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SHORT COMMUNICATIONS

Screening antioxidant activity by in-situ HPTLC-DPPH assay and in-vitro cytotoxic assessment of *Annona muricata* L. plant organ extracts on MCF-7 and SCC-40 cell lines

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Abstract
Annona muricata Linn exhibits immense array of medicinal and ethno-pharmaceutical benefits owing to the synthesis of secondary metabolites like alkaloids, phenols and acetogenins with prospective biological activity. In this work, we analyzed the potential antioxidant and cytotoxic efficacy through synergistic interaction possessed by phytoconstituents in crude methanol extracts of *A. muricata* plant organs. The rind, pulp, seed, leaf, bark and root extracts of *A. muricata* were evaluated by rapid free-radical scavenging activity of developed bioprofile using HPTLC method with post-chromatographic derivatization with DPPH reagent to assess the active antioxidant profile in plant parts. While, the in-vitro cytotoxicities of extract were studied employing MTT assay on MCF-7 and SCC-40 cancer cell lines; HPTLC-DPPH assay showed presence of distinct yellowish bands against violet chromatographic background confirming in-situ anti-oxidant activity in all plant organs. In-vitro anti-cancer assay demonstrated strong cytotoxic potential in all plant organs studied. The leaf extract showed better cytoxicity toward MCF-7 cells with IC_{50} value of 13.04 µg/mL while seed extract exhibited better efficacy with IC_{50} of 15.07 µg/mL against SCC-40 cell lines. The findings indicate that phytochemicals present in *A. muricata* organs might be responsible for bioactivity and indeed be used as a potential source of effective natural anti-oxidant and anti-cancer agent in nutrition and pharmaceutical industries.

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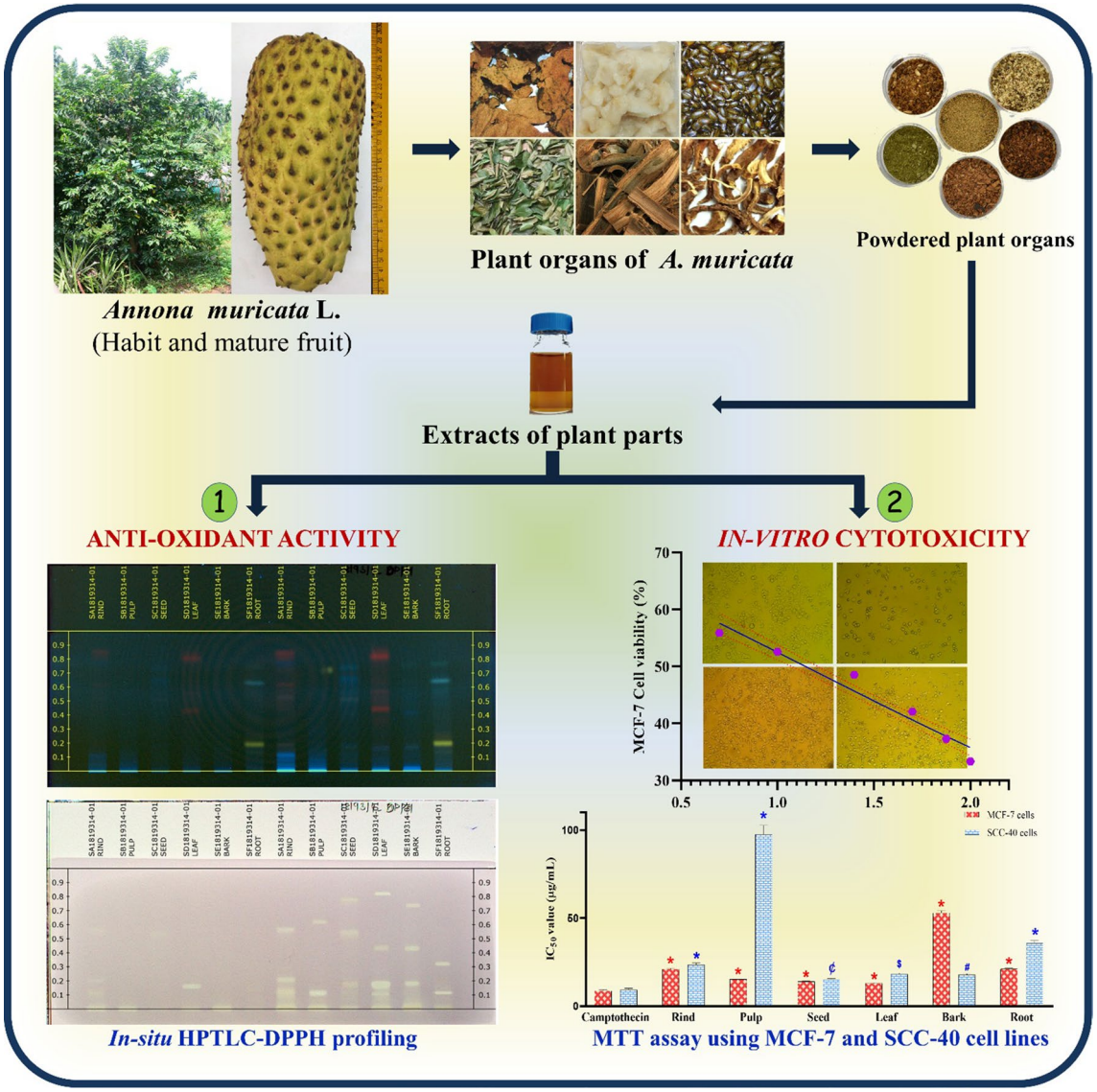
Graphic abstract



Keywords *Annona muricata* · Cancer · DPPH · HPTLC · MCF-7 · SCC-40

Introduction

Since the dawn of civilization natural products derived from plants, notably phytochemicals; have been used to resolve a variety of ailments (Yuan et al. 2016). With advances in science; medicinal plants have undergone comprehensive scientific evaluations for diverse pharmacological facets, including cancer. Plant produced bioactive compounds/extracts are studied extensively for their prospective anticancer derivatives as in the *Annona* sps. (Sharafi et al. 2014; Leite et al. 2020).

Annona muricata L. (Graviola) belonging to family Annonaceae comprises approx. 135 genera and 2500 species (Anuragi et al. 2016). It exhibits a broad spectrum of ethno-medicinal and efficacious properties toward cancer, accredited to various plant organs. As per reports, over 210 isolates of secondary metabolites consisting primarily of annonaceous acetogenins, phenolic compounds, alkaloids along with megastigmanes, flavanol triglycoside, cyclopeptide and essential oils have been identified from *A. muricata* having versatile pharmacological activity (Gavamukulya et al. 2017; Coria-Téllez et al. 2018).

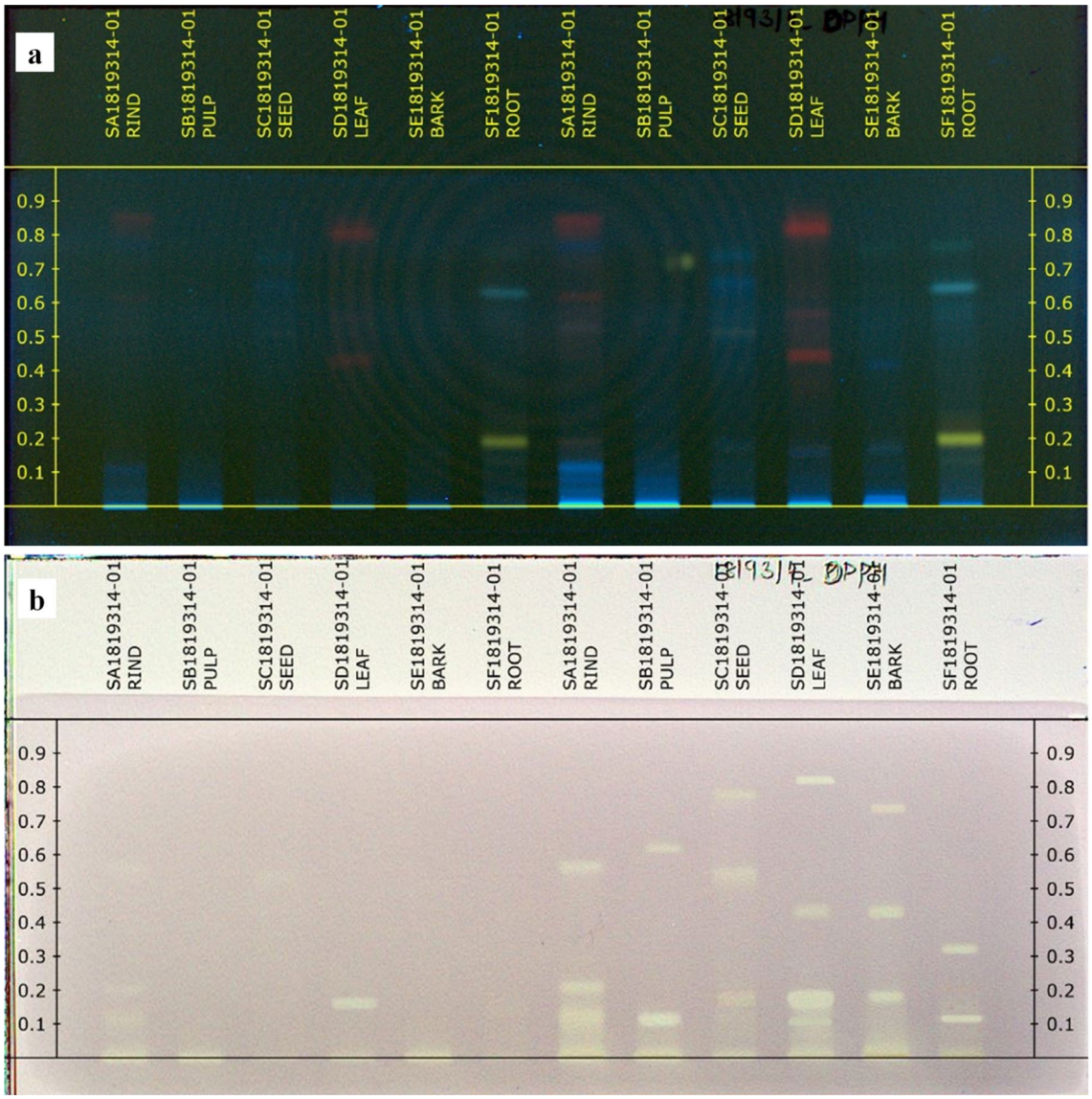


Fig. 1 HPTLC chromatogram profile of different organs of *Annona muricata* L. before and after derivatization with DPPH reagent. **a** Chromatogram before derivatization at 366 nm. **b** After post-chromatographic derivatization with DPPH antioxidant detection reagent at 540 nm. Tracks 1 and 7 (Rind); Tracks 2 and 8 (Pulp); Tracks 3 and 9 (Seed); Tracks 4 and 10 (Leaf); Tracks 5 and 11 (Bark); Tracks 6 and 12 (Root). Tracks 1–6 and tracks 7–12 represent 2 µL and 5 µL volume of extract respectively

Annonaine, asimilobine, annomuricin A, annonacin, annonacin A, muricin, annohexocin (Acetogenins); Reticuline, coclaurine, coreximine, anomuricine (Alkaloids); Quercetin, gallic acid, myricetin and lipophilic antioxidant compounds such as astocopherols and tocotrienols are some of the prominent phytochemicals found in *A. muricata* (Moghadamtousi et al. 2015; Gavamukulya et al. 2017; Coria-Téllez et al. 2018). Whereas, Annonacin is the foremost copious chemical found uniquely and predominantly in various plant organs with promising curative properties (Naik and Sellappan 2020a).

These metabolites are preferred over synthetic chemicals due to minimal cost, oral bioavailability and deemed safety in crude extracts. However, this plant grown under different cultivations does not necessarily exhibit the same curative benefits (Syed Najmuddin et al. 2017). Although major phytochemicals have been identified from *A. muricata*; the comparative anti-proliferative behavior of *A. muricata* plant part extracts remain quite obscure. Methanol, relative to several organic solvents and aqueous media used to retrieve plant-based compounds; extracts better compounds due to improved

solubility of these biochemicals in methanol relative to other solvents (Kallithraka et al. 1995; Fernández et al. 2017; Truong et al. 2019).

Being hitherto unexplored, the aim of this study was to assess the antioxidant potential with the help of in-situ HPTLC-DPPH assay and experimentally justify the comparative anti-proliferative capacity; possessed by *A. muricata* methanolic extracts of plant organs against MCF-7 (breast cancer) and SCC-40 (tongue squamous cancer) cell lines.

Materials and methods

Collection of plant material

Annona muricata L. ripened fruits, leaves, barks and roots were obtained from KOCL Research Farm, Kirbhatt, Nuvem, South Goa district, Goa, India located at 15° 18′ 12.77″ N and 73° 57′ 17.15″ E. Dr. S. Krishnan, Professor in Botany, Goa University, authenticated the plant. Voucher specimen (AVN, AM01516) was deposited at the Goa University Herbarium located at the Department of Botany, Goa University, Goa, India.

Preparation of plant part extracts

100 g of each powdered plant parts namely rind, pulp, seed, leaf, bark and roots were extracted with the aid of Soxhlet extractor with 300 mL of 95% methanol for 12 h at 45 °C. The miscella obtained after extraction was concentrated using rotary vacuum evaporator under reduced pressure at 45 °C. The miscella was lyophilized to yield methanol-free solid residue. After drying, the extracts were stored in a refrigerator at 4 °C, protected from light until further use.

HPTLC-DPPH analyses

Application and chromatography

HPTLC fingerprint was performed using 1 mg of concentrated extracts (rind, pulp, seed, leaf, bark and root). 10 mg of concentrated extract was dissolved in 10 mL of methanol to furnish solution of 1 mg mL^{-1} concentration. All samples in quantities 2 µL and 5 µL were spotted using a CAMAG ATS-4 sampler as 5 mm width bands on pre-activated HPTLC silica gel plates. Prior to setting in mobile phase, the sample loaded plates were dried. The chamber was saturated (20 min at room temperature) with solvent vapour. Then HPTLC plates loaded with samples were placed in TLC twin trough developing chamber (CAMAG, Mutenz, Switzerland) for chromatogram development up to 90 mm. Toluene: ethyl acetate: chloroform: formic acid: methanol (3:3:2:1:1 v/v) was employed as mobile phase for chromatogram or fingerprint development (Wagner and Bladt 1996; Pozharitskaya et al. 2007).

Derivatization and documentation

The HPTLC-DPPH assay for direct screening of antioxidant activity of separated chromatographic zones in plant parts was applied for visualization. The chromatogram developed was dried by electronic hot air blower (40–50 °C) to evaporate the solvents from the plate. Plates were then dipped into DPPH reagent for 2 s using a Chromatogram immersion system III (CAMAG). Plates were dried for 30 min in dark at RT. The developed plates were photo-documented using VisionCATS 2.5 Manager at 366 nm before derivatization and under white light illumination after derivatization via CAMAG TLC scanner 4 (Pozharitskaya et al. 2007). The work was conducted at M/s Anchrom Laboratories, Mumbai, India.

Procurement and maintenance of cell cultures

MCF-7 (human breast cancer) and SCC-40 (human tongue squamous cancer) cell lines were procured from National Centre for Cell Science (NCCS), Pune and maintained under controlled conditions. When cells attained approx. 70% confluence, they were regularly passaged. For seeding, confluent cells were washed with 3 mL phosphate buffer saline (PBS) and trypsinized with 1 × trypsin to detach the cells and made into supernatant. Following detachment of cells, FBS containing media was added to inhibit the trypsin action. The cell suspension was pelleted for 5 min at 1500 rpm and then washed with PBS to remove any cell debris.

In-vitro cytotoxicity assay using MTT

MTT assay was employed to determine cell proliferation and viability. Before treatment, 200 µL of the cell suspension at a density of 2×10^4cells/well were seeded in 96-well plates and maintained for 24 h. Following day, cells were treated with series of 5–100 µg/mL concentrations of rind, pulp, seed, leaf, bark and root extract and incubated for 48 h. 20 µL MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide) solution [5 mg/mL in phosphate-buffered saline (PBS)] was then added to each well and incubated in dark at 37 °C for 3 h. The purple formazan product produced was solubilized using 100 µL of DMSO in a gyratory shaker. Plates were read using ELISA plate reader (Thermo, Multiskan) at an absorbance of 570 nm and 630 nm as

Table 1 HPTLC-DPPH based anti-oxidant profile of aerial and underground plant organs of *A. muricata* L

R_f values	Plant organs of *A. muricata* L.						No. of bands
	Rind	Pulp	Seed	Leaf	Bark	Root	
0.11	+	+		+		+	4
0.18			+	+	+		3
0.21	+						1
0.32						+	1
0.44				+	+		2
0.56	+		+				2
0.62		+					1
0.76					+		1
0.79			+				1
0.82				+			1

identification of secondary metabolites, adulteration and authentication of *A. muricata* plant organs. HPTLC-DPPH assay performed for the explicit screening of in situ antioxidant activity in chromatographic zones was confirmed by the presence of yellowish bands appearing against light violet background in all tracks of plant organ extracts namely rind, pulp, seed, leaf, bark and root (Table 1).

All plant organs of *A. muricata* together showed the presence of anti-oxidant active zones with particular reference to R_f values ranging from 0.11 to 0.82. However, differences in active band intensities were also observed at the application position in all plant organs tested. The highest active band intensity was evidenced in leaf extract at R_f value 0.18 and concurrently lesser intensity bands were observed in seed and bark extracts. In general, more degree of active bands was observed in leaf extract with maximum four bands followed by rind, seed, bark showing three bands and two bands with anti-oxidant activity were observed in pulp and root crude extracts. Among the ten different active bands, anti-oxidant bands corresponding to R_f value 0.11 were commonly found in rind, pulp, leaf and root extracts. Band with R_f value 0.18 were present in seed, leaf and bark extracts. While leaf, bark and rind, seed jointly expressed bands with R_f values 0.44 and 0.56 respectively. Interestingly, anti-oxidant active bands with R_f values 0.21, 0.32, 0.62, 0.76, 0.79 and 0.82 were uniquely and intimately associated with rind, root, pulp, bark, seed and leaf respectively. Furthermore, based on DPPH spectroscopic assays, the IC_{50} value of plant parts ranged from 16.04 to 44.76 µg/mL with the highest antioxidant potential exhibited by leaf extract and least by root extract. Whereas, the IC_{50} value of ascorbic acid (standard) was found to be 15.72 µg/mL (unpublished data).

The cytotoxic effect of the crude extracts from *A. muricata* plant organs determined using MTT assay against the cancer cell lines MCF-7 and SCC-40 resulted in concentration dependent depletion in cell population when tested at a concentration range of 5–100 µg/mL after 48 h of treatment. The results also reveal morphological alterations and shrinkage of cells leading to cell bereavement induced by *A. muricata* plant organ extracts in the breast and tongue squamous cell lines (Figs. 2, 3, 4). As per the plant screening program of the United States National Cancer Institute (NCI), a plant extract is usually regarded to provide an active cytotoxic influence

reference. The medium with cells without experimental drug/extract was negative control, while medium with cells and 15 µM Camptothecin was positive control for all assays. (Alley et al.1986; Mosmann 1983). The maintenance of cell cultures and cytotoxicity studies were carried out at M/s Averin Biotech Pvt. Ltd., Nallakunta, Hyderabad, India. The viability of cells was assessed as percent viability of treated cells compared to untreated cells expressed using the formula:

$$\text{Percentage of viability} = \frac{(\text{mean abs. value of untreated cells} - \text{mean abs. value of treated cells})}{\text{mean abs. of untreated cells}} \times 100.$$

Using regression study, the correlation of cell viability and extract concentration was further examined and the value of IC_{50} (extract concentration resulting in cancer cell growth of 50%) was established.

Statistical analysis

The findings were interpreted as mean ± standard deviation. Descriptive statistics were used to analyze inter-group mean, standard deviation, variance, and level of statistical significance; where $p < 0.05$ was deemed statistically significant.

Results and discussion

The HPTLC fingerprint chromatogram used in this study showed unique profile and banding patterns (Fig. 1). The HPTLC chromatogram observed under 366 nm prior to chromatographic derivatization exhibited better separation of chemical zones. This may be well-suited for

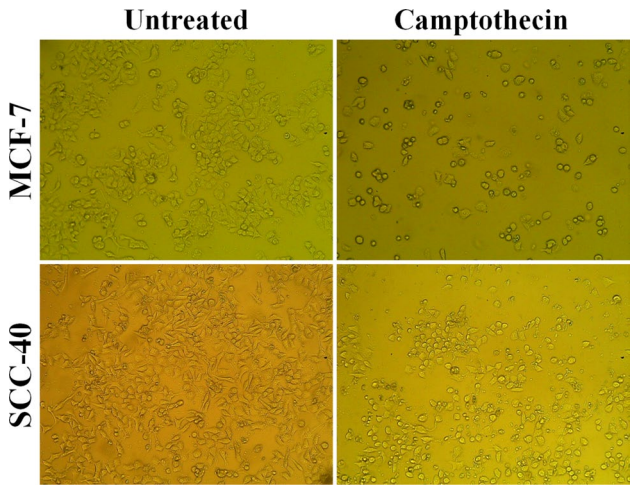


Fig. 2 Representative morphology images of untreated MCF-7 and SCC-40 cells in 0.1% DMSO and upon Camptothecin treatment after 48 h (magnification × 100)

if the IC_{50} value is 20 µg/mL or lower, after incubation between 48 and 72 h. Whereas, effective cytotoxicity is deemed for pure compounds with an IC_{50} value below 10 µg/mL (Lee and Houghton 2005; Roduan et al. 2019).

Based on the IC_{50} values; the pulp, seed and leaf extracts were shown to exert active cytotoxicity with IC_{50} values less than 20 µg/mL when treated against MCF-7 cells (Figs. 5, 6). Similarly the seed, leaf and bark extracts showed better cytotoxicity towards SCC-40 cells with IC_{50} values below 20 µg/mL. Both these findings revealed a differential mode of action by plant organs against the two cell lines tested. Camptothecin (positive control) showed 50% cellular inhibition at 8.76 µg/mL and 9.15 µg/mL concentrations when tested against breast and tongue squamous cancer cells respectively (Table 2). Though all extracts possessed moderate to high anti-proliferative activity, the leaf and seed extracts showed best efficacies against both MCF-7 and

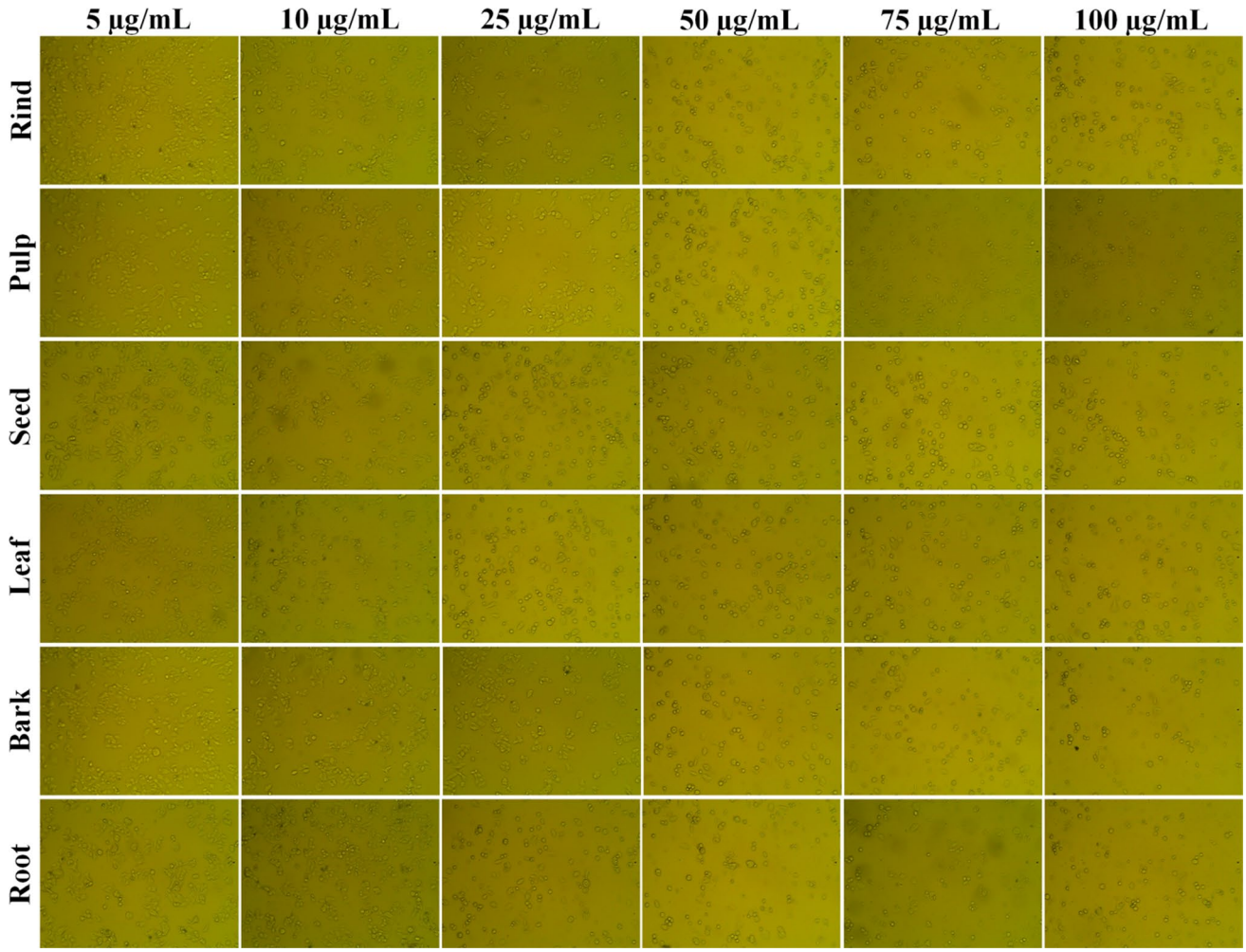


Fig. 3 Representative images showing dose dependent effect of *A. muricata* L. plant organ extracts (rind, pulp, seed, leaf, bark, and root) on the morphology of human breast cancer (MCF-7) cell lines (magnification × 100)

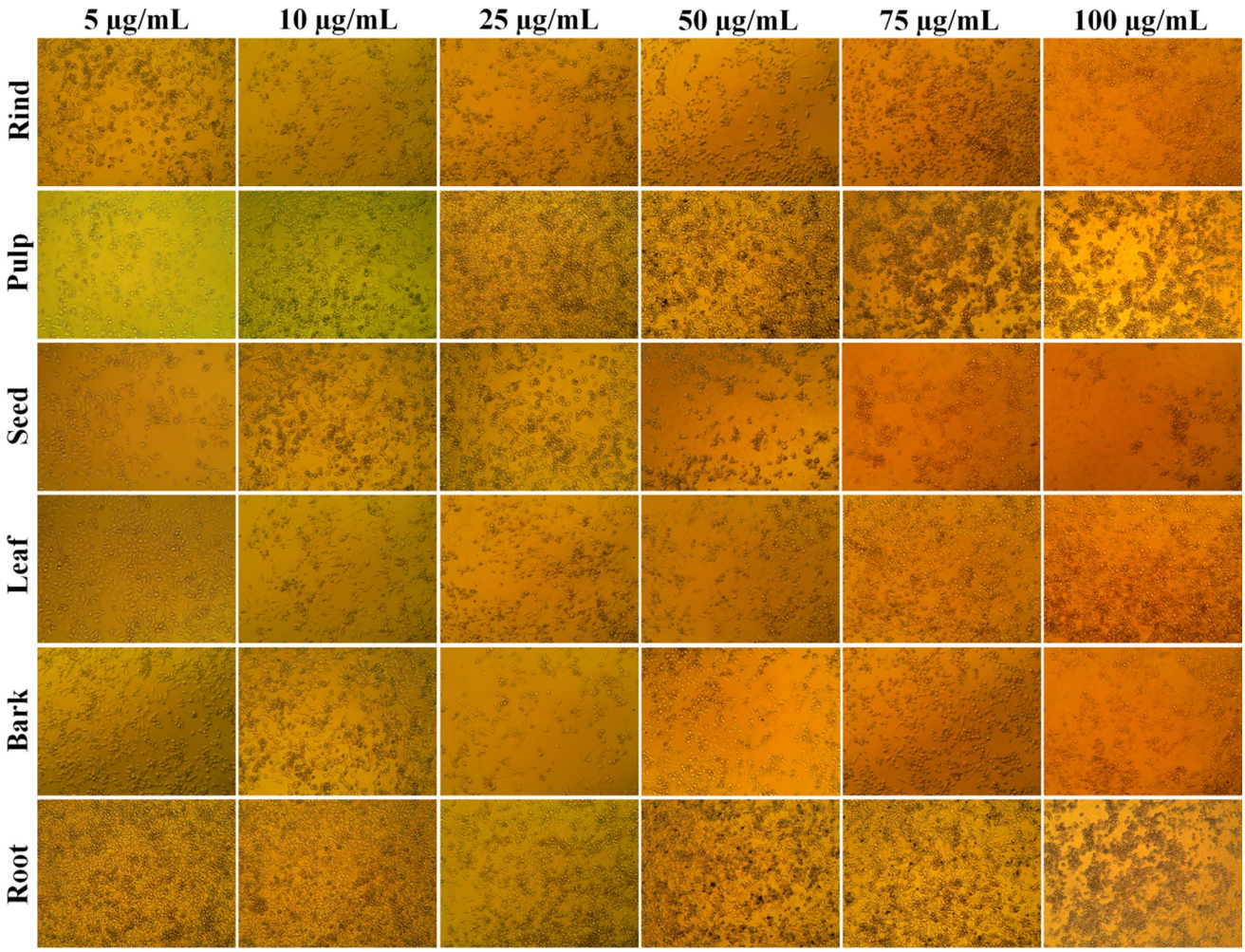


Fig. 4 Representative images showing dose dependent effect of *A. muricata* L. plant organ extracts (rind, pulp, seed, leaf, bark, and root) on the morphology of tongue squamous cancer (SCC-40) cell lines (magnification × 100)

SCC-40 cells. Further, the leaf and seed extract showed least IC_{50} value of 13.04 µg/mL and 15.07 µg/mL when tested against MCF-7 and SCC-40 cancer cells respectively. In comparison to different organs studied, bark extract showed least growth inhibition toward MCF-7 cells. While pulp extract exhibited better efficacy against MCF-7 cells but surprisingly showed least inhibitory activity when tested against SCC-40 cancer cells thus indicative of selective toxicity. The cytotoxic activity in MCF-7 cancer cells can be ranked as follows: camptothecin > leaf > seed > pulp > rind > root > bark. Whereas, the in vitro anti-proliferative trend in SCC-40 cells was found as follows: camptothecin > seed > bark > leaf > rind > root > pulp. Our work on preliminary phytochemical analysis of plant parts manifested the presence of salient classes of phytoconstituents largely extracted by methanol as solvent (Naik and Sellappan 2019, 2020b). The variations in cytotoxic activities of plant organ extracts from *A. muricata* can be explained by the difference in organ phytochemistry by which the solvent selectively extracts various hydrophilic or hydrophobic biochemical moieties with associative free-radical scavenging and corresponding anti-proliferative potential (Yang et al. 2015; Acésio et al. 2017; Fernández et al. 2017; Sheoran et al. 2019).

Imbalanced redox equilibrium may lead to development and progression of cancer. Antioxidants as therapeutic agents target the excessive production of intracellular ROS to prevent or inhibit the growth or progression of cancer cells (Oladimeji et al. 2016; Ghagane et al. 2017). However, *A. muricata* leaf extract as reported in our earlier study possessed strong ROS-scavenging activity (antioxidant response), which is correlated with anti-proliferative behavior in cancer cells and can

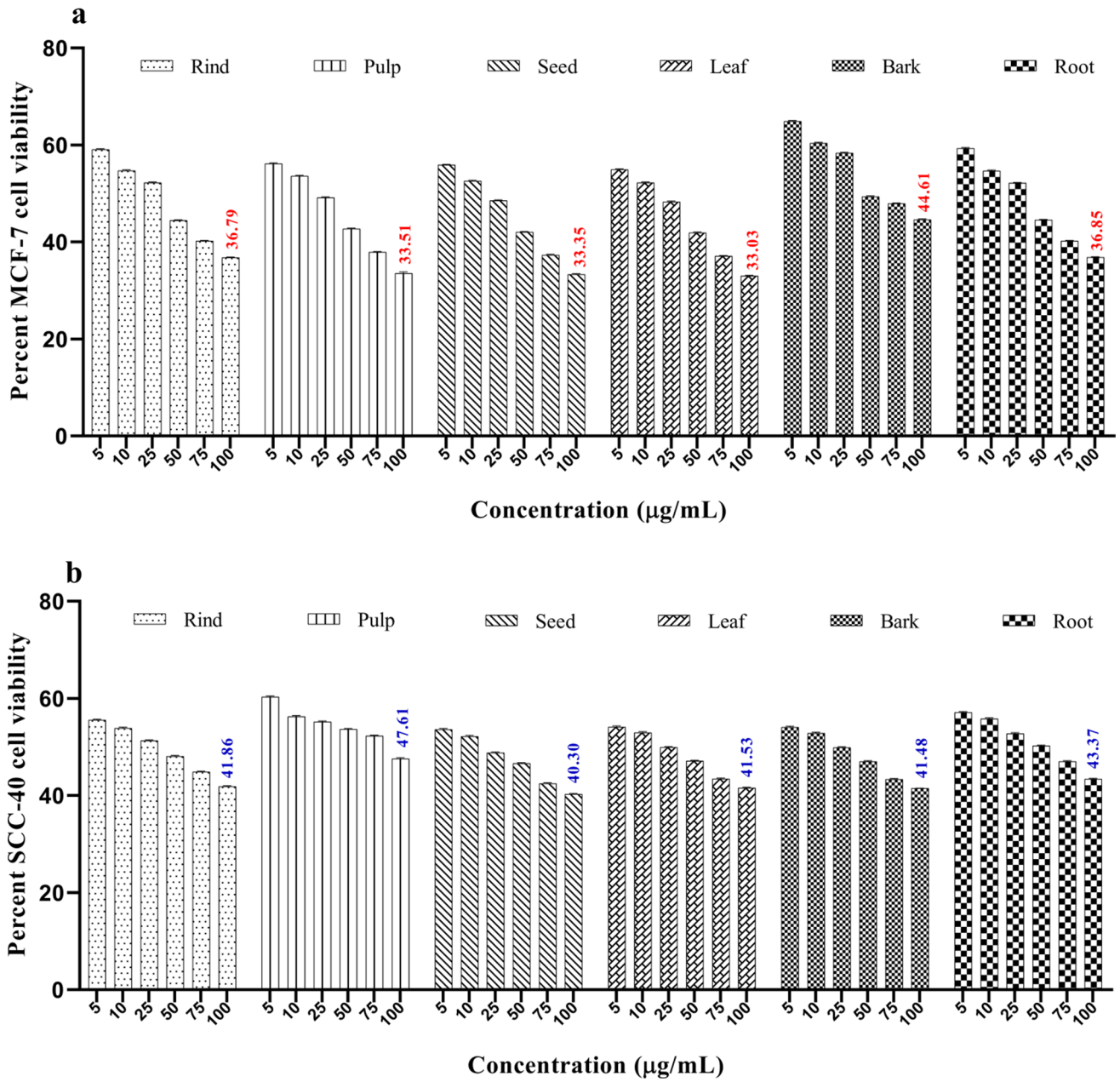


Fig. 5 Comparative cytotoxic assessments using MTT assay in **a** MCF-7 and **b** SCC-40 cells following 48 h exposure to various concentrations (5, 10, 25, 50, 75, 100 µg/mL) of rind, pulp, seed, leaf, bark and root methanol extracts of *A. muricata* L. Data is shown as mean ± standard deviation of three independent experiments

be extrapolated to other plant parts (Naik and Sellappan 2020a, 2020c; Naik et al. 2021). Thus, it may be used as a therapeutic and preventive agent in contrast to chemotherapy, which frequently causes severe side effects. Therefore, natural cancer treatments by using plant-derived products, may likely reduce some of these negative effects (George et al. 2015; Sheoran et al. 2019).

It is postulated that the cytotoxic and anti-tumour promoting activities stimulated by *A. muricata* plant organs in this study may be due to the synergistic activity of antioxidant compounds in extracts having differential selectivity and target specific mechanisms in certain cancer cells as also stated in our earlier studies (Renu et al. 2017; Vora et al. 2020; Naik and Sellappan 2020a; Naik et al. 2021). Morré et al. (1995), on the other hand reported that acetogenins contained abundantly in *A. muricata* demonstrates selective toxicity to cancer cells through its ability to inhibit the function of NADH oxidase enzyme by obstructing the

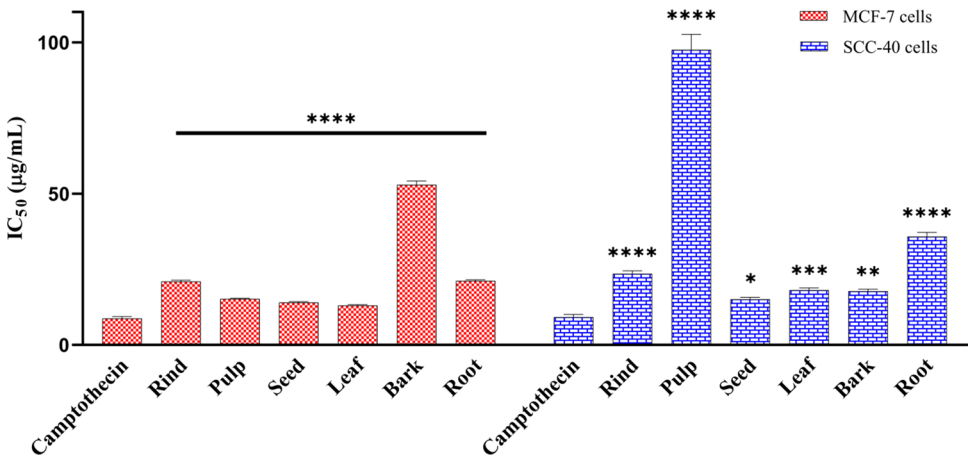


Fig. 6 In-vitro cytotoxic activity. The histogram plot represents the IC_{50} value of Camptothecin and plant organs of *A. muricata* L. carried out in MCF-7 and SCC-40 cell lines. All values are represented as mean ± SD and the experiments were carried out in triplicate. Statistically significant with respect to the control according to One Way ANOVA; Post hoc test: Dunnett's multiple comparison test. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$ versus control

Table 2 The IC_{50} values of *A. muricata* L. plant organs determined by MTT assay after 48 h exposure

Plant part or drug tested	IC_{50} value (µg/mL)	
	MCF-7	SCC-40
Camptothecin	8.76 ± 0.63	9.15 ± 0.93
Rind	20.997 ± 0.43	23.517 ± 0.97
Pulp	15.180 ± 0.27	97.513 ± 5.14
Seed	13.990 ± 0.28	15.067 ± 0.62
Leaf	13.037 ± 0.28	18.087 ± 0.77
Bark	52.950 ± 1.22	17.743 ± 0.69
Root	21.173 ± 0.39	35.760 ± 1.43

Values are represented in µg/mL

mitochondrial complex I leading to ATP starvation in cancer cells in particular which have higher ATP requirement than normal cells. Therefore, the inhibition of MCF-7 and SCC-40 cancer cell growth by *A. muricata* extracts could be credited to the capacity of solvent surpassing the effectiveness of many chemical constituents, the presences of different bioactive phytochemicals and natural antioxidant agents present in *A. muricata*.

Conclusion

Present study demonstrated anti-oxidant and cytotoxic capacity with significant variations in plant part extracts of *A. muricata* L. extracted using methanol. Amongst the studied plant parts, leaf extract possessed better cytotoxic activity toward MCF-7 cells with IC_{50} value of 13.04 µg/mL while seed extract showed better anti-proliferative efficacy against SCC-40 cells with IC_{50} of 15.07 µg/mL. This study adds empirical evidence for further studies and calls for demystification of lead compounds present in different organs having significant anti-cancer sensitivity to cancer type.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42535-021-00220-2.

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Declarations

Conflict of interest The authors declare no conflict of interest with respect to the authorship and/or the publication of this article.

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