WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 3, Issue 5, 970-980. Research Article ISSN 2277 – 7105

EXTRACTION AND THIN LAYER CHROMATOGRAPHY OF PIGMENT FROM THREE VARIETY OF EUCALYPTUS AND ANTIMICROBIAL EFFECT OF ESSENTIAL OIL

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Article Received on
22 May 2014,

Revised on 16 June 2014,
Accepted on 10 July 2014

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ABSTRACT

The genus Eucalyptus (Family- Myrtaceae) comprises well-known plants of over 600 species of trees. In all parts of the world, Eucalyptus leaves, bark, wood and Essential oils are utilized on a large scale. Eucalyptus essential oils could be grouped into three types- Medicinal, Industrial and Perfumery. The essential oils were highly used in the Aromatherapy and Antimicrobial activities. Eucalyptus essential oil compositions from various countries have been extensively investigated due to their numerous uses in the Pharmaceutical and Cosmetics Industries. The Essential oils isolated by the steam distillation using Clevenger-type apparatus from the leaves of the three Eucalyptus species- E. saligna, E.tetragona and E.terticornis. Antimicrobial activity of Eucalyptus sp. Essential oil againts pathogenic fungi and bacteria. Was evaluated following agar disc diffusion and poisoned food technique procedure both qualitatively and quantitatively. The essential oil of eucalyptus was found active toward gram-positive bacteria compared to gram –negative and showed activity towards drug resistans mutants of A.tunifuciens, Staphylococcus oureus, A. niger, C. lunata Eucalyptus oil may provide avaluable antimicrobial agent for microorganism

KEY WORDS: Extraction oil, Antimicrobial Activity, Eucalyptus, T.L.C.

INTRODUCTION

Eucalyptus is a tall evergreen tree. It attains the height of more than 300 feet. Leaves of the tree on juvenile shoots are opposite, sessile, cordate-ovate and covered with a bluish white bloom. The adult leaves are alternate, lanceolate and are 6-12 inches long and 1-2 inches

broad. Flowers are cream in colour. Eucalyptus belongs to the family Myrtaceae. The flowers tend to be groups into inflorescences. The flowers form distinctive fruits with a ribbed receptacle often topped by the remains of valves from the upper part of the ovary. Bark varies from ribbed to the smooth and can be distinctly deciduous - peeling off to leaving new bark of a strongly contrasting colour as is the case of E. Sp.. In contrast, the bark may be persistent and sometimes very hard as in the group of ironbark species, or it may be fibrous and deeply furrowed. The leaves are also variable in both shape and colour, but generally narrow and long in relation to their breadth i.e. lanceolate. They are positioned alternatively on the branchlets.

Eucalyptus is known for its use either as an essential oil or leaf tea for its ability to relieve congestion and ease breathing in colds. Its oil is also used as the pain reliever for sore and overextended muscles. The essential oil of Eucalyptus contains cineole, a potent antiseptic that helps in killing the bacteria and fungi. It helps in increasing cardiac action. It is taken in all types of fever. It helps in purifying the blood. It lowers the blood sugar. It brings relief to the patients of Asthma and bronchitis. It is the excellent topical remedy for aching joints and rheumatism. It helps in improving the blood circulation

Eucalyptus is used as the pulpwood in the manufacture of the paper as well as raw material. It is used as the poles for the construction of huts and houses. It is used in making plywood, doors and windows (www.ecoindia.com/flora/trees/Eucalyptus-tree.html).The genus Eucalyptus has provided foresters and farmers with a valuable resource of fast growing species able to grow under a wide range of conditions depending on the particular species being used. All Eucalypts are good for fuel wood and pole production. Their suitability for timber is somewhat mixed and depends on the species. One of the drawbacks of their rapid growth is the build up of stresses within the tree leading to distortion after the tree has been felled and sawn (Boland et al., 2003). Eucalypts have an important role in farm forestry (Brooker et al., 2000).

1. E. saligna.

These species are very similar botanically and in performance and growing requirements and consequently are frequently mixed up. They are suited to sites above 900 m in altitude where there are no excessive periods of drought, however within Africa these species can tolerate a dry season of 6-7 months, providing rainfall in the rest of the year is reliable and in excess of 1,250 mm. They are not tolerant of shallow soils and require moderately fertile land if they

are going to reach their optimum production levels. Both species coppice well and provide good poles and fuel wood; the wood can be sawn to provide acceptable construction timber if carefully seasoned and in Australia is regarded as acceptable for house building

2 .E. tereticornis

This species is more drought tolerant than E.grandis and can be used in semi-arid environment s where rainfall is down to 600 mm; it will also tolerate a long dry season. Unlike E. grandis and E. Sp., it is associated with lower altitudes and can be used from close to sea level up to 1,000 m. It is also tolerant of relatively poor sites in low rainfall areas (<600mm) it prefers alluvial flats subject to flooding. In areas of higher rainfall it grows on the lower slopes of hillsides and extends to mountain slopes and hillsides. Soils include rich alluvials, sandy or gravelly loams and seasonally waterlogged clays in forested wetlands. When planted as an exotic E. tereticornis appears to grow best on well-drained, fairly light-textured soils in areas receiving an annual rainfall of over 800 mm. E. tereticornis produces a hard heavy red timber with an S.G. of 0.8 -1.0 and is excellent at coppicing

3 . E. tetragona

The leaves on a mature Eucalyptus plant are commonly lanceolate, petiolate, apparently alternate and waxy or glossy green. In contrast, the leaves of seedlings are often opposite, sessile and glaucous. Four leaf phases are recognized in the development of a Eucalyptus plant: the 'seedling', 'juvenile', 'intermediate' and 'adult' phases. However there is no definite transitional point between the phases. The intermediate phase, when the largest leaves are often formed, links the juvenile and adult phases.The aim of present study was to investigate the Extraction and Thin Layer Chromatography of Pigment from three variety of Eucalyptus and Antimicrobial effect of Essential oil

Material and Method

Plant Material

Leaves from three different Eucalyptus species were collected from Jabalpur and its nearby areas in January, 2014.

Ex.1 Extraction of essential oil

Freshly collected 100 gm leaves were weighed. The leaves were shade dried and were subjected to hydrodistilled for three hours for complete extraction of essential oil, using a commercial Clevenger-type apparatus. The oil samples obtained from hydrodistillation were

freed from moisture by adding anhydrous sodium sulfate and absolute oil samples were obtained

Ex.2 Pigment extraction from leaves of Eucalyptus

Sample Preparation: (McLaughlin and Masters, 2004)

0.5 g of fresh Eucalyptus leaves was weighed**.** To this was added 0.5 g of anhydrous magnesium sulphate and 1 g of sand. Using a mortar and pestle, the mixture was grinded until it becomes fine, light green powder. This was transferred into a test tube and 2 ml of acetone was thereby added. The solution was stirred using a stir bar for 2 minutes. The mixture was allowed to settle for 10 minutes.The solid settled to the bottom, leaving a green liquid layer on top. Centrifugation was done at 5000 rpm for 30 minutes. The green layer was transferred to another test tube using a pipette.

TLC profiling of pigments present in Eucalyptus leaves

Preparation of TLC plate

(Wall, 2002; Girigowda and Mulimani, 2005)

The TLC plate was prepared by suspending 30 gm of Silica Gel- G powder in 45 ml of distilled water; the slurry was poured on to the glass plate to 0.2 mm thickness. The plates were then air-dried. Activation of the TLC plates was done at 100˚C for 1 hour.

Loading the Sample

3-5 µl of sample was spotted with a Hamilton Syringe on pre-activated TLC plates.

Solvent System for TLC (Chemicals: CDH, India) Pigments (McLaughlin and Masters, 2004)

Petroleum ether: cyclohexane: Ethyl acetate: Acetone: Methanol (6:1.6:1:1:0.4)

Preparation of the Chromatography Tank (PERFIT, India)

1. 100 ml of solvent system was added to a glass chromatography tank (25 cm x 27 cm x 10 cm) and was covered tightly applying grease.
2. Tank was equilibrated with solvent vapours for at least 2 hours.

Chromatography of the Silica Gel Plate

1. Using a pair of long forceps, the top of the plate was grasped and placed in the tank oriented with the spotted sample just above the level of the solvent.
2. The tank was tightly covered and the plate was allowed to remain undisturbed until the ascending solvent line reaches the top of the plate (approximately 30-45 min.).

3. The TLC plate was removed by grasping the top edge with forceps and was allowed to thoroughly air dry under chemical fume hood.

Determination of R_f values

R_f values of different amino acids separated as different spots were determined by measuring the movement of solvent and movement of solute molecules with a ruler. Movement of solute divided by movement of solvent gives the R_f value.

$$R_f = \frac{\text{Distance travelled by the Solute}}{\text{Distance traveled by the Solvent}}$$

Ex.3 Antimicrobial activity.

Preparation of the culture media

Nutrient agar media (Agrawal and Hasija, 1986) Nutrient broth (Agrawal and Hasija, 1986), Potato dextrose agar were used through the study.

Micro-organisms used

All bacteria and fungi mentioned below were obtained from Biodiversity Conservation and Rural Biotechnology Center Jabalpur.

Bacteria

1. Agrobacterium tunifaciens
2. Agrobacterium rhizogene
3. Xanthomonas sps.
4. Staphylococcus aureus
5. Pseudomonas aeruginosa
6. Salmonella typhii

A. Fungi

1. Aspergillus niger
2. Aspergillus flavus
3. Penicillium Sp.
4. Alternaria alternata
5. Fusarium oxysporum
6. Curvularia lunata
7. Malassaziz furfur

Antimicrobial activity

The essential oils extracted from Eucalyptus robusta, Eucalyptus tereticornis and Eucalyptus globulus were tested against various bacteria and fungi using following method

A. Filter paper disc diffusion method

Antimicrobial activity of essential plant extract was carried out following the filter paper disc diffusion technique (Vincent & Vincent, 1944). Antimicrobial properties of different oils were evaluated in pure form as well as in different dilutions. Dilution of the extracts was made by dissolving it into the solvents which were used for extraction purpose or by mixing with distilled water so as to get the final ratio. A small amount (l ml) of 18 hrs.old suspension of each bacterium was then separately added to Erlenmeyer flasks containing 100 ml sterilized and cooled (40°C) nutrient agar medium (NAM). Flasks were gently shaken to mix bacterial cells in the medium. Aliquots of 20ml seeded medium were poured in each sterile Petriplates. Sterile filter paper discs (5mm diameter) each impregnated with different dilution of each essentials oil were placed at equidistance on upper surface of seeded agar medium. The plates were left for 30min at room temperature. Antibiotic disc Gentamycin sulfates (40mg/ml) were used as a positive control, while discs soaked in respective solvent were used as a blank control. The zone of inhibition formed by each extract in different dilution and controls was recorded after 24 hour of inhibition at 35 ± 2°C for bacteria.

B. Poisoned food technique

The method of Grover and Moore (1962) was adopted to evaluate the effect of essential plant extract on the growth of microorganism. 20ml of sterilized and cooled growth media (PDA) with 10mg streptomycin were poured into pre sterilized Petri plates. Requisite amount of different concentration of plant extract like 100, 300, 500, 1000 mg was added into the plates. The assay plates were rotated carefully to ensure an even distribution of sterilized distilled water to compensate the volume instead of plant extract. After the solidification of the agar medium, inoculums of the 7 day old culture with the help of sterile cork borer) was placed aseptically in the centre of each Petri plates of treated and control sets. The assay plates were than incubated at 26 ± 2°C for 6 days. After the desire period of incubation diameter of the fungal colony of treated as well as control sets were measured. The experiment was conducted in the multiples of 3 triplicates.

The percentage of mycelial inhibition was calculated/ computated by mean value of colony diameter by the following formula:

$$\text{Percentage of mycelial inhibition} = \frac{dc - dt}{dc} X\ 100$$

dc - average diameter of fungal colony in control sets

dt - average diameter of fungal colony in treated sets

RESULTS AND DISCUSSION

Partial characterization of Eucalyptus leaves extract

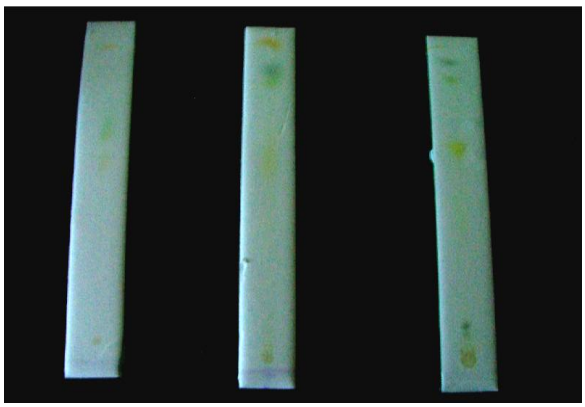
The TLC plate of the leaf extracts reveals – pigment lines.

Table 1: R_f values of Different fractions from leaf extracts of E. Sp., E. tereticornis and E. tetragona. (Bottom to top)

Serial Number	Name of Pigments	R_f values			Colour
		E. saligna	E. terticornis	E. tetragona	
1	Lutein	0.29	0.09	0.07	Light Yellow bands
2	Chlorophyll a	0.42	0.54	0.50	Dark Green bands
3	Pheophytin a	0.60	-	0.81	Light Yellow bands
4	Xanthophyll	-	0.15	0.11	Dark Yellow bands
5	Oil	0.73	0.81	0.87	Greyish spot
6	Chlorophyll b	-	-	0.38	Green Band
7	β- carotene	0.78	0.91	0.93	Dark yellow band

The literature reports the following R_f values for each component (Stahl, 1965)

S.No.	R_f values	Pigment
1.	0.16	Xanthophylls
2.	0.32	Chlorophyll-b
3.	0.44	Chlorophyll-a
	0.95	β- carotenes
5.	0.61	Pheophytin –a
6.	0.49	Pheophytin- b



TLC Pate of the leaves extract A typical separation of dyes in spinach shows the presence of (bottom to top) – Lutein, Chlorophyll- a, Chlorophyll-b, Pheophytin-b, Pheophytin-a and β-

carotenes.Remote sensing of chl a, chl b, chl a + chl b and total carotenoid content in Eucalyptus leaves- characterization and applications is being effectively done by Datt (1998). Usman et al., (2009) investigated retrieval of foliar information about plant pigment systems from high resolution spectroscopy.

Similarly, McLaughlin and Masters (2004) carried out experiments that focus on extraction and thin layer chromatography of chlorophyll a and B from spinach. Several solvent systems were tries before the five solvent system was finally employed. Petroleum ether (6 ml), Cyclohexane (1.6 ml), Ethyl acetate (1 ml), Acetone (1 ml) and Methanol (0.4 ml) comprised of the mobile phase yielding 5 pigment lines depicting five pigments (Bottom to top) viz., Lutein, chlorophyll b, chlorophyll a, Pheophytin a and β-carotene. TLC analysis of spinach extract reveals four pigment lines. From the bottom of the plate up , a yellow line for xanthophylls was observed , a green line for chlorophyll-b was observed a brighter green line was visible which corresponds to chlorophyll – a and for β- carotenes a yellow line was observed.

Antibacterial activity of Eucalyptus sp. leaves extract

The study showed that the solvent extracts investigated were active against various Gram positive and Gram negative bacteria .Acetone extract of Eucalyptus Sp. leaves exhibited stronger antimicrobial activity in comparison with Alcoholic extract. Among the Gram negative group of bacteria, acetone extract of the test plant inhibited the growth of A. tunifaciens to the maximum, followed by inhibition zones of A.rhizogene and Xanthomonas sp. Intermediate zone of inhibition was observed for Salmonella typhi while minimum zone of inhibition i.e. minimum antibacterial activity was reported with Pseudomonas aeruginosa and Staphylococcus aureus. The inhibition zones obtained were intermediate between those obtained for Gram negative bacteria and Gram positive bacteria. Alcoholic extract of Eucalyptus sp. leaves showed comparatively less antibacterial activity then the acetone extract. Similarly, among the gram negative, group of bacteria was observed on A. tunifaciens followed by A.rhizogene and Xanthomonas sp. showes maxmium effect. Mild antibacterial activity was observed with salmonella typhii. Less zone of inhibition were observed for Pseudomonas aeruginosa and Staphylococcus aureus. Good inhibition zones were observed for bacteria Eucalyptus Sp. leaves extract.

Table. 2 Antibacterial activity of Eucalyptus sp. Leaves Extract.

S. No.	Test Organisms	Zone of inhibition (Eucalyptus leaves extract)		
		Leave Alcoholic	Acetone	Genta
1	A. tunifaciens	18.2±0.33	19.6±0.12	38±0.50
2	A.rhizogene	14.8±0.33	14.1±0.16	33±0.04
3	Staphylococcus aureus	15.1±0.20	15.0±0.00	42±0.00
4	Xanthomonas sp.	14.1±0.11	15.8±0.12	40±0.11
5	salmonella typhii	13.2±0.25	14.6±0.12	36±0.02
6	Pseudomonas aeruginosa	8.0±0.20	3.2±0.12	36±0.04

Antifungal activity of Eucalyptus Sp. sp. leaves extract after different incubation period

As shown in methanolic extract of Eucalyptus Sp. leaves was subjected to antifungal activity with different fungal strains at different incubation periods. Maximum percentage inhibition was observed with 7 Days of incubation viz. Curvularia lunata, Alternaria alternata, Aspergillus niger and Malassazia furfur While maximum percentage inhibition at 3 days of incubation period was observed in case of Fusarium oxysporum followed by Penicillium sp. and Aspergillus flavus. Mild percentage inhibition was observed at 5 days of incubation.

Acetone extract of Eucalyptus Sp. leaves was subjected to antifungal activity with different fungal strains, at different incubation periods. Maximum percentage inhibition was observed with Malassazia furfur Aspergillus niger, Curvularia lunata and Fusarium oxysporum at 7 days of incubation. While maximum percentage of inhibition at 3 days of incubation was observed in case of Alternaria alternata followed by Curvularia lunata, Malassazia furfur and Aspergillus niger. Mild percentage of inhibition was observed at 5 days of incubation. Similarly mixture of methanolic and acetone extract of Eucalyptus Sp. leaves was also subjected to antifungal activity with different fungal strains, at different incubation periods. Maximum percentage inhibition was reported with Aspergillus niger followed by Aspergillus flavus, Malassazia furfur and Curvularia lunata at 7 days of incubation. While Maximum percentage inhibition at 5 days of incubation was recorded with Aspergillus niger, followed by Malassazia furfur Curvularia lunata and Fusarium oxysporum. As we have seen in earlier study maximum percentage inhibition was observed at 3 days of incubation. But in case of mixture of methanolic and acetone extract of Eucalyptus Sp. leaves there was moderate percentage inhibition recorded at 3 days of incubation.

Table 2: Antifungal activity of Eucalyptus sp. leaves extract

Plant Leaves extract (Pure)	Days	A. niger	A. flavus	Penicillium sp.	F.oxysporum	A. alternata	C. lunata	malassazia furfur
Methanolic extract	3	52.3±0.33	30±0.30	58.8±0.25	60.1±0.52	52.7±0.21	63.1±0.34	50.2±0.03
	5	30.3±0.33	51.2±0.31	62.0±0.35	67.2±0.50	49.9±0.25	57.1±0.34	30.1±0.03
	7	22±0.31	55±0.26	66.2±0.32	70.1±0.26	50.6±0.30	50.2±0.30	20.8±0.25
Acetone extract	3	80±0.40	63.7±0.30	69.8±0.28	70±0.30	52.8±0.28	74.3±0.30	90.7±0.25
	5	76±0.40	66.0±0.31	62.5±0.20	62.4±0.25	72.5±0.25	64.6±0.25	80.3±0.25
	7	69±0.31	57.8±0.30	68.2±0.25	67.7±0.22	78.1±0.25	77.9±0.24	72.8±0.20
Mixture of Methanolic and Acetone	3	100±0.34	100±0.36	91.6±0.28	100±0.30	94±0.22	95.4±0.19	100±0.03
	5	98.1±0.30	89±0.25	82.5±0.25	91.6±0.29	81.3±0.23	90.2±0.11	96±0.14
	7	90.5±0.31	80.1±0.30	82±0.30	80.3±0.33	78.1±0.23	89.2±0.10	91.8±0.11

SUMMARY AND CONCLUSION

The present study was conducted to obtain preliminary information on the antimicrobial activity of essential oil of Eucalyptus Sp. The disc diffusion and poisoned food method was applied to be used in this study. In our investigation, highest zones of inhibition and food poision were found in powder from fresh leave extract against all the bacteria & Fungi tested which was more than one and a half to twice as much effective as known antibiotic tetracycline (30 µg /disc) while fresh Leaves extract showed relatively higher inhibitory potency on all the tested bacteria and fungi this trend was also phenomenal against all the Micropes employed except These consequences suggest that leaves contain bio-components whose antimicrobial potentials are highly comparable with that of the antibiotic tetracycline against all Gram-negative and Gram-positive bacteria & Fungi tested. The activity of the plant against both Gram-positive and Gramnegative bacteria may be indicative of the presence of broad-spectrum antibiotic compounds in the plant.

The Extraction of pigments of the leaves of the three species of Eucalyptus was performed by Thin Layer chromatography (TLC) technique using Petroleum ether: Cyclohexane: Ethyl acetate: Acetone: Methanol (6:1:6:1:1:0.4). TLC technique was also performed for the

essential oils extracted from the leaves of three Eucalyptus species and their R_f values were determined. The main pigments identified were Chlorophyll a, Chlorophyll b, Carotenes, Pheophytin b and Lutein.

ACKNOWLEDGEMENT

The Authors acknowledgment to Director and Research Team of Research Institute, Biodiversity Conservation and Rural Biotechnology Center Jabalpur for Providing Research facilities.

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