CHAPTER – III

MATERIALS AND METHODS

The work on "**Enhancing *In vitro* Propagation Efficiency and Exploring Wound Healing Therapeutic Potential of *Rauvolfia tetraphylla* L.: A Multifaceted Approach"** is presented as follows.

Phase I - Plant tissue culture

Phase II - Phytochemical analysis

Phase III - Biological activities

Phase IV – Characterization of *RtTDC* gene

3.1. PLANT TISSUE CULTURE

General laboratory techniques recommend by Purvis *et al.* (1966), Tuite (1969) and Booth (1971) were followed for media preparation of inoculation and maintain of cultures.

3.1.1. Cleaning of Glasswares

All the glassware (Borosil) were immersed in cleaning solution (chromic acid) for 3 hours. Then, the glassware's were washed thoroughly with tap water, followed by detergent solution, and finally rinsed with distilled water. The cleaned glassware's were dried in a hot air oven and stored.

Preparation of Chromic Acid

Potassium dichromate	- 60 g
Conc. H_2SO_4	- 60 mL
Distilled water	- 1000 mL

To prepare chromic acid solution dissolved Potassium dichromate in distilled water and concentrated sulphuric acid was added slowly. It was mixed thoroughly and used for cleaning glassware. Increased the proportions to make larger volume. Use the mixture until it turns dark brown in color.

3.1.2. Sterilization

Dried glassware and media were sterilized in an autoclave at 15 Ibs pressure (15 min).

3.1.3. Plant Source

Rauvolfia tetraphylla L. saplings were collected from Top slip, Pollachi, Western Ghats, Coimbatore, Tamil Nadu, India, and maintained in Avinashilingam institute herbal garden. Plant

was identified in Botanical Survey of India, Southern region, Coimbatore, India, and its authentication number is BSI/SRC/5/23/2023/Tech-552.

3.1.4. Preparation of Tissue Culture Media

Murashige and Skoog (1962) medium were used for all the plant tissue culture experiments. Six individual stock solutions of macronutrients, micronutrients, iron, and vitamins were prepared and stored.

Iron stock was stored in a black bottle to prevent photolysis of chemicals. All the stock solutions were stored in refrigerator and used within one year. Meso-inositol, cytokinin, and auxin stock solution were freshly prepared and used every month. For preparation of medium, all the 6-stock solution were mixed thoroughly with required amounts of sterile distilled water. Sucrose 3% (30 g/L), 0.1% meso-inositol (100 mg/L) and required plant growth hormones were added to the medium and buffered by 1N HCl or NaOH to adjust the medium to 5.7 pH before autoclaving. The medium was solidified by adding 0.8% agar (8 g/L). Sterile distilled water was used to make the final volume. The medium was poured into culture vials and autoclaved at 15 Ibs pressure for 15 min at 121℃.

3.1.4.1. Chemical Composition of Murshige and Skoog (1962) Medium

S.NO	Component	Stock conc. (g)	Dissolved in water (mL)	For 1 liter
A	$(NH_4)\ No_3$ KNo_3 $CaCl_2.2H_2O$ $MgSo_4.7H_2O$ KH_2PO_4	16.500 19.000 4.400 3.700 1.700	500	50 mL
B	$MnSo_4.4H_2O$ $ZnSo_4.7H_2O$ H_3Bo_3	2.230 0.860 0.620	250	2.5
C	$Na_2\ MoO_4.4H_2O$ $CuSo_4.5H_2O$ $CoCl_2.6H_2O$	0.125 0.012 0.012	500	1.5
D	KI	0.083	250	2.5
E	**Iron** Na_2 EDTA $FeSo_4.7H_2O$	 1.862 1.392	250	5.0
F	**Vitamins** Thiamine HCl Pyridoxine HCl Nicotinic acid Glycine	 0.010 0.050 0.050 0.200	100	1.0

- Meso-inositol - 100 mg
- Sucrose - 30 g
- Agar - 8 g
- pH - 5.6 to 5.8

3.1.4.2. Preparation of Hormone Stock Solution

Auxins - 2,4-D (2,4-dichlorophenoxyacetic acid), IAA (Indole -3- acetic acid) and IBA (Indole -3- butyric acid) and cytokinin's - BAP (6 – benzyl amino- purine) and KIN –Kinetin (6-furfurylaminopurine) were prepared and stored at 4℃.

- **2,4-D -** (M.W. - 221.0), 100 mg was dissolved in 1.0 mL of 1N KOH, which was then heated, and made up to a final volume of 100 mL (1.0 mg/mL) with distilled water.
- **IAA -** (M.W. - 175.2), 100 mg was dissolved in 1.0 mL 1N KOH, warm distilled water was added and made up to a final volume of 100 mL (1.0 mg/mL).
- **IBA -** (M.W. - 203.2), 100 mg was dissolved in 1.0 mL 1N NaOH, warm water was added and made up to a final volume of 100 mL (1.0 mg/mL).
- **BAP -** (M.W. - 225.2), 100 mg was dissolved in 1.0 mL of 1N HCl and heated slowly and made up to a final volume of 100 mL (1.0 mg/mL) with distilled water.
- **KIN -** (M.W. - 215.2), 100 mg was dissolved in 1.5 mL of 1N KOH and made upto a final volume of 100 mL (1.0 mg/mL) with distilled water.

3.1.5. Seed Culture

Healthy seeds of *R. tetraphylla* were surface sterilized by adding three drops of Tween-20 and shaking them continuously for 10 minutes and washed with running tap water. Seeds were immersed with 1% Sodium hypochlorite solution for 10-20 minutes and thoroughly washed with sterilized double distilled water for three times in the laminar airflow cabinet (LAF). Subsequently, the seeds were treated with a 0.1% $HgCl_2$ solution by gently shaking them for 1-3 minutes. To remove the traces of $HgCl_2$, the seeds were washed thoroughly for three times with sterile double distilled water. Seeds of *R. tetraphylla* were incubated at 4℃ in refrigerator for 2 days. After two days the sterilized seeds were taken, and one fourth of the proximal side of each seed was cut to accelerate germination. Pre-cut seeds were inoculated on basal MS medium without PGRs.

3.1.6. Inoculation of Explants

Explants of leaf, stem (node, internode) and root were derived from *in vitro* derived plantlets of *R. tetraphylla*. Plantlets and inoculated on MS basal medium augmented with various combinations and concentrations of plant growth hormones.

3.1.7. Culture Condition

The cultures were incubated at 25±2℃, the culture vials were placed on the rack at a distance 25 cm from the light source. A 16/8 h. (light/dark) photoperiod of white light was provided from 2000 lux fluorescent tubes. The culture vials were kept for incubation at dark for 1 week to promote callus induction. Each experiment was repeated thrice for statistical evaluation.

3.1.7.1. Callus Formation of *Rauvolfia tetraphylla*

Callus culture was induced from the explants of leaves, stem (node, internode), and root from *R. tetraphylla* on MS medium supplemented with various concentration of plant growth regulators including auxin (IAA, IBA, 2,4-D), cytokinin's (BAP and KIN) and their combinations. The response of these inoculated explants was observed after 7^{th} day onwards. The callus cultures were subcultured on 21 days interval. Calli were healthy over a period on this medium, triplicates were maintained for data collection.

3.1.8. Direct Rhizogenesis

Leaf and nodal explants were inoculated on MS medium supplemented with IAA, 2,4-D+BAP, IAA+BAP and IBA+BAP" for direct root induction from the explants. The cultures were incubated at 25±2℃. Data on direct root induction from leaves and node from three replicates were recorded.

3.1.9. Multiple Shoot Formation

Nodal explant of *R. tetraphylla* was inoculated on MS medium amended with various concentration and combination of growth hormones such as BAP, 2,4-D+BAP, IAA+BAP and KIN+BAP are used for caulogenesis without intervening callus. Developed shoot were sub-cultured on fresh MS medium every 28 days with the same growth regulators for further shoot elongation. The percentage of shoot formation was recorded, and each experiment was observed in triplicate.

3.1.10. Histological Analysis

Histological studies were evaluated on somatic embryogenesis of leaf explant from *R. tetraphylla* at 10^{th}, 20^{th}, 30^{th}, 40^{th}, 50^{th}, 60^{th} and 70^{th} days. The samples were fixed on FAA

solution contain 95% ethyl alcohol: glacial acetic acid: formaldehyde: water (10:1:2:7) for 48 hours (Liao and Wu 2011). The samples were dehydrated in tertiary-butyl-alcohol and 70% alcohol (60%, 70%, 80%, 90% &100%) for 12 hours each and infiltrated by xylene in the oven at 50℃. Then the samples were embedded in paraffin wax and blocks were sectioned using microtome. The sections were stained with 1% safranin (Jensen 1962) and observed under light microscope.

3.1.11. Hardening

Rooted plantlets of *R. tetraphylla* were separated carefully from the culture tubes and the adhered medium was removed gently by washing under distilled water and then hardened in a plastic cup by using 1:2 ratio of sterilized garden soil: vermiculite.

3.1.12. Statistical Analysis

Data are presented as mean ± standard deviation and were analyzed using one-way analysis of variance (ANOVA). The different among means were tested by Duncan's Multiple Range Test at $p \leq 0.05$ level of significance.

3.2.1. PHYTOCHEMICAL ANALYSIS

3.2.2. Preparation of Extracts

Fresh and healthy leaves and fruits of *R. tetraphylla* were collected, washed, shade dried, and pulverized into fine course powder. The powder (400 g) of leaves and fruit were macerated into organic solvent for 5 days in the ratio of 4:1 and filtered using Whatmann filter paper. The filtrates were obtained by a sequential method from non-polar to polar solvents using hexane, chloroform, ethyl acetate and methanol. Using rotary evaporator, solvents were removed, crude extracts were obtained and stored at room temperature for further analysis (Tripathi *et al*. 2022).

3.2.3. QUALITATIVE PHYTOCHEMICAL ANALYSIS

Hexane, chloroform, ethyl acetate and methanol extracts were subjected to various phytochemical analyses to identify the phytoconstituents.

3.2.3.1. Detection of Alkaloids

Solvent free extracts (50 mg) were stirred with a few mL of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloid reagents as follows:

3.2.3.1.1. Mayers Test (Evans, 1997)

To a few mL of filtrate, one to two drops of Mayers reagents was added by the sides of the test tube. A white creamy precipitate indicated the test as positive.

Preparation of Mayers Reagent:

Mercuric chloride (1.358 g) was dissolved in 60 mL of water and potassium chloride (5 g) was dissolved in 10 mL of water. The two solutions were mixed and made up to 100 mL distilled water.

3.2.3.1.2. Wagners Test (Wagner, 1993)

To a few drops of filtrate, few drops of Wagner's reagent was added by the side of the test tube. A reddish-brown precipitate confirmed the test as positive.

Preparation of Wagners Reagent

Iodine (1.27 g) and potassium iodide (2 g) were dissolved in 5 mL of water and made up to 100 mL with distilled water.

3.2.3.1.3. Hagers Test (Wagner *et al*. 1996)

To a few mL of the filtrate 1or 2 mL of Hager's reagent (saturated aqueous solution of picric acid) was added. A prominent yellow precipitate indicated the test as positive.

3.2.3.1.4. Dragendorff's Test (Waldi, 1965)

To a few mL of filtrate, 1 or 2 mL of Dragendorff's reagent was added. A prominent yellow precipitate indicated the test as positive.

Preparation of Dragendorff's Reagent

Stock Solution

Bismuth carbonate (5.2 g) and sodium acetate (4 g) were boiled for a few min with 50 mL glacial acetic acid. After 12 hours the precipitated sodium acetate crystals were filtered using a glass funnel. Clear and red-brown filtrate (40 mL) was mixed with 160 mL of ethyl acetate and 1 mL of water stored in amber-bottle.

Working Solution: 10 mL of stock solution was mixed with 20 mL of acetic acid and made up to 100 mL with water.

3.2.3.2. Detection of Carbohydrates and Glycosides (Ramakrishna *et al*. 1994)

The extracts (100 mg) were dissolved in 5 mL of methanol and filtered. The filtrate was subjected to the following tests.

3.2.3.2.1. Molisch's Test

To 2 mL of filtrate, two drops of alcoholic solution of α-naphthol were added, the mixture was shaken well, and 1 mL of concentrated sulphuric acid was added slowly along the sides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

3.2.3.2.2. Fehling's Test

Fehling solutions I and II (1 mL each) was added to the one mL of filtrate was kept in boiling water bath. A red precipitated indicated the presence of sugar.

Preparation of Fehling's Solution

FEHLINGS SOLUTION I: Copper sulphate (34.66 g) was dissolved in distilled water and made up to 500 mL with distilled water.

FEHLINGS SOLUTION II: Potassium sodium tartrate (173 g) and sodium hydroxide (50 g) were dissolved in water and made up to 500 mL with distilled water.

3.2.3.2.3. Barfoed's Test

To one mL filtrate, 1 mL of reagent was added and heated on a boiling water bath for 2 mins, red precipitate indicated the presence of sugar.

Barfoed's Reagent

Copper acetate 30.5 g dissolved in 1.8 mL of glacial acetic acid.

3.2.3.2.4. Bendict Test

To 0.5 mL of filtrate, 0.5 mL of benedict's reagent was added. The mixture was heated on a boiling water bath for 2 min. A characteristic-colored precipitate indicated the presence of sugar.

Preparation of Bendict's Reagent

Sodium citrate (173 g) and sodium carbonate (100 g) were dissolved in 800 mL of distilled water and boiled to make it clear. Copper sulphate (17.3 g) dissolved in 10 mL of distilled water was added to it.

3.2.3.2.5. Borntrager's Test (Evans, 1997)

For detection of glycosides, 50 mg of extract was hydrolyzed with dilute hydrochloric acid for 2 hrs. on a water bath, filter, and the hydrolysate. To 2 mL of filtrate hydrolysate, 3 mL of chloroform was added, shake well and the organic layer was separated. Add 10 % strong ammonia solution to it, shake slightly and keep the test tube apart for few minutes. The lower ammoniacal layer showed pink or red color and indicated the presence of glycosides.

3.2.3.2.6. Legal's Test

Fifty mg of the extract was dissolved in pyridine, sodium nitroprusside solution was added and made alkaline using 10% sodium hydroxide. The formation of pink color indicates the presence of glycosides.

3.2.3.3. Detection of Saponins by Foam Test (Kokate, 1999)

The extract (50 mg) was diluted with distilled water and made up to 20 mL. The suspension was shaken in a graduated cylinder for 10 min. A two cm layer of foam indicated the presence of saponins.

3.2.3.4. Detection of Phytosterols (finar, 1986)

Libermann-burchard's test

The extract (50 mg) was dissolved in 2 mL of acetic anhydrate. To this, one or two drops of concentrated H_2SO_4 was added slowly along the sides of test tube. An array of color changes showed the presence of phytosterols.

3.2.3.5. Detection of Fixed Oils and Fats (kokate, 1999)

3.2.3.5.1. Spot test

A small quantity of extract was pressed between two filter paper. Oil stain on the paper indicated the presence of fixed oil.

3.2.3.5.2. Spanoification Test

A few drops of 0.5 N alcoholic potassium hydroxide solution were added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated in a water bath for 24 hrs. Formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

3.2.3.6. Detection of Phenolic Compounds and Tannins

3.2.3.6.1. Ferric chloride (Mace, 1963)

The extract (50 mg) was dissolved in 5 mL of methanol. To this a ferric chloride solution was added. A dark green color indicated the presence of phenolic compounds.

3.2.3.6.2. Gelatin Test (Evans, 1997)

The extract (50 mg) was dissolved in 5 mL of methanol and 2 mL of 1 % of gelatin containing 10 % sodium chloride was added to it. White precipitate indicated the presence of phenolic compounds.

3.2.3.6.3. Lead Acetate Test

The extract (50 mg) was dissolved in methanol and 3 mL of 10% lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds.

3.2.3.6.4. Alkaline Reagent Test

The extract was treated with a 10% ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavonoids.

3.2.3.6.5. Magnesium and Hydrochloric Acid Reduction (Harbone, 1998)

The extract (50 mg) was dissolved in 5 mL of alcohol and a few fragments of magnesium ribbon and concentrated hydrochloric acid (drop wise) were added. The presence of flavanol and glycosides were inferred by the development of pink to crimson color.

3.2.3.7. Test for Quinones

To 1 mL of extract, 1 mL of Conc. Sulfuric acid was added. Formulation of red color indicates the presence of quinones.

3.2.3.8. Test for Cardiac Glycosides (Keller-Kiliani Test) – To 0.5 mL of extract, 2 mL of glacial acetic acid and a few drops of 5% ferric chloride were added. This was under layered with 1 mL of Conc. Sulphuric acid. The formation of a brown ring at the interface indicates the presence of cardiac glycosides.

3.2.3.9. Test for Terpenoids (Salkowski test)

To 0.5 mL of extract, 2 mL of Chloroform was added, and Conc. Sulphuric acid is added carefully. The formation of red-brown color at the interface indicates the presence of terpenoids.

3.2.3.10. Test for Coumarins

To 1 mL of extract, 1 mL of 10% NaOH was added. The formation of yellow color indicates the presence of coumarins.

3.2.3.11. Test for Phlobatannins

To 1 mL of plant extract a few drops of 2% HCl was added, the appearance of red color precipitate indicates the presence of phlobatannins.

3.2.3.12. Test for Steroids

To 1 mL of plant extract equal volume of chloroform is added and subjected with a few drops of the Conc. Sulphuric acid, appearance of brown ring indicates the presence of steroids.

3.2.3.13. Test for Anthraquinones

To 1 mL of plant extract, a few drops of 10% ammonia solution were added, appearance of pink color precipitate indicates the presence of anthraquinones.

3.2.3.14. Test for Flavonoids (Shinoda Test)

To 2 mL of plant extract, 1 mL of 2N sodium hydroxide was added. The appearance of yellow color indicates the presence of flavonoids.

3.2.3.15. Test for Protein (Xanthoproteic test)

One mL of the extract was taken, and a few drops of nitric acid were added and shaken. The emergence of yellow color indicates the presence of protein.

3.3. QUANTITATIVE ANALYSIS

Ethyl acetate and methanol crude extracts of leaves and fruits were subjected to quantitative analysis of tannin, alkaloid, flavonoid and phenols.

3.3.1. Estimation of Total Tannin Content

Folin – Ciocalteu Method

Total tannin content (Amorim *et al*. 2008) was determined by colorimetry method using Folin-Ciocalteu reagent. Take 0.1 mL of sample extract to a volumetric flask (10 mL) containing 7.5 mL of distilled water, 0.5 mL of Folin-Ciocalteu phenol reagent, 1 mL of 35% sodium carbonate solution and dilute to 10 mL with distilled water. The mixture was incubated at room temperature for 30 mins. Gallic acid was used as a standard and prepared at various concentration (20, 40, 60, 80 and 100 µg/mL) in the similar method. Absorbance was measured at 725 nm with an UV/Vis Spectrophotometer. The amount of tannin content was calculated, and the result was expressed as µg gallic acid equivalent (µg/GAE) per mg plant extract.

3.3.2. Estimation of Alkaloids (Ajanal *et al*. 2012**)**

Standard Preparation

Atropine standard were aliquots (0.4, 0.6, 0.8, 1 and 1.2 mL) and transfer in separating funnel. Then add mixture of 5 mL phosphate buffer (pH 4.7) and 5 mL of bromocresol green solution in separating funnel and shake well. Chloroform was added in parts 1 + 2 + 3 + 4 up to 10 mL gradually separate the extracts and collected extracts were make up into 10 mL volumetric flask. The absorbance was measured at 470 nm in UV/Vis Spectrophotometer.

Preparation of Sample

Extracts were dissolved in 2N HCl and then filtered (up to pH 2). 1 mL of this solution was transferred to separatory funnel and washed with 10 mL chloroform (3 times). The pH 7 was adjusted with 0.1N NaOH. Then 5 mL of bromocresol green solution and 5 mL of phosphate buffer were added to this solution. Shake the mixture and extract yellow-coloured extracts with 1-, 2-, 3- and 4-mL chloroform by vigorous shaking in separating funnel, then collect extract in

10 mL volumetric flask and it required dilute with chloroform. The extracted complex was measured at 470 nm in UV/Vis Spectrophotometer.

The amount of alkaloid content was calculated from the calibration curve of atropine and the result was expressed as µg atropine equivalent per mg plant extract.

3.3.3. Estimation of Flavonoid

Aluminium chloride colorimetric method (Chang *et al*. 2002) was used for flavonoids estimation. Extracts (0.5 mL) were mixed with 1.5 mL of methanol, add 0.1 mL of 10% aluminium chloride, 0.1 mL of potassium acetate and 2.8 mL of distilled water. Incubated in room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with an UV/Vis Spectrophotometer. Rutin is used as a standard with different concentrations (0.05, 0.15, 0.25, 0.35, 0.45, 0.55, 0.65 and 0.75 µg/mL). The value was represented as µg rutin equivalent (µg/RUT) per mg plant extract.

3.3.4. Estimation of Total Phenolic Content

Total soluble phenolics in the extracts were determined using FC reagent according to the Slinkard & Singleton, (1977) method. Extracts (1 mL) were mixed with 1.0 mL of diluted FC reagent and incubate for 5 mins at room temperature. Add 3.0 mL of 2% sodium carbonate and the mixture was allowed to stand for 2 hrs at dark. The absorbance was measured at 760 nm with an UV/Vis Spectrophotometer. Gallic acid used as a standard at various concentration (10, 20, 40, 60, 80 and 100 µg/mL). The concentration of total phenolic compounds in the extracts were estimated in µg of gallic acid equivalent (µg/GAE) per mg plant extract (Lu *et al*. 2011).

3.4. Mass Spectra

The methanol extract of leaves from *R. tetraphylla* were subjected to mass spectra analysis such as GCMS and LCMS using GC-MS NIST (2008) library.

3.5. CHROMATOGRAPHIC ANALYSIS

3.5.1. Column Chromatography

Column chromatography is a technique used for purifying bioactive compounds from mixture of compounds present in plant extracts. It is a glass tube with a diameter of 50 mm and a height of 50 cm with stop cock at end to elute the compounds gradually. Silica or alumina is used, and mixture of solvents is used for mobile phase.

3.5.2. Packing the Column

The adsorbent (Silica gel) can be mixed with the liquid (Hexane) to form a thin slurry, which is then poured and rinsed into the column. The separation will be best if the bottom of the bed of adsorbent is flat. All traces of adsorbent have been rinsed down i.e., solvent will usually have to be drained off during this process and top of the bed of adsorbent has been flattened by jiggling the tube.

3.5.3. Adding the Sample

Once the column has been prepared, the solvent should be allowed to drain until it's level that covers the adsorbent. The sample is then added. A crude extract should be dissolved as possible of a solvent of low eluting power. After the addition, the column is allowed to drain again until the level of liquid has fallen just to top of the adsorbent. Rinse the sample down from the walls of the column with additional small portions of solvent, draining the column each time.

3.5.4. Elution

After the sample has been added, the column is eluted using a series of solvents as recommended. Start with a solvent of low eluting power (Toluene) and work up through solvents of increasing eluting power (Methanol) as mixtures. When the level of liquid in the column is low, the eluting solvent should be added very cautiously to avoid disturbing the top of the bed of adsorbent.

3.5.5. Collecting the Fractions

If the substances to be separated are colored, their progress down the column can be followed visually, and the eluant that contains each component can be collected separately. If the substances are colorless, the eluant must be collected in successive fractions and the presence of components of the original mixture must be determined by some analytical procedure.

3.5.6. Fractions

The Methanol leaf extract (2 g) was subjected to column chromatography using a series of increasing polarity solvents. The sample was loaded onto the silica gel packed column in a minimum volume of 100% hexane, then eluted sequentially with the following solvent systems:

- 100 mL Hexane – I
- 80 mL Hexane: 20 mL Toluene – II
- 60 mL Hexane: 40 mL Toluene – III

- 40 mL Hexane: 60 mL Toluene – IV
- 20 mL Hexane: 80 mL Toluene – V
- 100 mL Toluene – VI
- 80 mL Toluene: 20 mL Chloroform – VII
- 60 mL Toluene: 40 mL Chloroform – VIII
- 40 mL Toluene: 60 mL Chloroform – IX
- 20 mL Toluene: 80 mL Chloroform – X
- 100 mL Chloroform – XI
- 80 mL chloroform: 20 mL Ethyl acetate – XII
- 60 mL chloroform: 40 mL Ethyl acetate – XIII
- 40 mL chloroform: 60 mL Ethyl acetate – XIV
- 20 mL chloroform: 80 mL Ethyl acetate – XV
- 100 mL Ethyl acetate – XVI
- 80 mL Ethyl acetate: 20 mL Methanol – XVII
- 60 mL Ethyl acetate: 20 mL Methanol – XVII
- 40 mL Ethyl acetate: 20 mL Methanol – XIX
- 20 mL Ethyl acetate: 20 mL Methanol – XX
- 100 mL Methanol – XXI

3.5.7. Thin Layer Chromatography

TLC was carried out on precoated silica gel aluminium sheets (Merck TLC, silica gel 60 F254 (20 x 20 cm). The chromatogram was developed by placing the TLC plate in a TLC apparatus containing suitable solvent system. The developed TLC plates were dried at room temperature. The separated spots were observed under visible as well as UV light (254 and 365 nm) (Raaman, 2006). The R_f values were evaluated.

$$Rf = \frac{Distance\ travelled\ by\ the\ compound}{Distance\ travelled\ by\ solvent\ front}$$

Based on the TLC comparison and analysis, the fraction one was used for further characterization.

3.5.8. Characterization of Isolated Compounds

The isolated compounds were subjected to characterized by spectral data using UV (Labman UV-visible double beam spectrophotometer), FTIR (Shimadzu), GC-MS/MS (Perkin Elmer Clarus 680 GC) and ^{1}H NMR and ^{13}C NMR (Bruker 500).

3.6. ANTIMICROBIAL ACTIVITY

3.6.1. Microorganisms and Culture Conditions

The Microbial Culture Collection, Chandigarh, India, provided bacteria such as *E. coli* [MTCC-1687] (Gram-negative bacteria), *S. aureus* [MTCC-96] (Gram-positive bacteria) and *Enterococcus faecalis* [MTCC-439] (Gram-positive bacteria). All the cultures are then sub-cultured on Mueller-Hinton agar medium for bacterial culture on a regular basis and stored at 4℃ for subsequent studies.

3.6.2. Preparation of Inoculum

3.6.2.1. Test for Antibacterial Activity of *Rauvolfia tetraphylla*

Ethyl acetate and methanol extracts of leaf and fruit were evaluated against bacterial strains by agar well plate diffusion technique. 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℃ until needed. To check for viability and ensure the inoculum was not contaminated, dilutions of the inoculum were grown on nutrient agar.

3.6.3. *In vitro* Antimicrobial Activity (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 Mueller-Hinton Agar plates. A 10 mm uniform wells were made by sterile borer on all culture plates for 1 mg/mL concentrations of various extracts, namely, ethyl acetate and methanolic extracts of leaf and fruit. Furthermore, *R. tetraphylla* extracts were diluted and added to the wells according to their concentrations (25, 50, 75 and 100 µg/mL) and the control experiments without plant extracts. Tetracycline and dimethyl sulfoxide were “used as positive and negative control. Treated culture plates were incubated overnight in a microbial incubator at 37℃ and inhibition zones were measured.

3.6.4. Determination of Minimum Inhibitory Concentration

MIC is an antibiotic reduces observable bacterial growth. When a MIC value is compared to a breakpoint value, it may be determined if bacteria virulence of antibiotic. MIC of an ethyl acetate and methanol extract of *R. 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 extracts of leaf and fruit that inhibits visible bacterial growth under defined conditions. Agar dilution is mixing varied quantities (200 μg/ mL, 100 μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, 6.25 μg/mL, 3.125 μg/mL, 1.56 μg/mL, and 0.78 μg/mL) of extracts into a nutritional agar medium containing *E. coli*, then spreading a standardised number of cells throughout the agar plate's surface.

3.7. ANTIOXIDANT ASSAY

3.7.1. DPPH Radical Scavenging Activity (Hatano *et al*. 1988)

3.7.1.1. Principle

DPPH (1,1-Diphenyl-2-picrylhydrazyl) assay is used for scavenging the free radicals from the plant extracts to determine antioxidant capacity of the plant-based drugs. The antioxidants can reduce the stable radical DPPH to the yellow- coloured diphenyl picrylhydrazine. To quantify the antioxidant capacity of the plant extracts absorbance is measured at 517 nm.

3.7.1.2. Chemicals and Reagents

DPPH (1,1-Diphenyl-2-picrylhydrazyl), ascorbic acid, methanol and all other chemicals were used in analytical grade reagents.

3.7.1.3. Procedure

Ethyl acetate and methanol of leaves and fruits of *R. tetraphylla* (0.3 mL) were mixed with 2.7 mL of 0.1 mM solution of DPPH in methanol. The mixture was shaken vigorously and incubate in dark for 30 min at room temperature. Absorbance was measured at 517 nm Spectrophotometer. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the following equation.

$$Scavenging\ \% = \frac{Control\ OD - Sample\ OD}{Control\ OD} \times 100$$

The results were expressed as mean value ± standard deviations and percentage of inhibition (IC_{50}) is calculated. Ascorbic acid was used as standard.

3.7.2. TOTAL Antioxidant Activity (Prieto *et al*. 1999)

3.7.2.1. Principle

The phosphomolybdenum method is a widely used technique for assessing the total antioxidant activity of substances. Its principle involves the reduced from Mo (VI) to Mo(V).

3.7.2.2. Chemicals

Sulphuric acid, Sodium Phosphate, Ammonium molybdate, ethanol and ascorbic acid were used in analytical grade.

3.7.2.3. Procedure

Total antioxidant capacity of the leaf and fruit extracts of *R. tetraphylla* were evaluated according to the method described by Prieto *et al*. (1999). An aliquot of 0.5 mL of extracts were mixed with 4.5 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Ascorbic acid is used as a control. The mixtures of sample solution were incubated in a boiling water bath at 95°C for 90 min and the absorbance was measured at 695 nm. The higher absorbance value indicated higher antioxidant activity and inhibition percentage were calculated.

$$Scavenging\ \% = \frac{Control\ OD - Sample\ OD}{Control\ OD} \times 100$$

Results were given as mean ± standard deviation of 3 replicates and percentage of inhibition (IC_{50}) was calculated. Differences between means were determined using one-way ANOVA with Tukey test. The level of statistical significance was set at $P \leq 0.05$.

3.7.3. FRAP Assay (Mohti *et al*. 2020)

3.7.3.1. Principle

The method involves reducing the colourless Fe^{3+} tripyridyltriazine complex to the blue-coloured Fe^{2+} tripyridyltriazine complex using electron-donating antioxidants under acidic conditions.

3.7.3.2. Chemicals and Reagents

Phosphate buffer, potassium ferricyanide, trichloroacetic acid, ferric chloride, and ascorbic acid

3.7.3.3. Procedure

The antioxidant capacity in extracts from *R. tetraphylla* leaves and fruits was evaluated using the FRAP assay (Mohti *et al*. (2020) and Liaqat *et al.* (2021)). The procedure involved the preparation of a sample aliquot (100 µL) to which 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide solution (1%) were added. Following a 20-minute incubation at 50°C, 2.5 mL trichloroacetic acid solution (10%) was added and centrifuged at 3,000 rpm form 10 mins. From the mixture, 2.5 mL of supernatant and mixed with 2.5 mL of distilled water and

add 0.5 mL of 1% ferric chloride solution. The bluish colour will confirm the antioxidant activity at 700 nm. Ascorbic acid was used as the standard, with its FRAP value set at 2 µM. Triplicate analyses were performed for both the standard and each extract to ensure the reliability of the results. The FRAP value was calculated using the following equation.

$$\text{FRAP value} = [(A_1 - A_0) / (A_c - A_0)] \times 2 \text{ concentration of standard (µmol/L)},$$

where A_c is the absorbance of the positive control, A_1 is the absorbance of the sample, and A_0 is the absorbance of the blank. FRAP activity was quantified in ferrous equivalents (FE) measured in µM.

3.8. EVALUATION OF *IN VITRO* ANTI-INFLAMMATORY ACTIVITY

3.8.1. Principle

The egg albumin denaturation assay aims to assess whether agents can impede protein denaturation. Denaturation involves structural changes and loss of biological activity in proteins. Egg albumin, a model protein is denatured by exposure to heat, pH extremes or denaturing agents. The assay evaluates a substance's ability to reduce egg albumin denaturation, indicating potential anti-inflammatory properties. Agents inhibiting denaturation may have anti-inflammatory effects by stabilizing protein structures.

3.8.2. Chemicals

Egg albumin solution, Phosphate buffer saline and DMSO

3.8.3. Equipment

Clean pipettes and puppet tips, Test tubes, Incubator, Spectrophotometer, Water bath.

3.8.4. Procedure

In vitro anti-inflammatory activity of egg albumin assay (Chandra *et al*. (2012); Madhuranga & Samarakoon (2023)) was evaluated. Egg albumin solution (1%) was prepared from hen's egg. Reaction mixture of 5 mL was prepared, 0.2 mL of bovine serum albumin, 2.8 mL of phosphate buffered saline (PBS, pH 6.4) and 2 mL of various concentrations (20 to 120 µg/mL) of ethyl acetate and methanol crude extracts of leaves and fruits from *R. tetraphylla*. The mixtures will be incubated at 37±2℃ in a BOD incubator for 15 min, followed by heating at 70±2℃ for 15 min in a water bath. The absorbance was measured at 660 nm using a UV

spectrophotometer. Diclofenac sodium was used as a reference drug. The percentage inhibition of protein denaturation was calculated using the formula:

$$Percentage\ inhibition = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

The inhibition concentration (IC_{50}) value was calculated, and data were expressed as mean ± Standard deviation. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Turkey post hoc test.

3.9. CYTOTOXICITY ACTIVITY

3.9.1. Preparation of Media for Cell Culture

Ingredients	Growth medium (10%)	Growth medium (5%)	Maintenance medium (2%)
MEM	870 mL	920 mL	95 mL
P&S (penicillin & streptomycin) solution	1 mL	1 mL	1 mL
Phenol red (0.4%) solution	1 mL	1 mL	1 mL
Kanamycin solution	1 mL	1 mL	1 mL
Fungizone solution	1 mL	1 mL	1 mL
3% L-glutamine solution	1 mL	1 mL	1 mL
Fetal Bovine serum	100 mL	50 mL	20 mL
75% sodium bicarbonate solution	20 mL	20 mL	20 mL
Herpes buffer	5 mL	5 mL	5 mL
Total volume	1000 mL	1000 mL	1000 mL

All the ingredients were mixed well by shaking gently, pH was calibrated and adjusted to 7.2 to 7.4.

3.9.3. Cell Lines

African Green Monkey Kidney cell line (Vero) and mouse embryonic fibroblast cell line (3T3) were procured from the National Centre for Cell Sciences (NCCS), Pune, India.

3.9.3.1. Cell Culture

The cells were cultured in open vented 75 cm^2 culture vessels in a standard horizontal laminar flow hood and incubated in a NUAIR cell incubator at 37℃ in an atmosphere of 5% CO_2 and 95% air. Cell lines were provided with growth media of 90% minimal essential medium (Eagle), 10% fetal bovine serum (Gibco) as well as 5% streptomycin - penicillin (sigma-Aldrich). Media were refreshed at least three times a week (15 mL) under aseptic techniques. Cell lines were sub-cultured when 80% confluency was reached sub-culturing consisted of

dispensing old media with a vacuum and addition of 5 mL of 0.25% Trypsin - 0.03% EDTA solution (Gibco) to remaining cell layer followed by incubation for 6 min at 37℃ TPVG was removed and the bottle was incubated at 37℃, until all the cells detach from the surface. The cells were resuspended in 5 mL of growth media. The suspensor was aspirated a few times to break cell clumps. The cell concentration was determined by counting the cells in a hemocytometer.

3.9.4. Samples

Ethyl acetate and methanol extracts of leaves and fruits of *R. tetraphylla* were used to determine the cytotoxicity activity.

3.9.4.1. Preparation of Samples

To prepare stock solution, 5 mg of each sample was dissolved in 0.5 mL of 2% MEM (minimal essential medium eagle). From this working solution with different concentrations (10 - 100 µg/mL) were prepared in 96 well plate for cytotoxic study.

3.9.6. MTT Assay

3.9.6.1. Principle

MTT is a calorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tertazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazon product. The cells are then solubilized formazon reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

3.9.6.2. Procedure

The MTT method as described by Mosmann (1983) was used to quantitatively detect living but not dead cells. In brief approximately 2×10^4 cells/well were seeded onto 96 well plate, 100 µL of MEM medium was added and incubated at 37℃ for 24 hrs. Then, the medium was discarded, and fresh medium was added with different concentrations of extracts (5, 10, 25, 50 and 100 µg/mL). The setup was incubated for 1-3 h at 37℃ in CO_2 incubator. After respective incubation period the medium was discarded and 100 µL of fresh medium was added with 10 µL of MTT. After 2 hours of incubation, the medium was discarded and 100 µL of DMSO was added to dissolve the formazon crystals. Then, the absorbance was noted using spectrophotometer at 570 nm.

$$Viable\ cells\ (\%) = \frac{Sample\ OD}{Control\ OD} \times 100$$

A dose-response curve depicting cell viability (%) in relation to the concentration of *R. tetraphylla* extracts (µg/mL) was generated and the concentration at which 50% of Vero and 3T3 cells were killed CC_{50} (Cytotoxicity concentration-CC) was determined. All the *in vitro* experiments were done in triplicate. *p* value ≤ 0.05 was considered significant.

3.10. SCRATCH ASSAY (Liang *et al*. 2007**)**

3.10.1. Principle

The fundamental principle of scratch assays involves damaging a confluent cell monolayer to create a cell-free area. The assay involves three main steps such as cell injury (wounding), observing the subsequent healing process and acquiring data through microscopy. Subsequently, data evaluation is conducted.

3.10.2. Reagents

Dulbecco's modified Eagle's medium with supplements (serum, antibiotics), Versene (EDTA) with trypsin, Phosphate-buffered saline (PBS), 2 mg/mL bovine serum albumin (BSA), 1 mg/mL poly-L-lysine stock

3.10.3. Equipment

Tissue culture dishes (60 mm size), Fine marker, p200 Pipet tips, Haemocytometer, Phase-contrast microscope, Camera, Stage incubator, CO_2 supply, Image analysis software.

3.10.4. Procedure

3.10.4.1. Coating of Cell Culture Dishes

Prepare 60-mm dishes, cells are coated with 10 µg/mL fibronectin to the dishes and allow it to adhere either by keeping the dishes overnight at 4℃ or for 2 hours at 37℃. Make sure not to rotate or shake the dishes during this process. After the coating phase, remove any excess unbound substrate. Next, block the coated dishes by adding 3 mL of a 2 mg/mL bovine serum albumin (BSA) solution. Allow the blocking to occur for 1 hour at 37℃ and perform a single wash of the dishes using PBS and add 3–5 mL of appropriate cell culture media.

3.10.4.2. Passaging the Cells in Culture

To detach sub-confluent growing cells from a tissue culture dish, begin by rinsing the cells twice with PBS. Introduce versene supplemented with trypsin and then combine the cells

with medium containing serum. Gently employ a pipette to softly agitate the solution, ensuring even dispersion of the cells. Extract a small sample from the cell suspension and employ a hemacytometer to determine the cell counts. Following this, seed the cells onto the previously prepared 60 mm dish to establish a fully populated monolayer. Allow the dishes to be incubated for approximately 6 hours at 37℃, permitting the cells to adhere and spread thoroughly on the substrate.

3.10.5. Scratch Assay

The scratch assay was performed (Roy *et al*. 2023) to determine the impact of extracts of *R. tetraphylla* on cell migration and wound closure against Vero and 3T3 cells. To make a "scratch" on monolayer cells using a p200 pipette tip, forming a straight line. Eliminate debris and refine the scratch edge by rinsing cells growth medium (1 mL), followed by replacement growth medium (5 mL). The initial scratch image was captured. Subsequently, the dish was placed in an incubator at 37℃ for 24 hours. Migration was evaluated by counting the quantity of cells that moved away from the border of the wound after 24 hours incubation in DMEM with 0.5% serum and capture the image under phase-contrast microscope.

$$\text{Wound closure (\%)} = \left[\frac{At - Ah}{At}\right] \times 100$$

A_t – Area of the wound measured immediately after scratching, A_h – Area of the wound measured *h* hours after the scratch is performed.

3.11. CHICK CHORIOALLANTOIC MEMBRANE (CAM) ASSAY (Zwadlo-Klarwasser *et al*. 2001)

3.11.1. Incubation of Eggs

Eight-day old chicken eggs (hen eggs) were carefully selected for this assay. The eggs were thoroughly cleaned using 70% ethanol to remove dirt and debris. All the eggs were then incubated at 37℃ with 65 % of humidity. Candling processes were carried out to examine the quality of eggs before the treatment given.

3.11.2. Drilling of Hole on the Eggs

On the 8th day of incubation, the surface of the eggs was disinfected. Small 1x1 cm holes were carefully drilled at the blunt ends of the eggshells.

3.11.3. Abscission of the Eggs

Ethyl acetate and methanol extracts (100 µg/mL) obtained from the leaves and fruits of *R. tetraphylla* were prepared. Sterilized filter paper discs were soaked with the extracts and these discs were used to abscise the egg membranes. The extracts and a control substance were applied directly to the CAM membrane of the eggs by absorbing them onto the filter paper discs. Approximately 100 µL of the extract and control were placed onto the CAM and the manipulation window was covered with parafilm. The eggs were then returned to the incubator and allowed to continue incubating for an additional three days.

3.11.4. Visual Assessment of the CAM

On the 12^{th} day of incubation, the reactions of the CAM were visually assessed. The CAM was carefully harvested by removing the hard shell, while keeping the soft membrane intact. The branching points of blood vessels present in the CAM was observed.

3.12. HEAT STRESS ASSAY

Ceanorhabditis elegans strains used in this study were N2 wild type for most lifespan assay and stress resistance assay were maintained in incubator at 20℃. Worms were grown on nematode growth medium (NGM) agar plates with *E. coli* (OP50) as a food source. The ethyl acetate and methanolic extracts of leaves and fruits of *R. tetraphylla* was added during the preparatory process of the agar plates. Each day the dishes were evaluated for survival and animals were scored dead when they did not move after repeated taps with a pick. Per experiment, a minimum of 100 worms divided over 3–4 dishes, was used to evaluate the effect of each single concentration of adaptogen. In stress resistance assays, the number of surviving animals was monitored for several days following exposure to the indicated stressor (heat shock at 35℃ during 180 min) with a dose of 1mg/mL. The effect of the stress conditions on survival in the presence or absence of adaptogens was further compared with survival values in control cultures. The maximum lifespan and percentage effect of an experiments were calculated.

3.13. *IN VIVO* STUDIES ON WOUND HEALING

In vivo wound healing analysis were carried out using Zebra fish model (Shaibi *et al*. 2022). Adult wounding experiments utilized 6- to 12-month-old wild-type TL/Ek strain fish, by introducing manual wounds in embryonic caudal fins. The fins were carefully collected and preserved in 10% formalin for subsequent analysis. Ethyl acetate and methanolic extract of *R. tetraphylla* leaves and fruit were administered at varying concentrations (25 µg/mL, 50 µg/mL,

75 µg/mL and 100 µg/mL) for wound healing. The fish were fed with feed combined with the extract for the treated group and without extracts for the control group for the regeneration study. The fishes were divided into control and treated groups and were closely monitored for 21 days. The grown fins were collected and analysed for histological studies. Hematoxylin and eosin-stained slides were examined under the light microscope and the cellular changes of tissue regeneration were recorded.

3.13.1. Histological Studies

The assessment of regenerated fins involves examining specific histological aspects. These criteria include evaluating the re-epithelialization within the epidermis, assessing the maturity and arrangement of epidermal cells, measuring the thickness of the granular cell layer, evaluating the extent of tissue formation within wounds, and making comparisons between the regenerated tissue and the corresponding normal tissue section.

3.14. CHARACTERISATION OF *RtTDC* GENE

3.14.1. Isolation of DNA from *R. tetraphylla*

Healthy tender leaf tissues of *R. tetraphylla* was used for DNA isolation using DNeasy plant Mini Kit (Qiagen, Germany). DNA was purified, quantified and its quality will be verified using 1% agarose gel electrophoresis. Sequence information of the target gene (*R. verticillata*) was obtained from NCBI. Subsequently, primer was intended based on the coding DNA sequence, 5'-ATGGGCAGCATTGATTCAACAG-3' and 5'-TCAAGCTTCCTTGAGCAAATCA-3' forward and reverse primer respectively. A total volume of 20 µL containing 1µL template DNA, 0.5 µL Forward Primer, 0.5 µL Reverse Primer, 10 µL master Mix and 8 µL of molecular gradient water used for PCR amplification (Dharshini *et al*. 2020; Ashwin *et al*. 2019; Manoj *et al*. 2019). Amplified TDC gene were confirmed by using agarose gel electrophoresis.

3.14.2. Elution of TDC Gene

TDC gene was extracted from the agarose gel using QIAquick Gel Extraction Kit (Qiagen, Germany) and InsTAclone cloning kit (Thermo Fisher Scientific, USA) will be used for ligating in $pTZ_{57}R/T$ vector. Interest of target was cloned into the $pTZ_{57}R/T$ vector using standard molecular biology techniques. The $pTZ_{57}R/T$ vector is commonly used for cloning and allows for the amplification and sequencing of the inserted DNA fragment (Mirahmadi *et al*.

2015). The cloned DNA will be transformed into *E. coli*, a well-known bacterial host for DNA amplification and cloning (Froger and Hall, 2007).

3.14.3. Transformation of Cloned Vector into Bacterial System

The heat shock method was employed to transform *E. coli* competent cells by introducing 2.5 µL ligated product. The cells were initially incubated on ice for 30 minutes, followed by a brief heat shock at 42℃ for 1 minute and immediate transfer back to ice for 2 minutes. To recover the heat-shocked cells, LB medium (1 mL) was added, and incubated at 37℃ for 1 hour. Transformation efficiency will be determined by counting the average number of transformed colonies after spreading 100 µL of the heat-shocked culture onto an ampicillin-containing plate, along with a control plate (Saleh *et al*. 2023). Colonies will be isolated by Colony PCR and DNA fragments was confirmed by gel electrophoresis. Desired DNA will be isolates from the cloned bacteria using the QIAprep Spin Miniprep kit (Qiagen, Germany) and plasmid will be isolated (Sambrook *et al*. 1989).

3.14.4. Gene Sequencing

Plasmid DNAs were sequenced by the addition of ddNTPs to the reaction mixture to terminate DNA synthesis at each nucleotide using Sanger's DNA sequencing method. (Crossley *et al*. 2020). M13 primer will be used to anneal with $pTZ_{57}R/T$ vector, after sequencing complete coding sequence of TDC gene length will be analysed.

3.14.5. Analysis of TDC Gene Sequence

Similarities of TDC gene sequence of *RtTDC* will be validated using BLAST tool provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast) and Nuclotide and aminoacid will be aligned using CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). Open reading frame (ORF) can be used for graphical analysis (www.ncbi.nlm.nih.gov/projects/gorf) and ExPASy is used to detect the amino acids from cloned *RtTDC.*

3.14.6. Elucidation of Protein

To gain insights into the protein's structural characteristics and interactions, a comprehensive homology search was conducted using the NCBI Blast search tool on both the amino acid sequence and structural databases. The 3D structure of the protein was modelled using SWISSMODEL (http://swissmodel.expasy.org), employing various 3D templates with identity ranges from 98% to 47%. SWISS -MODEL is used for homology-based 3D model for

the *RtTDC* protein will be generated and 6eew.1.A of *C. roseus* used as a template structure. Additionally, to identify conserved regions and potential functional motifs, multiple sequence and structural homologues were aligned using MUSCLE (Sievers and Higgins, 2018). Multiple sequence alignment will be analysed TDCs from *R. verticillata*, *R. tetraphylla*, and *C. roseus*. It provides a valuable insight of structural aspects of *RtTDC* and its potential roles in enzymatic activity. Phylogenetic tree will be developed by Mega11 software (Kumar *et al*. 2018). The Neighbour-Joining (NJ) method is used for phylogenetic analysis. Expasy's ProtParam server (http://us.expasy.org/tools/protparam.html) to be used for physiochemical properties such as theoretical pI, molecular weight, ¬R and +R (total number of positive and negative residues), extinction coefficient (Gill *et al*. 1989), instability index (Guruprasad *et al*. 1990), AI (aliphatic index), and GRAVY (grand average hydropathy) (Kyte and Doolittle, 1982). The secondary structure of TDC gene *R. tetraphylla* protein sequences is calculated by HNN (https://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html) (Hierarchical Neural Network) (Kashani-Amin *et al*. 2019). Hydropathy plot will be generated by ProtScale program found at http://web.expasy.org/protscale/, a Kyte and Doolittle Kyte and Doolittle (1982).

3.14.7. Interaction of Ligand -Receptor

Interaction of gene protein and ligand is to be elucidated the TDC receptor structure (3D model) and L-Tryptophan as a ligand structure from the pubchem database (https://pubchem.ncbi.nlm.nih.gov/). Docking (Autodock vina) is used to predict how the ligand binds to the receptor, generating potential binding geometries and interactions (Morris *et al*., 2008). PyMOL is a graphical software used for governing the interaction between a ligand and its receptor (Rauf *et al*. 2015). Ligplot+ is used to analyse the two-dimensional structure at binding site (Wallace *et al*., 1995; Laskowski and Swindells, 2011).

3.14.8. Enzymatic Assay for TDC (Islas *et al*. 1994; Jadaun *et al*. 2017)

Two grams of frozen leaves will grind in pre-cold pestle and mortar make it fine powder and add 1.25 mL of 0.1 M HEPES (pH 7.5). The mixture will be centrifuged for 30 minutes (12,000 rpm) and collect the supernatant, it is used as enzyme. The complex of assay consists of enzyme source, 4 µM Pyridoxal 5-phosphate, 5 mM L-tryptophan, 50 mM HEPES buffer (25 µL of each) and incubate for 30 minutes (37°C). The enzyme activity will be stopped by addition of

pre-chilled methanol (100 µL), again centrifuge it for 10 minutes (12,000 rpm). The supernatants will be analysed by high-performance liquid chromatography (HPLC), gradient solvent system (Acetonitrile and water) used in C_{18} column and tryptamine used for standard.

3.15. MOLECULAR DOCKING ANALYSIS

3.15.1. Preparation of Protein Structure

The crystal structure of the IL-1β receptor (PDB ID: 6Y8M, resolution 1.9 Å), TGF-β receptor (PDB ID: 6B8Y, resolution 1.65 Å) and MMP 9 receptor (PDB ID: 1GEN, resolution 2.15 Å) was obtained from the RCSB Protein Data Bank (https://www.rcsb.org/). The enzyme was 3D protonated and hydrogen atoms were added in their standard positions. The system was then optimized, and partial charges were computed. Co-crystallized water molecules were removed, and the binding pocket was isolated and defined. It will be envisioned using PyMOL software.

3.15.2. Preparation of Ligand Structure

Some of the compounds (GCMS and LCMS) retrieved from PubChem database (**https://pubchem.ncbi.nlm.nih.gov/**) for 3D structure. Optimization of ligand will be docked into distinguished model using Ligand Fit theory. Structure files will be converted to the required format by using Open Babel.

3.15.3. *In silico* Docking analysis

The MGL Tools of AutoDock Vina (version 4.6.2) (http://autodock.scripps.edu) will be utilized to convert PDB files into PDBQT files. The receptor file is prepared by adding polar hydrogens, Kollman charges, and solvation parameters. Precalculated grid maps to be generated with dimensions to encompass all the amino acid residues present in the receptor. For the docking process, the Lamarckian genetic algorithm (LGA) to be selected, and maximum of 10 conformers, with a population size of 150 individuals.