studied by tail-flick method using paracetamol 100mg/kg oral

dose as standard on male Wistar mice. The anti-inflammatory activity was studied by carrageenan induced paw edema using diclofenac sodium at a oral dose of 120mg/kg as standard on male Wistar rats. The results indicated that the hexane extract of *Clausena dentata* exhibited more significant activity at a dose of 150 mg/kg than methanol and chloroform extracts in the treatment of pain and inflammation [12].

Malarvannan S *et al.*, (2009) have reported that the petroleum ether, chloroform, hexane, acetone and water extracts of the leaves of *Cipadessa bacifera* (Meliaceae), *Clausena dentata* (Rutaceae) and *Dodonaea angustifolia* (Sapindaceae) found in Western Ghats have ovicidal activity. Among the four plants *Clausena dentata* reduced the egg hatchability and proved to be highly ovicidal compared to others [13].

Rajkumar S and Jebanesan A., (2010) have isolated the volatile oil from C*lausena dentata* by steam distillation and the oil was tested for prevention of dengue fever through plant based mosquito repellent against *Aedes aegypti* I, (Dipetera: culicidae) mosquito. The result indicated that the use of plant based repellent for the control of dengue fever would replace the currently used synthetic repellent which causes many side effects [14].

Rajkumar S and Jebanesan A., (2010) have isolated the essential oil from the leaves of *Clausena dentata* (Willd). The larvicidal activity of the oil was tested against early fourth instar *Aedes aegypti* larvae. The GC-MS analysis of essential oil revealed the presence of fourteen compounds of which the major compounds are sabinene (21.27%), bioflorotriene (19.61%), borneal (18.34%), and β-bisabolol. Four major compounds

sabinene, biofloratriene, borneal, and β-bisabolol are potent sources of natural larvicides has also been reported. [15]

Krishnappa K *et al., (*2010) have isolated the essential oil from the leaves of *Clausena dentata* and its chemical composition was analyzed by GC-MS. The chemical constitution of 12 compounds was identified in the oil representing 99.17% and the major chemical composition of oil is sabinene (28.57%), borneol (14.62%), δ-cadinol (12.49%), β-bisabolol (15.56%) and biofloratriene (18.54%). The percentage composition of remaining seven compounds ranged from 0.59-2.38%. The oil exhibited significant larvicidal and ovicidal activity against armyworm, *Spodoptera litura* (Fab). Lebidoptera:(Noctuidae) [16].

OTHER SPECIES

Clausena heptaphylla

Chakraborty DP *et al.,* (1978) have reported the isolation of 2-methyl anthraquinone from the stem bark of *Clausena heptaphylla* and its biogenesis has been suggested [17].

Pathaik GK and Dhawan BN (1983) have evaluated the spasmolytic activity of crude ethanolic extracts from the aerial parts of *Clausena heptaphylla.* The extract showed maximum activity when compared with that of papaverin[18].

Bhattcharyya P *et al.,* (1985) have isolated a new carbazole alkaloid 'heptzjolocine' from the roots of *Clausena heptaphylla* and its structure has been identified by spectral and chemical methods [19].

Lockwood GB *et al.,* (1985) have isolated the essential oil from the leaves of *Clausena heptaphylla*. The oil contains anethole, anisaldehde, estragole, anisayl ketone and alpha-elemene as reported [20].

Nath SC and Bordoloi (1992) have reported that methyl chavicol (75.6%) as the major constituent followed by anethole (21.7%) in the leaf essential oil of *Clausena heptaphylla* [21].

Chakraborty A *et al.,* (1995) have isolated a new carbazole alkaloid designated as clausenal from the root of *Clausena heptaphylla.* The alkaloid was found to be active against both gram-positive and gram-negative bacteria and fungi as reported. [22]

Nath SC *et al.,* (1996) have reported the essential oil composition of the leaf and fruit oil of *Clausena heptaphylla*. The oil was analyzed by GC-MS and eight components were identified in both the leaf and fruit oil [23].

Sohrab MH *et al.,* (1997) have reported the isolation of coumarins, clausmarin-A, from the leaves of *Clausena heptaphlla,* and their structure was determined by the spectral methods [24].

Sohrab MH *et al.,* (1999) have isolated the two new coumarins Lunamarins A and B from the methanol extracts of the leaves of *Clausena heptaphylla* and their structure was analyzed by spectral methods [25].

Thuy *et al.,* (1999) and Yusuf *et al.,* (1994) have reported the plants of the genus ***"Clausena"*** are known to be useful in paralysis, ulcerated nose, colic, stomach trouble, fever, and headache , muscular pain and malarial fever [25].

Sohrab MH *et al.*, (2001) have demonstrated the antibacterial activity of different crude extracts from the leaves of *Clausena heptaphylla* and also isolated three coumarins from cold methanol extract [26].

Sohrab MH *et al.*, (2002) have reported the isolation of a new coumarin-Lunamarin C from the petroleum ether extract of the leaves of *Clausena heptaphylla* and its structure was determined by spectral analysis [27].

Chowdhury JU *et al.*, (2008) have reported the composition of the leaf oil of *Clausena heptaphlla* and *Clausena suffruticosa*. The GC-MS analysis of the oils revealed the presence of thirty six components in *Clausena heptaphylla* oil. The main components were 3-carene (64%), a-phellandrene (5.74%) and 1,4-terpeniol (5.15%). Twenty two terpene components were identified in *Clausena suffruticosa* oil. The main constituents being estragole (58.23%), anethole (33.20), linalol (3.38%) and β-ocimene (1.40%) [28].

Ahmad A *et al.*, (2008) have isolated the essential oil from the leaves of *Clausena heptaphlla*. The GC-MS analysis of oil showed the presence of 16 components. 97.8% of the total oil was identified; of which (E) anethole (92.2%), (Z) anethole (1.2%) and methyl chavicol (1.6%) are the major components. Eight minor components identified were unreported so far [29].

Rokeya Begum *et al.*, (2011) have isolated limonoid clausenolide-1-methyl ether from the stem bark of *Clausena heptaphylla* and its structure was analyzed by spectral methods [30].

Clausena anisata

Emerole G *et al.,* (1982) have done the pharmacological studies which include assay of glucose-6-phosphate, ethyl morphine-n-deamylase, protein, liver DNA, aniline hydroxylase, reduced glutathione, and blood clotting time, toxicity studies, from the isolated furanocoumarins of *Clausena anisata* [31].

Adesina SK and Ette EI (1983) have isolated heliettin, imperatorin from the methanolic extract of stem and root bark of *Clausena anisata* and its anticonvulsant property has also been discussed [32].

Makanju OAA *et al.,* (1984) have reported that the aqueous extract of *Clausena anisata* root bark depresses the central nervous system in mice and also exhibited mild to moderate anti-convulsant action [33].

Lakshmi V *et al.,* (1985) have isolated the mono terpene furanocoumarin from the aerial parts of *Clausena anisata* and its structure was analyzed by spectral methods [34].

Ekundayo O *et al.,* (1987) have isolated volatile oil from the leaves of *Clausena anisata* and the essential oil was analyzed by GC-MS [35].

Ngadjui BT *et al.,* (1990) have isolated a carbazole alkaloid from the root and stem bark of *Clausena anisata* and its structure was analyzed by spectral methods [36].

Ngadjui B.T *et al.,* (1991) have isolated two new geranyl coumarins from the leaves of *Clausena anisata* and its structure was analyzed by spectral methods [37].

Gundidza M *et al.*, (1995) have isolated the essential oil from the leaves of *Clausena anisata* and analyzed by GC-MS. The oil showed antibacterial activity against *Beneckea natriegens, Flavobacterium suaveolens, Enterococcus faecalis, Bacillus subtilis, Serratia marcescens, Alcaligenes faecalis and Leuconostoc cremoris* and exhibit antifungal activity against *Geotricum candidum, Aspergrillus parasiticus, Candida albicans, Penicillium citrinum and Alternaria alternate* [38].

Chakraborty A *et al.*, (1996) have isolated the two new carbazole alkaloids clausenol and clausenine from an alcoholic extract of the stem bark of *Clausena anisata* and its structure was analyzed by physical and chemical methods. Clausenol was found to be active against gram-positive and gram-negative bacteria and fungi as reported [39].

Addae Mensah I *et al.*, (1997) have reported that the essential oil of *Clausena anisata* contains (E)-anethole as one of the major constituents (85-100%) [40].

Ayedown M.A *et al.*, (1997) have reported the leaf and fruit essential oil of *Clausena anisata* contains methyl chavicol, E-anethole, limonene, and myrcene as main components [41].

Katsuno C *et al.*, (1998) have isolated three novel carbazole alkaloids named clausamaine A, B, C from *Clausena anisata* and its structure was analyzed by spectroscopic methods [42].

Ito C *et al.*, (2001) have isolated four new carbazole alkaloids from the acetone extract of the dried bark of *Clausena anisata* and have been reported to be an anti-tumour promoting agent [43].

John A O Ojewole (2002) have reported the hypoglycemic effect of *Clausena anisata* (Willd) root methanolic extract in normal (normoglycaemic) and in streptozotocin-treated diabetic rats [44].

Hamza OZM *et al.,* (2007) have reported the antifungal activity of methonolic extract of *Clausena anisata* [45].

Clausena excavata

Wu TS and Furukawa H (1983) have isolated a new compound-clausenidinaric acid from the root bark of *Clausena excavata* and its structure was elucidated by spectral methods. These compounds showed antibacterial activity [46].

Khan NU and Naqvi SWI (1984) have isolated a new furocoumarin from root bark of *Clausena excavata* and its structure was analyzed by spectral methods [47].

Wu C.C *et al., (*1994) have reported the antiplatelet effect of clausine-D from *Clausena excavata* and is due to inhibition of the formation of thromboxane A2 [48].

Wu TS *et al.,* (1997) have isolated five new carbazole from the stem bark of *Clausena excavata* and its structure was determined by spectral methods. These components showed significant inhibition of platelet aggregation and caused vasoconstriction as reported [49].

Ito C *et al.,* (1997) have reported the isolation of seven new carbazole alkaloids namely clauszolin A ,B, C, D, E, F, G and a new coumarin named 5-geranyl-7-hydroxy coumarin from the stem bark of *Clausena excavata* and their structure was elucidated by spectral methods [50].

Wu T.S *et al.,* (1997) have reported the isolation of binary carbazole alkaloids from the stem and root bark of *Clausena excavata* and its structure was determined by spectral methods [51].

Haung P *et al.,* (1997) have isolated two new pyranocoumarins and two flavanoid from the acetone extract of the root bark of *Clausena excavata*. Their structure was analyzed by spectral methods and it has been used in the treatment of snake-bite and detoxification agent [52].

Ito C *et al.,* (1997) have isolated a seven new carbazole alakaloids, named clauszolin-A,B,C,D,E,F and G a new coumarin named 5-gernyloxy-7-hydroxycoumarin from the stem and root bark and leaves of *Clausena excavata* and their structure were elucidated by spectral methods [53].

Wu T.S *et al.,* (1997) have isolated five carbazole alkaloids, clausines B,E,H,I and K, as well as 22 known compounds from the stem bark of *Clausena excavata* and their structure was analyzed by spectral methods [54].

Ito C *et al.,* (2000) have isolated of 10 new furanone-coumarins named clauslactones A (1), B (2), C (3), D (4), E (5), F (6), G (7), H (8), I (9), and J (10), together with a known carbazole, clauszoline M, and a coumarin, umbelliferone from *Clausena excavata*. These furanone-coumarins were found to exhibit inhibitory activity against 12-O-tetradecanoylphorbol-13-acetate-induced Epstein-Barr virus early antigen activation in Raji cells [55].

Arunrat Sunthitikawinsakul *et al.,* (2003) have isolated a limonoid, clausenolide-1-ethyl ether (1) and two coumarins, dentatin (2) and nor-dentatin (3) from

Clausena excavata. Limonoid 1 was obtained from the crude ethanol extract of the rhizomes and the roots but had not previously been isolated from *C. excavata* and exhibited HIV-1 inhibitory activity. Coumarins 2 and 3, with their structures related to an anti-HIV-1 substance, (+)-calanolide A (4), were obtained from the crude chloroform extract of the rhizomes. Both induced toxicity to cells used in a syncytium assay for anti-HIV-1 activity as reported [56].

Rahman M.T *et al,* (2003) have reported that the ethanolic extract of *Clausena excavata* leaves, given orally at doses of 125.25 and 500 mg/kg body weight, showed significant antinociceptive activity on acetic acid induced writhing in mice. [57].

Manosroi A *et al.,* (2003) have demonstrated the *in vitro* immunomodulatory activities of aqueous extract, acetone extract and the Thai folklore extract of *Clausena excavata* Burm. F. on the mouse immune system [58].

Manosroi A *et al.,* (2005) have done the *in vivo* immunomodulating activity of wood extracts from *Clausena excavata* Burm. [59].

Potterat O *et al.,* (2005) have isolated a new carbazole alkaloid, named clausine Z, from the stems and leaves of *Clausena excavata* Burm (Rutaceae) and its structure was established by spectroscopic methods. The compound exhibits inhibitory activity against cyclin-dependent kinase 5 (CDK5) and showed protective effects on cerebellar granule neurons in an *in vitro* method [60].

Taufiq-Yap YH *et al.,* (2007) have isolated a new carbazole alkaloid, 3-carbomethoxy-2-hydroxy-7-methoxycarbazole; Clausine-TY (1), together with two known carbazole alkaloid, Clausine-H (2) and Clausine-B (3) from the ethyl acetate

extract of the stem bark of the Malaysian *Clausena excavata* and their structure was analyzed by spectral methods. The new carbazole alkaloid shows significant cytotoxicity against CEM-SS cell line [61].

Sen-Sung Cheng *et al.,* (2009) have reported the mosquito larvicidal activities of leaf and twig essential oils from *Clausena excavata* Burm. and their individual constituents against *Aedes aegypti* L. and *Aedes albopictus* Skuse larvae. The yields of essential oils obtained from hydrodistillation were compared, and their constituents were determined by GC-MS analyses [62].

Clausena lansium

Dhan Prakash *et al.,* (1981) have isolated heptaphylline, lansamide, from the leaves of *Clausena lansium* and its structure was determined by spectral methods [63].

Kong YC *et al.,* (1984) have isolated dehydroindicolactone a new furanocoumarin from roots of *Clausena lansium* and its structure was determined by spectral methods [64].

Yang MH *et al.,* (1988) have isolated clausenamide from the leaves of *Clausena lansium* and its structure was analyzed by spectral methods. The compound was used in the treatment of acute and chronic viral hepatitis and also lowering elevated SGPT level [65].

Yang MH *et al.,* (1988) have isolated three novel cyclic amides, clausenamide, neoclausenamide and cycloclausenamide , that lower the elevated blood SGBT levels from the leaves of *Clausena lansium* [66**]**

Lakshmi V *et al.,* (1990) have isolated a new tetracyclic triterpene alcohol from the aerial parts of *Clausena lansium* [67].

Lu WS et *al.,* (1991) have isolated new carbazole alkaloids from root bark of *Clausena lansium* and its structure was analyzed by spectral methods [68].

Liu Y *et al.,* (1992) have isolated clausenamide from *Clausena lansium* (Lour) with the structure similar to piracetam. Clausenamide at the concentration of 10^{-5}mol/L inhibited the contraction of basilar artery caused by 5-HT, PGF2 alpha and arachidonic acid, indicating that clausenamide is a cerebral protective agent [69].

Rao EC *et al.,* (1995) have synthesized clausenamide which is one of the active constituents from the leaves of *Clausena lansium*. The compound has been reported to be used in treatment of liver injury [70]

Yao Q and Wang M (1998) have demonstrated the metabolic transformation of (-)-clausenamide in rat liver microsomes. (-)-Clausenamide, isolated from the leaves of *Clausena lansium* (Lour.) was studied *in vitro* with phenobarbital-induced rat liver microsomal incubate containing the NADPH-generating system [71].

Tzi B Ng *et al.,* (2003) have demonstrated the homodimeric sporamin-type trypsin inhibitor with antiproliferative, HIV reverse transcriptase-inhibitory and antifungal activities from wampee (*Clausena lansium*) seeds [72].

PLANT PROFILE [73-76]

BIOLOGICAL SOURCE : *Clausena dentata (Willd)*, Roem.

FAMILY : Rutaceae

SYNONYMS : *Clausena willdenowii, Amyris dentata*

DESCRIPTION : *Clausena dentata* is a deciduous shrub or tree belonging to the family rutaceae. It grows up to five meter heigh. The leaves are odd-pinnate, to 15×8 cm, leaflets 4-6 pairs.

SYSTEMIC POSITION

Kingdom : Plantae

Phyllum : Magnoliophyta

Class : Magnoliopsida

Order : Sabindales

Family : Rutaceae

Genus : *Clausena*

Species : *Dentata*

COMMAN NAME

English : Dentata clausena

VERNACULAR NAMES

Malayalam : Kariveppila, Karuvepella, Korakatta, Kurakol, Morakkuranni, Morkurangee, Morkurangi, Potti.

Tamil : Aanai chedi, Kattu veppilai, Ana, Anam, Anantalai, Anantalaiccetci, Karikkaracceti, Karikkaram, Kattukariveppillai,Kattukkariveppilai,Petica, Petikacceti,Potti.

Kannada : Chidae, Kadu karabevu, Kaadu karibevu

Oriya : Mohisiamoricingi

Telugu : Boyyaval, Kondakarivepaku

GEOGRAPHICAL DISTRIBUTION & OCCURRENCE

The shrub is distributed in Ceylon, India Northwestern Burma, Thailand, and South western China. In India it is distributed throughout the western peninsula and eastern Himalayan region. It is found in several regions of Tamil Nadu like Pudukkottai, Thanjavur, Arcot, Narthamali hills and Thiruvanamalai.

BIOLOGY & SPREAD

The shrub bears small white coloured fruits which contains germination ability. The fruit falls down on the ground and break opens the seeds. The rainy season is optimum for the seeds to grow into shrubs.

HABIT AND HABITAT

Clausena dentata is an evergreen shrub found in India. It is popularly known as anai chedi in Tamil. It is used by people of Tamil Nadu for its medicinal and nutritive values. It requires moist to wet soil conditions to germinate. The leaves are opposite or alternate, simple or compound, exstipulate and usually bear numerous oil glands. It is a perennial, multi-stemmed plant that is usually less than 4 to 5m (13 to 16 feet) in height. The shrubs have several stems arising from or near the ground, but may be taller than 5m or single-stemmed under certain environmental conditions.

Ethno medical information for whole plant [74,75,76]

1. The stem bark of the plant possesses hepatoprotective action.
2. The root bark of the plant has analgesic and anti-inflammatory activity.
3. The leaves are used as fertilizer
4. The essential oil of the leaves contains ovicidal, larvicidal activity.
5. Leaf paste applied in area to heal cut wounds.

PHARMACOGNOSTIC EVALUATION

Pharmacognostical study is the preliminary step in the standardization of crude drugs. The detailed pharmacognostical evaluation gives valuable information regarding the morphology, microscopical and physical characteristics of crude drugs. Pharmacognostic studies have been done on many important drugs, and the resulting observations have been incorporated in various pharmacopoeias. There are a number of crude drugs where the plant source has not yet been scientifically identified. Hence pharmacognostic study gives the scientific information regarding the purity and quality of crude drugs. **[77].**

Sensory evaluation [78]

The term sensory evaluation refers to organoleptic evaluation. Characters such as color, odor, taste, texture, touch etc. are evaluated with the help of sense organs. The sensory characteristics of organized as well as unorganized drugs gives an idea about the quality, identity, purity of drugs.

Macroscopical evaluation

Morphology: Some of these gross morphological characters of drugs such as shape, size, margin, apex, venation, are identification features of drugs. These features give valuable information about the drugs.

Cell morphology: It includes the study of morphological characteristics of particular cells.

Microscopical evaluation

Microscopical study of organized crude drugs is an important parameter for evaluation. Another important aspect of microscopical evaluation is the study of surface constants. These evaluations allow more detailed examination of the phytodrug to identify the organized drug by its histological character. It provides detailed information about the crude drugs by virtue of its property to magnify the fine structures of minute objects to be visualized and thereby confirm the structural details of the plant drugs under evaluation. It can also be used in the determination of the optical as well as micro chemical properties of the crude drug.

MATERIALS AND METHODS

Collection of specimens

The plant specimens were collected from Arimalam, a remote village in Pudukkottai District. The specimen was authenticated by the Director, The Rapinat Herbarium and Centre for Molecular Systematic, St. Joseph's College Campus, Tiruchirappalli-620002. A voucher specimen is kept in the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai **(Fig. 1).**

MACROSCOPICAL EVALUATION

The various aerial parts of *Clausena dentata* collected for macroscopical evaluation were leaves, flowers, fruits and seeds. The photographs of the macroscopic features of the plant are shown in **Fig. 2.1 to 2.5.**

MICROSCOPICAL EVALUATION

The leaves of the plant were subjected to microscopical evaluation. The samples of leaves were cut and removed from the plant and fixed in FAA (formalin, 5mL; acetic acid, 5mL; ethyl alcohol, 90mL). After 24h of fixing, the specimens were dehydrated with graded series of t-butyl alcohol **[79]**. Infiltration of the specimens was carried by gradual addition of paraffin wax (M.P-58-60^{o}C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with help of rotary microtome. The thickness of the sections was 10-12µm. De-waxing of the sections was carried out by customary procedure **[80]**. The sections were stained with toluidine blue **[81]** since it is a polychromatic stain. The staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink color to the cellulose walls, blue to the lignified cells, violet to the mucilage, and blue to the protein bodies and also stained with safranin.

Leaf clearing

Two methods were used for studying the stomatal morphology, venation pattern and trichome distribution. Paraffin embedded leaf was used for para-dermal sections. From these sections, the epidermal layers as well as vein islets were studied. Another method employed was clearing leaf fragments by immersing the material in alcohol (to remove chlorophyll) followed by treating with 5% sodium hydroxide. The material was rendered transparent due to loss of cell contents. Epidermal peeling by partial maceration

employing Jeffrey's maceration was also done. Glycerin mounted temporary preparations were made for cleared materials.

For study of elements of xylem, small fragments of leaves were macerated with Jeffery's maceration fluid.

Powdered materials of different parts were cleared with sodium hydroxide and mounted in glycerin medium after staining. Different cell component were studied and measured.

Photomicrographs

The photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For normal observations bright field was used and for the study of starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized they appear bright against dark background. The magnifications of the anatomical features are indicated by the scale-bars in the photographs.

The microscopic features of the various parts of the shrub namely leaf and stem are presented in **Fig. 3 to 10.**

QUANTITATIVE ANALYTICAL MICROSCOPY [82]

Quantitative analytical microscopy is useful for measuring the cell contents of the crude drugs, which help in their identification, characterization, and standardization. A clear idea about the identity and characteristic features of the drug can be obtained after several numbers of determinations; the characteristic's number obtained is noted and compared with a standard value to find out whether it is within the range.

Determination of stomatal number and stomatal index

Stomatal number: The average number of stomata/sq.mm area of each surface of a leaf epidermis is termed as stomatal number [82].

Stomatal index: The stomatal index is the percentage which the number of stomata formed to the total number of epidermal cells, each stoma being counted as one cell.

To study the stomatal morphology (type of stomata), stomatal number and stomatal index of leaf, the leaf was subjected to epidermal peeling.

Procedure [83]

The leaf was cleared by boiling with chloral hydrate solution or alternatively with chlorinated soda. The upper and lower epidermis was peeled out separately by means of forceps. The cleared leaf was placed on a slide and mounted in glycerin. A camera lucida and drawing board was placed and a stage micrometer was inserted for making the drawing scale. A square of 1mm was drawn by means of stage micrometer. The slide with cleared leaf (epidermis) was placed on the stage of the microscope and examined under 45X objective and 10X eye piece. The epidermal cell and stomata was traced. The numbers of stomata present in the area of 1sq. mm. including the cell if at least half of its area lies within the square was counted. The result for each field was calculated and the average number of stomata per sq. mm was determined and their values are tabulated in **Table 1**.

For stomatal index, the glycerin mounted leaf peeling as mentioned above was made and circle (O) like mark for each stomata and a cross (X) like mark for each

epidermal cells was marked on the chart paper. The stomatal index was calculated by using the formula,

Stomatal index = S/(E + S) x 100 where S was the number of stomata in 1sq mm area of leaf and E was the number of epidermal cells (including trichomes) in the same area of leaf. The values are tabulated in **Table 1.**

Determination of Vein Islets and Vein Terminations

The term vein islet is used to denote the minute area of photosynthetic tissue encircled by the ultimate divisions of conducting stands. The number of vein islet/sq.mm of leaf fragments is known as **vein islet number**. An ultimate free end or termination of a veinlet is termed as veinlet termination. The number of vein terminals present in one sq.mm area of leaf fragment is termed as **veinlet termination number** [82 & 78].

Procedure [83]

The fragment of leaf lamina with an area of not less than 1sq mm excluding the midrib and the margin of the leaf was taken. The fragments of leaf lamina were cleared by heating in a test tube containing chloral hydrate solution on a boiling water bath until clear. The cleared fragments were stained with saffranin solution and a temporary mount was prepared with glycerol solution. The stage micrometre placed on the microscopic stage, examined under 10X objective and 6X eye piece and an area of 1 sq mm square was drawn. The cleared leaf piece was placed on the microscope stage, the vein islets and vein terminals included in the square was drawn. The number of vein islets and terminals within the square were counted. The results obtained for the number of vein islets and terminals in1sq mm are tabulated in **Table 1**.

POWDER ANALYSIS

The behavior of the powder with different chemical reagents was carried out as mentioned by Kay 1938 [84] and Johansen 1940 [80]. The observations are presented in **Table 2**.

Fluorescence analysis

The fluorescent analysis of the plant extracts of *C. dentata* was carried out by using the method of Chase and Pratt (1949) [85]. The observations are tabulated in **Table 3.**

STANDARDIZATION PARAMETERS

The determination of the ash values, loss on drying, foreign organic matter and extractive values etc. gives a clear idea about the specific characteristics of crude drug under examination, besides its macro-morphological or cyto-morphological, microscopical nature in both its entire and its powder form. These diagnostic features enable the analyst to know the nature and characteristic of crude drugs. The procedures recommended in Indian Pharmacopoeia, 1996 and WHO guidelines, 1998 [86, 87] were followed to calculate total ash, water-soluble ash, acid-insoluble ash and loss on drying at 110^{o}C. The percentage of extractive values for different solvents was also calculated.

Determination of foreign organic matter [87]

The part of organ or organs other than those specified in the definition or description of the crude drugs is defined as foreign organic matter. An accurately weighed 100g of air dried coarse drug and spread out in a thin layer. The sample drug

was inspected with the unaided eye or with the use of 6X lens and the foreign organic matter was separated manually as completely as possible and weighed. The percentage of foreign organic matter was calculated with reference to the weight of the drug taken. The result is presented in **Table 4.**

Determination of Moisture Content (Loss on Drying) [86, 87]

An accurately weighed 10g of powdered drug was placed in a tared weighing bottle. Then the bottle was dried at 105^{o}C for 5h and weighed. The drying and weighing was continued at one hour intervals until the difference between the two successive weighing is not more than 0.25%. The loss on drying was calculated with reference to the amount of powder taken. The readings are tabulated in **Table 4.**

Determination of Ash values [86, 87]

Ash Content [84]

The residue remaining after incineration is the ash content of crude drug, which simply represents inorganic salts naturally occurring in the drug or adhering to it or deliberately added to it as a form of adulteration. The total ash, acid insoluble ash and water soluble ash are generally determined.

Procedure

Determination of Total Ash

An accurately weighed 2g of air dried powdered drug was taken in a tared silica crucible and incinerated at a temperature not exceeding 450^{o}C, upto 6hrs until free from

carbon then allowed to cool and weighed. The percentage of ash was calculated with reference to the air dried drug.

Determination of Acid Insoluble Ash

The total ash obtained from the previous procedure was mixed with 25mL of 2M hydrochloric acid and boiled for 5min in a water bath, and then the insoluble matter was collected in an ashless filter paper (Whatmann) and washed with hot water, dried and ignited for 15min at a temperature not exceeding 450^{o}C, cooled in a dessicator and weighed. The percentage of acid insoluble ash was calculated with reference to the air dried drug.

Determination of Water Soluble Ash

The total ash obtained from the previous procedure was mixed with 25mL of water and boiled for 5min in a water bath, and then the insoluble matter was collected in an ashless filter paper and washed with hot water, dried and ignited for 15min at a temperature not exceeding 450^{o}C, cooled in a dessicator and weighed. The insoluble matter was subtracted from the weight of the total ash; the difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried drug.

The values in respect of the total ash values, acid insoluble ash, water soluble ash and water insoluble ash are tabulated in **Table 4.**

Determination of Extractive Values [85, 87]

The extractive values are the important factor to determine the amount of active principle or phytocostituents present in the plant materials, when extracted with suitable solvents. The extraction of crude plant materials with various solvents gives a solution containing different phytoconstituents. Composition of the phytoconstituents in a particular solvent depends upon the nature of drugs and solvents used. This is an important tool for analysis of plant crude materials for its identity, purity and quality [78].

Procedure

Determination of ethanol soluble extractive

An accurately weighed 5g of the air dried coarsely powdered drug was macerated with 100mL of ethanol in a closed flask for 24h, shaking frequently during the first 6h and allowed to stand for 18h. Thereafter filtered rapidly, taking precautions against loss of ethanol. Then evaporate 25mL of the filtrate to dryness in a tared china dish dry at 105^{o}C and weighed. The percentage of ethanol soluble extractive was calculated with reference to the air dried drug.

Determination of water soluble extractive:

An accurately weighed 5g of the air dried coarsely powdered drug was macerated with 100mL of chloroform water in a closed flask for 24h, shaking frequently during the first 6h and allowed to stand for 18h. Thereafter filtered rapidly, taking precautions against loss of chloroform water. Then evaporate 25mL of the filtrate to dryness in a tared china dish dry at 105^{o}C and weighed. The percentage of water soluble extractive was calculated with reference to the air dried drug.

Determination of petroleum ether soluble extractive:

The procedure adopted under ethanol soluble extractive was followed using petroleum ether as a solvent.

Determination of hexane soluble extractive:

The procedure adopted under ethanol soluble extractive was followed using hexane as a solvent.

Determination of methanol soluble extractive:

The procedure adopted under ethanol soluble extractive was followed using methanol as a solvent.

Determination of chloroform soluble extractive:

The procedure adopted under ethanol soluble extractive was followed using chloroform as a solvent.

Determination of benzene soluble extractive:

The procedure adopted under ethanol soluble extractive was followed using benzene as a solvent.

Determination of ethyl acetate soluble extractive:

The procedure adopted under ethanol soluble extractive was followed using ethyl acetate as a solvent.

The extractive values obtained for different solvents are presented in **table 4.**

Determination of Foaming Index [87]

Some plant materials when shaken with water cause persistent foam which may be attributed to the presence of saponins in that material. The foaming ability of an aqueous solution of plant materials and their extracts is measured in terms of foaming index.

Procedure

An accurate quantity of about 1g of the coarse plant material was weighed and transferred into an Erlenmeyer flask containing 100mL of boiling water. The flask was boiled at moderate heat for 30min. The solution was cooled and filtered into a 100mL volumetric flask and sufficient distilled water was added to dilute to volume. The solution was poured into ten stoppered test tubes in successive portions of 1mL, 2mL, etc. upto 10mL, and the volume of the liquid in each tube was adjusted with water upto 10mL. The tubes were then stoppered and shaken in a length wise motion for 15sec (two shakes/sec) and allowed to stand for 15min. The height of foam was measured. If the height of the foam in every tube was less than 1cm the foaming index was less than 100. If a height of foam of 1cm was measured in any test tube, the volume of the plant material decoction in this tube (a) was used to determine the index. If the height of the foam was more than 1cm in every tube, the foaming index was over 1000. In this case, the determination was repeated using a new series dilution of the decoction in order to obtain a result. The foaming index was calculated by using the following formula 1000/A where A was the volume in mL of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm was observed. The result obtained is presented in **table 4**.

Determination of Volatile Oil (Essential oil)

Essential oils are the odours of the plants due to specific mixture of volatile substances, which in general are liquid at room temperatures. They occur in special cells, glands or ducts, either in one particular organ of the plant or distributed over many parts of the plant namely the leaves, barks, roots, flowers and fruits. Occasionally they are also present in combination with sugars as glycosides. They are composed of number of chemical compounds- hydrocarbons (terpenes, sesquiterpenes, diterpenes) alcohols, esters, aldehydes, ketones, oxides and occasionally compounds of nitrogen and sulphur.

Essential oils may be isolated from plant materials by one or other of following methods

1) Distillation (water, water and steam, live steam)
2) Extraction by volatile solvents, hot oils or fats (maceration) or cold neutral fats.
3) Expression by hand or machinery

Procedure [87]

The essential oil was isolated from *Clausena dentata* by hydro-distillation method. A 200g of fresh leaves of *Clausena dentate* was weighed and transferred into a round bottom flask and 500mL water was added and fitted with condenser for carrying out the determination by hydro-distillation keeping the temperature 40°C on a heating mantle to give slight heating to the flask. The distillation was continued for 3h.The oil was collected in a volatile oil container. The oil was dried over anhydrous sodium sulphate and stored at 40°C. The percentage yield of the oil was calculated using the formula [Volume of oil obtained / Weight of sample taken] x 100. The result is presented in **Table 4.**

RESULTS AND DISCUSSION

MACROSCOPICAL OBSERVATIONS

Clausena dentata is *a s*trongly aromatic shrub or tree about 5-8ft and occurs in the grasslands and rainforests. It requires moist to wet soil condition to germinate. It is a perennial, multi-stemmed woody plant that is usually less than 4 to 5m (6 to 10ft) in height. The shrubs are evergreen and have several stems arising from or near the ground, but may be taller than 5m or single-stemmed under certain environmental conditions. **(Fig. 2.1)**.

Leaf (Fig 2.2)

The leaves were dark green in color with imparipinnate leaves having strong aromatic odor and the texture was fine. The leaves were arranged opposite one another. They were odd-pinnate to 5-13 × 2.5-6cm. The leaflets were 4-6 pairs 1-2 in. [2.5-5 cm] long, oblique, oblong-ovate or ovate-lanceolate, acute or obtusely caudate-acuminate, crenulate and membranous. Crenulate margin with acuminate apex, petiole to 3.5cm, petiolate to 3mm, usually grey pubescent on nerves and midrib on both surfaces, sometimes glabrescent. The midrib was raised above, secondary nerves 7-11 pairs and tertiary nerves broadly reticulate.

Flower (Fig 2.3)

The flowers were bell-shaped and white in color. They were arranged in panicles. The flowers were bisexual, tetramerous to 1cm across. Calyx -lobes 4, ovate (1mm) and pubescent. Petals 4, white, oblong or obovate, 5mm, clawed, concave. Stamens 8, free, inserted round the disc. The filaments were unequal, 3-4mm dilated below the anthers

2mm, glandular on the back. Ovary stipitate, 4-angled, 4-celled and the ovules 2 to 4 per cells, collateral or superposed, style to 1.5mm furrowed, stigma 4-lobed.

Fruits and seeds (Fig. 2.4)

The shrub contained spherical, berry, globose or ovoid fruits with oblong seeds. The fruits were white in color; 8mm in diameter smooth and shiny in nature. The fruit were edible and flavourous, sweet, juicy and nutritious. The seeds oblong, solitary, ovoid, 7×4mm compressed. They were covered with white colored membrane.

MICROSCOPICAL OBSERVATIONS

Leaf (Fig. 3.1)

The leaf comprised of a prominent midrib and leathery lamina. The midrib consisted of an adaxial wide and thick semicircular part and an abaxial wider and fairly thick hemispherical part (**Fig. 3.1)**. The midrib was 700µm thick. The adaxial hump was 500µm wide and the abaxial part was 650µm wide. The adaxial part consisted of thin epidermal layer and an inner zone of sclerenchyma arc. The abaxial part also had a thin layer of epidermis and homogenous compact paranchymayous ground tissue.

The vascular system was double stranded and included an adaxial biconvex vascular segment and an abaxial shallow arc of thick and wide segment. Both adaxial and abaxial strands had long compact parallel lines of xylem elements and wide continuous strands of phloem on the outer part of the xylem **(Fig. 3.1).**

Lamina (Fig. 3.2; 4)

The lamina was smooth on the both sides. It was dorsiventral and hypostomatic 250µm thick. The adaxial epidermis was thicker and consists of wide square shaped cells

with prominent cuticle. The adaxial epidermis was 20µm thick. The abaxial epidermis was thick and the cells were cylindrical, measuring 10µm thick.

The mesophyll tissue was differentiated into adaxial zone of long, cylindrical columnar palisade cells which were 50µm in height. The abaxial part included 6 or 7 layers of lobed loosely arranged spongy parenchyma.

The stomata were located on the outer abaxial epidermis **(Fig. 4.2).** It was raised slightly above the level of epidermis. The guard cells had beak shaped pointed ledges which were formed by the extended cuticle of the epidermal cells.

Lysigenous secondary cavities were abundant in the lamina **(Fig. 5.)** and they were circular and wide measuring 120µm in diameter. The cavities were random in distribution. The cavity was lined by a thin layer of secretory substance.

Epidermal cells and Stomata (Fig. 5, 6)

The stomata type and epidermal cells was studied from the paradermal sections. The abaxial epidermal cells were polygonal in outline with thick and straight and anticlinal walls **(Fig.5.2).** The stomata were cyclocytic type. A stoma was surrounded by a ring of four or more subsidiary cells. The guard cells were broadly elliptical measuring 25 × 35µm in size.

Calcium oxalate (Fig.5.2)

Calcium oxalate crystals of prismatic type were located in line along the veins and the crystals were of rhomboidal or pyramidal type. Apart from the veins, the crystals were not evident in the other regions **(Fig. 5.2).**

Venation pattern (Fig.7)

The lamina consisted of well developed reticulate venation pattern. The major veins were thick and the minor veins become gradually thin and they were straight. The vein islets were distinct and they were well demarcated by vein boundaries. The vein terminations were distinct and they were seen in all islets. The terminations were un-branched, short or long and slender. The terminations were also branched once or twice (**Fig. 7.1).**

Stem (Fig. 8)

The stem was circular and measures 1.8µm thick. It consisted of epidermis, narrow cortex with secretory cavities, discontinuous masses of cortical sclerenchyma, thin layer of phloem and thick hollow cylinder of xylem enclosing the pith **(Fig. 8.1,2).**

The epidermal layer was thin and continuous. The cells were small and circular **(Fig. 8.2).** The cortex was narrow comprising of 6 or 7 layers of small compact parenchyma cells. Along the inner boundary of the cortex was a discontinuous cylinder of sclerenchyma cells. The vascular cylinder comprised of a wide zone of diffuse distributed phloem elements. The phloem elements included sieve elements and phloem parenchyma. The xylem cylinder consisted of radial fields of vessel and fibers. The vessels were thick walled, they were circular ovate or elliptical in sectional view. They were mostly solitary or in short radial multiplies. The xylem fibers were thick walled lignified and had narrow lumen. The vessels were 20µm wide.

Powder microscopy (Fig.9, 10)

The leaf powder of *Clausena dentata* was studied under the microscope and the following inclusions was observed.

a) Adaxial epidermal peeling (Fig. 9.1)

Small fragments of adaxial epidermal peelings were seen very frequently and the cells appeared in surface view. The epidermis lacked stomata. The cells were polygonal. The anticlinal walls were thick and straight. They appeared beaded due to presence of dense simple pits on their walls **(Fig. 9.2).**

b) Adaxial epidermal fragments (Fig. 10)

Small irregular fragments of abaxial epidermis were also common. The epidermis bears stomata which were of cyclocytic type. A guard cell was encircled by a ring of six or more cells (**Fig. 10.2).** The epidermal cells were polyhedral, smooth and thin walled. The guard cells were thick and elliptical.

c) Foliar sclereids (Fig.10.3)

The veins possessed xylem elements with annular or spiral scalariform lateral wall thickenings closely associated with the xylem elements. The xylem elements were long unbranched thread like foliar sclereids. The sclereids had thick walls with dense canal like simple pits and narrow lumen. Sometimes the sclereids protrude into the vein islets.

QUANTITIATIVE ANALYTICAL MICROSCOPY

The results obtained for the determination of leaf constants are presented in **Table 1.** From the table, it can be observed that the number of stomata in the upper epidermis was found to be 30.6±1.14 while in the lower epidermis it was 45.41±1.55. The stomatal index in the upper epidermis and lower epidermis was 16.45 ± 0.71 and 18.55±0.51 respectively. The vein islet number was found to be 15.33 ± 1.25 and the vein termination

number was 29.66 ± 1.40. These values help in identification of leaf of *Clausena dentata* from other species of the genus *Clausena* since these values are unique for each plant.

Table 1: Quantitative analytical microscopical parameters of the leaf of *Clausena dentata* (Willd)

S. No.	Parameters*	Values obtained
1	Stomatal number in upper epidermis	30.6±1.14
2.	Stomatal number in lower epidermis	45.41±1.55
3.	Stomatal index in upper epidermis	16.45±0.71
4.	Stomatal index in lower epidermis	18.57±0.51
5.	Vein islet number	15.33±1.25
6	Vein termination number	29.66±1.40

*** mean of 6 readings ± SEM**

POWDER ANALYSIS

The behavior of crude leaf powder of *C. dentata* with various reagents is presented in **Table 2.** From the table, it can be observed that the powder with 10% potassium hydroxide and 1M sodium hydroxide under UV light at 254nm exhibited a greenish florescence which will be useful for identification of the plant material in crude form.

Fluorescent analysis of the Extracts

The behavior of various extracts in natural light and under UV light at 254nm and 365nm are presented in **Table 3.** From the table, it was observed that the ethanolic, methanolic, chloroform, benzene and ethyl acetate extracts were orange under UV light at 365nm. These parameters are useful for quality control and purity checking of the plant n powder form.

Table 2: Behavior of the *Clausena dentata* powder with various chemical reagents

Drug powder + reagent	Colour in day light (Visible)	Colour in UV light	
		254nm	365nm
Powder	Pale green	Green	Black
Powder + 1M sodium hydroxide	Yellowish green	Fluorescent green	Greenish black
Powder + Iodine	Yellowish green	Dark green	Greenish black
Powder + 10% potassium hydroxide	Yellowish green	Fluorescence green	Greenish black
Powder + 1M Hydrochloric acid	Pale green	Dark green	Black
Powder + Glacial acetic acid	pale green	Green colour	Black
Powder + 50% sulphuric acid	Yellowish green	Green colour	Greenish black
Powder + 50% nitric acid	Brownish green	Green colour	Black
Powder + 50% hydrochloric acid	Pale green	Green colour	Bluish black

Note :- Colour reactions are viewed under natural light by naked eye

Table 3: Fluorescence Analysis of extracts of *Clausena dentata*

Extract	Consistency	Colour in day light	Colour in UV light	
			254nm	365nm
Ethanol	Semisolid	Yellowish green	Green	Orange
Methanol	Semisolid	Dark green	Green	Orange
Chloroform	Semisolid	Yellowish brown	Greenish black	Orange
Ethyl acetate	Semisolid	Yellowish green	Olive green	Orange
Water	Semisolid	Brownish green	Dark green	Pale green
Benzene	Semisolid	Brownish green	Green	Orange
Hexane	Semisolid	Yellowish green	Green	Greenish black
Acetone	Semisolid	Green	Dark green	Greenish black

STANDARDIZATION PARAMETERS

The results obtained for various standardization parameters are presented in **Table 4.** From the **Table 4,** it can be seen that the foreign organic matter present in the crude material was very low. The percentage of total ash was found to be 15.29 ± 0.04 and the percentage of water soluble ash was found to be 2.29 ± 0.04 while the acid insoluble ash was 6.24 ± 0.59. The determination of ash values helps to find out where the powdered material was adulterated with sand and other inorganic material. The water soluble ash helps us to find the amount of inorganic material present in the crude drug while acid insoluble ash helps us to find the amount of sand and other debris in the crude material.

Table 4: Standardization parameters of *Clausena dentata* (Willd)

S. No	Parameters*	Values*expressed as %
1	**Foreign organic matter**	0.04 ± 0.18
2	**Moisture content**	2.070 ± 0.04
3	**Ash values**	
	Total ash	15.29 ± 0.04
	Acid insoluble ash	6.24 ± 0.59
	Water soluble ash	2.29 ± 0.04
4	**Extractive Values**	
	Petroleum ether	3.74 ± 0.12
	Chloroform	5.84 ± 0.89
	Ethyl acetate	2.77 ±0.18
	Ethanol	25.30 ±0.14
	Methanol	21.48 ± 0.06
	Water	29.86 ± 0.80
	Hexane	14.46±1.07
	Benzene	7.65±0.41
5	**Foaming index**	100
6	**Volatile oil**	1.04 ± 0.02

*** mean of three readings ± SEM**

The various extractive values with different solvents have been determined. A maximum extractive value was found with water (29.86 ± 0.80) followed by ethanol (25.30 ±0.14). The extractive values help us to decide what solvent will be useful for extraction of maximum active principles and also helps to decide whether the crude material has already been exhausted or not.

The pharmacognostic evaluation which includes macroscopical, microscopical, analytical parameter evaluations helps in identity, quality and purity of the plant material either as a whole or in the form of powder.

PHYTOCHEMICAL EVALUATION

Phytochemistry is widely used in the field of herbal medicine. Phytochemical technique mainly applies to the quality control of Ayurvedic medicine (Indian traditional medicine) or herbal medicine of various chemical components such as saponins, alkaloids, volatile oils, flavonoids and anthraquinones. Therefore it is important to screen and analyze bioactive components, not only for the quality control of crude drugs, but also for the elucidation of their therapeutic mechanisms **[88].**

MATERIALS AND METHODS

QUALITATIVE CHEMICAL TESTS FOR THE LEAF POWDER AND CRUDE EXTRACTS [89, 90, 91]

Qualitative chemical tests are carried out for the purpose of specific identity of the substances in the crude extracts. The color reaction or precipitate usually observed can identify a class of compound. Chemical tests can be useful for the investigation of the chemical compounds and to observe the efficiency of an extraction process. The petroleum ether, ether, ethyl acetate, methanol, ethanol and aqueous extracts were subjected to qualitative chemical tests. The various chemical tests performed on the extracts were for steroids, terpenoids, flavones, anthraquinones, sugars, glycosides, alkaloids, quinones, phenols, tannins and saponins and the results were recorded.

1**. Test for sterols**

The powdered leaf was first extracted with petroleum ether and evaporated to a residue. Then the residue was dissolved in chloroform and tested for sterols.

a. Salkowski's Test: A few drops of concentrated sulphuric acid were added to the above solution, shaken well and set aside. The lower chloroform layer of the solution turning red indicates the presence of sterols.

b. Liebermann – Burchard's Test: To the chloroform solution a few drops of acetic anhydride and 1mL of concentrated sulphuric acid were added through the sides of the test tube and set aside for a while. At the junction of two layers a brown ring and the upper layer turning green indicates the presence of sterols.

2. **Test for carbohydrates**

a. Benedict's test: The aqueous extract of the powdered was treated with Benedict's reagent and boiled on water bath and cooled. An orange colour precipitate indicates the presence of carbohydrates.

b. Fehling's Test: The aqueous extract of the powdered leaf was treated with Fehling's solution I and II and heated on a boiling water bath for half an hour. A red precipitate indicates the presence of free reducing sugars

3. Test for glycosides

The substance was mixed with a little anthrone on a watch glass. One drop of concentrated sulphuric acid was added, made into a paste and warmed gently over a water bath. A dark green coloration indicates the presence of glycosides.

4. Test for Cardiac Glycosides

a. Keller Killiani Test: The substance was boiled with 10% alcohol for 2min, cooled and filtered. To the filtrate, lead sub acetate was added and filtered. The filtrate was then extracted with chloroform. The chloroform layer was separated and evaporated to dryness. The residue was dissolved in glacial acetic acid with traces of ferric chloride. To this few drops of sulphuric acid was added slowly along the sides of the test tube. A reddish brown layer changes to green colour on standing.

b. Legal test: The substance was dissolved in pyridine, sodium nitroprusside solution was added to it and made alkaline. A pink or red colour indicates the presence of cardiac glycosides.

c. Baljet test: To the substance sodium picrate solution is added. A yellow to orange colour indicates the presence of cardiac glycosides.

4. Test for Proteins and free amino acids

a. Millon's Test : A small quantity of aciduous – alcoholic extract of the powdered drug was heated with Millon's reagent. A white precipitate turning red on heating indicates the presence of proteins.

b. Biuret Test: To the alcoholic extract of powdered drug, one ml of dilute sodium hydroxide(10%) solution was added followed by this one drop of very dilute copper sulphate solution was added. A violet colour indicates the presence of proteins.

5. Test for Mucilage

A few mL of aqueous extract was prepared from the powdered drug was treated with ruthenium red. A pinkish red colour indicates the presence of mucilage.

6. Test for Flavonoids

a. Magnesium turning- con HCl test: A little of the powdered drug was heated with alcohol and filtered. To the test solution magnesium turnings and few drops of concentrated hydrochloric acid were added and Boiled for five minutes. A red colour indicates the presence of flavonoids.

b. Alkali Test: To the small quantity of test solution 10% aqueous sodium hydroxide Solution was added. A yellow orange color indicates the presence of flavonoids.

7. Test for terpenoids

The powdered leaf was shaken with petroleum ether and filtered. The filtrate was evaporated and residue obtained was dissolved in small amount of chloroform and the chloroform solution tin and thionyl chloride were added. A pink color indicates the presence of terpenoids.

8. Test for Tannins

A small quantity of the powdered drug was extracted with water. To the aqueous extract, few drops of ferric chloride solution was added. A bluish black color indicates the presence of tannins.

10. Test for Alkaloids

About 2gm of the powdered material was mixed with 1gm of calcium hydroxide and 5mL of water into a smooth paste and set aside for 5min. It was then evaporated to dryness in a porcelain dish on a water bath. To the residue, 20mL of chloroform was added, mixed well and refluxed for half an hour on a water bath. Then it was filtered and the chloroform was evaporated. To the residue, 5mL of dilute hydrochloric acid was added. The solution was divided into four parts and 2mL of each of the following reagents were added and the colour noted below indicates the presence of alkaloids.

a) Mayer's Reagent - Cream precipitate produced

b) Dragendorff's Reagent - Reddish brown precipitate produced

c) Hager's Reagent - Yellow precipitate

d) Wagner's Reagent - Reddish brown precipitate

Test for purine group (Murexide test)

The residue obtained after the evaporation of chloroform as described above was treated with 1mL of hydrochloric acid in a porcelain dish and 0.1gm of potassium chlorate was added and evaporated to dryness on water bath. Then the residue was exposed to the vapour of dilute ammonia solution. A purple colour indicates the presence of purine group of alkaloids.

Test for carbazole alkaloids

To the alcoholic extract of the leaf powder, conc. H_2SO_4 was added. A violet color indicates presence of carbazole alkaloids.

11. Test for Coumarins [92]

a. $Fecl_3$ test: To the alcoholic extract of the drug, few drops of alcoholic ferric chloride were added. The formation of deep green color turning yellow on addition of concentrated nitric acid indicates the presence of coumarins.

b. Flourecence test: The alcoholic extract of crude extract was mixed with 1N sodium hydroxide solution. A blue-green fluorescence indicates the presence of coumarins.

The above chemical tests were carried out using leaf powder and different plant extracts and the results were tabulated in **Tables 5 & 6** respectively**.**

QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

Medicinal plants have great importance to prepare herbal medicine are free from side effects and they are low cost medicines, which will be beneficial for the people who are suffering from with chronic diseases. The quantitative estimation of phytoconstituents is very essential for identifying and quantifying the phytochemicals present in the medicinal plants which is important for therapeutic action. A particular

group of compound like flavanoids, polyphenols and alkaloids present in the crude extracts can be quantified by means of using standard or reference marker compound and then reporting them as equivalent to that much amount of compound present in that extract as per standard compound. **[93]**

1. Determination of total phenolic content [94,95,96]

Principle

The total phenol content of *Clausena dentata*., was determined by the Folin-Ciocalteu colorimetric method **[94].** The Folin Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate. The method measures the amount of substance needed to inhibit the oxidation of the reagent **[95].**

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

10% sodium carbonate

1N Folin-Ciocalteu reagent (diluted with equal volume of water and made 1N)

Procedure

The compound gallic acid was used as standard **[97]** and was weighed and dissolved in distilled water to produce 1mg/mL stock solution. The stock solution was further diluted to get concentrations ranging from 2-10µg/mL. To these solutions, 0.5mL of Folin-Ciocalteu reagent and 0.5mL of sodium carbonate was added and the final volume was made up to 10mL with distilled water. The absorbance was measured at 760nm after incubation at room temperature for 30min. The ethanolic extract (0.5mL of 500µg/mL) was mixed with 0.5mL of Folin-Ciocalteu reagent and 0.5mL of 10% sodium carbonate and final volume was made up to 10mL with distilled water, and the

absorbance was measured at 760nm after incubation at room temperature for 30min. A calibration curve was constructed by plotting concentration versus absorbance of gallic acid (**Fig. 11).** A linear regression equation was formed and the amount of phenolic compounds was determined using this equation. The total phenolic content was expressed as mg gallic acid equivalents (GAE)/g of extract. The results are tabulated in **Table 7.**

Determination of total flavanoid content [98, 99, 100]

Principle

The aluminum chloride colorimetric technique was used for estimation of total flavonoid estimation [98]. Aluminum ions form stable complexes with C4 keto group and either to C3 or C5 hydroxyl groups of flavones and flavonols in acidic medium. It also forms acid labile complexes with ortho hydroxyl groups in the A or B rings of flavanoids [99]. These complexes showed a strong absorption at 415nm which is used for the estimation of flavanoids.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

10% aluminum chloride

1M potassium acetate

Procedure

A known quantity of quercetin [100] was dissolved in ethanol to get a stock solution of 1mg/mL. Further dilutions were made to get concentrations ranging from 20-100µg/mL. 1ml of the above standard solutions were taken in different volumetric flasks,

0.1mL of aluminum chloride solution, 0.1mL of potassium acetate solution and 2.8mL of ethanol were added and the final volume was then made up to 5mL with distilled water. After 20min the absorbance was measured at 415nm. A sample without aluminium chloride was used as a blank. From the absorbance obtained, a calibration curve was constructed by plotting concentration versus absorbance of quercetin (**Fig. 12**). 1mL of ethanolic extract at concentrations 40µg/mL and 80µg/mL were taken and the reaction was carried out as above and the absorbance was measured at 415nm after 20min and the readings were tabulated in **Table 8.** The amount of flavonoids present can be determined by linear regression analysis. The total flavonoid content was expressed as mg querecetin equivalents /g of extract.

Estimation of vitamin C [101, 102]

Principle

The estimation of Vitamin C was carried out using the method of *Sarojini et al.,* with slight modifications. The keto group of ascorbic acid undergoes a condensation reaction with 2,4 dinitro phenyl hydrazine to form a hydrazone which is orange yellow and has an absorbance of about 520nm.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

0.2% dinitro phenyl hydrazine

85% sulphuric acid

Sample Preparation

1gm of fresh plant material was cut into pieces and soaked in 70% ethanol for 24h. The plant extract was filtered and used as a sample.

Procedure

Ascorbic acid was weighed and dissolved in water to get stock solution of 1mg/mL. Further dilutions were made to get the concentrations ranging from 40-200µg/mL. To 1mL of sample 0.5mL of dinitro phenyl hydrazine solution was added and incubated for 3h at 37ºC. After 3h, 2.5mL of 85% sulphuric acid was added and the absorbance was measured after 30min at 520nm. A calibration curve was constructed by plotting concentration versus absorbance of ascorbic acid **(Fig. 13).** The procedure was repeated for the plant extract as above and the absorbance was measured at 520nm after 3h and the readings were tabulated in **Table 9.** The amount of vitamin C present can be determined by linear regression analysis. The vitamin C content was expressed as mg/g of extract.

CHROMATOGRAPHY

Plant contains many active constituents which are separated for the purpose of analysis of its chemical nature. Chromatography is one of method for separating complex mixtures that depends on the differential affinities of the solutes between two immiscible phases. One of the phases with a large surface area is called the stationary phase, while the other is fluid, which moves over the surface of the fixed phase called the mobile phase. The chromatographic methods can be classified based on the nature of the stationary and mobile phases used. If the stationary phase is a solid, the principle of

separation is called as adsorption chromatography and if the stationary phase is a liquid, it is termed as partition chromatography.

The various types of chromatography include paper chromatography (PC), thin layer chromatography (TLC), column chromatography (CC), gas chromatography (GC), high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) is widely used for plant analysis.

Thin Layer Chromatography [88]

Thin layer chromatographic technique is most widely used for the separation and identification of active components from plant sources. The principle of separation involved here is adsorption. The adsorbent should show a maximum of selectivity toward the substances being separated so that the differences in rate of elution will be large. For the separation of any given mixture, some adsorbents may be too strongly adsorbing or too weakly adsorbing. Depending on the distribution coefficients, the compounds are distributed on the surface of the adsorbent. The compound, which is readily soluble but not strongly adsorbed, moves up along with the solvent and that not so soluble but more strongly adsorbed move up less readily leading to the separation of compounds.

Preparation of TLC Plates

The adsorbent (silica gel G) slurry was prepared in water in the ratio of (1: 2). The glass plates (20cm x 5cm) were cleaned and laid in a row as a template, the suspension was poured into Stahl TLC spreader, which was adjusted to 0.25mm thickness and coated in a single passage of the spreader over them. These plates were air dried and activated in hot air oven at 105ºC for 30min and kept in a dessicator. The plates were

used as the stationary phase or Pre-coated aluminum plates coated with silica gel G F_{254} (Merck) were also used for analysis.

Sample application

The sample was prepared by dissolving the ethanolic extract of *C. dentata* in ethanol to get 5mg/mL. The sample was applied as a spot with the help of capillary tube.

Development of the chromatogram

The plates are dried after sample application. After drying of the spot, the plates were developed in a chromatographic chamber containing varying solvent system as a mobile phase. After one third of the plate was developed the plates were taken outside and dried. The TLC plates were observed under UV light.

(a) Under UV light at 365nm

The R_f values were calculated using the formula R_f = Distance traveled by solute/ Distance traveled by solvent. The results are presented in **Table 10.**

High Performance Thin Layer Chromatography [103, 104]

High performance thin layer chromatography (HPTLC) is a modern adaptation of TLC with improved versatility, separation efficiency and detection limits. HPTLC is useful for identification of plants and their extracts because each plant species produces a distinct chromatogram, with unique marker compounds used for plant identification. It is used a quality control tool since comparison of chromatograms of different lots can demonstrate the similarities and differences between the test samples and the standard chemical markers. HPTLC is a reliable method for quantitation of nanogram level even when present in complex formulation. HPTLC finger print analysis is used for rapid identity check, for monitoring purity of drugs, for detection of adulterants, for

determining whether a material is derived from a defined botanical species and also to known whether the constituents are clearly characterized [104].

Instrument

CAMAG TLC Scanner 3 "Scanner3-070408"S/N 070408(1.41.21) was used for detection and CAMAG Linomat 5 sample applicator was used for the application of the track. Twin trough plate development chamber was used for development of chromatogram. Software used was winCATS 1.4.3

Sample

The ethanolic extract of *C. dentata* was dissolved in ethanol to get a concentration of 10mg/mL and 2µL of this solution was used for taking HPTLC fingerprint.

Stationary Phase

Aluminium sheets pre-coated with silica gel Merck G F_{254}, 0.2mm layer thickness were used as the stationary phase.

Mobile phase

Toluene: ethyl acetate: 100% formic acid (7: 3: 0.2) was used as the mobile phase for development of chromatogram. The mobile phase was taken in a CAMAG twin trough glass chamber.

Detection wavelength

The developed plates were examined at wavelength 254nm and 366nm in Densitometry TLC scanner3. The TLC visualization, 3D display of the finger print profile and peak display at 254nm and 366nm are presented in **Fig.14 to 16.** The R_f values and area under curve for each peak of are presented in **Table 11.**

EVALUATION OF THE VOLATILE OIL ISOLATED FROM THE LEAVES OF *C. dentata* [105]

The volatile oil obtained from the leaves of *C. dentata* by hydro-distillation was subjected to organoleptic evaluation, physical and chemical evaluation.

Organoleptic evaluation

The organoleptic evaluation is an important factor to consider for the analysis quality and nature of the compounds. This evaluation includes the study of color, odor, taste, and solubility, of the material. The organoleptic studies have provided information about the originality of the material isolated and mainly to detect the presence of any adulterated material. The volatile oil of *Clausena dentata* was analyzed for its organoleptic nature and the results are tabulated in **Table 12.**

Determination of refractive index [105]

The refractive index (n) of a substance with reference to air is the sine of the angle of incidence to the site of the angle of refraction of a beam of light passing from air into the substance. It varies with the wavelength of light used in its measurement. The refractive index, n_D^{20} is measured at $20^{\circ} \pm 0.5^{\circ}C$ with reference to the wavelength of the D line of sodium (λ=589.3 nm). The temperature should be carefully adjusted and maintained since the refractive index varies significantly with temperature.

Instrument

Abbe's Refractometer model ATAGOTRA1

Procedure

The prism assembly of the refractometer was opened and the sample was applied onto the surface of the prism as a thin film and the prism assembly was closed. The lamp was adjusted so that the light shines on the prism and the hand wheel of the instrument

was rotated until a half dark at the bottom and light at the top was seen and the index of the refraction was read. Triplicate readings are obtained. The results obtained are presented in **Table 12.**

Determination of weight per millilitre

The weight per millilitre of a liquid is the weight, in gm, of 1ml of a liquid weight in air at 25°C unless otherwise specified.

Procedure

A clean and dry pycnometer was selected and weighed. It was filled with recently boiled and cooled water at 25°C and the contents weighed. The capacity of the pycnometer was calculated by assuming that the weight of the 1mL of water at 25°C when weight in air of density 0.0012g/mL was 0.99602g. The temperature of the substance being examined was adjusted to about 25°C and any excess of the substance was removed and weighed. The tared weight of the pycnometer was subtracted from the filled weight of the pycnometer. The weight per millilitre was calculated by dividing the weight in air, in g, of the quantity of liquid which filled the pycnometer at the specified temperature, by the capacity expressed in ml, of the pycnometer at the same temperature.

Gas Chromatography/Mass Spectrometric analysis of the volatile oil

Gas chromatography [104]

Gas chromatography is the most important spearation technique which uses gases as mobile phase at suitable temperature and pressure and a solid or a liquid as a stationary phase. The carrier gas containing the sample is passed through the injection point and heated. The sample get vaporized and passed through the packed column. The immediate vaporization of sample and the detector produce an electrical signal is directly proportional to the amount of compound arising from the packed column.

Mass Spectrometry

Mass spectrometry is an analytical technique that measures the mass of individual molecules and atoms. The neutral analyte molecules are converted into gas-phase ionic species. The excess energy transferred to the molecule during ionization leads to fragmentation. A mass analyzer separates these molecular ions and their charged fragments according to their m/z ratio. Finally the ion current due to these mass separated ions is detected by a suitable detector, and displayed in the form of a mass spectrum. Each of these steps is carried out under high vacuum (10-4 to 10-8 torr)

Gas chromatography/mass spectrometry

A powerful analytical method is needed for identification of every compound which can be achieved by linking GC with mass spectrometry. The separation of a complex organic mixture into individual components by using gas chromatography is an important method. Many gases and volatile oils are introduced in to the source through a small trickle from the gas reservoir. Hence GC-MS is the introduction of GC effluents without most of carrier gas into a mass spectrometer has its increasing utility in structural organic chemistry, pharmaceutical analysis and biochemistry. Here the fraction which elutes from GC column is condensed into a capillary or onto a small metal surface and these fractions are introduced into the MS source. A combination of GC/MS has many advantages in structure elucidation of a compound. All the process for identification and interpretation of collected data manually from spectrum is the time consuming process. The compound identification in gas chromatography is essentially comparative, in that the spectrum of the unknown are compared with the spectrum of known library

compounds. Recently modern mass spectrometer provides spectral data for many components to identify the components by spectral matching.

Instrument features

Name	: Hewlett Packard
Model	: GCD-1800 A
Specification	: EI source, quaterpole analyzer
Mass range	: 10 – 425 amu
Column	: fused capillary column (HP-1)
Column length	: 30 m, internal diameter -0.25mm, film thickness – 0.25µm
Temperature	: The instrument was programmed at 50-200°C at a rate of 4°C/min; and then increased at 20°C/min up to 280°C.
Carrier gas	: Helium
Flow rate	: 0.8ml/min
Split ratio	: 1:60
Ionization energy	: 70 eV
Scan time	: 1s

- Integrated gas chromatograph – electron ionization detector operated through a data system.
- Split/splitless capillary injection port.
- Generate traditional retention times and abundance information as also the mass spectral data for each sample component.
- Library search using NIST library of about 75,000 compounds

Identification of the components:

The components were identified by comparing the retention time of the peaks on HP-1 column matching against the standard library spectra of pure substances and components of known essential oils, and finally confirmed by comparison of mass spectral fragmentation patterns with the published data.

The gas chromatogram of the various components separated out of the column is given in **Fig.17** and the R_t of the eluates are presented in **Table 13** and the mass spectrum of the compounds that eluted out of the column are presented in **Fig. 18.**

RESULTS AND DISCUSSION

QUALITATIVE PHYTOCHEMICAL TESTS

The preliminary phytochemical investigation of the leaf powder and various extracts of plant material are presented in **Tables 5 & 6.** The leaf powder revealed the presence of sterols, carbohydrates. proteins, alkaloids esp. carbozole alkaloids, glycosides, coumarins, tannnis, flavonoids and phenolic compounds.

Table 5: Preliminary phytochemical screening for the leaf powder of *Causena dentata*

S.NO	TEST	RESULTS
1.	**TEST FOR STEROLS**	
	a. Salkowski's test	+
	b. Libermann- burchard's test	+
2.	**TEST FOR CARBOHYDRATES**	
	a. Benedict's test	+
	b. Fehling's test	+
3.	**TEST FOR PROTEINS**	
	a.Biuret tect	+
	b. Xanthoprotic test	+
4.	**TEST FOR ALKALOIDS**	
	a. Mayer's reagent	+
	b. Dragendroff's reagent	+
	c. Hager's reagent	+
	d. Wagner's reagent	+
5.	**TEST FOR GLYCOSIDES**	
	a. Anthraquinone glycosides	
	i) Borntrager's test	-
	ii) Modified Borntrager's test	-
	b. Cardiac glycosides	
	i) Keller Killiani test	+
	ii) Baljet test	+
6.	**TEST FOR SAPONINS**	+
7.	**TEST FOR TANNINS**	
	a) $FeCl_3$ test	+
	b) Lead acetate test	+
8.	**TEST FOR FLAVONOIDS**	
	a. Shinoda test	+
	b. Alkali test	+
9.	**TEST FOR TERPENOIDS**	+
10.	**TEST FOR VOLATILE OILS**	+
11.	**TEST FOR COUMARINS**	
	a) $FeCl_3$ test	+
	b) Fluorescence test	+

(+) indicates positive reaction (-) indicates negative reaction

Table 6: Preliminary Phytochemical Screening for the various extracts of leaf powder of *Clausena dentata* (Willd)

Tests	Petroleum ether extract	Hexane extract	Ethyl acetate extract	Chloroform extract	Methanol Extract	Ethanol Extract	Aqueous Extract
1. Test For Sterols							
a. Salkowski's test	+	+	+	-	-	-	-
b.Libermann-Burchard's test	+	+	+	-	-	-	-
2. **Test for carbohydrates**							
a. Molisch's test	-	-	-	-	-	-	+
b. Fehling's test	-	-	-	-	-	-	+
c. Benedict's test	-	-	-	-	-	-	+
3**.Test for protein**							
a. Millon's test	-	-	-	-	-	-	+
b. Biuret test	-	-	-	-	-	-	+
3.Test for Alkaloids							
a.Mayer's reagent	-	-	-	-	+	+	-
b. Dragendroff's reagent	-	-	-	-	+	+	-
c. Hager's reagent	-	-	-	-	+	+	-
d. Wagner's reagent	-	-	-	-	+	+	-
e. Test for purine group (Murexide test)	-	-	-	-	-	-	-
4.**Test for glycosides**							
a. Anthraquinone glycosides							
i) Borntrager's test	-	-	-	-	-	-	-
ii) Modified Borntrager test	-	-	-	-	-	-	-
b. Cardiac glycosides							
i) Keller Killiani test	-	-	-	-	+	+	-
ii) Baljet test					+	+	
iii) Cyanogenetic glycosides	-	-	-	-	-	-	-
iv) Coumarin glycosides	-	-	-	-	+	+	-
VI. Test for Saponins	-	-	-	-	-	-	+
VII. Test for Tannins							
i) $FeCl_3$ test	-	-	-	-	-	-	+
ii) Lead acetate test	-	-	-	-	-	-	+
VIII. Test for Flavonoids							
a. Shinoda test	-	-	-	-	+	+	+
b. Alkali test	-	-	-	-	+	+	+
IX. Test for Terpenoids	-	-	+	-	-	-	-
X. Test for Volatile Oils	-	-	-	-	-	-	-
XI. Test for Mucilage	-	-	-	-	-	-	-

(+) indicates positive reaction **(-) indicates negative reaction**

The ethanolic extract of *C. dentata* showed the presence of alkaloids (carbazole alkaloids), glycosides (cardiac glycosides), coumarins, flavones, carbohydrates, phenols, tannins, saponins and proteins. The petroleum ether extract and ethyl acetate extracts showed the presence of steroids. None of the extracts showed the presence of anthraquinones and terpenoids.

QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

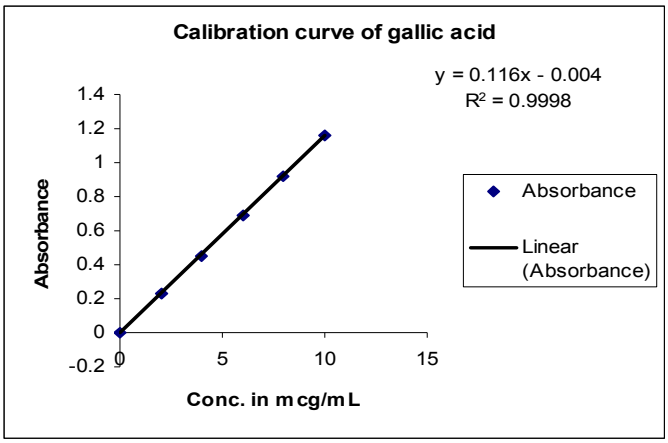
The results for the total phenolic content of 70% ethanolic extract of *Clausena dentata* are tabulated in **Table 7.**

Table 7: Total phenolic content in ethanolic extract of *Clausena dentata* in terms of gallic acid equivalents

S. No.	Conc. of gallic acid in µg/mL	Absorbance at 760nm	Conc. of ethanolic extract in µg/mL	Absorbance at 760nm*	Amount of total phenolic content in terms mgGAE/g of extract*
1	2	0.229 ± 0.010	50	1.114±0.006	141.04 ± 4.77
2	4	0.452 ± 0.006	100	1.641±0.021	198.9 ± 1.56
3	6	0.695 ± 0.005		**Average**	**169.96 ± 2.75**
4	8	0.918 ± 0.031			
5	10	1.162 ± 0.028			

*** mean of three readings ±SEM**

Fig.11: Calibration curve of gallic acid



The linear regression equation was found to be y = 0.116x-0.004 while the correlation was found to be 0.9998. The amount of phenolic content present in the extract in terms mg GAE/g of extract was found to be 169.96±12.75 by using the above linear regression equation.

Total flavonoid content

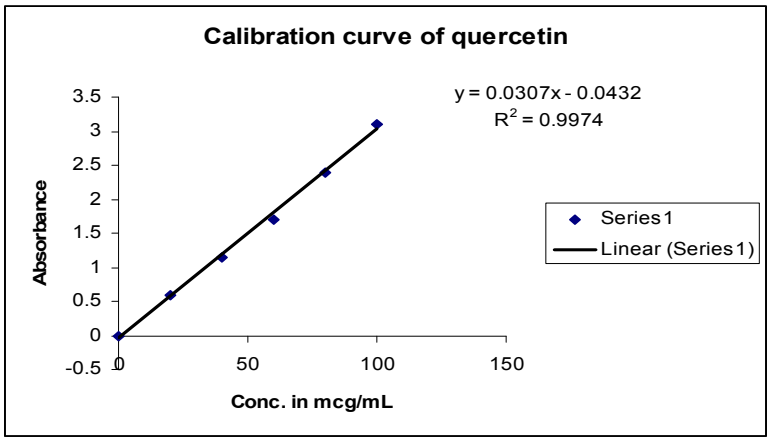
The results for total flavonoid content of 70% ethanolic extract of *Clausena dentata* are presented in **Table 8.**

Table 8: Total flavonoid content per gram of extract in terms of quercetin by aluminium chloride method

S. No.	Conc. of quercetin in µg/mL	Absorbance at 415nm	Conc. of methanolic extract in µg/mL	Absorbance at 415nm	Amt of total flavonoid content in terms mg quercetin equivalent/ g of extract
1	20	0.589 ± 0.01	50	0.064 ± 0.002	32.13±0.73
2	40	1.151 ± 0.04	100	0.196 ± 0.008	38.98±0.14
3	60	1.710 ± 0.09		**Average**	**35.56± 1.57**
4	80	2.390 ± 0.03			
5	100	3.112 ± 0.03			

***mean of three readings** ± SEM

Fig. 12: Calibration curve of quercetin



The linear regression equation was found to be y = 0.0307x-0.0432 while the correlation was found to be 0.9974. The amount of flavonoid content present in the ethanolic extract of *C. dentata* in terms mg quercetin equivalent/g of extract was found to be 35.56 ± 1.57mg/g of extract by using the above linear regression equation.

Vitamic C Content

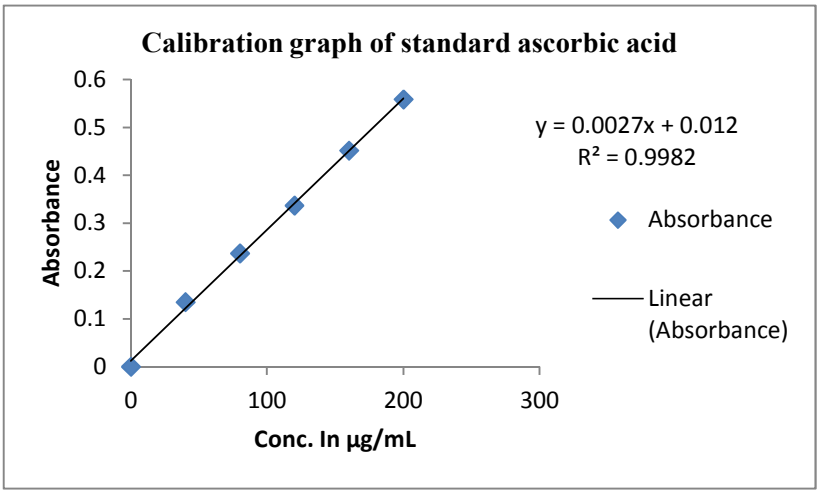
The results for vitamin C content of ethanolic extract of *Clausena dentata* are presented in **Table 9.**

Table 9: Estimation of Vitamin C in *Clausena dentata*

S. No.	Conc. of ascorbic acid in µg/mL	Absorbance at 520nm	Conc. of methanolic extract in µg/mL	Absorbance at 520nm	Amt of vitamin C present / g of extract
1	40	0.135 ± 0.000	40	0.286 ± 0.004	98.6 ± 0.006
2	80	0.265 ± 0.015	80	0.321 ± 0.007	125.80 ± 0.006
3	120	0.346 ± 0.010	120	0.456 ± 0.003	99.78 ± 0.002
4	160	0.468 ± 0.011	160	0.568 ± 0.007	132.34 ±0.002
5	200	0.525 ± 0.010	200	0.698 ± 0.008	189 ± 0.006
				Average	**129.36 ± 0.44**

***mean of three readings** ± SEM

Fig. 13: Calibration curve of ascorbic acid



The linear regression equation was found to be y = 0.003x + 0.002 and a correlation coefficient of 0.989. The amount of vitamin C content present in the ethanolic extract of *Clausena dentata* was found to be 82.89± 0.45mg/gm of fresh leaves by using the above linear regression equation.

Natural ascorbic acid is essential for the body since lack of the same impairs the normal formation of intercellular substances throughout the body including bone matrix, collagen and tooth dentine [106].

The clinical manifestation of scurvy, hemorrhage from the mucous membrane of the mouth and the gastro intestinal tract, anemia, pain in joints can be related to the lack of ascorbic acid. It is also required for normal wound healing [107]. The presence of high amount of ascorbic acid in the leaves may be useful for the above purposes and the nutritive value of the leaves has been proved by this study .

Chromatography

Thin layer chromatography of ethanolic extract of *Clausena dentata*

The results obtained are presented in **Table 10.** The extract showed 2 fluorescent spots at an R_f value of 0.8 and 0.77 when viewed under UV at 365nm after development in the mobile phase namely toluene:ether (1:1). The spots may be due to the presence of courmarin derivatives. The extract also showed 2 spots at an R_f value of 0.57(dark green) and 0.5 (organe) when viewed under UV at 365nm after development in the mobile phase namely n-butanol : n-glacial acetic acid : water (4: 4 : 1). The spots may be due to the presence of alkaloidal derivatives.

Table 10: TLC of the ethanolic extract of *C. dentata*

S. No	Solvent system	Detecting agent	No of spots	Color of spots	R_f values
1	Toluene: ether (1 : 1)	Under UV at 365nm	**2**		
			I	Fluorescence	0.80
			II	Fluorescence	0.77
2	n-butanol: Glacial acetic acid:Water (4:4:1)	Under UV light at 365nm	**2**		
			I	Dark green	0.57
			II	Orange	0.50

High Performance Thin Layer Chromatography

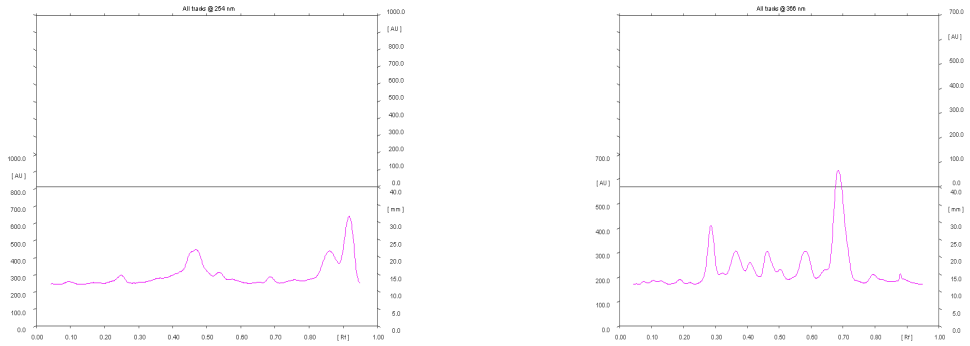
The visualization of the TLC plate of ethanolic extract of *C.dentata* at 254nm and 366nm is presented in **Fig. 14.** The photo of plate at 254nm shows the presence of 10 spots while at 366nm shows the presence 13 spots.

Fig. 14: Visualization at 254nm and 366nm

@254nm **@366nm**

Fig. 15: 3D Display of the fingerprint profile at 254nm and 366nm

@ 254nm **@366nm**



The 3D display of the fingerprint profile and the peak display of ethanolic extract of *C. dentata* at 254nm and 366nm is presented in **Figs. 15 & 16.** The display at 254nm shows the presence of 10 peaks while at 366nm shows the presence 13 peaks. The R_f values of the peaks along with the area under the curve for each peak at 254 and 366 nm are tabulated in **Table 11.**

Fig. 16: Peak display of ethanolic extract of *C.dentata* at 254nm and 366nm

@ 254nm **@366nm**

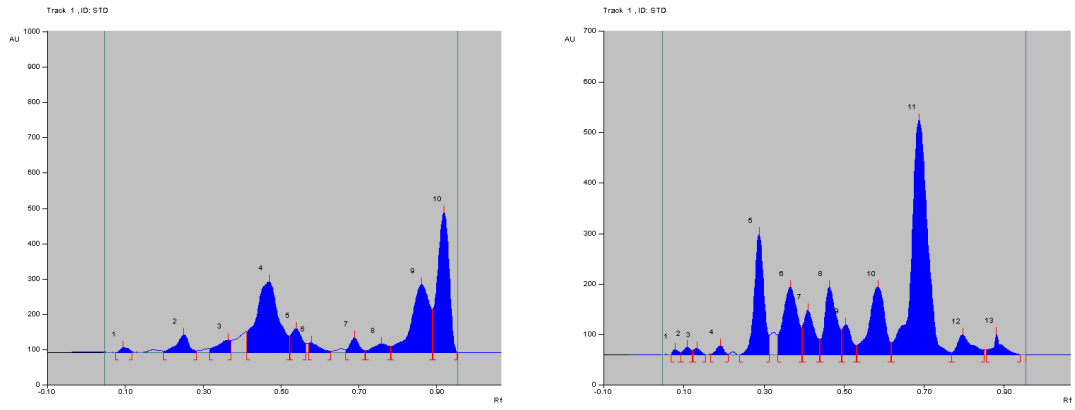


Table 11: R_f **values and area under the curve for each peak at 254 and 366nm**

S. No	@254nm		@366nm	
	R_f Value	AREA (AU)	R_f Value	AREA (AU)
1	0.09	279.7	0.08	131.8
2	0.25	1411.7	0.11	236.1
3	0.36	1049.2	0.13	189.8
4	0.47	10081.8	0.19	300.2
5	0.54	1675.8	0.29	4984.9
6	0.58	695.9	0.36	4166.7
7	0.69	868.0	0.41	2082.3
8	0.76	853.3	0.46	3399.2
9	0.86	7910.2	0.50	1219.0
10	0.92	10378.7	0.58	4572.1
11			0.69	16285.2
12			0.79	1264.2
13			0.88	832.9

The HPTLC finger print profile of the ethanolic crude plant extract of *Clausena dentata* showed the Rf values indicating the plant contains many medicinally active compounds were responsible for its therapeutic activity. The peak display and finger print profile can be utilized for the quality control for different test samples by comparing with different chromatogram.

EVALUATION OF VOLATILE OIL OF *Clausena dentata*

Organoleptic and Physical parameter evaluation

The organoleptic parameters of the volatile oil of the *Clausena dentata* which includes color, odor, taste, solubility, refractive index, weight/mL were carried out and the observations were tabulated in **Table 12.**

Table 12 : Organoleptic & Physical evaluation of the volatile oil of *Clausena dentata*

S. No	Parameters*	Results
1	Color	Yellowish brown
2	Odor	Fragrant and aromatic
3	Taste	Spicy
4	Solubility	Soluble in ether, ethyl acetate, ethanol, methanol and DMSO
5	Refractive index	1.53 ± 0.00
6	Weight per milliliter	0.7476 ± 0.02

GC/MS evaluation of the volatile oil

The gas chromatogram obtained for the volatile oil of the leaves of *Clausena dentata* is depicted in **Fig. 17**. The mass spectrum of the various eluates along with the retention times are presented in **Fig. 18.** The retention time, the peak width and peak areas for the major eluates are presented in **Table 13.**

Table 13: Chemical composition of leaf essential oil from *Clausena dentata*

S.No.	Retention time in (min)	Peak width (min)	Peak Area
1	3.9	0.0199	15461983.5
2	7.2	0.0226	7344692.70
3	12.9	0.0299	83375634.8
4	13.3	0.0274	53571427.4
5	13.6	0.0255	22568775.6

The chemical constituents present in the oil were found by matching the mass spectrum with the existing library of compounds and were found to be E-anethole, estragole (methyl chavicol), α-elemene. α-pinene, borneol and asarone.

PHARMACOLOGICAL EVALUATION

The ethanolic extract of *C. dentata* was subjected to pharmacological screening and the following activities were carried out namely *in vitro* antioxidant activity, antibacterial, antifungal, mosquito larvicidal activities.

ANTIOXIDANT ACTIVITY

Antioxidant compounds in plants play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenol acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Some compounds, such as gallates, have strong antioxidant activity, while others, such as the mono-phenols are weak antioxidants. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases [108, 109].

Antioxidants are chemicals compounds that donate their own electrons to free radicals, thus preventing cellular damage. Continuous ingestion of Antioxidants reduces the cellular damage and ageing process [110].

Current evidence suggests that the search for new antioxidant compounds from natural sources has increased. This is due to health concerns regarding the potential toxic and side effects generated from synthetic antioxidants like BHA and BHT as well as changes in consumer preferences for natural products [111].

Method 1: Free radical Scavenging activity using diphenyl picryl hydrazyl (DPPH) free radical

The free radical scavenging activity of the extracts is evaluated by assessing their ability to reduce the colour of DPPH in ethanol according to Brand Williams [112]. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of specific compound or plant extracts [113].

Principle

A simple method that has been developed to determine the antioxidant activity of plants utilizes the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The odd electron in the DPPH free radical gives a strong absorption maximum at 517nm and is purple in color. The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 51 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured [113].

$$DPPH^{\cdot} + AH \rightarrow DPPH - H + A^{\cdot}$$

Instrument

Shimadzu UV Visible spectrometer, Model 1800

Reagents

0.1mM Diphenyl Picryl Hydrazyl Radical in ethanol.

Procedure [114,115]

A stock solution of 1mg/mL concentration of ethanolic extract of *C. dentata* was prepared. To the 1mL of various concentrations of test samples, 4mL of DPPH was added. Control was prepared without sample in an identical manner. DPPH was replaced by ethanol in case of blank. The reaction was allowed to be completed in the dark for about 30min. Then the absorbance was measured at 517nm. Vitamin C was used as standard. The percentage scavenging was calculated using the formula [(Control-Test)/Control] x 100. A graph was constructed by plotting concentration versus percentage inhibition and a linear regression equation calculated. The concentration of the sample required for 50% reduction in absorbance (IC_{50}) was calculated using linear regression analysis. A triplicate reading was taken and average was calculated. The results obtained are presented in **Table14** and **Fig. 19.**

Method 2: Nitric oxide scavenging activity assay [116 & 117]

Principle

Nitric oxide scavenging activity was determined according to the method reported by Green *et al*., (1982) [116]. The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using

Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. The nitrite ions produced diazotizes sulphanilamide and then the diazonium salt reacts with NN naphthyl ethylene diamine dihydrochloride to give a pink colour chromophore which has a maximum absorption at 546nm.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

10mM sodium nitroprusside

Phosphate buffered saline pH 7.4

2% sulphanilamide in ortho phosphoric acid

0.1% naphthyl ethylene diamine dihydrochloride

Procedure

To 1mL of freshly prepared solution of sodium nitroprusside, 2.5mL phosphate buffered saline pH 7.4 was added and mixed with 1mL of extracts at various concentrations (44.44 to 266.67µg/mL), then the mixture was incubated at 25^{o}C for 30min. From the incubated mixture 1.5mL was taken. To this, 1mL of sulphanilamide in phosphoric acid and 0.5mL of naphthyl ethylene diamine dihydrochloride were added and the absorbance was measured at 546nm using reagent as blank. A control without the extract was also prepared and the subjected to the above procedure. Ascorbic acid was used as a standard. The percentage inhibition of nitric oxide radical generated was calculated using the following formula: % inhibition = [(Control-Test)/Control] x 100. A graph was constructed using concentration versus percentage inhibition and the linear regression equation calculated. The IC_{50} was calculated using linear regression analysis.

The results obtained for the nitric oxide scavenging activity assay are presented in **Table 15** and **Fig. 20**.

Method 3: Determination of scavenging activity against hydrogen peroxide [118]

Principle:

The free radical scavenging activity of plant extract against hydrogen peroxide was determined by using the method of Ruch *et al,* 1989 [118]. The principle was based on the capacity of the extracts to decompose the hydrogen peroxide to water.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

6% hydrogen peroxide diluted with water in the ratio of 1:10

0.1M phosphate buffer pH 7.4

Procedure

The ethanolic extract of *Clausena dentata* was dissolved in ethanol to get a stock solution containing 1mg/mL. Varying quantities of the stock solution were added to 3.8mL of 0.1M phosphate buffer solution (pH 7.4) and then 0.2mL of hydrogen peroxide solution was added and the absorbance was measured at 230nm after 10min. The reaction mixture without sample was used as blank. Ascorbic acid was used as standard. The percentage inhibition of hydrogen peroxide was calculated using the formula = $(A_{control} - A_{sample}) / A_{control} \times 100$. The concentration of extract to produce 50% inhibition was found using linear regression analysis and the results obtained are presented in **Table 16** and **Fig. 21.**

Method 4: Ferric Reducing Antioxidant Power (FRAP) Assay [119, 120]

Total antioxidant activity is measured by FRAP assay of Benzie *et al.,* 1999 (119). The ferric reducing antioxidant power assay measures the potential of antioxidants to reduce the Fe^{3+} and 2,4,6 tripyridyl-s-triazyne (TPTZ) complex present in stoichiometric excess to the blue coloured Fe^{2+} complex which increases the absorption at 593nm.

Principle

At low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue colour) can be monitored by measuring the change in absorption at 593nm. The reaction is non specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance is therefore, directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

FRAP Reagent

a) Acetate buffer 30mM pH 3.6: Weigh 3.1g sodium acetate trihydrate and add 16 ml of glacial acetic acid and make the volume to 1 L with distilled water.

b) TPTZ (2, 4, 6-tripyridyl-s- triazine) (M.W. 312.34) 10mM in 40mM HCl

c) $FeCl_3$. $6H_2O$ (M.W. 270.30) 20mM

The working FRAP reagent was prepared freshly by mixing a b & c in the ratio of 10:1:1 at the time of use.

Procedure

50µl, 100µl, 150µl, 200µl, 250µl, 300µl of 2mg/mL concentration of methanolic extract of *Clausena dentata* were taken and mixed with 3mL of working FRAP reagent and absorbance was measured at 0min after vortexing at 593nm. Thereafter samples were placed at 37ºC in water bath and absorption was again measured after 4min. Ascorbic acid was used as standard. The FRAP value of the sample was calculated using this equation: [Change in absorbance of sample from 0-4min/ change in absorbance of standard from 0-4min] * Frap value of standard. The result obtained for the FRAP assay are presented in the **Table 15** and **Fig. 22 & 23.**

ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACT AND VOLATILE OIL OF *CLAUSENA DENTATA* [121, 122, 123, 124]

The ethanolic extract of *C.dentata* was screened for antibacterial activity on eight bacterial strains and the zone of inhibition, minimum inhibitory concentration was determined.

Preparation of extract

The ethanolic extract of *Clausena dentata* was dissolved in DMSO solution and to produce a stock solution of 50mg/mL. A quantity of 10, 20 and 30µL were impregnated on the plain sterile disc and dried.

Preparation volatile oil suspension

The volatile oil dissolved in DMSO solution to produce 20 %v/v solution and was used for antibacterial activity. A quantity of 10µL, 20µL, 30µL was applied on to the sterile disc in the cultured plates.

Preparation of MH agar medium

Muller Hinton agar (MH, HI media) was used for culture of bacterial strains. It consists of Beef 2g, casein acid hydrolysate 17.5g, starch 1.5g and agar 17g (pH 7.4 ± 0.2). MH agar (38g) was weighed and dissolved in 1000ml of distilled water and adjusted to pH 7.3 ± 0.2, sterilized by autoclaving at 121^{o}C for 15min at 15psi pressure and was used for sensitivity tests. This medium was used for screening *Staphylococcus aureus, Staphylococcus albus, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Escherichia coli.*

Preparation of Blood agar medium

Blood agar medium (BAM) contains mammalian blood usually at a concentration of 5–10%. Blood agar medium are enriched, differential media used to isolate fussy organisms and detect hemolytic activity. β-hemolytic activity will show lysis and complete digestion of red blood cell contents surrounding colony.

The media contains nutrient substrate (heart extract and peptones) 20.0; sodium chloride 5.0: agar-agar 15.0. 40g of the above media was suspended in 1000mL of distilled water and autoclaved for 15min at 121°C and cooled to 45-50°C. The prepared medium was clear and yellowish-brown and then 5-8% defibrinated blood was added.

The blood agar medium was used for the antibacterial activity against the bacterial strains namely *Streptococcus pyogens* and *Streptococcus vridans*

Preparation of bacterial cultures

The various bacterial strains like *E. coli, P. aeruginosa, K. Pnemoniae* etc. were utilized for screening antibacterial activity. A few colonies of the bacterial strains picked from the agar slopes and were inoculated into 4mL peptone water in a test tube. They were incubated for 24h to form suspensions. The suspension was diluted with saline if necessary.

The visual density equivalent to standard prepared by adding 0.5ml of 1% barium chloride to 99.5mL of 1% sulphuric acid. These suspensions were then used for seeding.

Disc diffusion technique

The MH media was poured aseptically into sterilized petridishes and the petridishes were swirled to settle the agar and allowed to cool. The bacterial strains were the seeded on the MH agar media by streaking the plate with a sterile swab containing the strain. The plain sterile discs were impregnated with various volumes of the extract, volatile oil and DMSO (negative control) and dried. Amikacin (30µg/disc) was used as standard. The discs were then placed on the plate with the help of sterile needle and the plates were incubated at 37°C for 24h.

Similarly the blood agar medium was used for antibacterial activity against the *Streptococcus* species and the above procedure was adopted.

The results were read and the zone of inhibition was then measured and they are presented in **Tables 18 & 19.** The photographic representations of the antibacterial activity are presented in **Figs. 24 & 25.**

ANTI FUNGAL ACTIVITY ETHANOLIC EXTRACT AND VOLATILE OIL OF *CLAUSENA DENTATA* [123,124,125, 126]

Disc diffusion method

Inoculum preparation

The fungal colony to be tested was grown in potato dextrose agar slant at 35° C to induce the conidium and sporangiospore formation. After 7-10 days of incubation with well grown spores, the culture was taken for testing. 5ml of 0.85% sterile saline was added to the culture tube and the suspension were made by gently probing the colonies with the tip of Pasteur pipette. With the help of sterile pipettes, the saline with conidia was transferred in to a sterile screw cap tube. The tube was then vortexes for 30sec to 1min and allowed to stand at room temperature for 5 to 10min for the heavier particles to settle down. The upper homogenous suspensions were collected and the densities of the conidial suspensions were read and adjusted the optical density (OD) to be between 0.09 and 0.11 for *Aspergillus* species, 0.15 to 0.17 for *Fusarium* species by using UV Visisble spectrophotometer at 530nm. The suspensions were diluted 1:50 in RPMI 1640 medium. The final concentration of the conidia was $0.2 – 1 \times 10^4$ cfu/mL.

Preparation of media

Sabourand Dextrose Agar (Hi- media) media (SDA) was used for cultivation of fungi and particularly pathogenic fungi associated with skin infections. Formula gm/litre Peptone - 10g, dextrose 40g and agar 15 g; pH 5.6 $\pm$ 0.2. SDA (65g) was dissolved in

1000ml of distilled water. The medium was sterilized by autoclaving at 121^{o}C for 15 minutes at 15 psi pressure.

Preparation of volatile oil suspension

A 20%v/v of the volatile oil of *Clausena dentata* solution was prepared by dissolving the required quantity of the oil in DMSO. A quantity of 10µL, 20µL, 30µL was applied on to the sterile disc in the cultured plates.

Preparation of extract

A stock solution of 400mg/mL of the ethanolic extract of the plant *Clausena dentata* was prepared by dissolving the required quantity of the extract in DMSO. 20, 25 and 30µL were impregnated on the plain sterile disc and dried.

Procedure

The entire dried agar surface was evenly streaked in three different directions with a sterile cotton swap dipped into the inoculum suspension. The plate was allowed to dry for 20min. Using a pair of flame sterilized forceps the extract containing discs were applied on to the surface of the inoculated plates. The plates were incubated at 35°C for 48h. The plates were read at 24h and 48h.

The MIC and zones of inhibition were read and presented in **Table 20 to 22.** The photographic representations are presented in **Figs. 26 & 27** while the graphical representation of the zone of inhibition is presented in **Figs. 28 & 29.**

MOSQUITO LARVICIDAL ACTIVITY OF ETHANOLIC EXTRACT AND VOLATILE OF *Clausena dentata*

The infectious human diseases like malaria, filariasis, Japanese encephalitis, dengue hemorrhagic fever, chikungunya and yellow fever are transmitted by mosquitoes which cause millions of deaths every year. This is an important factor to consider destroy mosquitoes for prevention of infections. Nowadays many chemical insecticides being used to control mosquito vector which has many inconsistence like fighting resistance to vectors and adverse environmental hazards etc., and for this reason plant products are one of the best remedy to control for mosquito [127, 128].

Preparation of stock solution of ethanolic extract

A stock solution of 400mg/mL of the ethanolic extract of the plant *Clausena dentata* was prepared by dissolving the required quantity of the extract in distilled water.

Preparation of stock solution of volatile oil

A 20%v/v of the volatile oil of *Clausena dentata* solution was prepared by dissolving the required quantity of the oil in DMSO.

Procedure for extract

The method was adopted by Arivoli S and Samuel Tennyson 2012 (129). The larvicidal activity of plant extract was carried out on late 3rd and early 4th instar larvae of ***Anopheles stephensi,*** a primary vector of urban malaria, ***Culex quinquefaciatus***, and a common vector of filariasis. The mosquito larvae were obtained from ICMR, Madurai. Twenty larvae were released in 500mL beaker containing 200mL distilled water with varying concentration of plant extract (1, 2, 4, 10, 20, 40, 80 & 100mg). The larvae were provided with dog biscuit and yeast powder in a ratio of 3:2 as nutrients. The experiments

were carried out at room temperature (26^0C± 2°C). Three replicates of each concentration were run under the same microclimatic conditions along with untreated control. The mortality was monitored for 24h. The results obtained are presented in **Table 23 & Figs. 30 & 31.**

Procedure for volatile oil [130]

The method of Momin and Nair (2001) was modified and employed to conduct the mosquito larvicidal effect test. Ten fourth-instar mosquito larva were placed in 24.5mL distilled water, followed by addition 500µL of DMSO solution containing the test essential oil in a 30 mL beaker, with gentle shaking to ensure a homogenous test solution, and each beaker was left at the ambient temperature concentrations of 400, 200, 100, 50, and 25µg/mL of essential oil were tested at 50, 25, 12.5 and 6.25µg/mL. The control was prepared with 24.5mL of distilled water and 500µL of DMSO solution. The mortality was recorded after 24h of exposure and larvae were starved within this period. The toxicity and effect were reported as LC_{50} and $LC_{90,}$ representing the concentration in µg/ml with 50 and 90% larvae mortality rate in 24h, respectively. The mortality was monitored for 24h. The results obtained are presented in **Table 24 & Figs. 32 & 33.**

RESULTS AND DISCUSSION

Method I: Free radical Scavenging activity using diphenyl picryl hydrazyl (DPPH) free radical

The results obtained for the free radical scavenging activity against DPPH radical is presented in **Table 14.**

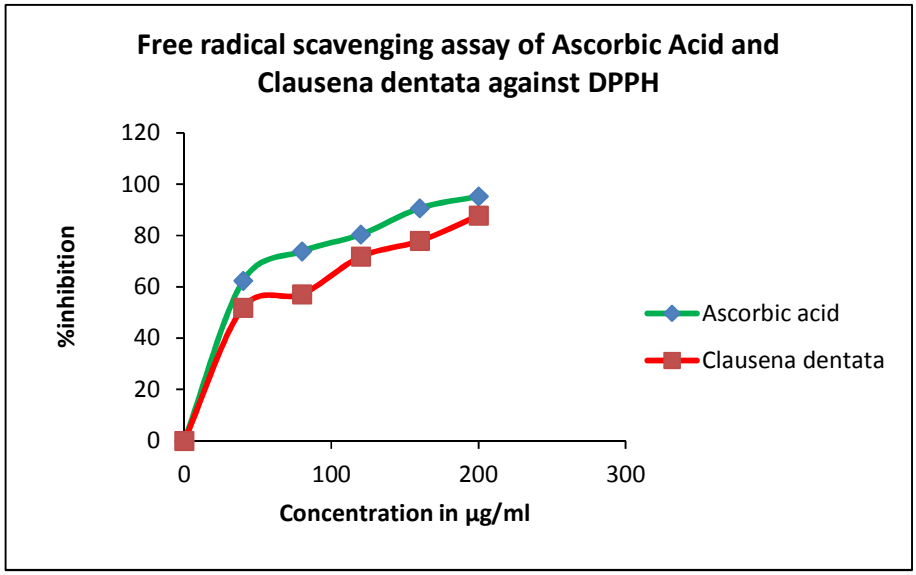
Table 14: Percentage inhibition of ethanolic extract of *C. dentata* and standard ascorbic acid against DPPH at 517nm

S. No.	Conc. in µg/mL	Percentage inhibition by ascorbic acid	Percentage inhibition by *Clausena dentata*
1	40	62.42 ± 1.25	51.93 ± 1.85
2	80	73.90 ± 0.75	57.18 ± 0.45
3	120	80.43 ± 0.52	71.83 ± 0.37
4	160	90.66 ± 0.32	77.94 ± 0.32
5	200	95.33 ± 0.50	87.82 ± 0.15
	IC_{50}	**57.09µg/mL**	**79.7µg/mL**

***mean of three readings ± SEM**

.

Fig. 19: Free radical scavenging assay of ascorbic acid and ethanolic extract of *C. dentata* against DPPH at 517nm



From the table, it can been seen that the ethanolic extract of *C. dentata* showed a percentage inhibition of 87.82 ± 0.15 while ascorbic acid showed a percentage inhibition of 95.33 ± 0.50 at a concentration of 200µg/mL. The IC_{50} value calculated using the linear regression analysis was found to be 79.7 and 57.09µg/mL for ethanolic extract and ascorbic acid respectively. The extract possessed a good radical scavenging capacity

Method 2: Nitric oxide scavenging activity

The results obtained for the free radical scavenging activity against nitric oxide radical are presented in **Table 15.**

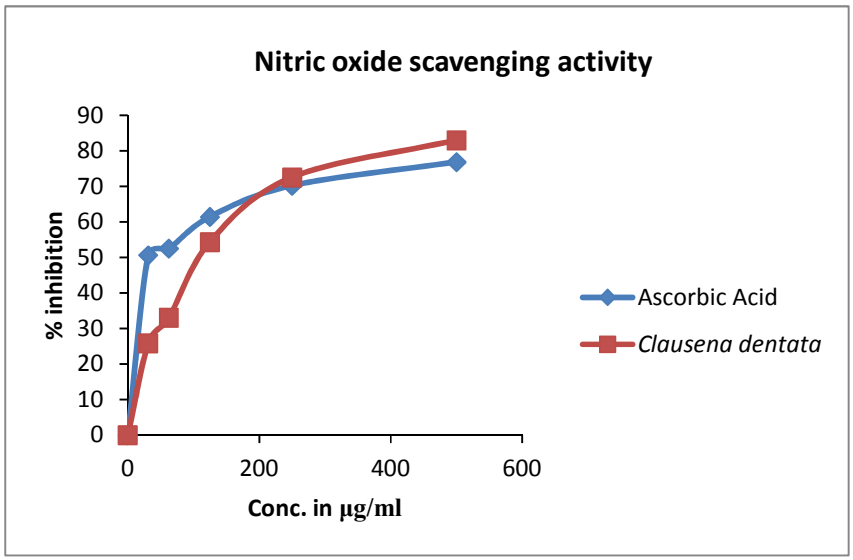
Table 15: Percentage inhibition of ascorbic acid and ethanolic extract of *C. dentata* against nitric oxide at 546nm

S. No.	Conc. in µg/mL	Percentage inhibition by standard ascorbic acid	Percentage inhibition by extract
1	31.25	50.68±1.02	25.88 ± 1.09
2	62.5	52.56 ± 2.57	33.08 ± 0.60
4	125	61.45 ± 0.06	54.36 ± 0.64
5	250	70.21 ± 2.12	72.56 ± 1.11
6	500	76.88±1.04	83.00±2.42
	IC_{50}	**142µg/mL**	**197µg/mL**

***mean of three readings ± SEM**

From the table, it can been seen that the ethanolic extract of *C. dentata* showed a percentage inhibition of 83.00 ± 2.42 while ascorbic acid showed a percentage inhibition of 76.88 ± 1.04 at a concentration of 500µg/mL. The IC_{50} value calculated using the linear regression analysis was found to be 197 and 142µg/mL for ethanolic extract and ascorbic acid respectively.

Fig 20: Nitric oxide scavenging activity of ascorbic and ethanolic extract of *C. dentata*



Method 3: Determination of scavenging activity against hydrogen peroxide

The results obtained for the scavenging activity against hydrogen peroxide are presented in **Table 16.**

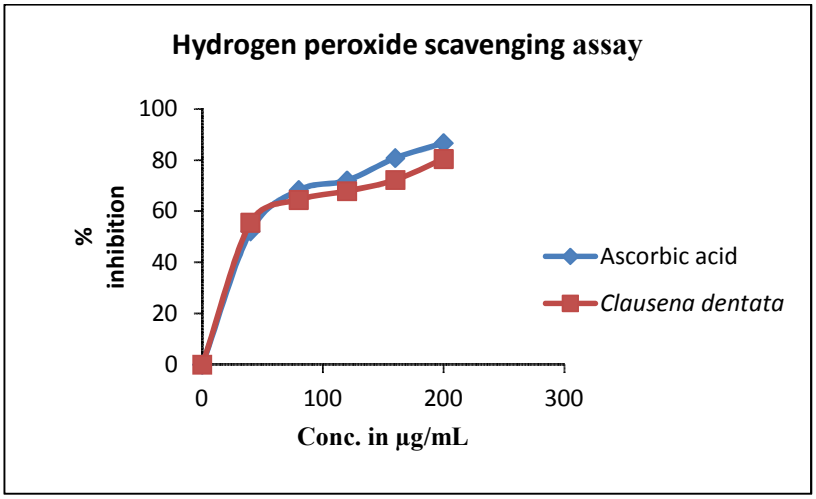
Table 16: Percentage inhibition of hydrogen peroxide of ascorbic acid and the ethanolic extract of *Clausena dentata*

S.No.	Conc. in µg/mL	Percentage inhibition	
		Ethanolic extract of *C. dentata*	Ascorbic acid
1	40	55.52 ± 0.32	52.22±0.32
2	80	65.48 ±0.03	68.37±0.12
3	120	67.88±0.55	72.05±0.17
4	160	72.26 ± 1.21	80.82±0.29
5	200	80.47 ± 0.30	86.70±0.09
	IC_{50}	**73.35 µg/mL**	**79.41 µg/mL**

***mean of three readings ± SEM**

From the table, it can been seen that the ethanolic extract of *C. dentata* showed a percentage inhibition of 80.47 ± 0.30 while ascorbic acid showed a percentage inhibition of 86.70±0.09 at a concentration of 200µg/mL. The IC_{50} value calculated using the linear regression analysis was found to be 73.35 and 79.41µg/mL for ethanolic extract and ascorbic acid respectively. The extract possessed a better hydrogen peroxide scavenging activity comparable to that of ascorbic acid.

Fig 21: Percentage Inhibition of hydrogen peroxide by ethanolic extract of *C.dentata*



Method 4: Ferric reducing anti oxidant assay

The results obtained for the scavenging activity against hydrogen peroxide are presented in **Table 17.** From the table, it can be seen that the ethanolic extract of *C. dentata* showed an absorbance of 0.616 ± 0.004 for a concentration of 500µg/mL while ascorbic acid showed an absorbance of 0.252 ± 0.004 at a concentration of 30µg/mL. The extract shows a dose dependent reducing ability. The graphical representations of the reducing power activity of the ethanolic extract of *C. dentata* and ascorbic acid are presented in **Figs. 22 & 23.**

Table 17: Ferric reducing anti-oxidant assay of ascorbic acid and ethanolic extract of *Clausena dentata*

S. No	Conc. in µg/mL	Absorbance of ascorbic acid	Conc. in µg/mL	Absorbance of ethanolic extract
1	10	0.098±0.006	31.25	0.362±0.05
2	15	0.176±0.007	62.5	0.378±0.002
3	20	0.198±0.002	125	0.439±0.008
4	25	0.232±0.009	250	0.498±0.003
5	30	0.252±0.004	500	0.616±0.004

***Mean of three readings ± SEM**

Fig. 22: Ferric reducing anti-oxidant assay of ethanolic extract of *Clausena dentata*

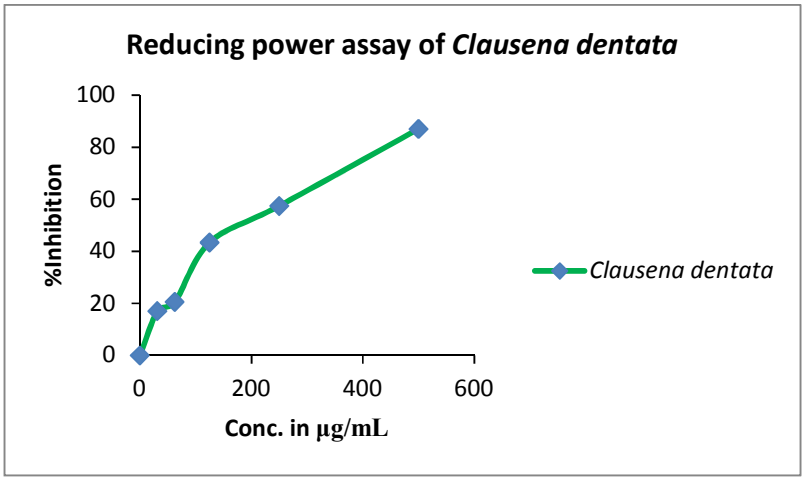
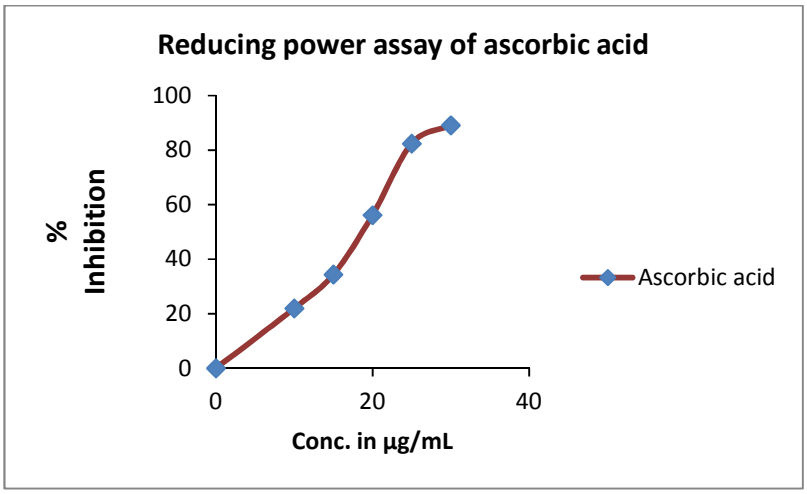


Fig. 23: Ferric reducing anti-oxidant assay of ascorbic acid



ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACT AND VOLATILE OIL OF *C. dentata*

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration was defined as the lowest concentration of the extract that allows no more than 20% growth of microbes after incubation on agar at 37ºC for 18-48h. The minimum inhibitory concentrations for the ethanolic extract and the volatile oil against various organisms are presented in **Table 18.** and the photographs of the antibacterial activity are presented in **Figs. 24 & 25.** From the table, it can be observed that there was no growth of the microorganisms at a concentration of 1.5mg/disc of ethanolic extract and 20µL/disc of a 20%v/v solution of the volatile oil in DMSO.

Table 18 : Susceptibility test for ethanolic extract and volatile oil of *C. dentata* against various microorganisms

S. No.	Name of the extract and volatile oil	Conc.	1	2	3	4	5	6	7	8
1	DMSO	--	+	+	+	+	+	+	+	+
2	Amikacin	30µg/disc	-	-	-	-	-	-	-	-
3	Ethanolic extract of *C. dentata*	0.5mg/disc	+	+	+	+	+	+	+	+
		1.0mg/disc	+	+	+	+	+	+	+	+
		1.5mg/disc	-	-	-	-	-	-	-	-
4	20% Volatile oil in DMSO	10µL/disc	+	+	+	+	+	+	+	+
		20µL/disc	-	-	-	-	-	-	-	-
		30µL/disc	-	-	-	-	-	-	-	-

NOTE: (+) indicates growth (-) indicates no growth

1. *Staphylococcus aureus 2. Staphylococcus albus 3. Escherhia coli 4. Klebsiella pneumonia 5. Proteus mirabilis 6. Pseudomonas aeruginosa 7. Streptococcus vridans 8. Streptococcus pyogens*

Zones of inhibition

The zones of inhibition obtained for various concentrations of the ethanolic extract and volatile oil of *Clausena dentata* are presented in **Table 19.**

Table 19: Zone of inhibition against various microorganisms

S. No	Name of the organism	Zone of inhibition (in mm)*			
		Standard	70% ethanolic extract	Volatile oil	
				20µl	30µl
1.	*Escherichia coli*	24.0 ± 0.0	22.0 ± 0.5	9.0 ± 1.0	20.0±0.0
2.	*Klebsiella pneumonia*	24.0 ± 3.0	18.0 ± 0.5	17.0 ± 3.0	20.0±0.0
3.	*Proteus mirabilis*	26.0 ± 0.0	26.0 ± 0.5	12.0 ± 0.0	22.0±0.0
4.	*Staphylococcus aureus*	26.0 ± 1.0	20.0 ± 0.5	06.0 ± 1.0	20.0±1.0
5.	*Staphylococcus albus*	26.0 ± 2.0	20.0 ± 2.5	10.0 ± 2.0	20.0±2.0
6.	*Pseudomonas aeruginosa*	25.0 ± 0.5	18.0 ± 0.5	10.0 ± 0.5	20.0±0.5
7.	*Streptococcus vridans*	22.0 ± 0.1	15.0 ± 0.5	06.0 ± 0.1	18.0±0.2
8.	*Streptococcus pyogens*	26.0 ± 0.2	09.0 ± 0.5	06.0 ± 0.2	18.0±0.1

*** mean of 2 readings ± SEM**

The zone of inhibition of both the extract and oil were compared to that of standard drug amikacin. The pictorial representations of the same are presented in **Figs. 24 & 25.** The antibacterial activity of the ethanolic extract and volatile oil of *C. dentata* was evaluated on four Gram positive and six Gram negative bacteria by disc diffusion method using amikacin (30µg/disc) as standard antibiotic disc. The plant extract showed zone of inhibition to almost all the strains (at a dose 1.5mg). The crude extract at the concentration of 1.5mg/disc showed 15, 9, 20, 20mm diameter zone of inhibition against Gram positive *Streptococcus vridans, Streptococcus pyogens, Staphylococcus aureus,* and *Staphylococcus albus* respectively and 18, 26, 18 & 22mm diameter against Gram negative *Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pnuemoniae* and *Escherichia coli* **(Table 19).**

On the other hand, the volatile oil showed zone of inhibition to almost all the strains (at a dose 4µL/disc). The volatile oil at a concentration of 6µL/disc showed 18, 18, 20, 20 mm diameter zone of inhibition against Gram positive *Streptococcus vridans, Streptococcus pyogens, Staphylococcus aureus,* and *Staphylococcus albus* respectively and 20, 22, 20 & 20 mm diameter against Gram negative *Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pnuemoniae* and *Escherichia coli* **(Table 19).**

The standard antibiotic amikacin (30µg/disc) showed significant antibacterial activity against all tested Gram positive and Gram negative bacteria. Amikacin at a concentration of 30µg/disc showed 22, 26, 26, 26 mm diameter zone of inhibition against Gram positive *Streptococcus vridans, Streptococcus pyogens, Staphylococcus aureus,* and *Staphylococcus albus* respectively and 25, 26, 24 & 24 mm diameter against Gram negative *Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pnuemoniae* and *Escherichia coli* **(Table 19).**

The results implicated that the Gram negative bacteria were more sensitive to the extract than the gram positive bacteria. This is possibly because of the presence of outer membrane that serves as an effective barrier in gram-negative species (131). In the case of the volatile oil, the activity was equal in both gram positive and gram negative bacteria.

An important characteristic of essential oils and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable [132,133]. Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death [134]. In general the gram-positive bacteria are more resistant to the

essential oils than gram-negative bacteria [135] but in the present study the gram-positive bacteria was equally susceptible as gram negative bacterial to volatile oil of *C. dentata*.

Although the antibacterial activities of the essential oils from many herb species have been extensively surveyed for anti bacterial activity (136), their antimicrobial mechanisms have not been reported in great detail. Since the active antimicrobial compounds of essential oils are terpenes and phenolics in nature, it seems reasonable to suppose that their modes of action might be similar to those of other phenolic compounds (137). Any individual essential oil contains complex mixtures of such compounds, however, little is known about the effect of the interaction between the individual constituents on the antimicrobial activity. Interactions between the constituents may lead to additive, synergistic, or antagonistic effects (138).This study has shown that essential oil of *C. dentata* possesses a significant activity against human pathogens. These results confirm the potential use of *C. dentata* essential oil as an alternative antimicrobial agent in natural medicine for the treatment of numerous infectious diseases(139).

ANTI FUNGAL ACTIVITY ETHANOLIC EXTRACT AND VOLATILE OIL OF *CLAUSENA DENTATA*

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration was defined as the lowest concentration of extract and volatile oil that allows no more than 20% growth of microbes after incubation on SDA media at 37°C for 24h. The results obtained for the MIC of antifungal activity of the extract and volatile oil are presented in **Table 20** and the photographs of the same are presented in **Figs. 26 & 27.**

Table 20: MIC of ethanolic extract and volatile oil of *Clausena dentata* against various fungal isolates

S. No	Name of the fungal isolate	MIC of the standard Itraconazole (µg/disc)	MIC of volatile oil 20% in DMSO (µl /disc)	MIC of ethanolic extract in (mg/disc)
1	*Microsporum gypseum 16 15*	10	30	8
2	*Aspergillus flavus 16 15*	10	20	8
3	*Aspergillus fumigatus 14, 13*	10	10	8
4	*Candida albicans 22 24*	10	10	8
5	*Trichophyton mentagrophytus 16*	10	20	Not tested

From the **Table 20,** it can observed that the minimum inhibitory concentration of ethanolic extract of *Clausuna dentata* against the fungi *Microsporum gypseum, Aspergillus flavus, Aspergillus fumigates, and Candida albicans* was found to be 8mg/disc.

It was also observed that MIC was 10µL/disc against the fungal isolates *Aspergillus fumigates* and *Candida albicans* and 20µL/disc against the fungal isolates *Aspergillus flavus* and *Trichophyton mentagrophytus* and 30µL/disc against the fungal isolate of *Microsporum gypseum.* The antifungal activity of extract and volatile oil was determined by using standard drug itraconazole (10µg/disc).

Zone of inhibition

The results obtained for zones of inhibition for various concentrations of extract against various fungal isolates are presented in **Table 21.** From the table, it can be observed that the ethanolic extract showed a higher zone of inhibition against *C. albicans.* The zones of inhibition for the other three fungal isolate namely *M. gypseum, A.*

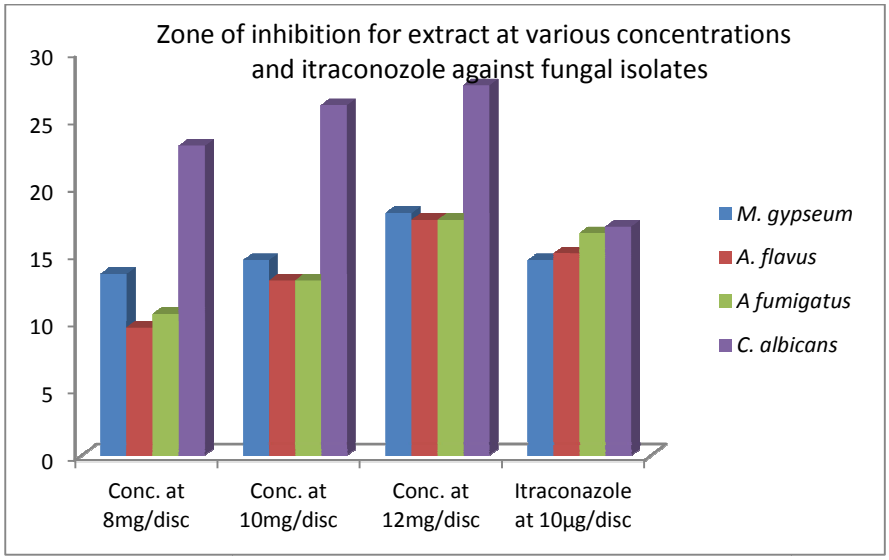
flavus and *A. fumigates* were nearly the same. The graphical representation of the same is presented in **Fig. 28.**

Table 21: Zone of inhibition for ethanolic extract against various microorganisms

S. No	Name of the organism	Itraconazole	Zone of inhibition (in mm)*		
			Conc. of extract in mg/disc		
			8	10	12
1.	*Microsporum gypseum*	14.5±0.0	13.5±0.5	14.5±0.5	18.0±0.0
2.	*Aspergillus flavus*	15.0±3.0	09.5±0.5	13.0±1.0	17.5±0.5
3.	*Aspergillus fumigatus*	16.5±0.0	10.5±0.5	13.0±0.0	17.5±0.5
4.	*Candida albicans*	17.0±1.0	23.0±1.0	26.0±0.0	27.5±0.0

*** mean of 2 readings ± SEM**

Fig. 28: Zone of inhibition for extract & itraconozole against various microorganisms



The results obtained for zones of inhibition for various concentrations of the volatile oil of the leaves of *C. dentata* against various fungal isolates are presented in **Table 22.** From the table, it can be observed that the volatile oil showed nearly the same

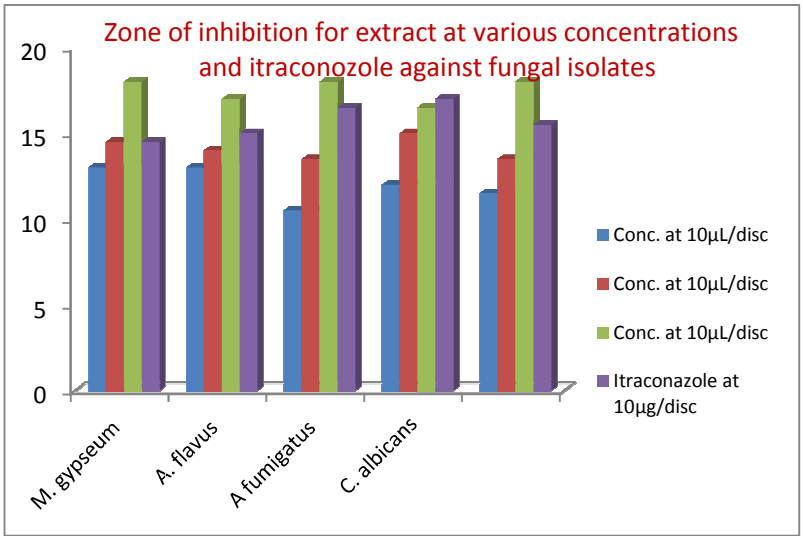
zones of inhibition for all the five fungal isolates tested namely *C. albicans, M. gypseum, A. flavus A. fumigates* and *T. mentagrophytus.* The graphical representation of the same is presented in **Fig. 29.**

Table 22: Zone of inhibition for volatile oil of *C. dentata* against various microorganisms

S. No	Name of the organism	Zone of inhibition (in mm)*			
		Itraconazole	Volatile oil of *C. dentata*		
			10 µl	20µl	30µl
1.	*Microsporum gypseum*	14.5±0.0	13.0±1.0	15.5±0.5	18.0±0.0
2.	*Aspergillus flavus*	15.0±3.0	13.0±0.5	14.0±0.0	17.0±1.0
3.	*Aspergillus fumigatus*	16.5±0.0	10.5±0.5	13.5±0.5	18.0±0.0
4.	*Candida albicans*	17.0±1.0	12.0±1.0	15.0±1.0	16.5±0.5
5.	*Trichophyton mentagrophytus*	15.50±0.5	11.5±2.5	13.5±2.0	18.0±2.0

*** mean of 2 readings ± SEM**

Fig. 29: Zone of inhibition for volatile oil of *C. dentata* and itraconazole against various microorganisms



Plant natural compounds are important source of fungi toxic compounds and they may provide a new source of useful fungicides. The effect of the extract against *C. albicans* was higher implying that this plant can be utilized against infection of *C.*

albicans There are, however, alarming reports of opportunistic fungal infections (140) which describe that the resistance of the organisms increased due to indiscriminate use of commercial anti-microbial drugs commonly used for the treatment of infectious disease. This situation forced the researchers to search for new anti-microbial substance from various sources including medicinal plant (140). The present research findings revealed that medicinal plant *C. dentata* can play a vital role in combating fungal resistance (141).

LARVICIDAL ACTIVITY OF THE EXTRACT AND VOLATILE OIL

The results obtained for the larvicidal effect of ethanolic extract of *Clausena dentata* are presented in **Table 23** and the graphical representations are presented in **Figs. 30 & 31.**

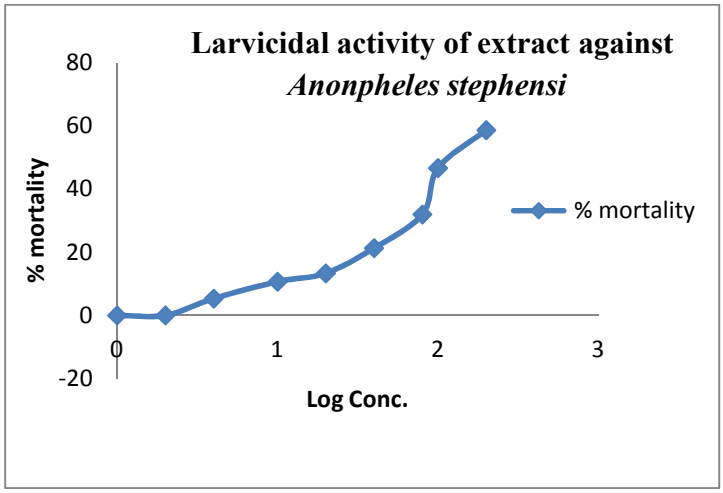
Table 23: Larvicidal activity of ethanolic extract of *Clausena dentata* against *Anopheles stephensi, Culex quinquefasciatus*

S. No	Conc. in ppm	% mortality of *Anopheles stephensi*	% mortality of *Culex quinquefasciatus*
1	1	00.00 ± 0.00	00.00 ± 0.00
2	2	14.67 ± 1.31	00.00 ± 0.00
3	4	32.0 0± 2.30	05.33 ± 1.33
4	10	39.33 ± 1.76	10.66 ± 1.33
5	20	44.00 ± 2.30	13.33 ± 1.33
6	40	64.00 ± 2.30	21.33 ± 1.33
7	80	65.33 ± 3.52	32.00 ± 2.30
8	100	70.66 ± 1.33	46.66 ± 1.33
9	200	98.66 ± 1.33	58.66 ± 1.33
LC50		**19.3912 (0.002%)**	**268.68 (0.027%)**
LC90		**233.24 (0.023%)**	**12177.24 (1.218%)**

From the **Table 23,** it can be observed that a mortality of 98.66 ± 1.33 and 58.66 ± 1.33 was observed for *Anopheles stephensi* and *Culex quinquefasciatus* respectively. The LC_{50} and LC_{90} values were calculated using Probit analysis. The percentage mortality calculated using Abbott's formula versus log concentration was plotted and

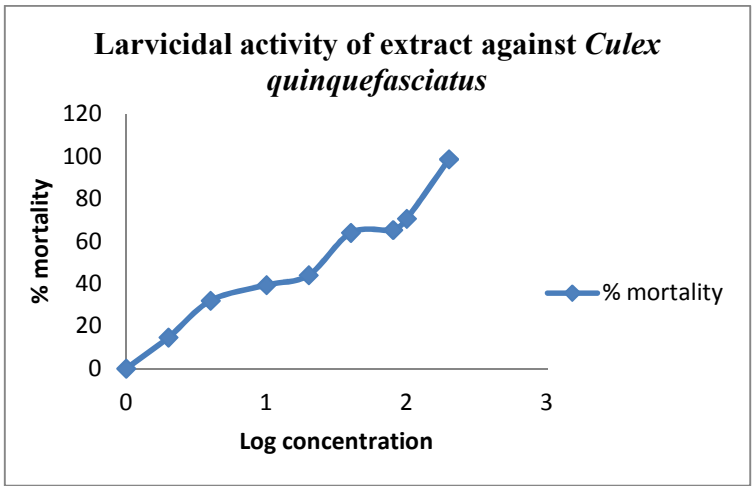
Y=50 is substituted in the resulting linear equation to obtain the X value. The linear regression equation was found to be y = 24.15x - 8.666 for activity against *Anopheles stephensi* while it was found to be y = 37.03x + 2.320 for activity against *Culex quinquefasciatus.* The antilog of X was then the LC_{50} (conc. of 50 mortality) or LC_{90} (conc. of 90% mortality) value.

Fig. 30: Larvicidal activity of ethanolic extract of *C. dentata* against *Anonpheles stephensi*



The LC_{50} was found to be **19.39ppm or 0.002%v/v** and **268.68ppm or 0.027% v/v** for *Anopheles stephensi* and *Culex quinquefasciatus* respectively. The LC_{90} value was found to be **233.24ppm or 0.023%v/v** and **12177.24ppm or 1.218% v/v** for *Anopheles stephensi* and *Culex quinquefasciatus* respectively. One hundred percent mortality was not observed for the concentrations tested against both the organisms. The extract was not as effective against *Culex quinquefasciatus* as it was for *Anopheles stephensi* which has been seen from the very low concentration required ie. 0.002% v/v solution in DMSO.

Fig. 31: Larvicidal activity of ethanolic extract of *C. dentata* against *Culex quinquefasciatus*



Hence the ethanolic extract of *Clausena dentata* possessed a good larvicidal activity against *Anopheles stephensi* than against *Culex quinquefaciatus.*

The results obtained for the larvicidal effect of volatile oil of the leaves of *Clausena dentata* against *Anopheles stephensi* and *Culex quinquefaciatus* are presented in **Table 24** and the graphical representations are presented in **Figs. 32 & 33.**

Table 24: Larvicidal activity of volatile oil of *Clausena dentata* against *Anopheles stephensi* and *Culex quinquefasciatus*

S. No	Conc. in ppb	% mortality of *Anopheles stephensi*	% mortality of *Culex quinquefasciatus*
1	10000	00.00 ± 0.00	00.00 ± 0.00
2	20000	40.00 ± 5.77	23.33 ± 3.33
3	30000	70.00 ± 5.77	50.00 ± 5.77
4	40000	96.67 ± 3.33	96.66 ± 3.33
5	50000	100.0 ± 0.00	100.0 ± 0.00
LC50		**4100.62 (4.1ppm)**	**5202.355 (5.20ppm)**
LC90		**1122018 (1122.02ppm)**	**1669168 (1669.17 ppm)**

From the **Table 24,** it can be observed that a hundred percent mortality was not observed in the concentrations tested. A mortality of 98.66 ± 1.33 and 58.66 ± 1.33 was observed for *Anopheles stephensi* and *Culex quinquefasciatus* respectively. The LC_{50} and LC_{90} values were calculated using Probit analysis. The percentage mortality calculated using Abbott's formula versus log concentration was plotted and Y=50 is substituted in the resulting linear equation to obtain the X value. The linear regression equation was found to be y = 16.41x - 9.287 for activity against *Anopheles stephensi* while it was found to be y = 15.96x - 9.312 for activity against *Culex quinquefasciatus.* The antilog of X was then the LC_{50} (conc. of 50 mortality) or LC_{90} (conc. of 90% mortality) value.

The LC_{50} was found to be 4.1 and 5.2ppm for *Anopheles stephensi* and *Culex quinquefasciatus* respectively. The LC_{90} value was found to be 1122 and 1669 ppm for *Anopheles stephensi* and *Culex quinquefasciatus* respectively. One hundred percent mortality was observed for concentrations ranging above 50ppm for both the organisms tested. When concentrations above 2µg/mL were used, a hundred percent mortality was observed within 2h of the start of the experiment.

Fig. 32: Larvicidal activity of volatile oil of the leaves of *C. dentata* against *Anopheles stephensi*

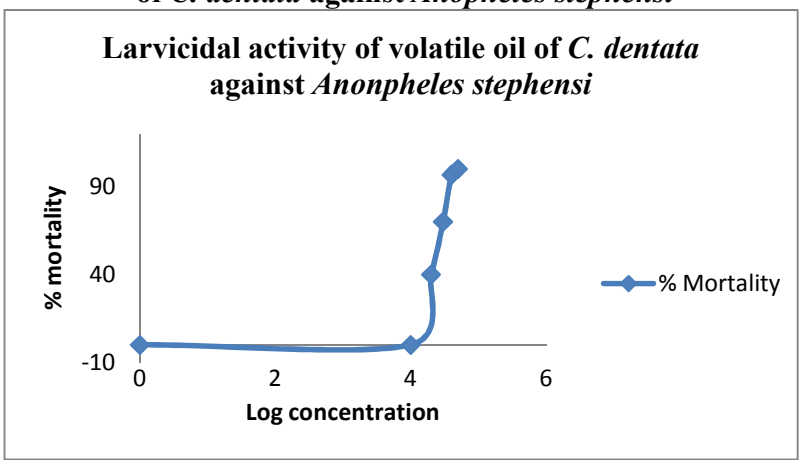
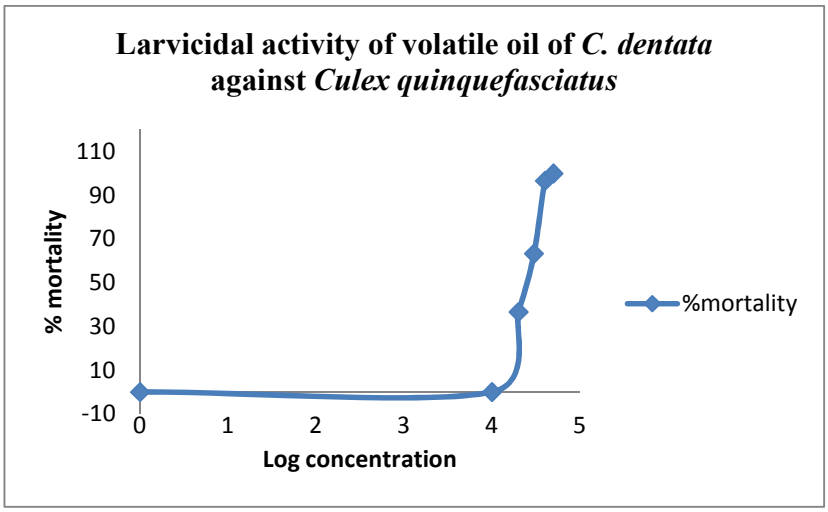


Fig. 33: Larvicidal activity of volatile oil of the leaves of *C. dentata* against *Culex quinquefasciatus*



The mosquito *Culex quinquefasciatus* (Diptera : Culicidae) acts as the vector for *Wuchereria bancrofti* responsible for filariasis (142) which is an endemic disabling, disfiguring disease (143) and is the most abundant house mosquito in towns and cities of the tropical countries (144). The mosquito *Anopheles stephensi* (Diptera : Culicidae) acts as the vector for *Plasmodium* species which is responsible for malaria. The control of *Culex* and *Anopheles* is essential since it is a serious public health problem in India and many developing countries.

One of the strategies adopted by World Health Organization in combating tropical diseases is to destroy the vectors or intermediate hosts. Since no effective vaccine available for filarial fever, it is essential to minimize the incidence of this disease by eradicating and controlling mosquito vectors mainly by application of insecticidal to larval habitats.

Vector control is by far the most successful method for reducing incidence of mosquito-borne diseases, but the emergence of widespread resistance and the potential environmental issues associated with some synthetic insecticide (such as DDT) has indicated that to control the proliferation of mosquito population, urgent research on alternative ways is necessary. Large number of products of natural origin, especially the secondary metabolites from the plants, has received considerable attention as potentially bioactive agents used in insect vector management. (145)

The search for herbal preparation that does not produce any side effect in the non-target organisms and are easily biodegradable remains a top research issue for scientist associated with alternative vector control (146).

The mosquito control program generally targets the larval stage at their breeding sites before they emerge as adults with larvicides (147). The screening of local medicinal plants for mosquito larvicidal activity may lead to their use in natural product based mosquito abatement practices.

Plant extracts have been reported to be eco-friendly mosquito control agents [148]. One hundred percent larval mortality was recorded by Daniel *et al* (149) in the study using *Acalypha indica* and Karmegam *et al* (150) using *Pergularia extensa, Argemone mexicana* and *Withania somnifera* and ethyl acetate leaf extract of *Strychnos nuxvomica* against the larvae of *Culex quinquefasciatus,* Similarly, Arivoli *et al* [151] studied the effect of hexane, diethyl ether, dichloromethane, ethyl acetate and methanol extracts of shoot with leaves of *Leucas aspera* and leaves of *Vitex negundo* against the larvae of *Culex quinquefasciatus* and proved that ethyl acetate extract of both plants provided maximum mortality.

The toxicity to the third instar larvae of *Culex quinquefasciatus* by ethanolic leaf extract of *Clausena dentata* showed LC_{50} value of --ppm after 24 hours of exposure against the third instar larvae of *Culex quinquefasciatus*.

The findings of the present investigation revealed that the leaf extracts of *Clausena dentata* possess larvicidal activity against *Culex quinquefasciatus*. It may be concluded that natural products as extracts from parts of plants of insecticidal and medicinal values have higher efficiency in reducing mosquito menace due to their larvicidal toxicity.

SUMMARY AND CONCLUSION

In the universe all the pharmacologically active substances derived from chemical synthesis and most widely used for the treatment of infectious diseases and the herbs is only the origin of many phytoconstituents. Recently there has been an increase in the use of herbals and its formulations for the treatment of diseases and to promote and safeguard the health of the people.

In the present study, an attempt was made to explore the medicinal values of the plant *Clausena dentat*a (Willd) especially for treating tropical infections.

The dissertation is entitled **''Pharmacognostic, Phytochemical and Pharmacological evaluation of the leaves of *Clausena dentata* (Willd). (Rutaceae)''**

The chapter on **Review of literature** of the plant *Clausena dentata* provides information on the Pharmacognosy, phytochemical and pharmacological activities of the various species of *Clausena.*

The chapter on **Phamacognostic Evaluation** deals with the macroscopical study of plant, the microscopical studies of the leaves, standardization parameters, quantitative microscopy, cell morphological characters, powder microscopy etc. and the results helps in achieving a trouble-free identification and authenticity of the plant as a whole or in powder form in future.

The chapter on **Phytochemical Evaluation** deals with the preliminary phytochemical evaluation and quantitative estimation of phtoconstituents present in the ethanolic extract of the plant which give information on the identify the presence of the secondary metabolites present in *Clausena dentata*. The amount of phenols and flavonoid content determination and quantification gives the information about the amount of secondary

metabolite present and responsible for the therapeutic or pharmacological activity of the plant. The chromatographic studies of the plant which includes TLC and HPTLC fingerprint profile and the results obtained may be used a standard and utilized to establish its quality, purity and authentication of the plant *Clausena dentata.* The evaluation of the volatile oil provides information on the physical parameters like refractive index and weight/mL. The chemical analysis of the oil by GC/MS has revealed the presence of E-anethole, estragole (methyl chavicol), α-elemene. α-pinene, borneol and asarone which may be responsible for the antibacterial, antifungal and larvicidal activity of volatile oil of the plant.

The chapter on **Pharmacological Evaluation** deals with the antioxidant studies of the ethanolic extract, the antibacterial, antifungal and larvicidal activity of the both ethanolic extract and volatile oil of the plant.

The extract was screened for **antioxidant property** by various methods and the extract possessed good antioxidant properties due to the presence of Vitamin-C, poly phenolic, flavonoid content.

The **antimicrobial and antifungal activity** of the both extract and volatile oil of the leaves of *Clausena dentata* was carried out by antibiotic disc diffusion method. Antibacterial activity of volatile oil showed good growth inhibiting activity against the human pathogenic bacteria. The volatile was also screened for antifungal activity and the results obtained showed that the oil had an excellent antimicrobial property and may be attributed to the presence of terpene compounds. The extract was also screened for antibacterial and antifungal activity and good results were obtained. Hence the presence

of secondary metabolites like coumarins, glycosides, carbazole alkaloids may collectively be responsible for the wide range of biological activities.

The **larvicidal effect** of both extract and volatile oil of the plant on mosquito larvae was carried out by standard procedures. The volatile oil has a strong larvicidal effect against the malaria and filarial larvae when compared with that of the extract.

The present investigation has revealed that the volatile oil has good antioxidant, larvicidal, antifungal and antibacterial activity. Further studies on the screening, isolation and purification of bioactive phytochemical constituents/compounds followed by in-depth laboratory and field bioassays are needed as the present study shows that there is scope to use *C.dentata* leaf extracts to control the larval stages

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