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COMPARATIVE ANALYSIS OF THE PHYTOCHEMICALS, ANTIOXIDANT AND ANTICANCER POTENTIAL OF LEAVES OF AVERRHOA BILIMBI LINN AND QUISQUALIS INDICA L

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Abstract:

In the present study, the methanolic leaf extracts of Averrhoa bilimbi and Quisqualis indica were evaluated to investigate Phytoconstituents, antioxidant and anticancer activity. The total carbohydrates, protein and flavonoid in leaf extracts of Averrhoa bilimbi Linn and Quisqualis indica L was also estimated. The findings also demonstrated that the extracts have stronger antioxidant and anti-cancerous effects. During preliminary phytochemical screening of the crude extracts of Averrhoa bilimbi and Quisqualis indica leaves, important therapeutic principles such as Alkaloids, Flavonoids, Phenols, Proteins etc., were detected. Therefore, the current findings can be attributed to these groups of chemical compounds. Leaf extract of Averrhoa bilimbi and Quisqualis indica have shown the potent free radical scavenging and antioxidant property. Significant correlation is seen in antioxidant and cytotoxic activity of leaf extract of both the extracts. The results suggested that the anticancer properties are influenced by the concentration of the extracts.

Key Words: Phytochemical Extraction, Averrhoa Bilimbi, Quisqualis Indica, Anti-Oxidant, Anti-Cancer Activity

Introduction:

Natural products are valuable sources of structurally diverse chemical compounds. Many such compounds have been isolated from different part of sources and applied into clinical medical practice. Among the natural resources, fruits and vegetables have been widely studied for the discovery of antimicrobial, anticancer, antioxidants, anti-inflammatory and others. Recently, underutilized fruits have drawn attention of many researchers as a natural source of treatment for curing various diseases. Moreover, antibacterial activities of extracts have been reported by many scientists (Mokhtar *et al*., 2016).

Averrhoa bilimbi from Oxalidaceae family is an edible and underutilized fruits, native from South-East Asia and cultivated in some parts of India. It is easily available and cost effective. It holds a great value in complementary medicine as evidenced by the substantial amount of research on it. Traditionally, the leaves are used as paste on itches, swelling, skin eruptions, cough, sites of poisonous, etc. The decoction of fruits is also treating inflammatory conditions including hepatitis, fever and diarrhoea. In some villages in India, the fruit has been used in folk medicine to control obesity. In Java, the fruits combined with pepper are eaten that cause sweating when people are feeling under the weather. It is used in the treatment of fever, syphilis, stomach ache, ulcer, hypertension, obesity and diabetes (Anandhalakshmi *et al*., 2018).

Quisqualis indica, also named as Rangoon creeper, is one of the universal valuable medicinal plants worldwide and it comes under the genus combretum, which belongs to the family of Combretaceae. Its flowers have a soft sweet aroma and they blossom in the spring, early summer or mid fall (Welsh, 1998). Fruits of this plant are used to delight ascariasis and oxyuriasis, although the decoction of the fruit is helpful in toothache and nephritis. The roasted ripe seeds are helpful in diarrhoea, fever and rickets. In addition, seeds macerated in oil can be used for parasitic skin troubles. The leaf decoction is useful for abdominal pain and the leaf juice is cure for ulcer. The leaves are used to treat various kinds of infantile disorders and skin diseases, somewhere the roots are used in rheumatism and diarrhoea (Bakyalakshmi *et al*., 2021).

There are many reports on various aspects of fruits of *bilimbi* and flowers of *Quisqualis*, however no work has been carried out on its leaves except the phytochemical profile. Hence the present study has been aimed to investigate the effect of antioxidant and anticancer activities of methanolic leaves extracts of *A. bilimbi* and *Q. indica*.

Methods and Materials:

Plant Collection and Preparation of Extract:

The leaves of *A. bilimbi* and *Q. indica* were collected from Chennai, Tamilnadu. The plant was authenticated by Department of Department of Plant Biology and Plant Biotechnology, Quaid-E-Millath Government College for Women, Chennai, Tamilnadu, India. The leaves materials were shade dried, powdered mechanically, stored in airtight containers. The powdered material (600 g) was refluxed successively with the

solvent Methanol in the ratio of 1:10 for 24 hours in the rotary shaker at room temperature and was filtered through whatmann's filter paper No.1. The filtrate was allowed to condense at room temperature; the resultant crude extract was stored in refrigerator at 4°C until further use.

Preliminary Phytochemical Screening:

Phytochemical analysis of extracts was conducted in accordance with the standard procedure (Harborne, 1998). By this analysis, the presence of the several phytochemicals like alkaloids, flavonoids, amino acids, carbohydrates, reducing sugar, Phenolic compounds, proteins, saponins, steroids, coumarin and cardiac glycosides were tested.

- **Test for Alkaloids - Mayer's Test**

To a few ml of plant sample extract, 2 drops of Mayer's reagent are added along the sides of test tube. Appearance of white creamy precipitate indicates the presence of alkaloids.

- **Test for Amino Acid - Ninhydrin Test**

The extract (100 mg) is dissolved in 10 ml of distilled water and filtered through Whatmann No. 1 filter paper and the filtrate is subjected to test for Amino acids. Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) are added to 2 ml of aqueous filtrate. Appearance of purple color indicates the presence of amino acids.

- **Test for Carbohydrates - Molisch' S Test**

To 2 ml of plant sample extract, 2 drops of molisch reagent is added. The mixture is shaken well and few drops of concentrated sulphuric acid is added slowly along the sides of test tube. A violet ring indicates the presence of carbohydrates.

- **Test for Reducing Sugar - Benedict's Test**

To 0.5 ml of filtrate, 0.5 ml of Benedict's reagent is added. The mixture is heated on a boiling water bath for 2 minutes. A characteristic-colored precipitate indicates the presence of sugar.

- **Test for Phenolic Compounds - Ferric Chloride Test**

The extract (50 mg) is dissolved in 5 ml of distilled water. To these few drops of neutral 5% ferric chloride solution are added. A dark green color indicates the presence of phenolic compound.

- **Test for Flavonoids - Alkaline Reagent Test**

An aqueous solution of the extract is treated with 10% ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavonoids.

- **Test for Phytosterols - Libermann - Burchard's Test**

The extract (50 mg) is dissolved in of 2 ml acetic anhydride. To this, 1 or 2 drops of concentrated sulphuric acid are added slowly along the sides of the test tube. An array of color change shows the presence of phytosterols.

- **Test for Proteins - Biuret Test**

The extract (100 mg) is dissolved in 10 ml of distilled water and filtered through Whatmann No. 1 filter paper and the filtrate is subjected to test for proteins. 2 ml of filtrate is treated with 1 drop of 2% copper sulphate solution. To this 1 ml of ethanol (95%) is added, followed by excess of potassium hydroxide pellets. Pink color ethanolic layer indicates the presence of protein.

- **Test for Saponins - Foam Test**

The extract (50 mg) is diluted with distilled water and made up to 20 ml. The suspension is shaken in a graduated cylinder for 15 minutes. A two cm layer of foam indicates the presence of saponins.

- **Test for Coumarin - HNO_3 Test**

To 2 mg of the extract is dissolved with distilled water and made up to 5ml. To this, few drops of Con. HNO_3 is added. Appearance of yellow fluorescence indicates the presence of coumarin.

- **Test for Cardiac Glycosides - Keller Kilani Test**

To the 0.5ml of extract, 2 ml of glacial acetic acid and a drop of 2% $FeCl_3$ are added. To this, 1 ml of con.H_2SO_4 is added along the sides of the test tube. Formation of brown ring at the junction indicates the presence of Cardiac glycosides.

Quantitative Estimation Phenols and Flavonoids:

Estimation of total Phenolic Content (Folin-Ciocalteu Assay):

Total phenolics were determined using Folin-Ciocalteu reagents (Singleton *et al.,* 1965). The plant extracts or gallic acid standard (40 μL) was mixed with 1.8 mL of Folin-Ciocalteu reagent (prediluted 10-fold with distilled water) and allowed to stand at room temperature for 5 min, and then 1.2 mL of sodium bicarbonate (7.5%, w/v) was added to the mixture. After standing for 60 min at room temperature, absorbance was measured at 765 nm. Aqueous solutions of known gallic acid concentrations in the range of 10-500 mg/L were used for calibration. Results were expressed as mg gallic acid equivalents (GAE)/g sample.

Estimation of Total Flavonoid (Aluminium Chloride Method):

The total flavonoid content was determined by aluminium chloride method (Siddique *et al.,* 2010). 50 µL of each test sample, 1.25 mL distilled water and 150 µL $NaNO_2$ were added in test tubes and kept at room temperature for 10 minutes. After 10 mints added 300 µL $AlCl_3$ after 5 mins, 1 mL NaOH was added and

incubated at room temperature for 20 mints. After incubation absorbance was read at about 510nm. The total flavonoid content was expressed in terms of quercetin equivalent (mg/g).

In Vitro Antioxidant Activity:

Averrhoa bilimbi and Quisqualis indica extracts were assessed for its *in vitro* antioxidant activity.

DPPH Assay:

The scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical by extracts is based on the method described by Alothman et al., 2009. 1 mL of the sample solutions containing different concentrations were mixed with 3 mL of 0.1 mmol/L solution of DPPH. The mixture was stored for 30 min in the dark. The absorbance was estimated at 517 nm after incubation, against an ethanol blank without DPPH. The control solution was a mixture of 1 mL of ethanol and DPPH. In case of aqueous extracts distilled water was used instead of ethanol for blank and control. Gallic acid was used as a standard. Results were expressed as percentage of inhibition of the DPPH radical. The percentage of DPPH radical inhibition was determined using the following equation.

Inhibition of DPPH = Abs of control-Abs of sample /Abs of control *100%

Scavenging of Nitric Oxide Radicals:

Sodium nitroprusside (10 mg) in phosphate buffer saline was mixed with different volume levels of test sample (100, 120, 140, 160, 180 and 200 µl) made 200 µl of each dose level by dilution with methanol. Incubate the solution at room temperature for 150 minutes. The same reaction mixture without the extract but equivalent amount of methanol served as control. After the incubation period 5ml of Griess reagent was added. The absorbance was taken in UV visible spectrophotometer at 546 nm. Ascorbic acid was used as positive control. The % reduction was calculated (Bisht *et al*., 2010)

Superoxide Scavenging Activity:

Superoxide radical (O2-) was generated from the photoreduction of riboflavin and was deducted by nitro blue tetrazolium dye (NBT) reduction method. Measurement of superoxide anion scavenging activity was performed based on the method described by Winterbourne et al (1975). The assay mixture contained sample with 0.1ml of Nitro blue tetrazolium (1.5 mM NBT) solution, 0.2 ml of EDTA (0.1M EDTA), 0.05 ml riboflavin (0.12 mM) and 2.55 ml of phosphate buffer (0.067 M phosphate buffer). The control tubes were also set up where in DMSO was added instead of sample. The reaction mixture was illuminated for 30 min and the absorbance at 560 nm was measured against the control samples. Ascorbic acid was used as the reference compound. All the tests were performed in triplicate and the results averaged. The percentage inhibition was calculated by comparing the results of control and test samples.

Anticancer Activity:

Cell line and Culture:

*U87*cell line was obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO2 at 37 °C.

In Vitro Assay for Anti-Cancer Activity: (MTT Assay) (Mosmann, 1983)

Cells (1 × 105/well) were plated in 24-well plates and incubated in 370C with 5% CO2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells .The absorbance at 570nm was measured with UV- Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC50) was determined graphically. The % cell viability was calculated using the following formula:

% Cell viability = A570 of treated cells / A570 of control cells × 100

Graphs are plotted using the % of Cell Viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments.

Results:

Preliminary Phytochemical Analysis:

Phytochemical constituents were tested in the extracts of *Averrhoa bilimbi* and *Quisqualis indica* leaves. Phytochemical constituents like Alkaloids, Amino acid, Carbohydrates, Reducing Sugar, Phenol, Flavonoid, Steroids, Protein, Saponin, Coumarin, Glycosides and Terpenoids were tested and presented in table.1. Alkaloid, Carbohydrate, Phenol, Flavonoid, Protein and Glycosides were observed in both the extracts.

Estimation of Total Phenolic, Flavonoid, Alkaloid and Alkaloidal Content:

Total phenol content was found to be 30.67 and 23.19 mgGA/g for *Averrhoa bilimbi* and *Quisqualis indica* whereas the flavonoidal content was found to be 61.43 and 37.52 mgQE /g for *Averrhoa bilimbi* and *Quisqualis indica* respectively. Standards used for phenolic estimation and flavonoidal estimation were Gallic acid and Quercetin respectively. The results were shown in the Table 2. From the quantification study, *A. bilimbi* showed the highest flavonoid content than the *Quisqualis indica*.

Table 1: Phytochemical screening of *Averrhoa bilimbi* and *Quisqualis indica*

No	Primary and Secondary Metabolites	Name of the test	Observation	Inference	
				Averrhoa bilimbi	Quisqualis indica
1	Alkaloid	Mayer's test	White creamy Precipitate	+	+
2	Amino acids	Ninhydrin test	Purple colouration	-	+
3	Carbohydrates	Molisch's test	Violet colouration	+	+
4	Reducing Sugar	Benedicts test	Orange brown ppt.	+	-
5	Phenol	$FeCl_3$ test	Dark green colour appears	+	+
6	Flavonoids	Alkaline reagent test	Yellow fluorescence	+	+
7	Phytosterols	Libermann-Burchard test	Yellow colouration	-	+
8	Protein	Biuret test	Pink colouration	+	+
9	Saponin	Foam test	No foam appearance	-	-
10	Cardiac glycoside	Keller killani test	Brown ring at the junction	+	+
11	Coumarin	Con. HNO3test	No Yellow fluorescence	+	-

+ Presence of compounds - Absence of compounds

Table 2: Quantitative Phytochemicals

No	Phytochemical	Bilimbi (mg/g)	Quisqualis (mg/g)
1	Phenols	30.67	23.19
2	Flavonoid	61.43	37.52

Antioxidant Assay:

DPPH activities of both the extracts of 5 different concentrations are represented in the table 3. While NO_2 activity of both the leaf extracts were represented in the table 4. Results of the antioxidant activity of both leaf extracts as measured by the SOD assay are represented in table-5. They showed no significant difference with each other. The extract produced dose dependent antioxidant action in the DPPH, superoxide method and the nitric oxide method. Ascorbic acid was used as standard in both the methods and the percentage inhibition and IC50 values were calculated. The results were shown in the Table 3, 4 & 5.

Table 3: Antioxidant Activity - DPPH Assay

No	Concentration µg/ml	% of Scavenging Activity	
		Bilimbi	Quisqualis
1	C_1 (500)	55.7	48.1
2	C_2 (400)	46.6	36.3
3	C_3 (300)	39.8	30.8
4	C_4 (200)	34.3	25.6
5	C_5 (100)	28.7	19.3

Table 4: Antioxidant Activity - NO_2 Assay

No	Concentration µg/ml	% of Scavenging Activity	
		Bilimbi	Quisqualis
1	C_1 (500)	69.5	58.37
2	C_2 (400)	61.49	52.81
3	C_3 (300)	50.77	47.41
4	C_4 (200)	47.41	42.36
5	C_5 (100)	41.98	36.23

Table 5: Antioxidant Activity - SOD Assay

No	Concentration µg/ml	% of Scavenging Activity	
		Bilimbi	Quisqualis
1	C_1 (500)	52.3	55.7
2	C_2 (400)	46.3	46.8
3	C_3 (300)	38.8	39.1
4	C_4 (200)	32.6	34.7
5	C_5 (100)	27.3	28.3

In Vitro Cytotoxicity Activity:

In the *in vitro* cytotoxicity studies, the cells were treated with various concentrations of the both the extracts. The percentage cytotoxicity progressively increased in a concentration dependent manner. The IC50 values were calculated for both the extracts studied and the results were given in Table - 6. The leaf extract of *A.bilimbi* exhibited an IC_{50} value at 7.8 µg/ml and *Quisqualis indica* exhibited an IC_{50} value at 125 µg/ml.

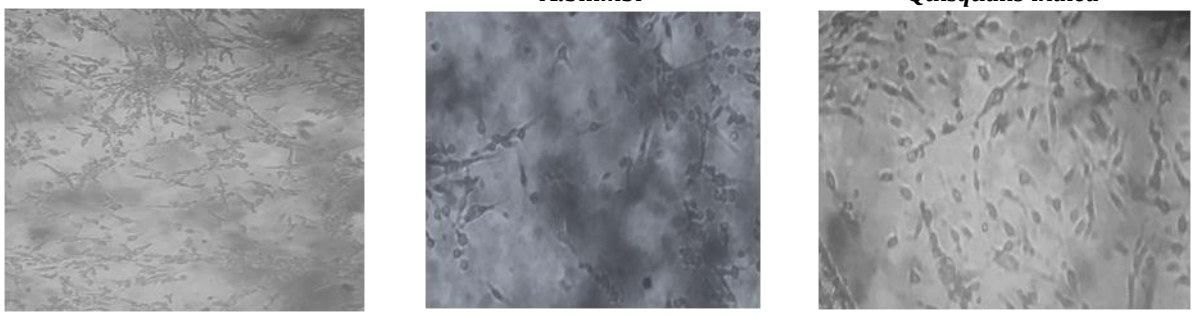
Table 6: Anticancer effect of Plant extracts on *U87* cell line

S.No	Concentration (µg/ml)	Cell Viability (%)	
		Bilimbi	Quisqualis
1	1000	11.76	26.32
2	500	15.63	37.58

3	250	19.36	44.61
4	125	24.67	51.21
5	62.5	30.41	57.81
6	31.2	35.29	63.84
7	15.6	41.03	70.58
8	7.8	48.49	77.76
9	Cell control	100	100

Normal Cells **Treated cells (IC50)**

A.bilimbi ***Quisqualis indica***



Discussion:

Medicines derived from plants have made immense contribution towards the betterment of human health and act as a source of inspiration for novel drug compounds. From the above research it can be concluded that this plant has immense potential to be used in the area of pharmacology and as a prospective source of valuable drugs. Due to the presence of various compounds that are essential for good health, it can also be used to improve the health status of society. The data clearly depicts the presence of compounds used for treating various diseases, indicating its use in the traditional system of medicine since ancient times.

Antioxidants may guard against reactive oxygen species (ROS) toxicities by the prevention of ROS construction, by the description of ROS attack, by scavenging reactive metabolites & converting them to less reactive molecules (Lauer *et al.*, 1998). The antioxidant activity of the plants might be due to inactivation of free radicals or complex forming with metal ions or combination thereof. Flavonoids and Tannins are a major group of compounds that act as primary antioxidants or free radical scavengers (Polterait *et al.*, 1997).

DPPH assay is a stable free radical method. It is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extract (Koleva *et al.*, 2002). The principle of DPPH method is based on the reduction of DPPH in the presence of a hydrogen donating antioxidant due to the formation of diphenyl picryl hydrazine. Extracts reduce the color of DPPH due to the power of hydrogen donating ability (Rajan *et al.*, 2011). Discoloration of violet DPPH to Yellow clearly demonstrated the effect of extracts as an antioxidant. The results suggested that the plant extract is an efficient Free radical scavenger.

Superoxide anion is a harmful reactive oxygen species as it damages cellular components in biological systems (Hazra *et al.*, 2008). The results suggested that the plant extract is a good superoxide radical scavenger. PMS-NADH coupling reaction accelerates the yield of superoxide radicals from dissolved oxygen.

The antioxidant constituents provide a fillip to the antioxidant defense mechanism in cancer and increase the uptake of glucose by muscles by modulating lipid peroxidation, scavenging free radicals and reducing the generation of reactive oxygen species (ROS) (Sachin Kumar *et al.*, 2016).

Over the past years, there was a major shift in the development of cancer drugs from screening of cytotoxic drugs to the development of molecularly targeted drugs. The conceptual idea is that the knowledge of the mechanism(s) of action of a drug provides a better approach to reach improved clinical results based on patient's molecular characteristics. The complexity of this task requires the interaction of scientists from different fields. This was the starting point of many efforts on the bioactivity-guided screening for natural products derived from medicinal plants of traditional Chinese medicine.

In the present study, the cytotoxic assay was performed with the extracts of *Averrhoa bilimbi* and *Quisqualis indica* leaves with a range of concentrations. The present findings exhibited a concentration dependent inhibition by the test extracts throughout the concentration range of 7.8-1000 µg/ml.

The data showed that the activity of the extracts increases with increased concentration of extracts. The extracts showed potent antioxidant and cytotoxic activity.

Conclusion:

Based on the result in the study, it may be concluded that the *Averrhoa bilimbi* and *Quisqualis indica* extracts of the leaves were found to be a good natural antioxidant *in vitro* system. Further studies are required to identify specific active principles of this plant for the significant antioxidant and cytotoxic effect and they may be incorporated into existing antioxidant and anti-cancer herbal compositions to improve their efficacy.

Conflict of Interest:

The authors declare that there is no conflict of interests regarding the publication of this paper.

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