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Research Article

Phytochemical Screening and *in vitro* Angiotensin Converting Enzyme Inhibition Assay of Green Leaf Extracts of *Artocarpus altilis* (Parkinson) Fosberg

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ABSTRACT
Objective: To screen the presence of various phytochemical constituents in green leaf extracts of *Artocarpus altilis* (Parkinson) Fosberg (breadfruit) and to test the ability of phytocompounds to potentially inhibit Angiotensin Converting Enzyme (ACE) under *in vitro* condition.
Methods: Qualitative phytochemical screening of green leaf of *Artocarpus altilis* (Parkinson) Fosberg was done by standard procedures. ACE inhibiting capacity of the plant leaf fractions was assayed after column chromatography, using N-Hippuryl-His-Leu hydrate as substrate and the compound present in better active fraction was confirmed by TLC analysis.
Results: The results revealed the presence of many important phytochemicals such as carbohydrates, quinones, aminoacids, proteins, reducing sugars, flavonoids, gums and mucilages, tannins, resins, terpenoids, phenols, saponins, cardiac glycosides, volatile oils, starch, steroids, emodols and fatty acids. All the six fractions (F1, F2, F3, F4, F5 and F6) obtained by column chromatography revealed their efficacy to inhibit ACE.TLC analysis of most active fraction F5 indicated the presence of a single compound with an RF vale of 0.55
Conclusion: The ACE inhibitory potential of *A. altilis* leaf extracts observed in the present results will shore up its utilization in the folk medicine for the better treatment of hypertension.

Keywords: *Artocarpus altilis*; Phytochemicals; Angiotensin Converting Enzyme (ACE)

INTRODUCTION

Hypertension is one among the major life-threatening symptoms or disease across the world, especially in the developed societies. Prevalence of patients with hypertension was confirmed recently in rural Tamil Nadu in a large scale [1]. Medication for hypertension includes diuretics, includingides, chlorthalidone and indapamide, beta-blocker and alpha-blocker, calcium channel blockers, peripheral adrenergic inhibitor, vasodilators, angiotensin-converting enzymes (ACE) inhibitors, angiotensin receptor lockers. All these constitute an established therapy in the management of high blood pressure [3].

ACE inhibitors alter the balance between the vasocontractive and the salt retentive properties of angiotensin II and the degradation of bradykinin. Since the original discovery of ACE inhibitors in snake venom, pharmacologically active ACE inhibitors such as captopril, enalopril, lisinopril, benazepril, fasinopril, ramipril, perindopril, quinapril and many more compounds have been developed and are currently in use and were established themselves in the hypertension and congestive heart failure therapy. Synthetic ACE inhibitors are remarkably effective, but they cause adverse side effects such as cough, angioedema, taste disturbance, skin rashes and allergic reaction

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Therefore, in recent times, the trend has been set towards the event of natural, safe and effective ACE inhibitors with minimized adverse effects. Most of the plants in the universe are known to possess therapeutic properties and have been used since ancient times to treat various human diseases effectively and efficiently. The extracts and metabolites from leaves, stem, fruits and bark of A. altilis (commonly referred as breadfruit) belonging to Moraceae family contains numerous beneficial biological active compounds and these are used in various biological activities. The preparation of tea leaves of breadfruit can be called herbal tea. The yellowing leaves of A. altilis are brewed into tea and used as a folk medicinal treatment for hypertension [11]. The tea is also thought to control diabetes. Hypertension and diabetes medications are prepared from a mix of the boiled leaves of this species combined with avocado, papaya and sour soup. The leaf contains the phenols quercetin and camphorol, plus gamma- 6 amino butyric acid, which are the lowers the blood pressure. In view of this, the present study was conducted to estimate the ACE inhibition property of breadfruit leaf extracts A. altilis (Parkinson) Fosberg commonly referred as breadfruit is a traditional starch rich crop of Moraceae family whose extracts and metabolites from leaves, stem, fruits and bark contains numerous beneficial biological active compounds and these are used in various biological activities. Much ongoing research suggest that leaf extract of A. altilis exhibits potent ACE inhibition.

MATERIALS AND METHOD

2.1 Chemicals used

All the reagents used were of standard grade and purchased from Sigma Aldrich – Merck, Bangalore, India.

2.2 Collection and processing of plant material

Green leaves of A. altilis (Parkinson) Fosberg were collected from coastal areas of Thengapattanam Village, Kanyakumari district, Tamil Nadu and brought to the laboratory, washed thoroughly, air dried in shade and reduced to coarse powder in a mixer grinder. The plant was authenticated by Dr. S. Mutheeshwaran, Entomology Research Institute, Loyola College, Chennai, Tamil Nadu, India.

1.3 Preparation of green leaf extracts of A. Altilis (Parkinson)

Green leaf powder of A. altilis (Parkinson) Fosberg was sequentially extracted (Fig. 1) with different organic solvents such as hexane, ethyl acetate, acetone, methanol and water in increasing polarity [18].The crude extracts obtained were evaporated under reduced pressure at 40oC and finally concentrated and stored in a refrigerator at 2-8oC for use in subsequent experiment.

2.4 Qualitative phytochemical analysis of A. altilis (Parkinson) Fosberg green leaf extracts

The solvent extracts of A. altilis leaves were subjected to phytochemical studies to screen the presence of phyto-constituents such as carbohydrates, quinones, alkaloids, aminoacids, proteins, reducing sugars, flavonoids, gums and mucilages, tannins, resins, terpenoids, phenols, saponins, cardiac glycosides, volatile oils, starch, betacyanins, coumarins, steroids, anthraquinones, phlobatannins, emodols and fatty acids as per the standard procedures [5].

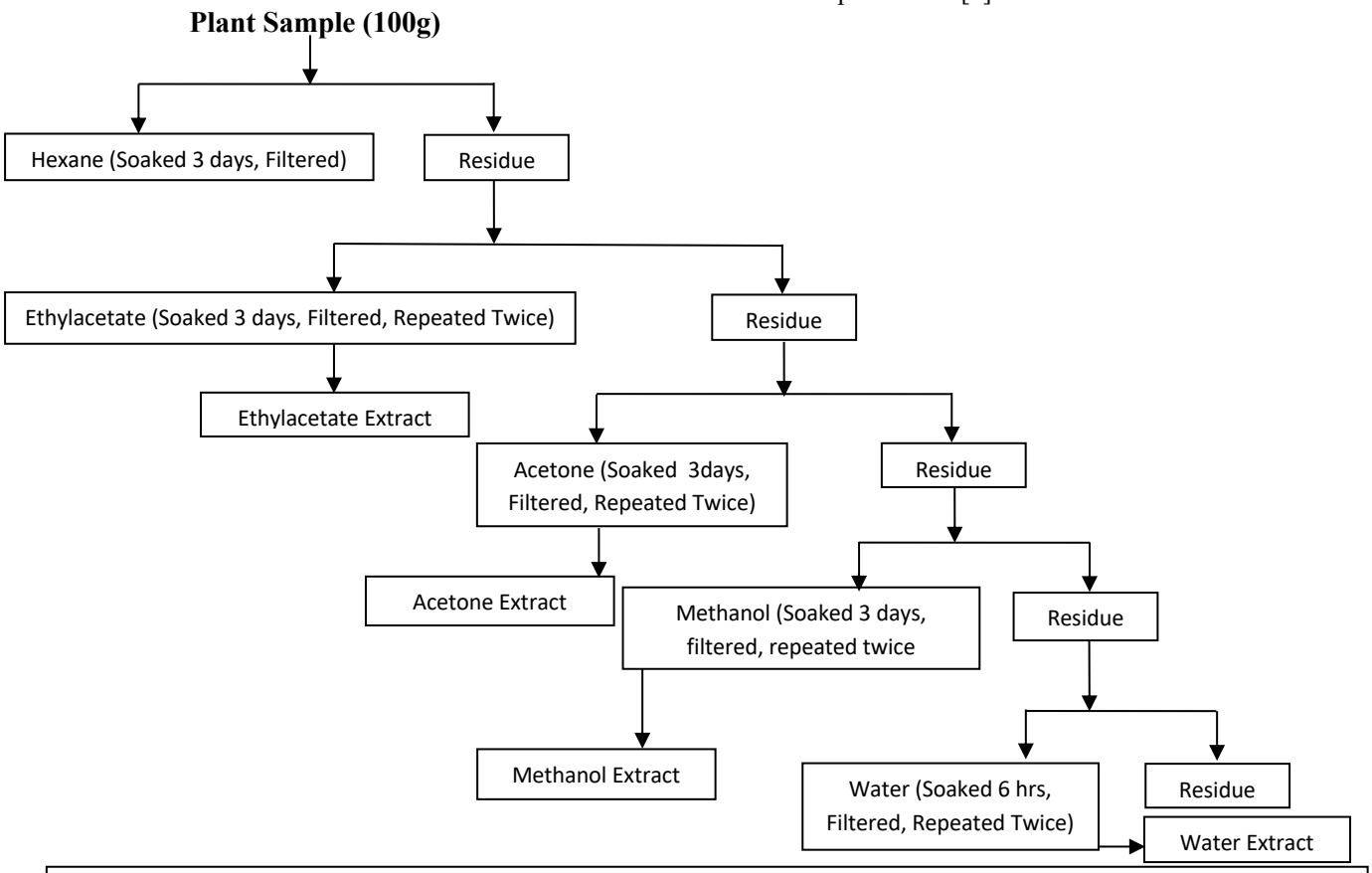

Figure1: Steps of sequential extraction of *A. altilis* (Parkinson) Fosberg green leaf sample using different solvents

2.5 Column Chromatography of *A. altilis* (Parkinson) Fosberg green leaf extracts

Crude water extract of breadfruit leaves was subjected to column chromatography using silica gel as stationary phase. A glass column (2.5×100cm) was taken and dried. The lower end of the column was plugged with absorbent cotton wool. The column was clamped and fitted in vertical position on a stand. Activated silica gel was packed on to the column using 90% chloroform and allowed to settle gently until the necessary length of the column was obtained. For the isolation of compounds, the prepared crudeaqueous extract of *A. altilis* green leaves was loaded on the top of the prepared column and eluted a flow rate of 1ml/min using a combination of solvents such as chloroform: ethyl acetate : methanol(5:3:2) selected from a list of solvent combinations after initial screening using thin layer chromatography. The resulting fractions were collected and concentrated using the rotary evaporator [4].

2.6 *In vitro* Angiotensin Converting Enzyme inhibitory assay using column chromatographic fractions of *A. altilis* (Parkinson) Fosberg green leaves.

ACE inhibition assay was done after heat evaporating and re-dissolving all the collected column chromatography fractions [14]. Angiotensin Converting Enzyme from rabbit lung (0.1 U, Product Number-A6778) and N-Hippuryl-His-Leu hydrate (25mg, Product Number-H1635) purchased from Sigma was used for the assay. A sample solution of 1mg/ml concentration (20 µL of borate buffer or 20 µL of ACE inhibitor) with 30 µL of ACE solution (0.04 U/mL in borate buffer) was pre-incubated for 10 min at 37°C, and the mixture was incubated with 50 µL of substrate (5 mM HHL in borate buffer) for 60 min at the same temperature. The enzymatic reaction was terminated by immersing the test tubes in a 95°C water bath for 10 min and the mixture was allowed to stand for 30 min at room temperature in the darkness and diluted with ethanol to 5 ml. The absorbance was then determined at 478 nm spectrophotometrically against the blank reagent and positive control (Lisinopril 1mg/ml). The inhibitory percentage was then calculated by the following equation

$$\% \text{ of Inhibition} = \frac{\text{OD of blank} - \text{OD of sample}}{\text{OD of balnk}} \text{X } 100$$

2.7. Data analysis

One way Analysis of Variance (ANOVA) was carried out using SPSS statistics data package. Means were compared at 0.001% levels and subsequent post-hoc multiple comparison with SNK test (One way ANOVA).

2.8 Thin Layer Chromatography

TLC analysis was done using silica gel G as the stationary phase and developed using solvents such as chloroform: ethyl acetate: methanol in ratios of 5:3:2. The spot obtained was measured for Rf value using the formula

$$\text{RF} = \frac{\text{Distance travelled by th solute}}{\text{Distance travelled by the solvent}}$$

RESULTS

3.1 Phytochemical analysis of *A. altilis* (Parkinson) Fosberg green leaf extracts

Among the 23 phytochemicals tested on five different extracts of *A. altilis* green leaves, strong presences of quinones and starch was noted in all except the absence of quinones and moderate presence of starch in hexane extract respectively. Abundance of starch was noticed in water extracts of leaf. But carbohydrates could not be detected in aqueous, ethyl acetate and methanol extracts of leaves while hexane and acetone extracts revealed their strong presence. Moderate and weak presence was shown by terpenoids and cardiac glycosides in all extracts respectively except in ethyl acetate and hexane where both could not be detected.

Aqueous extract of leaves showed strong intensity of aminoacids, flavonoids, gums andmucilages, saponins and cardiac glycosides while modest presence of tannins and weak presence of resins and phenols was observed. proteins, reducing sugars, carbohydrates, anthroquinones, phlotoblatannin and fatty acids could not be traced in theaqueous extract of leaf sample. Steroids showed uniformly weak presence in all the extracts of leaves except in ethyl acetate fraction of leaves which revealed the presence of steroids at moderate intensity. Strong presence of aminoacids was also noted in the methanolic extract of leaves. Hexane extracts of leaves reported weak presence of aminoacids, while in ethyl acetate and acetone extracts, the presence of aminoacids could not be detected. The ethyl acetate, acetone and methanolic leaf extracts showed strong intensity of protein followed by its weak presence in hexane extract. Ethyl acetate and hexane fraction of leaves extract did not show the presence of reducing sugars whereas it was moderately reported in acetone and methanol fraction.

Flavonoid in the ethyl acetate and hexane failed to show its presence, while acetone and methanol extracts showed its weak presence. However, strong presence of this biomolecule was noticed in aqueous extract. Hexane extract of leaves revealed strong intensity of gums and mucilage. Methanol fraction was found to possess lowlevel of gums and mucilage, while in acetone and ethyl acetate extract of leaves failed to show its presence. The leaf extract of acetone and methanol responded with strong intensity of phenols, followed by ethyl acetate with moderate intensity. Hexane extract responded hardly towards phenol test. Strong presence of tannins in ethyl acetate, acetone and hexane in leaf sample was noted. All leaf samples

responded negatively for both resins and saponins except for aqueous extracts. Aqueous, acetone and methanol extracts of leaves revealed moderate presence of terpenoids uniformly and its absence in ethyl acetate and hexane extract. Leaf extract showed negative report for volatile oil except for water.

Total absence of alkaloids and phlobatannins was reported in all the five extracts of leaf sample. All the extracts of leaf samples failed to show the presence of betacyanins, coumarins, athraquinones and emodols except the presence of emodols in aqueous and methanol extract. Fatty acids exhibited its strong occurrence in the methanolic extract of leaf. The present investigation showed that the better extracting solvent of phytochemicals from *A. altlis* was aqueous extract with the presence of fourteen out of twenty three phytochemicals screened followed by methanol extract which tested n positive for twelve phytochemicals, acetone nine phytochemicals, seven phytochemicals in hexane and ethyl acetate six phytochemicals.(Table 1)

Table 1: Phytochemical analysis of *A. altilis* (Parkinson) Fosberg green leaf extracts

Sl. No	Compounds	Aqueous	Ethyl acetate	Hexane	Acetone	Methanol
1.	Carbohydrates	-	-	+++	+++	-
2.	Quinones	+++	+++	-	+++	+++
3.	Alkaloids	-	-	-	-	-
4.	Aminoacids	+++	-	+	-	+++
5.	Proteins	-	+++	+	+++	+++
6.	Reducing sugars	-	-	-	++	++
7.	Flavonoids	+++	-	-	+	+
8.	Gums &Mucilages	+++	-	+++	-	+
9.	Tannins	++	+++	++	+++	+++
10.	Resins	+	-	-	-	-
11.	Terpenoids	++	-	-	++	++
12.	Phenols	+	++	-	+++	+++
13.	Saponins	+++	-	-	-	-
14.	Cardiac glycosides	+++	-	-	+	+
15.	Volatile oils	+	-	-	-	-
16.	Starch	++++	+++	++	+++	+++
17.	Betacyanins	-	-	-	-	-
18.	Coumarins	-	-	-	-	-
19.	Steroids	+	++	+	+	+
20.	Anthroquinone	-	-	-	-	-
21.	Phlobatannins	-	-	-	-	-
22.	Emodols	+	-	-	-	+
23.	Fatty acids	-	-	-	-	+++

++++ abundantly present; +++ strongly present; ++ moderately present; + weakly present; - absent

3.2 Angiotensin Converting Enzyme inhibitory assay using column chromatographic fractions of *A. altilis* (Parkinson) Fosberg leaves.

A total of six fractions obtained through column chromatography were individually tested for their inhibition capacity and the results were presented in table no 2. Lisinopril, used as positive control drug in this assay showed an inhibition of 90±0.1. The average inhibition percentage noted was 47±0.2, 43±0.1, 1.1±0.4, 26±0.3, 48±0.1, 15±0.2 in fraction 1 to 6 respectively. The compound present in the most active fraction F5 that was then analysed through TLC revealed a single greyish spot with an RF vale of 0.55 when sprayed with $FeCl_3$ solution (Fig:2).

Figure 2: Separation of compounds in Fraction F5 by Thin Layer Chromatography

3.2.1 Calculation of IC_{50} value

IC_{50} value of the standard drug lisinopril and the most active test fraction (F5)was calculated using the linear equation (y = mx+n) or parabolic equation ($y = ax^2+bx+c$) for y=50 on graphplotted with inhibitor concentration (I) against average inhibition percentage was 1.041mg/ml (1041ug/ml) for fraction 5 and the IC_{50} value of positive control (Lisinopril) was 1.388 mg/ml (1388ug/ml) to inhibit 50% of ACE activity. The percentage of ACE inhibition exerted by all fractions significantly ($P < 0.001$) differed as shown in table 2 and the ACE inhibition percentage of different concentrations of fractions F5 was presented in Fig:3.

Table 2 : ACE inhibition percentage of Column chromatography fractions of *A. altilis* (Parkinson) Fosberg green leaf extracts

Sl. No.	Samples	Average % of ACE Inhibition
1.	Standard (1mg/ml)	36 ± 0.1^a
2	F1	47 ± 0.2^b
3	F2	43 ± 0.1^c
4	F3	1.1 ± 0.4^d
5	F4	26 ± 0.3^e
6	F5	48 ± 0.1^a
7	F6	15 ± 0.2^f

Means with different lowercase letters are statistically different from each other (one-way ANOVA, $P < 0.001$ and subsequent postdoc multiple comparison with SNK test).

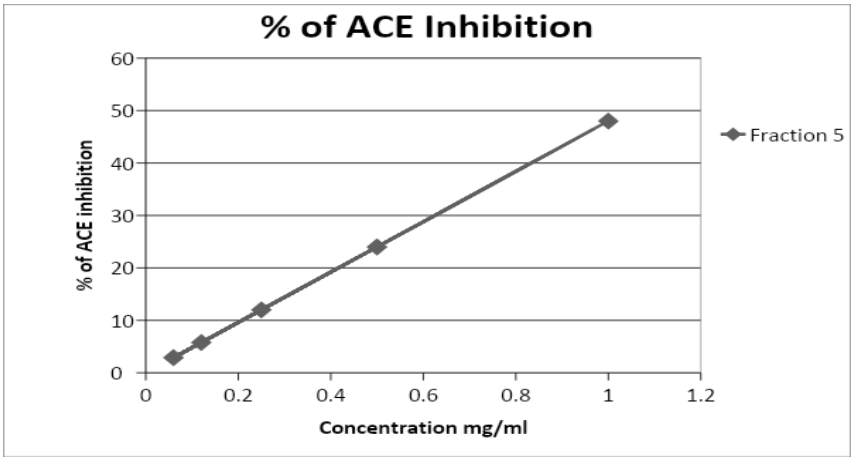

Figure 3: ACE inhibition percentage of F5 at different concentrations

DISCUSSION

Hypertension, a worldwide illness is a major factor in cardiovascular diseases that affects a large population of adults. One of the most effective medications for the treatment of hypertension is angiotensin converting enzyme inhibitors. Therefore, they can be important resources to develop new drug candidates. Active substances derived from medicinal plants can also be a source of new ACE inhibitors. Different species of Australian and Iranian medicinal plants with ACE inhibition capacity was introduced [12,13]. Plants utilized as traditional medicines in India had also been investigated for their ability to inhibit the angiotensin converting enzymes (ACE). The medicinal uses of breadfruit are being actively researched, however still there is a huge dearth of information regarding the angiotensin converting enzyme (ACE) inhibition of leaves of breadfruit. The traditional use of *A. altilis* leaves in folk medicine for the treatment of high blood pressure was earlier reported [8]. Findings of the present study also revealed the antihypertensive nature of breadfruit leaves under *in vitro* condition which might be attributed to the ACE inhibitory activity of the plant. Flavonoids, flavonols, anthocyanin, isoflavones, flavanols, flavones, and other phenolic compounds have proved to be effective in decreasing the ACE activity [12]. The high content of glycosidic and phenolic compounds could be involved in exerting ACE inhibitory activity [16]. Cold and hot extracts of *A. altilis* leaves possessed highest ACE inhibitory activity under in vitro condition. Phytochemical screening of all the extracts indicated the distribution of tannins, phenolics, glycosides, saponins, steroids, terpenoids and anthroquinones and significant correlation of phenolics and glycosides in ACE inhibitory activity of breadfruit leaf [15]. A number of different classes of compound with ACE inhibitory activity were isolated from plants including phenolics, glycosides, tannins, flavonoids, alkaloids, xanthones, terpenes, peptides [6]. In this sense, present phytochemical analysis revealed a varied distribution of compounds such as flavonoids, phenols, tannins, resins, terpenoids, saponins, cardiac glycosides, steroids, emodols etc in the initial analysis, whereby tannins were found associated with the most active fraction. The interaction of tannins with the catalytic site of ACE allosterically inhibits the enzyme thus affecting the enzyme conformation and its interaction with the substrate [2]. Therefore, the above-mentioned secondary metabolitescould be responsible for the ACE inhibition activity of *A. altilis* observed in the present study.

CONCLUSION

Natural products have been the aim of many investigations and the direct use of these products has been encouraged in the pharmaceutical and agricultural industries. *A. altilis* might have several applications as pharmaceutical products including ACE inhibition capacity. In conclusion, *in -vitro* studies indicate that *A. altilis* green leaves could be utilized to lower blood pressure. The role of phenolics, glycosides, terpenoids, tannins and any other compounds in ACE inhibitory activity of fractions obtained in the present study will be determined further in future.

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