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Secondary metabolites screening from in-vitro cultured *Rauvolfia tetraphylla* by HPTLC-MS: A special emphasises on their antimicrobial applications

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Abstract

The current study designed at evaluating the phytochemical, trace metal concentration and antimicrobial properties were screened by the ethanolic extracts of in-vitro cultured medicinal plant *Rauvolfia tetraphylla*. The In-vitro shoots proliferation from nodal explants of *R. tetraphylla* using Murashige and Skoog (MS) medium containing 0.1mM of NAA and 0.25mM of BAP was effectively induce the shoot buds. The phytochemical analysis of cultured plant extracts revealed the presence of steroids, reducing sugars, sugars, alkaloids, phenols, flavonoid, saponins, tannins and amino acids. In continuously, we assessed by HPTLC coupled with mass spectrum, based on the mass spectrum were easily identified the major compounds such as 3-isoreserpine, ajmalicine, ajmaline, reserpine and yohimbine from *R. tetraphylla*. Metal contents of plant samples, Cd, Cr, Cu, Fe, Ni, Pb and Zn concentrations are BDL, BDL, 0.12, 0.68, BDL, BDL and 0.62 mg kg-1, respectively. The ethanol extraction of in-vitro *R. tetraphylla* inhibits the growth of bacteria and fungi to a greater extent.

Keywords: *Rauvolfia tetraphylla*, Micro propagation, HPTLC-MS, Trace metals, Antimicrobial activity

1. Introduction

The ecological plunder as well as difficulties associated with seed germination in this biotechnologically significant plant has led *R. tetraphylla* to figure in the Red Data Book. Plants are a major source of bioactive substances used as pharmaceuticals, agrichemicals, colour, flavor and fragrance ingredients, food additives, etc.[1] The use of medicinal plants will steadily, increase in the coming years because of a shift in, attitude of people towards 'natural drugs' *R. tetraphylla* L. (Apocynaceae) is an endangered evergreen woody medicinal shrub, has great therapeutic potential due to the presence of alkaloids like ajmaline, ajmalicine, reserpine, serpentine and tetraphyllincine. Reserpine is a potent alkaloid that depresses the central nervous system and lowers blood pressure. The root is also used to stimulate uterine contraction and is recommended for use in difficult childbirth cases. Plants of *R. tetraphylla* are becoming critically endangered due to its extensive indiscriminate collection from wild, poor seed germination and lack of adequate commercial plantation.[2] Over exploitation of medicinal plants has led to their rapid depletion from the wild besides the conventional methods of propagation and conservation.[3] *in vitro* culture techniques can help salvage the solution.[4] Many bacteria and fungi produce human diseases which are currently controlled through the massive use of synthetic bactericides and fungicides.[5] Some of them are resistance to synthetic drugs and caused therapeutic problem. Plants extracts are one of the options that have recently received attention and expected that it will be active against synthetic drug resistant pathogens.[6] Therefore, the search for plant based new antibacterial and antifungal agents are imperative. *R. tetraphylla* extract have been used to treat infections for thousands of years in Indian system of medicines.

2. Materials and Methods

2.1 Plant material

Young shoots of *R. tetraphylla* were collected from 2 years old plants in Thanjavur district of Tamil Nadu in India during the period from January 2014 – February 2014.

2.2 Preparation of sterile explants

The nodal segments were washed thoroughly under running tap water for 30 min, then immersed in 5% (v/v) detergent (Labolene TM; Qualigens, Mumbai, India) for 5 min, rinsed three-times with sterile distilled water, surface sterilized with 0.1% (w/v) $HgCl_2$ under sterile conditions for 3 min, and rinsed four-times with sterile distilled water to remove all traces of the sterility. Nodal segments, approx. 3 to 5 mm long, were excised for encapsulation.

2.3 Culture medium

MS medium was used in the study. Depending upon the culture, different growth regulators were added to the medium.[7] the composition of MS medium (readymade: Product code: PT100).

2.4 Preparation of solid medium

Suspended 42.41 g of dehydrated medium in 600 ml of distilled water and rinsed media vial with small quantity of distilled water to remove traces of powder. Added desired heat stable supplements prior to autoclaving. Adjusted the medium pH 5.8 using 1N HCL/ 1N NaOH/ IN KOH and made up to final

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volume to 1000 ml with distilled water. Boiled the medium to dissolve agar completely and sterilized the medium by autoclaving at 121ºC for 15 minutes.

2.5 Growth regulators

The following growth regulators were used at various concentrations: Auxins -Naphthalene acetic acid and Cytokinins- Benzl aminopurine.

2.6 Direct shoots regeneration

The nodal of initial raised explants were cut into small pieces (0.5-1 CM) using sterile blade and were cultured on MS solid medium containing 3.0% (w/v) sucrose, 0.8% (w/v) agar along with combination of NAA (0.25-1 mM) and BAP (0.5 - 2.0 mM) as standard for proliferation of shoot induction.

2.7 Sample preparation for phytochemical screening

About 500 mg of in-vitro leaf material taken from plant cultured tube. The material was extracted with the help of mortar and pestle using ethanol and the dried substance was dissolved in same solvent and stored in cold room for future use.[8]

2.8 Phytochemical screening

The solvent extracts were subjected to qualitative chemical analysis to identify the nature of phytochemical constituents present in them.[9]

Steroids: A 3 ml of test solution and minimum quantity of chloroform was added with 3-4 drops of acetic anhydride and one drop of concentrated H_2SO_4. Purple color thus formed changes into blue or green color indicating the presence of steroids.

Triterpenoids: A 3 ml of test solution was added with a piece of tin and 2 drops of thionyl chloride. Formation of violet or purple colour indicates the presence of triterpenoids.

Reducing Sugars: A 3 ml of test solution was added with a 2 ml of Fehling's reagent and 2 ml of water. Formation of reddish orange color indicates the presence of reducing sugar.

Sugars: A 3 ml of the test solution was added with very small quantity of anthrone reagent and a few drops of concentrated H_2SO_4 and heated. Formation of green or purple color indicates the presence of sugars.

Alkaloids: A 3 ml of test solution was taken with 2N HCl. Aqueous layer formed was decanted and then added with one or a few drops of Mayer's reagent. Formation of white precipitate or turbidity indicates the presence of alkaloids.

Phenols: A 3 ml of test solution in alcohol was added with one drop of neutral ferric chloride (5%) solution. Formation of intense blue color indicates the presence of phenols.

Flavonoids: A 3 ml of test solution in alcohol was added with a bit of magnesium and one (or) two drops of concentrated HCl and heated. Formation of red or orange color indicates the presence of flavonoids.

Saponins: A 3 ml of test solution was added with H_2O and shacked. Formation of foamy lather indicates the presence of Saponins. Tannins: A 3 ml of test solution was added with H_2O and lead acetate. Formation of white precipitate indicates the presence of tannins.

Anthroquinones: A 3 ml of test solution was added with magnesium acetate. Formation of pink color indicates the presence of anthroquinones. Amino Acids: A 3 ml of test solution was added with 1% ninhydrin in alcohol. Formation of blue or violet color indicates the presence of amino acids.

Catechins: A 3 ml of test solution in alcohol was added with Ehrlich reagent and a few drops of concentrated HCl. Formation of pink color indicate the presence of catechins.

2.9 Quantitative analysis of phytoconstituents

The chlorophyll pigments in the leaves were estimated following the methods. After pre-cleaning, weighted fresh leaf material was homogenized and extracted thrice in chilled 80% acetone (v/v). The volume of the acetone extract was made up to a known one and the optical density was read at 645nm and 663nm wavelengths on a spectrophotometer. The concentration of the chlorophyll pigments was calculated and is expressed in mg/g fresh weight. Amino acids were estimated by Ninhydrin method which is calorimetrically measured at 570nm.[10] Proteins were estimated by Bradford method and the absorbance was measured at 595 nm against blank/ sample.[11] Carbohydrates were estimated by anthrone method which can be measured by using colorimetrically at 620nm (or) by using a red filter.[12] All the trials were performed thrice and the mean values were presented.

2.10 Mass analysis of High Performance Thin-layer chromatography (HPTLC) zones

HPTLC was performed on silica gel 60 f 254, 20X10 cm HPTLC stationary phase plate (Merck, Germany-5642), with mobile phase chloroform: toluene: ethylacetate: diethylamine (7:7:4:1). The 5.0 µL of 1 mg/mL concentrated sample was applied to the plate as 10 mm band, sample application with CAMAG-Linomat IV automated spray on band applicator equipped with a 100 µL syringe and operated with following settings: band length 10 mm, application rate 10 sec/ µL, distance between 4 mm, distance from the plate side edge 1.5 cm and distance from the bottom of the plate 2 cm (10, 14). CAMAG TLC Scanner 3 was used to densitometrically to quantify the bands using WIN CATS software (Version 4 X). The scanner operating parameters were: (Mode: absorption / reflection; Slit dimension; 5 x 0.1 mm; scanning rate: 20 mm/s and monochromator band width: 20 nm at an optimized wavelength 254nm and in visible range). The source of radiation was deuterium lamp emitting a continuous UV Spectrum in the range of 190 to 400 nm. The HPTLC Interface band eluate is transferred online into the mass spectrometer for structural confirmation and impurity control.

2.11 Trace metal analysis

The *R. tetraphylla* plant sample was collected from the Thanjavur district, Tamil Nadu. The leaves were carefully removed and washed with sterile distilled water. The cleaned leaves were dried in shadow area and were grinned with mortar and pestle. After drying, 1 g of plant samples was treated

with aqua-regia mixture in Teflon bomb and was incubated at 140 °C for 2-3 days. After incubation, the reaction mixture was filtered with Whatman No.1 filter paper. Then the extraction was test for trace metals (Fe, Cu, Zn, Pd, Cd, Cr and Ni) analysis. The extraction of the studied metals in the solutions was determined by the 797 VA Computrace voltametry, Metrohm. To avoid the contamination, the devices were rinsed with acidified water (10% HNO_3) and weighted to dissolve metals before analysis. And all the equipments and containers were soaked in 10% NHO_3 for 24 h then rinsed thoroughly in deionized water before use. Also find the below detectable limit of the instruments.

2.12 Testing of antimicrobial activity

The test strains were: *Aeromonas liquefactions* MTCC 2645 (B1), *Enterococcus faecalis* MTCC 439 (B2), *Klebsiella pneumonia* NCIM 2883 (B3), *Micrococcus luteus* NCIM 2871 (B4), *Salmonella typhimurium* NCIM 2501 (B5), *Vibrio cholerae* MTCC 3906 (B6), *Candida albicans* MTCC 1637 (F1), *Cryptococcus* sp. MTCC 7076 (F2), *Microsporum canis* MTCC 3270 (F3), *Trichophyton rubrum* MTCC 3272 (F4). The cultures were obtained from MTCC, Chandigarh and NCIM, Pune, India. Microbial strains were tested for antimicrobial sensitivity using the disc diffusion method.[13-16] The antibacterial and antifungal activity of test samples was analyzed against certain microorganisms on muller hinton agar (MHA) and potato dextrose agar (PDA), respectively.[17,18] A sterile cotton swab was used to inoculate the bacterial suspension on surface of agar plate. The 15 and 30 µL of sample coated disc were placed in agar plates, separately. For negative control study, the sterile triple distilled water was used. The plates were incubated at 37±1°C for 24–48 h (for bacteria) and 25 ±1°C for 48-72 h (for fungus). After incubation, the zone of inhibition was measured with ruler/ HiAntibiotic ZoneScale-C.[19]

3. Results and Discussion

3.1 Direct organogenesis from shoot explant

The growth regulators of BAP and NAA used to successfully regenerate the shoots from the explant. The healthy shoots proliferation occur in 2.0 mM BAP and 1.0 mM NAA concentrated semi solid medium but the length of the shoots was not sufficient. The BAP 1.0mM and NAA 0.25mM contained medium was observed 3.1 cm length of shoots (Table 1). There are earlier reports on the Micro propagation of *R. tetraphylla* via axillary bud sprouting and shoot tip culture with plant growth regulators such IAA, IBA, NAA, BA and Kinetin.[20-22] The correlation between earlier studies we were proliferate the healthy shoots from the explants in low concentration of growth regulators in the medium.

Table 1: Effect of BAP and NAA on Shoot proliferation from *in vitro* raised micro shoots of *R. tetraphylla* in MS medium

Growth Regulators (mM)		No. of Shoots per Explant	Length of Shoots (cm)	Shoot Quality
BAP	NAA			
-	-	1.0±0.01	1.1±0.3	Healthy
-	0.25	1.1±0.1	0.5±0.2	Healthy
-	0.5	1.1±0.2	2.5±0.1	Healthy
-	1.0	1.3±0.3	2.5±0.04	Healthy
0.5	-	1.3±0.1	1.2±0.1	Healthy
1.0	0.25	1.2±0.2	3.1±0.2	Healthy
1.5	0.5	1.1±0.2	0.7±0.1	Healthy
2	0.5	1.3±0.1	1.4±0.1	Healthy
2	1.0	2.0±0.4	1.5±0.1	Healthy

BAP= 6-Benzylaminopurine: NAA= 1-Naphthaleneacetic acid

3.2 Phytochemical Screenings

The ethanol extracts of in-vitro culture plants samples contain various secondary metabolites that phytoconstituents were screened by qualitative and quantitatively methods and the results are presented in Table 1 and 2. The presence or absence of colour change indicates positive and negative results. In these screening, steroids, reducing sugars, sugars, alkaloids, Phenols, flavonoids, saponins, tannins and amino acids were gave the positive results while triterpenes, catechins, and anthroquinones gave negative results. A phytochemical investigation of *R. tetraphylla* has revealed the presence of several alkaloids. The alkaloids are concentrated mostly in the bark of the roots, the quantity being much less in the wood; the bark is reported to yield about 90 % of the total alkaloid content.[23]

Table 2: Quantitative phytochemical constituent of *Rauvolfia tetraphylla*

Phytochemical Constituents	Result	Phytochemical Constituents	Result
Steroids	+	Catechins	-
Triterpenes	-	Flavonoids	+
Reducing sugars	+	Saponins	+
Sugars	+	Tannins	+
Alkaloids	+	Anthraquinones	-
Phenolics	+	Amino acids	+

+ = Present; - =Absent;

3.3 Phytoconstituents mass detection

High performance thin layer chromatography (HPTLC) was used for the estimation of phytoconstituents from *R. tetraphylla*. The samples were spotted in the form of band on the TLC plates and run in different solvent systems. The mobile phase consisting of chloroform: toluene: ethylacetate: diethylamine (7:7:4:1) gave well defined bands and sharp peaks. The compact mass spectrometer expresses the fast mass determination for TLC/HPTLC zones in a concentration dependent and reliable manner. Measurements can be performed directly from selected zones by online elution. The mass spectrum data were reviewed totally 5 compounds are presented in in-vitro cultured *R. tetraphylla* ethanolic extract, that are 3-Isoreserpine, Ajmalicine, Ajmaline, Reserpine and Yohimbine (Fig. 1-3).

Table 3: Quantitative phytochemical constituent of *Rauvolfia tetraphylla*

Biochemical constituents	*Rauvolfia tetraphylla* (mg/g)
Chlorophyll A	0.256
Chlorophyll B	0.927
Total Chlorophyll	1.183
Amino acid	160
Protein	2.521
Carbohydrate	1.416
Phenol	0.024

Figure 1: HPTLC Chromatograms of *in vitro* cultured *R. tetraphylla*

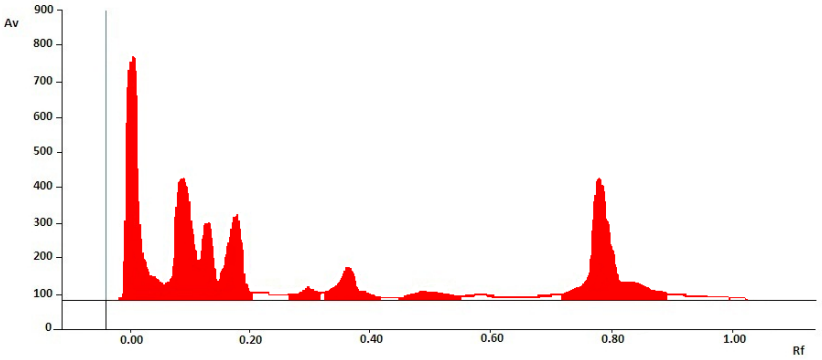
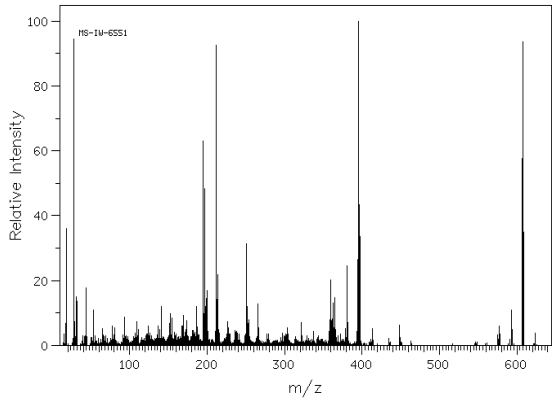
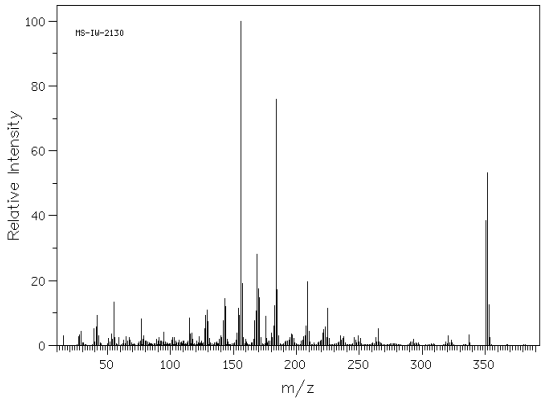


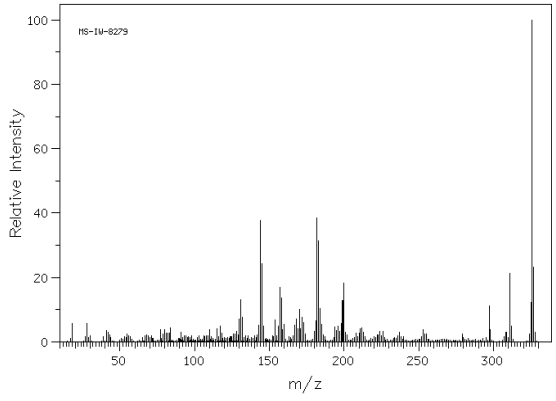
Figure 2: Mass Spectrum of various secondary metabolites isolated from *R. tetraphylla*



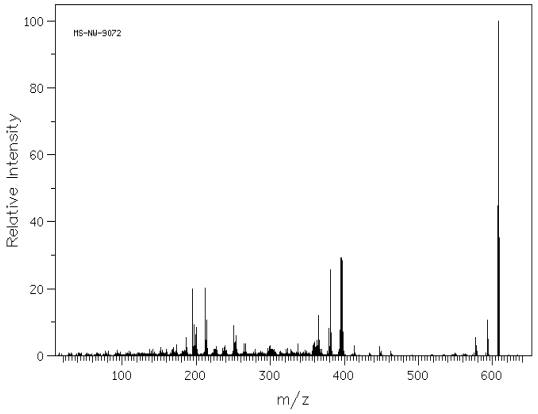
3-Isoreserpine



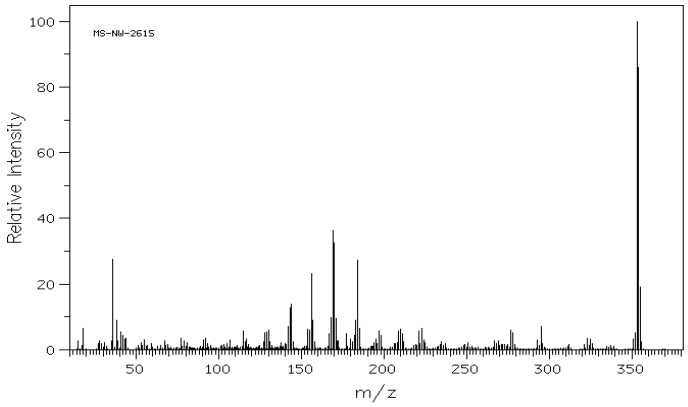
Ajmalicine



Ajmaline

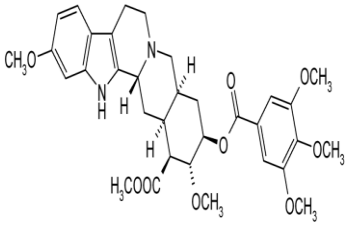


Reserpine

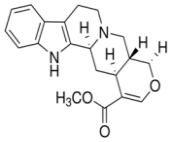


Yohimbine

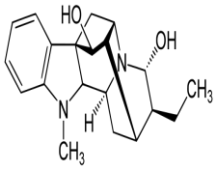
Figure 3: Structures of various secondary metabolites isolated from *R. tetraphylla*



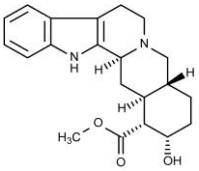
3-Isoreserpine



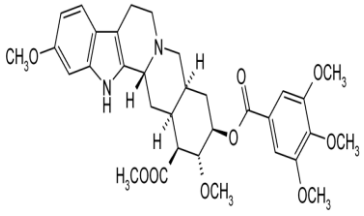
Ajmalicine



Ajmaline



Reserpine



Yohimbine

3.4 Trace metals analysis

Commonly, excessive metal concentration and toxic metals are also affected the photosynthetic ability of leaves, closure of leaf stomata, plant growth, physiology and productivity of plants. But, some of the trace metals (Cu, Zn and Fe) are essential for plant growth and remaining metals do not play any significant role in the plant growth. Metal contents of plant samples, Cd, Cr, Cu, Fe, Ni, Pb and Zn concentrations are BDL, BDL, 0.12, 0.68, BDL, BDL and 0.62 mg kg-1, respectively. The plants receiving/ drained metals are an avenue of their entry into the food web and it cause some deleterious effect on the human and animal health, which also affects their growth and proliferation.[24,25] But, some of the plants can survive in the trace metal contaminated regions due to the accumulation of high metal concentrations in the plants. In the matter of fact, the plants are able to tolerate high metal concentrations.

3.5 Antibacterial and Antifungal screening

The antimicrobial screening of in-vitro *R. tetraphylla* extraction (ethanol) was examined with different gram positive, gram negative and fungal strains by using of disc diffusion test. Both the concentrations (15 and 30 µL /disc) were produced zone of inhibition on the MHA and PDA plates for bacteria and fungi, respectively while the higher concentration produced greater zone than the low concentration. In bacteria, the test sample was most effective against *Aeromonas liquefactions* MTCC 2645 (B1) while smaller effect was noticed from *Vibrio cholerae* MTCC 3906 (B6). In fungi, which was effective against *Trichophyton rubrum* MTCC 3272 (F4) whereas smaller effect was observed in *Microsporum canis* MTCC 3270 (F3). The results of the antimicrobial activities are summarized in table 4. The higher concentration showed effective zone than the positive control, except B3, B4 and B6. The negative control produced nil effect on the agar plates. Suresh et al. reported the best antimicrobial activity of *R. tetraphylla*, which showed maximum activity against *E. coli* and *Enterobacter aerogenes*, and various tested fungi such as *A. niger* and *Penicillium* sp, were found to be more sensitive to

crude extract when compared to others.[27] But this is the first time, the in-vitro *R. tetraphylla* was screened for the antimicrobial studies. The result may be concluded that our ethanol extraction of in-vitro *R. tetraphylla* inhibit the growth of bacteria and fungi to a greater extent.

Table 4: Antimicrobial screening of in-vitro *R. tetraphylla*

S. No	Test Microorganisms	Zone of inhibition (mm) Sample (15 & 30) µL / disc				Diseases	Route of Transmission
Bacteria		**15 µL**	**30 µL**	**PC**	**Remarks**		
1.	*Aeromonas liquefaciens* B1	14	17	14	> PC	Wound Infections / Gastroenteritis	Water / Food
2.	*Enterococcus fecalis* B2	12	14	8	> PC	Endocarditis / Bladder, Prostate, and Epididymal Infections / Nervous system Infections	Water / Food
3.	*Klebsiella pneumoniae* B3	13	15	28	< *PC*	Acute diarrhoea / Dysentery	Water / Food
4.	*Micrococcus luteus* B4	13	14	38	< *PC*	Skin & Pulmonary infections/ Septic shock / Pneumonia endocarditis	Soil / Dust / Water/ Airways / Food
5.	*Salmonella typhimurium* B5	13	15	0	> PC	Typhoid	Water / Food
6.	*Vibrio cholarae* B6	12	13	16	< *PC*	Cholera	Water / Food
Fungi							
7.	*Candida albicans* F1	12	14	10	> PC	Skin (Integument) Infections/ Gastrointestinal tract Infection	Airways / Wound / Soil / Water
8.	*Cryptococcus* sp. F2	12	15	9	> PC	Cryptococcal disease / Bronchiectasis / Endophthalmitis.	Airways / Wound / Soil / Water
9.	*Microsporum canis F3*	12	13	9	> PC	Tinea capitis /Ringworm	Airways / Wound / Soil / Water
10.	*Trichophyton rubrum* F4	14	18	7	> PC	Tinea corporis / Tinea cruris/ Tinea pedis / Onychomycosis	Airways / Wound / Soil / Water

PC - Positive Control (Using antibiotic disc; Bacteria - Methicillin (10mcg/disc); Fungi – Itraconazole 10mcg/disc)
Samples - 15 µL / disc & 30 µL / disc; > PC – greater than positive control; < PC – less than positive control

4. Conclusion

This study concluded that the essential trace metals are highly present in the plants which indicated that the medium contains rich Cu, Fe and Zn metals. The remaining metals (Cd, Cr, Ni & Pb) were not observed in the plants. In micro propagation, the growth regulator concentrations (BAP 1.0mM and NAA 0.25mM) were highly support the healthy growth of shoot. The five metabolites (3-Isoreserpine, Ajmalicine, Ajmaline, Reserpine and Yohimbine) were screened from the in-vitro *R. tetraphylla* by HPTLC-MS. In phytochemical screening rich alkaloids were observed in the ethanol extraction. In antimicrobial activity, the test sample was most effective against *Trichophyton rubrum* MTCC 3272 (F4) while smaller effect was noticed from *Vibrio cholerae* MTCC 3906 and *Microsporum canis* MTCC 3270 (F3).

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