

## Exploring the Phyto-Constituents of *Ruellia patula* (Acanthaceae) as Antibacterial Agent

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### Abstract

The present study aims to identify a suitable solvent system for extracting high yield biologically active phyto-constituent in *R. patula*. Dried and then powdered plant material was subjected to extraction using different solvents based on the order of polarity and profiled for total phenol and flavonoid content along with antioxidant, which yielded high and represented the antioxidant ability in terms of DPPH and reducing power assay. Antimicrobial activity revealed chloroform, ethanol and methanol extracts to perform better compared to other extracts and exhibited a significant broad-spectrum antibacterial activity against both gram-positive and gram-negative bacteria. In quorum sensing activity, these extracts showed high anti-biofilm adhesion activity than anti-biofilm disturbance activity. Through GC-MS analysis, eleven, nine and six compounds each were identified from highly bio active extracts of ethanol, methanol and chloroform respectively. This perhaps supports the claim of efficacy reported in the divergence use of plants in the treatment of disease caused by some human pathogens, further providing scope for extending research in the direction of purifying the compounds as a futuristic study.

**Keywords:** *Ruellia patula*; Phytochemical Analysis; Antioxidant; Antimicrobial; Anti-Quorum Sensing Activity; GC-MS Analysis

### Introduction

Scientific research in herbs have received enormous attention due to their numerous health benefits at cheaper production cost and minimum side effects [1]. The presence of phenols and flavonoids render properties such as antibiotic, disinfectant, antioxidant, anti-inflammatory, anti-estrogenic, anti-carcinogenic, and anti-mutagenic activities [2-5]. They have the ability to scavenge the free radicals and donate hydrogen atom [6,7] which protects from oxidative damages [8]. In fact, processing through solvent extraction is stable, reliable, and reproducible to determine the bioactive compounds, because, the solubility of the bioactive compounds depends on the polarity of solvent, degree of polymerization of compound and the interaction between the solvent and compound [9,10]. Moreover, polarity of the solvents significantly affect the polyphenol contents, finding the suitable extracting solvent becomes necessary [3] and therefore was aimed in the present study to explore the suitable and standard solvent system ideal for high recovery of bioactive compounds in the plant of interest.

*Ruellia patula* Jacq. Syn. *Dipteracanthus patulus* Nees (vernacular names includes kiranthinayagam, chilanthinayagam, kayappacchilai, Tutadi, and sisodi) of *Acanthaceae*, distributed around tropical and subtropical regions of Africa, Arabia, Sri Lanka, Pakistan, Burma, and India (especially in the pockets of Rajasthan, Haryana, Tamil Nadu, Western Ghats, and Andhra Pradesh) is generally used as medicine

for many diseases viz., gonorrhoea, syphilis, eye sore, renal infection, cough, scalds, stomach ache, kidney stones, tumors, rheumatism, dental problems, itches, insect bites, paronychia, and ulcers [11]. Since the juice of leaves acts as sedative, roots as antipyretic, leaf paste as anti-inflammatory and flower and raw fruits as anti-diabetic in siddha medicine is considered as an important plant in folk medicine [12]. However, scarce information is available on the bioactive principles especially under different extracts based on solvent polarities. Therefore, it was aimed to evaluate the yield of total phenol and total flavonoid content along with antioxidant, antimicrobial, and anti-quorum sensing potential under different solvent system selected. In fact, the Industrial Botanical Drug Products guidelines of US Food and Drug Administration (FDA) indicates that characterization of drugs using spectroscopic or chromatographic fingerprints and chemical assay are necessary for identification of potential drug compound and marketing purpose also [13], hence, the study was further extended for chemical fingerprinting through GC-MS of highly bioactive extractions.

### Materials and Methods

#### Chemicals and Instruments

Analytical grade of Petroleum ether, chloroform, ethyl acetate, acetone, ethanol, methanol, Muller Hinton agar and broth (Himedia). Folin-Ciocalteu reagent, sodium carbonate ( $\text{Na}_2\text{CO}_3$ ), gallic acid, aluminum chloride, rutin, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Dimethyl sulfoxide (DMSO), potassium phosphate buffer, potassium ferricyanide, Trichloroacetic acid (TCA), Ferric chloride, and ascorbic acid were purchased from Sigma Aldrich, Bangalore, India. Spectra Max M2e Microplate reader, Spinco Biotech, India was used for microplate analysis. Gas Chromatography Mass Spectroscopy (GC-MS) analysis was done by JEOL GCMATE II operating in EI mode at 70 eV (Agilent Technologies 6890N Network).

#### Microbial strains and culture maintenance

Gram positive bacteria (*Staphylococcus aureus* MTCC 96, *Micrococcus luteus* MTCC 106 and *Bacillus cereus* MTCC 441), gram negative bacteria (*Pseudomonas aeruginosa* MTCC 4673, *Escherichia coli* MTCC 739 and *Vibrio vulnificus* MTCC 1145) were collected from Department of Microbiology, Pondicherry University, Pondicherry. The bacterial culture stocks were maintained on Muller Hinton agar plate and sub cultured on Muller Hinton broth (pH 7) at 37°C for 6 hours and adjusted to a turbidity of 0.5 prior to each antibacterial experiment.

#### Source of plant material

Plants were collected from the Madurai District of Tamilnadu during flowering stage in November 2014 for extraction and analysis, whose voucher specimen (No: Binfo 001 2014/4) was deposited in the herbarium of the Department of Ecology and Environmental Sciences, Pondicherry University, Pondicherry, India.

#### Collection and extraction of plant

Collected fresh, well grown and healthy whole plant of *R. patula* was washed with running water to remove soil particles and other adhered debris and finally washed with sterile distilled water, drained, shade dried and pulverized to coarse powder in a mechanical grinder. Extraction of secondary metabolites was performed according to the protocol of [14], where 5 gm of powder was transferred to flasks containing 200 ml of different solvents [in the order of polarity as Petroleum Ether (1PE), Chloroform (2CH), Ethyl acetate (3EA), Acetone (4A), Ethanol (5E), Methanol (6M), and water (7W)] and kept for 3 days at room temperature while shaking the flask for every 6 hours. The remnant left after filtering with whatman filter paper No.1 was extracted further until it turned pale. The pooled extracts were evaporated to complete dryness using a rotary evaporator at 40°C and stored at 4°C for further use.

#### Total phenol content (TPC)

Total phenolic content of the extracts was measured [15], by adding 25  $\mu\text{l}$  of diluted (1:10 folds) Folin-Ciocalteu Reagent in 96 well

plate each seeded with the mixture of 25 µl extracts (1PE-7W) and 75 µl of distilled water and incubated for 6 min. 100 µl of Na<sub>2</sub>CO<sub>3</sub> (7.5% w/v) was added and incubated for 90 min in the dark. After which the plate was shaken for 60 seconds. The optical density was measured at 765 nm. The mixture without extracts was used as negative control while Gallic acid was used as positive control and expressed as GAE mg/G.

### Total Flavonoid Content (TFC)

Total flavonoid content of the extracts was measured by aliquoting 100 µl of extract (labeled as 1PE to 7W) to 100 µl of 2% aluminum chloride and incubated for 1 hour at room temperature to measure optical density at 420 nm [16]. Rutin was used as standard and total amount of flavonoid was expressed as rutin mg/G.

### Antioxidant Assay

#### 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The addition of 100 µl of DPPH (200 µl of DPPH was prepared as stock in 80% methanol) to equal amount of different extracts of varying concentration (25 - 800 µg/ml) in the 96 well plate containing reaction mixture was incubated in dark at 37°C for 30 min to measure the OD at 517 nm [16]. Ascorbic acid was used as a positive control, while methanol and DMSO were used as blank for polar and non-polar solvent system respectively, DPPH without sample was used as negative control. Scavenging activity was calculated using the formula:

$$\text{Percentage of radical scavenging activity} = 1 - \frac{(\text{sample} - \text{blank})}{(\text{control} - \text{blank})} \times 100$$

### Reducing power Assay

For the reducing power assay, 300 µl of 0.1 M potassium phosphate buffer (pH 6.6) and 500 µl of potassium ferricyanide (1% w/v) was added to 200 µl of sample and incubated for 20 min at 50°C. After cooling the mixture, 500 µl of TCA (10%) was added and centrifuged for 10 min at 1200g. To 100 µl of the supernatant, 100 µl of water and 20 µl of FeCl<sub>3</sub> (0.1% w/v) were added and absorbed at 700 nm using ascorbic acid as the standard [17].

### Antibacterial Disc Diffusion Assay

Antibacterial activities of seven different extracts of *R. patula* were assayed against three Gram-negative and three Gram-positive bacteria using the disc diffusion method [18]. Briefly, 100 µl of bacterial suspension was spread on Muller Hinton agar plates. 1 mg/ml of each extracts was prepared by dissolving in 10% dimethyl sulfoxide. The discs (5 mm Whatman filter paper in diameter) were impregnated with 10 µl (1 mg/ml) of each extracts and placed on the inoculated agar plates to incubate at 37°C for 24 h. The diameters of the zone of inhibition were measured in millimeter, excluding the diameter of the disc. Equivalent quantities of DMSO was used as control.

### Minimum Inhibitory Concentration (MIC)

In order to measure the minimal inhibitory concentration (MIC) of the active extracts against the microbes [19], method was followed with slight modification. Based on the antibacterial results of disc diffusion assay, two-fold serial dilutions of highly active extracts 2CH, 5E, and 6M were prepared by dissolving 12.5, 25, 50, 100, 200, 400, 800, and 1600 µg/ml each in DMSO. Further, 100 µl of prepared extracts in defined concentration was added to the mixture of 100 µl of Muller Hinton broth culture inoculums into 96 well plate. Later, 10% DMSO was added instead of plant extracts to serve as a blank, while 200 µl of Muller Hinton broth with 20 µl of each inoculum was maintained as a control. The entire set up was maintained in triplicate and incubated at 37°C for 24 hours to observe OD at 540 by using Microplate reader.

## Ani-quorum sensing

### Biofilm Disturbance Assay

In order to ascertain the anti-biofilm disturbance potential of active extracts of *R. patula* against biofilm forming bacteria (Gram positive - *Staphylococcus aureus*, Gram negative - *Pseudomonas aeruginosa*), crystal violet staining method was followed with some modification as described by [20]. Accordingly, to 200  $\mu$ l of MHB medium, 20  $\mu$ l of the 6 hours grown cultures were seeded and incubated for 24 hours at 37°C, where, the non-adherent cells were gently removed by washing three times with sterile distilled water and fixed (dried). Later 200  $\mu$ l of different plant extracts (1mg/ml) was introduced into the wells and incubated for another 24 hours at 37°C, thereafter the plates were washed with distilled water thrice and air dried to further add 200  $\mu$ l of 0.1% crystal violet and incubated for 20 minutes. After 20 minutes, the plate was washed with sterile distilled water to remove excess stain and air dried followed by adding 200  $\mu$ l of 90% ethanol to release the stain. Control was prepared by adding medium and bacterial culture into the well, while wells containing medium and extracts alone were considered as blank, which were maintained for each test batch. The entire set was maintained in triplicate and disturbance of biofilm was measured by observing OD at 490 nm, while percentage of disturbance was calculated by the formula.

$$\text{Inhibition \%} = (\text{OD control} - \text{OD sample}) / (\text{OD control}) \times 100.$$

### Biofilm Adhesion Assay

To determine the adhesion effect of extracts, 200  $\mu$ l (1 mg/ml) of each extracts were transferred into a sterile 96-well polystyrene microtiter plate and dried completely, to add 200  $\mu$ l of MHB medium and 20  $\mu$ l of cultures (6 hours) and incubated for 24 hours at 37°C. Subsequently, non-adherent cells were removed and washed with distilled water for three times [21]. Above mentioned procedure of crystal violet staining was followed and the adhesion effects of the extracts were calculated using above formula.

## GC-MS Analysis

Based on the phenolic contents, antioxidants, anti-microbial and quorum sensing activities, highly bioactive solvent extracts were selected for chemical finger printing by GC-MS. Thus, 1  $\mu$ l of each extracts (0.1% w/v) was subjected to GC-MS analysis using JEOL GCMATE II GC-MS operating in EI mode at 70 eV (Agilent Technologies 6890N). The HP5 MS column was prepared with fused silica (30 m x 0.25 mm). Analyzing condition was fixed as 50 - 250°C at the rate of 10°C min<sup>-1</sup> with 220°C for injector temperature and high pure helium as the carrier gas. Molecular weight, structure, and other parameters were ascertained by interpretation of mass spectrum with widely used mass spectral library NIST EI-MS database [22]. The biological activity of the identified compounds was searched against *Dr. Duke's* Phytochemical and Ethnobotanical database (<https://phytochem.nal.usda.gov/phytochem/search>).

## Calculating the Peak area

The area of the peak is proportional to the amount of available compound, which was approximated by treating the peak as triangle and the area of a triangle by multiplying the height of the peak times to its width at half height. Using area, the percent of each compound in the sample was calculated using the formula:

$$\text{Percent compound 1} = \frac{\text{Area of compound 1}}{\text{Total area}} \times 100$$

## Statistical Analysis

All experiments were done in triplicates and results were expressed as mean  $\pm$  SD, using Predictive analytics software and solutions (SPSS 16.0 from SPSS Inc. Chicago, IL) and Prism Graph Pad 7.

## Results and Discussion

### Phytochemical Analysis

In pharmaceuticals, nutraceuticals, and cosmetic products, pulling out of phytochemicals from plant material is the foremost step [23]. Among the different strategies of milling, grinding, extraction, and homogenization [13], extraction is commonly applied due to its simplicity, efficiency, and wide applicability. However, picking out the appropriate solvent system is most important [24] in extraction for effective quantification. In this study, we used seven different solvents separately to identify the best solvent suitable for high yield of bio-active compounds in *R. patula*. More yield of the extracts per gram of dried plant material was obtained in ethanol (636.5 mg/G) followed by petroleum ether (589.5 mg/G) (Table 1). However, more or less equal quantity in both chloroform and ethyl acetate (338.6 mg/G and 328 mg/G) respectively was yielded, while least amount of 50.1 mg/G was recorded in acetone, which was approximately 12 fold reduced. These results suggest the interplay of the polar solvents to influence in higher recovery of the crude extracts indicating the fact that the yields depend on the parameters such as polarities of the solvent, time given for extraction, temperatures, chemical, and physical features of the compounds present in the plant material [25]. Thus, the difference in the yield might have been due to the solvents used [26] as well as solubility of various phytochemicals like oils, carbohydrates, and polyphenols etc., [27].

Extracts	Yield percentage (%)
1PE	58.95
2CH	33.86
3EA	32.8
4A	5.01
5E	63.65
6M	16.39
7W	20.24

**Table 1:** Yield of extracts per gram of dried plant material.

The values are in percentage; 1PE: Petroleum Ether; 2CH: Chloroform; 3EA: Ethyl Acetate; 4A: Acetone; 5E: Ethanol; 6M: Methanol; 7W: Water.

### Total phenol content (TPC) and Total Flavonoid content (TFC)

Phenolic compounds are abundant in plants [28], and commonly found as esters and glycosides [29]. Among the different extracts, highest phenol content of 61.67  $\pm$  2.4 mg/G was observed in ethanol (5E) extract followed by acetone extract (4A - 53.01  $\pm$  8 mg/G) (Figure 1). Perhaps, this is in agreement with the results of [30,31], where, methanol, ethanol, and acetone extracted higher levels of phenolic compounds typically from plant materials. However, the lowest amount of TPC (1PE - 0.89  $\pm$  0.07 mg/G) extracted by petroleum ether ensured the ability of polar solvents to extract more phenolics. Phenols are easily soluble in polar protic solvents, some prefer water, dichloromethane, and acetone. Phenols have non-polar aromatic ring attached by one or more polar hydroxyl group. Stereochemistry and the intermolecular forces like hydrogen bonds between phenols and solvent determine the tendency of the solubility. Hydrogen bonds formed between the electronegative oxygen of ethanol, methanol or acetone, and hydroxyl group or oxygen atoms, present inside the phe-

nol molecules might have contributed to the fluctuation in the dissolution of phenols in the different solvents [32]. Ethanol and methanol, although are less polar than water, but more efficient than water in denaturing the cell walls, which aids in the release of polyphenols from cell [33], thus, this property has determined the lowest yield by water (7W-13.15 mg/G). Methanol is effective in extraction of lower molecular weight polyphenols. Acetone, which preferred higher molecular weight flavanols [23], has resulted in higher yield of 53.01 mg/G than methanol 30.15 mg/G. According to the suggestion of [34], bigger molecules like phenols with longer aliphatic fragments prefer ethanol and non-polar solvents like petroleum ether, subsequently in the present study also the solubility of the phenol preferred intermediate polarity solvents like alcohols and acetone.

In concordance to the above represented factors, equitable measure of total flavonoid contents was absorbed, where a maximum of  $86.14 \pm 1.7$  mg/G and  $71.4 \pm 2.6$  mg/G TPC was recovered in ethanol (5E) and acetone (4A) respectively (Figure 1). Interestingly, trace amount of  $0.72 \pm 0.02$  and  $0.91 \pm 0.11$  mg/G was witnessed in ethyl acetate and Pet ether proving that whether TPC or TFC, both exhibited steric interactions with strong hydrogen and hydroxyl bonds that perhaps increases the dissolution rate in determining their contents in the solvents used. Thus, varying solubility of chemical constituents are greatly influenced by the solvent polarity [3]. Less polar flavonoids like flavanones and flavonols prefer chloroform and ethyl acetate, however, flavonoid glycosides and more polar aglycones prefer alcohol or alcohol water mixtures solvents, while flavan-3-ols like catechins and condensed tannins prefer water alone. Hence, the present observation resulting in highest records of both TPC and TFC in polar solvents (5E, 4A and 6M) is in synchrony with the above review and suggest that the sample of interest may contain more aglycones and flavonoid glycosides than others.

### Antioxidant Assays

Antioxidants are compounds which can delay, inhibit or prevent the oxidation by scavenging the free radicals [23] and the antioxidant potential of the plant material commonly appears to associate with the phenolic content [35]. Phenols balancing the oxidants by hydroxyl groups as well as by electron-donating and electron-withdrawing substituents are present in the ring structure [8,36]. Although, different bio-analytical reduction methods are available for evaluation of antioxidant capacity, DPPH and reducing power assays provide sensitive and stable results [37]. Though, they differ in their principle and experimental conditions [38], the former is classified into fast-kinetics, fast + slow-kinetics, and slow-kinetics based on the antiradical groups which they possess [39], while the latter is linearly related to antioxidant concentration in sample [38]. In the presented study the antioxidant activity was determined by DPPH which defines the ability to quench free radicals and reducing power.

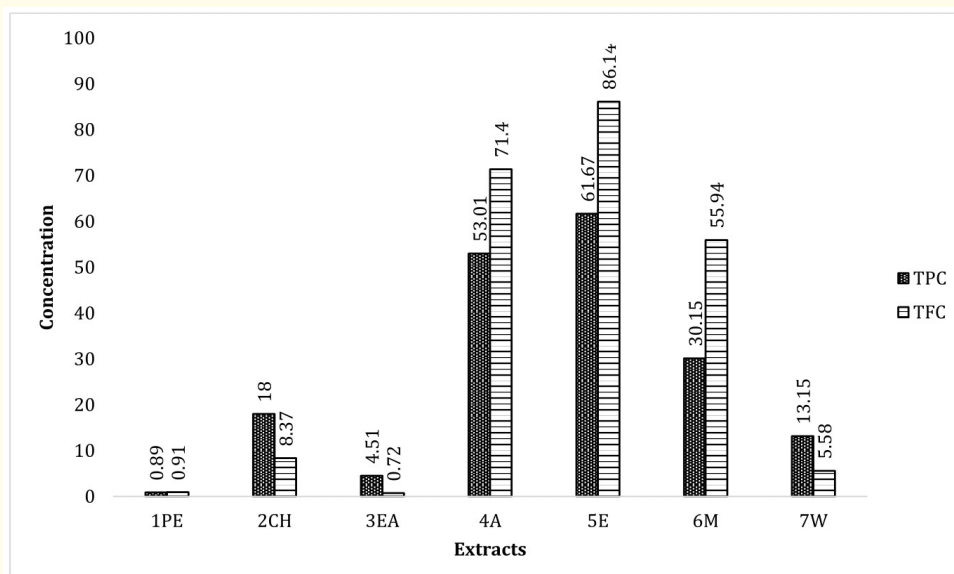
### DPPH 2, 2-Diphenyl-1-picrylhydrazyl Assay

DPPH method is widely applied to estimate antioxidant activity by the degrees of the polarities of the solvent systems [8,40]. Different solvent extracts of *R. patula* (1PE-7W) in varying concentration of 25 - 800  $\mu\text{g/ml}$  showed different inhibitory concentration (IC<sub>50</sub>) values based on their scavenging capacity (Table 2), Ethanol extracts showed robust reducing capacity of 70  $\mu\text{g/ml}$ , whereas no activity was observed in petroleum ether even above 800  $\mu\text{g/ml}$ , probably due to the presence of high contents of both phenols and flavanoids detected (Figure 1), indicating the fact that solubility and stereo selectivity of the radicals are proportionate to the presence of both phenol and flavonoid. 5E, 2CH and 6M had high antioxidant activity probably due to the presence of better antioxidant molecules. The yield of the extract is influenced by the selection of solvent present in the plant sample, however, it does not imply the fact that higher yielded solvent extracts will have higher biological (antioxidant) activity [41,42]. Thus, Acetone (4A - 300  $\mu\text{g/ml}$ ) was higher in TPC and TFC than methanol (6M) but was less effective in scavenging activity compared to methanol. Small phenolics prefer methanol and they are easily oxidized and possess pro-oxidant activity, which might be the cause for high antioxidant activity of methanol with less phenolic content [23]. Even though chloroform (125  $\mu\text{g/ml}$ ), ethyl acetate (320  $\mu\text{g/ml}$ ), and water (350  $\mu\text{g/ml}$ ) extracts exhibited less TPC and TFC, their scavenging activity was higher than methanol (195  $\mu\text{g/ml}$ ) relating the effect of solubility and stereo selectivity of the radicals in both polar and non-polar compounds present in the extracts [8] to be responsible for diversity of outcome [40].

Extracts	1PE	2CH	3EA	4A	5E	6M	7W
IC <sub>50</sub> values (µg/ml)	ND	125	320	300	70	195	350

**Table 2:** Effect of solvent system on DPPH Assay of *R. patula*

The values are in µg/ml; 1PE: Petroleum Ether; 2CH: Chloroform; 3EA: Ethyl Acetate; 4A: Acetone; 5E: Ethanol; 6M: Methanol; 7W: Water.



**Figure 1:** Effect of solvents system on Total phenol content (TPC) and Total Flavonoid content (TFC) in *R. patula*. The values are in µg/ml; 1PE-Petroleum Ether; 2CH- Chloroform; 3EA- Ethyl Acetate; 4A- Acetone; 5E- Ethanol; 6M- Methanol; 7W- Water.

### Reducing power Assay

Reducing power assay, which gives various green and blue shades by antioxidants, based on the reduction of the Fe<sup>3+</sup> to Fe<sup>2+</sup> was absorbed at 700 nm [43], which revealed the proportional increment in extracts concentration with activity. Polar solvents had high reducing power capacity compared to non-polar solvent extracts (Table 3). However, among the polar solvents examined, although all of them (5E, 6M, 4A and 7W) exhibited marginal differences, ethanol was identified to lead, which ranged from 0.16 + 0.002 to 0.20 + 0.003, signifying its role in accordance to the standard used. In the meanwhile, the non-polar solvents showed negligible activity compared to polar solvent extracts, that ranged from 0.03 + 0.002 to 0.07 + 0.004, indicating the fact that the reductions present in the polar solvents assisted in stabilizing and blocking the chain reaction of free radicals.

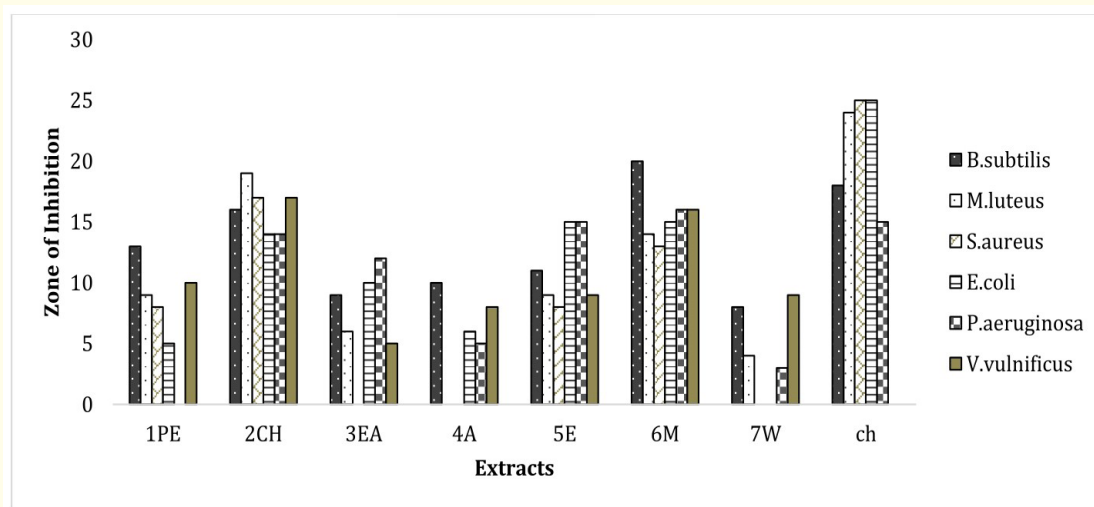
Extracts conc. µg/ml	25	50	75	100	125	150	175	200
1PE	0.04 ± 0.005	0.04 ± 0.005	0.04 ± 0.004	0.05 ± 0.005	0.05 ± 0.002	0.05 ± 0.003	0.06 ± 0.005	0.07 ± 0.004
2CH	0.03 ± 0.002	0.04 ± 0.003	0.05 ± 0.002	0.06 ± 0.003	0.06 ± 0.005	0.06 ± 0.003	0.06 ± 0.004	0.10 ± 0.002
3EA	0.04 ± 0.001	0.04 ± 0.004	0.05 ± 0.002	0.05 ± 0.002	0.06 ± 0.002	0.07 ± 0.003	0.08 ± 0.001	0.09 ± 0.010
4A	0.14 ± 0.016	0.17 ± 0.003	0.18 ± 0.004	0.18 ± 0.004	0.18 ± 0.004	0.18 ± 0.004	0.19 ± 0.001	0.19 ± 0.013
5E	0.16 ± 0.002	0.16 ± 0.008	0.17 ± 0.007	0.17 ± 0.008	0.18 ± 0.007	0.18 ± 0.013	0.19 ± 0.009	0.20 ± 0.003
6M	0.15 ± 0.004	0.16 ± 0.002	0.16 ± 0.004	0.16 ± 0.002	0.17 ± 0.005	0.17 ± 0.004	0.17 ± 0.003	0.19 ± 0.020
7W	0.15 ± 0.006	0.15 ± 0.006	0.16 ± 0.005	0.16 ± 0.005	0.16 ± 0.004	0.16 ± 0.001	0.17 ± 0.004	0.18 ± 0.006
Standard	0.53 ± 0.018	0.56 ± 0.006	0.64 ± 0.011	0.74 ± 0.007	0.77 ± 0.017	0.85 ± 0.127	1.25 ± 0.011	1.29 ± 0.039

**Table 3:** Effect of different solvent extracts of *R. patula* on reducing power assay

The values are in OD and expressed as mean ± SD (n = 3); 1PE: Petroleum Ether; 2CH: Chloroform; 3EA: Ethyl Acetate; 4A: Acetone; 5E: Ethanol; 6M: Methanol; 7W: Water.

**Disc diffusion antibacterial activity of extracts of *R. patula***

*In-vitro* anti-bacterial activity of different extracts of *R. patula* was studied against three gram positive and three gram negative bacteria using the disc diffusion assay. Interestingly, all extracts had inhibitory activity, if not for all, at least for few pathogen tested (Figure 2). Among the extracts methanol, chloroform, and ethanol showed non-selective antibacterial activity. However, methanol and chloroform although recorded high zone of inhibition against gram positive bacterium *B. subtilis* and *M. Luteus* respectively, they were considerably on par (20 mm and 19 mm) with the standard used. In contrast, ethanol extract was very active towards gram negative bacteria compared to gram positive bacteria. Petroleum ether, ethyl acetate, acetone, and water extracts showed selective antibacterial activity, where petroleum ether showed moderate inhibitory activity (ranged from 5 - 13 mm) against all gram positive bacteria, while ethyl acetate exhibited (5 - 12 mm) moderate activity against gram negative bacteria. Acetone extract was partially active against gram positive *B. subtilis* (10 mm), while inactive against all selected gram negative bacteria. Water extract was totally inactive against all the tested bacteria (3 - 9 mm), signifying the fact that the bioactive composition of extracts and its mechanism of action [26], the structure of the compounds present in the extracts and bacterial strain [44] to determine the antimicrobial activity. Values of the inhibition zone produced in our results also suggest that the high activity of the extracts on gram positive bacteria could be attributed to the compounds present in the extracts, which may disturb the cell wall to disturb the organism. While, it lower activity against gram negative bacteria may be attributable to the presence of outer hydrophilic membrane which makes it difficult to penetrate due to the high portion of lipid content. However, its moderate activity against gram negative bacteria could be due to the presence of lipophilic molecules which disturb the membrane of gram negative bacteria. 2CH, 5E, and 6M showed high activity against all selected organisms irrespective of gram positive and gram negative bacteria which was further investigated through MIC.



**Figure 2:** Disc diffusion antimicrobial activities of different extracts of *R. patula*.

Values are in mm; 1PE-Petroleum Ether; 2CH- Chloroform; 3EA- Ethyl Acetate; 4A- Acetone; 5E- Ethanol; 6M- Methanol; 7W- Water; ch- Chloramphenicol.



**Minimum inhibitory concentration (MIC) of extracts of *R. patula***

The MIC is the lowest concentration for complete inhibition of microbial growth [45]. However, the activity of the compound depends on diffusion nature of the compound, which exhibits higher activity at lower concentration or may show lesser activity at higher concentration too. Hence, it is essential to check whether the zone of inhibition is due to the polarity of the extracts or diffusion of compound present in the extracts [46]. Based on the promising high antimicrobial activity of chloroform, ethanol, and methanol extracts, MIC ( $IC_{50}$ ) was determined, which differed among the solvent extracts against the different organisms (Table 4). Chloroform extract indicated its positive role in combating the gram positive bacteria *M. luteus* and *S. aureus* by exhibiting  $IC_{50}$  values of 67.71 and 111.9  $\mu\text{g/ml}$  respectively. Likewise ethanol extract also exhibited a positive response against gram positive bacteria *B. subtilis* (106.5  $\mu\text{g/ml}$ ) and gram negative bacteria including *P. aeruginosa* and *V. vulnificus* (52.32 and 150.7  $\mu\text{g/ml}$ ) respectively. Perhaps, the results obtained through disk diffusion assay is in contrast to the present result which may be ascertained due to inappropriate diffusion of ethanol extract through agar substrata [47]. However, the poor performance of the methanol extract against the tested bacteria could be clearly defined by the principal of [48], according to whom the failure in the contact of organism may be attributed to extensive microbial growth.

Extracts	Gram Positive			Gram negative		
	<i>B. subtilis</i>	<i>M. luteus</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>V. vulnificus</i>
2CH ( $\mu\text{g/mL}$ )	170.3	67.71	111.9	114.8	53.72	329
5E ( $\mu\text{g/mL}$ )	106.5	218	138.2	479	52.32	150.7
6M ( $\mu\text{g/mL}$ )	1390	972.5	142.3	232.6	554.5	351.5
Ch ( $\mu\text{g/mL}$ )	12.5	12.5	12.5	12.5	12.5	ND

**Table 4:** Minimum inhibitory concentrations (MIC) -  $IC_{50}$  value of highly active extracts

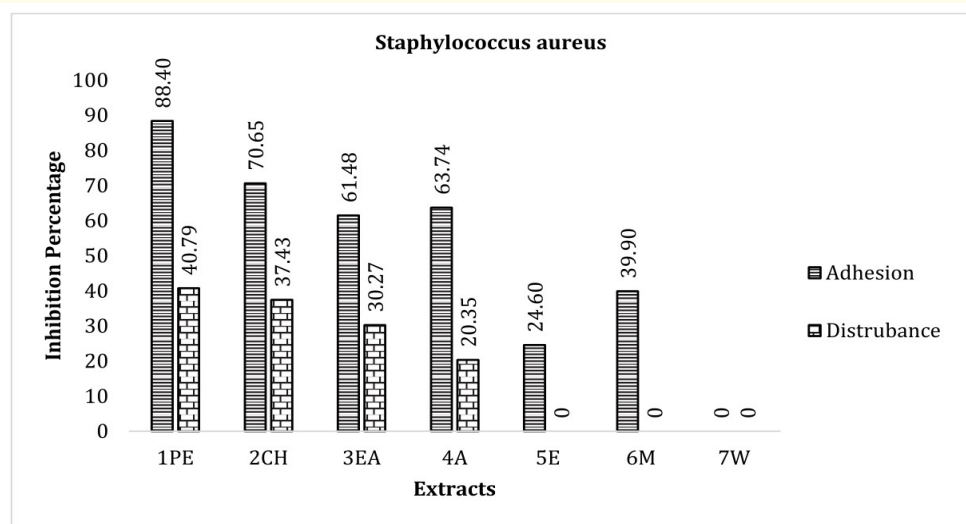
The values are in  $\mu\text{g/ml}$ ; 2CH: Chloroform; 5E: Ethanol; 6M: Methanol; Ch: Chloramphenicol;  $IC_{50}$  values are calculated using GraphPad Prism 7.

**Anti-quorum sensing activity**

Biofilm is an immobilized aggregate of microorganisms, which contribute to antibiotic and disinfectant resistant [49]. Thus, anti-quorum sensing activity of different extracts of *R. patula* was explored, but restricted to one gram positive (*S. aureus*) and one gram negative (*P. aeruginosa*) bacterium.

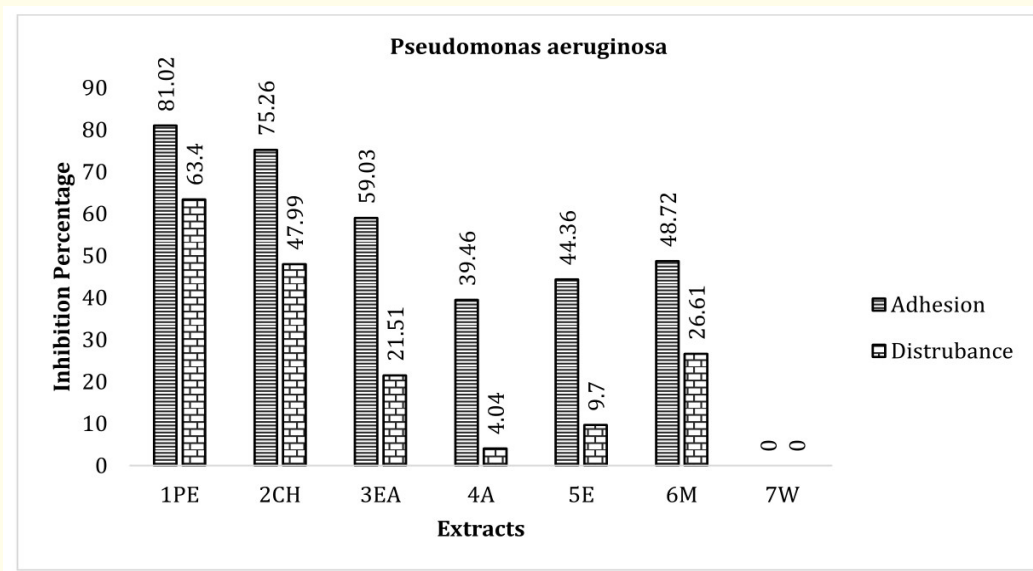
**Anti-biofilm Adhesion and Disturbance Assay**

All extracts except water had some degree of anti-biofilm adhesion property, however, none of the extracts had complete inhibition of cell attachment (Figure 3 and Figure 4). Petroleum ether extract had high anti-biofilm adhesion (88.40 and 81.04%) as well as disturbance activity (40.79 and 63.4%) against both gram positive *S. aureus* and gram negative bacterium *P. aeruginosa* respectively. Subsequently, intermediate solvents chloroform and ethyl acetate extracts showed better activity to both the organisms compared to the others. Perhaps, the efficacy in restricting the biofilm's activity by petroleum ether may be due to the presence of oil droplets which basically prevents the cell communication. Similarly, high activity of the intermediate solvent extracts could also be justified in respect of prevention of biofilm mediated by the presence of oil, which influences the reduction in biofilm thickness thereby control of bacterial count [50]. Meanwhile, acetone extract had positive selection towards *S. aureus* in term of adhesion and disturbance. However, ethanol and methanol extracts behaved contradictory by targeting gram negative bacterium than positive bacterium which is contradictory to the results of disk diffusion assay. In fact, the results obtained are in supports of [51] report, according to whom the performance of the extracts could be due to the mitigating effect of cell adhesion.



**Figure 3:** Anti-quorum sensing activity of *R. patula* extracts on *Staphylococcus aureus*.

Values are in percentage; 1PE- Petroleum Ether; 2CH- Chloroform; 3EA- Ethyl Acetate; 4A- Acetone; 5E- Ethanol; 6M- Methanol; 7W- Water.



**Figure 4:** Anti-quorum sensing activity of *R. patula* extracts on *Pseudomonas aeruginosa*.

Values are in percentage; 1PE- Petroleum Ether; 2CH- Chloroform; 3EA- Ethyl Acetate; 4A- Acetone; 5E- Ethanol; 6M- Methanol; 7W- Water.

Moreover, non-polar and intermediate solvent extracts could be potent anti-biofilm agents. As they showed high anti-adhesion capacity than disturbance capacity, which makes us to understand the capacity of these extracts in challenging the process of adhesion with limitations to disturbances, which is in support of the observations by [21]. Accordingly, the adhesion and formation of biofilms are diminished by the extracts applied on the surface area, which is essential for adsorption of substances leading to biofilm formation. Further, unfavorable plant extract film also reduces the effectiveness surface adhesion by repelling the cells back to fluid phase [52]. However, the effectiveness of those extracts in the disturbance assay could be attributable to the compounds present in the extracts, which possess the capacity to reduce the biofilm by suppressing the cell to cell communication [46]. Hence, the presence of such compounds in these extracts might have responded for the anti-biofilm activity confirming *R. patula* extracts to be applied as a potent anti-biofilm agent, however leaving a lacuna in understanding the mechanisms of inhibition with specificity which are proposed as futuristic study.

### GC-MS Analysis

Based on the above promising results, the highly active extracts such as ethanol, methanol, and chloroform were subjected to chemical fingerprinting by GC-MS, which enabled to identify twenty-seven chemical constituents. Among these, eleven were from ethanol, nine were from methanol, and six were from chloroform respectively. Since, chemical fingerprinting is used to evaluate the quality of herbal medicines [53] and knowing the nature and size of the compounds would generally help in identifying the hit to target proteins or DNA involved in diseases, it necessitated to identify the molecular formula, molecular weight, retention time and peak area % (concentration), which were calculated and tabulated (Table 5a, 5b, and 5c). Perhaps, relating the biological functions for those identified compounds is essential [54], hence the GC-MS eluted structures were exported to Dr. Duke's Phytochemical and Ethnobotanical Databases to assess their biological properties (Table 6).

S. No	Compound Name	Molecular formula	Molecular Weight	RT	Peak area %
1	d-Mannose	$C_6H_{12}O_6$	180	8.98	1
2	9-Octadecenoic acid, [2-phenyl-1,3-dioxolan-4-yl] methyl ester, cis	$C_{28}H_{44}O_4$	445	11.55	12
3	Dodecanoic acid, 2-(acetyloxy)-1-[(acetyloxy)methyl] ethyl ester	$C_{19}H_{34}O_6$	359	13.43	4
4	Tetradecanoic acid	$C_{14}H_{28}O_2$	229	15.8	15
5	Cholestan-3-ol, 2-methylene-, (3a,4A)-	$C_{28}H_{48}O$	401	16.25	13
6	Cyclopropanebutanoic acid, 2-[[2-[[2-[[2-pentylcyclopropyl)methyl]cyclopropyl]methyl]cyclopropyl]methyl]-, methyl ester	$C_{25}H_{42}O_2$	375	17.15	1
7	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	17.88	33
8	9-Hexadecanoic acid	$C_{16}H_{30}O_2$	254	19.13	7
9	Oleic acid	$C_{18}H_{34}O_2$	282	19.62	8
10	Octadecanoic acid	$C_{18}H_{36}O_2$	284	19.83	4
11	3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone	$C_{28}H_{25}NO_7$	488	23.37	2

**Table 5a:** Representation of compounds from 5E (Ethanol extract).

S. No	Compound Name	Molecular formula	Molecular Weight	RT	Peak area %
1	Dodecanoic acid, 10-methyl-, methyl ester	C <sub>14</sub> H <sub>28</sub> O <sub>2</sub>	228	15.22	3
2	Hexadecanoic acid, butyl ester	C <sub>20</sub> H <sub>40</sub> O <sub>2</sub>	313	15.93	25
3	Hexadecanoic acid, octadecyl ester	C <sub>34</sub> H <sub>68</sub> O <sub>2</sub>	509	17.63	41
4	9-Octadecenoic acid[Z],2,3,-dihydroxypropyl ester	C <sub>21</sub> H <sub>40</sub> O <sub>4</sub>	357	20.05	2
5	Hexadecanoic acid, 1-[hydroxymethyl]-1,2-ethanediyl ester	C <sub>35</sub> H <sub>68</sub> O <sub>5</sub>	569	21.28	8
6	9-Octadecenoic acid[Z],2-hydroxy-1[hydroxymethyl] ethyl ester	C <sub>21</sub> H <sub>40</sub> O <sub>4</sub>	357	22.92	13
7	2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosapentaene	C <sub>25</sub> H <sub>42</sub>	342	23.82	1
8	4,5,6,7-Tetrahydroindoxazen-5-ol-4-one, 3-[10-phendecyl]-	C <sub>23</sub> H <sub>31</sub> NO <sub>3</sub>	370	25.78	4
9	α-tocopherol	C <sub>29</sub> H <sub>50</sub> O <sub>2</sub>	430	27.68	3

**Table 5b:** Representation of compounds from 6M (Methanol extract).

S. No	Compound Name	Molecular formula	Molecular Weight	RT	Peak area %
1	1H-Inden-1-one,2,3-dihydro-5,6-dimethoxy-3-methyl-	C <sub>12</sub> H <sub>14</sub> O <sub>3</sub>	206	12.83	4
2	Aspidospermidin-17-ol, 1-acetyl-19,21-epoxy-15,16-dimethoxy-	C <sub>23</sub> H <sub>30</sub> N <sub>2</sub> O <sub>5</sub>	414	16.6	12
3	9-Octadecene, 1-methoxy, (E)-	C <sub>19</sub> H <sub>38</sub> O	283	16.82	59
4	n-Hexadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256	18.3	1
5	9-Hexadecanoic acid	C <sub>16</sub> H <sub>30</sub> O <sub>2</sub>	254	19.63	4
6	B(9a)-Homo-19-norpregna-9(11), 9a-dien-20-one, 3(dimethylamino)-4,4,14-trimethyl-(3a,4A)-	C <sub>26</sub> H <sub>41</sub> NO	384	23.52	20

**Table 5c:** Representation of compounds from 2CH (Chloroform extract).

S. No	Compound Name	Activity
1	Dodecanoic acid, 10-methyl-, methyl ester	Cosmetic; flavor and/or fragrance agents.
2	Hexadecanoic acid, butyl ester	Cosmetic; flavor; flavor and/or fragrance agents.
3	9-Octadecenoic acid[Z],2,3,-dihydroxypropyl ester	Flavor; antifoam; moisturizer; emulsifier; flavoring agent.
4	9-Octadecenoic acid[Z],2-hydroxy-1[hydroxymethyl] ethyl ester	Antioxidant; anti-atherosclerotic; emulsifier.
5	α-tocopherol	Antioxidant; antidiabetic inflammation; antidermatitic; antileukemic; antiulcerogenic; antispasmodic; antibronchitic; antitumor; apoptotic; antiaging; anti cataract and anticancer.
6	Hexadecanoic acid, octadecyl ester	Anti-inflammatory; antifibrotic.
7	d-Mannose	Cures Urinary tract infections
8	Tetradecanoic acid (fatty acid)	Antioxidant; cancer preventive; cosmetic; hypercholesterolemi; lubricant; nematocide.
9	Cyclopropanebutanoic acid, 2-[[2-[[2-[[2-pentylcyclopropyl)methyl]cyclopropyl)methyl]cyclopropyl)methyl]-, methyl ester	Fatty acid protein kinase C activator and anticoagulant for the treatment of stroke.
10	n-Hexadecanoic acid	Anti-Inflammatory; 5-Alpha-Reductase-Inhibitor; Antiallopecic; Antian-drogenic; Antifibrinolytic; Antioxidant; Flavor; Hemolytic; Hypercholes-terolemic; Lubricant; Nematocide; Pesticide; Propecic; Soap.
11	9-Hexadecanoic acid	5-Alpha-Reductase-Inhibitor; Soap.
12	Oleic acid	Cancer preventive; anti inflammation; anemiagenicinsectifuge; dermatiti-genic; antiandrogenic.

**Table 6:** Detection of Bio-activities of the Phyto-compounds referenced against Dr. Duke's library and literature survey.

Although, the solvents subjected to elution, showed a unique pattern of compounds, which varied from one another, decanoic acid in esterified forms was observed in highest concentration in all three extracts and is basically reported to encode as a flavoring agent (Dr. Duke's Phytochemical and Ethnobotanical Database). Interestingly ethanol extract generated approximately eleven peaks, where the

deconoic acid in the form of ethyl ester or methyl ester is observed. However, this extract revealed the presence of unique compounds (mannose and Cyclopropanebutanoic acid – encoding for urinary tract infection and anticoagulant properties) which were not reported from other extracts of methanol and chloroform. However, methanolic extract besides deconoic acid exemplified the presence of  $\alpha$ -tocopherol, which is widely used as an antioxidant, antidiabetic, inflammation, anti-dermatitic etc. Unlike the above two extracts, chloroform extract identified only six compounds, among which three belonged to unsaturated fatty acids, which might have been responsible for influencing the anti-quorum property in the present study in support of the proof made by [48]. Therefore, it is concluded that the compounds determined and identified in all of these extracts could potentially contribute to the medicinal value of *R. patula*.

### Conclusion

The difference in the total phenolic content, total flavonoid, antioxidant, antimicrobial and anti-quorum sensing capacity of the different solvent extracts observed in *R. patula* revealed the herb to be potentially explored in future to understand the mechanism of actions. The data presented is of interest and gives scope for research in particular ethanol, methanol and chloroform extracts as they revealed the presence of numerous but unique compounds that are responsible for anti-microbial and anti-quorum sensing properties. Additionally, the positive role played by the extracts could be claimed by the presence of higher concentrations of anti-oxidants in terms of phenols and flavonoids. Thus, culminating the interest in purifying these compounds, particularly from these extracts, which is basically designed as our future research.

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### Conflict of Interest

The authors have no conflicts of interest to declare.

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