A Study on Extraction of Essential Oil from the Leaves of *Eucalyptus citriodora* Hook. (Eucalyptus) and Its Antimicrobial Activity

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Abstract

Eucalyptus citriodora Hook. (Eucalyptus), popularly known as Lemon-Scented Gum, is widely used in food flavors, creams and ointments. The oil of Eucalyptus is known for its anti-inflammatory, antibacterial, antimicrobial, antiseptic and antioxidant. The purpose of this research is to study the investigation of phytochemical constituents, elemental contents, extraction of essential oil and its antimicrobial activity from the leaves of *Eucalyptus citriodora* Hook. (Eucalyptus) and its antimicrobial activity. The sample was collected from Kason Village, Kume, Myittha Township, Mandalay Region. Firstly, the preliminary phytochemical tests of this selected sample were carried out. The phytochemical investigation of Eucalyptus leaves indicated that the presence of alkaloids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, tannins, terpenoids, organic acids, steroids and α-amino acids were present in Eucalyptus leaves. But starch was absent in the sample. Then the elemental analysis of dried powder sample was carried out by EDXRF technique. The high contents of Ca and K were observed. In addition, the essential oil was extracted from Eucalyptus leaves by Hydro-distillation method. The yield percent of essential oil was found to be 0.4 % based on the powder sample. Furthermore, three compounds: 5-ethoxy-6-methoxy-8-nitroquinoline,2-propanone-1-[(3-hydroxy-6-methyl-2-pyridinyl) thio] and 1,3,2-Dioxaphosphorinane, 4-(hydroxymethyl) 2-oxo-2 phenoxy were detected in essential oil from Eucalyptus leaves by GC-MS method. In addition, investigation of antimicrobial activity on various crude extracts and essential oil of Eucalyptus leaves was done by agar well diffusion method against six microorganisms: *Bacillus subtilis, Staphylococcus aureus, Pseudomonas fluorescens, Bacillus pumilus, Candida albicans* and *Escherichia coli*. Moreover, PE, EtOAC, EtOH and H_2O extracts of Eucalyptus leaves were also exhibited the higher antimicrobial activity (inhibition zone diameters ranged between 15 ~ 27 mm) against six microorganisms tested. The essential oil of Eucalyptus leaves was found to exhibit antimicrobial activity (inhibition zone diameters ranged between 19- 22mm).

Keywords: *Eucalyptus citriodora* Hook., elemental analysis, EDXRF, essential oil, GC-MS method, antimicrobial activity, agar well diffusion method

Introduction

Eucalyptus is a large genus belonging to the family Myrtaceae. It comprises more than 600 species and subspecies of evergreen tree and shrubs, mainly native to Australia. *Eucalyptus citriodora* commonly known as Lemon-scented gum is an evergreen tree that grows up to 50 m tall at a fast rate. It is also known as blue spotted

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gum and lemon Eucalyptus. Its leaves are used as herbal remedy. The oil is extracted from leaves showing antibacterial, antiseptic, antioxidant, anti-inflammatory and anticancer activities. A tree for honey production and refined oil from the leaves is used as an insect repellant. *Eucalyptus citriodora* is among the economically important species of Eucalyptus available worldwide. Its essential oil is used, especially in perfumery but also medicinally. Nowadays, the plant is used in forestory (timber, fuel, paper pulp), environmental planting (water and wind erosin control), as a source of essential oil (cosmetic and aromatheraphy), for arts and craft. Essential oil is widely used in many countries like China, India, South Africa, Portugal, Brazil and Tasmania for many purposes. This plant was used traditionally as antiseptic and for the treatment of respiratory tract infections, the herb is, in fact, very helpful for cold flu, sore throats and chest infections including bronchitis and pneumonia. (Abd EI Mageed, 2011).

Figure 1. Photographs of (a) buds (b) flowers(c) leaves (d) bark and (e) trees of Eucalyptus

Eucalyptus

Eucalyptus contains citronellal, citronellol, 1,8- cineole, γ- terpenyl acetate, citronellic acid and citronellyl acetate. In both fresh and dried form, leaves of Eucalyptus are used as air fresheners and in medicinal teas. Eucalyptus leaves tea is high in flavonoids, which are antioxidants that may relive risk of cancers, heart disease and dementia. Eucalyptus leaves provide aroma that can be useful in dealing with stress and fatigue. As an ingredient in many products, it is used to reduce symptoms of coughs, colds and congestion. It also features in creams and ointments aimed at relieving muscle and joint pain. An oleo-resin obtained from the trunk. This resin contains tannins and is powerfully a stringent, it is used internally in the treatment of diarrhea and bladder inflammation, externally it is applied to cuts etc. The timer of Eucalyptus is used for light and heavy construction (i.e., bridge construction, flooring, cladding, tool handles and case manufacturing). The wood of young trees has been successfully used for pulp and paper. In Brazil, large plantations have been established for charcoal production. The oil of eucalyptus is known for its anti-inflammatory, antibacterial, antimicrobial, antiseptic, antifungal, antispasmodic, analgesic, antitussive and antioxidant.

Materials and Methods

Sample Collection

Eucalyptus leaves were collected from Kason Village, Kume, Myittha Township, Mandalay Region. After collection, it was identified by authorized botanist

at the Department of Botany, Kyaukse University. They were cleaned, cut into small pieces and then ground into purely fine powder by using an electric grinder.

Preliminary Phytochemical Tests

The dried powder sample was subjected to preliminary phytochemical tests in order to find out the types of organic constituents such as alkaloids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, tannins, starch, terpenoids, steroids and polyphenols according to appropriate reported methods.

Determination of Elemental Contents in Eucalyptus Leaves

Elemental compositions in Eucalyptus leaves were qualitatively determined by using EDXRF-7000 Spectrometer Shimadzu Co., Ltd. Japan. Elemental contents in Eucalyptus leaves were measured at the Department of Chemistry, Monywa University, Sagaing Region, Myanmar by applying EDXRF Method.

Extraction of Essential Oil from Eucalyptus Leaves by Hydro-distillation Method

The dried powder sample of Eucalyptus leaves (100 g) and distilled water (1000 mL) were placed in the round bottomed flask. The flask was fitted to clevenjar set which was joined to water condenser. When the flask was heated, the condensed oil and water coming out from condenser were collected in the receiver flask. The oil was extracted with pet-ether in a separating funnel. The pet-ether extract was dried over anhydrous sodium sulphate. After filtering, the pet ether was evaporated to get the essential oil which was then weighed until to be constant weight and kept in air tight bottle (Srivastava, 2003). The apparatus used for extraction of essential oil from Eucalyptus leaves is shown in Figure (2).

Figure 2. Extraction of essential oil from Eucalyptus leaves

Identification of Organic Compounds in Essential Oil of Eucalyptus Leaves by GC-MS method

Gas chromatography – mass spectrometry (GC-MS) is a method that combines features of gas chromatography and mass spectrometry to identify different substances with a test sample (Silverstein, 1998). Application of GC-MS includes identification and quantitation of volatile and semi-volatile organic compounds in complex mixtures. For identification of organic constituents in of Eucalyptus essential

oil, GC-MS is one of the hyphenated analytical techniques. Additionally, it can identify trace elements in materials that are previously thought to have disintegrated beyond identification (James, 1952). Organic constituents in essential oil of Eucalyptus leaves were detected by GC-MS spectroscopic method at Department of Chemistry, University of West, Yangon.

Preparation of Crude Extracts and Essential Oil from Eucalyptus Leaves

The dried powder samples of Eucalyptus leaves (10 g) were extracted with (50 mL) of pet-ether, ethyl acetate, ethanol and water in separate conical flask respectively for at least 7 days and then filtered to give the respective pet-ether, ethyl acetate, ethanol and watery extract. The essential oil from Eucalyptus leaves was extracted by Hydro-distillation method.

Antimicrobial Activity of Crude Extracts and Essential Oil of Eucalyptus Leaves

The antimicrobial activity of various crude extracts such as pet-ether, ethyl acetate, ethanol, watery and essential oil from Eucalyptus leaves were determined against six strains of microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli* by employing agar well diffusion method at Department of Chemistry, Meiktila University, Mandalay Region, Myanmar.

Procedure > Meat extract (0.5 g), peptone (0.5 g) and sodium chloride (0.25 g) were mixed with distilled water and the solution made up to 100 mL with distilled water. The pH of this solution was adjusted at 7.2 with 0.1 M sodium hydroxide solution and 1.5 g of agar was added. The nutrient agar medium was put into sterilized conical flask and plugged with cotton wool and then autoclaved at 121 °C for 15 min. After cooling down to 40 °C, one drop of suspended strain was inoculated to the nutrient agar medium was poured into the sterilized petri-dishes and left 10-15 min in order to set the agar. After that the agar wells were made with a 10 mm sterilized cork borer and the wells were filled with 0.1 mL of each extract to be tested. And the plates were incubated at 37 °C for 24 hr. After incubation, the diameters of inhibition zones including 10 mm wells were measured (Dorman *et al*., 2000).

Results and Discussion

Types of Phytochemicals Present in Eucalyptus Leaves

In order to know the types of phytochemical constituents present in Eucalyptus leaves, preliminary phytochemical tests were carried out according to the test tube method. From these results it was observed that alkaloids, flavonoids, glycosides, phenolic compounds, steroids, terpenoids, α- amino acids, carbohydrates, reducing sugars, tannins, saponins and organic acid were present in Eucalyptus leaves. But starch was absent in the sample.

Determination of Relative Composition of Some Elements by EDXRF Method

Table 1. Relative Composition of Some Elements in Eucalyptus Leaves

No	Symbols	Relative Composition (%)
1	Ca	0.671
2	K	0.396
3	S	0.113
4	Fe	0.011
5	Mn	0.003
6	Cu	0.002
7	Ti	0.001
8	Zn	0.001
9	Br	0.001

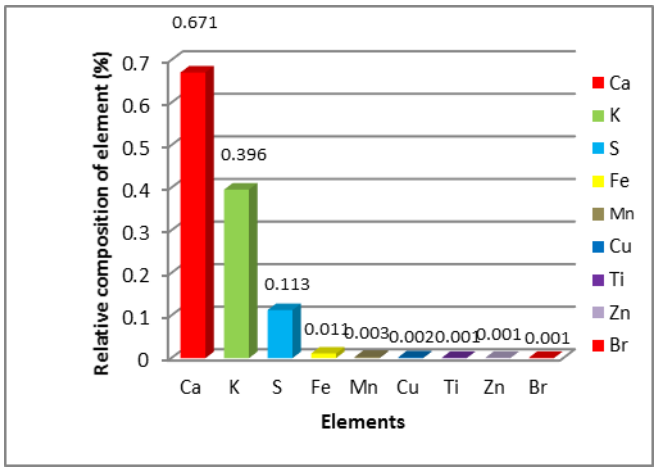


Figure 3. A bar graph of elemental contents in Eucalyptus leaves

Extraction of Essential Oil in Eucalyptus Leaves

Essential oil from Eucalyptus leaves was extracted by Hydro-distillation method. The yield percent of essential oil was found to be 0.4 % based on the powder sample.

Detection of Organic Compounds in Essential Oil of Eucalyptus Leaves by GC-MS Method

Gas chromatographic mass spectrometry (GC-MS) is the single most important tool for identification of unknown organic compounds by matching with reference spectra. The GC-MS chromatogram of essential oil from Eucalyptus leaves is shown in Figure 4. According to GC-MS chromatogram, the peak appears at the

retention time 3.74 min with 100 % relative abundance. At this retention time, the GC-MS spectrum Figure 5 shows the molecular ion peak at m/z 248 indicating the molecular weight of compound A to be 248 with the molecular formula $C_{12}H_{12}N_2O_4$. Therefore, it can be referred that the compound A is 5-ethoxy-6-methoxy-8-nitro quinolone. At the retention time 9.89 min, the GC-MS spectrum Figure 6 shows the molecular ion peak at m/z 197 which indicates the molecular weight of compound B to be 197 with the molecular formula $C_9H_{11}NO_2S$ and compound is 2-Propanone-1-[(3-hydroxy-6-methyl-2-pyridinyl) thio]. At the retention time 10.69 min, the GC-MS spectrum Figure 7 shows the molecular ion peak at m/z 244 which indicates the molecular weight of compound C to be 244 with the molecular formula $C_{10}H_{13}O_5P$ and compound is 1, 3, 2-Dioxophosphorinane, 4-(hydroxymethyl)-2-oxo-2-phenoxy. Therefore, the structures of organic compounds in essential oil extracted from Eucalyptus leaves are showed in Figure 8.

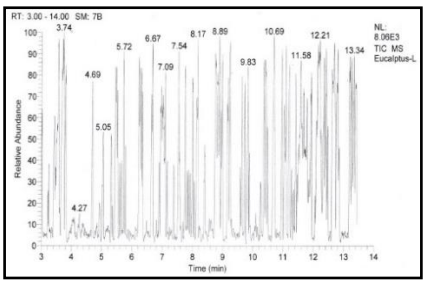


Figure 4. GC-MS chromatogram of essential oil of Eucalyptus leaves

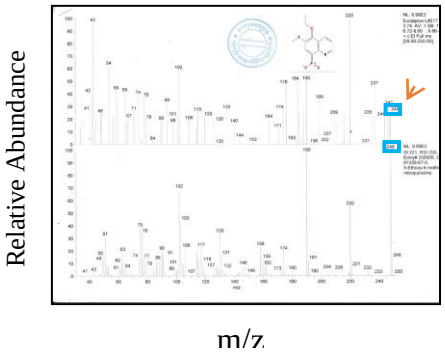


Figure 5. Matching of mass spectra of compound A at Retention Time 3.74 min and 5-ethoxy-6-methoxy-8-nitro quinoline

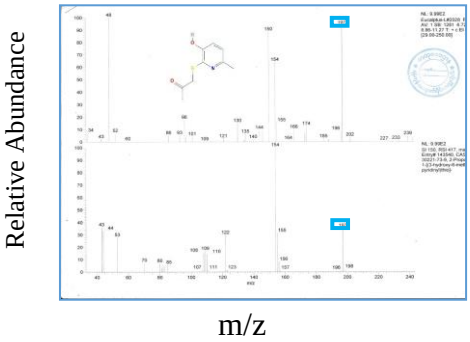


Figure 6. Matching of mass spectra of compound B at Retention Time 9.89 min and 2-Propanone-1-[(3-hydroxy-6-methyl-2-pyridinyl)

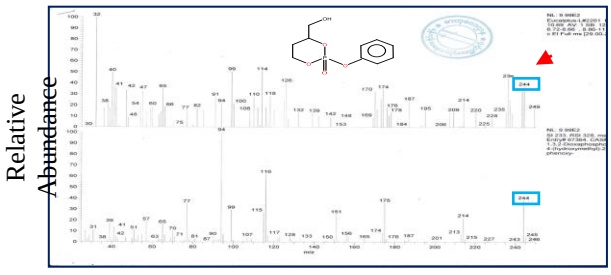
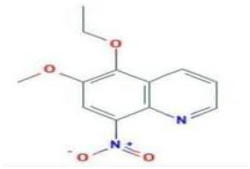
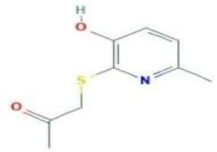


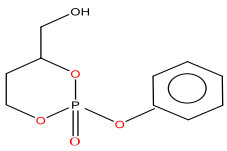
Figure 7. Matching of mass spectra of compound C at Retention Time 10.69 min and 1,3,2-Dioxaphosphorinane, 4-(hydroxymethyl)-2-oxo-2-phenoxy



5-ethoxy-6-methoxy-8-nitro quinoline



2-Propanone-1-[(3-hydroxy-6-methyl-2-pyridinyl) thio]



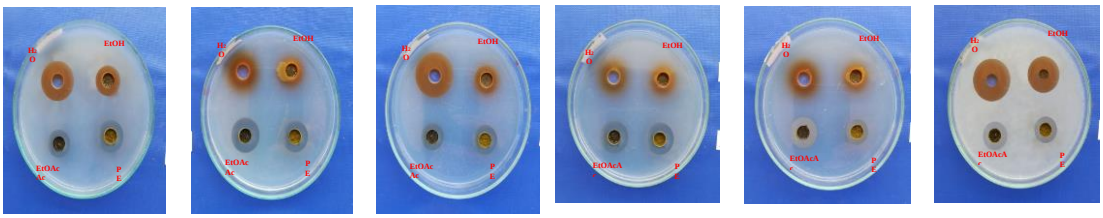
1,3,2-Dioxaphosphorinane, 4-(hydroxymethyl)-2-oxo-2-phenoxy

Figure 8. The structures of organic compounds in essential oil extracted from Eucalyptus leaves

Antimicrobial Activity of Crude Extracts and Essential Oil from Eucalyptus Leaves by Agar Well Diffusion Method

Screening of antimicrobial activity of various crude extracts such as PE, EtOAc, EtOH, watery and essential oil from Eucalyptus leaves sample was investigated by employing agar well diffusion method. In this study, the samples were tested on six species of microorganisms such as *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas fluorecscens*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli* species. The inhibition zone diameter shows the degree of the antimicrobial activity. The larger the inhibition zone diameters, the higher the antimicrobial activity. The resultant inhibition zone diameters are described in Table 2, Figure 9 and 10.

From these results, it was found that essential oil from Eucalyptus leaves showed the most pronounced antimicrobial activity against six microorganisms: *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas fluorecscens*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli* with the inhibition zone diameter ranged between 19 mm - 22 mm. In addition, PE extract (inhibition zone diameter 16 mm to 21 mm), EtOAc extract (inhibition zone diameter 15 mm to 22 mm) and EtOH extract (inhibition zone diameter 20 mm to 24 mm) H_2O extract (inhibition zone diameter 20 mm to 27 mm) exhibited antimicrobial activity against all six species of microorganisms tested.



Bacillus pumilus ***Bacillus subtilis*** ***Candida albicans*** ***Escherichia coli*** ***Pseudomonus fluorescence*** ***S. aureus***

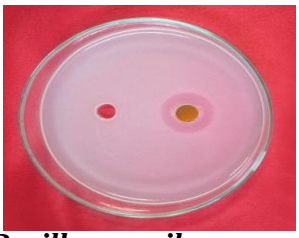
Figure 9. Inhibition zones of various crude extracts (PE, EtOAc, EtOH, H_2O) from Eucalyptus leaves against six microorganisms

Table 2. Antimicrobial Activity of Various Crude Extracts and Essential Oil from Eucalyptus Leaves

No	Microorganisms	Inhibition Zone Diameter (mm)				
		I	II	III	IV	V
1	*Bacillus pumilus*	21.12 (+++)	15.50 (++)	22.87 (+++)	25.93 (+++)	20.57 (+++)
2	*Bacillus subtilis*	20.73 (+++)	22.25 (+++)	20.34 (+++)	21.73 (+++)	20.43 (+++)
3	*Candida albicans*	19.88 (+++)	16.12 (++)	21.18 (+++)	27.57 (+++)	20.63 (+++)
4	*Escherichia coli*	20.33 (+++)	20.61 (+++)	20.08 (++)	20.45 (+++)	22.16 (+++)
5	*Pseudomonus fluorescens*	17.61 (++)	20.35 (+++)	20.16 (+++)	22.68 (+++)	19.87 (+++)
6	*Staphylococcus aureus*	16.78 (++)	19.76 (+++)	24.67 (++)	26.71 (+++)	20.51 (+++)

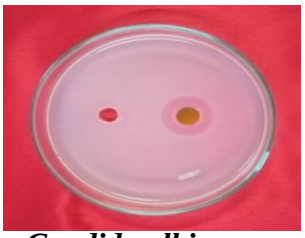
Agar well – 8mm
8mm ~ 12 mm (+)
13 mm ~ 17 mm (++)
18mm ~ above (+++)

I = PE extract of Eucalyptus leaves
II = EtOAc extract of Eucalyptus leaves
III = EtOH extract of Eucalyptus leaves
IV = Watery extract of Eucalyptus leaves
V = Essential oil from Eucalyptus leaves

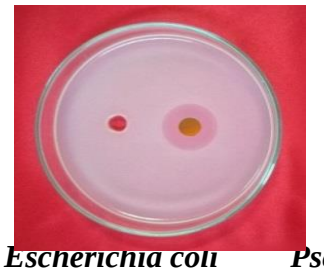


Bacillus pumilus

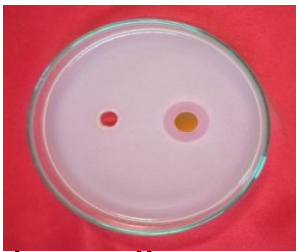
Bacillus subtilis



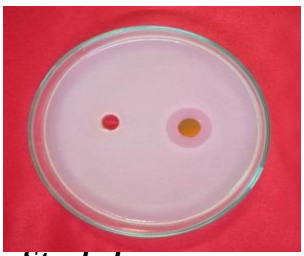
Candida albicans



Escherichia coli



Pseudomonus fluorescence



Staphylococcus aureus

Figure 10. Inhibition zones of essential oil from Eucalyptus leaves against six microorganisms

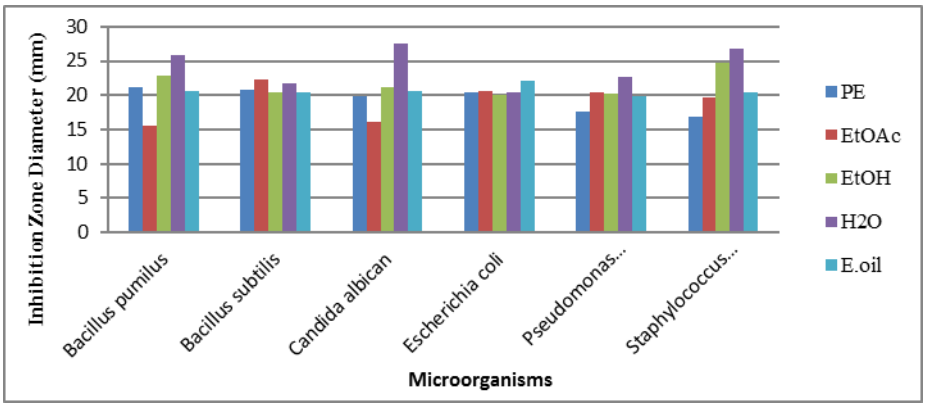


Figure 11. A bar graph of microbial inhibition zone diameters for various crude extracts and essential oil from Eucalyptus leaves against six microorganisms

Conclusion

From the overall assessment of the present work the following inferences could be deduced. *Eucalyptus citriodora* Hook. is a tall evergreen tree belonging to Myrtaceae. In this research, the preliminary phytochemical investigation of Eucalyptus indicated the presence of alkaloids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, terpenoids, steroids, α-amino acids, tannins and organic acids. But starch was absent in the sample. Relative composition of elements in *Eucalyptus citriodora* Hook. (Eucalyptus leaves) was determined by EDXRF spectrometer applying fundamental parameter method. It was found that there were contained Ca, K, S, Fe, Mn, Cu, Ti, Zn and Br in the sample. Among these elements, the calcium content was 0.671 % as major constituents in Eucalyptus leaves. The essential oil was extracted from Eucalyptus leaves by Hydro-distillation method. The yield percent of essential oil from Eucalyptus leaves was found to be 0.4 % based on the powder sample. Furthermore, three compounds: 5-ethoxy-6-methoxy-8-nitroquinoline, 2-propanone-1- [(3-hydroxy-6-methyl-2-pyridinyl) thio] and 1,3,2- Dioxaphosphorinane, 4-(hydroxymethyl)-2-oxo-2 phenoxy in essential oil of eucalyptus leaves were detected by GC-MS method. The antimicrobial activities of various crude extracts (PE, EtOAc, EtOH, and H_2O) and essential oil from Eucalyptus leaves were investigated by agar well diffusion method against six microorganisms: *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli*. In addition, PE, EtOAc, EtOH and H_2O extracts of Eucalyptus leaves also exhibited the higher antimicrobial activity with inhibition zone diameter between 15 mm – 27 mm against six microorganisms tested. Screening of antimicrobial activity showed that essential oil of Eucalyptus leaves was found to possess pronounced antimicrobial activity with inhibition zone diameters ranged between 19 mm – 22mm. Thus, the present work will contribute to scientific development of Myanmar traditional medicine formulation, especially for the treatment of diseases related to bacterial infections.

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References

Abd EI Mageed, A.A, Osma and A.K, Taw fik, A.Q, Mohammed H.A. (2011), "Chemical composition of essential oil of four Eucalyptus species (Myrtaceae) from Egypt". *Res.J. Phptochem.,* **5** (2): 115-122

Aye Hsu Khaing. (2018). "Study on extraction of essential oil from *Syzygium aromaticum* L. (Clove) and its antimicrobial activity", MSc (THESIS), Department of Chemistry, Kyaukse University

Berman, J. and P.E. Sudbery. (2002). "*Candida albicans* of Molecular Revolution Built on Lessons from Budding Yeast", *Nat. Rev. Genet*., **3**(12), 918-922

Dorman, H.J. and S.G. Deans. (2000). "Antimicrobial Agents from Plants and Antibacterial Activity of Plants Volatile Oils", *J., Applied microbial*, **88**, 308-316

Fingold, S.M. and W.J. Martin. (1978). "Diagnostic Microbilogy", The C.V. Mosby Co. Ltd., London, 11-12

Gershenzon, J. and N. Dudareva. (2007). "The Function of Terpenes Natural Products in the World", *Nature Chemical Biology*, **3**, 408-410

Gocho, S. (1991). "Antibacterial action of Aroma Compounds in Vapor State", *Journal of Antibacterial and Antifungal Agents*, **19**, 329-330

James, A.T. and A.J.P. Martin. (1952). "Gas-liquid Partition Chromatography; the Separation and Micro-Estimationof Volatile Fatty Acids from Formic Acid to Dodecanoic Acid", *Biochem. J.,* **50**, 679-680

Janssen, A.M., J.C. Scheffer and S.A. Baerheim. (1987). "Antimicrobial activity of Essential Oils", *Planta Medica*, **53**, 395-397

Karama, M. and P.V. Rossit. (2003). "Enter Oxytoxin Production by *Staphylococcus aureus* Isolated from Mastitic Cows", *Journal of Food Protection*, **66**, 169-172

Kipping, F.S. and F.B. Kipping. (1951). "Organic Chemistry", W & B Chambers Ltd., London, 323-325

Kirk, P.M., P.F. Cannon, D.W. Minter and J.A. Stapers. (2008). "Dictionary of the Fungi", 10th Edn., Bios Scientific Publishers Ltd., 452-453

Mander, Q., A. Hasseun, and Z. Isa. (2003). "Antimicrobial Activity of Certain Spice Extracts", *J. Spice Aromatic Crops.*, **122,** 146-453

Marini, B.G.B., M. Nicolettic and M. Patamia. (1981). "Plant Screening by Chemistry and Chromatographic Procedure Under Field Condition", *J. Chromato*., **121**, 213-214

Nakano, M.M. and P. Zuber. (1998). "Anaerobic Growth of a Strict Aerobe (*Bacillus subtilis*)", *Annu. Rev. Microbial*, **52**, 165-190

Paduch, R. and M. Trgtek. (2007). "Terpenes are Useful in Human Healthcare", *Archivum Immunologic*, **55**, 315-327

Parvathi, A. (2009). "Biochemical and Molecular Choracterization of *Bacillus pumilus*Isolated from Coastal Environment in Cochin", India., *Braz. J. Microbial*, **40**. 269-271

Perez, C., M. Pauli and P. Bazerque. (1990). "An Antibacterial Assay by Agar Well Diffusion Method", Acta. Bio. Et. Med. Exp., **15**, 113-115

Robinson, T. (1983). "The Organic Constituents of Higher Plant", 5th Ed., Wrdus Press, North Amberst, 285-286

Saeed, S. and P. Tariq. (2008). "*In Vitro* Antibacterial Activity of Clove against Gram-Negative Bacteria", *Pakistan. J. Botany*, **40**(5), 2157-2160

Schnaubelt, K. (1999). "The Science of Essential Oil of Therapy", Healing Arts Press, **5**, 112-115

Shriner, R.L. and R.C. Fuson. (1980). *The Systematic Identification of Organic Compounds, A Laboratory Manual*, John Wiley and Sons, New York, 385-425

Smith-Palmer, A. and J. Stewart. (1998). "Antimicrobial Properties of Plant Essential Oils and Essences against Five Important Food-Borne Pathogens", *Applied Microbial*., **26**, 118-112

Srivastava, A.K., S.K. Srivastava and K.V. Syamsudar. (2003). "Bud and Leaf Essential Oil Composition of *Syzygiumaromaticum*from India and Madagascar", *Flav. Fra.J*., **20**(1), 51-53

Tin-Wa, M. (1972). "Phytochemical Screening Methods and Procedure", Phytochemical Bulletin of Botanical Society of America Inc., **5**(3), 4-10

Zheng, G.Q., P.M. Kenney and L.K. Lam. (1992). "Sesquiterpenes from Clove *(Eugenia caryophyllata*) as Potential Anticarcinogenic Agents", *J. Nat. Prod*., **55**, 999-1001