PHYTOCHEMICAL SCREENING AND ANTIFUNGAL ACTIVITY OF BAY LEAF (*Laurus nobilis)* ON SELECTED PATHOGENS

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CERTIFICATION

This is to certify that this project work was carried out by **EGHOSA EGHE IMUWAHEN** in the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City under the supervision of

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APPROVAL

This project work is accepted in partial fulfillment for the award of Bachelor of Science, B. Sc (Hons) in the Department of Microbiology, University of Benin, Benin City.

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DEDICATION

This work is dedicated to God almighty for seeing me through and for his grace upon my life throughout the course of this project work, and to my parents Mr. & Mrs. Imuwahen for their love and support throughout the course of this study.

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ABSTRACT

Laurus nobilis, generally known as “bay leaf” belongs to *Lauraceae* family of plants. It contains compounds which have potential use for food safety because of the antimicrobial properties. Its leaves are widely used in traditional medicines and for food seasoning. This study was aimed at investigating the phytochemical constituents and antifungal effects of *Laurus nobilis* on selected pathogens; *Fusarium solani, Aspergillus niger, Aspergillus flavus*, and *Penicillium chrysogenum* which were obtained from hair, foot, and toenail samples from some students in the department of microbiology, University of Benin (UNIBEN) using swab sticks and confirmatory tests were carried out using cultural and biochemical methods. The fungi isolates were identified using cultural and morphological characteristics as well as the colour of spores, types of spores, and nature of hyphae. The phytochemical screening of the leaf was done using standard methods. Antifungal susceptibility was done using poisoned food method. Extraction was done after 2 days and 4 days. The percentage composition of saponin, alkaloid, tannin, flavonoid and total polyphenol in the *L. nobilis* leaves were 4.40%, 4.00%, 13.50%, 11.00%, and 0.010% respectively. In the *L. nobilis* leaf extract for 2 days and 4 days, *F. solani* had the highest radial growth of 44.00mm and 31.50mm obtained from ethanol and methanol at 300mg/ml and 400mg/ml respectively. The highest percentage mycelial growth inhibition for 2 days and 4 days were 40.74% and 39.06% obtained from *F. solani* and *P. chrysogenum* respectively. The results showed great antifungal activities of the leaves extracts against the selected isolates. From the antifungal activity, it could be noted that *L. nobilis* extracts in general, offers some potential in the combating of diseases caused by these fungal agents, and may be screened for activity against several other human and plant pathogens.

CHAPTER ONE

1.0 INTRODUCTION

One of the most well-known plants from the *Lauraceae* family is *Laurus nobilis*, which is also known as Bay or laurel leaves. Bay is one of the most frequently used cooking spices for flavoring meat products, fishes and soups. It has been used for 1000 years, and it is an essential ingredient in cooking and in many traditional practices (Parthasarathy *et al*., 2008). It is a native plant in the Southern Mediterranean area, found in warm climate regions, but it is used as a decorative plant in Europe and USA. In addition, it is commercially grown in, Algeria, Morocco, Portugal, Spain, Italy, France, Turkey and Mexico. *Laurus nobilis* is the member of family *Lauraceae* which comprises 32 genera and about 2,000-2,500 species. Laurus is also known as sweet bay, bay laurel, Grecian laurel, true bay and bay tree (Garg *et al*., 1992).

The natural plant products are chemical compounds extracted from plants which are synthesized by following pathways of primary or secondary metabolism. The study of natural products involves isolation of these compounds in a pure form by hydro-distillation, soxhlet extraction and chromatographic methods and analysis of their structure, formation, use and purpose, in living organisms. Essential oils are volatile secondary metabolites that are produced by plants for their own need other than for nutrition. Essential oils are also known as fragrant, volatile, ethereal and aromatic oils (Baser and Demirci, 2007). By virtue of their volatile nature these can be easily diffused into air and therefore responsible for wonderful scents of plants. An essential oil may contain 20-60 aromatic compounds and this advanced mixture of compounds offers the essential oil, its characteristic fragrance and flavor (Arora *et al.,* 2015). Essential oils are liquid at room temperature, generally have density lower than that of water and are often colored. These are slightly soluble in water but are highly soluble in organic solvents. Although essential oils are

only slightly soluble in water, the aqueous solubility of individual essential oil components varies with respect to polarity (magnetic activity). It also grows as an escapee, naturalized in eastern Bulgaria, along the Black Sea coast.

Moreover, from these leaves an essential oil is distilled, which is extensively used in the food industry for seasoning of meat products, soups and fish as well as in the fragrance industry. Furthermore, leaves and fruits of *L. nobilis* are used in folk medicine since antiquity against rheumatism, cough, cardiac diseases, viral infections, diarrhea, etc., and as a general gastric secretion stimulant, carminative, diaphoretic and antiseptic.

In folk medicine it is used for stomachic and carminative remedies as well as in the treatment of gastric diseases. *L. nobilis* leaf extracts have been investigated for their wound healing, cytotoxic, and trypanocidal properties. Laurel has attracted continuous and renewed interest because of its pharmacological and health beneficial properties related to several compounds present in the plant.

Aqueous extracts of laurel fruits and leaves have been used in herbal medicine as an astringent agent and for the treatment of several neurological, dermatological, and urological disorders. The laurel yield and composition were shown to be influenced by various factors, such as growth environment, harvest season, plant parts, extraction method, and others. The growing interest in natural products, and the inclusion of plant extracts in various cosmetic products is a prerequisite for an in-depth analysis of the chemical composition of laurel genotypes from various regions. According to some studies, certain constituents may cause allergic reactions when included in cosmetic products. Because of its biological activity, laurel leaf could be considered a natural supplement or antioxidant in cosmetics and medicine. The majority of the laurel genotype pool

in Bulgaria was probably introduced from Greece after the Second World War. However, some may have been transferred from Middle Eastern countries during the 15th to 18th century, or the Ottoman period. Generally, laurel fruits are not harvested and, therefore, not used although they have a potential to provide unique characteristics and other novel bioactivities. The leaves have been used as a spice and preservative in the food industry for making various products, as well as in folk medicine. According to Georgiev and Lazarov, the laurel trees found in Bulgaria had a sufficient content, which was higher in plants growing in the warmer parts of the country. The leaves of the middle part of the shoots had a higher content (1.9–3.35%). The twigs (less than 0.4%) allowed them to be used, together with the waste from the processing of the leaves (crushed and non-standard leaves), as a raw material for production. However, the latter authors did not analyze the composition. There has been a growing interest in biologically active substances from non-traditional and under explored plant species. Bulgaria is one of the largest producers in Europe; its distillation facilities and network could easily accommodate a novel from naturalized plants produced locally. Despite the interest demonstrated by the industry, there is no previous study on the composition and antimicrobial activity of laurel found in Bulgaria.

Nowadays, laurel tree is in addition an economically important plant that hides a lot of phytochemicals with interesting activities and potential applications that deserves to be reviewed Interestingly, there is a worldwide concern around that use of antibiotics to treat bacterial and fungal infections can lead to the rise and spread of organisms resistant to broad-spectrum antibiotics, opening ways to use plants as natural sources for novel antimicrobial agents with a similar activity. Natural medicinal plants, as *L. nobilis*, are rich sources of bioactive compounds. Thus, the biological properties of Bay extracts and its essential oil are documented, specifically their antimicrobial, antifungal and antioxidant effect.

1.1 Aim and objectives

The aim of this research was to investigate the phytochemical constituents and antifungal effects of bay leaf on selected pathogens.

The objectives include:

1. to investigate the phytochemical constituents of bay leaf
2. to determine the antifungal activity of the leaf extracts against selected pathogens
3. to compare the effect of methanolic, ethanolic and aqueous extracts on the selected pathogens

CHAPTER TWO

2.0 LITERATURE REVIEW

Laurus nobilis L. is a small tree, having alternate, narrowly oblong-lanceolate leaves. The flowers are small and four lobed; the male has 8-12 stamens and female 2-4 staminodes. The ripe fruit is 10-15 mm, ovoid and black when ripe. The smooth bark may be olive green or reddish-blue. The plant is hardly multi-branched, usually grows to a height of 20-30 feet in many warm regions of the world (Said and Hussein 2014). The leaves are plucked and dried under shade for use as a flavouring material in a variety of culinary preparations, especially in French cuisine. The fragrant leaves are sold commercially as bay leaf, a seasoning (Anon *et al.,* 2005)

Bay leaf is grown in different ecologic and climatic conditions. Wet, sandy soil that has a large quantity of water or some moist atmospheric conditions close to the ocean shore are optimum and the best conditions for rapid luxuriant growth (Patrakar *et al*., 2012).

Bay is widely growing in the following countries: India, Pakistan, other Southeast Asian countries, some Pacific islands, Australia, around the coast of the Mediterranean and Southern Europe, Greece, Portugal, France, Turkey, Spain, Algeria, Morocco, Belgium, Central America, Mexico, Southern United States, and the Canary Islands (Parthasarathy *et al*., 2008).

2.1 ECOLOGY

Bay leaf is native to South Europe (Patrakar *et al*., 2012). It is a multi-branched, deciduous shrub having height up to 8 m and diameter up to 40 cm with smooth, thin, and brown bark containing a shady crown (Patrakar *et al*., 2012). Leaves are alternate, lanceolate, and bipinnate compounds with smooth or sharp margins 30 cm long containing 24 leaflets that are lanceolate, 4.9 cm long, and 1.8 cm wide with 0.5 cm long petiole. Flowers are ebracteate, four-lobed, white, scented, and

small, having 8 -12 male stamens and 2-4 females taminoids, and the fruit is 15.00 mm, in small clusters, ovoid, thin pericarp enclosing spinach-green seeds and black when ripe. Calyx is pubescent having five clefts and five petals along with glabrous glands, free and white.

2.1.1 Chemistry

Bay leaf has a sharp and bitter taste. The difference in fragrance and aroma is due to the presence of essential oils in leaves and other parts of the plant. It has flavonoids, tannins, eugenol, citric acid, carbohydrate, steroids, alkaloids, triterpenoids, and essential oils. Antioxidant properties were discovered in the extract of bay leaf to have phenolic compounds. Each of these chemical constituents varies depending on the type of species. Tanine is a liquid glycoside derived from polypeptide and ester polymer that can be hydrolyzed by the secretion of bile (3, 4,5etrinidrokside benzoic acid) and glucose (Sumono *et al*., 2008). Tanine or tanatacid isolated from some part of plants can be found in the market. It is a cream-colored powder, aromatic, with astringent taste (Sumono *et al*., 2008). Tanine is used as an astringent for the gastrointestinal tract or skin and can cause precipitation of the cell membrane protein. It also has a little pene-tration activity, so it can influence the permeability of the cell membrane. Bay leaf has traces of fats; (that is, a low amount is present) so it has low caloric value. It is also known as a good and main source of vitamin A and many minerals. One ounce of bay leaf gives 54.00 calories, 1.20 g protein, 13.00 g carbohydrates, a trace of fat, 1.5 mg of iron (Fe), 53.00 mg of calcium (Ca), 3000 IU of vitamin A, 15.00 mg of vitamin C, and a small amount of potassium. Bay seeds are rich in dietary fibers. In bay leaf, compounds like eugenol (12.00%), methyl eugenol (12.00%), and elemicin (12.00%) are significant for the spicy aroma of bay leaves, and for determining effective quality of bay leaf, these are used as sig-nificant influencers (Biondi *et*

al., 1993). The essential oils in leaves vary from 0.80% to 3.00% and dry bay fruits from 0.60% - 10.00%.

Due to the commercial value of laurel leaves most of the reported work on laurel tree chemical composition is focused on them. The volatile fraction of laurel leaves has been particularly studied and reviewed. For that reason, this review will cover more comprehensively the non-volatile fraction of laurel leaves as well as that of the other laurel organs. Comparatively, laurel wood is clearly the part of the tree less studied. Volatile oils: A commercial laurel essential oil is obtained by steam distillation of leaves of *L. nobilis* as a yellow liquid with an aromatic, spicy odor. The trading interest of this oil justifies that its chemical composition has been widely studied and reviewed several times. More than 150 components have been identified in the oil by GC-MS, 1,8-cineole being usually the major component. Other important components are sabinene, α-terpinyl acetate, linalool, eugenol, methyl eugenol, α-pinene, among others. Other organs of the plant have also been explored regarding their volatile composition. Thus, essential oils from laurel fruits, seeds, flowers, and stems and bark have also been studied in several occasions, although to a lesser extent than laurel leaves.

2.1.2 Flavonoids

Flavonoids are the main phenolic constituents of laurel leaf's alcoholic extracts, chemically based upon a fifteen-carbon skeleton consisting of two benzene rings A and B linked via a heterocyclic pyrene ring. Flavones and flavonols are the main flavonoids in alcoholic extracts of leaves. Apigenin, kaempferol, quercetin and their glycosides are also frequently found. In the flavone group is mainly observed C-glycosidation at C-6 or C-8, while in flavonols predominates O-glycosidation at C-3. On the other hand, flavan-3-ols are the main flavonoids in laurel wood's ethyl acetate extracts, (−)-epicatechin, (−)-epigallocatechin, and (+)-catechin being recently

isolated from a Spanish sample. Moreover, four anthocyanins have been detected in laurel fruits from Italy. (Alejo-Armijo *et al.,* 2017).

- Proanthocyanidins: Proanthocyanidins are oligomers of flavan-3-ol units and are the main phenolic compounds of laurel wood's ethyl acetate extract. The trimeric procyanidin cinnamtannin B-1 is the major proanthocyanidin of laurel wood. This trimer has also been isolated from laurel leaves but in a lesser amount. Most of the works related with proanthocyanidin composition of laurel tree made just tentative elucidations by mass spectrometry. For instance, (Dias *et al*., 2017), have detected in leaves three dimeric, three trimeric and three tetrameric procyanidins. Unfortunately, they could not make the complete elucidation. However, the analysis of characteristic mass fragmentation pathways of proanthocyanidins has allowed a better accurate identification of them in laurel wood. In this way, it was possible to achieve the tentative identification of dimeric, trimeric and tetrameric proanthocyanidins, establishing the kind of monomer that conform them, its relative position in the structure and the kind of bond established among monomers. Moreover, dimeric trimeric proanthocyanidins have also been isolated from laurel wood. This work has revealed that laurel wood is a very interesting raw material for the isolation of proanthocyanidins.

2.2 ECONOMIC IMPORTANT OF BAY LEAF

Many herbs and spices contribute significantly to health despite low amounts of consumption, as they are full of antioxidants and certain mineral compounds. It is not clear how much bay must be consumed to get its health benefits. Researchers do not have particular recommendations about the specific amount of use. Nevertheless, bay is full of anti-oxidants and is a good source of minerals and dietary fibers. It complements food flavor, and bay tea is used to treat stomach aches, clear up mucus in the lungs, colds, and sore throat. Poultice of bay leaves is used for the

treatment of rheumatism and neuralgia (Goodrich *et al*., 1980). To treat headache, leaf of bay is kept in a nostril or under the head bands to relieve this pain. Traditionally, it has been used for the treatment of gastrointestinal problems such as impaired digestion, flatulence, eructation, epigastric bloating, diuretic and analgesic effects (Elmastas *et al*., 2006). Bay is great to add flavor and taste to food and many dishes with added health benefits. Bay has many uses ranging from culinary to religious. There are number of curious beliefs associated with the historical use of bay leaf. The Temple of Delphi, dedicated to Apollo, used many bay leaves. The roof was made of bay leaves, and priestesses would have to eat bay before giving their oracles. This may have been aided by bay's slightly narcotic qualities. Thus bay leaves are said to aid with psychic powers, particularly prophetic dreams, clairvoyance, protection, healing, purification, strength, wishes, magic, exorcism, divination, visions, inspiration, wisdom, meditation, defense, and accessing the creative world. Israelite society consider the bay leaf as a symbol of victory over misfortune; they were very impressed by this tree. Ancient Mediterranean said this tree radiates protective power and prevents them from misfortune, so it is planted near houses to keep lightning away. The Romans and Greeks used this as a head band mainly for their respected citizens, poets, heroes, and priests, and they consider sleeping with bay leaves to make a man a poet. It is an essential ingredient of many herbs and used in soups, stews, and stuffing, as well as fish, meats, vegetables, sauce, pickles, and sausages. It is easily blended with many other herbs such as garlic, mustard, pepper, parsley, rosemary, thyme, and oregano. Bay can also be an important ingredient in teas, oils, cheeses, and liquors, and its essential oil is used in the cosmetic industry for soaps, perfumes, prepared foods, beverages, and dental products. Bay has many traditional medical uses. Leaves are used for the treatment of skin rashes, earaches, and rheumatism. The leaves have aromatic fragrance, so they are kept in cloths and used to cover up

bad mouth odor. The leaves of this plant, having a pepper odor and clove-like taste, are used in cooking. Bay leaves have many sesquiterpene lactones that are responsible for inhibition of NO production, i.e., anti-inflammatory, inhibition of alcohol absorption, and may improve liver glutathione S-transferase activity (Fang *et al*., 2005). Using bioassay-directed isolation study, different cytotoxic and apoptosis-induced compounds are identified in bay leaf. Many components of essential oil of bay leaf such as eugenol, methyleugenol, and pinene have anticonvulsant activity, while eugenol, methyleugenol, and cineole produce sedation and motor impairment (Sayyah *et al*., 2002). Essential oil of this leaf also has analgesic and many anti-inflammatory activities (Barla *et al*., 2007).

Flavones, flavonol, and phenols are present in the methanolic extract of bay leaf and show antioxidative activity. Traditionally, it has been used as herbal medicine against number of diseases such as rheumatism, sprains, indigestion, earaches, and to enhance perspiration (Fang *et al*., 2005). It was reported by different researches that bay leaf can also be used to treat diabetes and migraine (Fang *et al*., 2005). It is used with warm water for drinking to treat internal ailments; as a result, excess water is removed by body by urination and acts as an emetic to induce vomiting. Fresh, mature leaves are used to treat blood dysentery, inflammation, and congestion of kidney. Bay leaf is also used to treat arthritis, headache, fungal diseases, anorexia, colds, cataracts, diarrhea, colic ulcer, appetizer, neuralgia, and digestive stimulant traditionally (Parthasarathy *et al*., 2008). Bay is found effective against many infections from fungi, viruses, bacteria, and protozoa. Bay is also helpful in inhibiting growth of carcinogenic cells. The leaves of bay are specific for many fevers, cough, flu, bronchitis, asthma, influenza, cough, cold, lowering blood cholesterol level, chicken pox, diarrhea, and anti-stress agents. Bay juice is an effective medication for sore eyes and night blindness, which is generally caused by deficit of

vitamin A. Bay seeds are mucilaginous and relieve indigestion, sore throat, constipation, and diarrhea.

Many herbs and spices contribute significantly to health despite low amounts of consumption, as they are full of antioxidants and certain mineral compounds. It is not clear how much bay must be consumed to get its health benefits. Researchers do not have particular recommendations about the specific amount of use. Nevertheless, bay is full of anti-oxidants and is a good source of minerals and dietary fibers. It complements food flavor, and bay tea is used to treat stomachaches, clear up mucus in the lungs, colds, and sore throat. Poultice of bay leaves is used for the treatment of rheumatism and neuralgia (Goodrich *et al.,* 1980). To treat headache, leaf of bay is kept in a nostril or under the headbands to relieve this pain. Traditionally, it has been used for the treatment of gastrointestinal problems such as impaired digestion, flatulence, eructation, and epigastric bloating and used as diuretic and has many analgesic effects (Elmastas *et al*., 2006). Bay is great to add flavor and taste to food and many dishes with added health benefits. Bay has many uses ranging from culinary to religious. There are number of curious beliefs associated with the historical use of bay leaf.

The aqueous extract of *L. nobilis* were compared with the aqueous extract of Allamanda and found to have better wound healing activity. Many excision and incision wound healing models were used to estimate the wound healing activity. Many factors were studied to assess the wound healing activity such as tensile strength, weights of the granulation tissue, rate of wound closure, period of epithelialization, histopathology of the granulation tissue, and hydroxyproline content of the granulation tissue. Animals treated with bay leaf were found to have a reasonably high rate of wound contraction, hydroxyproline content, and weight of granulation tissue. Bay leaf treated

animals showed a higher number of inflammatory cells and less collagen compared with the animals that were treated with Allamanda cathartica (Nayak *et al*., 2006).

2.2.1 Antioxidant activity

Ethanol extracts of *L. nobilis* showed powerful antioxidant activities. The antioxidant activity was determined by evaluating free radical scavenging, hydrogen peroxide scavenging, superoxide anion radical scavenging, reducing power, and metal chelating assays. Strong antioxidant activity of bay leaf was observed in linoleic acid emulsion at a concentration of 20, 40, and 60mg/mL (94.20%, 97.70%, and 98.60% inhibition of lipid peroxidation, respectively). The antioxidant activity of ethanol extract may be due to phenolic compounds present in the extract (Elmastas *et al*., 2006).

According to Al-Hashimi and Mahmood, 2016, the reducing power and antioxidant activity of alcoholic extracts of bay leaves was determined. The result showed the rates of antioxidant activity and reducing power increases as the concentrate of bay leaves extract increased.

2.2.2 Anticonvulsant Activity

The influence of aromatic *L. nobilis* on the development of two mycorrhizal species, *Glomus deserticola* and *Glomus intraradices* was investigated by (Hassiotis *et al.,* 2010). Both mycorrhizal fungi colonized successfully the host plants, positively influencing their growth. *G. deserticola* presented higher infection level than *G. intraradices*. Addition of *L. nobilis* oil into substrates resulted in mycorrhiza inhibition, and the level of inhibition was analogous with the amount of added essential oil. The fungi were benefited by the aromatic compounds up to 15.00 mg of essential oil per litre of soil. However 30.00mg/l and 60.00 mg/l of essential oil were able to create significant inhibition in mycorrhiza development and to restrict the host growth. The

leaf essential oil of *L. nobilis*, which has been used as an antiepileptic remedy in Iranian traditional medicine, was evaluated for anticonvulsant activity against experimental seizures (Sayyah *et al*., 2002). The essential oil protected mice against tonic seizures induced by maximal electroshock and especially by pentylenetetrazole. Components responsible for this effect may be methyleugenol, eugenol and pinene present in the essential oil. At anticonvulsant doses, the essential oil produced sedation and motor impairment. This effect seems to be related in part to cineol, eugenol and methyleugenol (Sayyah *et al*., 2002).

2.2.3 Analgesic and anti-inflammatory

The highest anti-inflammatory activities by using a carrageenan-induced hind paw edema model (Kozan *et al*., 2006). Bay leaves having antidiarrheal, anti-inflammatory, and anti-diabetic activity are used for the improvement of the immune system. Antioxidants such as *L. nobilis* essential oil showed analgesic and anti-inflammatory activities in mice and rats (Sayyah *et al*., 2003). Ethanol extract obtained from the leaves and seeds of bay leaf also show vitamin C, vitamin E, and carotenoids are used in many dietary sources and are used to lower blood cholesterol and uric acid level. Bay leaves have many sesquiterpene lactones that are responsible for inhibition of NO production, i.e., anti-inflammatory, inhibition of alcohol absorption, and may improve liver glutathione S-transferase activity (Fang *et al*., 2005). Using bioassay-directed isolation study, different cytotoxic and apoptosis-induced compounds are identified in bay leaf. Many components of essential oil of bay leaf such as eugenol, methyleugenol, and pinene have anticonvulsant activity, while eugenol, methyleugenol, and cineole produce sedation and motor impairment (Sayyah *et al*., 2002). Essential oil of this leaf also has analgesic and many anti-inflammatory activities (Barla *et al*., 2007).

2.2.4 Antimutagenic Activity

Ethyl acetate extract of bay leaf has 3-kaempferylp-coumarateantimutagen, which was identified experimentally and purified chromatographically. The antimutagenicity was due to a desmutagenic action that converted the Trp-P-2 metabolically activated form into its crucial carcinogenic form (Samejima *et al*., 1998).

2.2.5 Nematocidal activity

Plant parasitic nematodes are the most destructive group of plant pathogens worldwide and their control is extremely challenging. The root-knot nematodes, Meloidogyne spp, are one of the most economically damaging genera of plant parasitic nematodes on horticultural and field crops (Andres *et al*., 2012). Nematicidal activity of bay against Meloidogyne incognita was investigated in tomato and pepper. There were no significant differences between nematode inoculums level and essential oil concentration used. However, the plant extract treatments restrained nematode populations in both tomatoes and pepper host plants (Cetintas and Qadir 2014).

2.2.6 Immunostimulant activity

Immunostimulant effects of powder of bay leaf were shown on rainbow trout by giving them dietary constituents. Three groups of rainbow trout were fed with experimental diets. After 21 days, nonspecific immune parameters such as phagocytosis in blood leukocytes, extra- or intracellular respiratory burst activities, lysozymes, and protein levels were examined and showed immunostimulant activity (Bilen and Bulut, 2010).

2.2.7 Antiviral Activity

L. nobilis essential oil containing beta-ocimene, 1,8-cineol, alpha-pinene, and beta-pinene constituents were reported for inhibitory activity in vitro against SARS-CoV and HSV-1

replication. Essential oil has this activity with an IC50 value of 120 mg/mL and selectivity index of 4.16 (Bilen and Bulut, 2010).

2.2.8 Antifungal activity

Hassiotis *et al.* (2010) investigated the influence of aromatic *L. nobilis* on the development of two mycorrhizal species, *Glomus deserticola* and *Glomus intraradices*. Both mycorrhizal fungi colonized successfully the host plants, positively influencing their growth. G. deserticola presented higher infection level than G. intraradices. Addition of *L. nobilis* oil into substrates resulted in mycorrhiza inhibition, and the level of inhibition was analogous with the amount of added essential oil. The fungi were benefited by the aromatic compounds up to 15 mg of essential oil per litre of soil. However, 30.00 mg/l and 60.00 mg/l of essential oil were able to create significant inhibition in mycorrhiza development and to restrict the host growth. Essential oils of *Thymus vulgaris* (thyme), *Cymbopogon citratus* (lemongrass) and *L. nobilis* (bay) were chemically quantified (Millezi *et al*., 2012)

The extracts of *L. nobilis* showed the highest antifungal activity against *A. niger and C. albicans* with inhibition zone diameters of 20.00-32 mm/15ml (Erturk 2006). Essential oil of bay leaf was tested in vitro against two foodborne fungi belonging to the dominant mycobiota of stored rice, *Fusarium culmorum* and *F. verticillioides*, The result showed that bay essential oil possessed great potential to activity was tested at different water activity and pH conditions, and the fungal growth was followed by measuring the colony diameter during the incubation period. Bay leaf essential oil was more effective at pH 5, losing their activity as pH increased (Guynot *et al*., 2005). The potential of bay leaf essential oils against species belonging to *Eurotium, Aspergillus* and *Penicillium* genus was demonstrated (Geeta and Reddy 1990; Guynot *et al*., 2003). Biological assays showed that fungi toxicity against *Fusarium moniliforme* (Gibberella fujikuroi),

Rhizoctonia solani, Sclerotinia sclerotiorum and *Phytophthora capsici* was due to different concentrations of the phenolic fraction in the essential oils Muller Riebau *et al*. (1995) control both fungal pathogens (Rosello *et al*., 2015). The antifungal effect of twenty (20) essential oils against the most important moulds in terms of spoilage of bakery products (*Eurotium spp., Aspergillus spp*. and *Penicillium spp*.) was investigated at concentration in the range between 0-1,000 µg/ml.

The potential of bay leaf essential oils against species belonging to Eurotium, Aspergillus and Penicillium genus has been demonstrated (Geeta and Reddy, 1990; Guynot *et al*., 2003). Biological assays showed that fungi toxicity against *Fusarium moniliforme (Gibberella fujikuroi), Rhizoctonia solani, Sclerotinia sclerotiorum and Phytophthora capsici* was due to different concentrations of the phenolic fraction in the essential oils (Muller Riebau *et al*., 1995; Pandey, 1997; Pandey and Dubey, 1997).

2.3 Anticholinergic Activity

Essential oil, ethanolic extract, and decoction of *L. nobilis* were reported to have anticholinergic activity toward acetyl cholinesterase (AChE) enzyme and showed good anticholinergic activity. Ethanolic fraction of about 64.00% of bay leaf also shown this inhibitory activity (Ferreira *et al*., 2006).

2.3.1 Inhibitor of nitric oxide production

Two new metabolites were isolated by Marino *et al*. (2005) , 5αH,7αH-eudesman-4α, 6α,11, 12-tetraol 1 and 1b,15-dihydroxy-5αH, 7αH-eudesma-3,11-dien-12,6 α-olide , from the methanolic extract of *L. nobilis* leaves (collected from spontaneous plants grown in Avellino, Campania, Italy). Their structures were determined through analysis of their one- and two-dimensional NMR spectral data (1 H- and 13C-NMR, DEPT, COSY, HMQC, HMBC and ROESY). The

relative stereochemistry was proposed based on the combined J-based configuration analysis and ROESY data. In addition, three known sesquiterpene lactones, were isolated and identified by spectral means. The isolated compounds 1–6 inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-activated murine macrophages. The most active, compound 2, potently inhibited NO_2 release with an IC50 value of 0.80μM. The methanolic extract of leaves of *L. nobilis* (bay leaf) inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages (Matsuda *et al*., 2000). Through bioassay-guided separation, fourteen (14) known sesquiterpenes were isolated from the active fraction and were examined for ability to inhibit NO production. Seven sesquiterpene lactones (costunolide, dehydrocostus lactone, eremanthine, zaluzanin C, magnolialide, santamarine and spirafolide) potently inhibited LPS-induced NO production (IC50 values of 1.2–3.8 mm). Other sesquiterpene constituents also showed inhibitory activity (IC50 < or = > 21 mm). α-Methylene-γ-butyrolactone also showed inhibitory activity (IC50 = 9.6 mm), while mokko lactone and watsonol A, reductants of the α-methylene-γ-butyrolactone moiety by NaBH4 or DIBAL, and a 2- mercaptoethanol adduct of dehydrocostus lactone, showed little activity (IC50 < or = > 18 mm). These results indicated that the α-methylene–γ-butyrolactone moiety was important for activity. Furthermore, costunolide and dehydrocostus lactone inhibited inducible nitric oxide synthase (iNOS) induction in accordance. Almost complete inhibition of 3- nitrotyrosine formation (91.00%) was achieved with the essential oil obtained from the leaves of *L. nobilis* (at 300 mg/ml). 1,8-Cineole (eucalyptol), accounting for 50.00% of this essential oil, was inactive in this model, thus evidencing a major role for the minor volatile compounds present in the leaves (Matsuda *et al*., 2000; Chericoni *et al*., 2005).

2.3.2 Trypanocidal activity

Methanolic and chloroformic extracts of dried leaves of *L. nobilis* presented trypanocidal activity against *Trypanosoma cruzi*, the etiologic agent of Chagas' disease. This activity is mainly due to the guaianolides dehydrocostus lactone and zaluzanin D, and to a p-menthane hydroperoxide. Inhibition of ethanol in blood: Methanolic extracts of dried leaves of L.nobilis potently inhibited the elevation of blood ethanol level in ethanol-loaded rat. This activity is mainly due to costunolide, dehydrocostus lactone, and santamarine, being the α-methylene-γ-butyrolactone moiety essential for the preventive effect on ethanol absorption.

2.3.3 Insect Repellent Activity

L. nobilis essential oils extracted from seeds were reported to have insect repellant activity against Culex pipiens (Erler *et al*., 2006). Essential oils from laurel were evaluated for fumigant toxicity against all developmental stages of the confused flour beetle (*Tribolium confusum*). GC-MS analysis showed that 1,8-cineole was the major component of the essential oils. The vapours of laurel essential oil were toxic to all the stages of *T. confusum* (Isikber *et al*., 2006). Repellency and toxicity of essential oil from *L. nobilis* (*Lauraceae*) against the rust-red flour beetle (*T. castaneum Herbst*) were also reported by Andronikashvili and Reichmuth (2003). The toxicity of ethanol extracts from *L. nobilis* on the large diamond back moth*, Plutella xylostella*, was 55.00% (Erturk *et al*., 2004). The behavioural responses of adult female western flower thrips*, Frankliniella occidentalis,* to volatiles from meadowsweet (*Filipendula ulmaria*), bay laurel and sage (*Salvia officinalis*) were investigated in laboratory bioassays by (Chermenskaya *et al*., 2001). Volatiles collected by entrainment of a solvent extract of *F. ulmaria* were more attractive than was the original extract. *F. occidentalis* also was attracted significantly to volatiles from *L. nobilis* and *S. officinalis*. Analysis by gas chromatography and mass spectrometry identified 1,8-cineole (eucalyptol) as one of the main volatile components of all three plant species. In coupled

gas chromatography–electroantennography studies with *F. ulmaria*, both 1,8-cineole and methyl salicylate elicited responses from *F. occidentalis*. Eucarvone was identified as the major component of *F. ulmaria* volatiles, but showed no electrophysiological activity. The behavioural responses of thrips to a range of concentrations of 1,8- cineole and methyl salicylate were tested using a modified Pettersson 'star' olfactometer. 1,8-Cineole showed some attractant activity for the thrips at 0.01 mg, but methyl salicylate was repellent at all the concentrations tested. The bruchid, *Acanthoscelides obtectus*, is one of the most damaging pests of kidney beans (*Phaseolus vulgaris*) worldwide. However, aromatic plants from the families Lamiaceae, *Lauraceae*, Myrtaceae and Poaceae can protect *P. vulgaris* by a direct or delayed insecticidal effect, through increased adult mortality and inhibition of reproduction (both oviposition and adult emergence). The insecticidal effect is due to the presence of factors other than those in the essential oils as there is no significant difference between the efficacy of distilled and intact plant extracts. Inhibition of reproduction is particularly important. The results suggest that lipid, as well as nonlipid allelochemicals, such as phenolics, or non-protein amino acids or flavonoids may be involved in the toxicity of extracts of aromatic plants to *A. obtectus* (Regnault Roger and Hamraoui, 1995; Mackeen *et al*., 1997).

2.3.4 Acaricidal Activity

Acaricidal activity of bay leaf oils was observed against Psoroptescuniculi. Acaricidal activity of bay oil led to a mortality rate of 73.00% at a concentration of 10.00% and at 5.00% average activity was considerably reduced to 51.00% (Macchioni *et al*., 2006).

2.3.5 Anti-inflammatory Activity

One of the most important benefits of bay leaves is their ability to reduce inflammation throughout the body. These leaves contain a unique phytonutrient, called parthenolide, which can

quickly reduce inflammation and irritation when topically applied to affected areas, such as sore joints or areas affected by arthritis. This effect can also be achieved through normal consumption of bay leaf spice.

2.3.6 Antidiabetic activity

Diabetes is a disease that is characterized by high blood glucose (hyperglycemia) due to a total or partial insulin deficiency. This enhancement of blood glucose could produce several cardiovascular disorders, including platelet hyperactivity and hyperaggregability, which is associated to an increased oxidant production and abnormal cytosolic Ca^{2+} mobilization. One approach to decrease postprandial blood glucose levels is to postpone glucose absorption by inhibiting some enzymes like α-amylase or α-glucosidase, which are digestive enzymes that hydrolyze carbohydrates. To this end, several authors have checked hydroalcoholic extracts, acetone extracts and the essential oil of laurel leaves. In that way, Dearlove *et al*. observed that a hydroethanolic extract inhibited fructose-mediated protein glycation; (Yanardag and Can 1994) observed that the administration of an ethanolic extract produced a significant decrease in blood glucose levels in diabetic rabbits. Kazeem *et al.* (2015) reported that acetone extracts have glucose antyglycation activity in bovine serum albumin (BSA).

Moreover, in order to reduce the risk of thrombotic and ischemic events in diabetic disease, it has been observed that the trimeric procyanidin cinnamtannin B-1, isolated from a laurel wood ethyl acetate extract, reduces abnormal intracellular Ca^{2+} homeostasis and platelet hyperaggregability in type 2 diabetes mellitus patients. Antioxidant: *L. nobilis* extracts and essential oils have been extensively investigated in terms of antioxidant activity. Hydroalcoholic extracts of laurel leaves are the most investigated ones. For this purpose it has been used different methods, such as DPPH radical scavenging, hydroxyl radical scavenging , superoxide radical inhibition, hydrogen

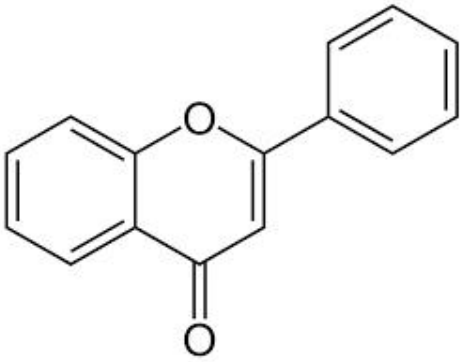
peroxide scavenging, lipid peroxidation inhibition, phosphomolybdenum method, ABTS radical scavenging, bovine brain peroxidation method, β -carotene–linoleic acid, ferric reducing antioxidant potential (FRAP), oxygen radical absorbance capacity (ORAC), superoxide dismutase (SOD), alkyl peroxy radical scavenging, and cyclic voltammetry. Moreover, the activity-guided isolation of antioxidant active compounds has been performed for several authors, achieving the isolation of the main contributors of the antioxidant power from hydroalcoholic extracts of laurel leaves. In that way four active flavonoids were isolated: kaempferol, kaempferol-3-rhamnopyranoside, kaempferol-3,7-dirhamnopyranoside and isoquercitrin . Other solvents have also been used for the extraction of antioxidants from laurel leaves: acetone, diethyl ether; chloroform and ethyl acetate. Among all these solvents, acetone and ethyl acetate get the most active extracts. Essential oils from laurel leaves have also been studied to determine their antioxidant activity. Moreover, some authors have used different extractive methods in order to enhance the antioxidant activity of the resulting essential oils. Thus, enzymes, supercritic CO_2 or microwave heating have been used to increase the antioxidant power of the essential oils. Other organs of laurel tree have also been analyzed for their antioxidant properties. In that way, hydroalcoholic extracts of fruits, bark and seeds, and laurel seed essential oil present antioxidant activities. Among all these extracts, the methanolic extract of laurel bark presented the most important antioxidant activity. Different laurel leaf extracts have shown anti-inflammatory effect through different mechanisms. For example, a dichloromethane-methanolic extract presented anti-inflammatory activity on the murine fibrosarcoma L929sA cells via regulation of NF κ B factor. A methanolic extract also presented anti-inflammatory effect in lipopolysaccharide (LPS)-activated mouseperitoneal macrophages via inhibition of nitric oxide production. In this case, the sesquiterpene composition of the methanolic extract is the main

responsible of such kind of activity, being the most active compounds the sesquiterpene lactones costunolide and dehydocostus lactone. Other compounds isolated from methanolic extracts like megastigmanes and flavonoids also inhibited the nitric oxide production in lipopolysaccharide-activated murine macrophages. An ethanolic extract exhibited anti-inflammatory effect in lipopolysaccharide monocytes RAW 264.7 macrophages. Also a dimethylsulphoxide extract presented this kind of anti-inflammatory activity in a lipopolysaccharide-stimulated macrophage model via reducing the expression of COX-2 and by the decrease of pro-inflammatory interleukin (IL)-6. Moreover, the essential oil of laurel leaves has important anti-inflammatory and anti-nociceptive activities in male NMRI mice and Wistar rats [198]. In control animals, subplantar injection of formaldehyde produced a local edema which is significantly inhibited in a dose-dependent manner by the essential oil. This anti-inflammatory action is in agreement with the reported anti-inflammatory activity of some constituents of the essential oil.

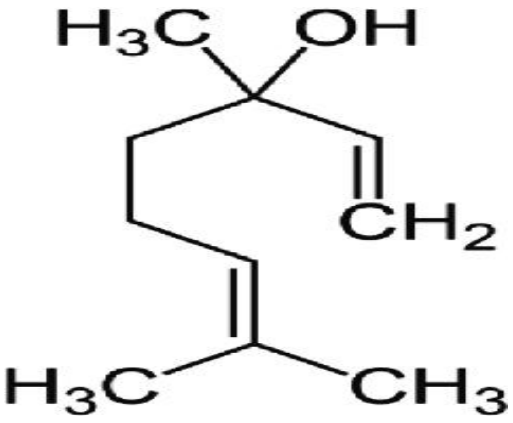
2.3.7 Side effects and toxicity

Bay leaf and bay leaf oil are likely safe for most people in food amounts. There is no choke possibility with ground bay leaf, as does exist with whole leaf. The whole leaf cannot be digested, so it remains intact while passing through the digestive system. There is not enough reliable information about the safety of taking bay leaf during pregnancy or breastfeeding. Bay leaf might interfere with blood sugar control and may not be safe to use during diabetes. Bay leaf might slow down the central nervous system (CNS). There is a concern that it might slow down the CNS too much when combined with anesthesia and other medications used during and after surgery. It is recommended to stop using bay leaf as a medicine at least 2 weeks before a scheduled surgery.

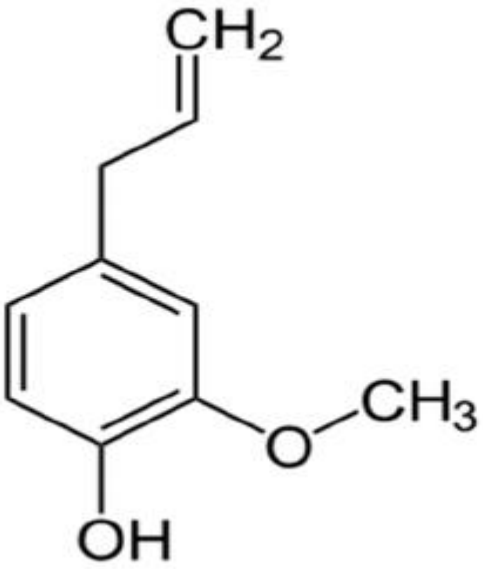
Figure 2.1: Structures of some compounds found in bay leaf



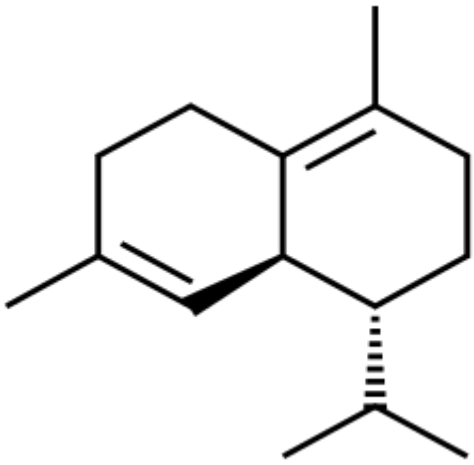
Linalool



flavonoid



eugenol



Cadinine

2.4 Storage

Leaves can be stored frozen for the sake of use for extended time beyond its fresh shelf life. For drying of bay leaf, different drying methods are available. Traditionally, it is dried in open air for 10-12 days. Sun drying has some disadvantages, like natural color loss and essential oil loss that

result in low market value of bay leaf. Hot air drying at 60^0C is the best method for preserving bay leaves. Steam distillation is the best method for the recovery of essential oils from the bay leaf plant. Essential oil extracted from bay leaf is in two forms, fixed oil and volatile oil, that are collected from bay fruits (Bozan and Karakaplan, 2007).

CHAPTER THREE

MATERIALS AND METHOD

3.1 SAMPLE COLLECTION

Dried *Laurus nobilis* (bay leaf) were purchased from the market, Benin City, and transported to the Department of Plant Biology and Biotechnology, University of Benin, Benin City, for taxonomical identification, then the leaves were taken to the laboratory for analysis.

3.2 TEST ORGANISM

The fungi used for this study include *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum* and *Fusarium solani*. Samples were gotten from hair, foot, and toenail samples from some students in microbiology, University of Benin (UNIBEN) using swab sticks.

3.3 STERILIZATION OF MATERIALS

Materials such as petri dishes, pipettes, glass containers {conical flasks, round bottom flasks etc.} and bottles were washed, drained and dried. They were wrapped in aluminium foil and sterilized

in a hot-air oven at 160°C for an hour. They were allowed to cool at about 40°C after sterilization. The working environment was swabbed with disinfectant.

3.4 PREPARATION OF PLANT ORGANIC EXTRACTS FOR ASSAY

The dried leaves of *Laurus nobilis* was grinded to fine powder, using manual grinder, to increase the surface area. 100g of bay leaf was soaked in 250 ml of solvents: methanolic, ethanolic and aqueous extracts in different conical flasks plugged with cotton plugs respectively and observed on a shaker for 48 and 72hrs. The stock concentration was 400 mg/ml. The extracts were filtered through a Whatman No.1 filter paper and Muslin cloth severally and concentrated to dryness with the aid of a rotary evaporator. The stocks were kept at 4 °C in a refrigerator until further use (Adeogun *et al.*, 2016).

The concentrated extracts of *Laurus nobilis* leaves were varied into different methanolic, ethanolic and aqueous concentrations: 100%, 75%, 50%, 25% according to the methods of Adeogun *et al.* (2016). Thus, making the concentrations 400 mg/ml, 300 mg/ml, 200 mg/ml, and 100 mg/ml.

3.5 PHYTOCHEMICAL ANALYSIS OF THE EXTRACTS

Phytochemical Screening of extract using the method described by Trease and Evans (1989) and Edeoga *et al* (2005) test for saponins, tannins, phenols, flavornoids and alkaloids were carried out. Phytochemical parameters of saponin, flavonoids, tannins, alkaloids and total polyphenol of *Laurus nobilis* was determined by standard methods (Alaa *et al.,* 2016). Phytochemicals in laurel tree: Due to the commercial value of laurel leaves most of the reported work on laurel tree chemical composition is focused on them. The volatile fraction of laurel leaves has been particularly studied and reviewed. For that reason, this review will cover more comprehensively

the non-volatile fraction of laurel leaves as well as that of the other laurel organs. Comparatively, laurel wood is clearly the part of the tree less studied.

3.5.1 Test for flavonoids

0.50 g of the grinded leaves was boiled in 50.00 ml of 2M concentrated hydrochloric acid (HCL) solution for 30 minutes under reflux. It was allowed to cool and then filtered through Whatman No. 42 filter paper. An equal volume of ethyl acetate was added to an equal measured volume of the extract. Red coloration shows the presence of flavonoids. The flavonoid precipitate was recovered by filtration using weighed filter paper. The resulting weight difference was taken as the weight of the constituent flavonoid in the sample. Flavonoids are the main phenolic constituents of laurel leaf's alcoholic extracts, chemically based upon a fifteen-carbon skeleton consisting of two benzene rings A and B linked via a heterocyclic pyrene ring. Flavones and flavonols are the main flavonoids in alcoholic extracts of leaves. Apigenin, kaempferol, quercetin and their glycosides are also frequently found. In the flavone group is mainly observed C-glycosidation at C-6 or C-8, while in flavonols predominates O-glycosidation at C-3.

3.5.2 Test for tannins

0.20 g of the powdered leaf sample was measured into a 50.00 ml beaker, followed by the addition of 20.00 ml of 50.00 % methanol was added and covered with paraffin and placed in a water bath at 77-80 ^{o}C for 1 hr and stirred with a glass rod to prevent lumping. The extract was quantitatively filtered using a double layered Whatman No. 1 filter paper into a 100.00 ml volumetric flask using 50.00 % methanol to rinse. This was made up to mark with distilled water and thoroughly mixed. 1.00 ml of sample extract was pipette into 50.00 ml volumetric flask, 20ml distilled water, 2.50 ml Folin-Denis reagent and 10.00 ml of 17 % Na_2CO_3 were added and

mixed properly. The mixture was made up to mark with distilled water, mixed well and allowed to stand for 20 min when a bluish-green colouration developed. Standard Tannic acid solutions of range 0-10 ppm were treated similarly as 1.00 ml of sample above. The absorbances of the tannic acid as well as the standard saponin solutions were read after colour development**.**

Percentage tannin was calculated using the formula:

$$\text{Tannin (\%)} = \frac{\text{Instrument reading x Colour volume x Extract volume x 100}}{\text{Aliquot taken x Weight of sample x 1,000,000}}$$

3.5.3 Test for alkaloids

The presence of alkaloids in laurel tree has been detected in several occasions by simple phytochemical tests. A measured weight of the powdered *Laurus nobilis* sample was dissolved in 10.00 % acetic acid solution in ethanol to form a ratio of 1:10. The mixture was left to stand for 4hrs at 28^{o}C, and then filtered using Whatman No. 42 grade filter paper. The filtrate was concentrated to one quarter of its original volume by evaporation and treated with conc. aqueous ammonium hydroxide (NH_4OH) added in drops until the alkaloid was precipitated. The alkaloid precipitate was collected in a weighed filter paper, washed with 1% ammonium solution and dried in the oven at 80 ^{o}C. Alkaloid content was calculated and expressed as a percentage of the weight of sample analyzed. Second method was done in a way where each 2 grams of the extract was extracted by warming with 20ml of sulphuric acid in 50ml conical flask on a water bath with intermittent shaking for 2 minutes. It was then centrifuged and the supernatant pipetted off into a small conical flask. One drop of Meyer's reagent was added to 0.1ml supernatant in a semi - micro tube. Cream precipitate indicated presence of alkaloids.

3.5.4 Test for Saponin

1.00 g of powdered leaf sample was weighed into a 250.00 ml beaker and 100.00 ml of isobutyl alcohol was added. The mixture was shaken on a UDY shaker for 5 hours to ensure homogenous mixture. Thereafter the mixture was filtered through a Whatman No. 1 filter paper into a 100.00 ml beaker and 20.00 ml of 40% standard solutions of magnesium carbonate ($MgCO_3$) added. The mixture obtained with saturated $MgCO_3$ was again filtered through a Whatman No. 1 filter paper to obtain a clear colourless solution. Then, 1.00 ml of the colourless solution was pipetted into 50.00 ml volumetric flask and 2.00 ml of 5% iron III chloride ($FeCl_3$) solution was added and made up to mark with distilled water. It was allowed to stand for 30 mins for blood red colour to develop. This was followed by the preparation of 0-10 ppm standard saponin solutions were prepared from saponin stock solution. The standard solutions were treated similarly with 2ml of 5% FeCl solution as done for 1ml sample. The absorbances of the sample as well as the standard saponin solutions were read after colour development on a spectronic 2ID spectrophotometer at a wavelength of 380 nm. Frothing are present, this indicates the presence of Saponin in the filtrate sample.

Percentage saponin was calculated using the formula:

$$\text{Saponin (\%)} = \frac{\text{Absorption of sample x Average gradient x Dilution factor}}{\text{Weight of sample x 10,000}}$$

3.5.5 Test for total polyphenols

200.00 µl of the leaf extract was added to 1 ml of 0.2 N Folin-Ciocalteau reagent and 0.80 ml of 7.50 % sodium carbonate solution. It was mixed well and allowed to stand for 30 mins at room temperature. Absorption at 765nm was read using a Shimadzu 300 UV-Vis spectrophotometer.

Quantification was based on the standard curve generated with 100.00 - 400.00 mg/ml of gallic acid.

Percentage total polyphenol was calculated using the formula:

$$\text{Total polyphenol (\%)} = \frac{\text{Instrument reading x Colour volume x Extract volume x100}}{\text{Aliquot taken x Weight of sample x 1,000,000}}$$

3.6 MEDIA PREPARATION

The media used for this work was Potato dextrose agar (PDA) for activity of the fungi. PDA (39g) was dissolved in 1000ml of distilled water in a conical flask then closed with a cork stopper. The suspension was first dissolved completely by shaking and then sterilized by autoclaving at 121°C for 15 minutes. The medium was allowed to cool then dispensed aseptically into sterile petri dishes. The petri dishes were covered and allowed to solidify.

3.7 PREPARATION OF EXTRACT CONCENTRATIONS

The concentrated extracts were reconstituted with 100ml of methanol and the concentrated extracts were later varied and diluted into different concentrations; 100%, 75% (7.5ml+2.5ml water), 50% (5.0ml+ 5.0ml water), 25% (2.5ml extract + 7.5ml water) according to Adeogun *et al.,* (2016).

3.8 ANTIFUNGAL ACTIVITY

The effect of solvent extracts on mycelial growth of test fungi was determined using poisoned food method. Sterile plates was loaded with extract (1ml) followed by addition of potato dextrose agar (19 ml) and the mixture was gently swirled to allow thorough mixing and allowed to solidify, after which a mycelial plug was placed centrally in the plate. Mycelial plugs were removed from the periphery of culture plates with actively growing mycelia. Culture plates were

then incubated at 25 ± 2°C. Measurements were taken after 24 hours of inoculation. Control plate contained media without any treatment (without extract). The assay was performed in duplicates and their mean values were noted.

3.9 PERCENTAGE MYCELIAL RADIAL GROWTH INHIBITION

Percentage inhibition of the growth of the test organisms was determined using the formula:

$$\frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100$$

CHAPTER FOUR

RESULT

The result shows the phytochemical and antifungal activity of *Laurus nobilis* against some selected fungi isolates (*Aspergillus niger*, *Aspergillus flavus, Penicillium chrysogenum and Fusarium solani).* The radius of growth was measured in millimeters (mm) in the result presented below:

Table 4.1, figure 4.1.a and figure 4.1.b shows the phytochemical composition of *Laurus nobilis* leaves extract. The percentage composition of saponin, alkaloid, tannin, flavonoid, and total polyphenol are 4.40%, 4.00%, 13.50%, 11.00% and 0.10% respectively. The highest percentage composition is tannin with a total composition of 13.50% and the lowest is total polyphenol with a composition of 0.10%.

Table 4.2 shows the cultural, morphological and biochemical characteristics of the isolates used in this study.

Table 4.3 shows the radius of growth of the fungal isolates after 2 days of inoculation. In the aqueous extract, the highest radial growth was obtained from *A. flavus* at 400 mg/ml concentration having 21.50 mm as the radius. In the methanolic extract, the lowest radial growth was obtained from *A. niger* at 400mg/ml and 300 mg/ml concentrations having 18.00 mm as the radius. In the ethanolic extract, the lowest radial growth was obtained from *A. flavus* at 100 mg/ml concentration having 16.00 mm as the radius.

Table 4.4 shows the Percentage mycelial growth inhibition (%) of *Laurus nobilis* against fungal isolates after 2 days of inoculation. In the aqueous extract, the highest mycelial growth percentage was 40.74% which was obtained from *F. solani* at 400mg/ml, 300mg/ml, 200mg/ml, and 100 mg/ml concentrations. In the methanolic extract, the highest percentage mycelial growth inhibition was 28.57% which was obtained from *P. chrysogenum* at 400mg/ml and 300 mg/ml concentrations. In the ethanolic extract, the highest percentage mycelial growth inhibition was 25.00% which was obtained from *P. chrysogenum* at 400 mg/ml concentration.

Table 4.5 shows the radius of growth of the fungal isolates after 4 days of inoculation. In the aqueous extract, the lowest radial growth was obtained from both *A. flavus* and *F. solani* at 400 mg/ml concentration having 16.00 mm as the radius. In the methanolic extract, the highest radial growth was obtained from *F. solani* at 400 mg/ml concentration having 31.50 mm as the radius. In the ethanolic extract, the highest radial growth was obtained from *P. chrysogenum* at 400 mg/ml concentration having 21.00 mm as the radius.

Table 4.6 shows the Percentage mycelial growth inhibition (%) of *Laurus nobilis* against fungal isolates after 4 days of inoculation. In the aqueous extract, the highest percentage mycelial

growth inhibition was 36.67% which was obtained from *P. chrysogenum* at 100 mg/ml concentration. In the methanolic extract, the highest percentage mycelial growth inhibition was 39.06% which was obtained from *P. chrysogenum* at 300mg/ml, 200mg/ml and 100 mg/ml concentrations. In the ethanolic extract, the lowest percentage mycelial growth inhibition was 0.00% which was obtained from *P. chrysogenum* at 100 mg/ml concentration.

Table 4.1: Phytochemical composition of *Laurus nobilis* leaves

Compounds	**Percentage (%) Composition**
Saponin	4.40
Alkaloid	4.00
Tannin	13.50
Flavonoid	11.00
Total polyphenol	0.01

Figure 4.1.a: Tannin Concentration

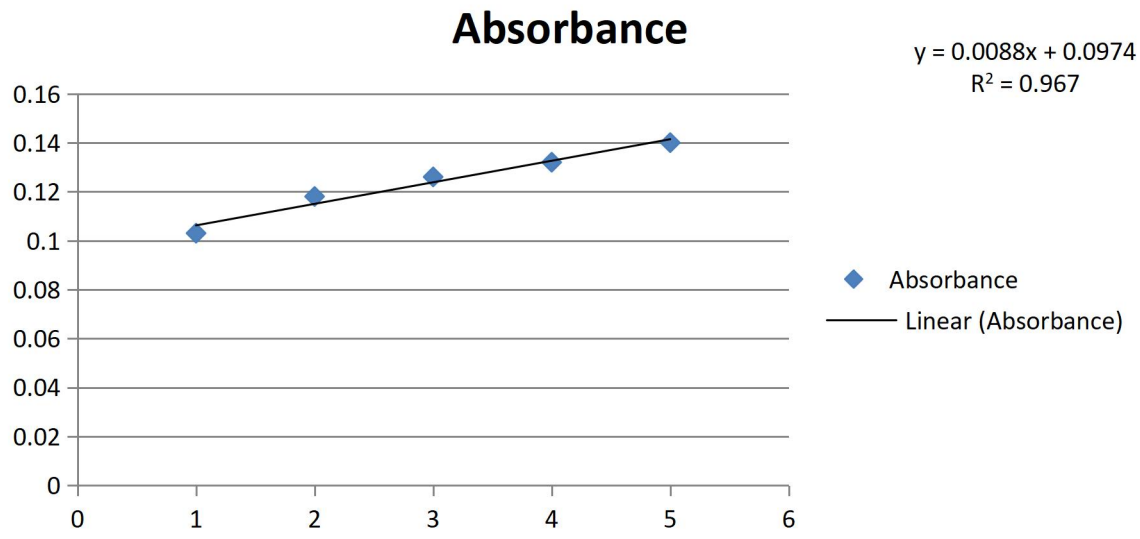


Figure 4.1.b: Total Polyphenol Concentration

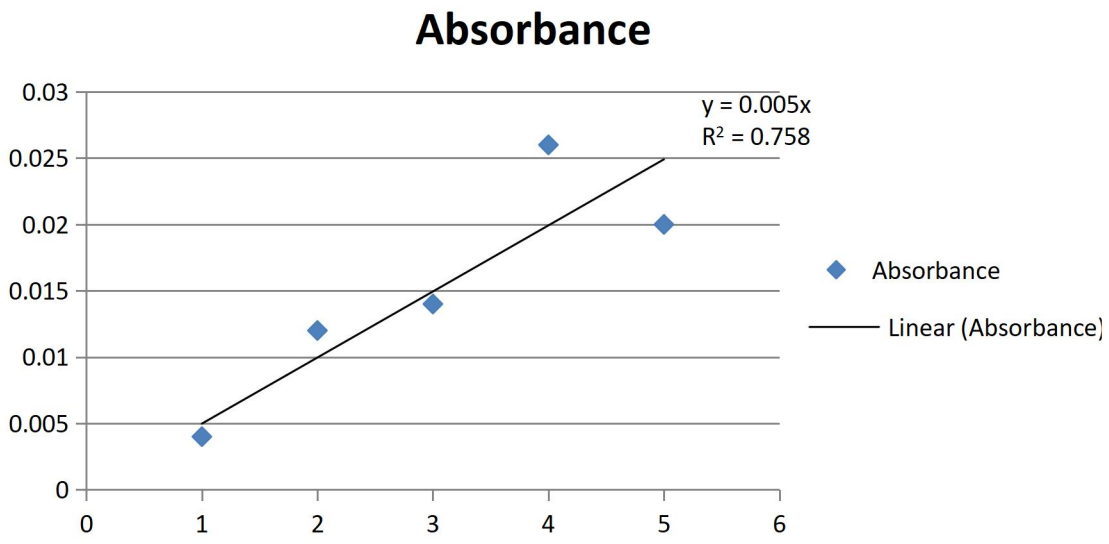


Table 4.2: Cultural and Morphological characteristic of isolates

Cultural	1	2	3	4
Colour of mycelium on agar plate	black	brown-black	green	white
Plate culture reverse	pale yellow	cream	yellow	Pale yellow
Morphological				
Nature of hyphae	septate	septate	septate	septate
Type of spores	conidiosphore	conidiospore	conidiospore	conidiosphore
Colour of spores	brown-black	colourless	blue-green	cream
Class of fungi	Eurotimycetes	Ascomycete	Eurotiomycetes	sordariomycete

Isolates	*Aspergillus niger*	*Aspergillus flavus*	*Penicillium chrysogenum*	*Fusarium solani*

Table 4.3: Radius of growth (mm) of fungal isolates after 2 days of inoculation

Extract	**Concentration**	*Aspergillus niger*	*Aspergillus flavus*	*Penicillium chrysogenum*	*Fusarium solani*
Aqueous	400 mg/ml	18.00	21.50	20.00	16.00
	300 mg/ml	18.50	21.50	18.50	16.00
	200 mg/ml	19.50	21.00	18.50	16.00
	100 mg/ml	18.50	20.50	18.00	16.00
	Control	21.00	21.00	28.00	27.00
Methanol	400 mg/ml	18.00	28.50	20.00	30.00
	300 mg/ml	18.00	28.50	20.00	25.00

	200 mg/ml	18.50	21.00	22.50	24.00
	100 mg/ml	19.50	21.00	20.50	21.00
	Control	21.00	22.00	28.00	25.00
Ethanol	400 mg/ml	18.50	22.00	21.00	43.00
	300 mg/ml	18.50	19.50	22.50	44.00
	200 mg/ml	20.00	18.50	24.00	42.50
	100 mg/ml	18.00	16.00	24.00	35.50
	Control	20.50	19.00	28.00	42.00

Table 4.4: Percentage mycelial growth inhibition (%) of *Laurus nobilis* extract against fungal isolates after 2 days

Extract	**Concentration**	*Aspergillus niger*	*Aspergillus flavus*	*Penicillium chrysogenum*	*Fusarium solani*
Aqueous	400 mg/ml	14.29	-2.38	28.57	40.74
	300 mg/ml	11.90	-2.38	33.93	40.74
	200 mg/ml	7.14	0.00	33.93	40.74
	100 mg/ml	11.90	2.38	35.71	40.74
Methanol	400 mg/ml	14.29	-29.55	28.57	-20.00
	300 mg/ml	14.29	-29.55	28.57	0.00
	200 mg/ml	11.90	4.55	19.64	4.00

	100 mg/ml	7.14	4.55	26.79	16.00
Ethanol	400 mg/ml	9.76	-15.79	25.00	-2.38
	300 mg/ml	9.76	-2.63	19.65	-4.76
	200 mg/ml	2.44	2.63	14.29	-1.19
	100 mg/ml	12.20	15.79	14.29	16.67

Table 4.5: Radius of growth (mm) of fungal isolates after 4 days of inoculation

Extract	**Concentration**	*Aspergillus niger*	*Aspergillus flavus*	*Penicillium chrysogenum*	*Fusarium solani*
Aqueous	400 mg/ml	17.00	16.00	20.50	16.00
	300 mg/ml	18.00	17.00	20.00	16.00
	200 mg/ml	18.00	18.00	19.50	25.00
	100 mg/ml	20.50	18.00	19.00	25.00
	Control	21.50	20.00	30.00	21.00
Methanol	400 mg/ml	17.00	17.00	20.50	31.50
	300 mg/ml	18.00	18.00	19.50	30.00
	200 mg/ml	18.50	19.00	19.50	30.50

	100 mg/ml	20.50	20.00	19.50	30.50
	Control	23.50	22.00	32.00	30.00
Ethanol	400 mg/ml	17.00	18.00	21.00	13.00
	300 mg/ml	18.00	18.00	19.50	13.00
	200 mg/ml	19.00	18.00	19.20	15.00
	100 mg/ml	20.00	18.00	19.00	15.00
	Control	23.00	20.00	19.00	17.70

Table 4.6: Percentage mycelial growth inhibition (%) of *Laurus nobilis* against fungal isolates after 4 days

Extract	**Concentration**	*Aspergillus niger*	*Aspergillus flavus*	*Penicillium chrysogenum*	*Fusarium solani*
Aqueous	400 mg/ml	20.93	20.00	31.66	23.81
	300 mg/ml	16.28	15.00	33.33	23.81
	200 mg/ml	16.28	10.00	35.00	-19.05
	100 mg/ml	4.65	10.00	36.67	-19.05
Methanol	400 mg/ml	27.66	22.73	35.94	-5.00
	300 mg/ml	23.40	18.18	39.06	0.00
	200 mg/ml	21.28	13.64	39.06	-1.67

	100 mg/ml	12.76	9.09	39.06	-1.67
Ethanol	400 mg/ml	26.09	10.00	-10.53	26.55
	300 mg/ml	21.74	10.00	-2.63	26.55
	200 mg/ml	17.39	10.00	-1.05	15.25
	100 mg/ml	13.04	10.00	0.00	15.25

CHAPTER FIVE

DISCUSSION

In some studies, bay leaf supports fungal growth, whereas it acts as a fungicide and fungistatic in others (Atanda, *et al*., 2007; De Corato, *et al*., 2010). These results showed that the inhibited growth of the studied fungi is related to the content of phenols, eugenol, carvacrol and thymol and the ester eugenyl acetate in *Laurus nobilis*, and also to the synergetic effect of the compounds found in a lower proportion to the majority compounds. In this study, the percentage composition of saponin, alkaloid, tannin, flavonoid, and total polyphenol in the *L. nobilis* leaves are 4.40%, 4.00%, 13.50%, 11.00% and 0.01% respectively. Tannin tend to have the highest percentage composition and total polyphenol the lowest.

In the *L. nobilis* leaf extract for 2 days, *F. solani* was the most inhibited by the aqueous extract but the methanolic and ethanolic extracts had no inhibitory effects on its growth because the 400mg/ml concentration was higher than the control. In the methanolic extract, radial growth of *A. niger* and *P. chrysogenum* were inhibited, but *F. solani* was not inhibited with the radial growth at 400 mg/ml (30.00 mm) growing higher than the control (25.00 mm). The ethanolic extract had no inhibitory effect on *A. flavus* and *F. solani*. *F. solani* had the highest radial growth from ethanol extract (43.00mm - 44.00mm from 400mg/ml – 300mg/ml respectively), growing higher than the control (19.00mm and 42.00mm). This could be due to different concentrations of the phenolics (Muller Riebau *et al*., 1995). In the *L. nobilis* leaf extract for 4 days, the aqueous extract had more inhibitory activity for all the isolates. *F. solani* was the most inhibited by the aqueous extract having a radial growth of 16.00mm at 400 mg/ml and 300mg/ml concentrations, the methanolic extract highly inhibited the growth of *A. niger* and *A. flavus* at 400 mg/ml with a radial growth of 17.00mm and 18.00mm respectively. *P. chrysogenum* was inhibited by the aqueous and methanolic extract but was not inhibited by the ethanolic extract having a radial growth of 21.00mm with a control of 19.00mm.

In the aqueous extract for 2 days, the highest percentage mycelial growth inhibition was 40.74% which was obtained from *F. solani* at 400mg/ml 300mg/ml, 200mg/ml and 100 mg/ml concentrations, showing higher inhibition than that of the 4 days obtained from *P. chrysogenum* (36.67%) at 100mg/ml. In the methanolic extract for 2 days, the highest percentage mycelial growth inhibition was 28.57% which was obtained from *P. chrysogenum* at 400mg/ml and 300 mg/ml concentrations, was lower than that of the 4 days showing 39.06% which was obtained from *P. chrysogenum* at 300mg/ml, 200mg/ml and 100 mg/ml concentrations. And in the ethanolic extract for 2 days, the highest percentage mycelial growth inhibition was 25.00%

obtained from *P. chrysogenum* at 400 mg/ml concentration, was lower than that of the 4 days showing 26.55% which was obtained from *F. solani* at 400mg/ml and 300 mg/ml concentrations . In general, the highest percentage mycelial growth inhibition was 40.74% obtained from *Fusarium solani* at 400mg/ml, 300mg/ml, 200mg/ml and 100 mg/ml concentrations. In comparison, more percentage mycelial growth inhibition of *L. nobilis* extract was observed in the 4 days extracts.

Generally, in this study, more inhibitory activity was observed against the isolates in the 4 days extracts than the 2 days extracts, and the aqueous extracts was seen to have more significant inhibitory activity against the isolates in the 4 days extract. This research was also in line with (Geeta and Reddy 1990; Guynot *et al*., 2003). It is acceptable that *Laurus nobilis* has the potential to control the development of fungi which contaminate food, as well as the production of mycotoxin by the toxin-producing species. However, the general ascertainment of researches who deal with antifungal testing of *Laurus nobilis* is that the methods of determining the antifiungal activity should be standardized.

L. nobilis has antifungal activity probably due to monoterpenes and sesquiterpenes in its composition. The antifungal activity of *L. nobilis* against microorganisms was determined qualitatively and quantitatively. In the result the inhibition zones was measured in millimeters (mm) against different pathogens (*Aspergillus niger*, *Aspergillus flavus, Penicillium chrysogenum and Fusarium solani.* The results indicate that *Laurus nobilis* exhibits strong antifungal activity against *Aspergillus niger*, *Aspergillus flavus, Penicillium chrysogenum and fusarium solani*. This activity might be due to 1.8-cineole and *α*-caryophyllene and their precursors (Sivropoulou *et al* 1997). In fact, 1.8-cineole alone has been shown to exhibit a better antifungal activity than the whole essential oil (Dammak *et al* 2019).

The phytochemical analysis includes tests such as test for flavanoids, saponins, tannins, phenols, and alkaloids were carried out. The result shows the presence of saponins, tannins, phenol, flavonoid and alkaloids in *Laurus nobilis*. This could be seen in table 4.1. All the test microorganisms were also sensitive to the extracts. It has been suggested that components of *Laurus nobilis* extracts cross the cell membrane and interact with the enzymes and proteins of the membrane producing a flux of protons towards the cell exterior, causing cell death of the isolates (Dammak *et al* 2019).

In general, the results indicate that the extracts of *Laurus nobilis* exhibits strong antifungal activity especially against *A. flavus*, *A. niger*, *P. chrysogenum* and *F. solani*. The observation of inhibitory activities after day four (4) was higher than that of day two (2). From here, it can be concluded that the longer the days, the lower the inhibition strength. The difference in antifungal efficacy is as a result of higher concentrations of the same chemical or a result of different chemical composition of the extract (Koji *et al*., 2004). Some composition of the extracts such as Pinene-type monoterpene hydrocarbons (α-pinene and β-pinene), limonene, and linalool are well-known chemicals having antifungal properties (Koji *et al* 2004). *L. nobilis* leaves are frequently used as a spice for cooking purposes. The leaf extract shows great activity against the pathogens. *L. nobilis* has antifungal activity probably due to monoterpenes and sesquiterpenes in its composition. The antimicrobial activity of *L. nobilis* against fungi was determined qualitatively and quantitatively. Folk medicine in many countries uses the infusion of the plant in stomachic and carminative remedies, as well as for the treatment of gastric diseases.

CONCLUSION

The results of this in vitro study suggest very important implications for the antifungal potential of *Laurus nobilis*. These results constitute an important step in the search for biologically active natural substances. *L. nobilis* exhibited important antioxidant and antifungal activities especially against all the selected fungi, suggesting its potential as a source of antifungal compounds identification. However, quantitative DPPH assay is needed to confirm the obtained results and more investigations on wide range of pathogen to assess the spectrum of bay leaves extracts are recommended.

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