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# Biochemical Characterization, Antioxidant and Cytotoxic Activity of Annona muricata L. Accessions Fruit

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***Abstract:*** ***Annona muricata L. is one of the important natural medicine yielding tree. Collection and Characterization of gremplasms for its active molecules variation and identify the elight germplasm is mandatory to help tree improvement programme and pharmaceutical industry. Survey has been carried out in different zones of Tamilnadu especially high rain fall zone like Kaniyakumari and Pondicherry, Southern zone Tirunelveli, Western zone Coimbatore. Nearly twenty accessions of A. muricata propagules and fruits were identified to establish field gene bank. Morphological variation and bio-chemical characteristics were measured and used to analyze to find out the superior accessions in this study their diversity remains under-explored for traits related to fruit quality. The investment in screening traits such as phytochemical compounds and antioxidant content and cytotoxic activity is very important to support fruit quality breeding efforts. Thus, the objective of this study was accessing the variations in content of fruit and antioxidant activity and cytotoxic activity (in vitro) of top five ranked A.muricata accessions. The results evidenced that M1 and M2 accessions showed highest stigmast-5-en-3-ol, Squalene and hexadeconoic acid methyl ester and acetogenin and ratio. Accessions M3, M4 and M5 showed highest concentration of polyphenolic components. In vitro antioxidant assays suggest the M1 from Kanyakumari with highest antioxidant activity. The results highlighted accessions that can be exploited in A.muricata breeding programs and can be conserved for long term benefit.***
***Keywords: Annona muricata, cytotoxic activity, antioxidant activity, conservation***

## I. INTRODUCTION

*Annona muricata* Linn is called as soursop, because of its sour an acidic nature of the fruit pulp, this fruit also known as Graviola. All parts of *Annona* are used in natural medicine in the tropics. Human benefits of this tree have been accounted to their specific phytochemical composition mainly the annonaceous acetogenins. The leaf, bark, roots and edible fruit has reported to contain various therapeutic components which are responsible for anti cancerous, anti diabetic, anti microbial and insecticidal activities. It is considered to be good source of natural antioxidants for various diseases. Therefore, attention in recent times has been focused on the isolation, characterization and utilization of natural antioxidants and conservation these valuable germplasms. The active metabolites of these medicinal plants play crucial role in drug discovery. In spite of these studies, a unique tree species needs through scientific exploration for its variation and components and our knowing is equivalently inadequate in connection with their valuable role in nature. Hence, the accomplishment of a logical interpretation of natural products demands indepth exploration on the pharmalogical activities of these plants and their unique active molecules [1].

*A. muricata* is a unique multipurpose tree which has medicinal properties and have enormous welfare for human being because of its active molecules . The medicinal benefits of the Annonaceae family was known from ancient period. Various minerals such as Mg, Cu, Na, Fe, K, Ca and Na makes this fruit a beneficial one [2]. More than 100 annonaceous acetogenins have been isolated from various parts of *A. muricata*. Annonaceous acetogenins, lactones and isoquinoline, alkaloids, phenolics, cyclopeptides, tannins, and coumarins are few important active compounds exisiting in the *A. muricata* leaves.

*Annona muricata* diversity remains under-explored for its valuable traits related to biochemical quality, interms of agronomic performances and in terms of yield and adaptation to biotic and abiotic stress. Even then, the forest genetic resources conservation and tree breeding programs have slowly been expanded to meet the new requirements specifically linked to consumer preferences, such as improved biochemical content and valuable traits mainly antioxidant content [3].

Antioxidants present in fruits and vegetables and its utilization has been linked with many health benefits in consequence to its fundamental role in prevent oxidative stress and chronic diseases caused by free radicals, which has been identified as a major causative factor in the development and progression of several life-threatening diseases [4]. *Annona muricata* are a good source of antioxidants including widespread compounds, such as acetogenins, lactones and isoquinoline, alkaloids, phenolics, cyclopeptides, tannins, and coumarins. In this study, the valuable traits, phytochemicals, and antioxidant activities and cytotoxic activities in a set of 5 accessions of *Annona muricata* from different zones were evaluated. The aim of this study was to reveal the biochemical variation to provide a superior germplasm which are valuable as other trees/crops.

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## II. MATERIAL AND METHODS

### *A. Plant material*

Survey has been carried out in different zones of Tamilnadu especially high rain fall zone like Kaniyakumari and Pondicherry, Southern zone Tirunelveli, Western zone coimbatore. Nearly twenty accessions of *A. muricata* propagules and fruits were identified from the forest area stretch to for germplasm collection and conservation. The fruits were washed with sterile water and wiped with ethanol. The pulp of the fruit is separated and allowed to dry at 45° C in BOD incubator for 3 days. The dried fruit pulp was grinded into fine powder and stored in air tight container.

### *B. Preparation of fruit extract*

The extraction of *A. muricata* fruit was done by soxhlet extraction method. 30g of powdered fruit were weighed and suspended in 300 mL of methanol and allowed to run for 48 hrs. The temperature was maintained at 50˚C. The fruit extract obtained was collected in a conical flask and stored in refrigerator for periodical analyses.

### *C. Quantitative Phytochemical Estimation* [5]

*1) Test for Phenol*

*a) Ferric chloride Test:* To 1 mL of extract, few drops of methanol and few drops of ferric chloride was added. The tubes are shaken well, the appearance of bluish black color confirms the presence of phenol.

*2) Test for Tannin*

*a) Braemar's Test:* To 1 mL of extract, few drops of 0.1% ferric chloride was added and mixed, the appearance of brownish green color indicates the presence of tannin.

*3) Test for Flavonoid*

*a) Alkaline reagent Test:* To 1 mL of extract few drops of sodium hydroxide was added to give intense yellow color. The presence of flavonoid is detected by the disappearance of yellow color soon after the addition of diluted hydrochloric acid.

*4) Test for Quinine:* To 1 mL of extract few drops of concentrated hydrochloric acid was added, appearance of green color indicates the presence of quinine.

*5) Test for Glycoside:* To 1 mL of extract 2mL of glacial acetic acid and one drop of ferric chloride was added, followed by 1mL of concentrated sulphuric acid was added and mixed well, appearance of brown color indicates the presence of glycosides.

*6) Test for Steroid:* 1 mL of extract was mixed well with a few drops of chloroform. A drop of acetic acid was added and the mixture was heated for few minutes after which few drops of concentrated sulphuric acid were added. Appearance of orange color confirms the presence of steroids.

*7) Test for Carbohydrate*

*a) Fehling's Test:* To 1 mL of extract few drops of Fehling's reagent was added and boiled for few minutes. Appearance of brown color indicates the presence of carbohydrate.

*8) Test for Alkaloid*

*a) Hager's Test:* 1 mL of extract was treated with few drops of hager's reagent. Yellow precipitation indicates the presence of alkaloids.

*9) Test for Terpenoid*

*a) Salkowski Test:* To 1 mL of extract 2 mL of chloroform and few drops of concentrated sulphuric acid was added along the walls of the test tube. Red color ring appears which confirms the presence of terpenoids.

*10) Test for Saponin*

*a) Foam Test:* To 1 mL of the extract add 1 ml of distilled water and shake vigorously. The appearance of the foam confirms the presence of saponins.

### *D. Total Protein Content*

The Total protein content was estimated by using Lowry's method. The phenolic group of tyrosine and tryptophan residues in a protein will produce a blue color complex with Folin's-Ciocalteau reagent. Different concentration (10, 20, 30, 40, 50 µg/µL) of standard BSA was prepared along with the blank. The volume was make up to 5 mL. 5 mL of alkaline copper solution was added and incubated at room temperature for 10 minutes. 0.5 mL of Folin's-Ciocaltaeu reagent was also added and kept in incubation for 30 minutes at dark condition. The absorbance was read at 660 nm using UV /Visible spectrophotometer. The total protein content was determined using the standard curve and expressed in terms of µg/µL of Bovine serum albumin equivalents.

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*E. Total Phenolic Content*

Total phenol content was estimated by using Folin's-Ciocalteau method [6]. Phenols react with phosphomolybdic acid present in Folin's-Ciocalteu reagent in alkaline medium to produce blue color compound**.**

Different concentration (2, 4, 6, 8, 10 µg/µL) of Standard (Gallic acid) was prepared along with the blank. The volume was make up to 1 mL. 1mL of 10% Folin's-Ciocalteau reagent was added and tubes were incubated for 5 minutes at room temperature, followed by the addition of 1mL of 20% sodium carbonate solution. After 30 minutes of incubation at room temperature, the absorbance was read at 695 nm using UV / Visible spectrophotometer. The total phenolic content was determined by using standard curve and expressed in terms of µg/µL of gallic acid equivalents.

*F. Total Tannin Content*

Total tannin content was estimated by using Folin's-Ciocaltaeu method [7]. Tannins reduce the phosphomolybdate present in Folin's-Ciocalteu reagent in alkaline solution to produce a blue color compound, the intensity of which is propotional to the amount of tannins present. The intensity is measured by spectrophotometer at 700 nm.

Different concentration (2, 4, 6, 8, 10 µg/µL) of Standard (Tannic acid) was prepared along with the blank. The volume was made up to 1 mL. 1 mL of 10% Folin's-Ciocalteau reagent was added, followed by the addition of 1mL of 20% sodium carbonate solution. After 30 minutes of incubation at room temperature, the absorbance was read at 700 nm using UV / Visible spectrophotometer. The total tannin content was determined by using standard curve and expressed in terms of µg/µL of tannic acid equivalents.

*G. Total Flavonoid Content*

Total flavonoid content was estimated by using Aluminium chloride colorimetric method [8]. Aluminium chloride forms acid stable complexes with the C-4 keto group either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminium chloride forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B- ring of Flavonoid.

Different concentration (10, 20, 30, 40, 50 µg/µL) of Standard (Quercetin) was prepared along with blank. The volume was made up to 1 mL. 0.3 mL of 5% sodium nitrite was added and incubated for 5 minutes followed by the addition of 0.3 mL of aluminium chloride and kept for 5 minutes at room temperature. 2 mL of 1% NAOH was added and incubated for about 10 minutes. The absorbance was read at 510 nm using Ultraviolet (UV) / Visible spectrophotometer. The total flavanoid content was determined by using standard curve and expressed in terms of µg/ µL of quercetin equivalents.

## III.SPECTRAL STUDIES

*A. FT-IR*

FT-IR is perhaps the most powerful tool for identifying types of chemical bonds (functional groups). The wavelength of light absorbed is characteristic of the chemical bond as can be seen in this annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. For the FT-IR study dried powder of methanolic extract 10 mg of each plant material was encapsulated in 100 mg of KBr (Potassium bromide) pellet, in order to prepare translucent sample discs. The powdered sample of plant specimens were treated for FTIR spectroscopy (Shimadzu, IR Affinity 1, Japan). Scan range: from 400 to 4000 cm−1 with a resolution of 4 cm−1.

*B. Chromatographic studies*

*Column chromatography*

Column chromatography was performed to isolate the compounds responsible for the antioxidant property present in the fruit extract by slurry method.

The silica gel of 100 to 200 mesh size was taken as stationary phase and methanol as mobile phase. 1ml of sample was loaded on to the column tube and the eluting components were collected in a test tubes (60 fractions are obtained each 1ml). This process was continued until the separation of all desired compounds present in the sample. Phosphomolybdenum assay was performed to all fractions and the intensity was read at 695 nm.

The fraction having maximum intensity and optical density was subjected to GC-MS analysis in order to find out the compounds responsible for the antioxidant property.

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*C. Gas Chromatography- Mass Spectrum Analysis (GC-MS)*

GC-MS technique was used in this study to identify the phytocomponents present in the *Annona muricata* fruit extract. GC-MS analysis of this extract was performed using GC SHIMADZU QP2010 system and gas chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with Elite-1 fused silica capillary column of 30 m length, 0.25mm diameter and 0.25 µm thickness and composed of 100% Dimethyl poly siloxane. For GC-MS detection, an electron ionization energy system with ionization energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1.51ml/min and an injection volume of 2µl was employed. Injector temperature was 200 °C and ion source temperature was 200 °C.

The oven temperature was programmed from 70 °C (isothermal for 2 min.), with an increase of 300 °C for 10 min. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds with scan range of 40 – 1000 m/z. Total GC running time was 35 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a GC MS solution ver. 2.53.

*D. Identification of Components*

Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technique (NIST08s) and WILEY8 having more than 62000 spectral patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST08s, WILEY8 library. The Name, Molecular weight, Molecular formula and Structure of the component of the test material was ascertained.

*E. In-vitro antioxidant assay*

Evaluation of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity

DPPH radical scavenging activity was determined by using [9]. Antioxidants react with DPPH which is a stable free radical and reduce it to DPPH-H and as consequence the absorbance decreased from the DPPH radical to the DPPH-H form. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at 517 nm. The degree of discoloration indicates the scavenging potential of the antioxidant compounds in terms of hydrogen donating ability.

Different concentrations (2, 4, 6, 8, 10 µg/µL) of fruit extract was prepared along with the control. The volume was make up to 1 mL. 2.0 mL of 0.1 mM DPPH solution was added. After 30 minutes of incubation in dark condition the absorbance was read at 517 nm using Ultraviolet (UV) visible spectrophotometer. Ascorbic acid was used as standard. Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation.

$$\% \text{ of DPPH scavenged} = [(A0 - A1)/A1] \times 100$$

( A0 - Absorbance of control; A1 - Absorbance of sample).

*F. In-vitro Anticancer Assay*

2-(4, 4-dimethyl-2-tetrazoyl)-2, 5-diphenyl-2, 4-tetrazolium salt (MTT) Assay

The *in-vitro* anticancer potential was determined by using MTT dye reduction assay. The 2-(4, 4-dimethyl-2-tetrazoyl)-2, 5-diphenyl-2, 4-tetrazolium salt (MTT) is converted into its formazan derivative by live cells and the amount of formazan formed is a measure of number of viable cells. The farmazan formed is then solubilized with suitable solvent and the cell viability is measured in a microtitre plate reader.

He La cell lines were procured from National Centre for Cell Science, research institute (Pune). 100 µL of treated cells were incubated with 50 µL of MTT at 37° C for 3 hours. After incubation, 200 µL of PBS was added to all the samples and aspirated carefully to remove excess MTT. 200 µL of acid-propanol was added and left overnight in the dark period for solubilization. The absorbance was read at 650 nm in a microtitre plate reader (Bio RAD U.S.A.). Doxorubicin was used as standard. The optical density of the control cells were fixed to be 100% viable and the percent viability of the cells in the other treatment groups were calculated using the formula

$$\% \text{ viability} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

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## IV. RESULTS AND DISCUSSION

Phytochemical compounds content in fruits of Annona muticata accessions are presented in Table 1 and Table 2. Analysis of variance showed a significant effect (P<0.05) among the five A.muricata accessions for most traits, except for titratable acidity (TA) and pH. These results reveal accessions with distinguished values for the development of A.muricata cultivars with highphytochemical fruit quality. Several studies have also indicated a wide variability in fruits for fruit quality traits and bioactive [10].

The phenolic content in the tree are associated with their antioxidant activities [11]. Almost certainly because of redox properties, this makes those molecules to act as reducing agents, hydrogen donors and singlet oxygen quenchers [12]. These kind of high total phenolics content in methanolic extract of *A.muricata* fruit establish its active molecules and the free radical scavenging ability.

Table 1: Qualitative analysis of Methanolic Fruit extract of a. Muricata

| Phytochemicals | Fruit | Leaf |
|---|---|---|
| Phenol | + | + |
| Tannins | + | + |
| Flavonoids | + + | + |
| Quinine | + | + |
| Glycoside | + + + | + |
| Steroids | – | + |
| Carbohydrates | – | + |
| Alkaloids | + | + |
| Terpenoids | + | + |
| Saponins | + + | - |

High level = (+++) Moderate = (++) Low level = (+) Absence = (-)

Table 2 biochemical compounds from *a.muricata* accessions.

| Accessions | pH | Tannin (mg TAE/g extract 100 $g^{-1}$) | Total phenolic content (mg GAE 100 $g^{-1}$) | Total flavonoid content (mg QE 100 $g^{-1}$) |
|---|---|---|---|---|
| M1 | 4.64 a | 109.12 a | 430 a | 208 a |
| M2 | 4.60 a | 47.54 cd | 270 bc | 201 a |
| M3 | 4.50 a | 88.22 ab | 410 a | 160 b |
| M4 | 4.60 a | 33.45 d | 292 b | 199 a |
| M5 | 4.62 a | 61.23 bc | 198 c | 200 a |
| CV (%) | 2.10 | 9.62 | 5.12 | 5.12 |

GAE *gallic acid equivalents*, TAE *tannic acid equivalents*, QE *Quercetin equivalents*

[a–d]Values (mean ± standard deviation, n = 3) in the same column with different superscripted letters are significantly different ($p < 0.05$)

Many tannin components were suggested to be anti-carcinogenic and have been shown to reduce the mutagenic activity of a number of mutagens. Numbers of carcinogens and/or mutagens produce oxygen free radicals for interaction with cellular macromolecules [13]. The anti-carcinogenic and anti-mutagenic potentials of tannins may be related to their anti-oxidative properties, which are important in protecting against cellular oxidative damage [14]. The large amount of tannins in the fruit of *A. muricata* could also inhibit free radicals and diseases associated with them.

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The results revealed higher amount of flavonoids in accessions (208 mg QE 100 $g^{-1}$**).** Flavonoids are the most common and widely distributed group of plant phenolic compound, which usually are very effective antioxidant because of the scavenging ability conferred by their hydroxyl group [15]. These compounds from trees are known to be good natural antioxidant [16]. The flavonoids in A.muricata fruits suggest that it may possess great antioxidant potential with significant biological activities.

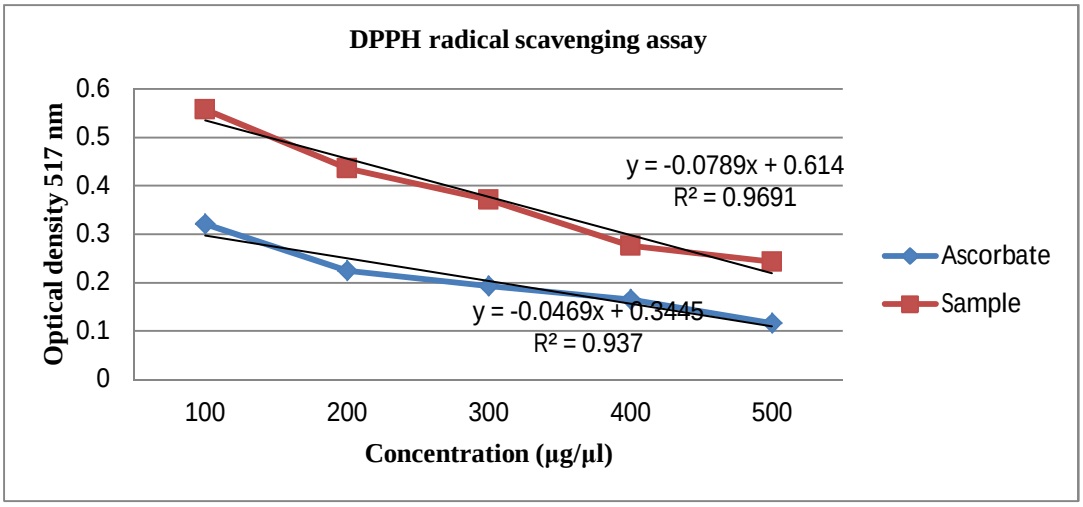
*A. In vitro Antioxidant Assays*

*1) DPPH scavenging activity:* The radical scavenging activity of different extracts was determined by using DPPH assay [17]. The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517nm. Ascorbic acid was used as standard. In this assay, the different concentrations of methanolic fruit extract of Annona muricata (20, 40, 60, 80 and 100 µg/µL) showed antioxidant activity in a dose-dependent manner (Table 3). Methanolic extract of A. muricata showed significant scavenging effect when compared with that of Standard (Ascorbic acid). The IC50 values were calculated by using linear regression curve which was found to be 92.421 and 33.138 µg/µL for A. muricata and standard respectively (Graph 1). Similar results were seen in Ethanolic bark extract of Annona muricata [18]. Ethanolic extract of A. muricata shows DPPH radical scavenging ability of the soluble constituents present in it. The IC50 values were found to be 109µg/ml and 3.5µg/ml for ethanolic bark extract of Annona muricata and Gallic acid respectively.

Table 3. % inhibition of dpph radical scavenging activity of *a. muricata* fruit and standard (Ascorbic Acid)

| Concentration (µg/µL) | Standard(Ascorbate) % inhibition | Sample %inhibition |
|---|---|---|
| 20 | 14.4 | 33.9 |
| 40 | 40.2 | 48.3 |
| 60 | 48.5 | 55.9 |
| 80 | 56.0 | 67.2 |
| 100 | 69.0 | 71.2 |

Graph 1. DPPH radical scavenging assay of *a. Muricata* fruit and standard (ASCORBIC ACID)




*B. In-vitro Anticancer Assay of methanolic fruit extract of A. muricata*

2-(4, 4-dimethyl-2-tetrazoyl)-2, 5-diphenyl-2, 4-tetrazolium salt (MTT) Assay

The test for *in-vitro* anticancer assay revealed the anticancer potential of methanolic fruit extract of *A. muricata*. The fruit extract showed severe cytotoxic reactivity to HeLa cells (cervical cancer cell line) after 24 hours contact. The % of cytotoxicity was decreased (94.6, 91.9, 87.8, 84.3, 81.2%) with increase in concentration of fruit extract (5, 25, 50, 75 and 100 µg/mL) (Table 8). The control (Untreated cells) shows no cytotoxic reactivity as expected. The graph showed the dose dependent response of the cells to the extract (Graph 2).

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Graph 2. % of cell viability of HeLa cell line treated with *A. muricata* fruit.

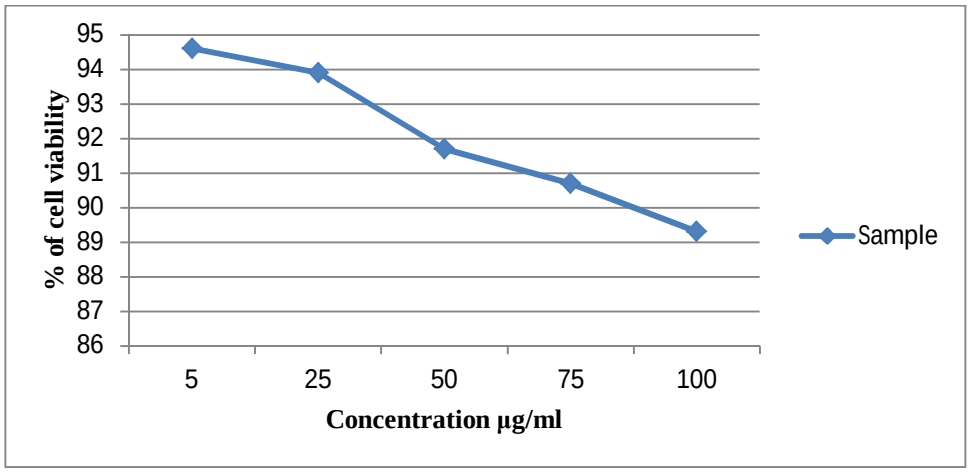



Fig 1. FT-IR analysis of methanolic fruit extract of *A. muricata*

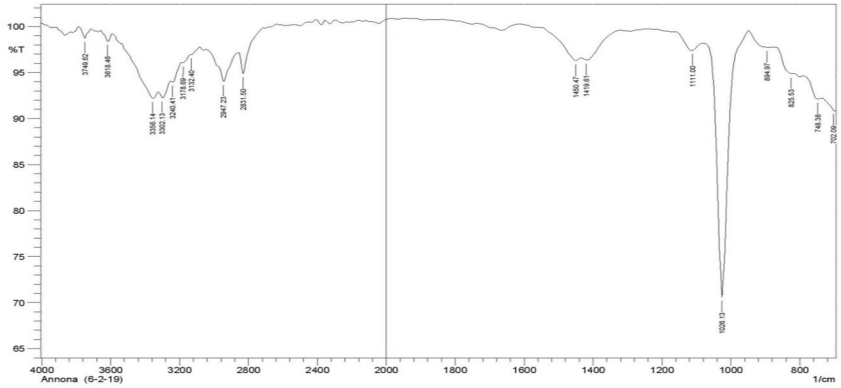
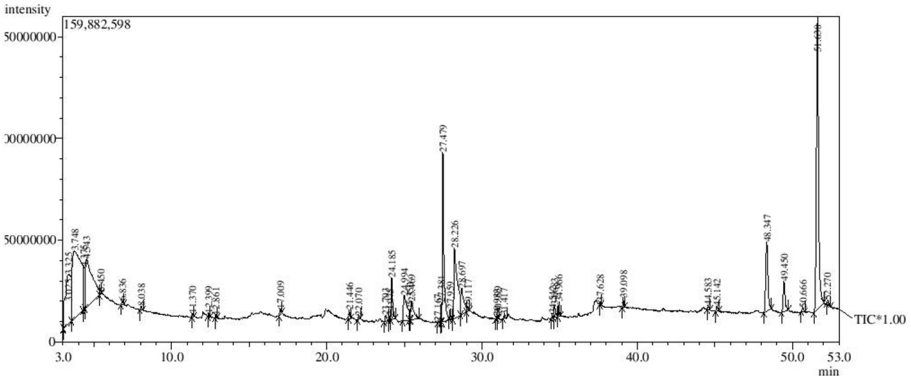



Fig 2. GC-MS analysis of methanolic fruit extract of *A. muricata*




Anticancer activity by MTT assay for the evaluation of cytotoxic activity of ethanolic leaves extracts of *Annona muricata* against two human breast cancer cell lines MDA and SKBR3 was reported similar to our result [19]. The IC50 values are obtained by plotting graph between concentrations of drug vs. % cell viability. It is found that IC50 values of MDA cell line by MTT assay is 248.77 µg/ml and IC50 values for SKBR3 cell line is 202.33. Cytotoxicity of the extract in MDA-MB435S, HaCaT and in WRL-68 cells by XTT assay was reported similar to this result [20]. Cells were treated with different concentrations of the extract and the respective IC50 values were found to be 52.4 µg (WRL-68), 29.2 µg (MDA-MB-435S) and 30.1 µg (HaCaT). The extract was found to destruct the cancer cells comparatively at lower doses than the normal cells. However, at the highest dose of 80 µg, the extract exhibited more or less similar cytotoxic effect on all the cell lines tested.

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## V. CONCLUSION

The accession of of *A. muricata* had high amounts of total phenolics, tannins and flavonoids. The crude extract and fractions demonstrated high in vitro antioxidant and antibacterial activities. These results explain the extensive bioactivity of *A.muricata* fruit. The higher antioxidant and cytotoxic activity of methanolic extract and its fractions may be due to the presence of active principles such as acetogenins, lactones and isoquinoline, alkaloids, phenolics, cyclopeptides, tannins, and coumarins its analogues in greater concentration compared to other extracts. The accession M1 and M2 was rich with stigmast-5-en-3-ol, Squalene and hexadeconoic acid methyl ester and acetogenin *comparing other accessions* fruit. *In vitro* antioxidant assays suggest the M1 from Kanyakumari has highest antioxidant activity comparing others. The results highlighted accessions that can be exploited in A.muricata breeding programs and can be conserved for long term benefit. This study appeals the need for better characterization of phytochemicals present in the wild fruits which would increase their commercial value. In conclusion, the antioxidant properties and phytochemical investigation of the *A. muricata* fruits are expected to increase the use of this fruits in pharmaceutical industry.

## VI. ACKNOWLEDGMENT

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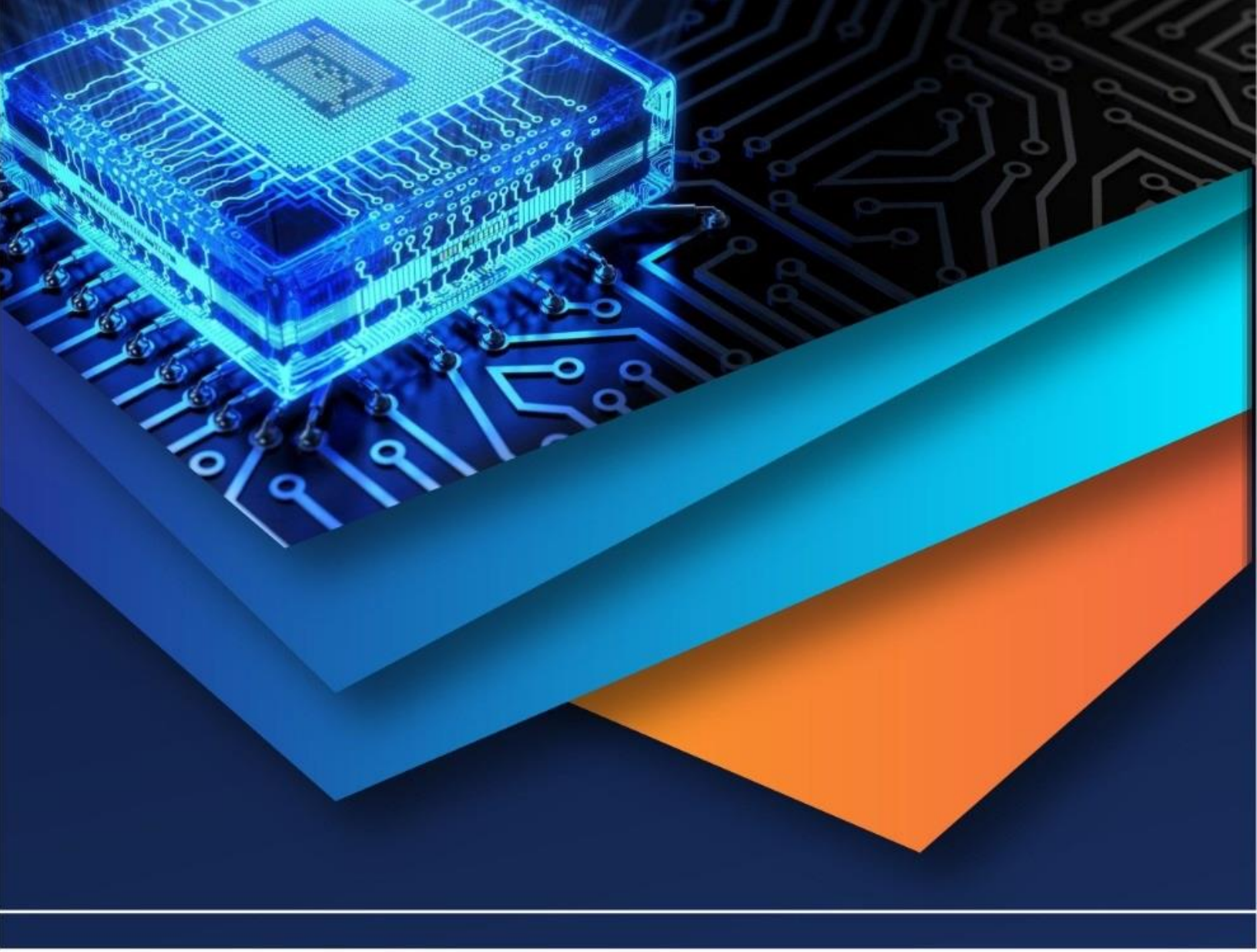

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