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Comparative studies on phytochemical and antimicrobial activity on aerial parts of *Careya arborea*

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Abstract
Careya arborea commonly called as kumbhi in Ayurveda. It is used as a astringent, demulcent, antipyretic and antipruritic, hepatoprotective, antimicrobial, antioxidant, anticoagulant activity, CNS activity, antileishmanial activity, antidiarrhoeal, analgesic, antitumour, cytotoxic activity. The authenticated barks were procured from Sri Venkateshwara University, Tirupathi. In the present study the driedand powdered drug was successively extracted with petroleum ether, chloroform, methanol, ethanol and water by continuous hot extraction using soxhlet assembly. The respective percentage yields were recorded with regular intervals. All the extracts were subjected to antimicrobial activity by agar diffusion method out of which the methanolic extract showed significant antimicrobial activity against bacteria and fungus.

Keywords: *Careya arborea*, antimicrobial activity, soxhlet assembly

Introduction
About eighty percent of the world population depends on herbal based alternative system of medicine (Ayurveda, Unani medicine, and Chinese traditional medicine). Except for homeopathy, the activities of these curative plants are evaluated by their chemical components. An estimated 70000 plants (including the lower plants) are used medicinally. The Chinese system depends on the 5757 plants listed in Encyclopedia of Traditional Chinese Medicinal Substances. Japanese and Korean system of medicine also includes a large number of medicinal herbs In WTO (World Trade Organization) perspective, all these plants are our common heritage [1]. Traditional plants which are available in India possess various pharmacological actions [2].
Careya arborea Roxb, commonly known as wild guava belonging to the family Lecythidaceae is known as Padmaka in Ayurveda. This plant extensively used in Indian-traditional medicine for the treatment of different diseases. *Careyaarborea* Roxb. was useful in tumours, cough, bronchitis, tooth ache, wounds, epilepsy, ulcer, colic, dysentery, leucoderma, smallpox, intestinal worms. [3]
The aerial parts of the plants will be considered to screen the various phytochemicals responsible for antimicrobial activity. This involves various methods of extraction for the crude plant material.
It contains terpenoids, flavonoids, coumarins, saponins, tannins. Particularly the stem-bark contains betulin and betulinic acid. Root bark contains-metformin as an active hypoglycemic principle. Leaves-contains triterpene ester, beta-amyrin, hexacosanol, taraxerol, beta-sitosterol, quercitin and taraxeryl acetate Seeds contains terpenoidsapogenol, sterols [3].

Materials and Methods
I. Collection and authentication of crude drug
The dried bark of *Careya arborea* was collected from Kerala. Identification and authentication of plant material was done by Dr. K Madhava Chetty, Asst. Professor, Dept. of Botany, Sri Venkateshwara University, Tirupathi. The respective part of plant materials were dried in shed and made into coarse powder for extraction.

II. Standardization of extract
The various standardization parameters studied were organoleptic properties, physico-chemical investigations, Fluorescence analysis and preliminary phytochemical analysis.
a) Pharmacognostic evaluation
- **Sensory characteristics:** In the present study the bark of *Careya arborea* were investigated for its macroscopy and microscopic characteristics as per WHO guidelines.

Macroscopic characteristics of bark of *Careya arborea* including appearance of outer surface, inner surface similarly the powder characteristics were studied [4].

b) Physico-chemical investigation

Physico-chemical investigation of extracts includes water soluble extractive value, Alcohol soluble extractive value, pet ether soluble extractive value, Chloroform soluble extractive value, total ash value, water soluble ash, and acid insoluble ash.

(i) Determination of water-soluble extractive

5 grams of air-dried bark was macerated with 100 ml of water in a closed flask, with shaking frequently during the first 6 hours and allowed to stand for 18 hours separately. Thereafter, it was filtered rapidly taking precaution against loss of water. Evaporated 25ml of filtrate to dryness in a tared flat bottom shallow dish dried at 105 ^{0}C and weighed. Percentage water soluble extractive was calculated with reference to the air-dried drug.

(ii) Determination of ethanol-soluble extractive

5 grams of air-dried bark were macerated with 100 ml of ethanol in a closed flask with shaking frequently during the first 6 hours and allowed to stand for 18 hours separately. Thereafter, it was filtered rapidly taking precaution against loss of ethanol. Evaporated 25ml of filtrate to dryness in a tared flat bottom shallow dish dried at 105 ^{0}C and weighed. Percentage ethanol soluble extractive was calculated with reference to the air-dried drug.

(iii) Determination of pet ether-soluble extractive

5 grams of air dried plant material were macerated with 100ml Pet. etherin a closed flask with shaking frequently during the first 6 hours and allowed to stand for 18 hours separately. Thereafter, it was filtered rapidly taking precaution against loss of chloroform. Evaporated 25ml of filtrate to dryness in a tarred flat bottom shallow dish dried at 105 ^{0}C and weighed. Percentage chloroform soluble extractive was calculated with reference to the air dried drug.

(iv) Determination of total ash

2-3 gm of bark powder was accurately weighed crude drug powder in a tared platinum or silica dish previously ignited and weighed. Scattered the powder drug on the bottom of the dish, incinerate by gradually increasing the heat not exceeding dull red heat until free from carbon, cool and weight. If a carbon free ash cannot be obtained in this way, exhaust the charred mass with hot water, collect the residue on an ash less filter paper, incinerate the residue and filter paper, add the filtrate, evaporate to dryness and ignite at a low temperature. Percentage of ash was calculated with reference to the air-dried drug.

$$\text{Total Ash value of the sample} = \frac{100\,(z - x)\,\%}{Y}$$

z = weight of the dish + ash (after complete incineration)
x = weight of the empty dish
y = weight of the drug taken

(v) Loss on Drying (LOD)

2-3 grams of powder was accurately weighed in a petridish and kept in a hot-air oven maintained at 110 ^{0}C for four hours. After cooling in desiccator, the loss in weight was recorded. This procedure was repeated till constant weight was obtained.

$$\text{Loss on drying (\%) (LOD)} = \frac{\text{Loss in weight}}{\text{Weight of the drug in gms}} \times 100$$

III. Preparation of extracts

Successive Solvent extraction

50gms of powdered drug was packed into a thimble each time and successively extracted with solvents in the increasing order of polarity viz. pet-ether 60-80 ^{o}C, chloroform, methanol and water until the solution in siphon tube was colourless. After the effective extraction, the solvent was distilled off, the extract was then concentrated on water bath and the extract obtained with each solvent was weighed. The extractive yield was calculated on air dried basis. The extract were concentrated by evaporating the solvent and stored at 4 ± 1.0 ^{o}C.

Preliminary phytochemical screening of extract

The various extracts of the selected plant materials were subjected to preliminary phytochemical screening to evaluate the primary and secondary cell metabolites like Carbohydrates, Proteins, Alkaloids, Glycosides, Tannins, Saponins, Flavanoids, Fixed oils, Phytosterols, Phenolic compounds etc [5, 6].

IV. Optimization of TLC system for methanolic extract

Various solvent systems were developed for TLC identification of constituents in the extract and the one showing efficient and clear separation was selected as mobile phase for the study.

Activation of the plates: The pre-coated plates were placed in a hot air oven and heated @ 105 °C for 2hrs.

Stationary phase: Precoated Silica gel 60 F_{254} (0.25µ) plates (Merk)

Mobile phase: n-butanol: Acetic acid: Water, Benzene: Metanol: Acetic acid, Hexane: Ethyl acetate, n-Butanol: Water, Pet ether: Ethyl acetate.

Sample preparation: 2mg of methanolic extract was dissolved in 5ml of methanol.

Visualization: UV at 254nm

Method: Sample solutions were applied in the form of bands on activated TLC plates using capillary tubes and developed in TLC chamber using suitable solvent system. Plates were air dried and observed under UV at 254nm. The solvent systems tried for the optimization of TLC solvent system are viz., Hexane: Ethyl acetate (1:1), Benzene: Methanol: Acetic acidv (45:8:40), n-butanol: Glacial acetic acid: Water (4:1:5), n-Butanol: Water (1:1), and Pet ether: Ethyl acetate (1:1).

V. Antimicrobial activity of extracts.

Antimicrobial activity of successive solvent extracts of *Careya arborea* was carried out by agar diffusion method. Bacterial cultures were grown on nutrient agar at 37±2^0 C 18 hours, fungal cultures were grown on potato dextrose agar medium at and colonies were suspended in sterile saline (0.85% Nacl) and its turbidity was adjusted to 10^8CFU/ml. Agar plates were seeded with bacterial cultures aseptically.

After solidification five wells were on agar plate by using cork borer of 6.0 mm size. Streptomycin was used as standard, 0.3ml of different concentrations of different extracts were introduced in each well respectively, DMSO was used as negative control and incubated for 24-48 hrs at 37±2 ^{0}C. Antimicrobial activity was carried out under strict aseptic condition in duplicate and zone of inhibition was measured in mm.

Microorganism used

Gram positive and Gram negative organisms *P. aeroginosa* (ATCC 9027), *E. coli* (ATCC 9837), S.aureus (ATCC 9886), *M. luteus* (ATCC 10240) and fungal organism such as *Aspergillus niger* (ATCC 16404), *Candida albicans* (ATCC 10231) were procured from KAPL Bengaluru India.

Preparation of Inoculums

Suspension of organism was prepared as per McFarland standard. A 24 hr old culture was used for the preparation of bacterial suspension. Suspension of organism was made in a sterile isotonic solution of sodium chloride (0.9% w/v) and the turbidity was adjusted such that it contained approximately 1.5X10 8cells/ml. It was obtained by adjusting the optical density of the bacterial suspension of 0.05ml of 1.175% of barium chloride and 9.95ml of 1%sulphuric acid.

(a) Procedure for preparation of bacterial inoculums

The medium was prepared by dissolving all the ingredients in distilled water and subjected to sterilization in an autoclave at 121^{0}C for 15 min. The petri plates were washed thoroughly and sterilized in hot air oven at 160 ^{0}C for 1 ½ hours. 30ml of sterile molten agar medium was seeded by organisms (about 2 ml according to McFarlands standard), in semihot conditions (40 ^{0}C) was poured aseptically in sterile petri plate and allowed to solidify at room temperature. Bores were made on the medium using sterile borer and 0.3 ml of the extracts at a different concentration were added to respective bore and 0.25ml of standard Streptomycin at a concentration of 100µg/ml was taken as a standard. The petri plate seeded with organisms, containing extracts and the standard were kept in refrigerator at 4 ^{0}C for 1 hour to facilitate the diffusion of the extracts and the standard into the media. After diffusion the petri plates were incubated at 37±2 ^{0}C for 24 hrs in BOD incubator and zone of inhibition was observed and measured using a scale.

(b) Procedure for preparation of fungal inoculums

The medium was prepared by dissolving all the ingredients in distilled water and subjected to sterilization in an autoclave at 121 ^{0}C for 15 min. The petri plates were washed thoroughly and sterilized in hot air oven at 160 ^{0}C for 1 ½ hours. 30ml of sterile molten agar medium was seeded by organisms (about 2 ml according to Mc Farlands standard), in semihot conditions (40 ^{0}C) was poured aseptically in sterile petri plate and allowed to solidify at room temperature. Bores were made on the medium using sterile borer and 0.3 ml of the extracts at a different concentration were added to respective bore and 0.25ml of standard Fluconazole at a concentration of 100µg/ml was taken as a standard. The petri plate seeded with organisms, containing extracts and the standard were kept in refrigerator at 4 ^{0}C for 1 hour to facilitate the diffusion of the extracts and the standard into the media. After diffusion the petri plates were incubated at 28 ^{0}C for 48hrs in BOD incubator and zone of inhibition was observed and measured using a scale.

Zone of inhibition

All extracts were dissolved in DMSO solution to get the different concentration (10, 20, 30, 40, 50 mg/ml). The antimicrobial activity was measured in triplicate analysis [7, 8].

Results and Discussion

1. The Pharmacognostic evaluation: The Pharmacognostic evaluation was carried out in *Careya arborea* by using leaves, bark and fruits for the appearance, outer surface, inner surface, colour, taste and texture (Table 1).

Table 1: Pharmacognostic Evaluation of *Careya arborea*

Macroscopic characteristics	Leaves	Barks	Fruits	Images
Appearance	Globrous, obovate, and small pieces	Thick, flat and small pieces	Round	 Leaves
Outer surface	Green, rough	Dark brown, rough	Lightish brown	
Inner surface	Light green	Whitish brown	Light red	 Bark
Colour	Green	Reddish brown	Greenish white	
Taste	Bitter	Bitter	Bitter	 Fruits
Texture	Rough and fibrous	Rough and fibrous	Rough	

2. Ash value in *Careya arborea*: The results of ash content quantification showed that the bark recorded highest total ash content (12.50) whereas leaves recorded least (8) (Table 2.0)

Table 2: Determination of ash Value in *Careya arborea*

Sl. No	Parts of the plant	Total ash	Water soluble ash	Acid insoluble ash
1	Leaves	8±12%	2.20±31%	1.40±10%
2	Barks	12.50±17%	2±32%	0.6±25%
3	Fruits	10±21%	2.12±11%	1.20±32%

3. Extractive value from different solvents: The extractive values is higher in of fruits in alcohol soluble extraction, water soluble extraction and acid soluble extraction whereas least in leaves (Table3).

Table 3: Determination of extractive Value from different solvents

Sl.no	Parts of the plant	Alcohol soluble extractive value (%)	Water soluble extractive value (%)	Petroleum ether extractive value (%)
1.	Leaves	4.3±22	13.66±10	8.75±22
2.	Barks	7.2±59	15±34	12±31
3.	Fruits	7.43±36	15.12±34	11.13±43

4. Moisture content of leaves, bark and fruits: The percent moisture contentquantification results showed that the percent loss on drying is more in bark (14.3) whereas least in fruits (10.0). The similar results were found in weight of china dish after drying (Table 4)

Table 4: Determination of moisture content leaves, bark and fruits

Sl.no	Parts of the plant	Weight of empty china dish (%)	Weight of drug (g)	Weight of china dish +drug	Weight of china dish after drying	Loss on drying	% loss on drying
1	Leaves	52.22	2.0	54.22	53.97	0.25	12.5±32
3	Barks	54.76	2.0	56.76	56.41	0.28	14.3±53
2	Fruits	52.22	2.0	54.22	54.02	0.20	10±11

5. Percentage yield of extracts obtained by successive solvent extraction: The percent yield of extracts by successive solvent extraction showed that the methanolic extract method gave highest percent yields of extracts in all bark, leaves and fruits compare to other solvents (Table 5)

Table 5: Percentage yield of extracts obtained by successive solvent extraction

Sl.no	Parts of the plant	Solvents	Weight of drug taken (g)	Weight of extract (g)	Yield (%)
1	Bark	Pet.ether	50	0.19	0.38
		Chloroform	50	0.47	0.94
		Methnol	50	2.43	4.86
		Ethanol	50	1.85	3.70
		Water	50	1.02	2.04
2	Fruits	Pet.ether	50	0.10	0.20
		Chloroform	50	0.12	0.24
		Methanol	50	1.54	3.08
		Ethanol	50	1.13	2.26
		Water	50	1.11	2.22

3	Leaves	Pet.ether	50	0.14	0.28
		Chloroform	50	1.20	2.40
		Methanol	50	2.10	4.20
		Ethanol	50	1.15	2.30
		Water	50	1.18	2.36

6. Preliminary phytochemical screening of successive extracts: To find the best extraction method in leaves, bark and fruits were quantified using different identification method in primary and secondary metabolites. The saponins, phytosterols, triterpinols, flavonoids and fixed oils and fats tests found best metabolic identification methods compared to other tests (Table 6).

Table 6: Preliminary phytochemical screening of extracts

Metabolites	**Parts of the plant**	**Leaves**					**Bark**					**Fruit**				
	Extracts	**P.E**	**C.E**	**M.E**	**E.E**	**A.E**	**P.E**	**C.E**	**M.E**	**E.E**	**A.E**	**P.E**	**C.E**	**M.E**	**E.E**	**A.E**
Test for carbohydrates	Molisch test	_	_	_	_	_	_	_	_	_	_	+	+	+	+	+
	Felhing test	_	_	_	_	_	_	_	_	_	_	_	+	_	+	_
	Benedicts test	_	_	_	_	_	_	_	_	_	_	_	_	_	+	_
	Barfoeds test	_	_	_	_	_	_	_	_	_	_	_	_	_	+	_
Test for proteins	Millons test	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
	Biuret test	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
	Ninhydrin test	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Test for alkaloids	Mayers test	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
	Wagners test	+	+	_	_	_	_	_	_	_	_	_	_	_	_	_
	Hagers test	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
	Dragendraffs test	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Test for glycosides	Brontragers test	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
	Legal test	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Test for saponin	Salkowaski test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Libermann test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Test for phytosterols	Buchard test	+	_	+	+	_	+	+	+	+	+	+	+	+	+	+
Test for fixed oils and fats	Spot test	_	_	+	+	+	+	+	+	+	+	+	+	+	+	+
	Saponification test	+	_	+	+	+	+	+	+	+	+	+	+	+	+	+
Test for phenols	$Fecl_3$ test	_	_	+	+	+	_	+	+	+	+	+	+	+	_	_
	Gelatin test	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Test for triterpinoids	Salkowaski test	_	_	+	+	+	+	+	+	_	+	+	+	+	+	+
	Libermann test	_	_	+	+	+	+	+	+	+	+	+	+	+	+	+
Test for flavonoids	Alkaline test	_	_	_	_	_	_	_	_	_	_	_	_	+	+	_
	Shinoda test	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_

Note: PE: Pet ether extract, CE:Chloroform extract, E.E.: Ethonolic extract, M.E.:Methonolic extract, A.E.:Aqueous extract,

7. Optimization of solvent system for active methanolicextact; Out of various solvent systems n n-butanol: Glacial acetic acid : Water(4:1:5) was found to be most suitable solvent system (Table 7).

Table 7: Optimization of solvent system for active methanolic extract

Sl. No.	Solvent system	Detection using UV at 254 nm	
		No. of bands	Rf value
1.	Hexane : Ethyl acetate	Single band	0.6
2.	Benzene : Methanol : Acetic acid (45:8:4)	No separation	-
3.	n-butanol : Glacial acetic acid : Water(4:1:5)	Three bands	0.7,0.82,0.74
4.	n-butanol : Water (1:1)	Single band	0.32
5.	Pet ether : Ethyl acetate (1:1)	No separation	_

8. Antimicrobial activity of methanolic extract: The of methanolic extract of bark of *carea arborea* showed highest antibacterial and antifungal activity by using streptomycin (100µg/ml) as a standard for bacteria and Fluconazole (100µg/ml) for fungi. The highest zone of inhibition for bacteria found in *E. coli* and in fungi showed in *candida albicans* (Table 8 and Fig 4).

Table 8: Antimicrobial activity of methanolic extract

Sl.no	Bacteria and Fungi	Concentration (mg)	Standards		Zone of inhibition (mm)
			Strptomycin 100µg/ml	Fluconazole 100µg/ml	
1.	M.luteus	50	8.3±33	-	8.2±16
2.	S.aureus	50	8.6±66	-	8.4±16
3.	E.coli	50	9.0±00	-	9.0±04
4.	P.aeroginosa	50	8.6±66	-	5.1±14
5.	A.niger	50	-	8.6±66	8.0±09
6.	C.albicans	50	-	9.0±00	9.0±04

Powder microscopy of bark, leaves and fruit: The powder microscopy characters were examined in bark, leaves and fruit and clear distinct appearance in cork cells, fibres, starch grains, stone cells, palisade cells (Fig 1, 2 and 3).

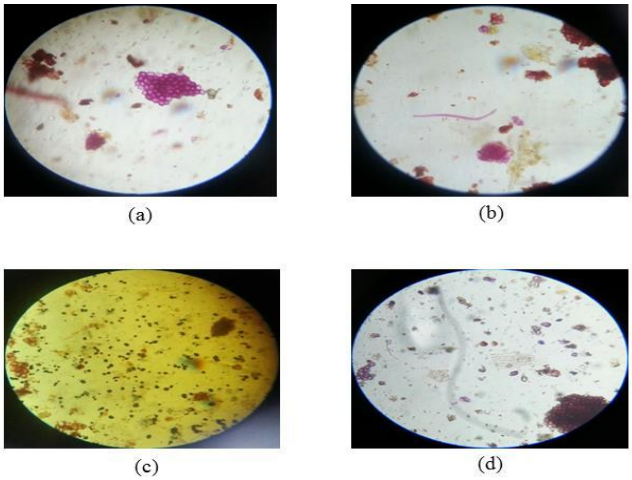

Fig 1. Powder microscopy of bark. (a) Cork cells and parenchymatous cells (b) lignified fibres (c) Starch grains (d) Calcium oxalate crystals.

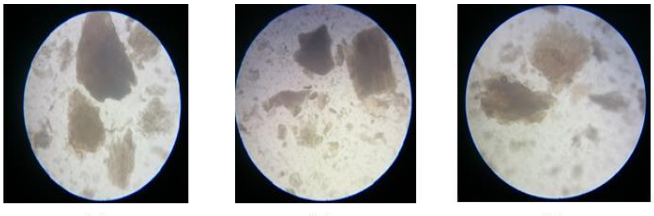

Fig 2. Powder microscopy of fruits. (a)Collenchyma (b) Stone cells (c) Parenchyma

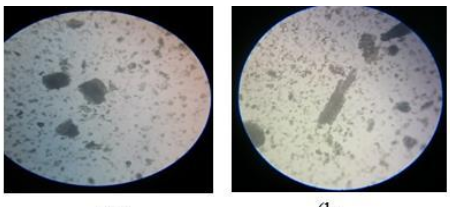

Fig 3. Powder microscopy of leaves (a)Fragments of Parenchyma (b)Palisade cells

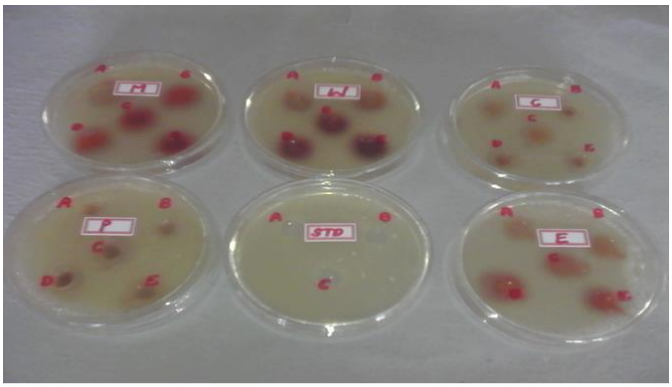

Fig 4. Antimicrobial activity of successive solvent extracts of *Careya arborea*

Preliminary photochemical screening of extract

The successive solvent extracts of the leaves, bark, fruit of *Careya arborea* were screened for various tests mentioned in methodology.

Conclusion

- The drug was subjected to preliminary pharmacognostic evaluation as per WHO guidelines. Microscopy, macroscopy, ash values, extractive values, loss on drying.
- Successive extraction was carried out with solvents viz., petroleum ether, chloroform, methanol, ethanol, water by continuous hot extraction using soxhlet apparatus followed by refluxation with water. The percentage yield of extracts was calculated.
- The preliminary phytochemical screening of extracts revealed the presence of tannins, flavonoids, saponins, phytosterols, fixed oil and fats, phenolics compounds, and triterpinoids in methanolic, ethanolic and aqueous extract of *Careya arborea.*
- Successive solvent extracts of barks, leaves, fruits of *Careya arborea* was screened for antimicrobial activity against two Gram +ve strains such as *staphylococcus aureus, Micrococcus luteus*and two Gram –ve strains such as *Escherichia coli, Pseudomonas aeurogenosa* and fungal strains such as *Aspergillus niger, Candida albican.*
- In comparison with standard antibiotic Streptomycin and Fluconazole. The DMSO was used to dissolve the various extracts respectively. The result showed that, methanolic extract could inhibit growth of all test organisms in 50mg/ ml concentration ranges.
- The methanolic extract showed significant antimicrobial activity. Hence this extract was chosen for separation of compounds by column chromatography.

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