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3.1. Materials

3.1.1. Plant material

Six plants including, *Abutilon indicum, Albizia julibrissin, Caesalpinia pulcherrima, Clitoria ternatea, Euphorbia hirta* and *Psidium guajava* were collected from Coimbatore area, Tamilnadu, India. The disease-free mother plants (3-4 year old) were well maintained in the institute campus. These were given prophylactic spray of 0.1% Bavistin at weekly intervals. Leaves and stems of all six selected plants, flowers of *Abutilon indicum, Albizzia julibrizzin, Caesalpinia pulcherrima,* and *Clitoria ternatea,* and roots of *Psidium guajava,* and *Euphorbia hirta* were used for the study (Plate.3.1). The plant specimens were identified, confirmed by Botanical Survey of India (BSI), Southern Circle, Coimbatore, India.

The selected plant parts were dried in the laboratory at room temperature to complete dryness. Once completely dried, the plant materials were ground separately to a coarse powder using blender. Materials were stored in a closed container at room temperature until required.

3.1.2. Chemicals

Chemicals and solvents used for extraction and estimation of flavonoids were AR grade and chemicals used for *in vitro studies* to prepare the stock solutions were of LR or equivalent grade. Plant growth regulators (PGRs) used in the study comprised auxins such as 2,4-dichlorophenoyacetic acid (2,4-D),I -Naphthaleneacetic acid (NAA), Indole- 3-acetic acid (IAA), and cytokinins such as Benzylaminopurine (BAP), 6-furfurylaminopurine (Kn), Organic supplements such as Yeast extract and Peptone, Elicitors such as Salicylic acid (SA), Gibberellic acid (GA3) and Abscisic acid (ABA), Copper Sulphate, Zinc sulphate, were procured from Sigma, USA or HiMedia Laboratories Pvt. Ltd., Mumbai. TLC plates K6F Silica gel 60A purchased from Whatman, USA. HPLC grade Methanol (Himedia, Mumbai) and standard quercetin (Sigma, UK) were used for quantification of quercetin by HPLC.

Plate. 3.1.

Plants selected for the study

Abutilon indicum

Albizia julibrissin

Caesalpinia pulcherrima

Clitoria ternatea

Euphorbia hirta

Psidium guajava

3.1.3. Equipment

HPLC was performed using a Shim-pack CLC-ODS C18 (150 mm x 6 mm) (Shimadzu, Japan) consisted of LC-10ADvp pump, UV detector, CTO-10Avp column oven, SIL-10Dvp auto sampler. All NMR experiments were conducted on a Bruker AVANCE III 500 MHz (AV 500) multi nuclei solution NMR spectrometer equipped with a cryoprobe.

3.2. Methods

3.2.1. *In vivo* screening of quercetin in selected plant parts

3.2.1a. Extraction and estimation of flavonoids

Hundred grams of air dried and powdered plant materials of the selected plants were taken separately. One hundred gram of each plant powder was extracted with 1 Litre of 8 different solvents with different polarity (petroleum ether, benzene, chloroform, acetone, ethanol, methanol, methanol: ethyl acetate (60:40), and methanol: ethyl acetate (80:20)). Extraction was done by swirling for 16 h at room temperature using an orbital shaker for 3 days. Extracts were then centrifuged and supernatants were filtered under suction and dried.

The weight of the residue remaining in the analytical portion of the extract after evaporation of the solvent was determined by weighing each vial containing the residue (B) and reweighing the vial after washing (A). Extractive value of the each solvent was calculated using the following formula (Khandelwal, 2007).

$$\text{Extractive value (g/100 g of dried plant material)} = \frac{(B - A)}{W} \times 100$$

W = Weight of the Plant material (g)

Extracted residues were used for estimation of total flavonoid (Appendix I) and purification of quercetin. Total flavonoids contents of the selected plant extracts were determined by colorimetric method (Chang *et al.*, 2002).

3.2.1b. Purification of quercetin by acid treatment

Extracts which showed maximum flavonoid content was used for purification of quercetin by acid treatment. One g of extract was then treated with 20 ml HPLC grade Methanol and 1.5M HCl (0.2ml), and the mixture was refluxed for 1 h to obtain the quercetin aglycons by hydrolysis of the quercetin glycosides (Wach *et al.*, 2007). The hydrolysate was diluted with methanol to 100ml and filtered (PTFE Syringe filter, Whatman, UK). Acid treated extracts were used for separation and quantification of quercetin aglycones by TLC and HPLC, respectively.

3.2.1c. Method development for quercetin analysis using TLC

TLC analysis was performed on precoated 20 X 20 cm (0.25 mm thick) TLC plates K6F Silica gel 60 A purchased from Whatman, USA. Ten µl of standard quercetin solution (concentration 0.1 mg /ml), was applied as spots onto TLC sheets. Extracted residues of selected plant parts using optimal solvent were dissolved in 100 ml appropriate solvent and from that 10 µl was taken for the study. Ten different mobile phases were selected (according to their polarity) to establish the R_F value for standard. The spotting line was about 0.5 cm from the developing solution. The plates were developed at room temperature in a vertical separating chamber to the height of approximately 18 cm from the start. The chamber was previously saturated for two hours with the appropriate mobile phase.

Standard quercetin and quercetin extracted from different plant parts were chromatographed on silica gel plates using the above solvent systems and the retention factor **(R_F)** was calculated.

$$R_F \text{ value} = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent}}$$

After drying, visualization was performed either without derivatization (under short wave UV light illumination - 254 nm) or by derivatization (by spraying 1% ethanolic ferric chloride).

Solvent systems used for TLC

Solvent system No	Solvent System	Ratio	Reference
1	Toluene: ethyl acetate: acetic acid	36:12:5	Medic-Saric *et al.,* 2004
2	Toluene: acetone: methanol: formic acid	46: 8: 5: 1	El Sohafy *et al.,* 2009
3	Toluene: ethyl acetate: formic acid	6:4:1	Vijayalakshmi *et al*., 2012
4	Toluene: acetic acid	40:20	Nikolova *et al.,* 2004
5	Toluene: acetone: Formic acid	30:60:10	Bendini *et al.,* 2002
6	Chloroform : benzene : Ethanol : acetic acid : water	11 : 4 : 2 : 1 : 2	Dashputre and Naikwade, 2010
7	n-Butanol: acetic acid: Water	40:10:50	Vijayalakshmi *et al*., 2012
8	n-Hexane: ethyl acetate : methanol : acetic acid	19 : 4 : 0.2 : 0.1	Chanda and Baravalia, 2011
9	n-Hexane: ethyl acetate: formic acid	31:14:5	Jasprica *et al.,* 2004
10	Petroleum ether: ethyl acetate: formic acid,	30:15:5	Medic-Saric *et al.*, 2004

3.2.1d. Quantification of quercetin by HPLC

HPLC Separation and Quantification of quercetin aglycone in plant extracts was carried out by a modified method of Olszewska (2007), as presented in Appendix II.

3.2.2. *In vitro* tissue culture studies

3.2.2a. Selection of explants

Plant leaves with higher content of quercetin were selected for callus culture. Tender leaves of *Abutilon indicum*, *Caesalpinia pulcherrima, Euphorbia hirta* were collected from new shoots, in the month of May.

3.2.2b. Composition of various culture media used

The Murashinge & Skoog (MS) medium (Murashige & Skoog, 1962) was used for callus induction and cell suspension culture studies. For preparing the MS medium, all stock solutions were kept in appropriate proportions (Appendix III). Elix-3 (Milli–Q) water was used for the preparation of culture media. After addition of all the media constituents, pH was adjusted to 5.8 using 0.1 N KOH or 0.1 N HCl. Gelling agent (agar-agar) at a concentration of 0.8% was added and the medium was steamed to melt

the gelling agent. It was then dispensed into test tubes (10ml per tube) or screw capped bottles (25 ml per bottle) or liquid medium without gelling agent into conical flasks (100 ml per 250 ml flask).

Carbon source was added and made up to required volume with double distilled water. Different concentrations of sucrose were tried for callus induction and secondary metabolite production. The sucrose concentration was varied from 3-7% (W/V). The medium was autoclaved at 121° C at a pressure of 1.1kg.cm^{-2} for 20 min.

All the plant growth regulators in required concentration used during the course of the present work were added before autoclaving the medium. All media used in the various studies are presented in Table.3.1, 3.2 and 3.3.

3.2.2c. Inoculation of leaves

The working table of the laminar airflow chamber was first surface sterilized with 70% ethanol. Sterile Petri dishes and tools (forceps, scalpels, sterile cotton, and sterile paper towels) that were used for inoculation were kept in the laminar airflow chamber. The ultra violet light was switched on for 20 min. Prior to inoculation, hands were sterilized with 70 % ethanol. The forceps and scalpels were dipped in 70% alcohol and flamed, cooled and used for inoculation.

For callus induction studies, the leaves were kept in running water for 20 minutes to remove dust particles. Inside the sterile laminar air flow the explants were treated with 0.1% bavistin for 3 minutes to remove fungal contamination. After treatment, they were washed with sterile distilled water for 3-5 times to remove the traces of fungiside. Further surface sterilization was carried out by immersing the explants into 0.1% $HgCl_2$ for 3-5 minutes and washing with sterile distilled water for 3-5 times. The treated materials were trimmed into pieces of about 1 cm^2 and inoculated on to the medium. All these cultures were incubated at 25 ± 2ºC. Each experiment had 15 replicates with three explants in each. Cool white fluorescent light of 2000lux intensity at 16/8-hour photoperiod (Ma *et al.,* 2009) and 55-60 percent relative humidity were maintained.

Table.3.1.

Hormone supplementation in MS media for callus and cell suspension culture

a. Callus induction

Treatment*	Growth regulators (mg/L)			
	2,4 D	**IAA**	**NAA**	**Kn**
C0	0	0	0	0
C1	0.5	-	-	-
C2	1.0	-	-	-
C3	2.0	-	-	-
C4	3.0	-	-	-
C5	4.0	-	-	-
C6	5.0	-	-	-
C7	-	0.5	-	-
C8	-	1.0	-	-
C9	-	2.0	-	-
C10	-	3.0	-	-
C11	-	4.0	-	-
C12	-	5.0	-	-
C13	-	-	0.5	-
C14	-	-	1.0	-
C15	-	-	2.0	-
C16	-	-	3.0	-
C17	-	-	4.0	-
C18	-	-	5.0	-
C19	0.5	-	-	0.5
C20	1.0	-	-	0.5
C21	2.5	-	-	0.5
C22	0.5	-	-	1.5
C23	1.0	-	-	1.5
C24	2.5	-	-	1.5
C25	0.5	-	-	2.5
C26	1.0	-	-	2.5
C27	2.5	-	-	2.5

* - effect of sucrose concentration (3%, 5% & 7% tested along with growth regulators)

Table.3.1.contd…

b. Cell suspension culture

Treatment*	Growth regulators (mg/L)			
	BA	NAA	2,4 D	Kn
S1	2.5	1.0	-	-
S2	2.5	2.0	-	-
S3	2.5	3.0	-	-
S4	2.5	-	-	1.0
S5	2.5	-	-	2.0
S6	2.5	-	-	4.0
S7	2.5	-	1.0	-
S8	2.5	-	2.5	-
S9	2.5	-	5.0	-
S10	2.5		1.0	1.0
S11	2.5		2.5	1.0
S12	2.5		2.5	1.5
S13	2.5		2.5	2.5

*- sucrose concentration at 5%

3.2.2d. Callus induction and establishment of cell suspension cultures

(i) Effect of different auxins on callus induction

Leaf explants were cultured in MS basal medium supplemented with auxins such as 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA) and indole 3 acetic acid (IAA), each at 0.5 to 5.0 mg/L to study their relative effects on callus induction and kept in 16/8-hour photoperiod in a culture room maintained at 25± 2^0 C. Observations were recorded as percentage of explants initiating calli, callus growth rate and period taken for the calli to initiate. Pieces of calli (100 mg FW) were transferred to fresh suitable MS media and incubated for 30 days. To determine the growth kinetics of the callus cells, calli grown on MS media supplemented with different concentrations of auxins (Table.3.1a MS media (C1 to C18)), were collected and Fresh Weight (FW) of calli was determined after 30 days.

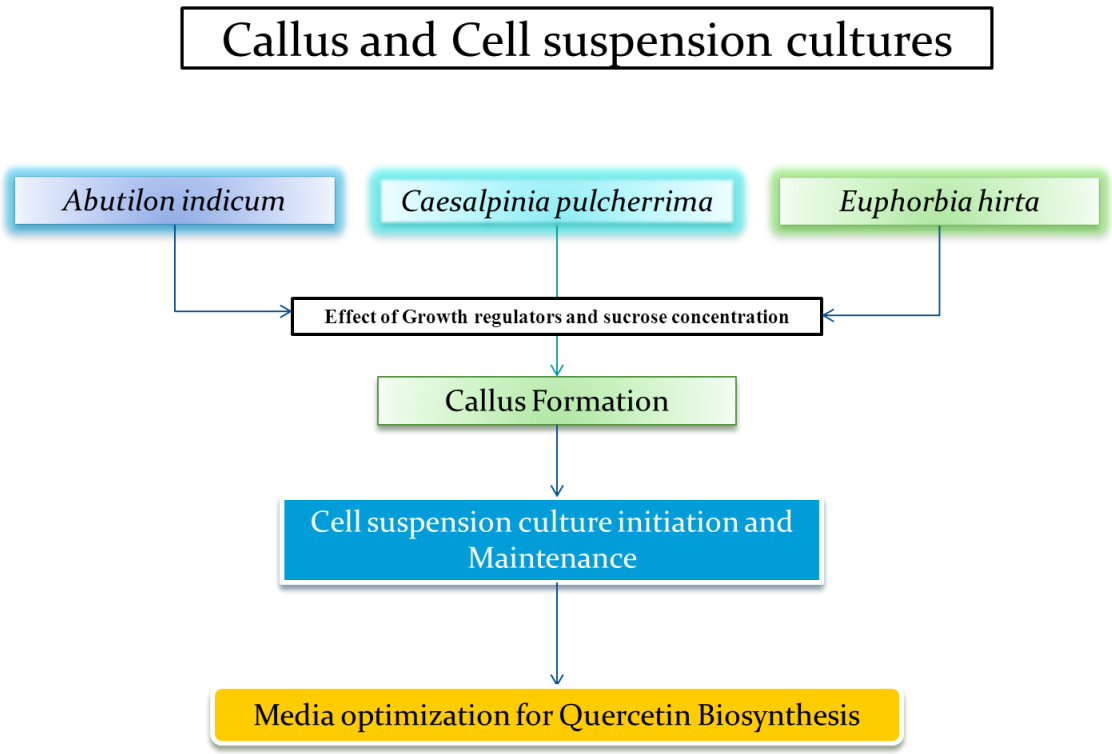
(ii) Effect of auxin and cytokinin on callus induction

Major cytokinin kinetin (Kn) at various concentrations was incorporated to the basal medium supplemented with auxin, (2,4-D), Response of explants to varying

concentrations of auxins and cytokinins (Table.3.1a. MS media (C19 to C27)), were evaluated after 30 days of inoculation with respect to Fresh Weight (FW) of calli.

(iii) Establishment of cell suspension culture

Plant cell cultures were initiated by transferring friable soft callus to liquid nutrient medium with the optimal growth regulator and sucrose as used for callus culture. Callus tissue was grown on solid medium (MS). Mother suspension cultures from friable callus of selected plant materials were established by dispersing approximately two gram of actively proliferating callus tissue in 50 ml of liquid MS basal media supplemented with appropriate/ optimum growth regulators.



3.2.2e. Media optimization for quercetin biosynthesis

To select the medium suitable for optimum growth and quercetin biosynthesis, MS medium supplemented with BA (2.5mg/L) in combination with NAA (1 - 3mg/L), 2, 4 - D (1-5 mg/L), Kn (1-4mg/L) or 2,4-D (2.5mg/L) with Kn (0.5 - 2.5mg/L) and Sucrose (5%) concentrations were employed (Table.3.1b. MS media (S1 to S13)).

The resulting cell suspension cultures were established in 250ml Erlenmeyer flasks at 100 rpm and 25^0C in the 16/8 hour photoperiod. FW, Dry weight (DW) and quercetin accumulation (mg/g of DW) of these cell suspensions were the parameters recorded at 6- day intervals for 30 days. Three replicates were harvested every week. The cell cultures were filtered under vacuum and weighed to determine FW. Fresh cells were dried at 60^0C in a vacuum oven overnight until they reached a constant weight, and then DW was recorded. For the extraction of quercetin from suspension cultures, one gram of dried cells were extracted with suitable solvent and hydrolyzed with acid as mentioned in section 3.2.1a, and section 3.2.1b, respectively. The residue was then dissolved in known aliquots of methanol and used for quercetin quantification by HPLC analysis (Appendix II).

(iv) Subculture of suspensions

Suspension cultures were subcultured at 30 days interval. Before subculturing, the culture suspension was allowed to stand for 30 minutes to settle the larger fragments. Then two g of cell biomass were transferred to respective fresh MS media for further studies.

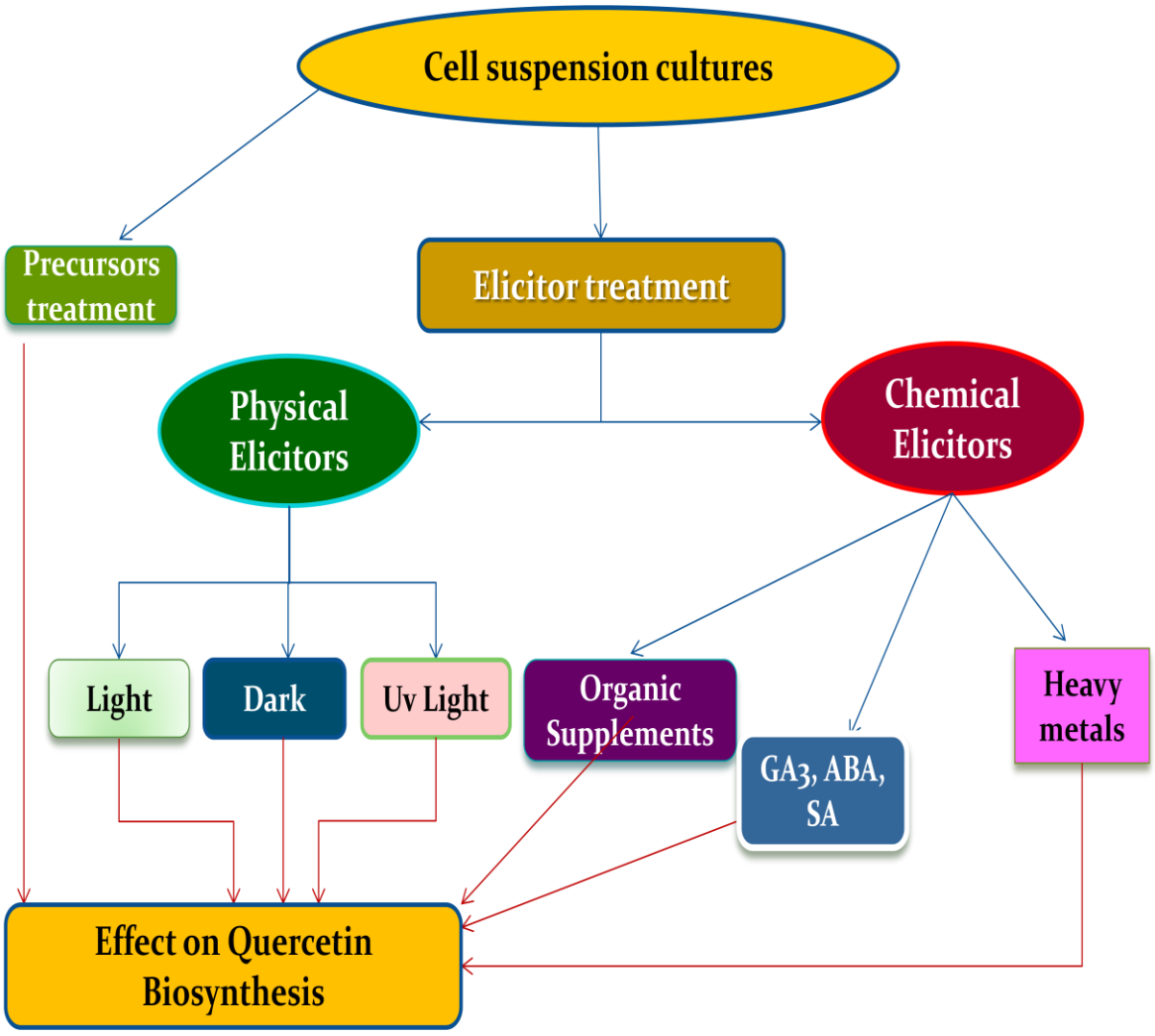
3.2.3. Enhancement of quercetin biosynthesis in *in vitro* cultures

Two gram of cell biomass from stock cell suspension cultures were aseptically transferred to 50 ml of sterilized MS medium. Cell cultures were subsequently collected at 4 days intervals up to 20^{th} day to determine the fresh weight and quercetin (Appendix II). For chemical elicitor treatments, elicitors were added on 4^{th} day of culture. All determinations were performed in triplicate.

3.2.3a. Physical elicitation using different light sources

One month old suspension cultures of selected plants were subjected to continuous light (24 hours), continuous dark (24 hours) and UV light regime over a period of one month. Exposure to UV-B radiation from lighting which specifically releases UV-B irradiation (UV-B-313bulbs) occurred on day 4. Flasks were opened in a laminar flow hood and exposed to UV-B light (254 nm, 10 $\mu W/cm^2$) for 60 min. The

cell suspension cultures were incubated at 25°C in the three different light treatments for 30 days. Controls were given 16/8 hours light photoperiod treatment.



3.2.3b. Organic supplements

Organic supplements such as Coconut water, yeast extract and peptone water were tested for enhancement of quercetin biosynthesis in cell suspension cultures. Preparations of organic supplements are given in Appendix IV. The various supplement media used in this study are listed in Table.3.2. (Media (CW1-CW6, YE1-YE6, PW1-PW6)). The pH of the medium was adjusted to pH 5.7 prior to the addition of agar and autoclaved at 121^0C for 30 min under 1 atm. The harvest time was determined based on the time courses of cell growth (24th day of culture) and quercetin production.

Table.3.2. Enhancement media used for quercetin biosynthesis

Treatment	Elicitors	Liquid MS Medium
Precursor Feeding	**Phenylalanine (mg/L)**	
Ph1	0.1	MS
Ph2	0.2	MS
Ph3	0.4	MS
Organic Supplements	**Coconut Water** (%)	
CW1	10	MS
CW2	10	½MS
CW3	10	¼ MS
CW4	25	MS
CW5	25	½MS
CW6	25	¼ MS
	Yeast extract (%)	
YE1	0.1	MS
YE2	0.1	½MS
YE3	0.1	¼ MS
YE4	0.25	MS
YE5	0.25	½MS
YE6	0.25	¼ MS
	Peptone Water (%)	
PW1	0.01	MS
PW2	0.01	½ MS
PW3	0.01	¼ MS
PW4	0.025	MS
PW5	0.025	½ MS
PW6	0.025	¼ MS
Chemical Elicitors	**GA_3 (mg/L)**	
G1	1.0	MS
G2	2.0	MS
	ABA (mg/L)	
A1	0.05	MS
A2	0.1	MS
A3	0.2	MS
	SA (µM)	
SA1	1	MS
SA2	10	MS
SA3	50	MS
SA4	100	MS

3.2.3c. Precursor feeding

l-Phenylalanine was dissolved in ethanol, sterilized and added to the cultures through a membrane filter (0.22 µmeter) to give final concentrations of 0.1-0.4 mg/l for l-phenylalanine (Table.3.2. Media (Ph1-Ph3)). Then it was added to the cell suspension on the first day of the culture cycle. Control experiment was run concurrently in which only ethanol was added.

3.2.3d. Chemical elicitation

Cell suspension cultures were maintained until 24 days were elicited for quercetin biosynthesis with various concentrations of Gibberellic acid, Abcisic acid and Salicylic acid (Table.3.2. Media (G1 & G2, A1- A3, and SA1-SA3, respectively)) and were subcultured in the same medium every 4 weeks for elicitation experiments.

3.2.3e. Heavy metals

Elicitation with heavy metals and in combination with salicylic acid was carried out on 4-day old cultures in MS medium supplemented with optimum growth regulators. Zinc Sulphate and Copper sulphate solutions were sterilized by filtration and added to the cultures at final concentrations of 50-150 µM. Meanwhile salicylic acid solutions (10-100µM) were added along with heavy metals to the 4^{th} day of cell suspension cultures (Table.3.3. Media (H1- H10)).

Table.3.3. Heavy metal media used for quercetin biosynthesis

Treatment	Elicitors	Salicylic acid (µM)	Liquid MS Medium
Heavy Metals	**Copper Sulphate (µM)**		
H1	50	-	MS
H2	100	-	MS
H3	150	-	MS
H4	50	10	MS
H5	100	50	MS
	Zinc Sulphate (µM)		
H6	50	-	MS
H7	100	-	MS
H8	150	-	MS
H9	50	10	MS
H10	100	50	MS

3.2.4. Structural analysis of quercetin derivatives isolated from leaves and cell suspension cultures

3.2.4a. Preparative thin layer chromatography (PTLC)

Glass plates (20 x 20cm) thickly coated (0.4-0.5nm) with silica gel 'G'(45g/80 ml water) and activated at 100°C for 30 minutes and cooled at room temperature were used for preparative thin layer chromatography (PTLC). The selected leaf extract fractions and cell suspension culture fractions were applied on separate plates and developed on n-Butanol: acetic acid: water (40:10:50). The developed plates were air dried and visualized under UV light. Each of the fluorescent spots coinciding with those of standard reference compound quercetin and other fluorescent spots were also marked. The marked spots were scrapped and collected separately along with the silica gel 'G' and eluted with methanol. Each of the eluted compounds was then crystallized with chloroform. The purified compounds were subjected to NMR spectral analysis.

3.2.4b. NMR spectral analysis of selected leaf and cell suspension fractions

^{1}H NMR (500 MHz) spectra of each purified material collected from PTLC were recorded in CD_3OD (30^0C) on Bruker Avance 500 MHz instrument (SAIF, IIT Madras, Chennai, Tamil Nadu, India). Briefly, fractions eluted from PTLC was redissolved in 1 mL of deuterated solvent containing a 1:1 ratio of methanol–d_4 and D_2O (KH_2PO_4 buffer, pH 6.0) with 0.005% TMS (w/v). An aliquot of 800 µlitre of sample was transferred to the 5 mm NMR tube. Residual solvent resonances were used as internal references relative to TMS at 0.00 ppm. Standard pulse sequences and parameters were used to acquire 1D ^{1}H, 1D ^{13}C, ID selective Rotating Frame Overhauser Enhancement (ROE), Double Quantum Filtered-Correlation (DQF-COSY), Heteronuclear Single Quantum Correlation (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC) data as required.

3.2.5. Antimicrobial activity of quercetin isolated from plant and cell suspension cultures.

Sample Preparation

Each acid treated plant extracts (leaves, stems, flowers and roots) and cell suspension culture extracts were separated by PTLC for purification of quercetin.

Purified compounds collected after separation with PTLC, were resuspended in DMSO. For testing antibacterial and antifungal activity, leaf extracts at 1-4mg/ml concentration and purified compounds 0.050-1.0mg/ml concentrations of were used.

Microorganisms used:

Clinical samples of bacterial strains including *Bacillus cereus, Escherichia coli, Enterococcus faecalis, Klebsiella pneumonia, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi, Staphylococcus aureus,* and *Staphylococcus epidermis* and fungal samples including *Aspergillus niger, Aspergillus flavus, Aspergillus fumigates, Candida albicans, Candida utilis, Fusarium oxysporum, Fusarium solani, Microsporum gypseum, Trichophyton metagraphytes, Epidermophyton floccosum,* and *Trichophyton rubrum* were obtained from Microbiology Department, Vivekanandha college of Arts and Sciences for women, Tiruchengode, Tamilnadu.

Standard Antibiotics

Gentamycin (0.030mg/ml) and Ketaconazole (0.030mg/ml) were taken as positive control (standard antibiotics) for bacterial and fungal species, respectively. Methanol and DMSO were used as negative control against all the species.

Preparation of inoculum

Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller-Hinton broth (MHB) for bacteria and Sabouraud dextrose broth (SDB) for fungi that were incubated without agitation for 24hrs at 37°C and 25°C respectively. The cultures were diluted with fresh Mueller-Hinton and Sabouraud dextrose broth to achieve optical densities corresponding to 2 X 10^6 colony forming units (CFU/ml) for bacteria and 2 X$\cdot 10^5$ spore/ml for fungal strains.

Disc diffusion method

In vitro antibacterial and antifungal screening was carried out by disc diffusion method (Duraipandiyan *et al.,* 2006), which is a qualitative to semiquantitative test. The Muller Hinton Agar plates were prepared by pouring 20 ml of molten media into sterile

petriplates. Filter paper discs (6 mm in diameter) impregnated with various concentrations of plant extract (refer sample preparation) was placed on the surface of test organism- seeded plates (pour plate method) and the compound was allowed to diffuse for 5 minutes and the plates were kept for incubation at 37°C for 24 hrs. At the end of incubation, inhibition zones formed around the disc were measured with transparent ruler in millimeter. The same procedure was followed against the fungal culture also. These studies were performed in triplicate.

3.2.6. Statistical analysis

The data obtained from the various experiments were subjected to statistical analysis by using the statistical software SIGMASTAT and AGRES, in completely randomized design (CRD). Each experiment was repeated twice with a minimum of 3 replicates in each.