Phytochemical and Thin Layer Chromatographic analysis of chloroform and methanol extracts of *Azadirachta indica* and *Eucalyptus globulus* leaves

ABSTRACT

The study focuses on phytochemical screening of *Azadirachta indica* and *Eucalyptus globulus* leaf extracts in chloroform and methanol solvents. The antibacterial compounds found in both leaf extracts of *Azadirachta indica* and *Eucalyptus globulus* plants were investigated using phytochemical studies. The extracts contained flavonoids, terpenoids, tannins, alkaloids, saponins, and phenolic chemicals, according to preliminary phytochemical screening. The solvent systems of hexane: ethyl acetate (1:1) and toluene: ethyl acetate (97:3) yielded the most chemicals from chloroform and methanolic extracts of *A. indica* and *E. globulus* plants, respectively. On TLC plates, these chemicals were separated, resulting in the discovery of different spots in both leaf extracts. The Rf values of chloroform extract of *A. indica* run under Hexane: Ethyl acetate (1:1) solvent system was 0.05, 0.11, 0.52, 0.58, 0.74, 0.82, 0.88, and 0.94, respectively, while Rf values of methanol leaf extract of *E. globulus* run under Toluene: Ethyl acetate (97:3) solvent system was 0.03, 0.07, 0.12, 0.25, 0.37, 0.43, 0.56, 0.62, 0.75, 0.81, 0.87, 0.88 and 0.94 respectively. The results of the investigation will be used to confirm the proper identification of *A. indica* and *E. globulus* crude plant extracts. The optimum solvents for extracting antibacterial components from *A. indica* and *E. globulus* leaves were chloroform and methanol.

Key words: *Azadirachta indica*, *Eucalyptus globulus*, Phytochemical analysis, Extraction, TLC, compounds, chloroform, methanol extract.

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1. Introduction

The finest source for a variety of newer herbal antibacterial chemicals is to be from different medicinal plants. These plants have provided civilization with useful, and occasionally lifesaving, pharmaceuticals for many generations. When a link between chemical structure and biological activity was discovered in modern agriculture, empirical research gave way to rational antimicrobial design. Because of the conceptual cooperation of chemistry and biology, this novel technique to identify and develop possible new fungicides is largely effective. As a result, such plants should be explored further to discover more about their properties, safety, and efficacy. Plant-derived fungicides have long been utilized in agriculture.

Eucalyptus is one of the most frequently planted genera on the planet of Myrtaceae family (Batish et al., 2008). The Tasmanian Blue Gum, *Eucalyptus globulus*, is a fast-growing, evergreen tree with hanging leaves that is native to Tasmania and southeast Australia (Oyedeji et al., 1999). The genus *Eucalyptus* contains a greater number of secondary metabolites such as terpenoids, flavonoids, tannins, alkaloids, monoterpenes, cyanogenic glycosides, and related polyphenols etc. (Brophy, 2002, Takahashi et al, 2004 and Santos et al, 2011). *Eucalyptus globulus* is a popular medicinal plant in India which have been utilized for medicinal purposes, including as the treatment of various ailments with its leaves, barks, and fruits. (Mei, 2005; Mei, 2005).

Azadirachta indica, a Meliaceae family tree, is well-known for bearing a varied source of bioactive compounds. Various chemical substances, including diterpenoids, triterpenoids, polyphenolics, tannins, and alkaloids have been isolated from various portions of this tree to date (Randhawa et al, 1993 and Prakash et al. 2004). With more than 140 chemicals extracted from various areas of the tree, neem produces a wide range of biologically active compounds that are chemically diverse and structurally changeable (Subapriya and Nagini 2005). For the control of plant diseases, many compounds have been produced. However, as people become more aware of the harmful side effects of these chemicals, greater emphasis is being placed on the use of biocontrol agents. In the study of plant pathology, there is currently a huge difficulty in introducing some environmentally acceptable and safe alternative control tactics for agriculture, which has encouraged researchers to focus on plants and microorganisms as a biocontrol agent (Choudhary et al., 2017). Neem has already risen to the top of the list of plants with the greatest promise as a source of biocontrol agents.

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Pharmacological studies have also established the importance of medicinal plants as a source of bioactive compounds. These bioactive chemicals are generally stored in all plant cells as secondary metabolites, although their concentration varies depending on the plant part, season, climate, and growth phase (Maji, et al., 2010). Scientists have now devised techniques to extract natural materials with desired biological activity and employ them against phytopathogenic microorganisms. Many plant-based therapies are utilized to treat various plant diseases in India, but little research has been conducted to provide the scientific validation of these plants' usefulness in the agrochemical business. The aim of this paper is to study the phytochemicals and partial characterization of a bioactive antimicrobial compounds from chloroform and methanol extract of *Azadirachta indica* and *Eucalyptus globulus* plants. In present work, novel antibacterial compounds are identified from *Azadirachta indica and Eucalyptus globulus* leaf extracts which possess various antibacterial activities against the citrus canker bacterium *Xanthomonas axonopodis* pv. *citri* as compared to the previously work done.

2. Material and methods

2.1 Collection and preparation of plant leaves-

The leaves of neem and nilgiri plants used in this study were collected from the campus of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola city of Maharashtra.

Collected leaves (*Azadirachta indica* and *Eucalyptus globulus*) were thoroughly washed under tap water to remove dust and other impurities. The leaves were dried separately under shade with occasional shifting for about 3 to 4 weeks. The dried leaves were powdered with grinder and stored in airtight container until further use (Rajinder *et al*. 2015).

2.2 Extraction of leaves using different solvents: -

For nilgiri leaf extracts, methanol, ethyl acetate, chloroform, and sterilized distilled water were utilized as solvents. The extraction was done using Soxhlet's apparatus. The 250 ml solvent was added to a round bottom flask, which is attached to a Soxhlet extractor and condenser on heating mantle. The powder (50g) of dried leaves was loaded into the thimble, which is placed inside the Soxhlet extractor. The solvent was heated using the heating mantle and began to evaporate, moving through the apparatus to the condenser. The condensate then

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drips into the reservoir containing the thimble. Once the level of solvent reaches the siphon it pours back into the flask and the cycle begins again. This process is run for a total of 16 hours.

The supernatant from flask was filtered separately through Whatman No. 1 filter paper and then was evaporated completely at 28-30°C. Air dried extracts were weighed separately and transferred into small vials and kept in refrigerator at 5˚C until further use. The percentage of extraction yield was calculated by using following formula (Khan *et* al. 2010).

Extraction yield % = Weight of extract/ Weight of ground floral material x 100

The resultant crude extracts were used for phytochemical analysis, growth inhibition assay against *Xanthomonas axonopodis* pv. *citri* and for chromatographic analysis.

2.3 Preliminary phytochemical screening of plant extract

Preliminary phytochemical analysis of nilgiri and neem crude leaf extract were performed for analysis of different phytochemicals like cardiac glycosides, saponins, fixed oils and fats, alkaloids, steroids, flavonoids, tannins, and phenolic compounds by following method given by Prashanth and Krishnaiah (2014).

i) Test for cardiac glycosides:

Take 2 ml of test solution, then 3 ml of glacial acetic acid and 1 drop of 5 % ferric chloride were added in test tube. Carefully take 0.5 ml of concentrated sulphuric acid and add it by the sides of test tube. Formation of brown ring in acetic acid layer indicates the presence of cardiac glycosides.

ii) Test for saponins:

Take 2 ml extract and dilute it with distilled water and shake in a graduated cylinder for 15 minutes. Then formation of layer of foam indicates the presence of saponins.

iii) Test for alkaloids:

To the extract, dilute hydrochloric acid was added, shaken well, and filtered. With the filtrate, few drops of Wagner's reagent were added in a test tube. Formation of reddish-brown precipitate indicates the presence of alkaloids.

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iv) Test for steroids and tri terpenoids:

The extract was treated with chloroform and filtered. The filtrate was added with few drops of concentrated sulphuric acid, shaken, and allowed to stand. If the lower layer turns red, sterol is present. Formation of reddish-brown color indicates the presence of terpenoids.

v) Test for flavonoids:

The extract was treated with few drops of sodium hydroxide solution separately in a test tube. Formation of intense yellow colour, which becomes colourless on addition of few drops of dilute acid indicates the presence of flavonoids.

vi) Test for tannins and phenolic compounds:

A small amount of extract was dissolved in distilled water. To this solution of 2 ml of 5 % ferric chloride solution was added. Formation of blue, green, or violet colour indicates presence of phenolic compounds.

2.4 Thin layer chromatography and Bioautography: -

Based on in vitro growth inhibition assay methanol extract of nilgiri and chloroform extract of neem were selected for chromatographic analysis. This method was used to study the preliminary phytochemicals. Thin layer chromatography for separation of different compounds presents in crude methanol and chloroform extract, bioautography for isolation of active compounds.

2.5 Thin layer chromatography

Thin layer chromatography was carried out to know the chemical profile of nilgiri and neem crude leaf extract.

2.6 Preparation of TLC plates

The TLC plates were prepared as described by Harborne (1998). Briefly, 25 g of silica gel-G (Hi media, Manufactured, India) was mixed with 50 ml of distilled water and the slurry formed was uniformly spread over TLC plates with a thickness of 0.25 mm using the spreader. The plates were allowed to dry at room temperature and heated in an oven at 110˚ C for 1 hr.

2.7 Standardisation of solvent system

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Each sample of the crude extract of nilgiri and neem were diluted in methanol and chloroform solvents. The prepared TLC plates were marked 1 cm from bottom and 10 µl each sample was applied on TLC plates at equal distance with the help of capillary tubes. For separation of maximum bands on TLC plates, different solvent systems were used according to polarity and from that toluene: ethyl acetate (93:7) for nigiri and hexane: ethyl acetate (1:1) for neem were selected as standard solvent system.

List 1 Solvent system used during experiment for *Azadirachta indica* chloroform extract:

Sr. No.	Solvent systems used	Proportion
1	Methanol: Toluene	8:2
2	Toluene: Chloroform: Acetone	45:25:15
3	Hexane: Ethyl acetate	1:1
4	Methanol: Formic acid	1;1
5	Acetic acid: Water	1:10
6	Ethyl acetate: Methanol: Water	81:11:8
7	Ethyl acetate: Methanol	9.5:0.5
8	Toluene: Ethyl acetate	7:3
9	Ethyl acetate: Methanol: Water	78:14:8
10	Ethyl acetate: Acetic acid: Formic acid: Water	10:1.1:1.1:2.7

List 2 Solvent systems used during experiment for *Eucalyptus globulus* methanol extract.

Sr. No.	Solvent systems used	Proportion
1	Toluene: Ethyl acetate	93:7
2	Ethyl acetate: Methanol; Water	81:11:8
3	Chloroform: Methanol	3:2
4	Toluene: Ethyl acetate: Water	7:2:1

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5	Petroleum ether: Toluene: Ethyl acetate	3:1:1
6	Hexane: Ethyl acetate	9:1
7	Petroleum ether: Ethyl acetate: Acetone	7.8:2.2:0.2
8	Hexane: Ethyl acetate	3:1
9	Chloroform: Methanol	8:2

TLC plate was kept in chromatography chamber, containing toluene: ethyl acetate (93:7) for nigiri and hexane: ethyl acetate (1:1) for neem as solvent system and allowed to run until it reaches as 3/4th position. The developed chromatogram on TLC plates was allowed to air dry and observed under visible, UV light (both at 360 nm and 254 nm). The bands were noted and the Rf value (Relative front) of separated bands were calculated by measuring the distance travelled by solute and the solvent. It is given by formula,

Rf value= Distance travelled by solute/ Distance travelled by solvent front

2.8 Isolation of active compounds by Bioautography

The TLC plate was developed in toluene: ethyl acetate (93:7) solvent system for *Eucalyptus globulus* and hexane: ethyl acetate (1:1) for *Azadirachta indica*. All the prepared chromatogram was dried for complete removal of solvent. The bioautography agar overlay method was used to analyze antibacterial component present in *Eucalyptus globulus* (nilgiri) and *Azadirachta indica* (neem) crude extract. The bacteria *Xanthomonas axonopodis* pv. *citri.* Was grown in the nutrient broth. This broth was distributed over a prepared TLC plate. After solidification of the suspension, the TLC-bioautography plate was incubated at 37°C for 24 h. The bioautogram that developed was sprayed with a 1% aqueous solution of 2,3,5-tri phenyl tetrazolium chloride (TTC) and incubated at 37°C for 4 h. Inhibition zones indicated as the white color zone against pink background as the presence of active compounds. Growth inhibition areas were compared with the Rf of the related spots on the reference TLC plates.

The bioactive compounds resulted from bioautography was scraped from TLC plates using preparative TLC. Purified samples were collected in separate eppendorf tube and dissolved in respective solvents. Then it was centrifuged at 10,000 rpm for 10 minutes in

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centrifuge machine. The supernatant was collected in small 2ml eppendorf tubes and were evaporated using vacuum evaporator for complete evaporation of solvent.

3. RESULTS

3.1 Extraction yield of plant leaf extracts

Extraction yield of plant leaves in each solvent were determined as described in "Materials and Methods". Extraction yields of each plant leaves in different solvents are presented in Table 1 and Plate 1.

3.1.1 Extraction yield of *Eucalyptus globulus*

Methanol exhibited (9.54%) maximum extraction from *Eucalyptus globulus* leaves whereas minimum extraction yield was observed in ethyl acetate (7.1%).

3.1.2 Extraction yield of *Azadirachta indica*

Maximum extraction yield (9.08%) of *Azadirachta indica* leaves was reported in distilled water solvent and minimum in petroleum ether (2.9%).

3.2 Preliminary phytochemical analysis

Preliminary phytochemicals present in methanol extract of *Eucalyptus globulus* and chloroform extract of *Azadirachta indica* were analysed by following standard procedure as explained under "Materials and Methods". Observations on presence or absence of phytochemicals namely, cardio glycosides, saponins, fixed oils and fats, alkaloids, steroids, flavonoids, tannins, and phenolic compounds were noted as + sign for presence and - sign for absence and are presented in Table 2.

Results presented in Table 2 revealed that, from all the tested phytochemicals, cardio glycosides, saponins, alkaloids, terpenoids, tannins and phenolic compounds were observed in methanolic extract *Eucalyptus globulus* while steroids and flavonoids were absent. While in case of chloroform extract of *Azadirachta indica* alkaloids, phenolic compounds, cardio glycosides, flavonoids were present and terpenoids, saponins, tannins and steroids were absent.

3.3 Chromatography

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Based on *in vitro* results, methanol extracts of *Eucalyptus globulus* and chloroform extracts of *Azadirachta indica* were selected for further partial purification by chromatographic analysis. All these methanol and chloroform extract of *Eucalyptus globulus* and *Azadirachta indica* were screened for preliminary phytochemical analysis and thin layer chromatography. Observations were recorded for presence or absence of phytochemicals, number and Rf values of bands (compounds) present in extract.

3.3.1 (TLC) Thin Layer Chromatography

Thin layer chromatography was used for separation of different chemical constituents present in methanol extract of *Eucalyptus globulus* and chloroform extract of *Azadirachta indica* respectively, as described under 'Materials and Methods'.

3.3.2 Standardization of solvent system

Various solvent systems were screened for efficient separation of bands according to polarity. Total 19 solvent systems were used in present investigation to know most suitable solvent system for separation of compounds in methanol and chloroform extract of *Eucalyptus globulus* and *Azadirachta indica*. The Rf values and colour of separated bands in different solvent systems under UV transilluminator are summarised in Table 3a and 3b.

It is observed from data presented in Table 3a and 3b, different solvent systems showed differences in number of bands and their Rf values in methanol and chloroform extract of *Eucalyptus globulus* and *Azadirachta indica*. Among all the tested solvent systems most promising solvent systems produced good results on TLC plates were toluene: ethyl acetate (93:7) and hexane: ethyl acetate (1:1) for methanol and chloroform extract of *Eucalyptus globulus* and *Azadirachta indica.*

The Rf values of chloroform extract of *Azadirachta indica* run under Hexane: ethyl acetate (1:1) solvent system was 0.05, 0.11, 0.52, 0.58, 0.74, 0.82, 0.88 and 0.94 (Table 3a). The Rf values of methanol extract of *Eucalyptus globulus* run under Toluene: ethyl acetate (93:7) solvent system was 0.03, 0.07, 0.12, 0.25, 0.37, 0.43, 0.56, 0.62, 0.75, 0.81, 0.87, 0.88 and 0.94 (Table 3b).

3.3.3 TLC Bioautography

TLC plates run in Toluene: Ethyl acetate (93:7) and Hexane: Ethyl acetate (1:1) system was used for bioautography technique to determine antibacterial activity of separated

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compounds against tested bacterium. The TLC plate after spraying with 2,3,5-tri phenyl tetrazolium chloride, showed a white colored inhibition against pink background around band which contain active principle responsible for antibacterial activity. The one compound from *Azadirachta indica* chloroform extract showed well resolved inhibition of *Xanthomonas axonopodis* pv. *citri* at Rf- 0.74 showing pink color under UV transilluminator (Plate 2).

Eucalyptus globulus methanol extract exhibited strong antibacterial activity on TLC plate with zone of inhibition at band with Rf 0.37 value against *Xanthomonas axonopodis* pv. c*itri* showing blue color under UV transilluminator. The compound from *Eucalyptus globulus* methanol extract and *Azadirachta indica* chloroform extract showing inhibition band was denoted as compound: 1.and compound: 2.

4. DISCUSSION

4.1 Extraction yield of plant leaf extracts

The maximum extraction yield in water extract (4.5g) was obtained by Rajapandiyan et al. (2011), indicating the presence of high polar components of alkaloids, flavones, and sugars. Highest extraction yield (0.6882g) from *Azadirachta indica* leaves in water extract was also reported in past (Babu *et al*. 2016). Furthermore, the methanol extract of *Eucalyptus globulus* leaves yielded the maximum yield of 12.90g (Ataollah et al. 2014). The current findings are similarly consistent with those of Badrunnisa and Pai (2017), who reported a 41.6 percent methanol extraction yield.

4.2 Preliminary phytochemical analysis

Similar observations were made by Ishnava et al. (2013), who discovered tannins, saponin, cardiac glycosides, steroids, phenolic compounds, and terpenoids in *Eucalyptus globulus* leaves. The presence of cardio glycosides, alkaloids, phenols, and flavonoids in *Azadirachta indica* leaves was detected earlier by Johnson *et al*. (2014).

4.3 (TLC) Thin Layer Chromatography

Mondali et al. (2014) measured the retention factors (Rf) of *Azadirachta indica* ethanol extracts in several solvent systems. Under hexane: ethyl acetate (1:1) solvent system, the ethanol extracts showed nine fractions with Rf 0.09, 0.10, 0.19, 0.22, 0.38, 0.48, 0.58, 0.66, and

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0.91 Rf value. TLC results shows that chloroform extracts contain a variety of chemical components. Obiorah et al. (2012) found that methanol extracts of *Eucalyptus globulus* eluted with a chloroform: petroleum ether: diethyl ether (10:7:3) solvent system had three Rf values of 0.15, 0.83, and 0.83. Using a thin layer chromatography technique eluted with a petroleum ether: ethyl acetate (2:1) solvent system, More et al. (2016) discovered ten active fractions of *Aegle marmelos* leaves extract.

4.4 TLC Bioautography

Guleria et al. (2011) used TLC-bioautography to evaluate the essential oil from *Eucalyptus teretecornis* leaves and analysed it using gas chromatography/mass spectrometry (GC/MS), revealing the presence of two main bioactive components, -fenchol (oxygenated monoterpene) and -eudesmol (oxygenated sesquiterpene) with Rf 0.27 and Rf 0.33, respectively. The detection of active compounds presents in *Azadirachta indica* oil by High Performance Liquid Chromatography (HPLC)-electrospray ionization mass spectrometry was done and named them as linoleic and oleic acid (Kruzselyi *et al.* 2016). Baghat et al. identified essential oils from different portions of the *Eucalyptus lanceolatus* plant using Gas Chromatography/Mass Spectrometry and TLC-bioautography and termed them alpha-pinene and limonene, respectively, with Rf 0.95 and 0.73. (2016). Shubham *et al*. (2016) also reported the presence of active compound in *Azadirachta indica* leaves using TLC-bioautography and spectroscopic analysis and identified them as tetranor-triterpenoid limonoid of Rf 0.56 showing retention time at 3.8 minute.

5. Conclusion:

The present study concluded that the highest extraction yield of *Eucalyptus globulus* leaves was obtained from methanol extract (9.54%) whereas lowest extraction yield was obtained from ethyl acetate (7.1%). While maximum extraction yield of *Azadirachta indica* leaves was reported in distilled water (9.08%) solvent and minimum in petroleum ether (2.9%). The phytochemical investigation revealed presence of cardio glycosides, saponins, alkaloids, terpenoids, tannins and phenolic compounds in methanolic extract *Eucalyptus globulus* while steroids and flavonoids were absent. And in the case of chloroform extract of *Azadirachta indica,* alkaloids, phenolic compounds, cardio glycosides, flavonoids were present and terpenoids, saponins, tannins and steroids were absent. Among all the tested solvent systems the most

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promising solvent systems produced good results on TLC plates were toluene: ethyl acetate (93:7) and hexane: ethyl acetate (1:1) for methanol and chloroform extract of *Eucalyptus globulus* and *Azadirachta indica*. The TLC-bioautography plates after spraying with 2,3,5-tri phenyl tetrazolium chloride, showed one compound from *Azadirachta indica* chloroform extract resembling well resolved inhibition of *Xanthomonas axonopodis* pv. *citri* at Rf- 0.74 which showed pink color under UV transilluminator. While the *Eucalyptus globulus* methanol extract exhibited strong antibacterial activity on TLC-bioautography plates with zone of inhibition at band with Rf 0.37 value against *Xanthomonas axonopodis* pv. *citri* showing blue color under UV transilluminator.

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A) Dry leaves powder of *Azadirachta indica*

B) Dry leaves powder of *Eucalyptus globulus*

C) Crude extract

D) Conservation of extract

Plate 1: Powder form *Azadirachta indica* and *Eucalyptus globulus* leaves and extraction.

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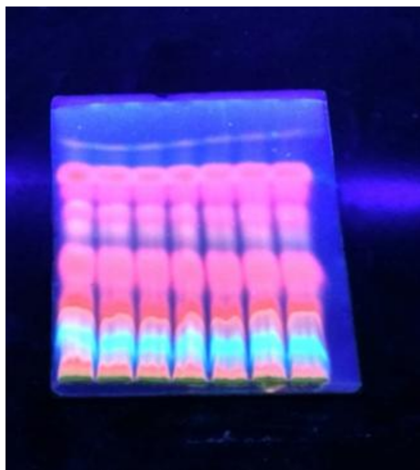
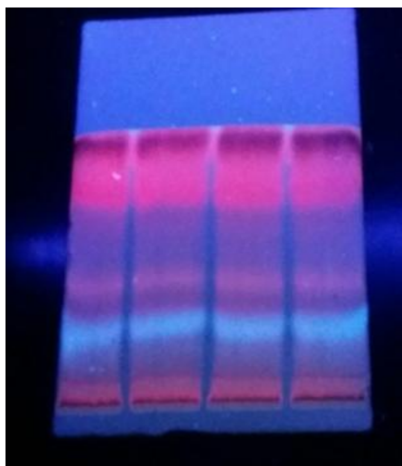


A) TLC of *Eucalyptus globulus*

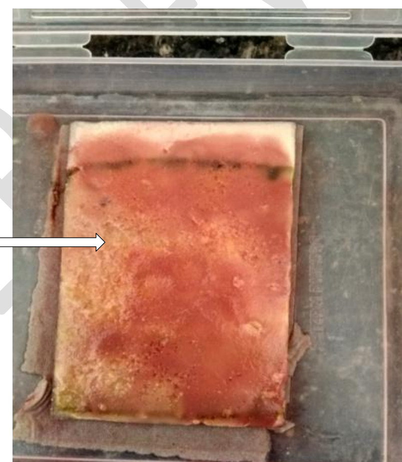
Rf – 0.37

B) Bioautography of *Eucalyptus globulus*



C) TLC of *Azadirachta indica*

Rf-0.74



D) Bioautography of *Azadirachta indica*

Plate 2: TLC and Bioautography of methanol and chloroform extract of *Eucalyptus globulus* and *Azadirachta indica*

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Table 1. Effect of different solvents on per cent extraction yield from dry weight of leaves

Plant	Solvent	Yield in %
Azadirachta indica	Petroleum ether	2.9
	Chloroform	6.7
	Dichloromethane	4.64
	Distilled water	9.08
Eucalyptus globulus	Chloroform	7.64
	Ethyl acetate	7.1
	Methanol	9.54
	Distilled water	7.2

Table 2. Preliminary phytochemical analysis of methanol extract of *Eucalyptus globulus* and chloroform extract of *Azadirachta indica.*

Test	*Azadirachta indica*	*Eucalyptus globulus*
Alkaloids	+	+
Terpenoids	_	+
Saponins	_	+
Tannins	_	+
Steroids	_	-
Phenolic compounds	+	+
Cardio glycosides	+	+
Flavonoids	+	-

+ presence, - absence.

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Table 3a. Standardization of solvent system for chloroform extract of *Azadirachta indica*

Sr. No.	Solvent system	Proportion	Chloroform extract of *Azadirachta indica*	
			Rf	**Color**
1	Acetic acid: Water	1:10		Smear of compounds No bands
2	Ethyl acetate: Acetic acid: Formic acid: Water	10:1.1:1.1:2.7	0.94	All pink
3	Methanol: Toluene	8:2	0.11	Brown
			0.66	Pink
			0.77	White
			0.88	Dark green
			0.93	Light brown
4	Methanol: Formic acid	1:1	0.70	Light pink
			0.82	Violet
			0.94	Light orange
5	Ethyl acetate: Methanol: Water	81:11:8	0.57	Pink
			0.71	Light green
			0.75	Blue
			0.85	Violet
			0.92	Pink
			0.97	Black

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6	Ethyl acetate: Methanol	9.5:0.5	0.11	Light orange
			0.35	Light pink
			0.70	Blue
			0.82	Light pink
			0.94	Black
7	Toluene: Ethyl acetate	7:3	0.11	Pink
			0.44	Light pink
			0.50	White
			0.83	Light pink
			0.88	Pink
			0.94	Black
8	Ethyl acetate: Methanol: Water	78:14:8	0.75	Pink
			0.87	White
			0.97	Light pink
9	Toluene: Chloroform: Acetone	45:25:15	0.11	Pink
			0.44	Light pink
			0.50	White
			0.83	Light pink
			0.88	Pink
			0.94	Black
10	Hexane: Ethyl acetate	1:1	0.05	Light orange
			0.11	Light pink
			0.52	Blue
			0.58	Light pink

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			0.74	Pink
			0.82	Light pink
			0.88	Violet
			0.94	Pink

Table 3b. Standardization of solvent system for methanol extract *Eucalyptus globulus*

Sr. No.	**Solvent system**	**Proportion**	**Methanol extract of *Eucalyptus globules***	
			Rf	**Color**
1	Chloroform: Methanol	3:2	0.18	Light orange
			0.50	Violet
			0.81	Light violet
			0.91	Pink
2	Ethyl acetate: Methanol: Water	81:11:8	0.78	Pink
			0.94	Violet
3	Hexane: Ethyl acetate	9:1	0.22	Pink
			0.31	Light violet
			0.50	Light pink
			0.55	Violet
			0.66	Light pink
			0.72	Black
			0.97	Light violet
4	Chloroform: Methanol	8:2	0.17	Light orange
			0.47	Violet

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			0.70	Light violet
			0.94	Pink
5	Petroleum ether: Toluene: Ethyl acetate	3:1:1	0.05	Light pink
			0.21	Blue
			0.42	Light pink
			0.61	Pink
			0.71	Light pink
			0.89	Pink
			0.94	Light violet
6	Hexane: Ethyl acetate	3:1	0.16	Light pink
			0.66	Pink
			0.72	Black
			0.93	Light violet
7	Toluene: Ethyl acetate: Acetone	7:2:1	0.11	Light green
			0.45	Light pink
			0.68	Light orange
			0.73	Light orange
			0.88	Pink
			0.96	Blue
8	Petroleum ether: Ethyl acetate: Acetone	7.8:2.2:0.2	0.05	Light green
			0.21	Light pink
			0.44	Pink
			0.66	Light blue

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			0.72	Black
			0.88	Light pink
			0.94	Black
9	Toluene: Ethyl acetate	93:7	0.03	Green
			0.07	Orange
			0.12	Pink
			0.25	Light pink
			0.37	Blue
			0.43	Pink
			0.56	Light violet
			0.62	Pink
			0.75	Light violet
			0.81	Pink
			0.87	Light violet
			0.88	Light pink
			0.97	Pink

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