Eco. Env. & Cons. 28 (October Suppl. Issue) : 2022; pp. (S440-S447)
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ISSN 0971–765X

DOI No.: http://doi.org/10.53550/EEC.2022.v28i06s.073

In vitro Callus Induction of *Rauvolfia tetraphylla* (L.) an Endangered Medicinal Plant and Evaluation of Biological Activities

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(Received 8 April, 2022; Accepted 22 July, 2022)

ABSTRACT

Rauvolfia tetraphylla (L.) is a critically endangered woody shrub that is one among the most significant Apocynaceae family species cultivated for its therapeutic benefits. The goal of this work is to standardize the micropropagation methodology for *Rauvolfia tetraphylla* (L.). Explants were used to make the callus (Leaf, Internode, and Anther). "When leaf explants were grown on Murashige and Skoog (MS) media supplemented with Indole-3-Acetic Acid (IAA) (0.5mg/ml), a maximum of 94.2 percent of callus initiation was observed"."On MS media with 2,4-dichloro phenoxy acetic acid (2,4-D) + Indole-3-Acetic Acid (IAA) (0.5+0.5mg/ml), internodal explants demonstrated a maximum of callus initiation of 77.9%"."On MS media with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (2,4-D) (0.5 mg/ml) alone, the greatest callus initiation (89.8%) was found in anther explants". The qualitative preliminary phytochemical analysis was carried out from leaf and fruit extracts. Extracts were obtained from various organic solvents by the sequential method. Alkaloids, carbohydrates, fixed oils and fats, phenolic compounds, and tannins were identified from various crude extracts such as Hexane, Chloroform, Ethyl acetate, and Methanol. In antibacterial activity, the maximum inhibition was observed in methanol extract of leaf and fruit against *Escherichia coli* (100 μg/ml).

***Key word**: Rauvolfia tetraphylla, Callus induction, Phytochemical analysis, Antioxidant and cytotoxicity*

Introduction

Medicinal plants play a significant role worldwide (Dwivedi *et al*., 2019). Folk medicine is the traditional medicinal practice with the knowledge of the medicinal properties of medicinal plants (Eatimony *et al*., 2019). In many countries, herbal remedies play a key role in the health system. According to Hao (2018), medicinal plant utilization was recorded from ancient times. The traditional knowledge, skill, and practices of medicinal plants nourished healthy communities. Srivastava (2018), analyzed medicinal plants as essential for human health and these medicinal plants cure almost all deadly dangerous diseases and lead to a healthy life. Many countries have rich biodiversity and traditional literature was recorded in India, China, Egypt, and African countries also have a vast knowledge of medicinal uses of natural products. Prakash *et al*. (2018), studied bioactive compounds derived from various plants.

Primarily, natural products usage provides a clue for the synthesis of many effective drugs production from plants. In the past few decades, medicinal plants' value and their usage have increased. Many advanced biotechnological techniques are gradually developed, through which identified a novel drug and their application is used for pharmaceutical purposes (Thatoi and Patra, 2011).

Rauvolfia tetraphylla is a shrub belonging to Apocynaceae. It's also known as the "devil pepper" or "be still tree". It stands at a height of 6 feet. Leaves are whorled, medium to dark in color, and each mode has a group of four unequally sized leaves. Flowers are white, 5-mm long, tube, and 3.7 mm, typically fruit is bright red, but they change to black when they ripen. Mexico, Central America, the West Indies, and South America are all home to this species. It is now naturalized throughout the tropics, including Australia, Indochina, and India. The plant is dispersed in various states in India, such as Karnataka, Madhya Pradesh, Orissa, West Bengal, Bihar, Andhra Pradesh, Kerala, and Tamil Nadu. Reserpine, rauvolscine, canescine, pseudoyohimbine, yohimbine, and ajmalicine are alkaloids found in the plant (Mahalakshmi *et al.*, 2019).

Anxiety, epilepsy, mental problems, cough, and cold are all treated using the plant's leaf decoction. *R. tetraphylla* is an antidote used to treat snakebite, insect sting, animal bite, insomnia, high blood pressure, and madness. The plant's leaves and flowers are consumed to treat asthma (Kalam Khan *et al.* 2013). The roasted fruit in the Kanyakumari district was used to treat Intestinal worms (Rani and Jeeva, 2017). The leaves and fruit of the plant in the Thiruvannamalai district were used against snakebite (Ranganathan *et al.*, 2012). In recent years, the Screening of medicinal plants for their biological activities and phytochemicals is important for discovering potential new compounds for therapeutic uses.

Overexploitation of this plant species by humankind and pharmaceutical industries, considering this medicinal property and to conserve this species, *R. tetraphylla* was selected for in-vitro propagation through plant tissue culture. The main objectives of the work include (i) Standardizing the in-vitro propagation technique for *R. tetraphylla*; (ii) To extract and analyze the secondary metabolites by preliminary qualitative phytochemical screening; (iii) To test the efficiency of crude extracts such as hexane, chloroform, ethyl acetate and methanol of *R. tetraphylla* for antimicrobial activity.

Materials and Methods

Surface sterilization and explant preparation for callus induction

From 2019 to 2021, the experiment was carried out in the Department of Botany, Avinashilingam Institute. Explants were collected from Top slip, Western Ghats (Tamilnadu, India). To eliminate dirt particles from the surface of the explants, healthy young plant leaves, nodes, and internodes were gathered and thoroughly washed in running tap water for 25 minutes. To minimize the interaction of microorganisms such as bacteria and fungus in plant tissue culture, explants were introduced to the laminar airflow chamber and surface sterilized with sodium hypochlorite (1.0%) and mercuric chloride (0.1–0.3%) for various time durations. Finally, the explants were washed 4-5 times with sterile distilled water. Dead sections of surface-sterilized explants were excised from all borders, and all explants were mildly injured on the surface to promote callus formation.

Collection and processing of planting material

The collected plant material (leaves and fruit) was dried in the shade followed by grinding to fine particles (powder). The powdered sample was soaked in the various organic solvent for about 5 to 6 days at room temperature. The extract is then filtered and concentrated to the final volume and subject to phytochemical analysis using the sequential method. Hexane, Chloroform, Ethyl acetate, and Methanol were used in a series of extractions with increasing polarity.

Qualitative Preliminary Phytochemical Analysis

Qualitative preliminary phytochemical investigations were used to screen phytoconstituents (Harborne 1998). Drangenddorff's reagent, Mayer's reagent, and Wagner's reagent detect alkaloids, whereas Salkowski's and Liberman Burchardt's tests detect steroids and triterpenes, and Legal's test detects glycosides, while Molisch's test, Fehling's test, Barfoed's test, and Banedict's test detect carbohydrates. The Xanthoproteic test was used to examine the proteins. The alkaline reagent test, lead acetate test, Shinoda test, magnesium hydrochloric

acid reduction test, and foam test are all used to identify flavonoids and saponins, respectively. The ferric chloride and lead acetate tests are used to identify phenolic compounds and tannins, while the gelatin test is used to identify tannins.

Antimicrobial activity of a methanol crude extract of *R. tetraphylla* L. leaf and fruit

Microoraganisms and culture conditions

The Microbial Culture Collection, Chandigarh, India, provided harmful bacteria such as *Escherichia coli* [MTCC-1687] (Gram-negative bacteria), *Staphylococcus aureus* [MTCC-96] (Gram-positive bacteria), *Enterococcus faecalis* [MTCC-439] (Gram-positive bacteria), and fungi *Candida albicans* [MTCC-183]. All of the cultures are then subcultured on nutritional agar medium for bacterial culture and potato dextrose medium for fungal culture on a regular basis and stored at 4 °C for subsequent studies.

Preparation of inoculum

Test for antibacterial activity

The antibacterial activity of the compounds was evaluated against harmful bacteria using the agar well diffusion method. To offer osmotic protection, the bacteria suspensions were adjusted with sterile saline solution (0.85–0.9 percent). Inoculum was made from a 24-hour-old culture and kept at 4 °C until needed. To check for viability and ensure the inoculum was not contaminated, dilutions of the inoculum were grown on nutrient agar.

Test for antifungal activity

The antifungal activity of the methanol extract of leaf and fruit was evaluated using an agar well diffusion technique. The culture was incubated at 35°C for 24 hours. The suspension was adjusted to a concentration of roughly 0.9 - 1.0 with a final volume of 100 L per well using sterile saline. To ensure that the inoculum was free of contaminants and viable, it was diluted and cultivated on potato dextrose agar.

***In vitro* antimicrobial activity was carried out using the agar well diffusion method (Janssen *et al.*, 1987; Magaldi *et al.*, 2004)**

Using sterile cotton swabs and 8 hours old broth, the antibacterial activity of *R. tetraphylla* extracts was evaluated on Nutrient Agar (NA) and Potato Dextrose Agar (PDA) plates. A 10mm sterile cork-borer was used to make uniform wells on all culture plates for 1 mg/mL concentrations of various extracts, namely, methanolic extracts of leaf and fruit. Furthermore, methanolic crude extracts of *Rauvolfia tetraphylla* were diluted and added to the wells according to their concentrations and the control experiments without plant extracts. Tetracycline, 30 mg, and dimethyl sulfoxide, 100% were used as a positive and negative control. At 37 °C, the treated plates were incubated overnight in a microbial incubator. Antimicrobial activity was measured by the diameter of the zone of inhibition.

Determination of Minimum Inhibitory Concentration

In the very minimal concentration of Minimum Inhibitory Concentration, an antibiotic reduces observable bacterial growth (MIC). When a MIC value is compared to a breakpoint value, it may be determined if bacteria are susceptible, intermediate, or resistant to a particular antibiotic. The MIC of a methanol extract of *Rauvolfia tetraphylla* leaf and fruit against *Escherchia coli* was determined using a two-fold broth dilution technique. The broth dilution method aims to find the lowest concentration of methanol extracts of leaf and fruit that inhibits visible bacterial growth under defined conditions. Agar dilution is mixing varied quantities of methanol extracts into a nutritional agar medium containing *E. coli*, then spreading a standardised number of cells throughout the agar plate's surface.

Results and Discussion

The present study describes an effective and rapid method for mass propagating *R. tetraphylla*. To investigate the levels of surface sterilisation, *Rauvolfia tetraphylla* leaf, internode, and anther explants were treated with 1 percent sodium hypochlorite for 20 minutes and 0.1 percent mercuric chloride for 2 minutes. Table 1 shows the maximum percentage of surface sterilization for leaf explants at 96.3%, while internodes had a percentage of 95%, and anthers had a percentage of 95%, as shown in Table 3. Murthy *et al*. (2019) used three different surface sterilising agents for *Bacopa monnieri* L. with different concentrations of ethanol (50 and 70%), mercuric chloride ($HgCl_2$; 0.1 and 0.5 percent), and sodium hypochlorite (NaOCl; 0.1, 0.5, and 1 percent) at different time intervals of 1, 3, 5, 8, and 10 minutes to culture an explant in an aseptic condition.

Callus induction was observed on seventh day of inoculation which shows maximum of 94.2% of cal-

lus was observed on MS media with IAA from leaves on the seventh day after inoculation, as shown in Table 4 (Fig. 1 and 2). Based on the callus derived from internode, a peak of 77.9% was observed in MS media augmented with IAA and 2,4-D as shown in Table 6 (Fig. 3). On MS media with 2,4-D, the maximum of 89.8% of callus initiation was recorded in anther, as indicated in Table 5 (Fig. 4). *R. tetraphylla* developed the most roots on MS medium with 1.0 mg/l IAA and 1.0 mg/l IBA, according to Rohela *et al*. (2019). Mamatha *et al*. (2021) looked at *R. tetraphylla* shoot tips and nodal segments and found that MS medium containing 2.5 mg/l BAP, 0.5 mg/l NAA, and 0.5 mg/l IAA produced the most shoot induction.

Table 1. Effect of Sodium Hypochlorite 1% and Mercuric Chloride 0.1% in Leaf

S. No.	NaOCl/ Min	$HgCl_2$/ Min	Germination %
1	10	1	94.4 ± 0.6
2	15	2	95.2 ± 1.15
3	20	3	96.3 ± 0.6

Table 2. Effects of Sodium Hypochlorite 1% and Mercuric Chloride 0.1% in Internode

S. No.	NaOCl/ Min	$HgCl_2$/ Min	Germination %
1	10	1	94.7 ± 0.1
2	15	2	95.0 ± 0.8
3	20	3	94.3 ± 0.2

Table 3. Effects of Sodium Hypochlorite 1% and Mercuric Chloride 0.1% in Anther

S. No.	NaOCl/ Min	$HgCL_2$/ Min	Germination %
1	10	1	95.0 ± 0.3
2	15	2	94.5 ± 0.6
3	20	3	95.0 ± 1.0

Qualitative Phytochemical Analysis

The present study used crude extracts of *R. tetraphylla* leaf and fruit in hexane, ethyl acetate, chloroform, and methanol to undertake qualitative preliminary phytochemical analyses (Tables 7 and 8). Secondary metabolites include alkaloids, carbohydrates, glycosides, oils and fats, phenolic compounds, tannins, quinones, cardiac glycosides, terpenoids, coumarins, phlobatannins, steroids, phytosteroids, anthraquinones, flavonoids, and protein were present in all the extracts. In the ethanol extract of *R. tetraphylla*, Nandhini *et al*., (2014) determined the presence of steroids, reducing sugars, sugars, alkaloids, flavonoids, saponins, tannins, and amino acids.The presence of amino acids, alkaloids, carbohydrates, flavonoids, phenols, terpinoids, oils, saponins, sterols, and lipids was revealed in several organic solvent extracts of *Rauvolfia serpentine* in petroleum ether, chloroform, acetone, and methanol by Ratnam (2021).

Antimicrobial activity

The antimicrobial activity of *Ravoulfia tetraphylla* methanolic extract against Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram-negative (*Escherichia coli*) bacteria, as well as the antifungal activity of the methanolic extract against *Candida albicans*, was studied using the agar well diffusion method. All the extracts showed the zone of inhibition which has been calculated and shown in Fig. 5&6. The maximum inhibition was observed in methanol extract of leaf and stem against *Escherichia coli* (100 µg/ml). The antifungal activity of methanolic extracts against *Candiada albicans* shown in Fig. 7.

Antimicrobial activities of *Rauvolfia serpentina*, *Adhatoda vasica*, and *Alstonia scholaris* belonging to the Apocynaceae family are tested in various solvents (water, ethyl acetate, chloroform, and methanol). The antibacterial activity of methanol extract

Table 4. Effect of Different Concentration of IAA on Callus Induction

Hormone	Concentration (mg/ml)	Leaf	Internode	Anther
IAA	0.1	78.6 ± 5.4	45.1 ± 3.2	65 ± 1.7
IAA	0.2	79.8 ± 4.09	55.6 ± 4.9	71.5 ± 5.4
IAA	0.3	82.8 ± 2.2	66.5 ± 0.8	79 ± 4.1
IAA	0.4	87.2 ± 1.4	74.6 ± 1.7	80.8 ± 32
IAA	0.5	94.2 ± 1.6	77.2 ± 2.0	84.6 ± 2.4

Values represent mean ± standard deviation of three replicates per treatment.

was highest against two bacterial strains (*Streptococcus pyogenes* and *Pseudomonas aeruginosa*) and two fungus strains (*Aspergillus niger* and *Vibrio cholerae*). Methanol extraction of *Rauvolfia serpentine* showed antimicrobial activity against *Vibrio cholerae* and other bacteria tested (Koti Ratnam, 2021).

Table 5. Effect of Different Concentration of 2,4-D on Callus Induction

Hormone	Concentration (mg/ml)	Leaf	Internode	Anther
2,4-D	0.1	76.2 ± 1.9	45.8 ± 3.0	64.4 ± 2.2
2,4-D	0.2	83.4 ± 2.2	56.2 ± 3.5	74.3 ± 2.2
2,4-D	0.3	86 ± 1.6	64.8 ± 2.1	83.3 ± 5.0
2,4-D	0.4	89.6 ± 1.4	72.9 ± 1.7	86.9 ± 2.2
2,4-D	0.5	92.1 ± 2.6	77.0 ± 1.3	89.8 ± 3.9

Values represent mean ± standard deviation of three replicates per treatment.

Table 6. Effect of Different Concentration of IAA + 2,4-D on Callus Induction

Hormone	Concentration (mg/ml)	Leaf	Internode	Anther
IAA + 2.4 D	0.1	74.7 ± 2.7	45.5 ± 3.1	72.9 ± 1.4
IAA + 2.4 D	0.2	80.8 ± 3.0	57.2 ± 3.8	79.5 ± 2.1
IAA + 2.4 D	0.3	87.5 ± 1.9	65.6 ± 2.0	84.7 ± 2.4
IAA + 2.4 D	0.4	91.2 ± 1.6	73.9 ± 2.1	87.8 ± 3.3
IAA + 2.4 D	0.5	93.9 ± 2.0	77.9 ± 3.0	89.5 ± 3.8

Values represent mean ± standard deviation of three replicates per treatment.

Table 7. Qualitative Preliminary Phytochemical Analyses of Fruit

S. No.	Tests	Hexane crude extract	Chloroform crude extract	Ethyl acetate crude extracts	Methanol crude extracts
1A	AlkaloidsMayer's test	+	"	+	+
B	Wagner's test	+	"	+	+
C	Drangendorff's test	+	"	+	+
2A	CARBOHYDRATESMolisch's test	+	+	+	+
B	Fehling test	+	+	+	+
C	Barfoed test	+	+	+	+
D	Bendict's test	+	+	+	+
E	Legal's test	+	+	+	+
3	Saponin test	"	"	"	"
4	Oils and fats testSpot Test	"	"	"	"
5A	Phenolic and tannin testferric chloride Test	"	"	"	++
B	Gelatin test	"	"	"	++
C	Lead acetate test	+	+	"	++
D	Alkaline reagent test	"	"	"	++
E	Magnesium and hydrochloric acid test	"	"	"	++
6	Quinones test	++	+	++	+
7	Glycosides test	++	+	++	++
8	Cardiac glycosides	+	+	++	+
9	Terpenoids	++	++	++	"
10	Coumarins test	"	+	"	++
11	Phlobatannins test	"	+	"	"
12	Steroids	++	++	"	"
13	Phytosteroids	"	"	++	"
14	Anthraquinones (Borntrager's test)	"	"	+	+
15	Flavonoids (Shinoda test)	"	++	+	++
16	Protein (xanthoproteic test)	+	"	++	+

Minimum Inhibitory Concentration

A two-fold broth dilution approach was used to determine the minimum inhibitory concentration against *E. coli*. The nutrient agar broth is utilised as a growth medium, and the methanol leaf and fruit extract of *R. tetraphylla* is diluted with DMSO. 200 g/ml, 100 g/ml, 50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml, 3.125 g/ml, 1.56 g/ml, and 0.78 g/ml are the concentrations. The solutions' turbidity is measured. The minimum growth of methanol extract of leaf of *Ravuolfia tetraphylla* is observed in the concentration 1.56μg/ml and the fruit contains 50 μg/ml.

Aqueous extracts of Apocynaceae and Solanaceae members from an Amazon rain forest and Atlantic forest native were tested for antimicrobial activity against *Staphylococcus aureus* and *Enterococcus faecalis* using the broth microdilution method, and they had shown some inhibition of bacterial growth at concentrations of 100g/ml (Suffredini *et al*., 2004). Two indole alkaloids were isolated from a *Rauvolfia caffa* Sond from stem barks such as rauvolfianoids A (1) and B (2). It was analyzed antimicrobial activity against three bacterial strains (*Escherichia coli* [ATCC 35218], *Shigella sp*, and *Salmonella sp*). Compound 1 exhibited moderate antimicrobial activity against *Salmonella sp* with 25 mg/ml MIC values compared to compound 2 (Bitombo *et al*. 2021).

Conclusion

The goal of this research was to standardise in vitro micropropagation procedures of *Rauvolfia tetraphylla* L. using Murashige and Skoog media and diverse explants such as a leaf, internode, and anther. *R. tetraphylla* is an important medicinal plant; various extracts including hexane, chloroform, ethyl acetate, and methanol were used for phytochemical analysis. It is a renowned medicinal herb for its pharmacological activities such as antioxidants, antibacterial, antifungal, and anticancer characteristics. Our results concluded that *Rauvolfia tetraphylla*is responsible for potential anticancer activity. Researchers have spent a tremendous amount of time and resources to find the importance of medicinal plants. Every plant contains special compounds which help

Table 8. Qualitative Preliminary Phytochemical Analysis of Leaf

S. No.	Tests	Hexane crude extract	Chloroform crude extract	Ethyl acetate crude extracts	Methanol crude extracts
1A	Alkaloids Mayer's test	+	"	+	+
B	Wagner's test	+	"	+	+
C	Drangendorff's test	+	"	+	+
2A	CARBOHYDRATESMolisch's test	+	+	+	+
B	Fehling test	+	+	+	+
C	Barfoed test	+	+	+	+
D	Bendict's test	+	+	+	+
E	Legal's test	+	+	+	+
3	Saponin test	"	"	"	"
4	Oils and fats testspot test	"	"	"	"
5A	Phenolic and tannin testferric chloride Test	"	"	"	+
B	Gelatin test	"	"	"	+
C	Lead acetate test	+	"	"	++
D	Alkaline reagent test	"	"	"	+
E	Magnesium and hydrochloric acid test	"	"	"	+
6	Quinones test	+	"	++	+
7	Glycosides test	"	+	+	++
8	Cardiac glycosides	++	+	"	"
9	Terpenoids	++	+	+	+
10	Coumarins test	"	+	"	++
11	Phlobatannins test	"	+	"	"
12	Steroids	+	+	"	+
13	Phytosteroids	"	"	++	"
14	Anthraquinones (Borntrager's test)	"	"	"	"
15	Flavonoids (Shinoda test)	"	"	+	++
16	Protein (Xanthoproteic test)	+	"	++	+

in various pharmacological purposes. The work carried out was a basic approach to find out the biological activity of *Rauvolfia tetraphylla*. Purification and isolation of a specific set of bioactive components from plant extracts, as well as the exact potential of pharmacological investigations, require more effort.

Data Availability

The data used to support the findings of this study are incorporated within the article and can be liberally available to authors with suitable reference in their research work.

Conflict of Interest

The authors declare that there is no conflict of interest regarding this research study.

Acknowledgement

Our sincere thanks to Avinashilingam Institute for Home Science and Higher Education for Women for providing moral support to complete our project. Many thanks to Tamil Nadu forest department for collecting this plant. I would like to express my gratitude to the Department of Botany, Zoology, Biochemistry and Biotechnology for their support in completing the project. My special thanks to my supervisor for their extraordinary support in this thesis process.

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