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ANTIOXIDANT ACTIVITY OF ISOLATED FLAVONOIDS FROM THE LEAVES OF *CORCHORUS AESTUANS* LINN.

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ABSTRACT

Preliminary phytochemical investigation of various extracts of leaves of *Corchorus aestuans* Linn showed the presence of flavonoids, carbohydrates, saponins, phytosterols, phenolic compounds, triterpenoids, cardiac glycosides and tannins. An attempt has been made to isolate flavonoids and perform antioxidant potential of the same. The antioxidant activity was performed by two *in-vitro* testing methods, such as scavenging activity on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals and hydroxyl radical scavenging activity by EDTA. The data obtained in these testing systems clearly established the antioxidant potency of *Corchorus aestuans* Linn.

INTRODUCTION: Free radicals are considered as important factors in the etiology of cancer, and components with antioxidant activity have received particular attention as potential inhibitors of several cancers [1]. BHA and BHT have been found to be anti-carcinogenic as well as carcinogenic in experimental animals. There are reports in the literature saying that BHA appeared to have tumor-initiating and tumor-promoting action [2].

Therefore, there has been considerable interest to develop natural antioxidants from botanical sources, especially edible medicinal plants, to replace synthetic antioxidants due to the long-term safety and negative consumer perception of synthetic antioxidants [3]. The natural antioxidants generally function as free radical scavengers and chain breakers, complexes of pro-oxidant metal ions and quenchers of singlet-oxygen formation [4].

Corchorus aestuans Linn (Syn. *Corchorus acutangulus* Lam.), family-Tiliaceae, is an annual herb occurring throughout the hotter parts of the Subcontinent, Indo-china, Australia, Tropical Africa, West Indies, and Central America [5, 6]. It is popularly known as Jute. The roots and leaves are said to cure gonorrhea and used in making an injection for urethral discharge. The seeds are stomachic and used in pneumonia [7, 8]. The plant is said to possess anticancer, antipyretic, anticonvulsant, stomachic and digitalis glycosides like action [9].

In the present study, an attempt has been made to isolate new flavonoids from the methanol extract of leaves of *Corchorus aestuans* Linn and perform the antioxidant potency of isolated flavonoids by two different established *in vitro* testing systems, such as scavenging activity on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals and hydroxyl radical scavenging activity by EDTA.

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MATERIALS AND METHODS:

Materials: DPPH, Methanol, Ascorbic acid, Vitamin E, Ethylene diamine tetra acetic acid (EDTA), Ferric chloride, Hydrogen peroxide, Deoxyribose, Phosphate buffer, Trichloro acetic acid, Thio-barbituric acid were purchased from Chemco Laboratories Ltd, Baroda, India. All chemicals and solvents used were of analytical grade.

Collection and identification of the Plant Material: The plant *Corchorus aestuans* Linn was collected from the local area of Kheda district, Gujarat, India, in the month of August 2009 and its authentication was confirmed by Dr. M. S. Jangid, Botany Department, Sir P. T. Science College, Modasa, Gujarat, India. Herbarium of the plant has been deposited at Department of Pharmacognosy, B. Pharmacy College, Rampura, Kakanpura, Dist. Panchmahal, Gujarat, India for future reference.

Preparation of Sample: The freshly cut leaves were washed with distilled water and air-dried to constant weight at room temperature for 15 days, after which the leaves were powdered into powdered form (using mixer grinder), labeled and kept in the air tight glass jars in a refrigerator.

Preparation of Extracts: 100 g of the air-dried powdered leaves of *Corchorus aestuans* L. were extracted with 500 ml of each of hexane, ethyl acetate, methanol and distilled water using Soxhlet apparatus. The extracts were concentrated in a rotary evaporator apparatus at approximately 60°C. The concentrated extracts were kept in a desiccator until analyses[10].

Isolation of Flavonoids: The methanol extract of leaves of *Corchorus aestuans* L. was subjected to PTLC to isolate flavonoids using toluene: ethyl acetate: acetone: formic acid (5: 2.5: 7.5: 0.5) as mobile phase [11]. The TLC plates were observed under UV-254 nm and UV-365 nm and sprayed with anisaldehyde-sulphuric acid reagent followed by heating at 100˚C till the colored bands appear. The R_f values and color of the resolved bands were recorded and shown in **Table 1 and Figure 1**.

TABLE 1: TLC DETAILS OF *CORCHORUS AESTUANS* LINN. LEAVES EXTRACT

R_f Values	Color of the band		
	UV 254 nm	**UV 365 nm**	**After derivatization**
0.10	Light Green	Greenish blue	Light yellow
0.15	Green	Blue	Light yellow
0.56	Green	Violet	Light brown
0.74	Green	Light blue	Light yellow
0.86	Green	Greenish yellow	yellow
0.92	Light green	Light blue	Light brown

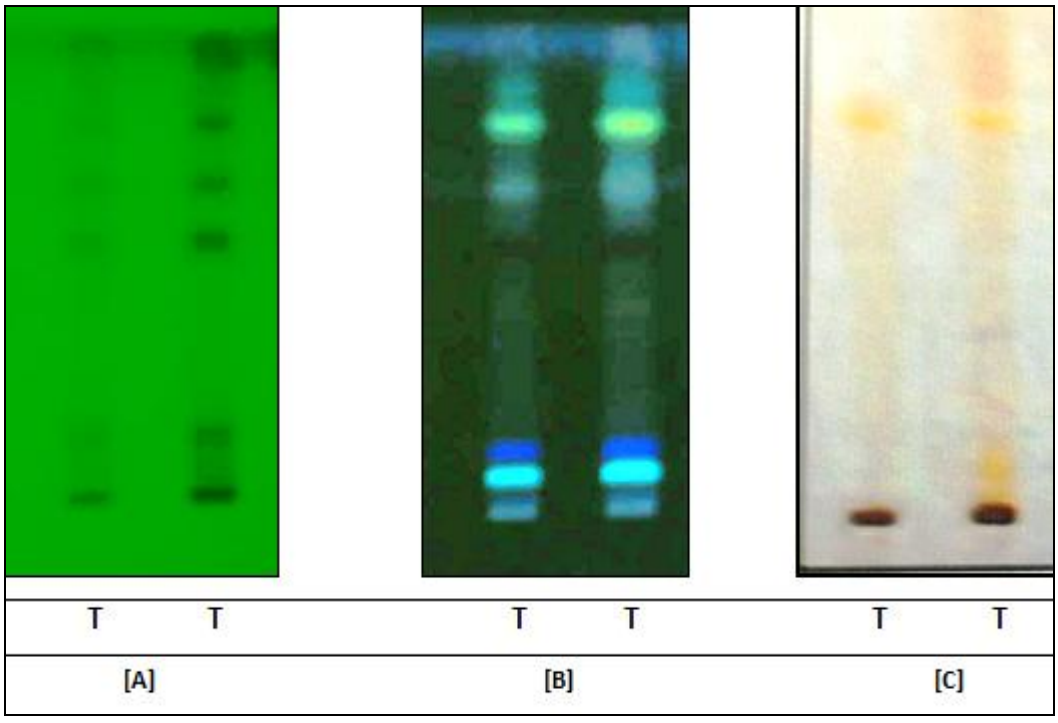

FIGURE 1: TLC PROFILE OF *CORCHORUS AESTUANS* LINN. LEAVES EXTRACT

A. UV 254 nm; **B**. UV 365 nm; **C.** After spraying with anisaldehyde-sulphuric acid reagent and heating at 100˚C; **T.** Test solution

Total six spots were found at R_f - 0.10, 0.15, 0.56, 0.74, 0.86 and 0.92. The bands corresponding with R_f – 0.86 and 0.74 were marked and scrapped. The scrapped materials were refluxed with 100 ml methanol, filtered and concentrated to dryness. They were re-crystallized from alcohol. The yellow colored crystalline compounds were subjected to general chemical tests (Shinoda test, Fluorescence test, $FeCl_3$ test) for flavonoids and TLC studies. The melting point of isolated compounds was taken. Then they were characterized with the help of instrumental techniques like UV spectroscopy, LC-MS spectroscopy, IR spectroscopy and NMR spectroscopy [12-13].

Working standard and Test solutions:

a. Test solution for DPPH Method: 50mg of isolated compounds were dissolved in 10ml of methanol and volume was made up to 50ml to give 1mg/ml stock solution. The above stock solution was further diluted to get the working test solution in the concentration range of 10-100 μg/ml.

b. Standard solution for DPPH Method: Different concentrations (10-100 μg/ml) of ascorbic acid solutions were prepared in methanol as similar to test solutions.

c. Test solution for Hydroxyl scavenging by EDTA method: 10mg of isolated compounds were dissolved in sufficient quantity of methanol and volume was made up to 100ml to give 100 μg/ml of the stock solution. The above stock solution was further diluted to get the working test solution in the concentration range of 1-10 μg/ml.

d. Standard solution for Hydroxyl scavenging by EDTA method: Different concentrations (1-10μg/ml) of Vitamin E solutions were prepared in methanol as similar to test solutions.

Determination of Antioxidant Activity by DPPH method: The free radical scavenging activity of isolated flavonoids from the methanol extract of leaves of *Corchorus aestuans* L. was measured by DPPH using the method of Cheung *et al*. A commercially available and stable free radical DPPH soluble in methanol was used. DPPH in its radical form has an absorbance peak at 517nm, which disappears on reduction by an antioxidant compound. 1ml of different concentrations of the isolated compound/standard (10-100 μg/ml) were added to 2ml of freshly prepared methanolic solution of 90 μM DPPH and the volume was made up to 4ml with methanol. The reaction mixtures were kept at room temperature in the dark and after 1 hour the absorbance was measured at 517nm using spectrophotometer. A blank was performed excluding the isolated compound. The optical density of the sample, and the blank was measured by comparing the methanol.

Ascorbic acid was used as standard. The percentage inhibition of DPPH in reaction mixture was calculated by comparing with the blank. %RSC was calculated by the following formula:

$$\text{Scavenging Activity (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100\%$$

Where, $A_{Control}$ = Absorbance of the control and A_{Sample} = Absorbance of the sample/standard.

All assays were conducted in triplicate. Ascorbic acid was used as positive reference standard in this study. From RSC values, the EC_{50} values was calculated which represents the concentration of scavenging compound that was 50% neutralization. EC_{50} values were obtained by linear regression method using %activity in y-axis and concentration in x-axis. RSC of the isolated compounds were determined and the activity was compared with standard ascorbic acid [14-16].

Determination of Hydroxyl Radical Scavenging Activity by EDTA: The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell. Stock solutions of all reagents were prepared in doubly distilled water. The assay was performed by adding 0.1ml of EDTA, 0.01ml of Ferric chloride, 0.1ml of Hydrogen peroxide, 0.35ml of deoxyribose, 1ml of the isolated compound /working standard solution (1-10 mcg/ml), 0.3ml of phosphate buffer (pH-7.4, 50mM) and 0.1ml of ascorbic acid in sequential order of addition. The mixture was then incubated at 37°C for 60 minutes. 1ml of incubated reaction mixture was mixed with 1ml of 1% Thio-barbituric acid (TBA) and 1ml of 5% Trichloro acetic acid in NaOH (0.025M) to develop the pink colored chromogen and absorbance was measured at 532nm.

Deoxyribose degradation was measured as TBARS and percentage inhibition was calculated. %RSC was calculated by the following formula:

$$\text{Scavenging Activity (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100\%$$

Where, $A_{control}$ = Absorbance of the control and A_{sample} = Absorbance of the sample/standard.

All assays were conducted in triplicate. Vitamin E was used as positive reference standard in this study. From RSC values, the EC_{50} values was calculated which represents the concentration of scavenging compound that was 50% neutralization. EC_{50} values were obtained by linear regression method using %activity in y-axis and concentration in x-axis. RSC of the isolated compounds were determined and the activity was compared with standard Vitamin E [17-19].

Statistical Analysis: All the data were represented as Mean ± S.E.M., n=3, and analyzed by One-way ANOVA followed by Dunnett's t-test. Values representing the concentrations of investigated compounds that cause 50% of neutralization/inhibition (EC_{50}) were determined by the linear regression analysis.

RESULTS AND DISCUSSION: The methanol extract of leaves of *Corchorus aestuans* Linn. was subjected to PTLC to isolate flavonoids using toluene: ethyl acetate: acetone: formic acid (5: 2.5: 7.5: 0.5) as mobile phase. Two yellow colored crystalline compounds C_1- 5.8 gm at R_f ~0.86 and C_2–6.5 gm at R_f ~0.74 were collected. Isolated compounds showed positive chemical tests (Shinoda test, Fluorescence test, $FeCl_3$ test) for flavonoids. Melting point of isolated compound C_1 was 230-232°C and C_2 was 212-214°C respectively.

Compound C_1 showed green and greenish yellow colored spots at R_f ~0.86 under UV-254nm and UV-365nm using toluene: ethyl acetate: acetone: formic acid (5: 2.5: 7.5: 0.5) as mobile phase. A UV spectrum of C_1 was taken in methanol which showed peaks at λ max - 259 nm, 375 nm. The LC-MS spectrum showed $RT_{(min)}$ – 44.0 and molecular weight – 464 with molecular formula $C_{21}H_{20}O_{12}$. The IR spectrum showed peaks (cm^{-1}) – 3439.82(O-H), 2925.92(C-H stretch), 1725.24 (C=O), 1630.06(C=C), 1383.98(C-H bend), 1119.28, 1075.93 (C-O stretch for alcohols and ethers).

The ^{1}H-NMR spectrum of the compound C_1 displayed five protons at δ - 6.19, 6.38 in ring A and δ - 7.65, 6.98 and 7.68 in the ring B. The ^{13}C NMR spectrum showed peaks at δ – 154.5, 131.9, 167.5, 160.6, 99.0, 164.0, 93.4, 156.3, 105.1, 122.3, 115.2, 144.7, 147.7 and 121.8. From the spectral data, it could be concluded that the isolated compound C_1 was Quercetin 3-o-galactoside with molecular formula $C_{21}H_{20}O_{12}$.

Compound C_2 showed green and blue colored spots at R_f ~0.74 under UV-254nm and UV-365nm using toluene: ethyl acetate: acetone: formic acid (5: 2.5: 7.5: 0.5) as mobile phase.

UV spectra of C_2 (λ_{max} - 256 nm, 372 nm) was taken with standard quercetin which was superimposed with standard quercetin (λ_{max} - 259 nm, 375 nm) and the peaks were found to be exactly matching to each other.

The LC-MS spectrum showed $RT_{(min)}$ – 50.6 and molecular weight – 302. This confirmed the identity and purity of the isolated compound to be quercetin with molecular formula $C_{15}H_{10}O_7$.

Determination of Antioxidant Activity by DPPH method: The reduction capacity of DPPH radical was determined by decreasing it absorbance at 517nm induced by antioxidants. The decreased absorbance by DPPH is due to progressive reaction between free radicals and antioxidant molecules which results in scavenging of radical by donation of hydrogen. It is visually noticeable by decrease in intensity of purple color.

Hence, the DPPH free radical is generally used as a substrate to evaluate antioxidant activity. Stable free radical DPPH was effectively scavenged by isolated flavonoids and the inhibition was found to be dose-dependent.

Table 2, 3 and Figure 2 illustrates the scavenging activity of quercetin 3-o-galactoside, quercetin and ascorbic acid on the DPPH radicals and the effects increase with increasing concentration range 10-100 µg/ml.

TABLE 2: DPPH RADICAL SCAVENGING ACTIVITY OF ISOLATED FLAVONOIDS OF *C. AESTUANS* L. EXTRACTS (ABSORBANCES OF TEST SAMPLES AND STANDARDS)

Concentration (µg/ml)	Absorbance		
	Quercetin 3-o-galactoside	Quercetin	Ascorbic acid (standard)
10	0.356	0.324	0.294
20	0.325	0.309	0.205
50	0.312	0.298	0.134
60	0.289	0.245	0.069
80	0.231	0.124	0.046
100	0.176	0.097	0.032
Blank	0.414	0.415	0.413

TABLE 3: DPPH RADICAL SCAVENGING ACTIVITY OF ISOLATED FLAVONOIDS OF *C. AESTUANS* L. EXTRACTS (% INHIBITION OF TEST SAMPLES AND STANDARDS)

Concentration (µg/ml)	DPPH Radical Scavenging activity (% inhibition)		
	Quercetin 3-o-galactoside	Quercetin	Ascorbic acid (Standard)
10	14.01±0.10	21.93±0.09	**28.81±0.08**
20	21.49±0.19	25.54±0.14	**50.36±0.17**
50	**24.64±0.14**	**28.19±0.22**	**67.55±0.10**
60	**30.19±0.21**	**40.96±0.05**	**83.29±0.20**
80	**44.20±0.29**	**70.12±0.09**	**88.86±0.08**
100	**57.49±0.18**	**76.63±0.14**	**92.25±0.26**
EC_{50} values	87	65	25

Values are Mean ± SEM, n=3, A value of P<0.0001 was considered statistically significant by One way ANOVA followed by Dunnett's t-test.

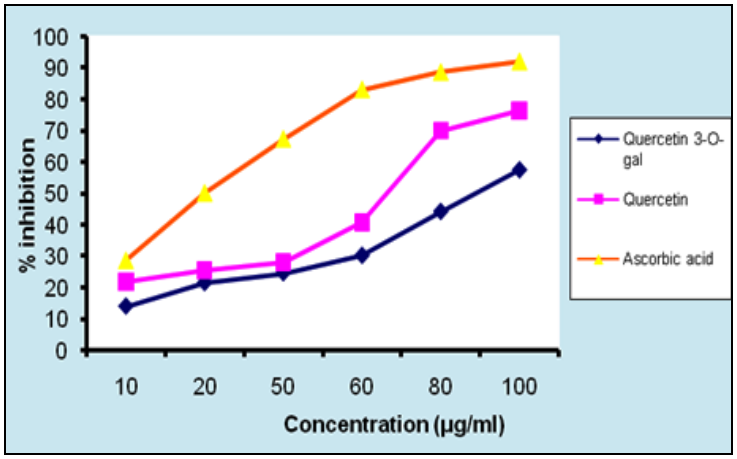

FIGURE 2: ANTIOXIDANT ACTIVITY (%) MEASURED BY DPPH METHOD

The scavenging activity of quercetin 3-o-galactoside, quercetin and ascorbic acid on the DPPH radical decreased in the order: Ascorbic acid > Quercetin > Quercetin 3-o-galactoside was of 67.55±0.10, 28.19±0.22 and 24.64±0.14 (P<0.0001) at the concentration 50 µg/ml respectively.

The concentration of quercetin 3-o-galactoside resulting in 50% inhibition of the free radical (EC_{50}) was 87 µg/ml and those of quercetin and ascorbic acid were 65µg/ml and 25µg/ml.

Determination of hydroxyl radical scavenging activity by EDTA: Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and test compound for hydroxyl radical generated from the Fe^{+3}/ascorbate/EDTA/H_2O_2 system. The hydroxyl radical attacks deoxyribose, which results in Thio-barbituric acid reacting substance (TBARS) formation based on the inhibition rate of 2-deoxyribose oxidation by hydroxyl radical.

Among the all oxygen radicals, the hydroxyl radical (OH) is the most reactive and damages diverse biomolecules. Hydroxyl radicals were generated in a reaction mixture containing ascorbate, H_2O_2 and Fe^{+3}, EDTA at pH 7.4 and measured by their ability to degrade the sugar deoxyribose into fragments that on heating with TBA at low pH form a pink chromogen.

Table 4, 5 and Figure 3 shows the scavenging capacity of quercetin 3-o-galactoside, quercetin and Vitamin E. Quercetin 3-o-galactoside showed high hydroxyl radical scavenging activity that intensify with increasing concentration, reacting 80.60±0.19 at 10 µg/ml, while at an equal concentration the quercetin and Vitamin E caused 83.50±0.19 and 85.91±0.26 inhibition of hydroxyl radical.

The decreasing order of antioxidant effect (expressed as EC_{50}) is isolated quercetin 3-o-galactoside 1.9µg/ml, quercetin 1.3µg/ml and vitamin E 0.8µg/ml.

TABLE 4: HYDROXYL RADICAL SCAVENGING ACTIVITY OF ISOLATED FLAVONOIDS (ABSORBANCES OF TEST SAMPLES AND STANDARDS)

Concentration (µg/ml)	Absorbance		
	Quercetin 3-o-galactoside	Quercetin	Vitamin E (standard)
1	0.162	0.156	0.150
2	0.147	0.139	0.137
4	0.124	0.108	0.094
6	0.104	0.098	0.089
8	0.093	0.079	0.064
10	0.058	0.049	0.042
Blank	0.299	0.297	0.298

TABLE 5: HYDROXYL RADICAL SCAVENGING ACTIVITY OF ISOLATED FLAVONOIDS (% INHIBITION OF TEST SAMPLES AND STANDARDS)

Concentration (µg/ml)	Hydroxyl Radical Scavenging activity (% inhibition)		
	Quercetin 3-o-galactoside	Quercetin	Vitamin E (Standard)
1	45.82±0.09	47.47±0.15	49.66±0.68
2	50.84±0.16	53.20±0.23	54.02±1.13
4	58.53±0.14	63.64±0.15	70.19±1.43
6	65.22±0.21	67.00±0.30	71.89±0.98
8	68.90±0.10	73.40±0.21	80.12±0.19
10	80.60±0.19	83.50±0.19	85.91±0.26
EC_{50} values	1.9	1.3	0.8

Values are Mean ± SEM, n=3, A value of P<0.0001 was considered statistically significant by One way ANOVA followed by Dunnett's t-test.

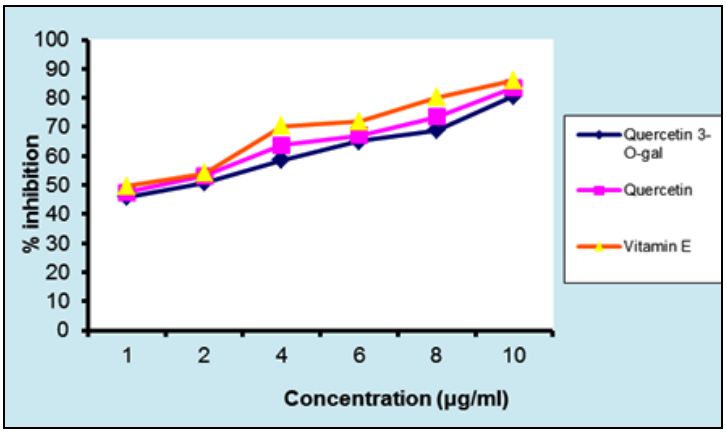

FIGURE 3: HYDROXYL RADICAL SCAVENGING ACTIVITY (%) OF ISOLATED FLAVONOIDS

CONCLUSION: The scavenging activity of isolated flavonoids quercetin 3-o-galactoside, quercetin and ascorbic acid on the DPPH radical decreased in the order: Ascorbic acid > Quercetin > Quercetin 3-o-galactoside was of 67.55±0.10, 28.19±0.22 and 24.64±0.14 (P<0.0001) at the concentration 50 µg/ml respectively. The concentration of quercetin 3-o-galactoside resulting in 50% inhibition of the free radical (EC_{50}) was 87 µg/ml and those of quercetin and ascorbic acid were 65µg/ml and 25µg/ml.

Quercetin 3-o-galactoside showed high hydroxyl radical scavenging activity that intensify with increasing concentration, reacting 80.60±0.19 at 10 µg/ml, while at an equal concentration the quercetin and Vitamin E caused 83.50±0.19 and 85.91±0.26 inhibition of hydroxyl radical. The decreasing order of antioxidant effect (expressed as EC_{50}) is isolated quercetin 3-o-galactoside 1.9µg/ml, quercetin 1.3µg/ml and vitamin E 0.8µg/ml.

In conclusion, antioxidant study of *C. aestuans* L. leaves suggested that *C. aestuans* L. is a potential source of natural antioxidants. However, further investigations on *in vivo* antioxidant activities are highly recommended.

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