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Qualitative and quantitative estimation of bioactive secondary metabolites present in fruit extracts of *Crescentia cujete*

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Abstract
Crescentia cujete is a small evergreen tree, commonly known as the calabash tree belonging to the family Bignoniaceae. The mature fruits of *Crescentia cujete* have a woody shell with a pulpy flesh. In folklore medicine, the fruit is used to treat several ailments especially respiratory diseases and problems related to infection and inflammation. In the present study the qualitative and quantitative evaluation of the major bioactive molecules present in the fruit extract of *Crescentia cujete* is investigated.
Preliminary phytochemical study detected the presence of alkaloid, flavonoid, phenol and saponin in all the fruit extracts. Quantitative estimation of these biomolecules indicated that these are present in moderate to high concentration in the fruit extract. These phytochemicals are known for their antibacterial, antioxidant and anti-inflammatory activities. Hence, the therapeutic potential of the fruit can be correlated with these bioactive phytochemicals detected in the current investigation.

Keywords: *Crescentia cujete*, calabash, bioactive, therapeutic potential, fruit extract

Introduction
Genus *Crescentia* (Bignoniaceae) is represented by 7 species distributed in tropical America and Asia. The species *Crescentia cujete* L. is found in different parts of India [1]. It is a small tree about 6 to 10 m tall with a wide crown and long branches covered with clusters of tripinnate leaves and gourd-like fruit. All parts of the tree have been found to be useful. The fruit is reported to have medicinal applications [2]. In folklore medicine of several countries all over the world this fruit is used to treat numerous diseases. Treating respiratory ailments is the most widely distributed medicinal use of the fruits. The fruit pulp and crushed seed is used for respiratory problems such as asthma and also used as laxative [3, 4]. The unripe fruit is used for curing patients bitten by snake as well as for managing inflammation, diarrhea, and hypertension. The consumption of *C. cujete* fruit and seed extracts evoke contractile response from the uterus [5, 6]. Fruit pulp, cooked on water and sometimes mixed with alcohol, is used to treat bruises and sprains. Fruit pulp and bark are used for treating dermatological conditions, such as burnings and rashes [4]. Secondary metabolites are the bioactive substances produced by plants. These compounds provide protection to plants against biotic and abiotic stresses and make them competitive in their own environment. Modern medicines rely on these metabolites for the treatment of various diseases [7, 8]. Realizing the medicinal importance of this fruit, the current study was undertaken to determine the predominant secondary metabolites present in the extracts of the fruit of *Crescentia cujete* that might be responsible for the therapeutic potential of the fruit.

Materials and Methods
Fresh fruit samples of *Crescentia cujete* were collected from Bharthana area of Surat, South Gujarat (Figure 1). Fruits were botanically identified with the help of local flora and authenticated by experts.

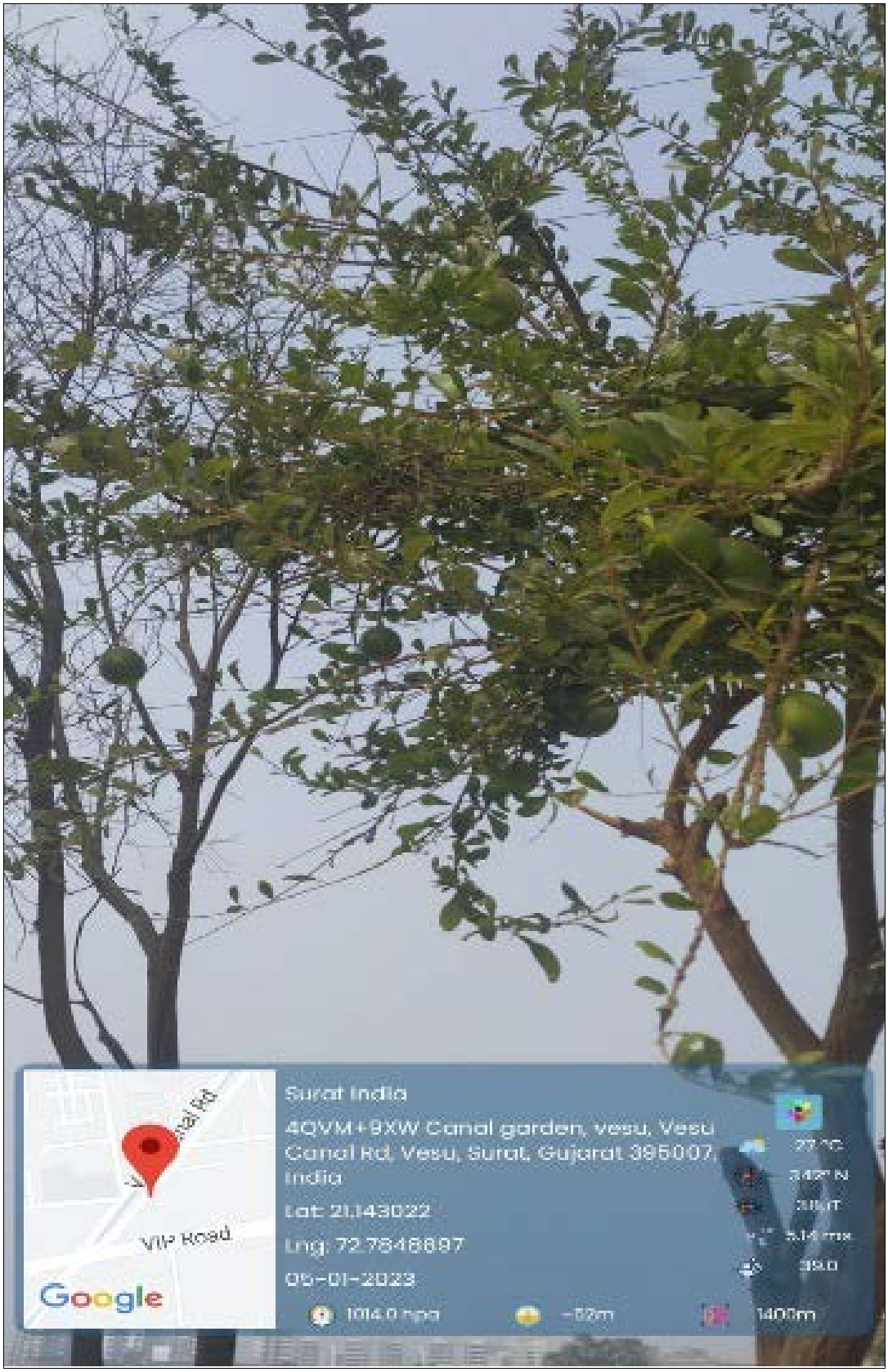

Fig 1: Tree of *Crescentia cujete*

Preparation of fruit extract

1kg of fruit powder was suspended in methanol (1500 ml) and the mixture was kept for 24hrs on shaker at room temperature. The residue was filtered through Whatman No.1 filter paper and filtrate was collected. The residue was again suspended in methanol twice in similar manner. The filtrates were combined and dried at room temperature by putting in petri plates to get methanol extract. 100 g of methanol extract was dissolved in 20% aqueous methanol (100 ml) and put into the separatory funnel. The hexane and chloroform were added to remove the fatty compounds from the methanol layer. Then ethyl acetate was added in separatory funnel and mixed properly. After some time the two layers were formed which were separated to get ethyl acetate filtrate and 20% aqueous methanol stock. The three filtrates of ethyl acetate were collected and dried at room temperature in petri plates to get ethyl acetate extract. After this, *n*-butanol was added serially in 20% aqueous methanol to get *n*-butanol extract [9].

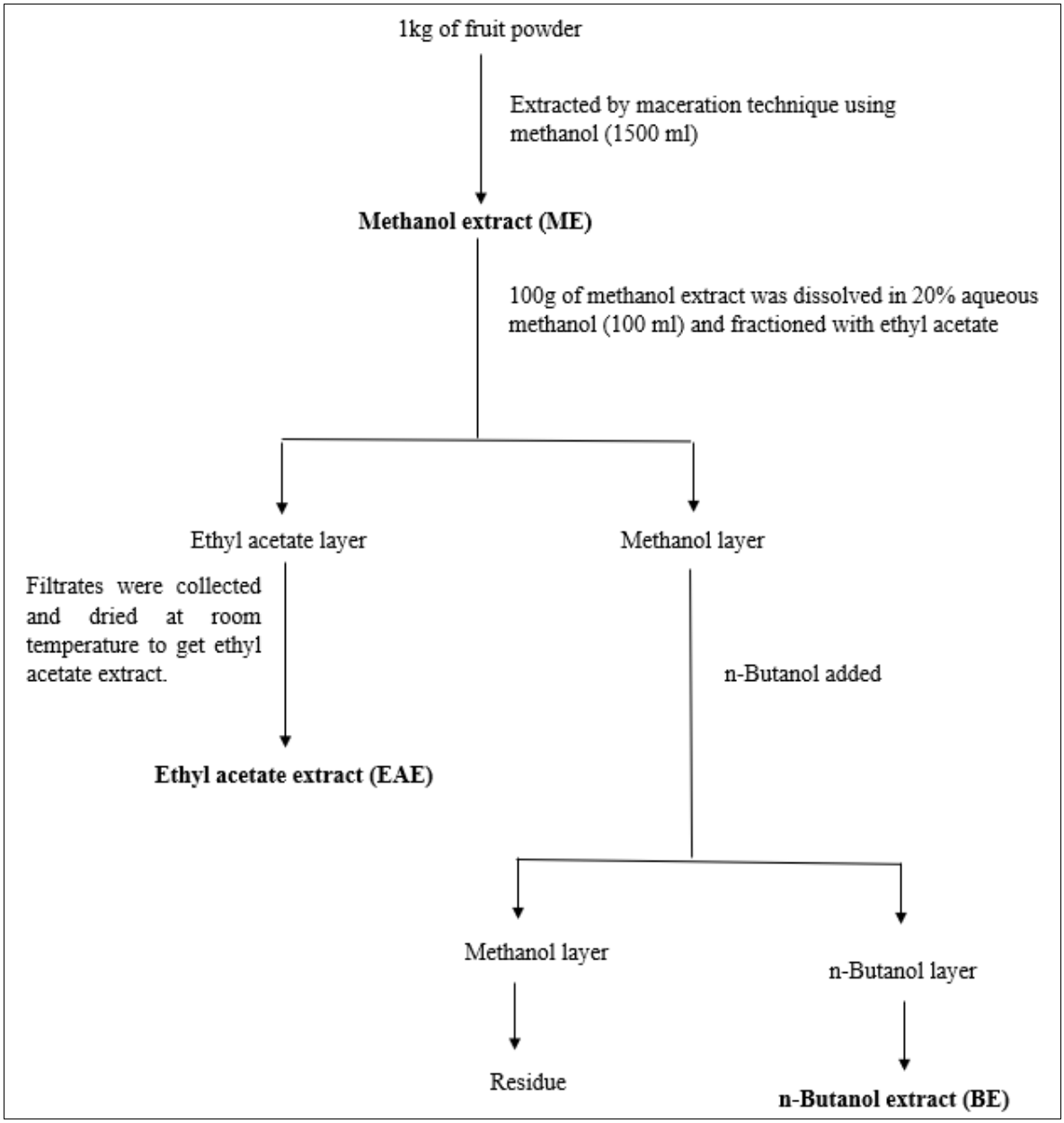

Fig 2: Schematic diagram of extraction of fruits of *Crescentia cujete* using various organic solvents.

Qualitative Detection of Phytochemical Constituents

Screening of the active phytochemicals for all the extracts was performed using standard procedures [10-13].

Detection of Alkaloid: Extracts were dissolved individually in dilute HCl and filtered.

1. Mayer's Test - To about 1ml of the filtrate I ml of Mayer's reagent was added Formation of a yellow coloured precipitate shows the presence of alkaloids.
2. Wagner's Test - Formation of brown/reddish precipitate after adding Wagner's reagent to the extract indicates the presence of alkaloids.
3. Dragendorff's Test - Few drops of Dragendorff's reagent were added to 1ml of the filtrate. Presence of alkaloids is indicated by the Formation of orange-red precipitate.

Detection of phenols

Ferric chloride test - Few drops of Ferric chloride solution were added to the extracts. Formation of green or blue colour indicates the presence of phenols.

Detection of flavonoid

1. Shinoda's test - Three to four pieces of magnesium fillings (ribbon) was added to 0.5ml of ethanolic extract followed by few drops of concentrated HCl. A pink, orange, or reddish colouration indicates the presence of flavonoids (Trease and Evans, 2002) [25].
2. Ferric chloride test - Few drops of 10% Ferric chloride solution was added to 1 mL of extract. Formation of green-blue or violet colouration indicated the presence of flavonoids.
3. Sodium hydroxide test - 1-2 ml of 10% aqueous sodium hydroxide was added to few ml. of the extract to produce yellow colouration. It became colourless on addition of dilute hydrochloric acid indicated the presence of flavonoids.

Detection of saponins

Foam test- 5ml of distilled water was added to the extract and shaken vigorously for a froth. Appearance of stable persistent froth indicates the presence of saponins.

Detection of tannins

Ferric chloride test- 0.5 g of the dried extract was boiled in 20ml of water in a test tube and then filtered. A few drops of 0.1% $FeCl_3$ was added and observed for brownish green-black

or a blue-black colouration.

Detection of steroids and terpenoid

Liebermann - Burchard test: 2ml of acetic anhydride was added to 200mg of extract, cooled on ice followed by addition of concentrated H_2SO_4 slowly. Development of colour from violet to blue or bluish-green indicated the presence of steroid. A small amount of extract was dissolved in ethanol. To it 1 ml of acetic anhydride was added. By adding concentrated H_2SO_4 a change colour from pink to violet showed the presence of terpenoids.

Detection of cardiac glycosides

Keller-Kiliani Test - 2 mL of glacial acetic acid containing one drop of 2.0% $FeCl_3$ was added to 2 mL of the extract. Formation of brown coloured ring between the layers indicates the presence of glycoside.

Quantitative determination of phytochemicals

Phytochemicals that were detected in all the extracts in preliminary screening were further estimated quantitatively according to the following methods.

Alkaloid

5g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed and expressed as mg/gm [14].

Saponin

20g of plant sample was dispersed in 200 ml of 20% ethanol. The suspension was heated over a hot water bath for 4 h with continuous stirring at about 55 ºC. The mixture was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90 ºC. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of normal butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the sample were dried in the oven into a constant weight. The saponin content was calculated in percentage [15].

% Saponin = Weight of saponin/Weight of sample × 100.

Determination of total phenols and flavonoids by Spectrophotometric method

Total phenolic content

Total phenolic content of each fruit extract were determined by the Folin-Ciocalteu method [16] with some modifications. The diluted aqueous solution of each extract and juice (0.5 ml) was mixed with Folin Ciocalteu reagent (0.2 N, 2.5 ml). This mixture was allowed to stand at room temperature for 5 min and then sodium carbonate solution (NA_2CO_3, 75 g/l in water, 2 ml) was added. After 2 hours of incubation, the absorbance was measured at 760 nm against water blank. A standard calibration curve was plotted using Gallic acid.

Determination of total flavonoid

Total flavonoid content

Total flavonoid was estimated by Aluminium trichloride colorimetric method [17]. A diluted methanolic solution (2 ml) of each fruit extract and juice was mixed with a solution (2 ml) of Aluminium trichloride ($AlCl_3$) in methanol (2%). The absorbance was read at 415 nm after 10 min against a blank sample consisting of a methanol (2 ml) and with $AlCl_3$. Quercetin was used as reference compound to produce the standard curve.

Statistical analysis

All the experiments were conducted in triplicates. The values are given as mean ± S.D. (Standard deviation).

Results and Discussions

Preliminary screening of phytochemicals showed the presence of Alkaloids, flavonoids, saponin and phenol in all the extracts. These phytochemicals were subjected to quantitative determination following standard procedures. Previous works have reported the fruits to contain flavonoids (flavones and flavanones), saponins, tannins, alkaloids, phenols. However in the present study, tannin was found to be present only in methanol extracts where as the presence of other phytochemicals are in accordance with the previous study [18, 19].

Table 1: Phytochemical screening of fruit extracts of *Crescentia cujete*

Test	Methanol extract	Ethyl-acetate extract	N-butanol extract
Alkaloids	+	+	+
Flavonoids	+	+	+
Saponins	+	+	+
Phenol	+	+	+
Steroids	-	-	-
Tannins	+	-	-
Terpenoids	-	-	-
Cardiac glycosides	-	-	-

Plus (+) indicates presence of compound; minus (-) indicates absence of compound

The fruit was found to be rich in flavonoid. Flavonoid concentration was expressed in Quercetin equivalent (mgQE/gm) using the calibration curve of Quercetin ($y=0.0067x+0.048$ $R^2=0.983594$). Quantitative determination of flavonoid following spectrophotometric methods indicated 86.72± 0.15mg/G QE flavonoid content (Table 2). Total phenol was estimated using Gallic acid as reference compound and expressed as mg/g Gallic acid equivalent (mg GAE/gm) using the standard curve equation ($y =0.885x +0.209$ $R^2=0.962468$). Total phenol content was found to be 69.34+ 0.8 mg/G GAE (Table 2). The most important use of the fruit in treating respiratory disorders and for treating inflammations, pain bruises and skin infection can be correlated with the flavonoid content of the fruit. Phenols and flavonoids are known for their antioxidant, anti-inflammatory, analgesic and anticancer properties. Reported research and review articles about biological activities of phenol and flavonoid the suggested that these phytochemicals in the fruit might possibly impede inflammation, oxidative stress and can play significant role in the treatment of pulmonary illnesses and any types of inflammations and infections [20-22].

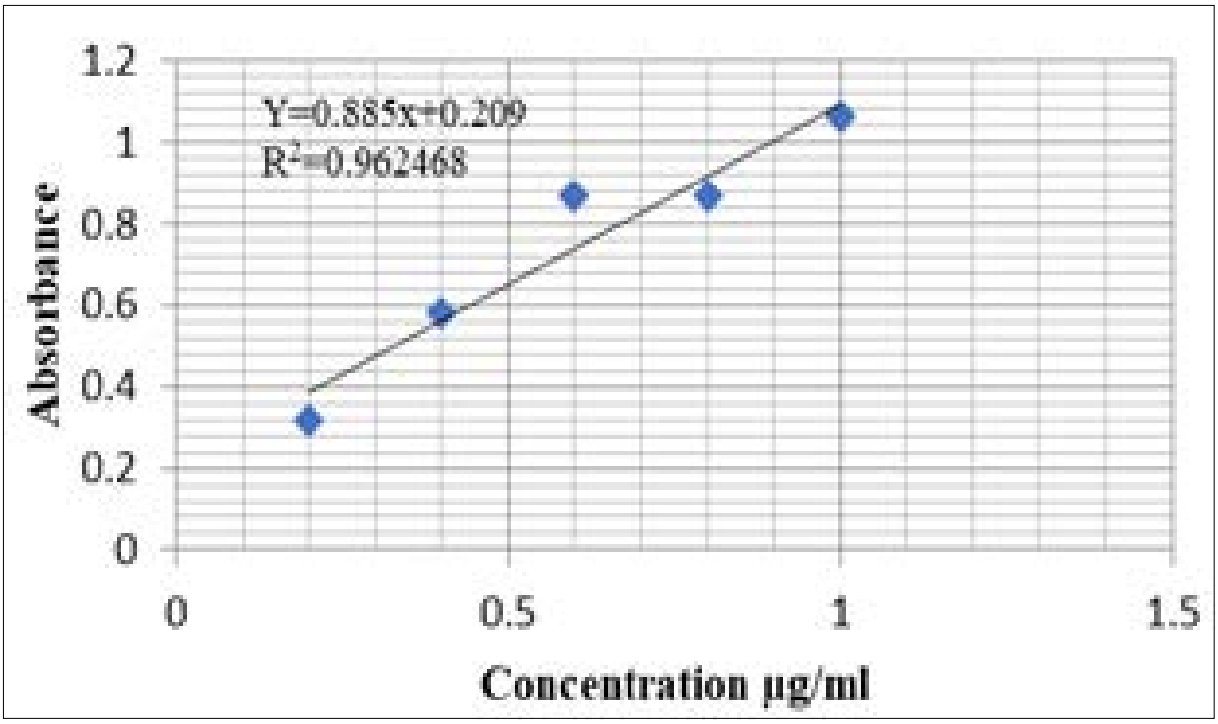

Fig 3: Standard curve for gallic acid

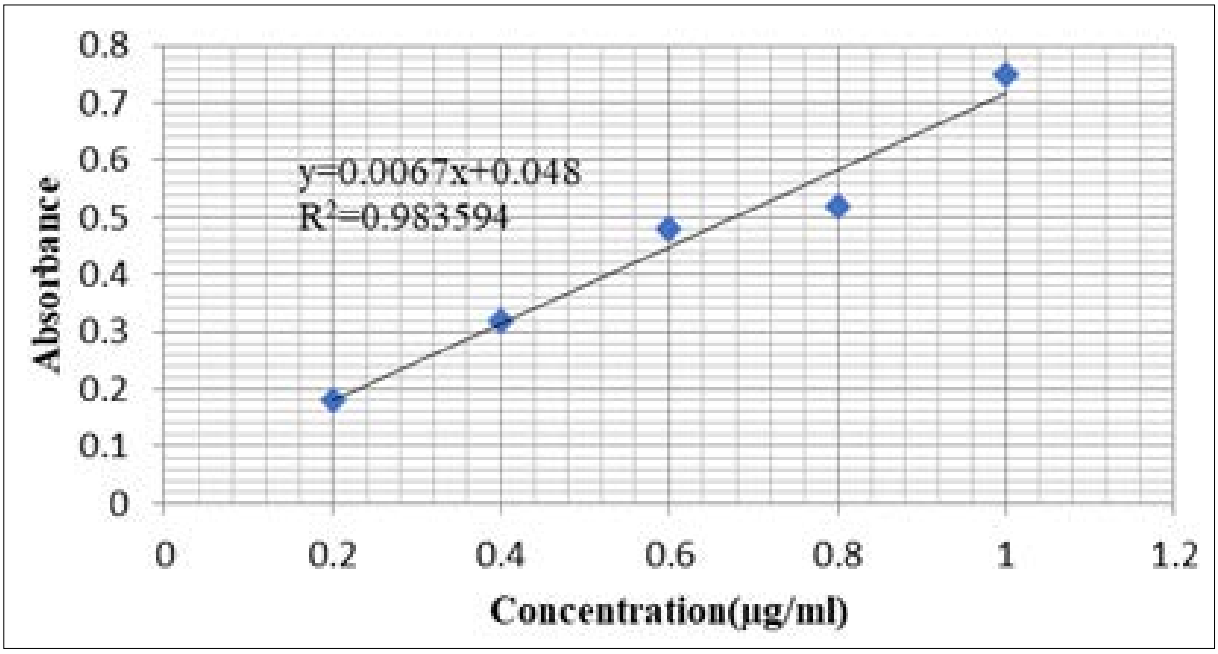

Fig 4: Standard curve for quercetin

Alkaloid was determined using Harborne, 1973 method [14]. Alkaloid content was found to be 57.15 ± 0.39 mg/gm (Table 2). Saponin content was determined by method as described by Nahapetian and Bassiri, 1974 [15]. Percentage of saponin was found to be 17.0±0.34% (Table 2). Both saponins and alkaloids are particularly well known as anti-inflammatory agents [23, 24]. Presence of these bioactive components in the extract validates the traditional use of the fruit in treating inflammations.

Table 2: Total phenol, flavonoid, alkaloid and saponin contents of fruit extracts of *Crescentia cujete*

Total phenol	Total flavonoid	Alkaloid (mg/gm)	Saponin (%)
69.34 ± 0.42 mg/G GAE	86.72 + 0.15mg/G QE	57.15 ± 0.39	17.0+0.34

Values are presented as mean ± S.D. (N=3), S.D.: Standard deviation

Conclusion

The fruit of *Crescentia cujete* L has wide range of medicinal uses. Most common is for the treatment of respiratory diseases, intestinal disorders and skin problems. The present study aimed at screening the biologically active molecules present in fruit of *Crescentia cujete*. The predominant phytochemicals detected were flavonoids, phenols, saponin and alkaloids. The biological activities of these phytochemicals can be correlated with the medicinal uses of the fruit. Further identification and scientific justification of

the medicinal use of the bioactive components is required for validating the traditional medicinal use of the fruit.

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