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Qualitative Phytochemical Screening, Anti-Bacterial Activity and TLC Profiling of Different Parts of Three Medicinal Plants

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ABSTRACT

The aim of this study was to carry out phytochemical screening Anti-bacterial activity and TLC profiling of sequentially extracted petroleum ether, dichloromethane, ethyl acetate and methanol extracts of leaf and barks of the Nerium oleander, Leaf, bark and seeds of Cascabela thevetia and Cerbera odollum. The phytochemical analysis of the extracts obtained from different solvents was carried and Ant-bacterial activity was carried out for all the extractions at 25 mg/ml and 50 mg/ml test concentration against E.coli and S.aureus bacteria by well diffusion method. All the tested extractions showed low activity against both bacteria at 25mg/ml compared with 50mg/ml. Results were subjected to one way (ANOVA) and followed by Tukey's test ($P < 0.05$). All the extractions of all plant materials showed in bioactivity against E.coli and S.aureus bacteria at 50 mg/ml among them. The statistical analysis revealed that anti-bacterial activity of the ethyl acetate extracts of C.thevetia leaves and N.oleander barks against E.coli bacteria and Methanol extract of Nerium oleander leaves and ethyl acetate extracts of C.thevetia bark against S.aureus bacteria were comparable with the positive control Amoxicillin. TLC profiling was carried out using a various solvent system of varying polarity for all sequential extractions and obtained different retention factor (Rf) values of different phytochemicals.

KEYWORDS: *Phytochemical analysis, Antibacterial activity, Thin-layer chromatographic profiling, Sequential extractions, Cascabela thevetia and Cerbera odollum*

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1 INTRODUCTION

Plants have the capability to biosynthesize a wide variety of chemicals, some of which play an important role in primary metabolic activities while others are part of plant's secondary metabolism. Phytochemicals are biosynthesized as secondary metabolites, which are being synthesized in various parts of the plant such as a leaf, bark, roots, fruits and stem etc. (Pichersky & Gang 2000) These phytochemicals are the basic sources of pharmaceutical industries and also used for identifying the crude drugs and also phytochemical screening is important for identification of therapeutically and industrially important compounds such as alkaloid, steroid, etc.(Akindele & Adeyemi 2007).

The phytochemical screening is mainly applied to the quality control of traditional medicine. Thus, it is anticipated that phytochemicals with adequate anti-bacterial efficacy will be used for the treatment of bacterial infections. (Renisheya *et al*. 2011). Even at present, medicinal plants play a major role in the primary health care of many developing countries. (Vaghasiya *et al*. 2009) Therefore, in order to isolate and characterize novel antimicrobial compounds, scientists are interested in screening on medicinal plant extracts.

Nerium oleander, *Cascabela thevetia*, and *Cerbera odollum* belong to Family Apocynaceae. All parts of these plants are poisonous. Also, they play a vital role in traditional medicine. The leaves of *Nerium oleander* are most commonly used for the treatment of Nervous system, Hemiplegia, Epilepsy and skin diseases like Leprosy or ringworm infection (Dey & Chaudhuri 2014). Extract of the plant also possesses anti-bacterial and anti-fungal activities. The bark of the plant is used as intermittent fever and cathartic, febrifuge and because of its toxic nature, the powerfully resolving roots are used in the form of plasters and is usually applied externally and have been used against corns, warts, cancerous ulcer, carcinoma, hard tumours. (Alobaidi 2014)

The cardiac glycosides obtained from bark, kernels and flowers of *Cascabela thevetia* are useful for heart diseases. The plant or its individual parts can be used for the treatment of various disorders in human beings such as diabetes, liver toxicity fungal infection, microbial infection, inflammation, pyrexia and relive pain (Laphookhieo *et al*. 2004)

Cerbera odollum is also known for various medicinal properties. The oil from the seeds is used as a cure for itching or applied to the hair as an insecticide. (Kirtikar & Basu 1918) The bark and leaf of the plant are traditionally used as emetic and cathartic. Kernels are used as an emetic. The fruit is used as a cure for hydrophobia. (Rollet 1981) Its bark and fruits are purgative and used for the treatment of rheumatism. (Ahmed *et al*. 2006), (Guruswami *et al*. 1970) The extraction methods of plant materials may influence the inhibitory potentiality when testing against microbes (Valgas & Machado 2007). The sequential extraction technique has become a popular method for the extraction of active components from natural sources as the sequential

extraction procedure involves different solvents of various polarities that can provide the optimum effect of extraction and better activity than direct solvent extraction (Ken & Stevenson 2007)

In the present study, it was attempted to test the antibacterial activity of different solvent extractions of leaves, seeds and bark of three plants in family Apocynaceae including *Nerium oleander*, *Cascabela thevetia*, and *Cerbera odollum* obtained by sequential extraction methods tested against gram positive and gram-negative bacteria and also studied the phytochemical constituents present in the test extracts and TLC profile of each extracts. The obtained results provide support for the use of these plants in traditional medicine and further investigation and isolation of Phytoconstituents.

2 MATERIALS AND METHODS

2.1 Samples collection

Three different plant species belonging to family Apocyanacea were collected from Puttalam district, Sri Lanka [Fig. 1]. The aerial parts of the plant (Leaves, Barks) in *Nerium oleander* plant and leaves, bark and seeds of the plants in *Cascabela thevetia* and *Cerbera odollum* plants were collected from Puttalam district during the month of November- December. The seeds of *Cascabela thevetia* and *Cerbera odollum* plants were carefully separated from the shell and removed remnants outer shell, twinges and pebbles. Then seeds were opened manually to obtain kernels. All the plant parts of plants were washed thoroughly with running tap water (freshwater) to remove dust particles, contaminants, impurities and then all the samples were transported to the laboratory at the Department of Chemistry, Eastern University Sri Lanka, and then, all the samples were finally cleaned with distilled water. Collected plant materials were authenticated using herbarium, at the Department of Botany, Eastern University Sri Lanka, and Voucher specimens were deposited in the herbarium of the Department of the Botany, Eastern University, Sri Lanka. Then all the samples were air-dried for ten days until a constant weight was achieved. Then the samples were ground to a 0.5 mm particle size powder and weighed using electrical balance and stored in airtight container.

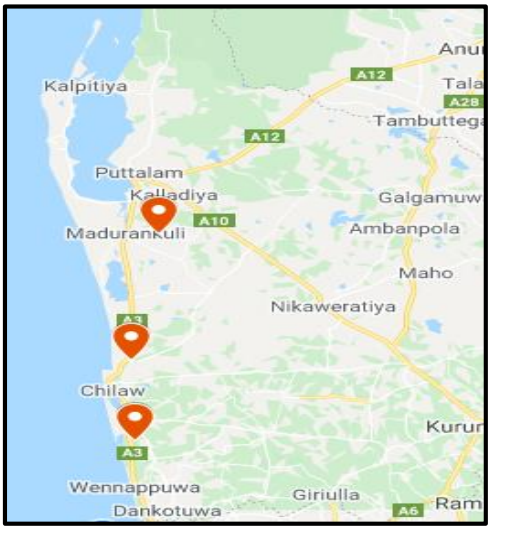


Figure 1: Map of plant samples collection locations (Bangadeniya, Mahawewa, and Madurankuliya)

2.2 Plant samples

Table 1: Abbreviation of Different part of different plant used for the study

Plant samples	Abbreviations
Nerium oleander leaves	NOL
Nerium oleander bark	NOB
Cascabla thevetia leaves	CTL
Cascabela thevetia bark	CTB
Cascabela thevetia seeds	CTS
Cerbera odollum leaves	COL
Cerbera odollum bark	COB
Cerbera odollum seeds	COS

2.3 Preparation of plant extracts

Each powdered plant part of NOL, NOB, CTL, CTB, CTS, COL, COB and COS (Table 1) was successively extracted with different organic solvents in increasing polarity order. 50 g of each powder was macerated separately in 250 ml of light petroleum ether (PE) with intermittent shaking for two days. Then they were first filtered through double-layer muslin cloth and then using Whatman no 1 filter paper. The residue was further extracted three times by using the same fresh solvent and all the filtrates were pooled together. The resulting residue was air-dried and further extracted with Dichloromethane (DCM) and followed by Ethyl acetate (EA) and Methanol (MeOH) similar to the procedure carried out for the PE extraction. Finally, from each 32 filtrates the solvent was removed by using a rotary evaporator under reduced pressure and low temperature. The yield of each crude extracts was weighed and stored at 4 °C until used (Giridharan & Somasundaram 2002). The yield percentage of each extract was calculated using the results obtained.

2.4 Phytochemical analysis

The phytochemical analysis involved standardized chemical test in evaluating presence or absence of several active phyto constituents such as alkaloids, flavonoids, Terpenoids, Steroids, Saponins, Phenols, Tannins, Quinone, Glycosides and Cardiac glycosides. All the test samples were subjected to analysis using modified and standard protocols. (Maung Tin-Wa *et al*. 1960), (Sofowora 1993), (Evans 1996).

2.5 Bacterial culture

Gram positive bacteria *Staphylococcus aureus* and Gram-negative bacteria, *Escherichia coli* were kindly provided by the Department of Microbiology, Faculty of Medicine, Eastern University, Sri Lanka. All the test bacteria were maintained on nutrient agar slope at 4 ºC. Then, these were subcultured for 24 hours before use.

2.6 Preparation of bacteria cultures

Stock cultures were maintained at 4 °C on slopes of nutrient agar. Active cultures of the experiment were prepared by transferring a loop full of cells from stock cultures to nutrient agar medium plate for preparation of subculture and those were incubated for 24 hours at 37 °C. A 18 to 24 hours. Single colonies of sub cultured agar plates were used to prepare bacterial suspension with turbidity of 0.5 McFarland (equal to 1.5×10^8 colony forming units CFU/ml).

2.7 Antibacterial screening

In vitro antibacterial activity of different crude extracts obtained by sequential extraction was studied using agar well diffusion method (Nair & Kalariya 2005). Autoclaved nutrient agar was cooled down to 40 °C, and around 15-20 ml of Nutrient agar was poured on glass Petri dishes and allowed to solidify. Then 1 ml of bacterial suspension (1× 60 cells/ml) was poured into agar surface and spread by sterile glass spreader. Wells were made on it using a sterile cork borer of 5 mm diameter, and each well was filled with 100 µl of each extract of test samples at 50 mg/ml test concentration. Then, the plates were allowed to standby for 30 min and were incubated at 37 °C for 24 hours. Amoxicillin was used as a reference. The antibacterial activity was recorded by measuring the zone of inhibition in mm. around the well. Each experiment was replicated twice and the mean value was calculated.

2.8 Statistical analysis

The diameter of zone of inhibition of replicates (four samples) was expressed as Mean ± standard deviation (SD). The data were subjected to One- way analysis of variance (ANOVA), followed by Tukey's test ($P < 0.05$) by using software Minitab 17 for windows version.

2.9 Thin layer chromatography analysis for sequential plant extracts

For TLC analysis, plate with Silica gel 60 F254 TLC (Merck, Germany), 10×10 cm was cut with ordinary household scissors. Plate markings were made with a soft pencil. Glass capillaries were used to spot the sample for TLC applied sample volume 1-µl of the sample by using capillary at a distance of 1 cm and developed in a TLC chamber using suitable mobile phase. The developed TLC plates were air-dried and observed under ultraviolet light UV at both 254 nm and 366 nm and exposed to freshly prepared anisaldehyde spaying reagent and dried for 1 minute for the development of colour in separate bands. The movement of the analyte was expressed by its retention factor (R_f).

The different solvent systems used to observe the TLC profiles were chloroform, DCM ethanol and 5 % methanol in acetonitrile, 2 % methanol in acetonitrile, 1 % methanol in acetonitrile and 0.5 % methanol in acetonitrile.

3 RESULTS & DISCUSSION

3.1 Weight of each plant material during ten days of air-dry.

Table 2: Weight of each plant material in each day during air dry.

Days	*Nerium oleander*		*Cascabela thevetia*			*Cerbera odollum*		
	Leaves	Bark	Leaves	Bark	Seeds Kernels	Leaves	Bark	Seed Kernels
Day 01	198.50	142.60	309.88	531.58	180.29	317.05	566.93	185.21
Day 02	167.55	130.64	289.09	516.45	178.15	315.05	547.52	174.91
Day 03	159.54	118.29	250.48	475.54	176.02	295.65	511.25	172.55
Day 04	149.39	99.89	214.31	432.79	175.30	195.65	478.35	170.66
Day 05	126.54	95.76	150.65	398.98	174.09	180.70	254.45	164.73
Day 06	115.65	93.20	147.98	248.97	173.00	120.87	198.67	162.02
Day 07	90.91	91.97	142.80	201.56	171.95	110.54	187.50	159.43
Day 08	89.39	90.90	138.98	190.87	169.30	105.98	173.87	158.40
Day 09	84.70	89.56	130.56	184.78	169.00	104.80	171.29	157.35
Day 10	80.65	89.19	129.98	183.01	169.00	101.15	170.56	157.30

Drying and weighing were continued at the same time interval until the constant weight was obtained. Normally, the air drying takes from 3-7 days to months depending on the types of samples dried. According to these results, weight loss of each plant material during the days 9 and 10 days were negligible, and they were taking almost 10 days to get constant value(Table 2). Drying is a process to remove moisture from fresh plant materials and reduce its water activity, which inhibits microbial growth and minimize deteriorative biochemical reactions (Buchaillot *et al*. 2009). However, drying can have adverse effects on phytochemical and nutritional components, especially heat-sensitive compounds (Hajimehdipoor *et al*. 2012). There are different drying methods such as freeze-drying, hot air drying, vacuum drying and microwave drying. These

drying methods are linked with various energy consumption, and have a significant effect on the phytochemicals of the samples (Nadi & Mey 2017). Therefore, it is necessary to identify the most suitable drying method and condition for specific type of plant samples. The present study used the air-dried (Shade drying) method to dry the plant materials.

Table 3: Yield percentage of crudes of sequentially extracted each plant extracts

Extract type	**Percentage yield (%)**							
	NOL	NOB	CTL	CTB	CTS	COL	COB	COS
PE	1.90	3.06	3.54	2.90	28.3	5.92	2.14	30.82
DCM	2.30	1.02	2.80	7.10	10.70	2.32	0.82	3.80
EA	4.20	1.08	3.02	1.28	2.78	1.50	2.44	3.66
MeOH	5.46	8.02	5.48	6.98	6.42	0.30	8.22	7.12

The objective of the sequential extraction process is to separate bioactive fractions according to the polarity of each compound from different solvents which have different polarity. In the present study, the extraction of each plant material was done sequentially using solvents from low polarity to high polarity in order of PE, DCM, EA, and MeOH, which is high polar solvent.

According to the present study, result yield percentage (Table. 3) of each plant material from sequential extractions demonstrated that various solvents bring about different extraction yields. This is because differences in polarity of the extraction solvents could cause a wide variation in the level of bioactive fractions in the extract. MeOH and EA extracts of NOL, NOB, CTL, CTB and COB showed higher percentage yield. This could be due to the presence of higher number of flavonoids because these plant materials contain high level of polar compounds that are soluble in the solvent with high polarity such as MeOH and EA. But in the case of CTS, COL and COS in PE extract showed higher yield percentage than other extracts solvents. This may be due to high level of non-polar compounds rather than polar compounds. Thus, the plant materials which were extracted from PE extract except CTS, COL and COS showed low yield and low yield percentage. This could be due to low level of non-polar compounds that are soluble in non-polar solvents such as PE. Yield percentage of different solvent extracts obtained from sequential extraction clearly revealed that compounds dissolved in polar solvents are quantitatively higher than compounds dissolved in low polar solvents in most of the plant materials. (Table 3)

3.2 Phytochemical analysis tests

Table 4. Results of phytochemical analysis of *Nerium oleander* Plant

Phytochemicals	Name of the test	NOL				NOB			
		PE	DCM	EA	MeOH	PE	DCM	EA	MeOH
Alkaloids	Wagner's reagent	+	+	+	+	+	+	+	+
	Mayer's reagent	-	-	-	-	-	-	-	-
	Confirmatory test	+	+	+	+	+	+	+	+
Flavanoids	Conc HCl	-	+	+	+	-	+	+	-
	NaOH	-	+	-	-	-	+	-	+
	Conc H_2SO_4	-	-	+	+	+	-	+	-
Tri-terphenoids	Liebermann-Burchard test	-	+	-	-	-	-	+	-
Unsaturated Steroids	Liebermann-Burchard test	+	-	+	-	-	+	-	-
	Salkowski test	+	+	+	+	+	+	+	+
Saponins	Froth test	-	-	-	+	-	-	-	-
Phenols	Lead acetate test	-	-	-	+	-	-	-	+
	Ferric chloride test	-	+	+	+	-	-	+	+
Tannins	Ferric chloride test	-	+	-	+	-	+	+	+
Glycoside	Liebermann's test	-	+	+	+	-	-	-	-
Cardiac Glycoside	Killer-kilani test	+	+	+	+	+	+	+	+
Quinone	Conc H_2SO_4	+	+	+	+	+	+	+	+
Total number of phytochemicals		5	9	7	9	6	6	8	7

Note. PE-Petroleum Ether, DCM-Dichloromethane, EA-Ethyl Acetate, MeOH-Methanol

Table 5: Results of phytochemical analysis of *Cascabela thevetia* Plant

Phytochemicals	Name of the test	CTL				CTB				CTS			
		PE	DCM	EA	MeOH	PE	DCM	EA	MeOH	PE	DCM	EA	MeOH
Alkaloids	Mayer's reagent	-	-	-	-	-	-	-	-	-	-	-	-

	Wagner's reagent	+	+	+	+	+	+	+	+	+	+	+	+
	Confirmatory test	+	+	+	+	+	+	+	+	+	+	+	+
Flavanoids	Conc HCl	-	+	+	-	-	-	+	+	-	-	+	+
	NaOH	-	+	-	-	-	+	+	+	-	+	-	-
	Conc H_2SO_4	-	-	-	-	+	-	-	-	+	-	+	-
Triterphenoids	Liebermann Burchard test	-	-	-	-	+	+	+	-	+	+	-	-
Unsaturated steroids	Liebermann Burchard test	+	+	+	-	-	-	-	+	-	-	+	+
	Salkowski test	+	-	-	-	+	+	-	-	+	+	-	-
Saponin	Froth test	-	-	-	+	-	-	-	-	-	-	-	-
Phenols	Lead acetate test	-	+	-	+	-	-	-	+	-	-	-	+
	Ferric chloride test	-	+	+	+	-	-	-	-	-	-	-	-
Tannins	Ferric chloride test	-	-	-	+	-	-	-	-	-	-	-	-
Glycoside	Liebermann's test	-	-	+	+	-	-	-	-	-	-	+	+
Cardiac Glycoside	Killer-kilani test	+	+	+	+	+	+	+	-	+	+	+	+
Quinones	Conc H_2SO_4	-	-	-	+	+	+	-	+	+	+	+	+
Total number of phytochemicals		3	5	7	8	6	6	4	5	6	6	6	7

Table 6. Results of phytochemical analysis of *Cerbera odollum* Plant

Phytochemicals	Name of the test	COL				COB				COS			
		PE	DCM	EA	MeOH	PE	DCM	EA	MeOH	PE	DCM	EA	MeOH
Alkaloids	Mayer's reagent	-	-	-	-	-	-	-	-	-	-	-	-
	Wagner's reagent	+	+	+	+	+	+	+	+	+	+	+	+
	Confirmatory test	+	+	+	+	+	+	+	+	+	+	+	+
Flavanoids	Conc HCl	-	-	-	+	-	-	-	+	-	-	-	+
	NaOH	-	+	+	-	-	+	+	-	-	+	-	-
	Conc H_2SO_4	-	-	-	-	+	-	+	-	+	-	+	-
Triterphenoids	Lieberman Burchard test	-	-	-	-	+	+	+	-	+	+	+	+
Unsaturated steroids	Lieberman Burchard test	+	+	+	-	-	-	-	+	-	-	-	+
	Salkowski test	+	-	-	-	+	+	+	-	+	+	-	-
Saponin	Froth test	-	-	-	+	-	-	-	-	-	-	-	-
Phenols	Lead acetate test	-	+	-	+	-	+	+	+	-	-	-	+
	Ferric chloride test	-	+	+	+	-	+	-	+	-	-	-	-
Tannins	Ferric chloride test	-	-	-	+	-	-	-	-	-	-	-	-
Glycoside	Liebermann's test	-	-	-	-	-	-	-	-	-	-	+	+

Cardiac Glycoside	Killer-kilani test	+	+	+	+	+	+	+	+	+	+	+	+
Quinones	Conc H_2SO_4	+	-	+	+	-	+	+	+	+	+	+	+
Total number of phytochemicals		4	5	6	7	5	7	7	6	6	6	6	7

According to the results of present study, alkaloids were present in all the sequential extractions of all tested plant materials. The results revealed that Alkaloids content of these plant materials was high. DCM extracts of all the tested plant materials showed presence of flavonoids and also most of the plant materials in EA and MeOH showed positive results for flavonoids when compared with PE extracts. It revealed that the presence of flavonoids in DCM, EA and MeOH extractions may be due to the polarity of flavonoids.

More terpenoids and steroids are existing in the PE extracts when compared with other extracts. In most cases, steroids and terpenoids are soluble in the non-polar solvent than in the polar solvents. For the Saponin test, only the MeOH extracts of NOL, CTL and COL showed positive results. It revealed that the solubility of saponins was more effective compared with polar solvents and also only the leaves of all the tested plant showed positive results for saponin which may be a high content of saponins in leaves of each plant compared with other plant parts. Phenol, tannin and glycoside showed more availability in the DCM, EA MeOH extracts when compared with PE extracts. It may be due to the polarity of the solvent as well as the polarity of both phytochemicals. But in the case of cardiac glycosides and Quinone, all the extractions of most of the plant part showed positive results for both phytochemicals. (Table 4)

According to the results of the study of qualitative phytochemical analysis among three plant Leaf of *N. oleander* showed more availability of phytochemicals in the tested samples. And also, some phytochemicals were available in low polarity solvent and some were available in a polar solvent. The present study of the qualitative phytochemical analysis revealed that availability of phytochemicals depends on plant type, part of the plant, the solvent used, the polarity of the solvent, plant extraction method, and also depends on the polarity of the phytochemicals. (Tables 4-6)

3.3 Anti-bacterial screening

The present study was carried out to evaluate the *in vitro* anti-bacterial activity of different parts of three different plant materials (Table 7) which were sequentially extracted with different organic solvents of PE, DCM, EA and MeOH in increasing polarity order using agar well diffusion method (Sukanya 2009) at 25mg/ml and 50mg/ml test concentration against the human pathogen such as gram-negative *E.coli* and gram-

positive *S.aureus*. (The 25 mg/ml test concentration was selected from the preliminary investigation). Amoxicillin was used as a standard positive control. The study showed that all plant extract utilized in this study showed a varying degree of antimicrobial activity against the gram-negative *E.coli* and gram-positive *S.aureus*.

Table 7: Mean diameters of inhibition of each solvent extract of each plant material against *E.coli* bacteria at 50 mg/ml

Plant type/ standard	The diameter of inhibition zone of inhibition(mm)			
	SE PE	SE DCM	SE EA	SE MeOH
NOL	19.750 ±1.708	18.000 ±0.816	26.500±0.577	25.500±0.577
NOB	14.750 ±0.957	12.500 ±0.577	30.500±1.291	21.000±0.00
CTL	12.750 ±0.957	13.500 ±0.577	32.000±0.00	20.250±0.957
CTB	17.750 ±0.957	17.750 ±0.957	20.000±0.816	20.000±0.816
CTS	13.750 ±0.957	14.500 ±0.577	13.250±0.957	20.000±0.816
COL	19.750 ±0.957	16.500 ±0.577	16.000±0.816	23.750±0.957
COB	14.500 ±1.291	18.500 ±0.577	12.500±0.577	19.000±0.816
COS	20.000 ±1.826	16.500 ±1.291	19.750±0.957	19.000±0.816
Amoxicillin	32.25± 0.957			

Note: Values are means of 4 replicates ± Standard deviation, SE-Sequentially extracted

Table 8. Mean diameters of Zone of inhibition against *S.aureus* bacteria of each solvent extract of each plant material at 50mg/ml

Plant type/ Standard	The diameter of inhibition (zone of inhibition(mm)			
	SE PE	SE DCM	SE EA	SE MeOH
NOL	18.250±0.957	18.000±0.816	26.500±0.577	29.250±4.50
NOB	12.500±1.291	18.000±0.000	30.500±1.291	22.000±0.816
CTL	12.500±1.291	33.000±0.00	32.00±0.00	21.500±1.291
CTB	17.500±0.577	25.250±1.258	34.000±0.816	20.750±1.708
CTS	12.500±1.291	33.000±0.000	13.250±0.957	21.000±0.816
COL	17.000±0.816	15.500±0.577	16.000±0.816	29.500±1.291
COB	13.000±0.816	16.000±0.000	12.500±0.577	24.000±0.816
COS	18.000±0.816	12.500±1.291	19.750±0.957	20.500±1.291
Amoxicillin	34.50±0.577			

Note: Values are means of 4 replicates ± Standard deviation

According to the statistical analysis from one way ANOVA test for anti-bacterial activity of different extracts which were sequentially extracted with above-mentioned solvent types against gram-negative bacteria of *E.coli* and gram-positive bacteria *S.aureus* at concentration of 50 mg/ml showed the p-value was lower than 0.05. So, there is a significance (p=0.00) at 95% confident level among the diameter of inhibition zone of each test sample which was extracted from the respective solvent for both bacteria. (Table 8)

According to the one-way ANOVA Tukey pairwise comparison, sequentially extracted PE extracts of the mean diameter of inhibition against *E.coli* bacteria, N. *oleander* showed zone of inhibition, but when considering leaves and bark, leaves showed highest inhibition zone (19.750 ±1.708). Among plant parts of *C. thevetia* also showed higher zone of inhibition considering leaves, bark and seeds, bark has shown highest diameter of (17.750 ±0.957). From the leaves, bark and seeds of the *C. odollum*, both leaves and seeds show a higher effect (19.750 ±0.957, 20.000 ±1.826). From that NOL, CTB, COL and COS extracted from PE solvent by sequential extraction process showed higher effect to inhibit *E.coli* when compared with other plant materials. From that revealed most of the non-polar phytochemicals extracted to PE extracts of NOL, CTB, COL and COS and these compounds might be more effective to inhibit the growth of *E.coli* bacteria when compared with compounds extracted to other PE extracts of NOB, CTL, CTS and COB.

Among the mean diameter of inhibition of SE DCM extracts against *E.coli* bacteria, *N. oleander* showed high zone of inhibition, but when considered leaf and bark, Leaf showed the highest zone of inhibition (18.000 ±0.816). Plant parts of *C. thevetia* showed zone of inhibition but considered leaf, bark and seeds; bark showed the higher diameter of inhibition (17.750 ±0.957). From the leaf, bark and seeds of *C. odollum*, bark showed higher effect (18.500 ±0.577). From that, sequentially extracted DCM extracts of NOL, CTB, COB shows higher effect to inhibit *E.coli* when compared with NOB, CTL, CTS, COL and COS. From that revealed most of the active semi-polar phytochemicals extracted to DCM solvent in each NOL, CTB, COB and these compounds effective to inhibit the growth of *E.coli* bacteria when compared with other plant materials.

Among the mean diameter of inhibition of SE EA extracts against *E.coli* bacteria, leaf and bark of *N. oleander* plant, NOB (30.500± 1.291) showed higher effect. Among the CTL, CTB and CTS in *C. thevetia* plant CTL (32.000±0.00) showed a higher diameter of inhibition. From the SE EA extracts of leaf, bark and seeds of *C. odollum*, COS (19.750± 0.957) showed a higher effect when compared with the other two. From that NOB, CTL and COS extracted from EA solvent by sequential extraction process showed highly effective to inhibit *E.coli* when compared with other plant materials. From that revealed most of the higher active polarity phytochemicals

extracted to EA solvent in each NOB, CTL and COS and these polar compounds might be effective to inhibit the growth of *E.coli bacteria* when compared with compounds those extracted EA extracts of NOL, CTB, CTS, COL and COB.

Among the mean diameter of inhibition of SE MeOH extracts against *E.coli* bacteria, leaf and bark of *N. oleander*, NOL showed (25.500±0.577) higher effect to inhibit the growth of *E.coli* bacteria when compared with NOB. Among CTL, CTB and CTS, from them, all the extracts showed a higher diameter of inhibition (20.250±0.957, 20.000±0.816, 20.000±0.816). From the COL, COB and COS, higher effect was showed in COL (23.750±0.957). From that SE MeOH extracts of NOL, CTL, CTB, CTS and COL showed higher effect to inhibit *E.coli* when compared with other plant materials. From that revealed most of the highest active polarity phytochemicals extracted to MeOH solvent in each NOL, CTL, CTB, CTS, COL and these polar compounds might be effective to inhibit the growth of *E.coli* bacteria when compared with compounds those extracted to MeOH extracts of NOB, COB and COS.

Among the mean diameter of inhibition of SE PE extracts against *S. aureus* bacteria, *N. olender* shows a high effect of inhibition, when considering leaf and bark, leaf shows the highest inhibition (18.25±0.96). Among *C. thevetia* plant, when considering leaf, bark and seeds from each extract CTB showed a higher diameter of inhibition (17.500±0.577). From the COL, COB and COS, higher effect showed in COS (18.000±0.816). From that SE PE extracts of NOL, CTB and COS show a higher effect to inhibit *S.aureus* when compared with other plant materials. From that revealed, most of the non-polar phytochemicals extracted to PE extract in each NOL, CTB and COS and these compounds might be more effective to inhibit the growth of *S.aureus* bacteria when compared with compounds those extracted to PE extracts of NOB, CTL, CTS, COL and COS.

Among the mean diameter of inhibition of SE DCM extracts against *S.aureus* bacteria of NOL and NOB of *N. oleander* plant, both of NOL and NOB shows a higher effect to inhibit (18.00±0.82 and18.000±0.00). Among CTL, CTB and CTS, from each extract, CTL and CTS showed a higher diameter of inhibition (33.000±0.00, 33.000±0.00). From the COL, COB and COS, higher effect showed in COB (16.000±0.000). From that NOL, NOB, CTL, CTS and COB extracted from DCM solvent by sequential extraction process showed highly effective to inhibit *S.aureus* when compared with other plant materials. From that revealed most of the active semi-polar phytochemicals extracted to DCM solvent in each NOL, NOB, CTL, CTS and COB and these compounds might be effective to inhibit the growth of *S.aureus* bacteria when compared with compounds extracted to DCM extracts of CTB, COL and COS.

Among the mean diameter of inhibition of SE EA extracts against *S.aureus* bacteria of NOL and NOB of *N. oleander* plant NOB shows higher inhibition zone against *S.aureus* bacteria (30.500±1.291). Among the SE EA extracts of CTL, CTB and CTS, from each extract CTB shows a higher diameter of inhibition against *S.aureus*

bacteria (34.000±0.816). From the COL, COB and COS higher effect shows in COS (19.750±0.957). From that NOB, CTB and COS extracted from EA solvent by sequential extraction process showed highly effective to inhibit *S. aureus* when compared with other plant materials. From that revealed most of the highly active polarity phytochemicals extracted to EA solvent in each NOB, CTB, COS and these polar compounds are effective to inhibit the growth of *S.aureus* bacteria when compared with compounds those extracted of EA extract of NOL, CTL, CTS, COL and COB.

Among the mean diameter of inhibition SE MeOH extracts of NOL and NOB of *N. oleander* plant, NOL shows a higher effect to inhibit the growth of *S.aureus* bacteria (29.250±4.50). Among the SE MeOH extracts of CTL, CTB and CTS, all the extracts of CTL, CTB and CTS show a higher diameter of inhibition against *S.aureus* bacteria (21.500±1.291, 20.750±1.708, 21.000±0.816). From the COL, COB and COS, higher effect was shown in COL (29.500±1.291) From that NOL, CTL, CTB, CTS and COL extracted from MeOH solvent by sequential extraction process showed highly effective to inhibit *S.aureus* when compared with other plant materials. From that revealed most of the highest polarity phytochemicals extracted to MeOH solvent in each NOL, CTL, CTB, CTS and COL and these polar compounds were effective to inhibit the growth of *S. aureus bacteria* when compared with NOB, COB and COS in MeOH extracts.

According to the Tukey test, among all the extracts compared with their mean diameter of inhibition against *E. coli* and *S. aureus* with standard amoxicillin and finally revealed that EA extracts of NOB, CTL against *E.coli* and MeOH extract of NOL and EA extracts of CTB for *S.aureus* were comparable with standard Amoxicillin.

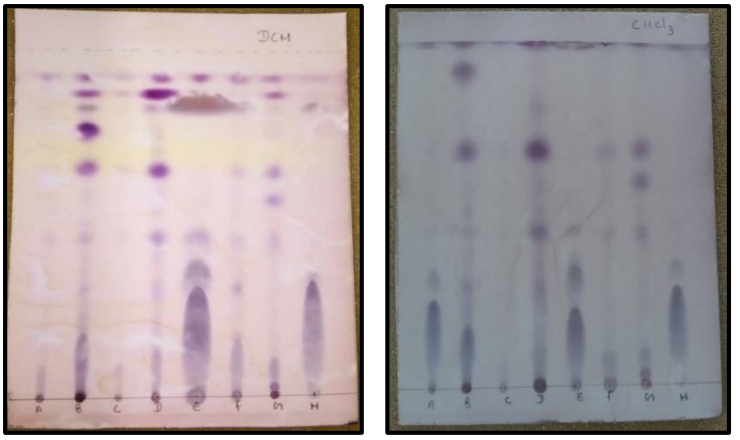
In the present study, the plant materials were sequentially extracted with different organic solvents in increasing polarity order. The sequential extraction method ensures the extraction of active compounds from plant material according to their polarity. Previous studies reported that Cold extraction and well diffusion method was better when compared with Soxhlet extraction and disc diffusion method. During the Soxhlet extraction, due to the high-temperature treatment, some of the active biomolecules might escape from the extract. It has also been reported that the agar well diffusion method is better than the disc diffusion method, because, the free hydroxyl groups present in the disc may prevent the diffusion of cationic polar molecules. The present study demonstrated the inhibitory effect of cold sequential extracts of present plant materials against test bacteria of *E.coli* and *S.aureus* at two different test concentrations of 25 mg/ml and 50mg/ml. When compared with the activity of both test concentrations, 25 mg/ml showed very poor activity against both bacteria when compared with 50 mg/ml test concentrations. Only the PE, EA and MeOH extracts of NOL and NOB, PE extracts of CTL showed somewhat higher

inhibition zone at 25mg/ml against *E.coli* bacteria when compared with other extractions. And also, the diameter zone of inhibition of all the solvent extracts of each plant materials at 25 mg/ml against *S.aureus* showed very poor results compared with *E.coli*. From those results, further statistical analysis was carried out only considering the anti-bacterial activity at 50 mg/ml.

According to the results of the present study, SE EA of CTL and NOB showed a higher diameter of inhibition for *E.coli* bacteria when compared with standard Amoxicillin and also SE MeOH of NOL and SE EA of CTB showed a higher diameter of inhibition for *S.aureus* bacteria when compared with standard Amoxicillin. From this result, it revealed that most of the polar solvent extracts of MeOH and EA showed better activity than low polar solvents such as PE and DCM. The higher inhibitory effect of polar solvent extracts is due to more solubility of active components in polar solvents and the polarity of solvents may play an important role in the inhibitory effect of plant extract. Yield percentage of different solvent extracts obtained from sequential extraction revealed that in the most cases, compounds that dissolve in polar solvents (Table 3) were quantitatively higher than compounds that dissolve in low polar solvents except CTS, COS. The efficacy of an anti-bacterial effect of plant material depends not only on the type of solvent, type of assay and the dose used but also on the method of extraction of plant materials.

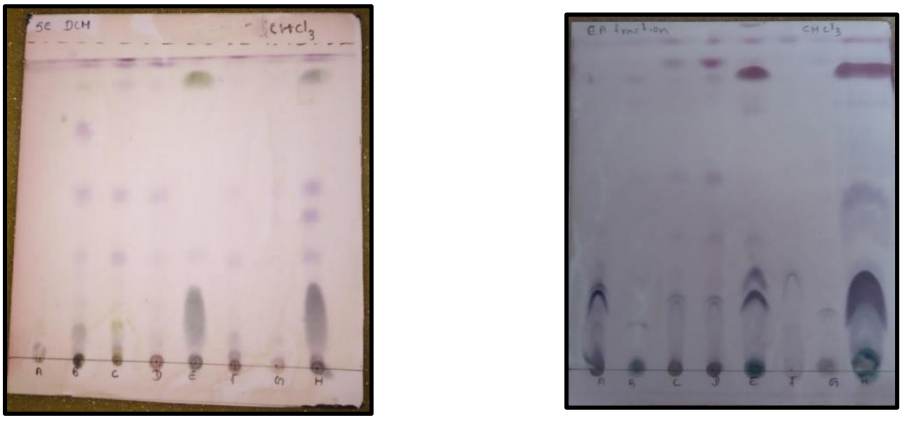
From the results of present study, all the examined plant parts of each three plants of family Apocyanacea such as *N. oleander*, *C. thevetia* and *C.odollum* showed anti-bacterial activity against *E.coli* and *S.aureus* bacteria. Among them, leaves and barks of *N. oleander* and Leaves and barks of *C. thevetia* showed more effects for each bacterium. From that, it revealed that these extracts have a good potential for developing bio-inspired anti-bacterial drugs. There is a good scope to develop natural drugs to fight against bacterial pathogens. But care should be taken as these plants have toxic properties also. Further investigation is recommended for isolation and development of non-poisonous anti-bacterial pharmaceutical compounds from crude extracts from these plant extracts.

3.4 Thin layer chromatography analysis



a **b**

Figure 2: TLC profile of each PE extracts of each plant material [a- Elute: Dichloromethane, b- Elute: Chloroform]



a **b**

Figure 3: TLC profile of each a- DCM extract [Elute: Chloroform], b- EA extracts [Elute: Chloroform]

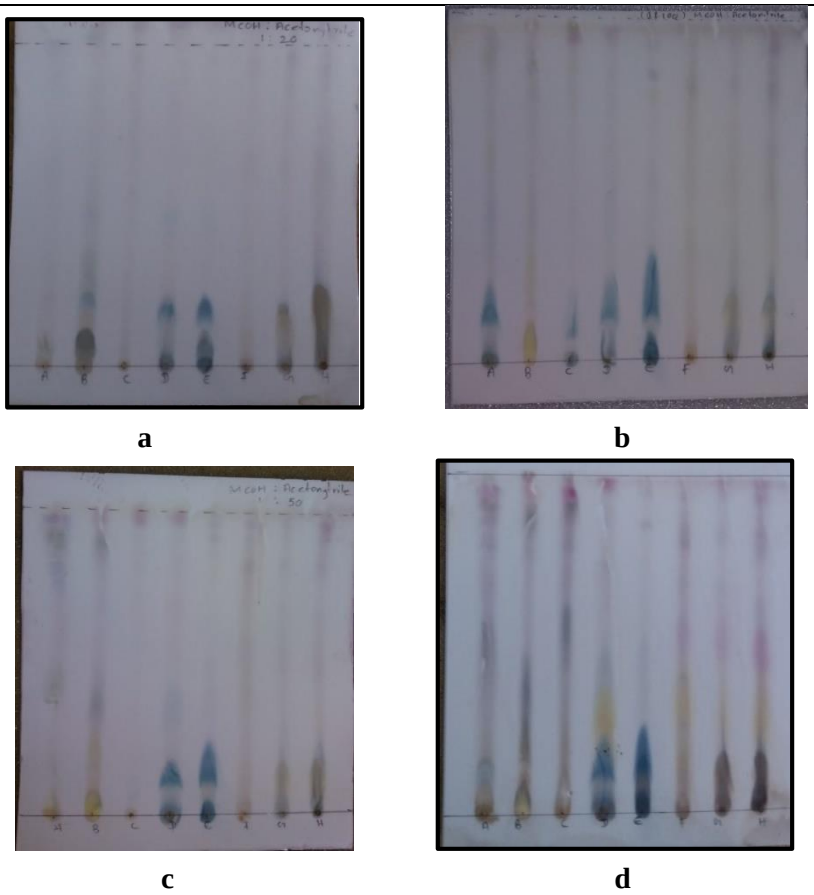


Figure 4: TLC profile of each MeOH extract [Elute: a- 0.5 % b- 1 % , c- 2 %, d- 5 % MeOH in Acetonitrile]

The sequential extraction method was carried out to confirm the nature of each plant material by analyzing TLC chromatograms and compared with bioactivity of each plant material in each plant extract.

According to the TLC profile (Figs.2-4) of each PE extract of each plant material eluted by dichloromethane, all the plant materials except NOB showed same compound with R_f value 0.928. And also, NOB, CTL, CTB and COB showed same compound with R_f value 0.880 and NOB, CTB, CTS, COL and COB showed the same compound with R_f value 0.824. Also, NOB, COL and COB showed the same compound with R_f value 0.686. NOL, NOB, CTB, CTS, COL and COB showed the same compound with R_f value 0.456. The same compound with R_f value 0.424 showed in NOB, CTB, and COL. Compound with R_f value 0.784 showed only in NOB. CTS and COS showed the same compound with R_f value 0.320.

According to the TLC profile (Figs. 2-4) of each plant extract of each plant material eluted by Chloroform; all the plant materials showed the same compound with R_f value 0.961. And also, NOB, CTB, COL and COB showed the same compound with R_f value 0.695. In the case of NOB, CTB,

CTS, COL and COB and COS showed the same compound with R_f value 0.495. CTS and COS showed the same compounds with the R_f values 0.291, 0.390, 0.495 and 0.961, respectively. Only NOB showed compound with R_f value 0.914.

According to the TLC profile (Figs. 2-4) of each DCM extract of each plant material eluted by chloroform, all the plant materials except CTB showed the same compound with R_f value 0.981. And also, NOL, NOB, CTB, COB and COS showed the same compound with R_f value 0.973. And also, NOB, CTL CTB showed the same compound with R_f value 0.928. NOB, CTL, CTB, COL and COB showed the same compound with R_f value 0.562. NOB, CTL, CTB, CTS, COL and COS showed the same compound with R_f value 0.348.

According to the TLC profile (Figs. 2-4) COL, COB and COS showed the same compounds with R_f values 0.404, 0.571 and 0.857. In the present study TLC profiling of 04 sequentially extracted PE, DCM and EA and MeOH extracts give an impressive result directing towards the presence of a number of phytochemicals. Various phytochemicals give different R_f values in a different solvent system. This variation in R_f values of the phytochemicals provides a very important clue in the understanding of their polarity and also helps in the selection of the appropriate solvent system. This information will help selection of appropriate solvent system for further separation of the pure compound from these plant extracts by column chromatography. In the results of the present study, in each extraction, some compounds might be the reason for the increasing or decreasing of the bioactivity. When compared with the TLC profile for each extract in respective solvents and bioactivity for each extract, availability of the compounds in each extract might be the reason to increase or decrease bio activity in each extract. For example, TLC profile of PE extracts of NOL and NOB eluted by dichloromethane shows a higher number of compounds compared with NOB and NOL which shows higher bioactivity compared with NOB against both bacteria. Antagonist effect of these compounds might be the reason to decrease the bioactivity of PE extracts of NOB compared with NOL. And also, some compounds in each extract might be the reason to increase the bioactivity compared with other extracts. When compared with TLC profile and bioactivity for each solvent extract for respective plant parts, some compounds showed in TLC might increase bioactivity against *E. coli* and some compounds might decrease the bioactivity against *S.aureus*. Therefore, these active extracts could be subjected to further isolation and purification of active compounds to discover novel lead anti-bacterial agents.

4 CONCLUSIONS

Phytochemical analysis of different solvent extracts of leaves and barks of *N. oleander* and leaves, barks and seeds of both *C. thevetia* and *C.odollum* of family Apocynaceae revealed the presence of alkaloids, flavonoids, steroids, cardiac glycosides, Quinone, phenol in all plant materials and saponins only presence in the

leaves of each plant. Glycosides absence in the bark of the *N.oleander* and *C.thevetia.* Tannins absence in the bark and the seeds of both *C.thevetia* and *C.odollum* plants. In vitro anti-bacterial activity of different solvents of each plant material revealed that all the solvent extracts of the tested plant materials show bio activity against both *E.coli* and *S.aureus* bacteria at 50 mg/ml and among them ethyl acetate extracts of *C.thevetia* leaves and bark, *N.oleander* bark and methanol extract of *N. oleander* leaves exhibit higher bio activity against tested bacteria. And also, most of the solvent extractions show very poor activity at 25mg/ml against both bacteria and among them petroleum ether extracts of leaves of *N.oleander* and *C.thevetia*, and Ethyl acetate and methanol extracts of *N.oleander* leaves and *N.oleander* bark shows higher bio- activity against *E.coli* bacteria and very poor activity against *S.aureus* bacteria. TLC profiling revealed that different phytochemicals contain different R_f values and most of the plant materials contain the same compounds with the same R_f values for the respective solvent phase. Therefore, these active extracts and using R_f values of respective compounds could be subjected to further isolation and purification of active compounds to discover novel lead anti-bacterial agents. But care should be taken as these plants have toxic properties also. Further investigation is recommended for isolation and development of non-poisonous anti-bacterial pharmaceutical compounds from crude extracts from these plant extracts.

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