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EVALUATION OF ANTIBACTERIAL ACTIVITY AND STANDARDIZATION OF FLOWER OF *MYRTUS COMMUNIS* L.

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ABSTRACT

The present study attempts to investigate *in vitro* antibacterial activity against bacterial strains and the main aspects included in the study are Phytochemical, physico-chemical studies, organoleptic characters, fluorescence analysis of dried flowers extracts, HPTLC, TLC profile, Heavy metal analysis and Aflatoxin estimation of the dried flowers of *M. communis.* The antibacterial activity of *M. communis* flowers was assessed against most prevalent microbes like *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumonia, Escherichia coli, Salmonella typhi* against five different extracts by using agar well diffusion method and the Phytochemical, physico-chemical studies, organoleptic characters, fluorescence analysis of dried flowers extracts, HPTLC, TLC profile, Heavy metal analysis and Aflatoxin estimation were analyzed as per the methods described in WHO guidelines. The evaluation of antibacterial activity on chloroform extract was found to be more active than other extracts and among the tested organism *Escherichia coli* was found to be most sensitive organism followed by *Salmonella typhi, Bacillus subtilis, Staphylococcus aureus* and *Klebsiella pneumonia.* When compared with streptomycin (10 mcg) as standard antibiotic. The flowers of *M. communis* might represents a new antimicrobial source with stable, biologically active components that can establish a scientific base for the use for developing medicine. Further it was standardized according to the methods described in WHO guidelines with respect to Phytochemical, physico-chemical studies, organoleptic characters, fluorescence analysis on flowers extracts were carried out along with HPTLC, TLC profile, Heavy metal analysis and Aflatoxin contamination.

KEYWORDS: ***Myrtus communis*** **Flowers, Antibacterial activity, Phytochemical screening, and HPTLC.**

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INTRODUCTION

Myrtus communis or common Myrtle (Myrtaceae) is an ever green shrub or small tree with evergreen leaves, 6-8 feet in height with small foliage and covered with a deep fissured bark Myrtus, the Greek name for Myrtle and communis means common plant growing in groups. The common Myrtle was introduced into Britain in around 1597 and was described by Linnaeus in 1753. Myrtus occupies a prominent place in the writings of Hippocrates, Pliny, Dioscorides, Galen and the Arabian writers[1,2]. The Myrtus is a genus of flowering plants with 1 or 2 species, native to Southern Europe and North Africa and widespread in the Mediterranean area. It is cultivated in North West India gardens for its fragrant flowers. The star-like flower has 5 petals and sepals, and numerous stamens. Petals usually are white. The fruit is a round blue-black berry containing several seeds. The flower is pollinated by insects, and the seeds are dispersed by birds that eat the berries. *M. communis* (Myrtaceae) has been used since ancient times for medicinal and food and purposes. Medicinal plants are very important one in human health, it will act as an antibactericide activity against the bacterial pathogens, this is followed from ancient times[3]. Over the past 2 decades, there has been a lot of interest in the investigation of natural materials as sources of new antibacterial agents[4]. According to the World Health Organization (WHO), medicinal plants would be the best source to obtain a variety of drugs and active compounds[5]. For a long time, *M. communis* has been used as a folk medicine for treatment of various conditions such as lung disorders and as an antiseptic, anti-inflammatory, mucolytic, carminative and astringent remedy[6]. *M. communis* possess several pharmacologic, biologic and medical activities such as antibacterial, antiviral, antifungal, anti-inflammatory, analgesic, antioxidant, antimutagenic, anti-hemorrhagic, hepatoprotective, wound healing and anti-hyperglycemic activities[7].The antimicrobial activity of the crude preparation of Myrtus, on *Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *P. vulgaris, P. mirabilis*, *Klebsiella aerogenes*, *Salmonella typhi* and *S. shigiella* was determined by Alem et al and preliminary study supported its traditional claim of effective anti-infective[8].The plant contains fibers, sugars and antioxidants and many biologically active compounds[9]. Phenolic compounds, flavonoids and anthocyanins are the major phytochemicals in berries. Seeds yield 12- 15% of a fatty oil (fixed oil) consisting of glycerides of oleic, linoleic, myristic, palmitic, linolenic and lauric acid[10]. Studies on fatty acid analysis of myrtle fruits showed that it contains 14 fatty acids, oleic acid being the dominant fatty acid (67.07%) followed by palmitic acid (10.24%) and stearic acid (8.19%)[11]. Myrtle oil is the essential oil of *M. communis* which is extracted from the leaves, branches, fruits and flowers through steam distillation. It is yellow or greenish yellow in colour with a characteristic refreshing odour. *M. communis* leaves were found to be the most effective in bacterial growth inhibition; Root is reported to have antibacterial property[12.]But according to the literature, there is no information available about the flowers *M.communis*. In the present study, the main objective is to evaluate the antimicrobial activity against the bacterial strain and standardization of flowers *M. communis.*This study scientifically supports the usage of flowers *M. communis.*As a remedy for various bacterial infections in traditional medicine.

COLLECTION OF MATERIAL

M. communis flowers were collected from the local market Hyderabad, and was identified by the botanist Dr. V.C. Gupta, former Deputy Director (Botany) at CRIUM, Hyderabad.

This article can be downloaded from www.ijpbs.net

Dried flowers of Myrtus communis L.

Antibacterial activity was performed in different bacterial strains with control as per WHO guidelines, Anonymous, 1998[13] and The present investigation includes parameters such as physico-chemical parameters and TLC fingerprint of different solvent extract. Physico-chemical parameters were determined according to the methods described in Anonymous, 2009[14]. Phytochemical screening was also performed in the different solvent drug extracts. Fluorescence analysis was carried out as per the method described by Trease and Evans, 1972[15].

ANTIBACTERIAL ACTIVITY

TEST ORGANISMS

To evaluate the antibacterial studies, the microorganisms used were *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumonia, Escherichia coli, and Salmonella typhi* All the organisms were confirmed using specific biochemical tests (Mackie & McCartney ,1996).They were sub cultured on nutrient agar for every 15 days and maintained on nutrient agar slants at 4^0C. Fresh inoculums were taken for the test.

PREPARATION OF EXTRACTS

The material was extracted with five solvents, independently viz. Methanol, Ethanol, Aqueous, Pet. Ether, and Chloroform extracts. Briefly, 100g of the powder was soaked into respective solvent for three days and followed by filtration of the solvent using Whattman's filter paper under aseptic condition. A stock solution of the extracts was prepared at the concentration of 200mg/ml and stored at 2^0C till further use.

SCREENING FOR ANTIBACTERIAL ACTIVITY

Anti-Bacterial activity of the extract was determined by agar diffusion assay[16,17]. Bacterial strains were first grown in Mueller Hinton broth (MHB) under shaking condition for 24 h at 37 ^{0}C and after the incubation period 0.1ml of the test inoculums was spread evenly with a sterile glass spreader on Mueller Hinton Agar (MHA) plates. In seeded plates, wells were made using sterile 6 mm cork borer in the inoculated MHA plate. The wells were filled with 150µl of the extracts. The concentration of stock extracts was 200 mg/ml. The inoculated plates were incubated at 37^0C for 24 h. The plates were observed for the presence of inhibition of bacterial growth that was indicated by a clear zone around the wells. The size of the zones of inhibition was measured and the antibacterial activity was expressed in terms of average diameter of the zone of inhibition in millimeters. The test was conducted in triplicate and the photograph was taken in UV Visible documentation system.

CHEMICAL ANALYSIS

Physico-Chemical parameters of the herbal drug were studied as shown in the table1. Such as total ash, acid insoluble ash, water soluble matter and alcohol soluble matter, pH, loss on drying at 105^0C, and Aflatoxins contamination were analyzed as per the methods described in WHO guidelines Anonymous, 1998. Phytochemical screening was carried out in the Petroleum ether, Chloroform, Ethyl acetate, methanol, Acetone and aqueous extracts as per the methods described by Evans and Trease, 1972; to know the nature of phyto-constituents present in the drug as shown in the table 2.

Table 1
Antibacterial screening of M. communis

S.No	Organisms	Zone of Inhibition (mm)					
		MeOH	Ethl	H_2O	Pet. Ether	$CHCl_3$	Standard Streptomycin(10 mcg)
1.	*Staphylococcus aureus*	15	13	18	18	21	-
2.	*Bacillus subtilis*	16	12	19	18	24	23±24
3.	*Klebsiella pneumonia*	15	12	18	17	21	21±22
4.	*Escherichia coli*	17	14	19	16	25	24±25
5.	*Salmonella typhi*	18	15	18	19	24	23±24

Table 2
Physico-chemical parameters of M. communis

Parameters	Results			Average
Total ash (% w/w)	5.0248	5.0652	5.1026	5.0642
Acid insoluble ash (% w/w)	0.8434	0.8815	1.1972	0.9740
Alcohol sol. Matter (%w/w)	12.5632	12.5763	12.5940	12.5778
Water sol. matter (% w/w)	20.4983	20.5002	20.5231	20.5072
pH of 1% Aqueous Solution	5.02	5.05	5.08	5.05
pH of 10% Aqueous Solution	5.01	5.03	5.06	5.0333
Loss of weight on drying at 105^0C	8.6307	8.7221	8.9552	8.7693

TLC FINGERPRINT PROFILE
PREPARATION OF EXTRACT

Five grams of powdered drug was dissolved in 100 ml of carried out in the Petroleum ether, Chloroform, Ethyl acetate, methanol, Acetone and aqueous separately in a stoppered conical flask and was kept for 2 hours while shaking at regular intervals. Later the contents were filtered through whattmann No. 41 paper and evaporate the solution to 20 ml. Thus obtained solutions were used as samples for the separation of components.

DEVELOPMENT AND DETERMINATION OF THE SOLVENT SYSTEM

The samples were spotted as 6mm band on Precoated Aluminum Sheets of Silica Gel 60 F_{254} (Merck). After trying with various solvent system with variable volume ratios, the suitable solvent system was selected in its proportional ratio and developed in the Twin through TLC chamber to the maximum height of the plate so that components are separated on the polar phase of silica gel and mobile phase of solvent system.

DETECTION SYSTEM

After the developing, the TLC plate was dried completely and detected under the UV visible chamber and also by spraying with anisaldehyde sulphuric acid on the plate heated at 105^0C for 5 minutes and then observed in the UV chamber for detection of spots and photographed as shown in figure 2.

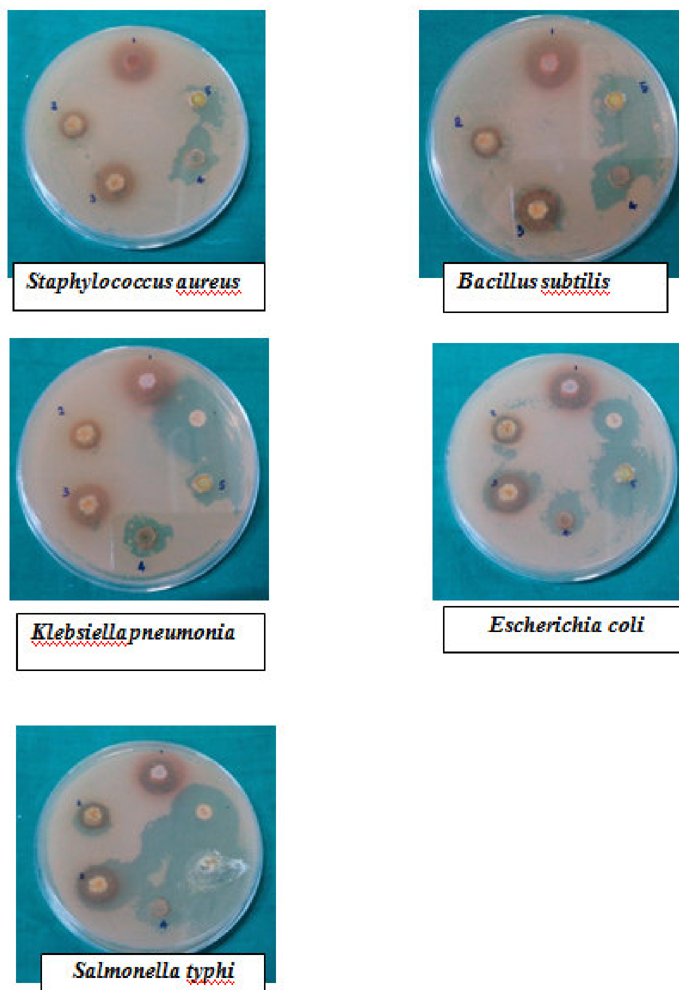
RESULTS AND DISCUSSION

ANTIBACTERIAL ACTIVITY

The Methanol, Ethanol, Aqueous, Pet. Ether, and Chloroform extracts of M. *communis* of flowers were screened for antibacterial activity against five bacterial strains i.e., *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumonia, Escherichia coli, Salmonella typhi* and the results obtained were illustrated in table1.*Escherichia coli* was found to be the more sensitive with better inhibition zone of 25mm in chloroform extract whereas in other extract the zone of inhibition ranges from 14mm to 19mm, *Salmonella typhi* and *Bacillus subtilis* showed similar sensitivity with inhibition zone of 24mm in chloroform extract whereas in other extract the zone of inhibition ranges from 12mm to 19mm. *Staphylococcus aureus* and *Klebsiella pneumonia* showed similar sensitivity with inhibition zone of 21mm in chloroform extract whereas in other extracts it is less

sensitive with inhibition zone ranges from 13mm to 18mm.Streptomycin(10 mcg) was used as a standard antibiotic with inhibition zone of 21± 25mm. as shown in figure 1.

Figure 1
Plates showing antibacterial activity of M. communis flower extracts.



Note: 1. Aqueous, 2.Ethanol, 3 MeOH, 4.Pet. Ether, 5.$CHCl_3$ extracts and standard antibiotic Streptomycin (10mcg).

ORGANOLEPTIC CHARACTERS

The crude drug consists of the dried flowers of M. *communis* of Myrtaceae Family. It is yellow in colour and having fragrance odour.

PHYSICO-CHEMICAL STANDARD

The Physico-Chemical Parameters data expressed here as mean values were shown in the table2.

PHYTOCHEMICAL SCREENING

Preliminary phytochemical screening was conducted to identify various secondary metabolites in *M. communis* Chemical tests for tannin, saponins, flavonoids, phenolic glycosides, chromophores, free anthra-quinones, anthraquinone glycosides, alkaloid and poly phenols were analysed and the nature of compounds present in the drugs is shown in table 3.

Table 3
Phytochemical screening of the nature of compounds present in the drug.

S.No	Phytoconstituents	Pet. ether	Chloroform	Ethyl acetate	Methanol	Acetone	Aqueous
1.	**Alkaloids** 1.Dragendroff's reagent.	-	-	-	-	+	-
	2.Mayer's test	-	-	-	-	+	-
2.	**Carbohydrates** 1.Benedict's test	-	+	-	+	-	+
	2.Molisch's test	-	+	-	+	-	+
3.	Resinified/ volatile oils	-	-	-	-	-	-
4.	Glycosides	-	-	-	+	+	+
5.	**Phenols:** FeCl3 test	-	-	-	+	+	+
6.	Saponins	-	-	-	+	-	-
7.	**Proteins :**Millon's test	-	-	-	+	+	+
8.	Starch	-	-	-	-	-	-
9.	**Phytosterols (Steroids)** Salkowskireaction Test	-	-	-	+	+	+
10.	**Tannins:** Ferric chloride test:	-	-	-	+	+	+
11.	**Flavanoids :**Shinoda test	-	-	-	-	-	-

TLC ANALYSIS

Different extract of the drug whose chromatogram was developed using the solvent toluene, ethyl acetate and MeOH (7:2:1) and detected under the UV 366nm has clearly shown different spots with Rf values and upon spraying with 5% methanolic Sulphuric acid and heated at 105^{0}C for 5 minutes and observed in UV 366nm revealing clear spots with Rf values as shown in the table- 4 and figure 2.

Aflatoxins were analyzed and found to be absent as given in the table-5 inferring the drug to be safe and non-toxic. Powdered drug was screened for fluorescence characteristic with or without chemical treatment. The observations pertaining to their colour in daylight i.e., visible region and under ultra-violet light were noticed and are presented in the table-6. Fluorescence analysis of powdered drug extracts in different solvents was observed and reported in the table-7.

Table 4
TLC profile of different extract of along with R_f values and detection system.
Solvent system : Toluene:Ethyl acetate:Methanol = 7:2:1

S.No	Name of the extract	Detection	No. of spots	R_f Values
1.	Pet.Ether extract	Observed under UV 366nm	2	0.88 (Black), 0.74 (Black)
		Spraying with 5% methanolic sulphuric acid and observed under UV 366nm	3	0.91 (Grey), 0.80 (Grey), 0.71 (Grey)
2.	Chloroform extract	Observed under UV 366nm	5	0.88 (Black), 0.74 (Black), 0.43 (Blue), 0.36 (Red), 0.15 (Purple)
		Spraying with 5% methanolic sulphuric acid and observed under UV 366nm	5	0.91 (Grey), 0.77 (Grey), 0.76 (Grey), 0.56 (Grey), 0.47 (Grey)
3.	Eth.acetate extract	Observed under UV 366nm	4	0.88 (Black), 0.45 (Blue), 0.43 (Red), 0.15 (Blue)
		Spraying with 5% methanolic sulphuric acid and observed under UV 366nm	4	0.91 (Grey), 0.77 (Grey), 0.70 (Grey), 0.49(Grey)
4.	Methanol extract	Observed under UV 366nm	5	0.88 (Black), 0.74 (Black), 0.45 (Blue), 0.36 (Red), 0.14 (Blue)
		Spraying with 5% methanolic sulphuric acid and observed under UV 366nm	5	0.91 (Grey), 0.77 (Grey), 0.70(Grey), 0.56 (Grey), 0.45 (Grey)
5.	Acetone extract	Observed under UV 366nm	5	0.88 (Red), 0.74 (Black), 0.56(Blue), 0.43 (Red), 0.14 (Blue)
		Spraying with 5% methanolic sulphuric acid and observed under UV 366nm	5	0.91 (Grey), 0.80 (Grey), 0.70 (Grey), 0.57 (Grey) ,0.45 (Grey)
6.	Aqueous extract	Observed under UV 366nm	1	0.24(Blue)
		Spraying with 5% methanolic sulphuric acid and observed under UV 366nm	1	0.23(Grey)

Table 5
Aflatoxin Contamination

S.No	Parameter analyzed	Results	Permissible limits as per WHO
1.	B1	Nil	Not more than 0.50 ppm
2.	B2	Nil	Not more than 0.10 ppm
3.	G1	Nil	Not more than 0.50 ppm
4.	G2	Nil	Not more than 0.10 ppm

Table 6
Fluorescence analysis of powdered drug

S.No	Reagents	UV light		Visible light
		Short 254nm	Long 366nm	
1.	Powder as such	Black	Black	Brown
2.	Powder treated with 1N NaOH in Methanol	Black	Black	Pale Yellow
3.	Powder treated with 1N NaOH in Water	Black	Black	Dark Brown
4.	Powder treated with 1N HCl	Black	Black	Brown
5.	Powder treated with 50% HNO3 aqueous	Black	Black	Yellow
6.	Powder treated with 50% H_2SO_4 aqueous	Black	Black	Black
7.	Powder treated with Glacial Acetic acid	Black	Black	Pale Yellow

Table 7
Fluorescence analysis of powdered drug extracts in different solvents

S.No.	Extraction Solvent	UV light		Visible light
		Short 254nm	Long 366nm	
1.	Petroleum ether extract	Black	Black	Light green
2.	Chloroform Extract	Black	Black	Green
3.	Ethyl Acetate	Black	Black	Light green
4.	Methanol	Black	Black	Light green
5.	Acetone Extract	Black	Black	Light green
6.	DCM MeOH Extract	Black	Black	Light green
7.	Distilled water	Black	Black	Brown

Figure 2
TLC chromatogram of M. communis L. flower extract at UV 366nm, 254nm, under expose to Iodine vapour and after derivatization with Anisaldehydesulphuric acid.

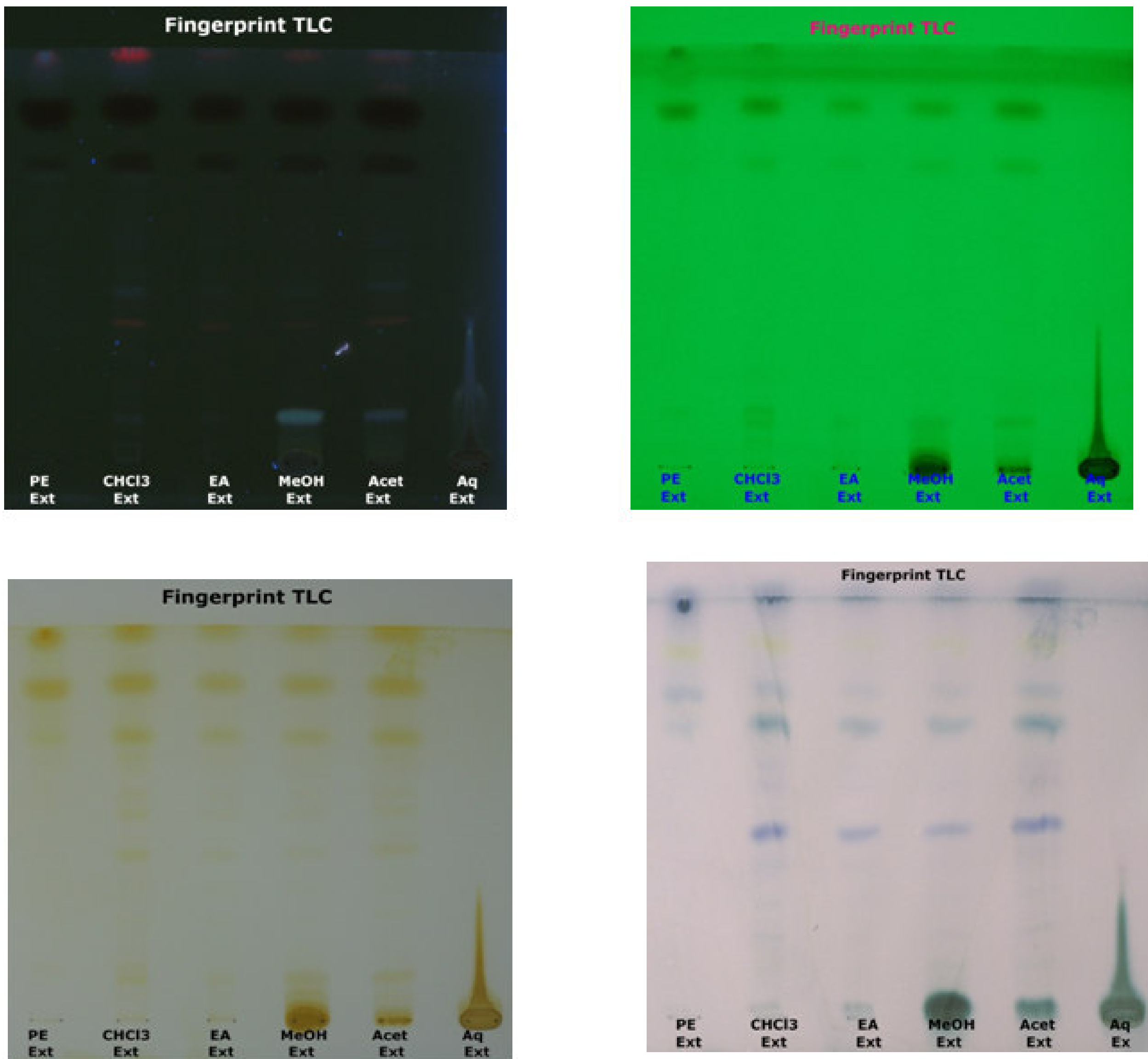


Figure 3
Over lay Densitogram showing the separation of peaks of M. communis L. flower at UV 366nm in 1.Petroleum ether extract 2.Chloroform extract 3. Ethyl Acetate 4. Methanol extract 5.Acetone extract 6. Aqueous extract.

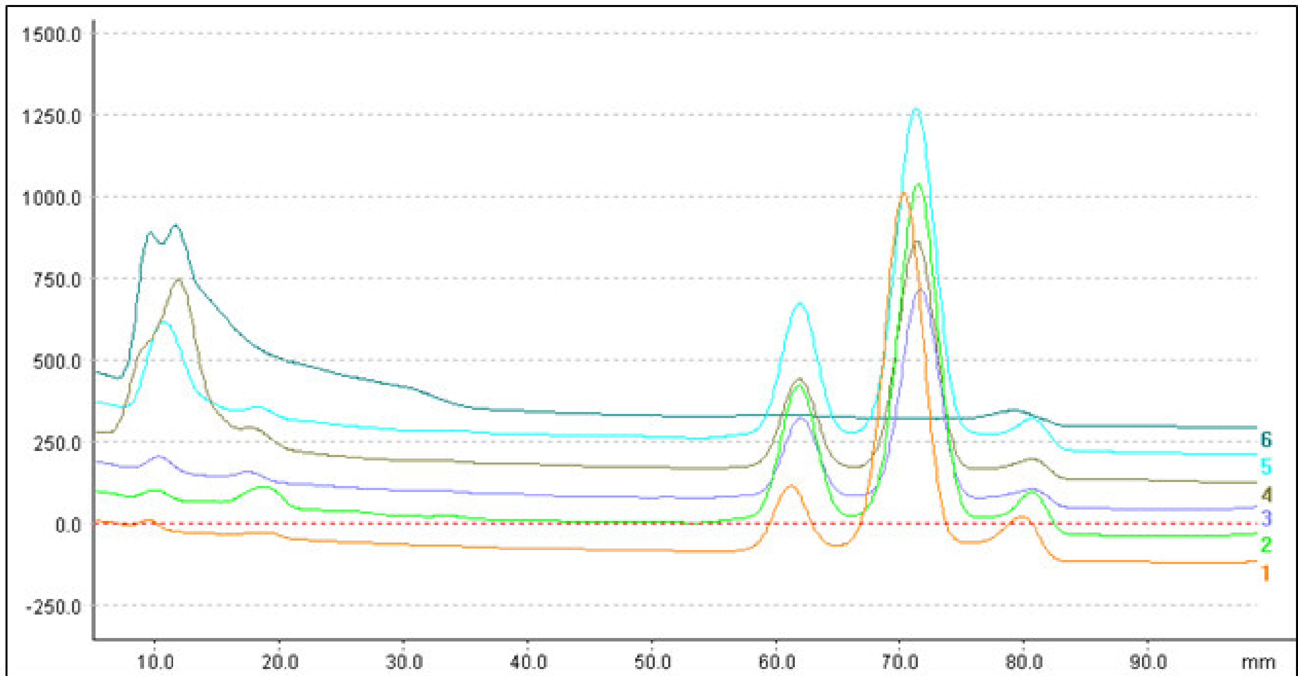


Figure 4
Over lay Densitogram showing the separation of peaks of M. communis L. flower at UV 254nm in 1. Petroleum ether extract 2. Chloroform extract 3. EthylAcetate 4. Methanol extract 5.Acetone extract 6. Aqueous extract.

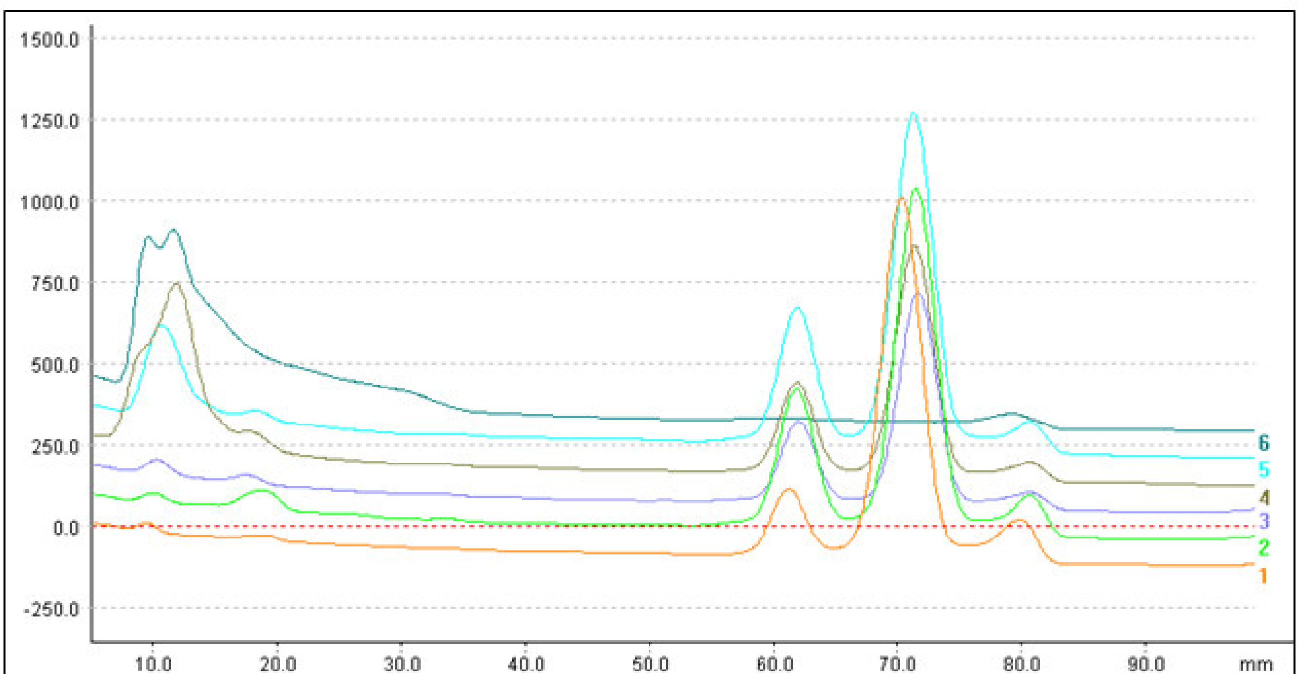


Figure 5
Over lay Densitogram showing the separation of peaks of M. communis L. flower at UV 580nm after exposed to Iodine vapours in 1. Petroleum ether extract 2. Chloroform extract 3.EthylAcetate 4. Methanol extract 5.Acetone extract 6. Aqueous extract.

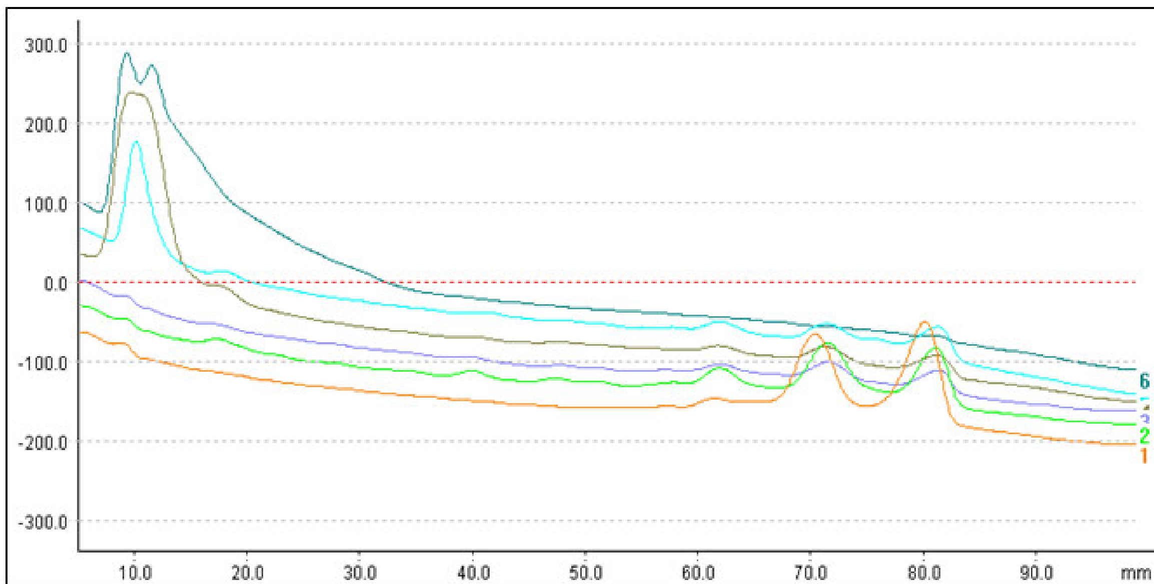


Figure 6
Over lay Densitogram showing the separation of peaks of M. communis L. flower at UV 580nm after derivatized with Anisaldehyde sulphuric acid in 1. Petroleum ether extract 2. Chloroform extract 3.EthylAcetate 4. Methanol extract 5.Acetone extract 6. Aqueous extract.