Anatomical, Histochemical and Biological Activities of Essential Oil from *Piper nigrum* L.: An Aromatic Spice Plant

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Mother

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PREFACE

In this study, we explore the anatomy, histochemistry, and pharmacological potential of *Piper nigrum*. Anatomical analysis and histochemical techniques using Sudan IV and Oil O Red stains enhance the reliability and specificity and underscore the importance of selecting appropriate staining techniques. Autofluorescence in plants refers to the natural emission of light by certain compounds or structures within plant tissues when they are excited by light of a specific wavelength, and it also serves as a versatile and valuable tool in plant biology research, providing insights into plant physiology, metabolism and responses to environmental stimuli.

Essential oils derived from spices often possess therapeutic properties, including antimicrobial, anti-inflammatory, antioxidant effects, insecticidal, and repellent properties, offering environmentally friendly alternatives. As per the literature review, *Piper nigrum* fruit has been widely explored as it is of commercial importance, whereas fewer reports are on the leaf essential oil. Dried leaves and fruit essential oil were extracted using hydro-distillation and were assessed for antioxidant and larvicidal activities to understand the potential applications.

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ABBREVIATIONS USED

Entity	Abbreviations
Absorbance of the control	A_0
Absorbance of the sample	A_1
Centimetre	Cm
Chi- square	X2
1,1-Diphenyl-2-picrylhydrazyl	DPPH
Degree Celsius	°C
Grams	g
Global Positioning System	GPS
Hours	Hrs
Lethal concentration killing 50%	IC_{50}
Lethal concentration killing 90%	LC_{90}
Lower Confidence Limit	LCL
National Institute of Malaria Research	ICMR
Meter	m
Milligrams	mg
Microgram	µg
Millilitre	mL

Millimeter	mm
Parts per million	Ppm
Percent	%
Replicate	R
Reactive Oxygen Species	ROS
Statistical Package for Social Sciences Software	SPSS
Transverse section	TS
Upper confidence limit	UCL
Weight in volume	w/v
World Health Organization	WHO

ABSTRACT

Piper species hold significant medicinal importance in traditional Indian medicine. Among these, the fruits of *Piper nigrum*, commonly known as Kali Mirch, are highly regarded. Belonging to the Piperaceae family, they are extensively used in culinary practices as a pungent condiment. Several lipid droplets and calcium oxalate crystals were observed in leaves and stem, indicating their role in defense strategies, mechanical support, and pollinators' attraction. Histochemical studies using Sudan IV and Oil O Red stain aid in localizing pharmacologically active compounds**.** Fluorescence microscopy revealed the autofluorescence in various parts (Fruit, leaves, stem, and petiole) of *Piper nigrum*. Powder microscopy analysis indicated the presence of parenchyma tissues, stomata, annular vessels, perisperm, and starch grains in the leaf and fruit powder of *Piper nigrum.*

The essential oil of dried leaves and fruits of *Piper nigrum* was extracted via hydro-distillation. These essential oils were subjected to antioxidant and mosquito larvicidal activities. The results of antioxidant efficacy of extracted leaf essential oil revealed good activity with IC_{50} = 166.29µg/mL as compared to fruit essential oil 214.16µg/mL. In the larvicidal activity, the leaf and fruit essential oil showed 100% larval mortality at 20 ppm and 30 ppm, respectively. Leaf essential oil depicted maximum larvicidal activity with $LC_{50=}$ 117.4 ppm and 91.43 ppm at 24 and 48 hours, respectively, while LC_{90} value was 181.5ppm and 181.43ppm at 24 and 48 hours, respectively, then fruit essential oil with 170.17ppm and 96.80ppm at 24 and 48 hours and their LC_{90} value was 564.37ppm and 267.36ppm at 24 and 48 hours respectively.

INTRODUCTION

1.1. BACKGROUND

Aromatic plants contain odorous volatile substances such as essential oil, exudate gum, and oleoresin from roots, bark, stems, flowers, and fruits. Medicinal aromatic plants have healing properties and pleasant aromas and have been used in traditional medicinal practices for centuries (Gupta, 2023). Essential oils are secondary metabolites that have many biological effects. They also provide a defence mechanism for plants against herbivores. Families such as Myrtaceae, Lauraceae, Piperaceae, Lamiaceae, and Asteraceae are well known for their potential to produce essential oils of commercial and medicinal value (Asadi *et al.,* 2018).

Spices and culinary herbs are essential in shaping a nation's cultural and economic landscapes. Their rich flavours and distinctive pungency make the dishes palatable and delightful in food preparation. In addition, spices are renowned for their invaluable medicinal and pharmacological properties (Parthasarathy *et al*., 2008). Spices are natural additives that contribute immensely to the taste of our foods and are used to prevent food deterioration and pathogenic diseases. They have been used since ancient times. Spices and herbs are recognized as natural sources of antioxidants and play an essential role in the aging and chemoprevention of diseases. Among the plants investigated to date, the pepper family is known to show enormous potential (Dodson *et al*., 2000). They have been utilized for thousands of years to increase food's flavour, colour, and aroma (Nagalingam and Arumugam, 2015). *Piper* species are known for their economic importance and are widely used as spices such as *P. nigrum* and *P. guineense*, as herbal medicine such as *P. betle,* or as condiments such as *P. auritum* (Dyer *et al.*, 2004). The world population relies on plants and plant extracts for healthcare, with *Piper nigrum* being widely used in Ayurvedic medicines (Srivastava *et al.,* 2000).

1.1.1. FAMILY: PIPERACEAE

The Piperaceae family, also known as the “pepper family” in the order Piperales, comprises mainly herbs, vines, shrubs, and trees, often aromatic. It comprises about 13 genera and 3600 species, mainly distributed in two main genera: *Piper* and *Peperomia*. The genus *Piper* is best known because of its scientific and commercial importance. It is widely distributed throughout the tropics and subtropics (Tamokou *et al.,* 2017)

The Piperaceae exhibits stems of herbaceous/woody aromatic climbers. Leaves are rarely opposite or whorled, entirely asymmetrical bilaterally, palmately, or pinnately veined. Flowers are small, bisexual, or unisexual, lacking a perianth dioecious, polygamous, arranged on a pendulous spike, and rarely racemose. Inflorescence occurs in the axillary. Fruit is usually a drupe or nutlet (Xu *et al.,* 2017). The essential and economic genus in this family is *Piper,* and the well-known species is *P. nigrum L*., a popular culinary spice; other species include *Piper betle*, *Piper longum*, *Piper capense*, *Piper retrofractum*, and *Piper guineese.*

1.1.2. Scientific Classification of *Piper nigrum* L (Damanhouri and Ahmad, 2014)

Kingdom : Plantae

Clade : Angiosperms

Order : Piperales

Family : Piperaceae

Genus : *Piper*

Species : *P. nigrum* L.

Black pepper is a tropical, medicinal plant belonging to the family Piperaceae and is cultivated for its fruit, known as a peppercorn, which is used as a spice (Jaramillo *et al.,* 2001). Based on the maturity and ripening stages of the fruit, pepper is classified into three types: white pepper, black pepper, and green pepper.

Piper nigrum L., commonly known as the pepper, is well known for its massive trade share in the global market (Srinivasan, 2007). The word “pepper” originates from the Sanskrit word “*pippali”, which* means berry; other Indian names are “Kali Mirch” in Hindi and Urdu, “Milagu” in Tamil, “Peppercorn” in English, “Miriyam” in Telugu (Kumar *et al.,* 2011; Kaliyaperumal *et al.,* 2021) **(Plate 1 and 2).**

1.1.3. Varieties of *Piper nigrum*:

Numerous varieties of pepper within the *Piper* genus exceed 600 in number. However, few are used as spices, typically categorized by their level of ripeness and the processing method employed. The market's common pepper is the fruit of *P. nigrum*, featuring small spherical green berries that mature into a vibrant red when fully ripe. Depending on the harvest time and specific processing techniques, we can distinguish the following types of pepper (Shukla *et al.,* 2018).

1. **BLACK PEPPER:** This pepper variety originates from relatively young pepper seeds, which are dried until they develop a black, wrinkled appearance. It is available in both coarse and finely ground forms for marketing and has been widely used as a spice in culinary dishes such as soups and beef steak.
2. **WHITE PEPPER:** These are derived from the same plant as black pepper. White pepper is crafted by harvesting fully ripened pepper fruits from plants soaked and stripped of their outer layer, resulting in a white colour. White pepper is often ground into powder form and is typically used in seasoning culinary dishes, which gives a distinctive flavour and aroma.
3. **GREEN PEPPER:** Harvested in its green stage, ensuring a vibrant appearance. It is often mixed with a seasoning solution to preserve its crispness and adds flavour to chicken and seafood dishes.

Plate 1. *Piper nigrum*: (a) Habit, (b) Fruiting twig, (c) Aerial roots, (d) Abaxial leaf surface, (e) Adaxial leaf surface.

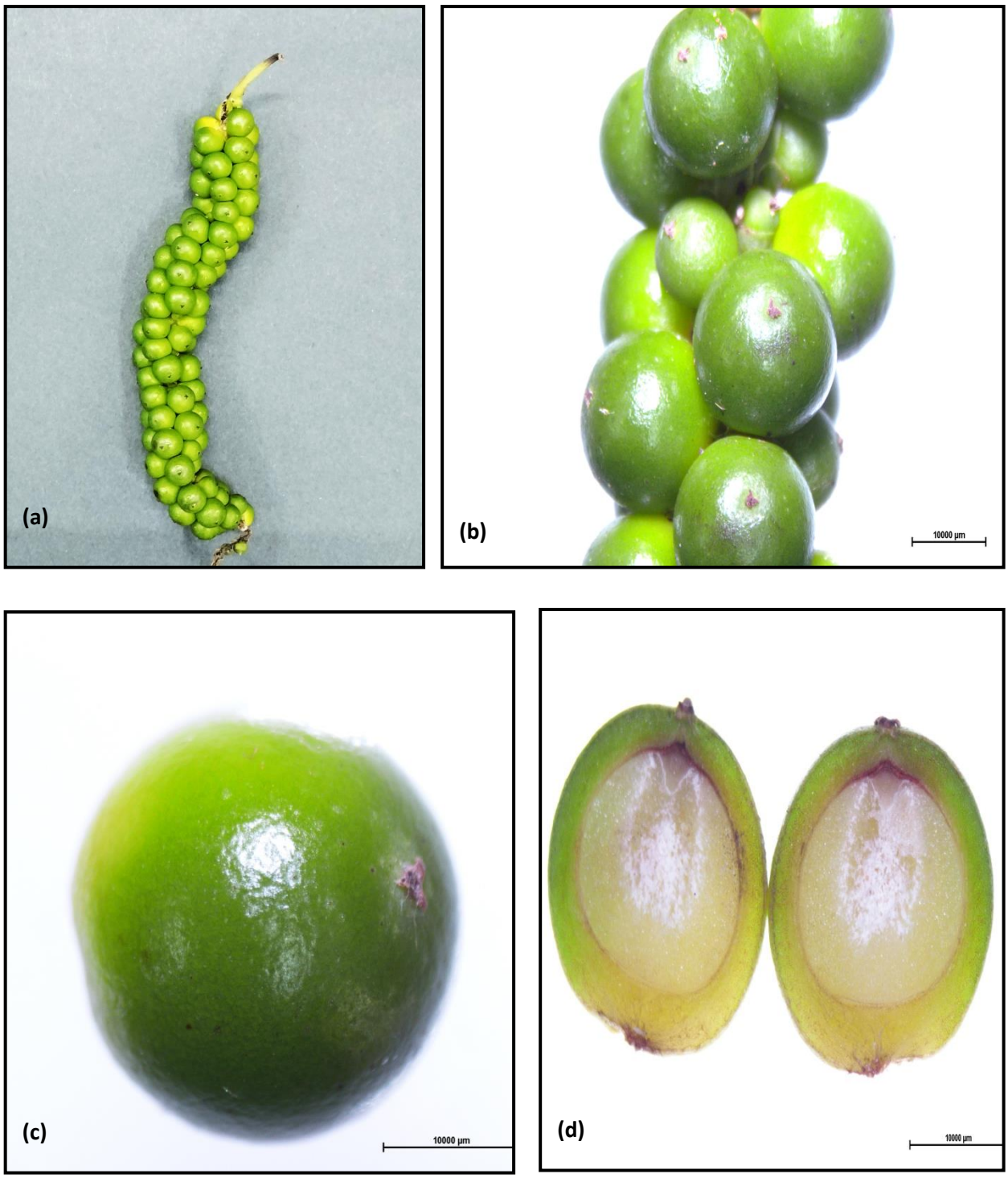


Plate 2: Macroscopic images of *Piper nigrum* fruit (a) Spike of Fruit, (b) Magnified image of fruit, (c) Single Fruit, (d) Cut open fruit.

4. **RED PEPPER:** It lacks the green pigment on its skin, producing vibrant red peppercorns. It has a unique flavour and offers a balance of spiciness and sweetness not found in other varieties. It is used to enhance seafood dishes, marketed both fresh and dried.

1.1.4. ORIGIN AND DISTRIBUTION

Black pepper, a popular spice worldwide, is rooted in the rich soil of South West India, particularly the Western Ghats region of South India. It was the first oriental spice to be introduced into the Western world. Initially confined to cultivation in the western coastal region of India, black pepper has since spread its aromatic influence to tropical countries across the globe.

It is grown in many tropical zones of the Asia-Pacific region, with critical producers India, Indonesia, Malaysia, Sri Lanka, Thailand, and Vietnam. Beyond these primary regions, black pepper is widely cultivated in approximately 26 countries worldwide, including Brazil and Mexico. It has evolved into a significant agricultural export commodity and the most widely traded spices globally (Ravindran and Kallupurackal, 2012).

1.1.5. BOTANICAL DESCRIPTION

The pepper plant is a perennial woody aromatic climber that grows on supporting trees, trellies, or poles up to 50-60 cm tall. Stems are herbaceous; as they grow, aerial roots sprout at nodes along the length that adhere to tree trunks, supporting the pepper plant as it grows and climbs. Nodes are enlarged and rooting (Bui *et al.,* 2017).

There are two types of roots: one type extends from nodes underground, serving as nutrient-absorbing anchors, while the other type grows above the ground, which acts as adhesive roots. The stem of the pepper is a long, cylindrical vine. Young stems are green, while old stems are woody with a diameter of 4-6 cm (Wulandari *et al*., 2021). The leaves are

glabrous, broadly ovate to ovate-oblong (5-10cm long and 3-6 cm wide), thick, acuminate, base usually rounded, more or less oblique, petiolate, and alternately arranged.

Flowers are small, white to pale yellow, polygamous, usually monoecious, and are produced on pendulous spikes of about 7-8cm long at the leaf nodes (Shariat, 2007). Spikes are of variable lengths and lengthens up to 7 to 15 cm as the fruit matures. Bracts of female spikes more or less adnate to the rachis, forming a short hemispheric cup beneath the ovary, and bracteoles forming a semilunar ridge above the ovary. Stamens-2 and stigmas 2-4 (Britannica, 2024).

The fruits are drupes about 0.2 inches in diameter, round in shape, dark green when unripe, and at maturity, the fruits become yellowish to dark red and bear a single seed. The fruits are aromatic, the taste is hot, and very pungent (Govindarajan, 1980). Flowering and fruiting are during the monsoon season. The pepper plant is cultivated for its fruits, usually dried and used as a spice.

1.1.6 IMPORTANCE AND USES

- **Culinary and Traditional uses:**
- *Piper nigrum* is mainly used as a spice in culinary dishes. In Western cuisine, it is the principal ingredient to enhance food flavour and as a natural preservative (Ravindran and Kallupurackal, 2012).
- In India, pepper is used in culinary dishes and folk medicine, whereas in European cuisines, it is only used as a seasoning ingredient to enhance food flavour. Whole peppercorns are used in stews and soups with thyme, parsley, and bay leaf. Crushed peppercorns are added to creamy sauces or to coat chicken breasts, adding spiciness. In

Chinese cuisine, white pepper is ground and added to salads and cream sauces (Takoore *et al.,* 2019).

- Different parts of the pepper plant (flower, fruit, seed, and leaf) are used in many ailments. Fruits are dried and used as a folk medicine to treat gastrointestinal disorders, flu, colds, and fever diseases (Nahak *et al*., 2011)
- Black peppercorns are used in Ayurveda, Siddha, and Unani medicine in South Asia and have been frequently used as an appetizer to aid digestion and treat digestive system problems, such as to exterminate parasitic worms.
- In Ayurvedic medicine, black pepper has been used to improve appetite and treat coughs, colds, flu, heart problems, piles, and anemia.
- Black pepper is also used in toothpaste for toothache, and an infusion is used to treat sore throat and hoarseness. Chewing black pepper reduces throat inflammation, and the paste is applied externally to treat boils, hair loss, and skin diseases.
- In Ayurveda and Siddha medicine, white pepper paste treats some eye diseases (Nalla *et al*., 2020).

1.1.7. ANATOMY

The study of anatomy stands as one of the earliest branches of plant science, boasting a vast reservoir of accumulated knowledge (Sokoloff *et al*., 2021). Plant secondary metabolites are accumulated within both external and internal secretory tissues. Internally, secondary metabolites can be found in specialized structures such as secretory cells, secretory glands, secretory canals, and secretory cavities. Externally, secretory tissues could be in the form of trichomes (Fahn, 1990).

1.1.8. HISTOCHEMICAL STUDIES

Plants serve as rich reservoirs of active compounds crucial for medicinal purposes and are primary targets for extracting natural substances. Their therapeutic value lies in diverse secondary metabolites such as flavonoids, alkaloids, tannins, and saponins. These compounds are important because they possess significant biological activities and are active constituents in many drugs. Histochemistry is a branch of science that combines biochemistry and histology methods to investigate the chemical composition within cells and tissues. This involves verifying chemical compounds' presence, density, and distribution in cells and tissues using staining techniques and photographic recordings under a microscope. The procedure includes the preparation of fixed, variably stained specimens and examining the sample under a microscope (Badria and Aboelmaaty, 2019).

1.1.9. FLUORESCENCE MICROSCOPY

Fluorescence is a unique property in certain substances, characterized by their ability to absorb shorter wavelength light, such as ultraviolet or blue, and then emit light of longer wavelength, such as green, yellow, or red. This fascinating phenomenon is commonly observed through a fluorescence microscope, where mechanisms are set up to allow only the emitted light to reach the observer’s eye. In contrast, the exciting light is blocked out. The fluorescence microscope can observe autofluorescence due to substances naturally present (Kiernan, 2015).

Plants possess an abundance of auto-fluorescent molecules that are valuable for various research purposes, including biochemical analysis, physiological investigations, and imaging studies. The two most studied molecules are chlorophyll, which stands out with its distinctive orange/red fluorescence, and lignin, which emits blue/green fluorescence. Chlorophyll fluorescence is a crucial tool for assessing plant's physiological condition using handheld

devices that can measure photosynthesis, linear electron flux, and CO2 assimilation by directly scanning leaves or through aerial reconnaissance using drones and satellites (Donaldson, 2020)

1.1.10. POWDER MICROSCOPY

Powder microscopy serves as a vital tool for assessing and ensuring the quality of medicinal plants by examining their distinctive microscopic features with the aid of various staining agents. It facilitates the identification of adulterants within a sample through comparative analysis with authenticated samples (Aeri *et al.,* 2019).

1.1.11. ESSENTIAL OIL

Essential oils are hydrophobic, volatile oils, and aromatic compounds and are secreted from oil cells, secretory ducts, and glandular hairs of plants; having a characteristic scent, and the oil is extracted from different parts of the plant like fruits, leaves, flowers, seeds, barks, roots (Rahman *et al*., 2017). Volatile oils are mixtures of hydrocarbon terpenes, sesquiterpenes, polyterpenes and oxygenated derivatives. They evaporate on exposure to air at ordinary temperature (Kulkarni, 2023). The complex composition of essential oils comprises saturated and unsaturated hydrocarbons, alcohols, aldehydes, esters, ketones, and terpenes, giving rise to their distinctive aromas (Ali *et al.,* 2015).

Essential oils extracted from many plants possess antioxidant, anti-inflammatory, anti-microbial, insecticidal, and other properties (Adorjan *et al*., 2010). Some are also used in the treatment of cancer treatment, aromatherapy, food preservation, and perfume (Dinh *et al*., 2020).

Essential oils serve many purposes for plants, including

1. Attracting or repelling insects,
2. Safeguarding against extreme temperatures,
3. Chemical ingredients in the oil are used as defence equipment.

These versatile oils have diverse applications, ranging from their use as resins and flavouring agents to serving as additives in foods and components in perfumes, cosmetics, and soaps (Arshad *et al.,* 2014).

The quality of essential oils relies heavily on the extraction method employed, which must align with the specific characteristics and components desired from the botanical extract. Hydro distillation, steam distillation, solvent extraction, supercritical fluid extraction, subcritical extraction liquid, and solvent-free microwave extraction are examples of techniques for extracting essential oils (Aziz *et al*., 2018).

Chemical characterization of essential oil uses Gas chromatography coupled with mass spectrometry (GC-MS). This analytical technique is widely adopted due to its simplicity, rapidity, and efficiency in determining the constituents of essential oils. A GC-MS report acts as a fingerprint for any particular batch of essential oil, revealing the chemical composition that unveils the oils' unique properties (Aziz *et al.,* 2018).

1.1.12. CHEMISTRY OF ESSENTIAL OIL

The complex composition of essential oils comprises saturated and unsaturated hydrocarbons, alcohols, aldehydes, esters, ketones, and terpenes, giving rise to their distinctive aromas (Ali *et al*., 2015). The constituents of essential oils may be broadly classified as volatile and non-volatile fractions. The volatile fraction includes mono and sesquiterpene components and several oxygenated derivatives, alcohols, aliphatic aldehydes, aldehydes, and esters. On the other hand, 1-10% of the isolated essential oils include carotenoids, fatty acids, flavonoids, and waxes (Aziz *et al.,* 2018).

1.1.13. HYDROCARBON

Hydrocarbon in essential oils consists of interconnected building blocks of hydrogen and carbon bonds. Isoprene is a fundamental hydrocarbon commonly found in essential oils (Aziz *et al*., 2018).

1.1.14. TERPENES

Terpenes encompass mono, sesqui, diterpenes within their group classification. Monoterpenes arise from the fusion of two isoprene units. Sesquiterpenes form when three isoprene units combine, while diterpenes are structured from four linked isoprene units (Aziz *et al.,* 2018).

1.1.15. ANTIOXIDANT ACTIVITY

Reactive oxygen Species (ROS) and Reactive Nitrogen Species (RNS), commonly known as free radicals, play pivotal roles in numerous diseases. These radicals are byproducts of metabolic processes, capable of oxidizing lipids. This oxidation leads to a decrease in the fluidity of bio-membranes, loss of different enzyme activities, and loss of receptor activities, ultimately resulting in cell dysfunction (Ahmad *et al*., 2011).

Antioxidants are agents that stop entirely or delay the process of oxidation. There are two groups of antioxidants: natural and synthetic. However, synthetic antioxidants are carcinogenic (Rahman *et al.,* 2011), and hence, naturally occurring antioxidants from plants and plant-derived products have gained importance in recent years (Gupta *et al.,* 2015).

They are classified as enzymatic and nonenzymatic. Enzymatic antioxidants include Superoxide dismutase, catalase, and glutathione peroxidase, which work within the body to neutralize harmful free radicals. On the other hand, nonenzymatic antioxidants encompass

essential nutrients like vitamin E, vitamin C, carotenoids, and plant polyphenols, which provide an additional defence against oxidative stress (Gupta *et al*., 2015).

Antioxidants can scavenge ROS and RNS, hindering the formation of harmful reactive oxidants; this action helps safeguard food against oxidative degradation, a common issue during storage and processing. As such, antioxidants are frequently employed as additives in food to maintain its quality and extend its shelf life by preventing undesirable changes caused by oxidation (Gulcin *et al*., 2005). Essential oil has been recommended as an alternative to prevent food spoilage and stabilize food lipids (Ordonez *et al*., 2008).

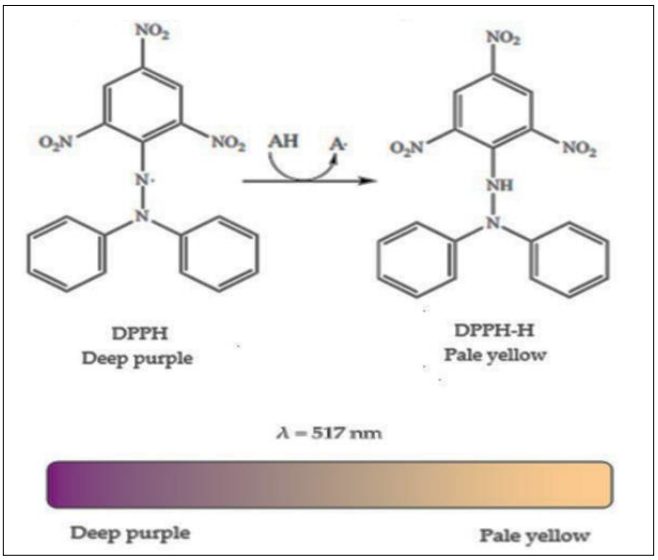


Figure 1.1 DPPH scavenging mechanism by an antioxidant (Munteanu *et al.*, 2021).

Numerous bioanalytical methods exist to measure antioxidant efficacy. One is the 1,1-dinpheyl-2-picrylhydrazil (DPPH) assay **(Fig 1.1)**, the most popular and commonly used to determine antioxidant ability (Gulcin *et al*., 2023).

The DPPH assay method relies on reducing DPPH, a stable free radical. DPPH possesses an odd electron, exhibits maximum absorbance at 517nm, and appears purple. Antioxidants interact with DPPH by providing a hydrogen atom, causing the stable free radical to pair off and reducing it to DPPH-H. Consequently, the absorbance decreases, leading to decolorization

(from purple to yellow) as the number of captured electrons increases. Greater decolorization indicates a higher reducing ability,

DPPH's radical form displays an intense UV-visible absorption spectrum. When mixed with a substance capable of donating a hydrogen atom, DPPH is converted to its reduced form, diphenyl picryl hydrazine, resulting in the loss of violet coloration (Dontha, 2016).

1.1.16. LARVICIDAL ACTIVITY

In many countries, vector-borne diseases pose a significant challenge to public health. Mosquitoes are the primary disease vectors responsible for annually infecting millions of people worldwide, with many pathogens transmitted through their bites (El-Bahnasawy *et al.,* 2013).

Controlling mosquitoes at the larval stage offers a crucial advantage by targeting these disease carriers before they can transmit illnesses to humans. Annually, more than millions of lives are lost to this relentless disease, encompassing malaria, filariasis, dengue, Zika fever, and chikungunya. By focusing on larva mosquito control, we can interrupt the transmission cycle and prevent the spread of disease (WHO, 2014). The persistent use of chemical insecticides disrupts natural biological control mechanisms, paving the way for outbreaks of certain insect species (Milam *et al*., 2000).

Plants are great alternative agents for managing mosquitoes because they possess bioactive chemicals. These compounds exhibit efficacy against selected species of mosquitoes and are eco-friendly. Traditionally, human communities have relied on plant–based solutions for insect management. Plants' diverse secondary metabolites are a natural defence mechanism against insect predation (Veer *et al*., 2016).

Culex quinquefasciatus, known as the southern house mosquito, acts as an urban bridge vector by connecting various hosts to humans through its interaction with different vertebrates

and serves as the primary carrier of bancroftian filariasis and is also a possible transmitter of Dirofilaria immitis. Additionally, this mosquito species poses a threat as a vector for numerous arboviruses like West Nile Virus (WNV), Rift Valley fever virus and avian pox and protozoa like *Plasmodium relictum*, the causative agent of avian malaria (Negi and Verma, 2018).

1.1.16.1 HABITAT OF *CULEX* MOSQUITO

Culex mosquitoes thrive abundantly in urban and tropical settings, particularly in towns and cities. They typically breed in stagnant water sources such as polluted ponds, marshes, tanks, tin cans, barrels, puddles, creeks, and ditches (Tennyson, 2007).

1.1.16.2. LIFE CYCLE OF *CULEX* MOSQUITO

The *Culex* mosquito goes through 4 separate and distinct stages of its life cycle- Egg, Larva, Pupa, and Adult **(Fig 1.2).**

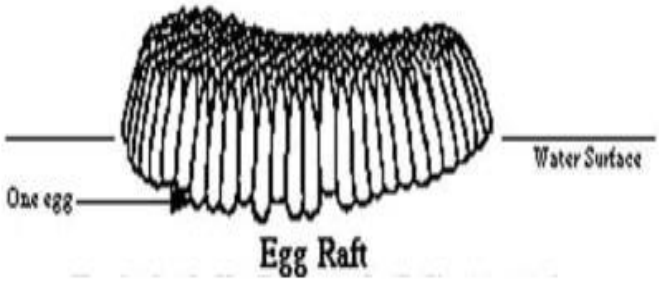


Figure 1.2. Mosquito Egg Raft (Tennyson, 2007).

Culex mosquitoes typically lay their eggs at night, with each female laying eggs every third night throughout its lifespan. These eggs are grouped in a raft containing 100-400 eggs, adhering closely to each other. Shaped like cigars, the eggs remain upright due to their buoyancy and lack of air space. This arrangement helps them float on the water's surface, resembling a speck of soot. The rafts, measuring about ¼ inch in length and 1/8 inch in width, hatch into larvae within 48 hours (Tennyson, 2007).

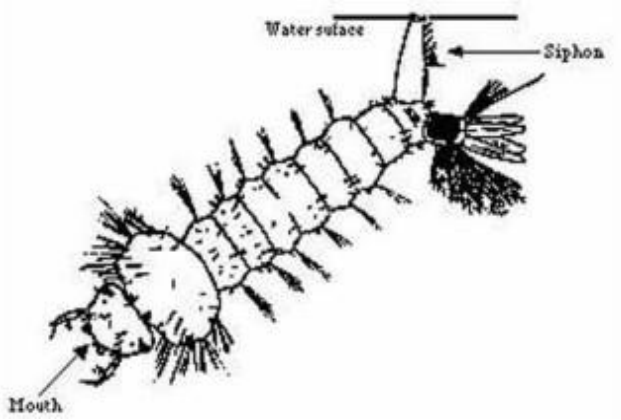


Figure 1.3. Mosquito Larva (Tennyson, 2007).

Mosquito larvae, referred to as “wranglers,” exhibit distinct characteristics and behaviours. Upon hatching from the egg after 2-3 days, the larva of the *Culex* mosquito positions itself obliquely, with its head downward. It has a distinct head but no clear demarcation between the thorax and abdomen. It lacks legs. Tufts of bristles line each side of its body. To breathe, the larva rises to the water’s surface using a respiratory siphon located at the end of its abdomen. This tube draws in atmospheric air, supplying it to two main tracheae extending from tail to head **(Fig 1.3)**.

Semi-transparent and propelled by wave-like body movements, the larva feeds on small aquatic vegetation, facilitated by feeding bristles near its mouth. During growth, the larva undergoes molting (shedding its skin) four times, growing larger after each molt. The stages between molts are called instars. After the fourth instar, the larva transforms into a pupa, typically taking around two weeks (Tennyson, 2007).

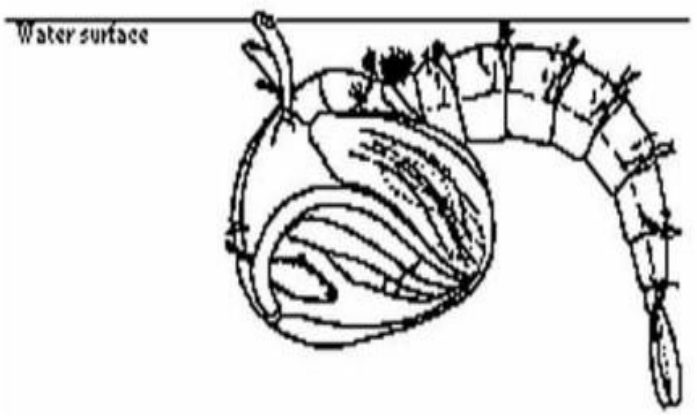


Figure 1.4. Mosquito Pupa (Tennyson, 2007).

Mosquito pupae are called “tumblers” and possess distinct features and behaviours. The pupa of the *Culex* mosquito is lighter than water and floats on the water's surface. Oxygen is acquired through two breathing tubes called trumpets. The pupa is in the non–feeding stage of development, but pupae are mobile and responsive to light changes **(Fig. 1.4)**.

The pupa is comma-shaped and comprises a cephalothorax and a slender abdomen. After about two days, the eyes, antennae, legs, and wings begin to form, becoming visible through the transparent outer covering.

In *Culex* mosquitoes, the respiratory trumpets are elongated and narrow. The pupa undergoes metamorphosis to form the adult mosquito. (Tennyson, 2007).

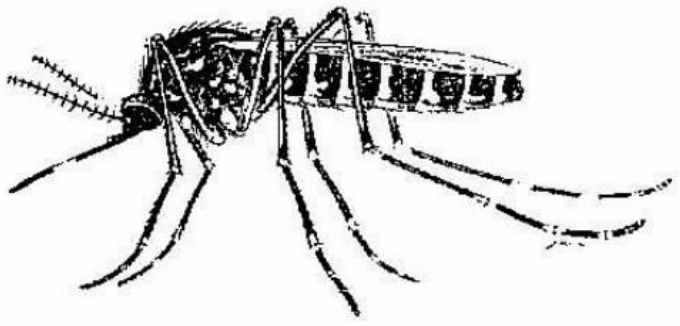


Figure 1.5. Mosquito Adult (Tennyson, 2007).

The transformation of a mosquito into an adult occurs entirely within the pupal case. Upon completion, the adult mosquito emerges by splitting the pupal covering **(Fig 1.5).** Initially, the newly hatched mosquito rests on the discarded pupal skin, then briefly on the water's surface to allow for drying and hardening of its body parts. Proper drying is essential for the wings to spread out and dry thoroughly before the mosquito can take flight. Adult Culex mosquitoes feature unspotted wings and typically rest parallel to surfaces when at rest. (Tennyson, 2007).

1.2. AIM AND OBJECTIVES

AIM: Anatomical, Histochemical and Biological activities of essential oil from *Piper nigrum*: An Aromatic Spice Plant.

OBJECTIVES:

1. Collection of different plant parts of *Piper nigrum* for anatomical, histochemical, investigation.
2. Autofluorescence and powder microscopical analysis of *Piper nigrum*.
3. Extraction and isolation of essential oil from leaves and fruits of *Piper nigrum.*
4. Determination of the antioxidant activity of essential oil of leaves and fruits of *Piper nigrum.*
5. Evaluation of larvicidal efficacy of essential oil against *Culex quinquefasciatus.*

1.3. HYPOTHESIS / RESEARCH QUESTION

The study hypothesizes that the anatomical study will reveal the presence of secretory structures, mucilage canals, trichomes and the histochemical study will aid the localization of Lipid bodies. There are few reports on the leaf essential oil of *Piper nigrum* as compared to other *Piper* species. In contrast, fruit essential oil has been widely explored as it is of economic value, and a comparison study of the antioxidant and larvicidal activity of dried leaves and fruit essential oil will reveal the potential of these oils.

1.4. SCOPE

Conducting anatomical studies on medicinal plants holds crucial significance for accurately characterizing species, particularly concerning the localization of

pharmacologically active compounds. Autofluorescence offers valuable insights into fluorescent compounds' composition, distribution, and physiological status within plant tissues. Further investigations using fluorescence microscopy, biochemical analysis, and spectral imaging can help characterize the specific fluorescent compounds and elucidate their roles in plant biology. Powder microscopy provides a comprehensive understanding of the plant's anatomy and composition. Essential oil studies offer a multifaceted exploration of these aromatic substances with opportunities for research, innovation, and practical application in various fields.

2. LITERATURE REVIEW

2.1. Anatomy of *Piper* species

Nugroho *et al*. (2019) did a comparative leaf and stem anatomy of ten *Piper* species and revealed the structural diversities and presence of a big secretory canal above the midrib, a distinctive character of *Piper* leaves. Also, the presence of glandular trichomes on the leaves is a distinctive feature as they play a significant role in their function as oil-producing organs. These specialized trichomes are directly involved in the secretion of essential oils. The vascular bundle found in the stem of *Piper* was closed collateral type.

Anatomical studies of the *Piper amalago* stem showed that vascular bundles are arranged in two concentric circles separated by sclerenchyma, which serves as protective and supportive functions (Santos *et al.*, 2015). Corti *et al*. (2021) showed the presence of peculiar secretory structures in the leaf of *Piper malgassicum.*

Raman *et al.* (2012) did a detailed microscopic analysis of the leaf, petiole, stem, and aerial root of *Piper sarmentosum* with that of *Piper betle,* stating both the species are distinct and that the leaves of both species can be compared with few differences. Anatomical studies on the stem and leaves of *Piper nigrum* carried out by Shethi *et al*. (2019) revealed the presence of non-glandular, unicellular trichomes, presence of thick cuticle, maximum number of cortical and medullary vascular bundle that is conjoint collateral and in leaf presence of big secretory canal in the midrib, unicellular trichomes.

The anatomical studies by Marinho *et al*. (2019) showed that the transverse section of *Piper umbellatum* leaves, when devoid of staining treatment, reveals secretory cells containing a translucent secretion with an oily appearance.

2.2. HISTOCHEMICAL STUDY

Corti *et al*. (2012) carried out histochemical localization of natural lipids using Sudan Red III-IV in the leaf of *Piper malgassicum*, which revealed its presence in leaf cuticle and glandular trichomes which appeared reddish, cutin components resulted positive for this stain, appearing brownish. Also, in the stem, Sudan III-IV positively stained the cuticle and lipid droplets occurring in the lumen of the phloem within the vascular bundles.

Marinho *et al*. (2019) investigated the secretory cells of *Piper umbellatum* leaves with different histochemical tests.

2.3. FLUORESCENCE MICROSCOPY

Corti *et al*. (2012) observed autofluorescence in green leaves of *Piper malgassicum* in blue light, which resulted in red fluorescence of the chloroplast due to chlorophyll in all chlorenchyma cells, including palisade and spongy mesophyll cells. Lipid droplets appeared as yellow fluorescence within chlorenchyma cells.

The fluorescence studies by Raman *et al*. (2012) in the transverse section of the *Piper betle* stem show autofluorescence of the secretory cells, cuticles, sclerenchyma, and endodermis. Microscopic analysis of *Piper umbellatum* was analysed by Marinho *et al*. (2011), wherein the transverse section of leaves showed blue autofluorescence of the secretory cells under UV light.

2.4. POWDER MICROSCOPY

Kadam *et al*. (2013) reported the powder microscopy characteristics of *Piper nigrum* fruit, which indicated the presence of epicarp, mesocarp, sclereid, stone cells, starch, and oil cells. Shaheen *et al.* (2019) did the microscopic evaluation of powder *Piper nigrum* fruit that indicated the presence of lignified endocarp cells, oil cells, hypodermal parenchyma cells, starch granules, testa with reddish brown pigments, stone cells, annular vessels. Microscopic

evaluation was carried out by Chaudhary *et al*. (2015) of *Piper longum* fruit that showed the presence of stone cells, oil globules, and brown content.

2.5. ISOLATION AND ANALYSIS OF ESSENTIAL OIL

Bagheri *et al.* (2014) extracted *P. nigrum* essential oil by supercritical carbon dioxide (SC-CO2) technique and stated that the main components isolated by this method under optimal conditions were beta-caryophyllene (25.38%), limonene (15.64%), sabinene (13.63%), 3-carene (9.34%), beta-pinene (7.27%), and alpha-pinene (4.25%).

Sasidharan and Menon (2010) studied the chemical composition of *P. nigrum*. They concluded that the main compounds of its leaf essential oil were alpha-bisabolol (24.3%), alpha-cubebene (20%), elemol (15%), bisabolene (15%), and alpha-guaiene (15%) that was completely different with seed essential oil. Parts of plants differ in terms of essential oil constituents.Li *et al*. (2020) analysed the chemical composition of black and white pepper essential oil collected from five different locations, and the oil was isolated using the Clevenger apparatus. *P. nigrum* essential oil showed strong antifungal and antibacterial activity. They also concluded that black pepper essential oil is slightly superior to white pepper oil.

Kapoor *et al.* (2009) extracted the essential and oleoresin (ethanol and ethyl acetate) of *Piper nigrum* using the Clevenger and Soxhlet apparatus. The major component of pepper essential oil was beta-caryophyllene (29.9%), limonene (13.2%), beta-pinene (7.9%), and sabinene (5.9%). The significant components of ethanol and ethyl acetate oleoresins were piperine (63.9 and 39.0%). Salleh *et al*. (2012) studied the chemical composition of leaf and stem essential oils from *Piper officinarum* and concluded that abundant components of leaf oil were beta-caryophyllene (11.2%), alpha-pinene (9.3%), sabinene (7.6%), beta-selinene (5.3%), limonene (4.6%). Stem oil consists of beta-caryophyllene (10.9%), alpha-phellandrene (9.3%), linalool (6.9%), limonene (6.7%), and alpha-pinene (5.0%).

Varughese *et al*. (2016) reported that the leaf essential oil of *P. longum* from the Western Ghats region of Kerala, India, had elemol (22.5%), β-caryophyllene (16.8%), and α-humulene (5.8%) as the major components, while the stem essential oil was dominated by β-pinene (34.8%), α-pinene (14.0%), limonene (10.3%), and β-caryophyllene (9.3%). Jeena et al. (2014) studied the chemical composition of *Piper nigrum* essential oil and concluded that the main constituents were beta-caryophyllene (23.98%), limonene (14.36%).

Parthasarathy *et al*. (2008) studied the spatial influence of beta-caryophyllene and nerolidol in the leaf oil of *Piper nigrum* collected from western ghats of Karnataka and Kerala. Utpala *et al*. (2014) studied *Piper* species' leaf volatile oil constituents using GC/MS from western ghats. They reported that the most abundant compounds in *Piper* leaf oil of western ghats were beta-caryophyllene, Nerolidol and beta-Elemene and the leaf oil is rich in sesquiterpenoids.

2.6. ANTIOXIDANT ACTIVITY

Bagheri *et al*. (2014) conducted a comparison between the radical scavenging of black essential oils extracted via supercritical CO_2 extraction and hydro-distillation. The result demonstrated that essential oil obtained through supercritical CO_2 extraction exhibited higher DPPH radical scavenging activity (IC_{50}=103.28 µg/mL) compared to those obtained through hydro-distillation (IC_{50}=316.27µg/mL). Kapoor *et al*. (2009) evaluated the antioxidant activities of essential oil and oleoresins and compared with its ethanol and ethyl acetate oleoresins and also with synthetic antioxidants like BHA, BHT and propyl gallate using various antioxidant assays in vitro. The study stated that pepper oil and its ethyl acetate extract showed better ferric reducing power than its ethanol extract compared to BHA and BHT but were lower than that of propyl gallate. The result also indicated pepper oil exhibited greater efficacy in

scavenging DPPH radicals compared to pepper oleoresins, BHA and BHT but lower than propyl gallate.

Andriana *et al*. (2019) evaluated the essential oils of *Piper nigrum* and *Piper cubeba* for antioxidant activities and concluded that high antioxidant activity was found in *Piper cubeba* essential oil than *Piper nigrum.* Salleh *et al*. (2012) evaluated the leaf and stem essential oils of *Piper officinarum* for antioxidant activities and concluded that they showed weak activity (IC_{50} = 777.4 µg/mL) in the DPPH system but showed moderate lipid peroxidation inhibition in the β-carotene-linoleic acid system (88.9 ± 0.35%) compared with BHT (95.5 ± 0.35%). Nahak *et al*. (2011) evaluated the antioxidant activity of ethanol extract of *Piper cubeba* and *Piper nigrum*. They stated that high antioxidant activity was found in *Piper cubeba,* i.e., 77.61±0.02% in comparison to *Piper nigrum* extracts with 74.61±0.02% with IC_{50} values 10.54±0.12µg/mg and 14.15±0.02 µg/mg respectively.

Kavitha *et al*. (2018) studies showed that the ethanol extract of *Piper nigrum* leaf has moderate activity (26.78 µg and 42.68 µg) in DPPH and ABTS when compared with the control ascorbic acid (2.54 µg and 4.39 µg) respectively. Sultana *et al*. (2022) studies revealed good antioxidant efficacy of *Piper nigrum* essential oil with an IC_{50} value of 35.83±2.92 µg/mL as compared to standard ascorbic acid (27.34±1.86 µg/mL).

2.7. MOSQUITO LARVICIDAL ACTIVITY

Escaline (2015) reported the chemical composition of leaf essential oil of *Piper nigrum* using the GC-MS method and evaluated crude extract (methanolic extract) of leaves of *Piper nigrum* for larvicidal activity against *Ae. aegyptii* (LC_{50} = 34.97 µg/mL).

Huong *et al*. (2019) reported the chemical composition of essential oils from *Piper* species (*P. caninum, P. longum, P. montium, P.mutabile*) collected from central Vietnam. They analysed larvicidal activity against *Ae.aegytii* with LC_{50} and LC_{90}= values less than 10µg/ml.

Dey *et al*. (2020) study assessed the larvicidal activity of *Piper longum* petroleum ether (PE), chloroform (CHL), methanolic and aqueous extract against *Aedes aegytii, Anopheles stephensi, Culex quinquefasciatus.* The water extract showed an LC_{50} = 50.81, 133.42, and 257.70 ppm and LC90 = 104.47, 279.60, and 582.44 ppm, respectively. Similarly, PE extract of *P. longum* showed LC_{50}= 213.88, 223.70, and 289.92 ppm and LC90 = 474.96, 424.92, and 596.908 ppm against 3rd instar larvae of *Ae. aegypti, Anopheles stephensi and Culex quinquefasciatus*. The CHL extracts also showed LC_{50}=389.33, 219.34, and 234.45 ppm and LC90= 986.07, 420.98, and 561.09 ppm, and ME extracts with LC50 =395.51, 134.712, 315.35 ppm, and LC_{90} = 878.33, 464.72 and 626.87 ppm, respectively.

Weluwanarak *et al.* (2023) investigated the larvicidal activity of hexane extracts from *Piper retrofractum* and its alkaloid compounds, piperine and piperanine, was investigated against *Culex quinquefasciatus* larvae. The results revealed significant toxicity with LC_{50} values of 0.9ppm from the extract, 0.27ppm for piperine, and 2.97ppm for piperanine after 24 hours of exposure. Upon further investigation, it was found that both the extract and the isolated alkaloids reduced the activities of carboxylesterase, glutathione-S-transferase, and acetylcholinesterase enzymes, which may be responsible for their larvicidal activity.

Vasudevan *et al*. (2009) investigated the crude extracts of dried ripened fruits of *P. nigrum* against *Culex quinquefasciatus.* The results indicated that the ethanolic extract showed better activity than the aqueous extract. Prabhu *et al*. (2022) investigated the aqueous and ethanol extracts of *Piper betle* L. leaves for mosquito larvicidal activity against *Culex quinquefasciatus.* The results indicated that the ethanol extract having LC_{50} =118.26 µg/mL and LC_{90} = 50.0 µg/mL at 24 and 48 hours respectively showed better activity than the aqueous extract with LC_{50} =52.63 µg/mL

3. MATERIAL AND METHODS

3.1. COLLECTION OF PLANT SAMPLES

The fresh plant materials of *Piper nigrum* were procured from the Local farm, Gimona, Betki, Goa. The details of the collection site are mentioned in **Table 3.1**. Mature and healthy leaves and fruits were collected in separate zip-lock polythene bags and brought to the laboratory for further studies.

Table 3.1: Plant collection site and their GPS Coordinates.

Sr. No.	Name of the plant	Collection site	GPS Coordinates
1.	*Piper nigrum*	Gimona, Betki, Goa	15° 29'56.9"N 73°58'52.0"E

3.2. ANATOMICAL STUDIES

Anatomical studies of plant species were carried out to understand the structure of the leaf, petiole, stem, and fruit. Fresh, mature leaves, petiole stems, and fruits were brought to the laboratory. Free-hand thin sections of all the above parts were taken and stained with 0.1% safranin, rinsed with distilled water, and mounted on the glass slide using 10% glycerine. Sections were observed and examined using bright-field microscopy. The desirable portions were photographed using a Nikon Eclipse E200 microscope, and images were captured using TC–capture software. The following standard procedures were adopted for the preparation of stains.

Preparation of stain: 0.1% w/v Safranin was prepared by dissolving 0.1g of safranin in 100 mL distilled water.

Protocol: Thin free-hand sections were placed in distilled water and then introduced in the stain for 2-3 minutes, and the excess stain was removed by washing the sections in distilled water. Then, the sections were mounted on a clean, stain-free glass slide using 10% and

observed using a bright–field Nikon Eclipse E200 microscope under 4X, 10X, AND 40X magnification.

3.3. HISTOCHEMICAL STAINING

The study was conducted to understand the localization of lipids in mature leaves, petioles, stems, and fruit. Free-hand thin sections of all the parts mentioned above were taken and stained with Sudan IV, Oil O red stain, and Iodine- Potassium Iodide. Sections were observed and examined using bright-field microscopy. The desirable portions were photographed using a Nikon Eclipse E200 microscope, and images were captured using TC–capture software. The following standard procedures were adopted for the preparation of stains.

3.3.1 Localization of Total Lipids

A. Sudan IV Method (Chiffelle and Putt, 1951)

Preparation of stain: 0.7g of the dye was dissolved in 100mL of ethylene glycol by heating the solution to 100-110℃ and stirred thoroughly and the stain was filtered and used.

Protocol: Free-hand thin sections were placed in pure ethylene glycol for 3-5mins. Then, the sections were stained in Sudan IV dye for 7-10 mins. Then, the sections were transferred to 85% ethylene glycol for 2-3 min, rinsed with distilled water, mounted on a clean stain-free glass slide using 10% glycerine, and observed using a bright-field microscope. Lipids are stained orange-red.

B. Oil O Red Method (Lillie, 1965)

Preparation of stain: 0.5g oil red O is added to 100 ml of 98% isopropanol. 6ml of stock solution is diluted with 4ml water, and after 30min, it is filtered.

Protocol: Free-hand thin sections were placed in water and then rinsed in 60% isopropanol (freshly diluted) and transferred to the stain for 10min and then differentiated in 60% freshly diluted isopropanol, washed in distilled water and mounted on a clean slide. Lipids are stained dark red.

3.4. FLUORESCENCE MICROSCOPY

Protocol: Free-hand thin sections were placed in water, mounted on a clean slide with dilute glycerine, and observed under a Fluorescence microscope using a UV excitation filter for autofluorescence.

3.5. POWDER MICROSCOPY

Preparation of *P. nigrum* leaf and fruit powder: The collected *P. nigrum* samples were shade-dried, coarsely powdered with the help of a mixture, and further used for microscopy studies.

Protocol: A small amount of coarse powder was taken on the slide, further stained with safranin for 1-2 minutes, and observed under a bright field microscope.

3.6. ISOLATION OF ESSENTIAL OIL

Fresh leaves and fruits were brought to the laboratory, washed with tap and distilled water, and shade-dried at room temperature for 6-7 days. Dried leaves were cut into small pieces with Secateur and ground in the mixture to form a fine powder. Similarly, dried fruits were ground to form fine powder. The powder was stored in closed containers for further use. 100g of each powder and 1000mL of distilled water were subjected to hydro-distillation using a Clevenger-type apparatus with 5L capacity for 4 hours at 70℃. The extraction of essential oil from its aqueous phase was obtained using n-hexane. The extract was further dried over anhydrous sodium sulphate and stored in amber vials at 4℃ until further analysis.

Essential oil yield was calculated using the formula:

$$\textbf{Yield \%} = \frac{\textit{weight of oil extracted (g)}}{\textit{weight of sample taken}} \times 100$$

3.7. ANTIOXIDANT ACTIVITY

The antioxidant study of *P. nigrum* leaf and fruit essential oils was carried out using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method.

A. **Preparation of DPPH:** 24mg of DPPH was dissolved in 100mL of ethanol to prepare the stock solution and stored in an amber-coloured bottle in the dark. The working solution was prepared by adding 10mL of stock solution in 45mL ethanol.

B. **Preparation of L-ascorbic acid solution:** 10mg of ascorbic acid was dissolved in 10 mL of ethanol. Serial dilution was carried out to prepare concentrations (12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL).

C. **Preparation of test solution:** 1 mL of leaf essential oil of *P. nigrum* dissolved in 1 mL of ethanol. Serial dilutions were performed to prepare concentrations (12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL). A similar procedure was followed for fruit essential oil.

D. **Preparation of control:** 2mL of DPPH was used as a negative control.

In the reaction mixture, 2mL of DPPH working solution was added to 1mL of each sample solution of different concentrations and incubated in the dark for 30 minutes at room temperature. The absorbance was measured at 517nm.

The following equation calculated the percent inhibition.

Inhibition % = $(A_0 - A_1)/A_0$ x 100

Here, A_0 is the absorbance of the control and

A_1 is the absorbance of the sample.

The antioxidant assay was performed in triplicate, and the mean value was expressed. IC_{50} values were calculated by plotting percent (%) inhibition against the essential oils' concentration (µg/mL).

3.8. MOSQUITO LARVICIDAL ACTIVITY

A. Source of mosquito larvae

Mosquito larvae of *Culex quinquefasciatus* were screened for larvicidal activity. The ICMR-National Institute of Malaria Research, Campal, Panaji, Goa, maintained the cyclic colony of this mosquito species in their insectary. 3rd instar larvae were used for the bioassay. Fish flakes were used in the laboratory as food to maintain mosquito larvae.

B. Larval Bioassay

Detection of susceptibility of larvae to the essential oil of *Piper nigrum* leaves and fruit: 20 larvae of *Culex quinquefasciatus* were selected and placed in separate plastic bowls containing 100mL distilled water. The essential oil was dissolved in Dimethyl sulfoxide (DMSO) to prepare various concentrations (50ppm, 100ppm, 150ppm, 200ppm).

The control was prepared using a desired concentration of DMSO in 100mL distilled water. For each concentration, four replicates were maintained. Larval mortality was assessed by direct observation of larvae movements 24 and 48 hours after exposure at room temperature.

The percentage mortality was calculated using the formula:

$$\textbf{Percentage mortality} = \frac{\textit{Average of dead larvae}}{\textit{Total number of larvae exposed}} \textbf{ x 100}$$

Calculation of LC_{50} and LC_{90} value

The activity of the essential oil against test mosquito larvae in terms of LC_{50} and LC_{90} values was calculated using SPSS software and associated with 95% fiducial limits, i.e., upper and lower confidence limits.

4. RESULTS AND DISCUSSION

4.1. Study of anatomical characteristics of *P. nigrum*

4.1.1. Leaf Anatomy

The leaf was dorsiventral with a prominent midrib. The transverse section of the leaf showed the presence of a single-layered epidermis with a thick cuticle. Epidermal cells were thick-walled and rectangular. Adaxial and abaxial epidermis consist of 1-2 hypodermal layers. The lower side of the epidermis consists of anomocytic stomata. The mesophyll tissue is differentiated into palisade cells, composed of compactly arranged elongated cells, which showed the presence of oil droplets, and no idioblasts were observed. Shethi *et al*. (2019) mentioned that idioblasts occasionally appeared in *P. nigrum*, and only oil droplets were observed. The spongy parenchyma cells are nearly circular and loosely arranged with small intercellular spaces **(Plate 3**).

In the transverse section of the midrib, both adaxial and abaxial epidermal layers were covered by a thick cuticle. The hypodermal layers of the adaxial epidermis are replaced in the ridge by a patch of angular collenchyma. Mesophyll tissue continues but is undifferentiated in the midrib region, made up of circular chlorenchyma on either side of vascular bundles. A large, single mucilage canal is present towards the adaxial surface. Unicellular trichomes are confined to the lower epidermis. Vascular bundles are solitary, collateral, and xylem adaxial. Similar observations were reported by Shethi *et al*. (2019) **(Plate 3).**

4.1.2. Stem Anatomy

The transverse section of the stem is round in shape. A thick cuticle covers a single layer of epidermis. The cortex follows the epidermal layer, which possesses three types of cells-collenchyma, chlorenchyma, and parenchyma. The outer cortex is collenchymatous, followed by a continuous band of sclerenchyma layer and 2-3 layers of chlorenchyma, which showed the presence of oil droplets, and the cells of the inner cortex are parenchymatous in nature. The

vascular bundle shows peripheral and cortical vascular bundle arrangements and a wavy strip of the sclerenchymatous ring is present, separating both arrangements. Vascular bundles are conjoint, collateral, and open. The Xylem of both the vascular bundles is highly lignified, but metaxylem elements are unlignified. The center of the stem is occupied by a large, lysigenous type of mucilage canal, similar to the observations reported by Shethi *et al*. (2012) **(Plate 4: a, b, c).**

4.1.3. Petiole Anatomy

The transverse section of the petiole showed a single epidermal layer, and the cells were tabular to squarish in shape, covered externally by the cuticle. Hypodermal layers 1-2, consisting of polygonal, circular parenchyma cells, show the presence of calcium oxalate crystals, followed by 5-8 layers of sclerenchyma present all around the petiole. Unicellular trichomes are present. Vascular bundles collateral, few, are arranged in a U-shaped arc in the ground tissue, and larger and smaller bundles alternate with each other. The xylem faces toward the petiole's center, and the phloem faces outwards. A large mucilage canal is present at the center of the petiole. Similar observations were reported in *P. sarmentosum* by Raman *et al*. (2012) **(Plate 4: d, e, f).**

4.1.4. Fruit Anatomy

The fruit consists of a thick pericarp, approximately one-third of its overall size, and an inner mass of perisperm. The pericarp consists of an external epicarp and a large parenchymatous mesocarp and endocarp.

The epicarp is the single epidermal outer layer comprising tabular cells with distinct cuticles. The mesocarp is a big band of tangentially elongated parenchymatous cells. Oil cells are seen in the outer region of the mesocarp, followed by the presence of vascular tissue, which facilitates the transport of water and nutrients during fruit development, and a regular row of

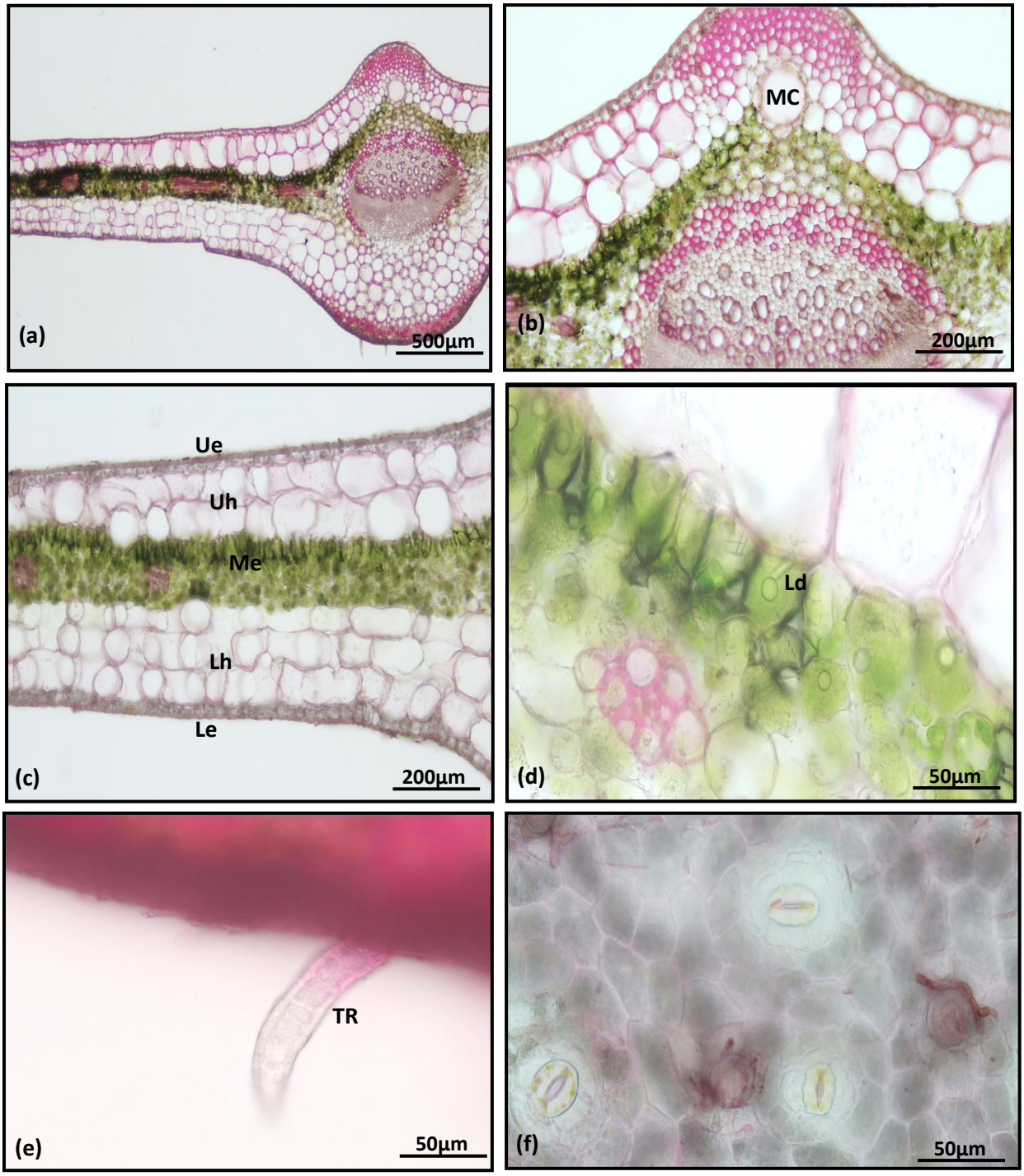


Plate 3: Anatomy of *Piper nigrum* leaf. (a) Overview of leaf, (b) Overview of leaf midrib, (c) T.S of leaf lamina, (d) Mesophyll cells showing the presence of Lipid droplets, (e) Leaf showing non glandular, unicellular trichome, (f) Leaf showing anomocytic stomata. MC=Mucilage canal, Ue=Upper epidermis, Le= Lower epidermis, Uh= Upper hypodermis, Lh= Lower hypodermis, Me= Mesophyll tissue, Ld= Lipid droplet, TR= Trichome.

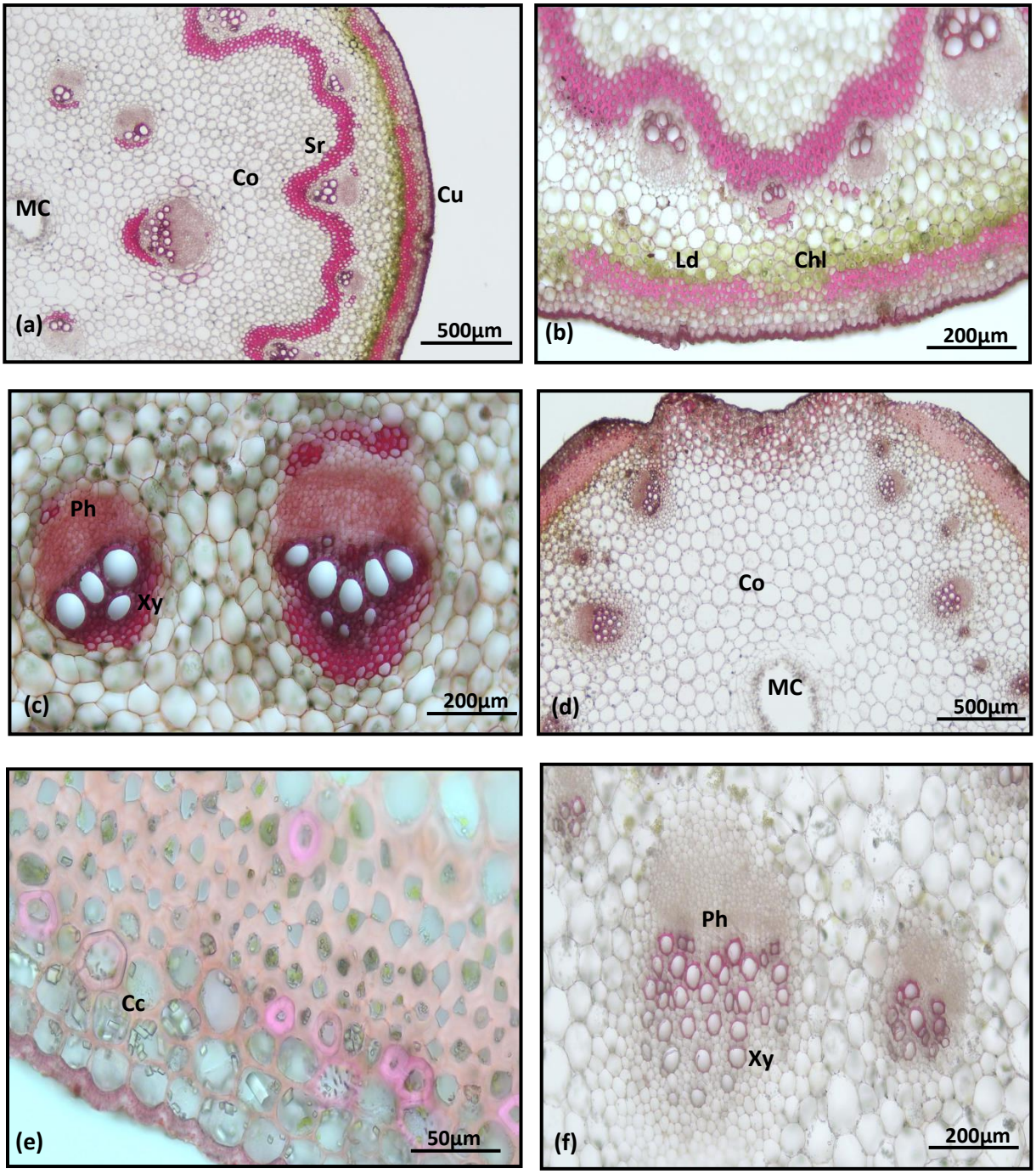


Plate 4:Anatomy of *Piper nigrum* stem and petiole. (a)Portion of overview of stem, (b) Minute quantity of Lipid droplets in chlorenchyma layer, (c) Section of stem showing vascular bundles, (d) Portion of overview of petiole, (e) Section of petiole showing the presence of Calcium oxalate crystals, (f) Section of petiole showing Vascular bundles.

Cu= cuticle, Sr= Sclrenchyma ring, Co= Cortex, MC= Mucilage canal, Cc= CaOx crytals, Xy= xylem, Ph= Phloem, Chl=Chlorenchyma.

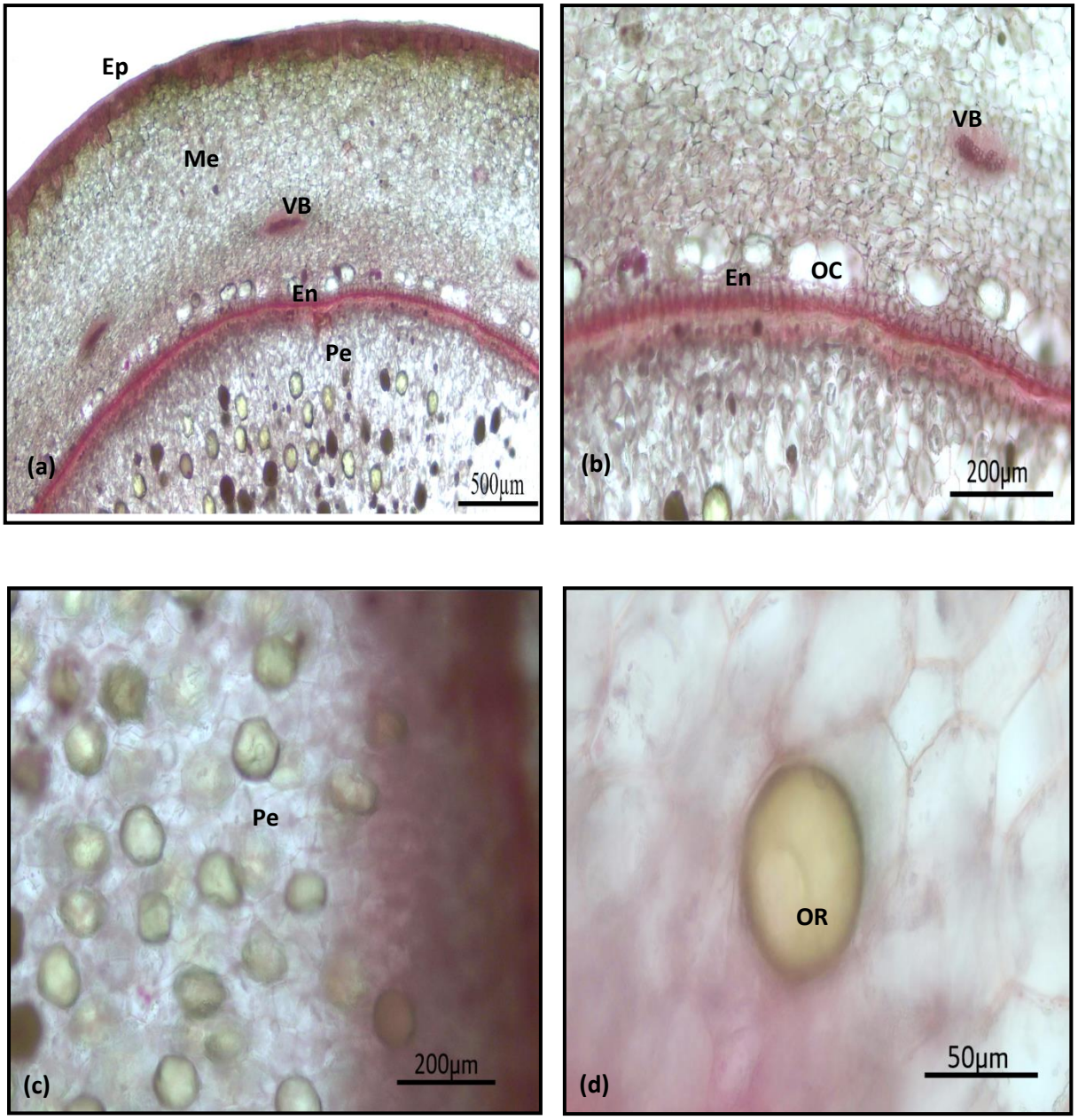


Plate 5: Anatomy of *P. nigrum* fruit. (a) Portion of overview of Fruit, (b) Section showing Endocarp and Oil cells, (c) Perisperm showing abundant oleoresins, (d) Oleoresin.

Ep- Epicarp, Me- Mesocarp, En- Endocarp, Pe- Perisperm, OC- Oil Cell, OR- Oleoresin.

oil cells is seen in the inner region of the mesocarp. The endocarp is the innermost layer of the fruit surrounding the seed; the testa is comprised of a single layer of thick-walled sclrenchymatous cells. Perisperm contains parenchymatous cells containing olio resins and is densely packed with abundant, oval-to-round-shaped, simple starch grains **(Plate 5).**

Shethi *et al*. (2019) investigated the anatomical description of the stem and leaves of *Piper nigrum* L. They found similar results to those obtained in this study. Their results showed that the leaves of *P. nigrum* possessed non-glandular trichomes, unicellular epidermis covered by a thick cuticle, presence of a big secretory canal in the midrib, Presence of cortical and medullary vascular bundle and mucilage canal at the center. Raman *et al*. (2012) reported that the secretory cells in mesophyll were less frequently observed in *Piper sarmentosum* and more frequently in *Piper betle.*

4.2. Histochemical studies

4.2.1. Localization of Lipids using Sudan IV stain

4.2.1.1. Localization of Lipids in the transverse section of fruit

Oil globules were seen in the secretory cells, which are present in the inner region of the mesocarp. Seed coat was stained red and positive for Sudan IV **(Plate 6: a, b, c).**

4.2.1.2. Localization of Lipids in the transverse section of the leaf

In the transverse section of the leaf lamina, lipids appeared to be present throughout the mesophyll tissue. Cuticle was stained slightly red in colour **(Plate 6: d, e, f).**

4.2.1.3. Localization of Lipids in the transverse section of the stem

In this, Sudan IV positively stained the cuticle. Lipid droplets were present in the chlorenchyma cells, stained orange-red. The vascular bundle showed reddish-brownish colouration **(Plate 7: a, b, c).**

4.2.1.4. Localization of Lipids in transverse section of petiole

In this section, a minute quantity of lipid droplets was observed in the cortex region. The cuticle layer was slightly stained red with Sudan IV, and the vascular bundle showed reddish-brown coloration **(Plate 7: d, e, f).**

4.2.2. Localization of Lipids using Oil O Red stain

4.2.2.1. Localization of Lipids in transverse section in fruit

Lipid globules were seen in the secretory cell, stained dark red. Seed coat was stained dark red and positive for Oil O Red **(Plate 8: a, b).**

4.2.2.2. Localization of Lipids in transverse section in leaf

Lipid droplets were stained dark red and appeared throughout the mesophyll tissue. The cuticle appeared slightly red and tested positive for Oil O Red stain **(Plate 8: c, d).**

Corti *et al*. (2012) investigated the localization of lipids using Sudan IV in the leaves of *Piper malgassicum*. They revealed its presence in leaf cuticle and glandular trichomes, which appeared reddish, and cutin components resulted in a positive for this stain, appearing brownish. Also, in the stem, Sudan III-IV positively stained the cuticle and lipid droplets occurring in the lumen of the phloem within the vascular bundles. Similar results were obtained in this histochemical localization using Sudan IV.

The localization of lipids in the transverse section of *Piper umbellatum* leaves was reported by Marinho *et al*. (2011), which resulted in orange, red, and black when stained with Sudan III, Sudan red B, and Sudan black B, respectively.

A histochemical study was conducted to localize lipids in different parts of *P. nigrum* using Sudan IV and Oil O Red stains and to observe the staining intensity of both stains. It was

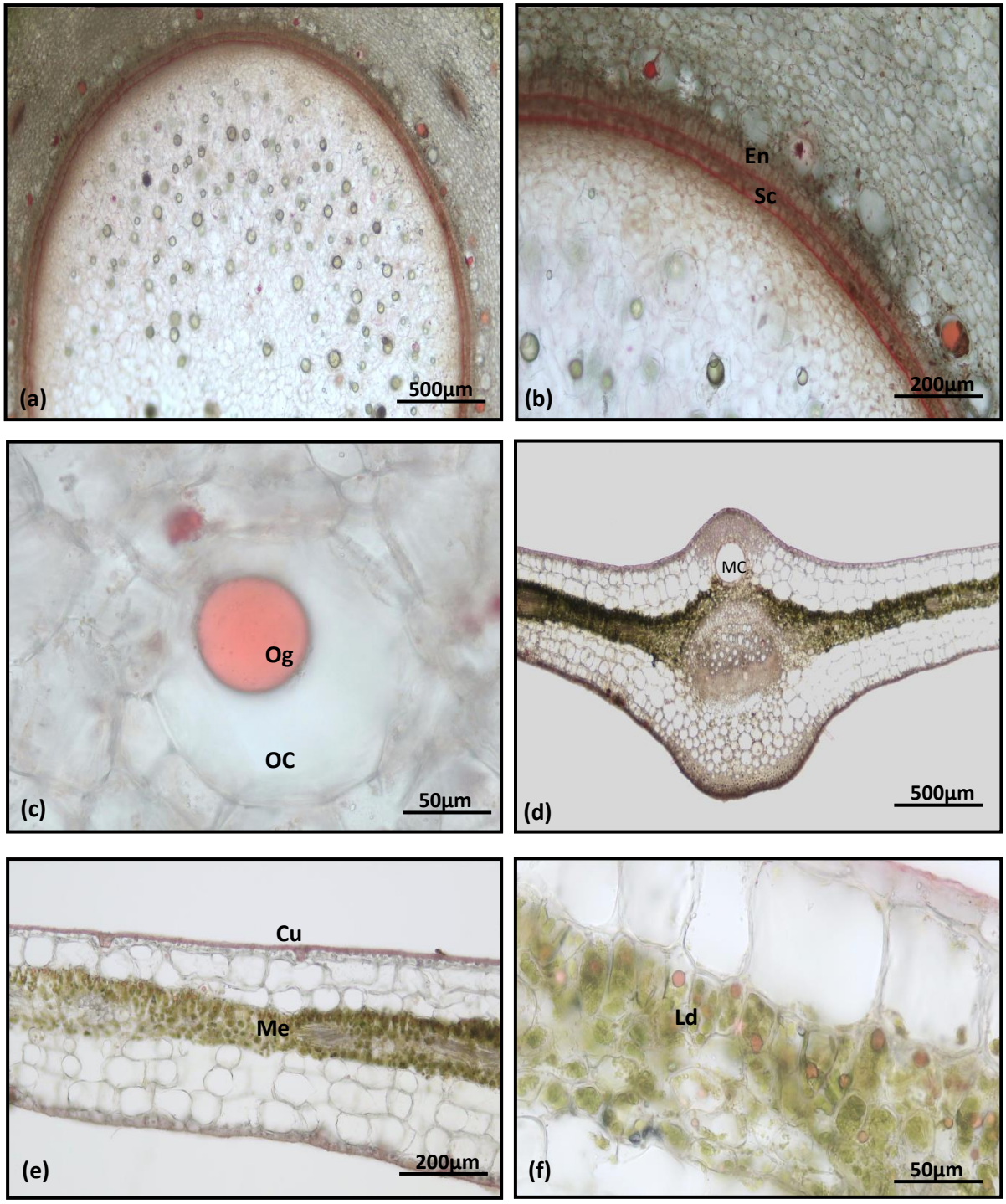


Plate 6: Localization of Lipids in T.S of fruit and leaf. (a) Overview of fruit, (b) Portion of the fruit stained with Sudan-IV, (c) Lipid globule in the oil cell, (d) Overview of leaf, (e) T.S of leaf lamina, (f) Mesophyll tissue showing stained Lipid droplets.

En= Endocarp, Ld=Lipid droplet, Og= Oil globule, OC= Oil cell, MC= Mucilage canal, Cu= Cuticle, Me= Mesophyll tissue, Sc= seed coat.

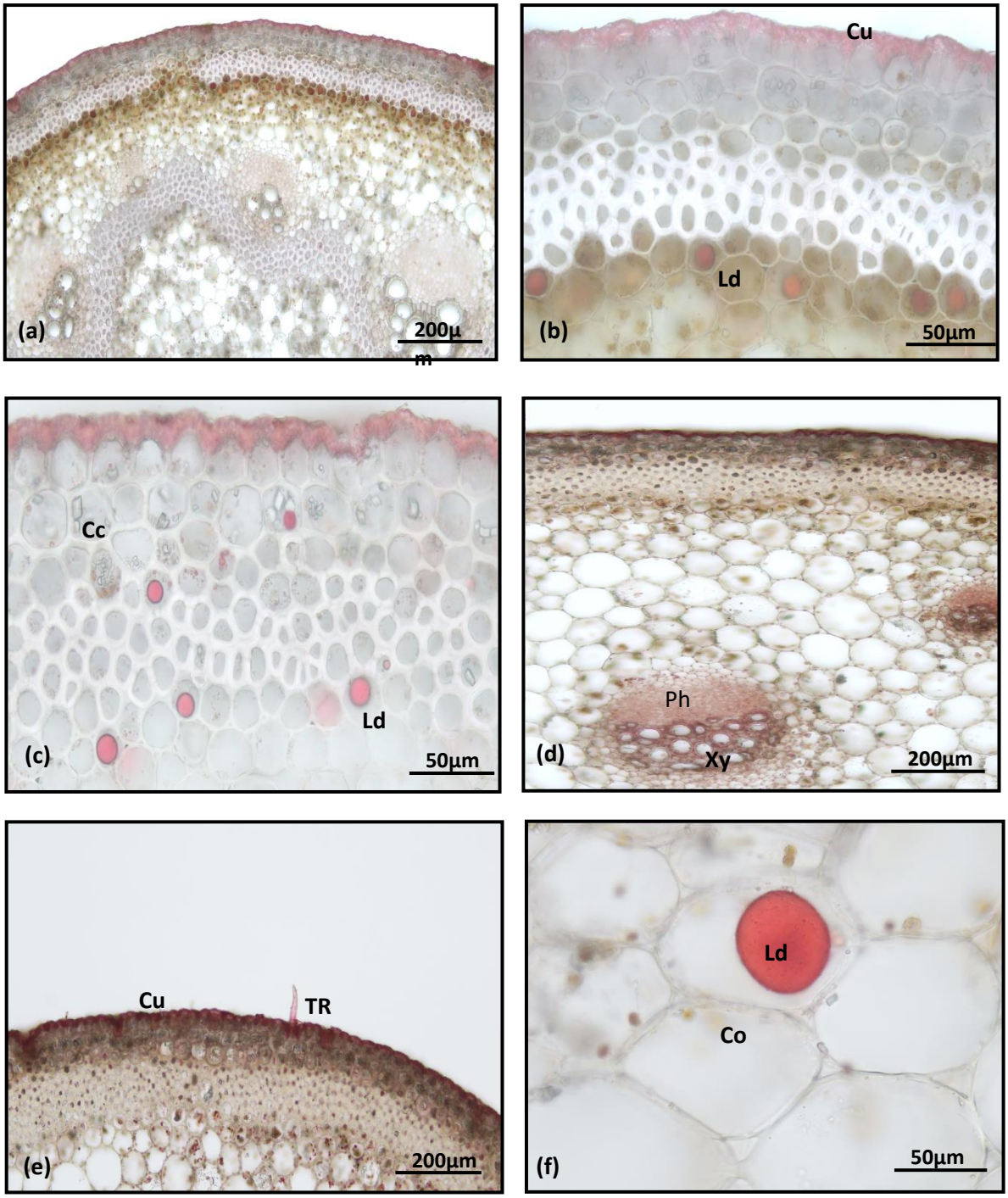


Plate 7: Localization of Lipids in T.S of stem and petiole. (a) Overview of T.S of stem stained with Sudan IV, (b) Section showing Lipid droplet, (c) Section showing the presence of Calcium oxalate crystals, (d) Portion of overview of petiole, (e) Section of petiole showing trichome and cuticle stained positive for Sudan IV, (f) Cortex showing the presence of minute Lipids. Cu= Cuticle, Ld= Lipid droplet, Cc= CaOx crystals, Tr= Trichome, Xy= xylem, Ph= Phloem, Co= Cortex.

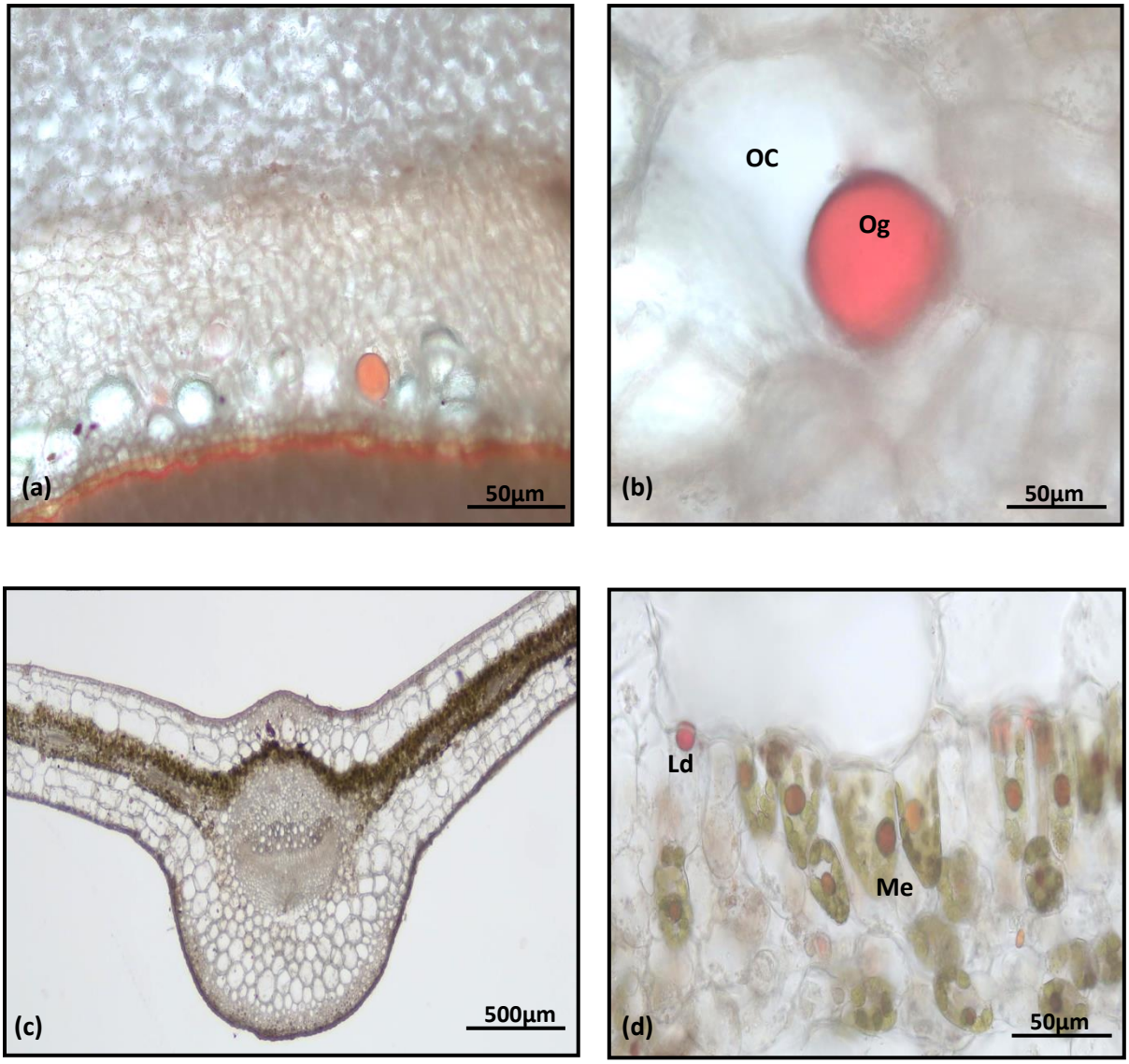


Plate 8: Localization of Lipids using Oil O Red stain in T.S of fruit and leaf of *P. nigrum.* (a) Portion of fruit stained with Oil O Red, (b) Lipid droplet stained positive for Oil O Red, (c) Overview of leaf, (d) Mesophyll tissue showing stained Lipid droplets. OC= Oil Cell, Ld= Lipid droplet, Og= Oil globule, Me= Mesophyll tissue.

observed that lipid droplets/globules appeared dark red with Oil O Red stain, whereas Sudan IV resulted in an orange-red colouration of the lipids.

4.3. Fluorescence study of *Piper nigrum*

Table 4.1: Autofluorescence of different parts of *Piper nigrum* under UV (330-380 nm).

Sr. No.	Plant part used	Excitation filter used UV (330-380)
1.	Transverse section of leaf	❖ Cuticle exhibits a blue autofluorescence. ❖ Chlorophyll autofluoresces in red. ❖ Vascular bundle autofluoresces in bluish-white **(Plate 9: c, d).**
2.	Transverse section of fruit	❖ Epicarp, the inner region of the mesocarp, endocarp exhibits a blue autofluorescence, and the perisperm shows bluish-white autofluorescence **(Plate 9: a, b).**
3.	Transverse section of stem	❖ Cuticle shows white autofluorescence. ❖ Chlorophyll autofluoresces in red. ❖ The continuous band of the sclerenchyma layer, sclerenchymatous ring, and xylem vessels shows bluish-white autofluorescence **(Plate 10: a, b).**
4.	Transverse section of petiole	❖ Cuticle layer, trichomes showed white autofluorescence. ❖ Chlorophyll autofluorescence in red. ❖ Sclerenchyma in the cortex xylem vessels showed autofluorescence in blue **(Plate 10:c, d).**

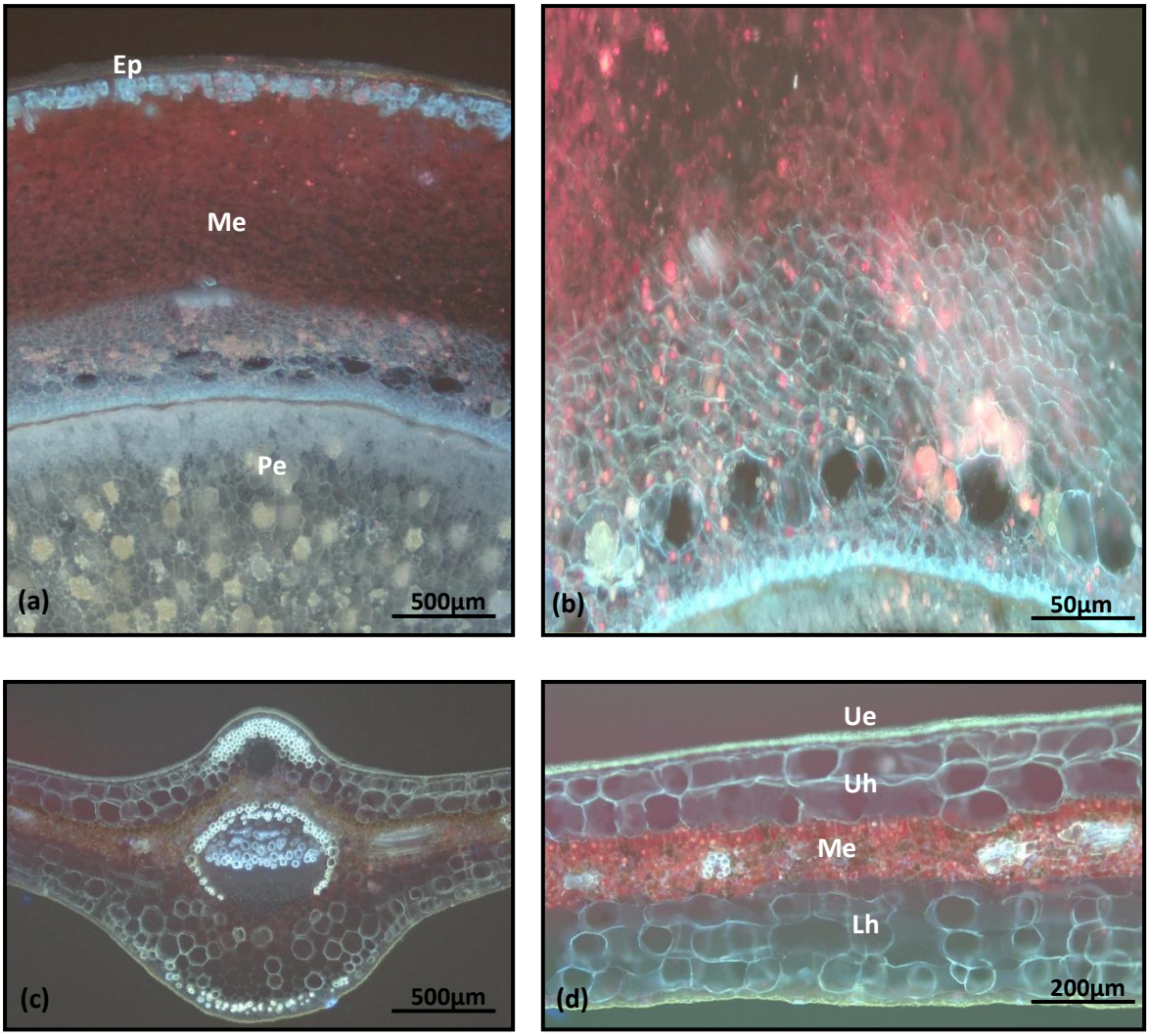


Plate 9: Autofluorescence of some tissues in T.S of fruit and leaf of *P.nigrum*. (a) Portion of overview of fruit, (b) Section showing inner region of mesocarp, (c) Overview of leaf, (d) T.S of leaf lamina.Ep= Epicarp, Me= mesocarp, Pe= perisperm, Ue= upper epidermis, Uh= upper hypodermis, Lh= lower hypodermis.

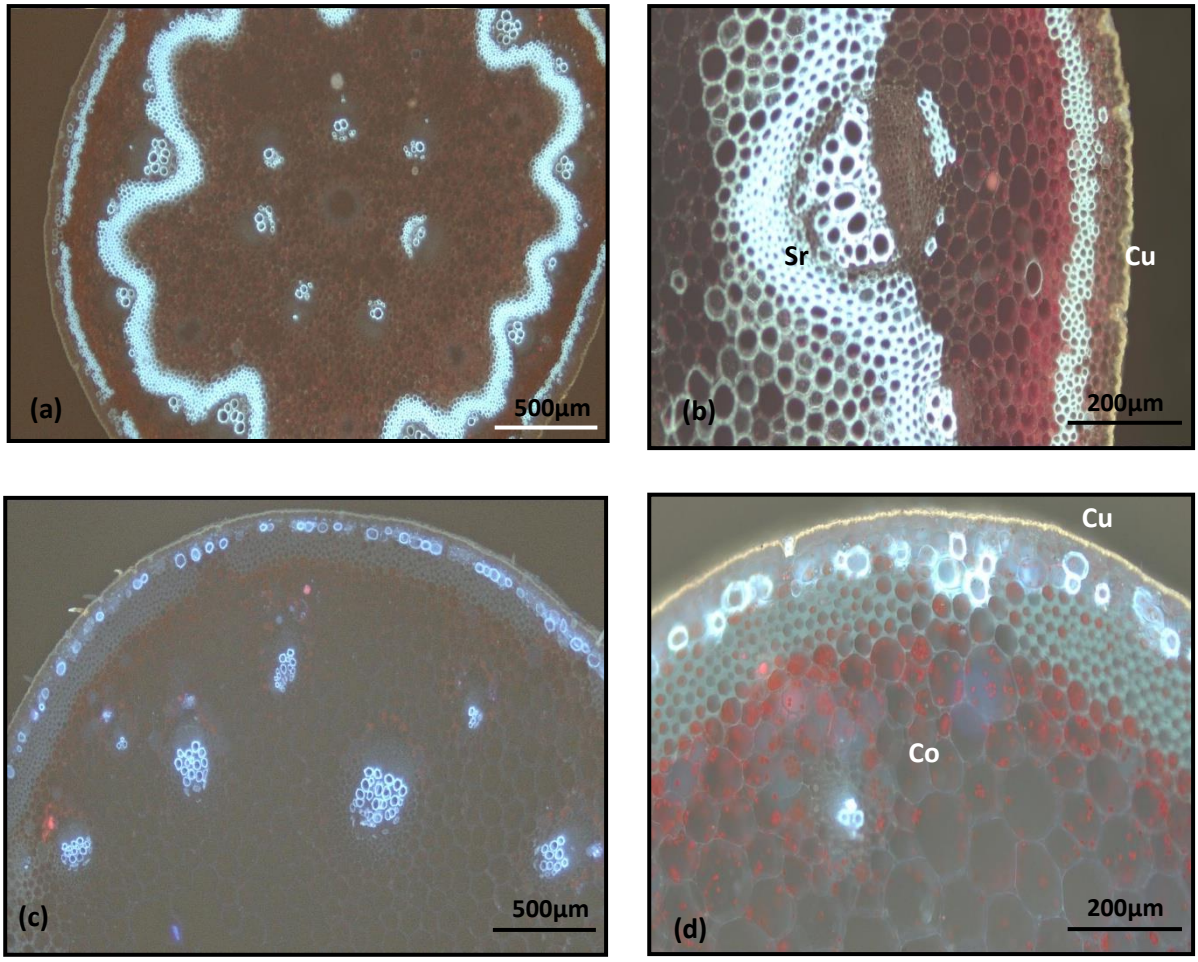


Plate 10: Autofluorescence of some tissues in T.S of stem and petiole of *P. nigrum.* (a) Portion of overview of stem, (b)Magnified image of (a), (c) Portion of overview of petiole, (d) magnified image of (c).

Corti *et al.* (2012) noted autofluorescence in the green leaves of *Piper malgassicum* when exposed to blue light, leading to red fluorescence emitted by chloroplasts containing chlorophyll in all chlorenchyma cells, including those in the palisade and spongy mesophyll layers. Dmitruk *et al*. (2019) reported fluorescence studies in the leaves of *Dracocephalum moldavic*a L., and analysis revealed the presence of phenolic compounds, characterized by blue-light blue autofluorescence in the sections and the presence of phenols in the cells of the tissues was confirmed by staining the sections with Toluidine blue O which results in green, blue colour.

According to Donaldson *et al*. (2020), the fluorescence in cutin may arise from phenolic acids or flavonoids attached to the cutin structure. Our study noted a comparable autofluorescence pattern in specific tissues of leaf, fruit, stem, and petiole of *Piper nigrum*.

4.4. POWDER MICROSCOPY

4.4.1. *P. nigrum* leaf powder:

The leaf powder is dark green in colour, fibrous, with a characteristic spicy odour. Microscopic examination of the powder reveals the presence of parenchyma tissue containing stomata, tubular cells with thick and lignified walls of xylem tracheid, and frequent observations of annular vessels **(Plate 11: a, b, c and d).**

4.4.2. *P. nigrum* fruit powder:

The fruit powder is dark brown with a spicy aroma and pungent taste. The microscopic examination of the powder reveals grey-coloured perisperm cells, abundant in starch granules **(Plate 11: e, f).** Similar observations were made by Thatipelli *et al*. (2019) and Rezvanian *et al*. (2016) in *P. nigrum* fruit powder.

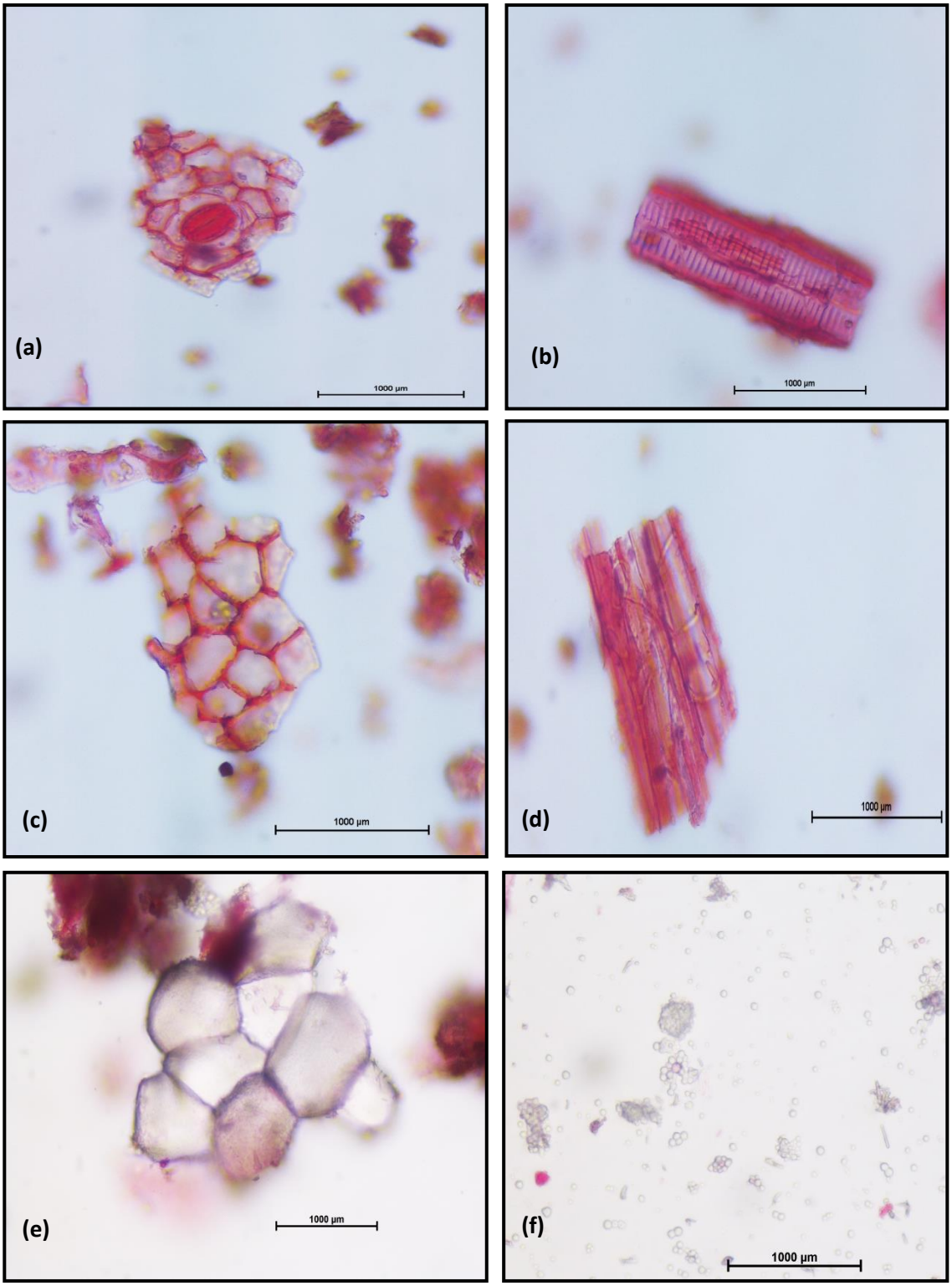


Plate11: Powder microscopy of leaves (a-d) and fruit (e-f) of *P. nigrum*. (a)Anomocytic stomata, (b) Xylem tracheids (c) Parenchymatous cells, (d) Annular vessels, (e) Perisperm cells, (f) Starch granules.

4.5. Extraction of essential oil

The dried leaves and fruits were ground into a fine powder **(Plate 12 b)**, and the essential oils of dry leaves and fruits of *P. nigrum* were extracted using a Clevenger hydro-distillation apparatus **(Plate 12 c)**. The essential oil yield of dry leaves was 0.7 %, whereas dry fruits yielded 3.07 % **(Plate 12 d)**. The essential oil isolated from the dry leaves was pale yellow with a spicy, peppery, aromatic scent. In contrast, fruit essential oil was colourless and had a strong, spicy, and peppery aroma, more intense and pungent compared to the leaves.

Several factors affect the quality of essential oils, such as Environmental conditions like temperature, humidity, rainfall, harvesting stage, plant material used, and storage conditions. All these factors also have an impact on the yield of essential oils.

Spice essential oils extracted from plants are known to possess medical and pharmacological activities such as antioxidant, antimicrobial, insecticidal, and various other properties, having diverse applications ranging from their use as resins and flavouring agents to serving as additives in foods and components in perfumes, cosmetics, and soaps (Arshad *et al.,* 2014). Pale yellow to orange, yellow essential oil from the fruits of *P. nigrum*, having a 5-6% yield, was extracted by steam distillation (Jeena *et al*., 2012).

4.6. Antioxidant activity of essential oil

Antioxidants are the agents that stop entirely or delay the process of oxidation. They can scavenge free radicals such as ROS and RNS (Rahman *et al*., 2011); in addition to plant extracts rich in non-volatile compounds, essential oils serve as valuable resources for investigating antioxidant properties as they are readily extractable and harbor a diverse array of compounds. *Piper*, a highly esteemed genus for its medicinal properties, is a prominent reservoir of essential oils. Consequently, the medicinally significant species *P. nigrum* was subjected to essential oil extraction, followed by antioxidant analysis.

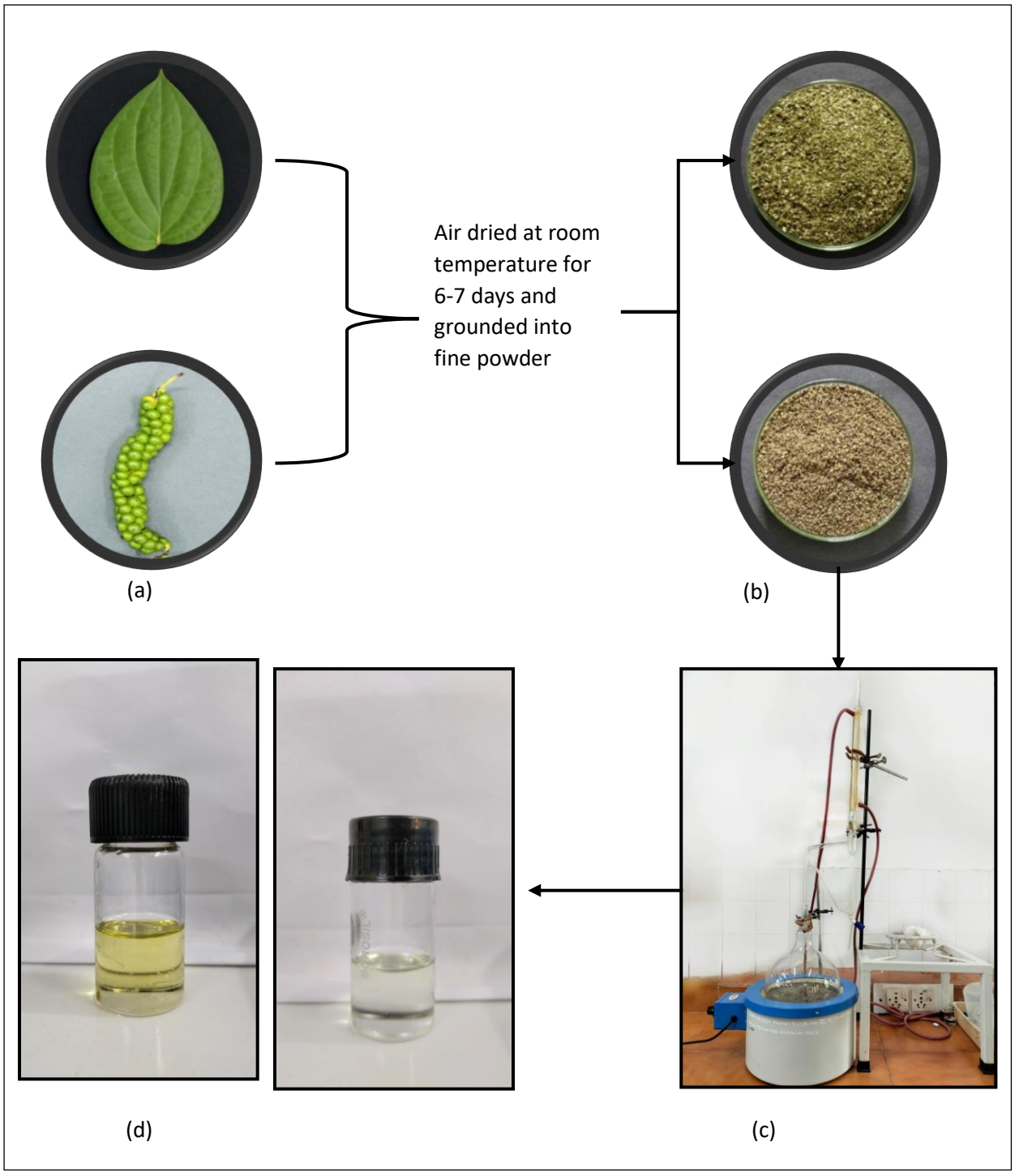


Plate 12: Extraction process of essential oil. (a) Fresh leaves and fruits of *P. nigrum*, (b) Dried and powdered form of leaves and fruits of *P. nigrum*, (c) Clevenger apparatus, (d) Essential oil extracted from the leaves (left) and fruits (right) from *P. nigrum*.

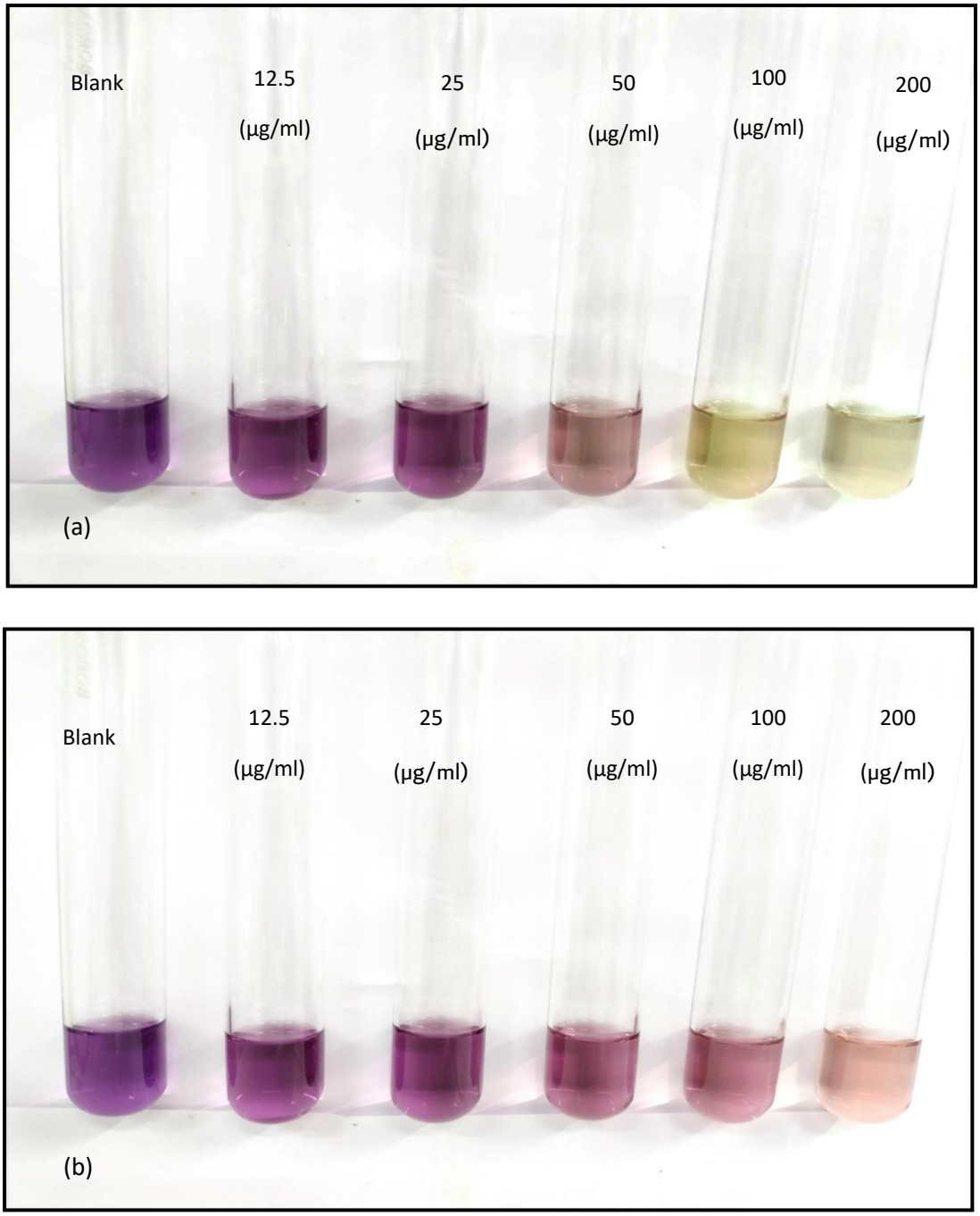


Plate 13: Antioxidant studies using DPPH method (a) L-ascorbic acid as standard, (b) Essential oil.

Leaves and fruit essential oils of *P. nigrum* were subjected to the 1,1- diphenyl-2-picrylhydrazyl (DPPH) test to determine their potential as free radical scavengers. This method measures the decrease in the absorption of the DPPH solution after adding an antioxidant at 517 nm **(Table 4.2)**, the standard of which was ascorbic acid. The experiment was carried out in triplicates **(Plate 13)**.

When concentrations increased, absorbance values decreased, and percentage inhibition increased. The linear regression of the percentage of inhibition v/s antioxidant activity demonstrates an inverse relationship between the antioxidant potential and the IC_{50} value. IC_{50} value represents the concentration at which a substance exerts half of its maximal inhibiting a specific biochemical process **(Fig 4.1 and 4.2)**.

The results were recorded in terms of IC_{50} value and oil with low IC_{50} showed high DPPH scavenging ability and vice versa. Fruit essential oil showed the lowest DPPH radical scavenging activity with high IC_{50} value of 214.16 µg/mL. In contrast, leaf essential oil showed maximum free radical scavenging activity with an IC_{50} value of 166.29 µg/mL **(Table 4.3; Fig 4.3)**.

Bagheri *et al*. (2014) compared the radical scavenging abilities of black pepper essential oils extracted using supercritical CO_2 extraction and hydro-distillation. They found that the essential oil extracted via supercritical CO_2 extraction showed more excellent DPPH radical scavenging activity (IC_{50}=103.28 µg/mL) than the oil extracted through hydro-distillation (IC_{50}=316.27 µg/mL).

A study by Nahak *et al*. (2011) assessed the antioxidant activity of ethanol extracts from *Piper cubeba* and *Piper nigrum*. They found that *Piper cubeba* showed higher antioxidant activity (77.61±0.02%) than *Piper nigrum* (74.61± 0.02%). The IC_{50} values were 10.54 0.12 µg/mL for *Piper cubeba* and 14.15 0.02 µg/mL for *Piper nigrum*.

In another study, Salleh *et al*. (2012) analysed the leaf and stem essential oils of *Piper officinarum* for antioxidant effects. They found weak activity of stem essential oil (IC_{50} = 777.4 µg/mL) compared to leaf essential oil (IC_{50} = 622.2µg/mL), and they stated that the low activity was attributed to the low phenolic content of essential oil.

Sruthi (2016) evaluated the antioxidant activity of essential oils extracted from *P. nigrum*, *P. longum, and P. chaba* using DPPH free radical scavenging activity. She reported that the *P. nigrum* oil showed the lowest DPPH scavenging activity with high (IC_{50} =79.5µg/mL) followed by *P. chaba* (IC_{50} =62.0µg/mL) whereas *P. longum* essential oil showed good antioxidant activity with low IC_{50} =21.0µg/mL. Kavitha *et al*. (2018) found that the ethanol extract of *P. nigrum* leaf exhibited moderate activity in both DPPH (26.78µg/mL) and ABTS (42.68µg/mL) assays compared to the control ascorbic acid (2.54 µg and 4.39 µg) respectively.

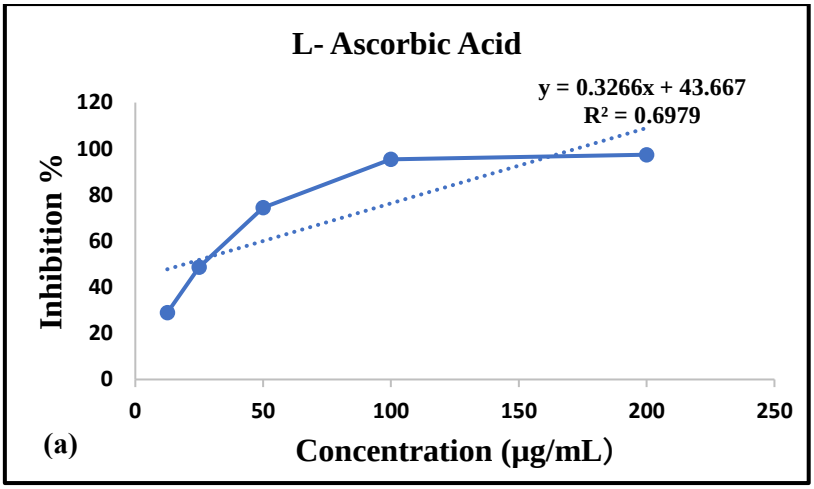
Table 4.2: DPPH free radical scavenging assay: % scavenging activity of DPPH by ascorbic acid and essential oils of leaves and fruit of *P. nigrum.*

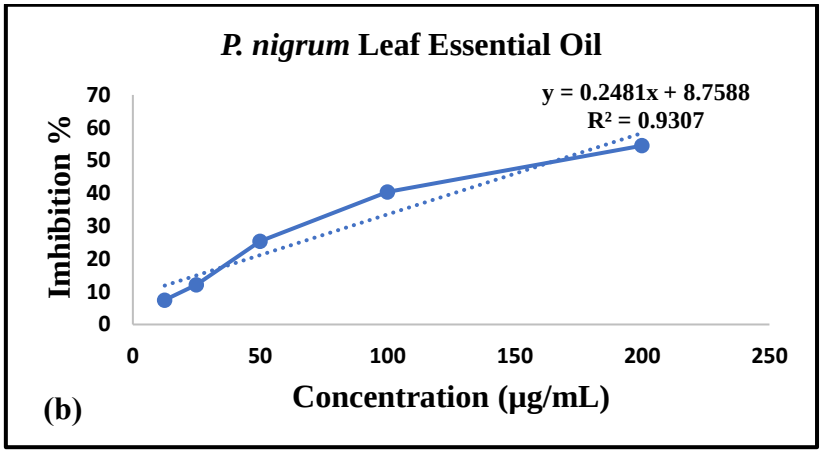
Sr No.	Concentration (µg/mL)	L- Ascorbic Acid	Essential oil of *Piper nigrum*	
			Leaves	Fruit
1.	12.5	28±0.044	7.46±0,018	3.37±0.001
2	25	48.7±0.044	12.16±0.014	9.06±0.004
3	50	74.5±0.02	25.37±0.019	20.32±0.018
4	100	95.4±0.005	40.41±0.023	34.37±0.058
5	200	97.4±0.001	54.53±0.025	42.85±0.003

Results are reported as % inhibition ± standard deviation of triplicate measurements.

Table 4.3: IC_{50} value of leaf and fruit essential oils of *P. nigrum*.

Sr. No.	Plant Name	IC_{50} Value of Essential Oils.	
		Leaves (µg/mL)	Fruit (µg/mL)
1.	*Piper nigrum*	166.29	214.16





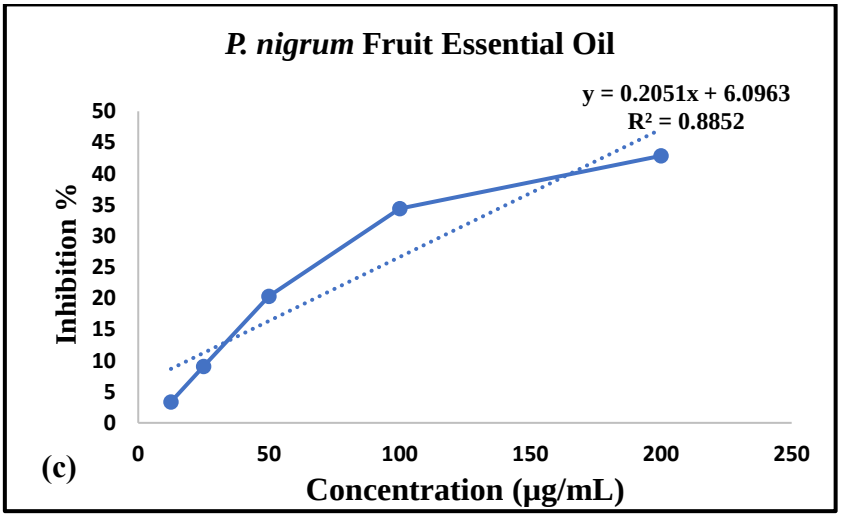


Figure 4.1: DPPH Radical Scavenging activity of (a) L-Ascorbic acid, (b) Leaf essential oil of *P. nigrum*, (c) Fruit essential oil of *P. nigrum.*

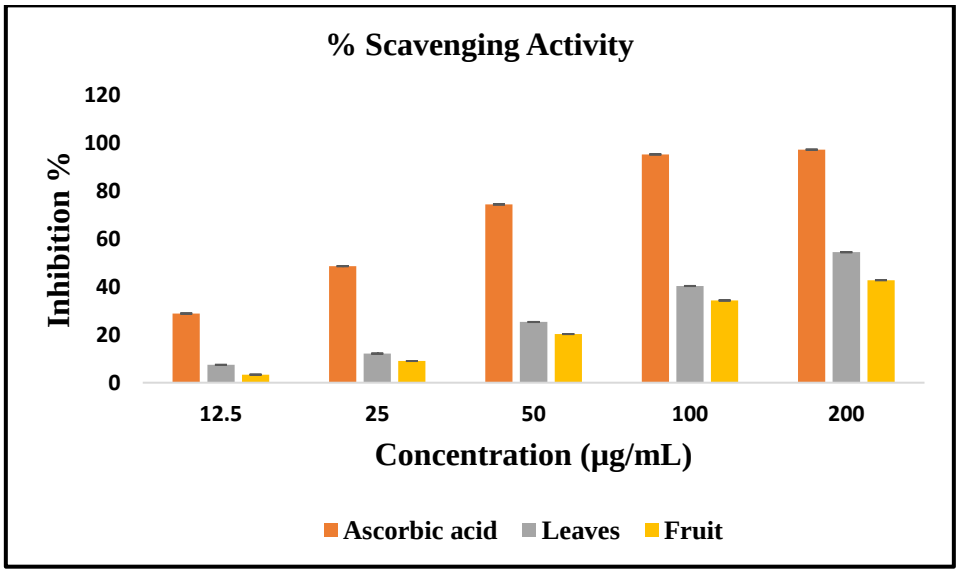


Figure 4.2: Percentage Scavenging activity of (a) L-Ascorbic acid (standard) with leaf and fruit essential oils of *P. nigrum*.

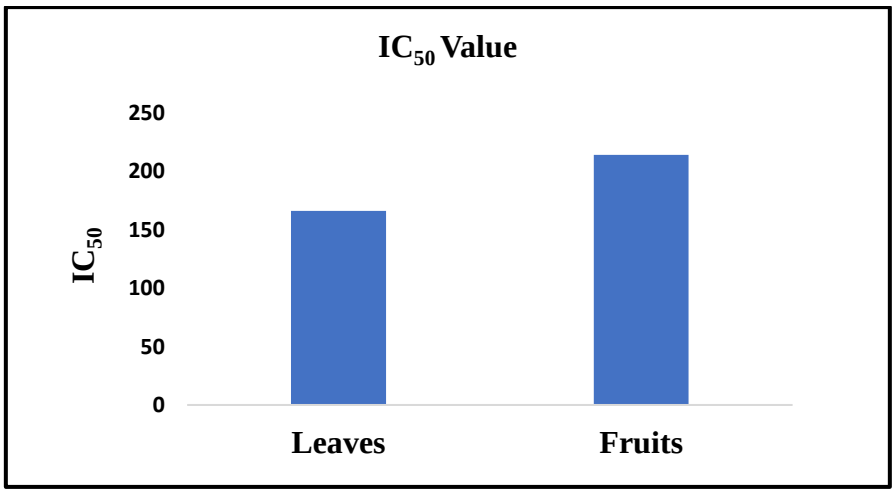


Figure 4.3: IC_{50} value of *P. nigrum* leaf and fruit essential oils.

4.7. Mosquito larvicidal activity

Mosquitoes have been a persistent nuisance throughout human history, and the threat they pose through the transmission of diseases is significant and growing. Efforts to control mosquito populations and mitigate the spread of mosquito-borne diseases are crucial. The most effective methods for reducing mosquito bites involve controlling adult mosquitoes and their larvae through repellents and insecticides. However, the widespread use of synthetic insecticides has led to insecticide resistance, environmental pollution, and health hazards to human health. Eco-friendly alternatives such as essential oils contain complicated mixtures of products such as phenols, sesquiterpenes, and monoterpenes and act as larvicides, insecticides, adulticides, and repellents (Khater *et al*., 2013).

Our study aimed to evaluate the lethal concentration of the larvicidal effects of leaves and fruits essential oil of *P. nigrum* and analyse the most effective ones against *Culex quinquefasciatus*. Four concentrations of leaf oil and five concentrations of fruit oil were prepared and tested against 3rd instar larvae of *Culex quinquefasciatus*. 20 3rd instar larvae were selected for the bioassay as they grow actively, are in the best feeding stage, are large enough to handle easily for experimental purposes, and are often more sensitive to toxins than older instars **(Tables 4.4 and 4.8)**. 1st and 2nd instar larvae are tiny and often less active, whereas 4th instar larvae are larger and more developed than the 3rd instar larvae and are less sensitive to toxins. The exposure time and concentration of the oil were shown to be directly associated with larval mortality in the study. After 24 hours **(Tables 4.5 and 4.9)** and 48 hours **(Tables 4.6 and 4.10)** of exposure, larval mortality was measured.

The LC_{50} value of essential oil extracted from the leaves of *P. nigrum* against *Culex quinquefasciatus* was 117.4 ppm and 91.43 ppm at 24 and 48 hours, respectively **(Fig 4.4a),** while LC_{90} value was 181.5ppm and 181.43ppm at 24 and 48 hours respectively **(Table 8; Fig 4.4 b).**

The LC_{50} value of essential oil extracted from the fruits of *P. nigrum* against *Culex quinquefasciatus* was 170.17ppm and 96.80ppm at 24 and 48 hours, respectively **(Fig 4.4a),** and their LC_{90} value was 564.37ppm and 267.36ppm at 24 and 48 hours respectively **(Table 4.11; Fig 4.4b)**. The leaf essential oil of *P. nigrum* was more toxic to *Culex quinquefasciatus* than the fruit essential oil.

The larvicidal activity against *Culex quinquefasciatus* larvae was evaluated by Vasudevan *et al*. (2009) using aqueous and ethanolic extracts of *P. nigr*um and reported that the *P. nigrum* ethanolic extract showed remarkable larvicidal potency than the aqueous extract. The diverse array of active constituents found in *Piper* species may be responsible for the variability in their potential against *Culex quinquefasciatus* larvae. A study conducted by Subsuebwong *et al*. (2016) reported that the essential oil extracted from *Piper retrofractum* showed good adulticidal activity against *Culex quinquefasciatus* (6.95% and 17.35%). The mosquito larvicidal activities of *P. nigrum* leaf essential oil have not been explored properly so far. A study conducted by Gupta, (2023) demonstrated that the *P. betle* leaf oil depicted good larvicidal activity against *Aedes aegypti*. Prabhu *et al*. (2022) reported that the ethanol extracts of *P. betle* leaves demonstrated better results than the aqueous extract.

Both *P. nigrum* leaf and fruit essential oils possess larvicidal properties against *Culex quinquefasciatus* larvae. The larvicidal efficacy of fruit essential oil of *P. nigrum* was notably lower than that of leaf essential oil in the study. The variability in the larvicidal potential of *P. nigrum* leaf and fruit essential oil against *Culex quinquefasciatus* may be attributed to the diverse chemical composition of these oils, which exhibited varying toxicity levels. However, this study, which used leaves and fruit essential oils of *P. nigrum*, showcased the potential of *P. nigrum* oils against 3rd instar larvae of *Culex quinquefasciatus*. Exploring these essential oils' benefits could create cost-effective and environmentally friendly larvicides for mosquito control **(Plate 14).**

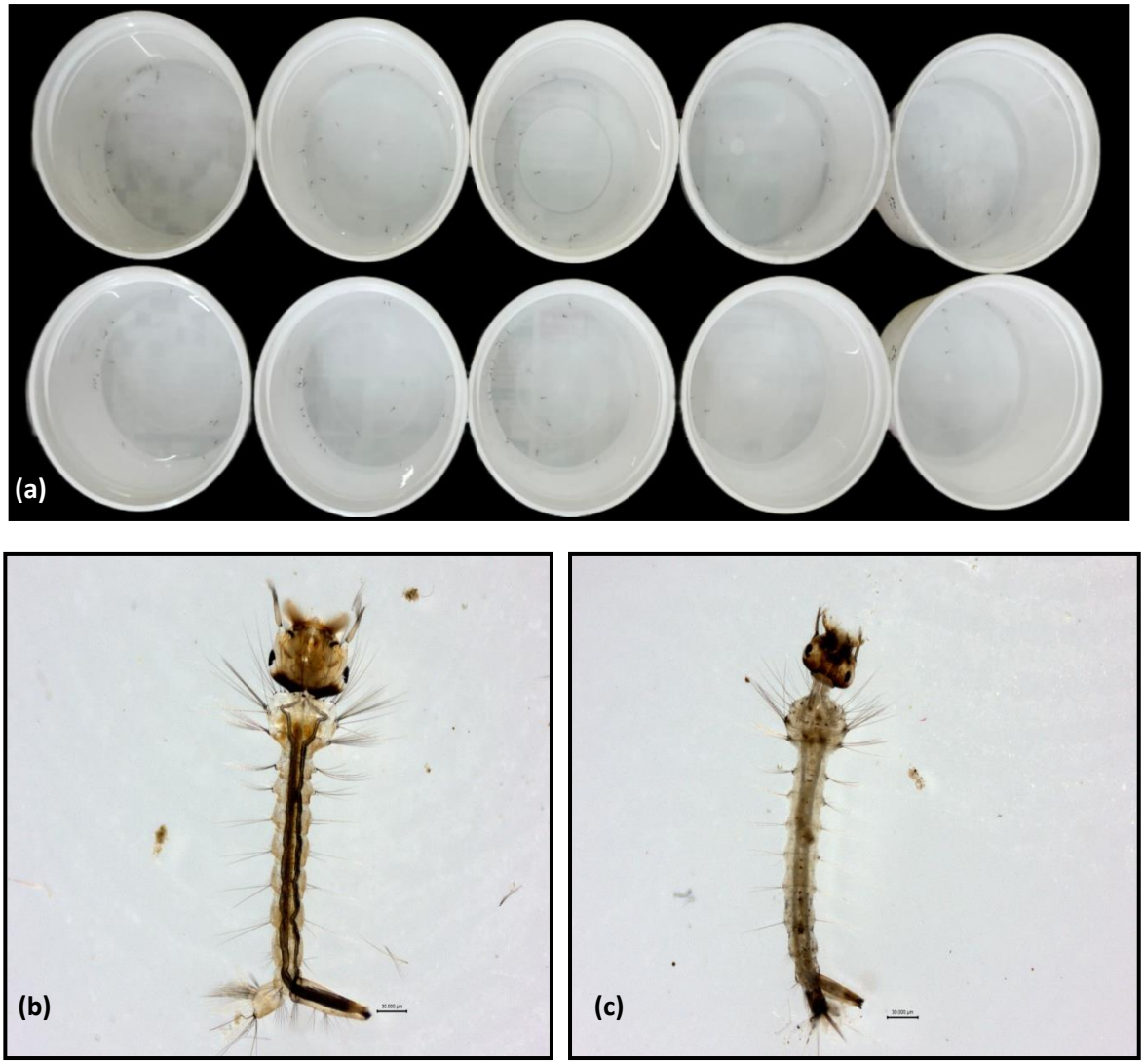


Plate 14: Larvicidal activity (a) Larvae in different concentrations of both the essential oil, (b) 3rd instar larvae of *Culex quinquefasciatus* (untreated), (c) 3rd instar larvae of *Culex quinquefasciatus* exposed to essential oil.

Table 4.4: Effect of leaf essential oil of *Piper nigrum* against 3rd instar of *Culex quinquefasciatus* larvae.

Name of sample	***Piper nigrum* (Leaf essential oil)**
Number of larvae exposed in each bowl	20
Volume of water	100mL
Replicates	4

Table 4.5: Larvicidal activity of essential oil from leaves of *Piper nigrum* against 3rd instar of *Culex quinquefasciatus* larvae after 24hours of exposure.

Sr. No.	**Dose (ppm)**	**Number of dead larvae**				**% mortality**
		R_1	**R_2**	**R_3**	**R_4**	
1.	**5**	1	0	1	0	2.5
2.	**10**	8	13	4	5	37.5
3.	**15**	14	15	18	14	76.25
4.	**20**	19	20	20	20	98.75
5.	**control**	1	2	0	0	3.75

Table 4.6: Larvicidal activity of essential oil from leaves of *Piper nigrum* against 3rd instar of *Culex quinquefasciatus* larvae after 48 hours of exposure

Sr. No.	**Dose (ppm)**	**Number of dead larvae**				**% mortality**
		R_1	**R_2**	**R_3**	**R_4**	
1.	**5**	3	5	5	2	18.75
2.	**10**	16	20	10	14	75
3.	**15**	18	19	20	18	93.75
4	**20**	19	20	20	20	98.75
5.	**control**	1	2	2	2	8.75

Table 4.7: LC_{50} and LC_{90} values of essential oil from leaves of *Piper nigrum* against 3rd instar larvae of *Culex quinquefasciatus* after 24 and 48 hours of exposure.

Mosquito species	Exposure period	LC_{50} (ppm) (LCL-UCL)	LC_{90} (ppm) (LCL-UCL)	Regression equation	Slope (±SE)	X^2
Culex quinquefasciatus	**24 hours**	117.4 (98.3-141.3)	181.5 (152-216.7)	Y=6.786x-9.043	6.786±0.039	0.031
	48 hours	91.43 (71.38-117.1)	181.43 (141.96-232.90)	Y=4.352x-3.532	4.352±0.055	0.616

LC_{50} = Lethal concentration killing 50% of the exposed larvae, LC_{90}= Lethal concentration killing 90% of the exposed larvae. LCL= Lower confidence limit, UCL= Upper confidence limit, SE= Standard error, X^2 = Chi square.

Table 4.8: Effect of fruit essential oil of *Piper nigrum* against 3rd instar larvae of *Culex quinquefasciatus.*

Name of sample	*Piper nigrum* (Fruit essential oil)
Number of larvae exposed in each bowl	20
Volume of water	100mL
Replicates	4

Table 4.9: Larvicidal activity of essential oil from fruit of *Piper nigrum* against 3rd instar of *Culex quinquefasciatus* larvae after 24 hours of exposure.

Sr. No.	Dose (ppm)	Number of dead larvae				% mortality
		R_1	R_2	R3	R_4	
1.	**5**	0	1	9	3	16
2.	**10**	5	6	6	6	28
3.	**15**	4	7	8	8	33
4.	**20**	13	14	13	12	65
5.	**30**	15	16	17	18	82
6.	**control**	1	2	0	0	3.75

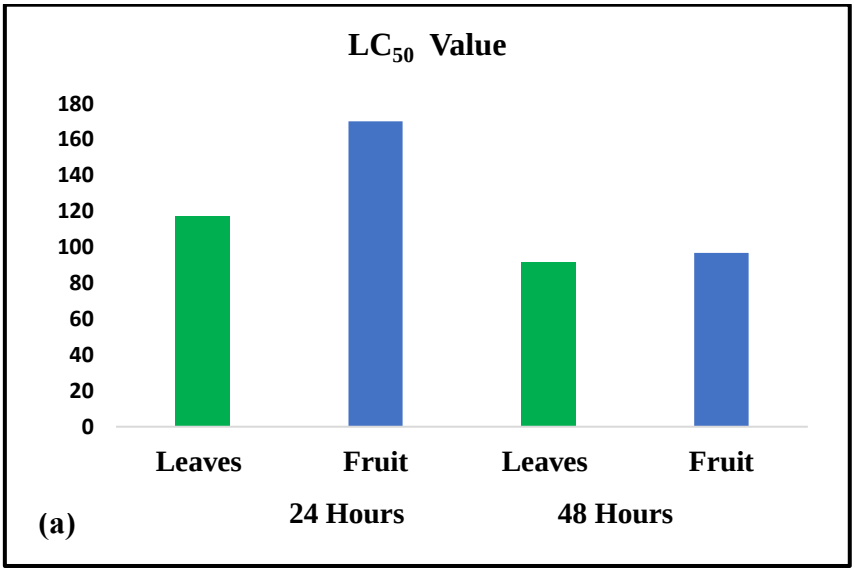
Table 4.10: Larvicidal activity of essential oil from fruit of *Piper nigrum* against 3rd instar of *Culex quinquefasciatus* larvae after 48 hours of exposure.

Sr. No.	Dose (ppm)	Number of dead larvae				% mortality
		R_1	R_2	R_3	R_4	
1.	**5**	2	2	10	9	28
2.	**10**	6	7	8	7	35
3.	**15**	17	14	19	18	85
4.	**20**	18	19	18	16	88
5.	**30**	17	20	19	20	95
6.	**control**	1	2	0	0	3.75

Table 4.11: LC_{50} and LC_{90} values of essential oil from fruit of *Piper nigrum* against 3rd instar larvae of *Culex quinquefasciatus* after 24 and 48 hours of exposure.

Mosquito species	Exposure period	LC_{50} (ppm) (LCL-UCL)	LC_{90}(ppm) (LCL-UCL)	Regression equation	Slope (±SE)	X^2
Culex quinquefasciatus	**24 hours**	170.17 (120.24-240.83)	564.37 (398.78-798.71)	Y=2.498x-0.577	2.61±0.89	0.895
	48 hours	96.80 (70.96-132.06)	267.36 (195.97-364.74)	Y=2.938x-0.837	2.939±0.069	0.232

LC_{50} = Lethal concentration killing 50% of the exposed larvae, LC_{90}= Lethal concentration killing 90% of the exposed larvae. LCL= Lower confidence limit, UCL= Upper confidence limit, SE= Standard error, X^2 = Chi square.



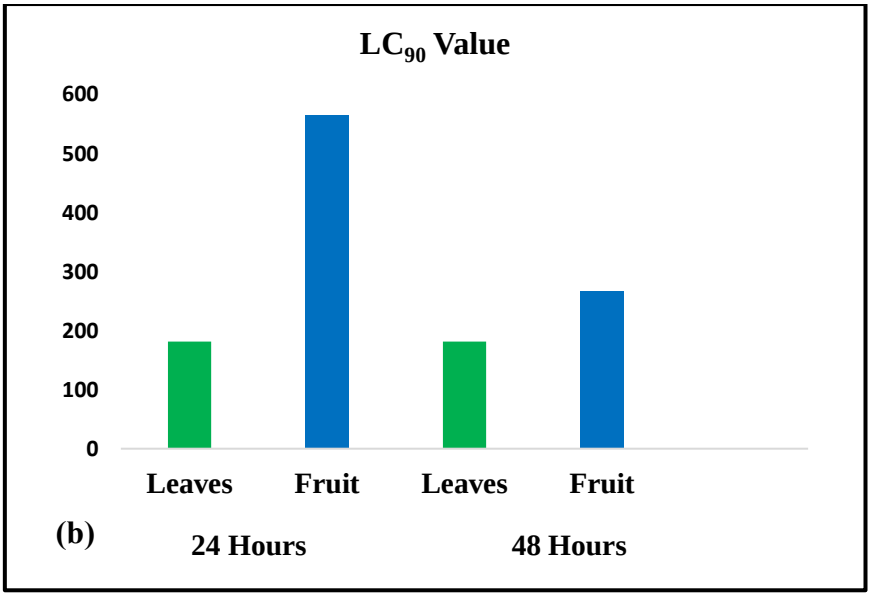


Figure 4.4: Mosquito larvicidal activity (a) LC_{50} Value, (b) LC_{90} Value.

5. CONCLUSIONS

Piper nigrum, belonging to the Piperaceae family, was studied for anatomical, histochemical, autofluorescence, powder microscopy, and biological activities. The essential oils were obtained from the dried leaves and fruits of *Piper nigrum*. The anatomical characterization of *Piper nigrum* indicated the existence of distinct anatomical traits. Oil cells were abundant in the fruit, and abundant translucent, oval structures of essential oil droplets were seen in the palisade layer of mesophyll tissues, showing the absence of secretory cells or oil glands. Similarly, oil droplets were observed in the chlorenchyma tissues in the stem, and a minute quantity of oil droplets were observed in the petiole. Histochemical studies were conducted using both Sudan IV and Oil O Red stains to localize essential oils in various parts of *Piper nigrum* (fruit, leaves, stem, and petiole) and to check for the difference in the staining intensity and the results depicted that essential oils were stained dark red with Oil O Red stain compared to Sudan IV.

Autofluorescence studies under the UV filter (330-380nm) were carried out in various parts of *Piper nigrum* (fruit, leaves, stem, and petiole), which resulted in red, blue, whitish blue autofluorescence of different tissues in the sections. Also, the present study aimed to investigate the anatomical features and composition of *Piper nigrum* leaves and fruits through powder microscopy analysis. Through carefully examining the powdered material revealed observations such as parenchyma tissues, stomata, sections of vascular bundles, and starch grains.

Many studies have documented the isolation of volatile chemicals from the fruit of *Piper nigrum* as it is the most common spice, and has been widely used in therapeutics, and economic value. Literature data indicated that its geographical location highly influences essential oil composition. The essential oil was extracted from the dried leaves and fruits of

Piper nigrum through hydro-distillation. *Piper nigrum* fruit essential oil extraction yielded more than leaf essential oil.

The leaf and fruit essential oil demonstrated good antioxidant and mosquito larvicidal activity. The antioxidant activity of the leaf essential oil was higher than the fruit essential oil. Thus, this property aids in mitigating food degradation and extending shelf life, thereby contributing to the advancement of food preservation strategies.

In today's context, environmental safety stands as a top priority, with the emphasis shifting towards eco-friendly solutions in pest control. Unlike traditional insecticides, which pose risks to both human health and the environment, modern approaches prioritize solutions that are not solely reliant on high mortality rates among target organisms but also demonstrate eco-friendliness. The leaf essential oil depicted the maximum effectiveness as larvicidal activity against the larvae of *Culex quinquefasciatus* compared to the fruit essential oil. The primary constituent compounds of essential oils are usually one of the critical elements responsible for particular essential oil insecticidal effect.

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