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Research Article

Evaluating the Anticancer Potentials of Methanol Extracted *Annona muricata* Fruit Pulp and Seed(s) Phytochemicals

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ABSTRACT

Annona muricata L. has been widely used in traditional medicine for the treatment of various diseases ranging from fever to cancer. In this study, we evaluate the *in vitro* anticancer potential of methanol extracted *A. muricata* fruit pulp (AMPM) and seeds (AMSM) phytochemicals against breast (MCF-7), cervical (HeLa), prostate (PC-3) and colorectal (HCT-116) cancer cell lines. Additionally, the *in vitro* anti-inflammatory and antioxidant activities of the extracts have been carried. The findings suggest that the AMSM is the most potent among the either extracts. Notwithstanding, both AMPM and AMSM showed significant dose and cell line-dependent anticancer potential(s).

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Introduction

Cancer, characterized by an uneven cell replication and dysregulated cellular programming, has been regarded a catastrophic biological event occurring in the higher multicellular organisms. What begins as an abnormal cell proliferation, eventually amounts to the development of a tumor with competencies to strike locally or migrate to the far-off vital organ systems within the body. Such a growth is ensued by a genetic drift inducing a sense of cellular heterogeneity, eventually resulting in antigenicity, metastatic potential known for the invasiveness, higher differentiation and proliferation capacities. Over a hundred varieties of this disorder have been identified based on the type of primary cell/tissue affected. Such types include breast adenocarcinoma, cervical cancer, melanoma, leukaemia, pulmonary carcinoma and prostate cancer to name a few [1-7]. Notwithstanding, any type of cancer is now regarded a serious medical condition, thereby implying that their treatments are of utmost clinical importance [8].

In the present scenario, the challenge that is faced worldwide is the increasing resistance by tumors to the prevailing therapeutic candidates. Directing the researchers to work tirelessly to identify potent anticancer agents which have a high sensitivity to such rogue cells, thereby acting locally. Phytocompounds have been considered the best prospects in this regard, adding more value to research focussed on mitigating cancer with their use. Endorsing this, any such effort is now cumulatively called cancer phytotherapy [9, 10]. Over the years, as many as thirty-five thousand plants have been evaluated for their probable tumoricidal potential by the National Cancer Institute (NCI), USA, alone. Plants like *Abrus precatorius* (Rosary Pea), *Albizia lebbek* (Woman's tongue), *Alstonia scholaris* (Devil Tree), *Anacardium occidentale* (Cashew nut), *Asparagus racemosus* (Indian Asparagus), *Boswellia serrata* (Indian Frankincense), *Erythrina suberosa* (Coral tree), *Euphorbia hirta* (Snakeweed), *Gynandropsis pentaphylla* (Wild Spider flower), *Nigella sativa* (Black cumin), *Paederia foetida* (Skunk Vine), *Picrorhiza kurroa* (Hellebore), *Withania somnifera* (Winter Cherry), *Annona muricata*

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(Soursop), to name a few, have been found to be of a great scientific interest [11-24].

Annona muricata (Linn.), belongs to the family *Annonaceae* of plant kingdom. Commonly called Soursop/Graviola, this tropical plant variety has been extensively studied for its therapeutic significance. Having said that, the use of *A. muricata* as a traditional medicine has been recorded in detail, apart from the botanical aspects [10]. The plant metabolites have been reported to possess effective curative properties, even against cancer cells [25]. Nearly 212 phytochemicals have been reported to be present in the *A. muricata* plant [10, 24]. Alkaloids, phenolic acids, cyclopeptides, flavonol triglycosides, cyclopeptides, megastigmanes and essential oils constitute a major portion of the phytochemical composition. Meanwhile, the essential nutrients calcium, sodium, iron, potassium, copper and magnesium are found in adequate quantities [26]. In addition, a special class of compounds called the annonaceous acetogenins are reported to be present in majority. Annonaceous acetogenins, called so due to their unique presence in only the plants belonging to *Annonaceae* family, are rendered responsible for the significant biological activities of the plant. Also, the *A. muricata* alkaloids and phenolics are believed value additions with regard to the medicinal significance [10].

Use of *A. muricata* plant and plant organs in ethnomedicinal practices has been widely reported. Literature suggests that all organs of *A. muricata* plant, viz. leaves, fruits, barks, roots, and seeds have been extensively used in preparation of traditional medicines to treat a range of diseases from fever to cancer [10, 24, 27]. Notwithstanding, the numerous traditional uses of *A. muricata* yet remain undocumented, thereby shedding little light on its medicinal benefits [28, 29]. The validation of these biomedical significances of *A. muricata* have been carried out since over eight decades now and substantial evidences ascertain their use in natural medicine. Decoctions of the plant organ phytochemicals have been reported to be widely used as cure for various diseases and disorders [10]. For instance, the *A. muricata* leaf decoction

was reportedly used as an analgesic as well as comforting agent in the event of cold, flu, asthma and malaria, while the fruit juice was consumed to promote lactation, to ease the discomforts arising from diarrhoea, cardiovascular and hepatic disorders, and against intestinal parasites [10, 24, 30].

Furthermore, *A. muricata* has been found to be extensively used to cure torment, respiratory and skin diseases, bacterial infections, hypertension, aggravation, inflammation, neuralgia, rheumatism, cystitis, diabetes and even cancer. In addition, records suggest *A. muricata* was used as a sedative, nervine, relaxant, and astringent [31]. Recent reports highlight the use of *A. muricata* capsules, concentrates and even the extracts of phytochemicals towards treatment of the major types of cancer [32-35]. However, limited insights are available with regard to the medicinal values of the plant's edible component, viz. the fruit, which is regularly consumed as a refreshment [10]. The current study focuses on evaluating the *in vitro* antioxidant, anti-inflammatory and anticancer potentials of the methanol extracts of *A. muricata* fruit pulp (AMPM) and seeds (AMSM).

Methods

I Plant Sampling: Procurement and Preparation of *A. muricata* Fruit and Seed Extracts

A. muricata fruits were procured from an organic farm at Kyathanahalli village in Mandya district, Karnataka, India (Geographical Coordinates: 12.46°N, 76.65°E). Obtained fruits were identified and authenticated at the Agricultural Technology Information Center (ATIC), Indian Council of Agricultural Research – Indian Institute of Horticulture (ICAR-IIHR), Bangalore, India (Annexure I). The fruits were washed at the laboratory using tap water and hand separated into the epicarp, pulp and seeds. The epicarp was discarded while the fruit pulp and seeds were utilized for extraction (Figure 1).

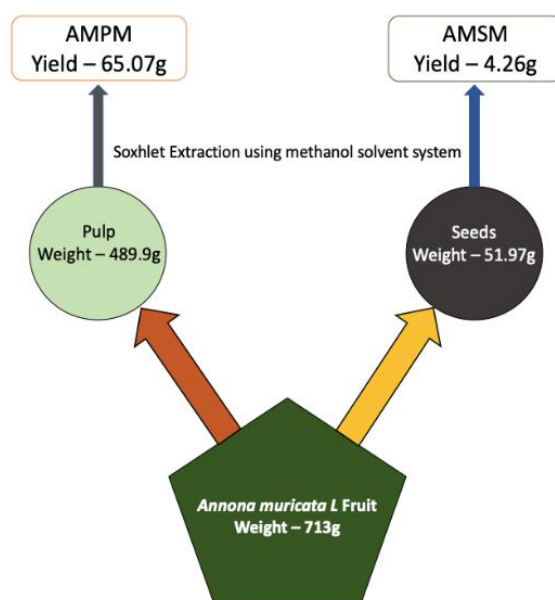


Figure 1: Schematic Representation of Phytochemical Extraction from *A. muricata* Pulp and Seeds, using methanol solvent system.

i Methanol Extract of *A. muricata* Fruit Pulp

60g of fruit pulp was subjected to Soxhlet extraction with 300ml methanol, according to Redfern *et al.* (2014), at 50°C [36]. After 4 hours, the mixture was cooled before filtering through the Whatman filter paper No.1 and dried at 40°C using a rotary evaporator. The concentrated filtrate obtained was labeled AMPM and stored at -20°C.

ii Methanol Extract of *A. muricata* Seeds

20g of dried seed powder was dissolved in 100ml methanol and extracted at 50°C for 4h using a Soxhlet apparatus. The mixture was cooled before filtering through the Whatman filter paper and dried at 40°C using a rotary evaporator. The obtained semi solid concentrate, was labeled as AMSM and stored at -20°C.

II Antioxidant and Anti-Inflammatory Activity Assay(s)

i Determination of Ferric Reducing Antioxidant Power (FRAP)

FRAP assay was performed experimentally according to Benzie and Strain (1999), where FRAP reagent, i.e. 2.5ml of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) (10mM) (Heated at 50°C for 5 minutes), 2.5ml ferric chloride (FeCl₃) (20mM) and 25ml of acetate buffer (pH 3.6) (300mM), was freshly prepared and warmed at 37°C just before use [37]. A volume of 190µl FRAP reagent, added alongside AMPM and AMSM derivatives with increasing concentrations of 10, 20, 40, 80, 160 and 320µg/ml, as well as Ferrous sulphate (standard: 200-1800µM) was incubated in dark for 30 minutes. The absorbance at 593nm and converted to FRAP units, viz. equivalent amount of ferrous sulphate, using the calibration curve.

ii Estimation of DPPH Radical Scavenging Activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity was evaluated as described by Aksoy *et al.* (2013) [38]. Experimentally, 140µl of DPPH solution (6.2mg in 100ml of absolute alcohol) was incubated with 20.0µl of increasing concentrations (10, 20, 40, 80, 160, and 320µg/ml) of AMPM and AMSM derivatives in dark for 30 minutes at room temperature and optical density was measured at 536nm. The dark purple coloured reaction mixture turned colourless in the presence of free radical scavenging activity. The calibration curve was constructed using vitamin C standard of increasing concentrations (0.25 – 4µM). % free radical scavenging potential was determined using the formula:

$$\text{Percentage free radical scavenging activity} = [A_0 - A_1/A_0] \times 100$$

Where,

A₀= Absorbance of DPPH incubated with solvent

A₁= Absorbance of DPPH incubated with increasing concentration of Vitamin C/Test samples

iii Determination of Anti-Inflammatory Property by RBC Membrane Stabilization

RBC membrane stabilization assay was performed according to Anosike *et al.* (2018) [39]. Experimentally, human RBC's were pelleted by centrifuging 5.0ml of blood, collected from the blood bank of JSS Medical College and Hospital, JSSAHER, Mysore (IEC No. ECR/387/Inst/KA/2013/RR-19), at 2000rpm at 4°C for 5 minutes. The RBC pellet was washed twice with 5ml of iso-saline (0.9% NaCl) and

resuspended in iso-saline to produce a 10% RBC suspension. Next, 500µl of 10% RBC suspension was mixed with the test samples, 10 – 320µg/ml concentration of AMPM and AMSM, and 1 ml of phosphate buffer (0.15M, pH 7.4). This reaction mixture was incubated at 37°C for 1 hour. The samples were then centrifuged at 2000rpm for 5 minutes to collect the supernatant and the absorbance read at 560nm in a UV-visible spectrophotometer. The RBC suspension incubated with distilled water was taken as a control for complete lysis, while aspirin (1mg/ml) served as positive control. The percentage RBC protection compared to water treated control was measured using the below formula.

$$\text{Percentage inhibition of RBC lysis} = [A_0 - A_1/A_0] \times 100$$

Where,

A₀= Absorbance of 10% RBC suspension incubated with Distilled water

A₁= Absorbance of 10% RBC suspension incubated with Test samples

III In vitro Anticancer Activity Assay

The anti-proliferative effects of AMPM and AMSM were determined, using the MTT assay, on breast cancer (MCF-7), cervical cancer (HeLa), prostate cancer (PC-3) and colorectal cancer (HCT-116) cell lines procured from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), containing 10% Fetal Bovine Serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin, in 5% CO₂, at 37°C, until confluent. The cells were trypsinized with 0.05% trypsin-EDTA solution for the hemocytometric cellular viability screening before 10,000 cells/well were plated and incubated in 5% CO₂ at 37°C, until confluent. The treatment was carried out at the AMPM and AMSM concentrations of 10, 20, 40, 80, 160 and 320µg/ml.

Measurement of % Inhibition Using MTT Assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed as previously described by Denizot and Lang (1986) to check for % inhibition [40]. Upon treatment for 24 h, the cells were fixed using 5mg/ml of MTT reagent per well, and incubated at 37°C for 1 h, then centrifuged for 5 minutes at 3000. Excess dye in the plates was washed using distilled water and kept for air drying. The so formed crystals were solubilized using 100µl of DMSO, to read the optical density at 570 nm. % inhibition was calculated based on the formula:

$$\% \text{ Inhibition} = [(OD \text{ of control} - OD \text{ of sample})/OD \text{ of control}] \times 100$$

The observations made were represented graphically and statistically using the Prism 8 statistical analysis tool (GraphPad Software, San Diego, CA, USA).

Results

I AMPM and AMSM Showed Dose-Dependent Antioxidant Activity with Moderate Anti-Inflammatory Activity

Antioxidant activity potential of AMPM and AMSM was determined using Ferric Reducing Antioxidant Power (FRAP) and DPPH free radical scavenging activity (Figure 2) assays. Experimentally, increasing concentrations AMPM and AMSM were incubated with DPPH/FRAP reagents as detailed in the methods section and the developed colour measured at 536nm and 593nm, respectively, using multimode plate reader. The data demonstrated a dose-dependent increase in antioxidant

activities of the extracts. Notwithstanding, AMPM exhibited limited free radical scavenging activity at concentrations < than 80µg/ml.

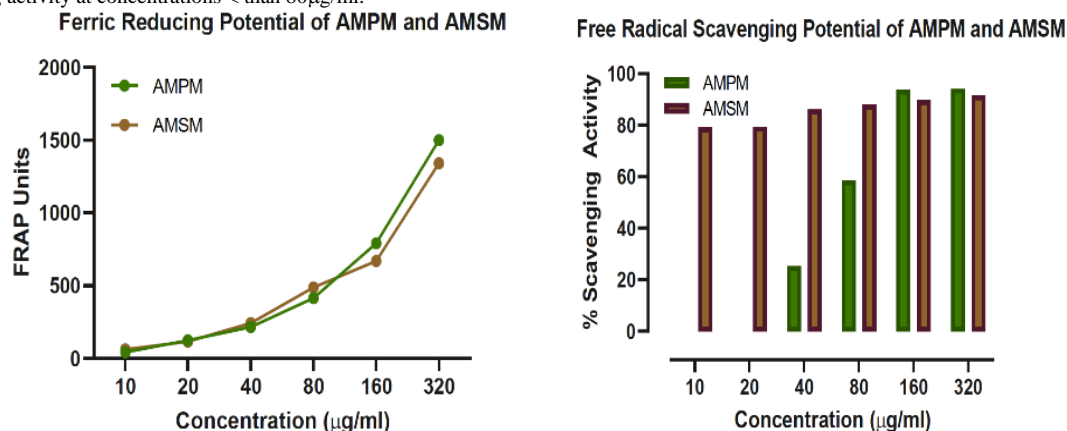


Figure 2: Graphical representations of Antioxidant potentials of AMPM and AMSM.

Antioxidants are known to exhibit potent RBC membrane stabilizing effect [41]. Since RBC membranes structurally resemble the membranes of lysosomes, it is important to study the effect of phytochemical extracts on RBC membrane stabilization. Experimentally, the anti-inflammatory effect was carried out by incubating increasing concentrations of AMPM and AMSM with the RBC followed by measuring the haemoglobin content in the medium (Figure 3). It was hypothesized that potent

antioxidants protect RBC from undergoing membrane damage. Analysis of the data showed that both AMPM and AMSM exhibited the moderate anti-inflammatory property. Nonetheless, all the test samples did demonstrate a dose-dependent anti-inflammatory activity, with the activity of AMPM in higher concentrations comparable to that of aspirin (100µg/ml) positive control.

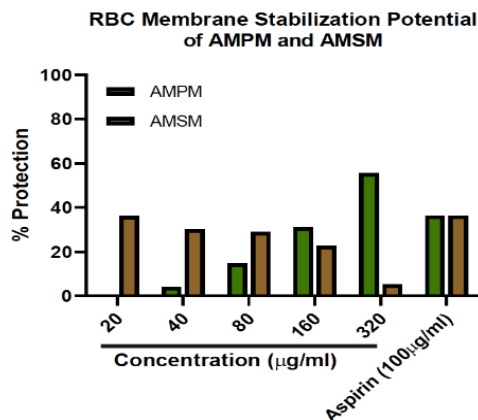


Figure 3: Graphical representation of Anti-inflammatory activity of AMPM and AMSM.

II AMSM Demonstrated a Higher Growth Inhibition of Cancer Cell Lines

Both the extracts showed dose-dependent antioxidant and anti-inflammatory properties, and their cytotoxic potential of *A. muricata* phytochemicals was determined using MTT assay. The *A. muricata* extracts, AMPM and AMSM, were treated on various cancer cell lines, MCF-7, HeLa, PC-3 and HCT-116, based on the total phytochemical yield or percentage (%) concentration of the extract, to check for potent tumoricidal properties. Each cell line(s) was treated for 24h with differential concentrations of the test samples ranging from 10µg/ml to 320µg/ml concentrations.

24h treatment of the AMPM as well as AMSM showed a dose-dependent cytotoxicity on all the cell lines. The AMPM extract was found to inhibit significantly all the cell lines, with a greater inhibition of the prostate and

colorectal cancer cells as compared to those of the breast and cervical cancers. The IC₅₀ values of AMPM treatment were 98.65 for MCF-7, 118.8 for HeLa, 103.7 for PC-3 and 122.5 for HCT-116 (Figure 4). Surprisingly, the IC₅₀ values for MCF-7 and HeLa remained significantly lower than the other two cell lines which were actually the most affected during the treatment. However, the growth inhibition potential of AMSM was notable in all the cell lines, with the breast and colorectal cancer cell lines being inhibited the most. Additionally, the seed extract significantly reduced the number of viable cervical cancer cell lines too. The IC₅₀ values for AMSM treatment were recorded to be 77.38, 82.94, 98.69 and 120.4 for MCF-7, HeLa, PC-3 and HCT-116 cell lines, respectively (Figure 5). Similar to the observation made above, the IC₅₀ value of AMSM for HCT-116 remained higher than the lesser inhibited cell lines, HeLa and PC-3.

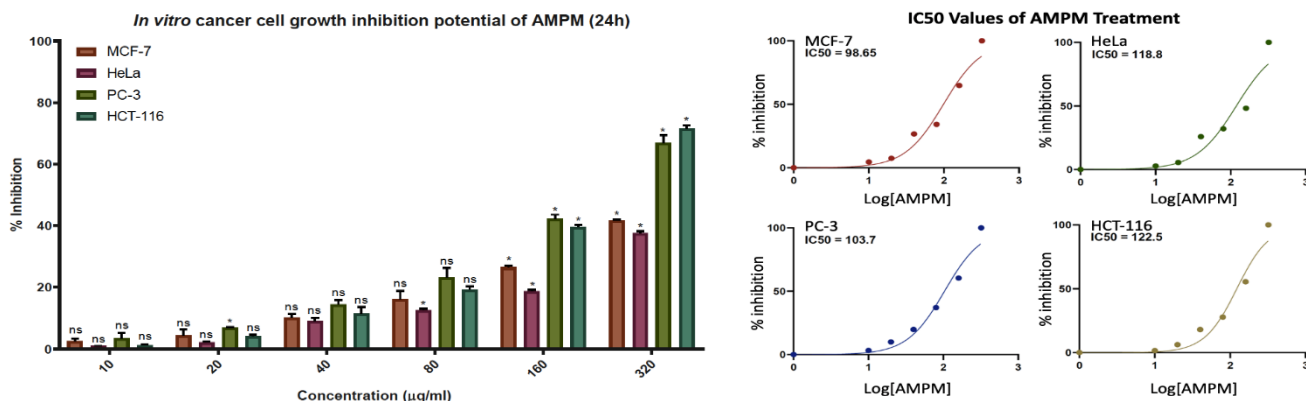


Figure 4: Graphical representation of *in vitro* anticancer activity and IC₅₀ values of AMPM treatment on MCF-7, HeLa, PC-3 and HCT-116 cell lines.

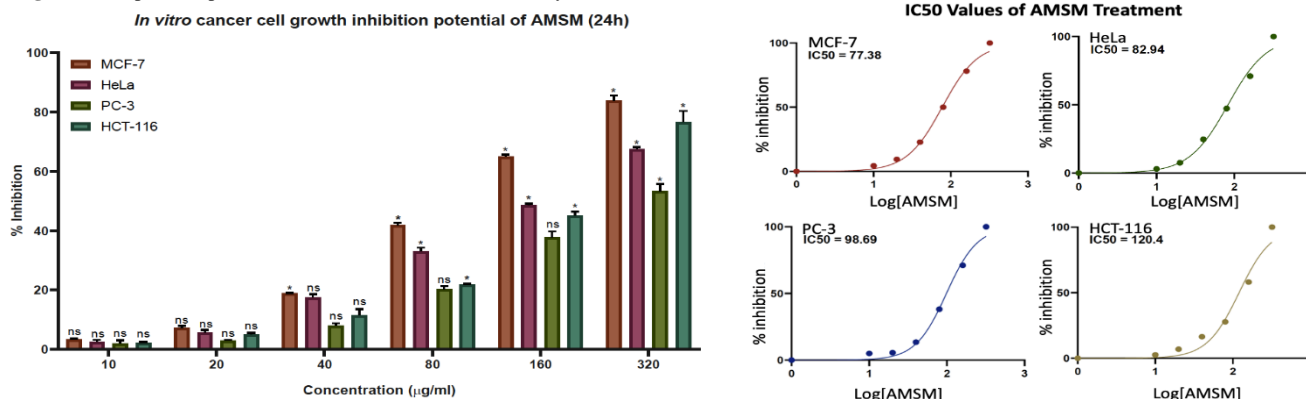


Figure 5: Graphical representation of *in vitro* anticancer activity and IC₅₀ values of AMSM treatment on MCF-7, HeLa, PC-3 and HCT-116 cell lines.

Discussion

Plant-based novel drug discovery has been one of the most preferred research topics since time unknown [42, 43]. Efforts to harness suitable anticancer candidates from natural sources have been occurring globally. With the advent of recent analytical and computational techniques, possibilities of processing complex natural products and establishment of the more accurate structure-activity relationships (SAR) has opened new avenues to derive novel anticancer agents [44]. *Annona muricata* (*L.*), a tropical fruit-bearing plant, is one such species used elaborately in ethnomedicinal practices as well as in the current researches focussed on identifying potent phytochemicals of biomedical significance [24, 45, 46]. Indicative of fact that cytotoxic potential of the plant was most studied among all the other biomedical activities. Nonetheless, not much evaluation has been conducted with regards to the tumoricidal activity of the *A. muricata* fruit, as compared to other organs of the plant. Addressing this lacuna, we have tried to identify the anticancer activities of the *A. muricata* fruit pulp and seeds in this study.

Antioxidants have been known for their indispensable role in the maintenance of cellular integrity, homeostasis of the immune system, essential to discourage tumor progression [47]. Earlier studies have also marked the *A. muricata* extracts to be having potent antioxidant and anti-inflammatory properties. In a study conducted by de Sousa *et al.* (2010), the antioxidant activity of the plant was attributed to the presence of proton donating lipophilic phytochemicals [48]. Additionally, Inhibition of inflammatory mediators, such as tumor necrosis factor- α (TNF- α), interleukin-1b (IL-1b), interleukin-6 (IL-6) and nitric oxide

(NO), by 96% ethanol extract of *A. muricata*, comparable to the non-steroidal anti-inflammatory drug, indomethacin, has been reported [49-52].

Affirming the mechanistic association of the antioxidant and anti-inflammatory potentials. With a cohesion between the antioxidant and anti-inflammatory properties as well as the cytotoxicity established in *A. muricata*, we carried out the MTT-based anti-breast cancer assay to validate the same in the extracts of our interest, AMPM and AMSM [49, 53-56]. Dose-dependent cytotoxicity was shown by both extracts in this study. Similar observations have been made by various research groups working on phytochemicals found in different organs of the plant *A. muricata*. In a report by Ko *et al.* (2011), it was shown that the plant favours apoptosis in ER-related pathways [57].

In addition, it was also noted that *A. muricata* had subsided the proliferation of MCF-7 cell lines while hindering nude mice ER-cyclin D1 and Bcl-2 protein expressions [58]. Parama *et al.* (2013) have demonstrated that acetogenins from *A. muricata* have growth inhibitory and cytotoxic effect on cervical cancer cell line [59]. Experiments have been performed to show that the fruit phytochemicals initiate necrosis in PC-3 cells by the inhibiting cellular metabolism and tumor mobility [60]. Zorofchian *et al.* (2014) had observed that leaf extract exerted a striking cytotoxic effect on HCT-116 cells. These observations were made using the cell viability assays using MTT and Lactate dehydrogenase (LDH) [61]. All of this asserting the possible beneficial effects of *A. muricata* fruit consumption towards mitigating cancer.

Conclusion

In conclusion, *A. muricata* fruit pulp and seeds are both comprised of potent anticancer agents which will need to be isolated and characterized for further evaluation to gain a mechanistic insight towards their working.

Conflicts of Interest

None.

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