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Original article

Phytochemical profiling of *Azima tetracantha* Lam. leaf methanol extract and elucidation of its potential as a chain-breaking antioxidant, anti-inflammatory and anti-proliferative agent

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ABSTRACT

Azima tetracantha, a traditional medicinal plant included in the order Brassicales and family Salvadoraceae, is widely used as a dietary supplement in folklore medicines. The plant is also used for the treatment of rheumatism, diarrhea and other inflammatory disorders. The present investigation focused on the phytochemical composition, radical scavenging, reducing potential and anti-proliferative activities of the *A. tetracantha* leaves. Quantitative estimation of the polyphenols and flavonoids revealed significantly elevated levels in the methanol extract. Corroborating with this, methanol extract exhibited higher *in vitro* anti-radical scavenging effect against 2,2-diphenyl-1- picrylhydrazyl (34.14 ± 2.19 μg/mL), and hydrogen peroxide (44.96 ± 1.77 μg/mL), as well as ferric reducing properties (58.24 ± 6.98 μg/mL). The methanolic extract also showed strong lipoxygenase (71.42 ± 6.36 μg/mL) and nitric oxide inhibitory activities (94.23 ± 8.11 μg/mL). Cytotoxic activity against MCF7 cells was found to be higher (IC50= 37.62 ± 2.94 μg/mL), than that of MDAMB231 cells (IC50= 69.11 ± 5.02 μg/mL). The qPCR-based analysis indicated dose-dependent increase in the expression of the pro-apoptotic genes such as executioner caspases and apoptotic protease activating factor-1. Overall, the results indicated the possible use of methanol extract of *A. tetracantha* leaves as a chain-breaking antioxidant molecule and are capable of inhibiting inflammatory enzymes and the proliferative potential of breast cancer cells.

1. Introduction

Free radical-mediated oxidative insults and inflammatory microenvironment are the underlying factors for the development of various diseases including cancers (Arfin et al., 2021). The prominent reactive molecules involved in oxidative stress are the reactive radical molecules including reactive oxygen species (ROS) as well as active reactive nitrogen species (RNS). They are highly reactive molecules and produce lipid peroxides, protein carbonyls, and DNA adducts and functionally inhibit enzyme activities by the interaction with cellular macromolecules. Apart from these oxidative stress mediators, inflammatory agents also play critical roles in the onset of cancers. These inflammatory molecules include nitric oxide, NF-kB, and other metabolic products of Arachidonic acid by lipoxygenase and cyclooxygenase (Wisastra and Dekker, 2014). These molecules further activate cellular pathways such as toll like receptors (Narayanankutty, 2019b), phosphoinositide 3 kinase (Narayanankutty, 2019a), and epidermal growth factor receptors (Roy et al., 2017); further, these pathways regulate the proliferation, growth, survival, and motility of the cancer cells.

The prevention of oxidative insults and inflammation are key events in the targeting of cancer development and progression. Plant-derived compounds, especially dietary agents, are under the limelight of drug development as anticancer agents. Salvado-

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raceae family comprises of some important biological active plants such as *Salvadora persica* that are known for their antioxidant activities *in vitro* and *in vivo* (Kumari and Parida, 2016; Mohamed and Khan, 2013). Likewise, some other plants belonging to this family are also known for their anti-proliferative activity in multiple cancer cells (Al Bratty et al., 2020; Farag et al., 2021). *Azima tetracantha* is a plant belonging to this family which is less studied as a dietary supplement (Sunil et al., 2013), despite its wide applications in traditional medicinal systems. A bioactive compound friedelin has been isolated from *A. tetracantha*, which is known to be efficient antioxidant (Sunil et al., 2013), inflammation inhibitory (Antonisamy et al., 2011), and ulceration preventive in nature (Antonisamy et al., 2015). However, no clear information is available on the anti-proliferative activity of the plant, and also studies with crude extracts are limited. Considering the possibility that there may be more bioactive compounds in the plant, the present study evaluated the radical scavenging, enzyme inhibitory, and anti-proliferative potentials of *A. tetracantha* leaf extracts.

2. Materials and methods

2.1. Chemicals, kits, reagents and cells

Laboratory chemicals, biochemical kits, and reagents were procured from Sisco Research Laboratories Pvt. Ltd. (Maharastra, India) and Sigma Aldrich (St. Louis, MO, United States); the reagents and chemicals used in the study were of cell culture/ molecular biology grade. Cell culture media, supplements, and plastic wares were purchased from Thermo Scientific (Massachusetts, United States). Human mammary carcinoma cell lines with variable ER expression status such as MCF7 (ER+) and MDAMB231 (Triple-negative) were procured obtained from the repository of DBT-National Centre for Cell Science, Pune, and maintained under standard conditions.

2.2. Plant collection, extraction, and phytochemical analysis

Azima tetracantha plant was collected from the Botanical garden of Malabar Christian College, Calicut, Kerala. The leaves were washed, shade-dried, powdered, and extracted with hexane, chloroform, acetone, and methanol in the Soxhlet apparatus. Total polyphenols (Ortiz-Cruz et al., 2020) and total flavonoids (Wang et al., 2020) were estimated according to standard procedures.

LCMS (LC- 8045, Shimadzu, Japan) analysis was employed for the phytochemical screening of the active extract. LCMS was conducted in gradient elution mode using a C18 column having the size of 4.6 × 15.0 cm and inner diameter of 5 μm; the 100% methanol (A) and formic acid in water (0.1%, v/v) had been used as the mobile phase at gradient elution mode. The HPLC elution ranges and MS setup were conducted as described in our previous publication by House et al. (2020).

2.3. Radical scavenging, ferric reducing, and anti-inflammatory potentials of A. tetracantha leaf extracts

The radical scavenging potential of the extracts was analyzed in terms of the two commonly utilized radicals such as DPPH or the so called diphenyl picryl hydrazyl (Liu et al., 2020) and peroxide radical source- hydrogen peroxide (Bi et al., 2014). The reduction capacity of the extracts was analyzed as the FRAP or ferric reducing antioxidant potential according to the method (Dutta and Ray, 2020). The inhibition of the pro-inflammatory enzyme, lipoxygenase, was carried out as detailed in the publications previously reported (Manga et al., 2020). The scavenging potential of the extracts on sodium nitroprusside-derived nitric oxide radical was conducted using the Griess reagent method (Tonisi et al., 2020).

2.4. In vitro anti-proliferative potential of A. tetracantha

Briefly, the human mammary tumor cell lines of passage number 24 and 37 (MCF7 and MDA-MB-231) were plated in 96 well culture tissue culture plate at a density of 10,00,000 cells/ mL of RPMI-1640 media and permitted to get adhered to the bottom of the flask; the different concentrations (0–0.2 mg/mL) of the methanol extract (methanol extract was estimated to be more active in phytochemical screening and in vitro assays) was added. The cells were further incubated for 48 h and the anti-proliferative activities of *A. tetracantha* were done using MTT assay (Al-Yousef et al., 2021).

The half-maximal inhibition concentration also known as the IC50 value of different extracts was estimated by plotting percentage inhibition of each assay on Y-axis against the corresponding concentrations (X-axis) and then determining the slope of the curve. Using the formula obtained, the IC50 values were calculated using Microsoft Office Excel (Probit analysis).

2.5. qPCR analysis

The MCF-7 cells were cultured and exposed to *A. tetracantha* extract at doses 10, 20, and 30 μg/mL, respectively. The cells were collected by mechanical scraping and cDNA synthesis was done using CellAmp™ cell to cDNA kit (Takara Bio, India) agreeing with the industrialist's guidelines. The analysis of gene expression was conducted using Applied Biosystem 7500 qPCR (Thermo Scientific, Massachusetts, United States). The thermal cycling was denaturation 95 °C, 60 °C, and 74 °C, for denaturation, annealing, and extension, with 40 cycles, and CT values were calculated by the software program. The amplification of genes was determined using specific primers as shown in the Table 1. The ΔΔCT values were calculated and fold change in expression against the untreated control to the $2^{-\Delta\Delta CT}$ method.

2.6. Data presentation and statistical analysis

The data obtained in the entire experiments were presented as Mean ± SD. The cell culture and in vitro studies were conducted out in 96 well plates and each dose was repeated six times and in five independent plates. The statistical comparison of the available data was carried out using one-way ANOVA followed appropriate post hoc test. The difference of $p < 0.05$ is considered to have significant variation between each other.

Table 1
The primer sequence of different genes involved in apoptosis and internal standard-β-actin used in the experiment.

Gene	Direction	Sequence
Caspase-3	Forward	5′-GTGGAACTGACGATGATATGGC-3′
	Reverse	5′-CGCAAAGTGACTGGATGAACC-3′
Caspase-7	Forward	5′- GGACCGAGTGCCCACTTATC-3′
	Reverse	5′-TCGCTTTGTCGAAGTTCTTGTT-3′
Apaf-1	Forward	5′-CTGGCAACGGGAGATGACAATGG-3′
	Reverse	5′-AGCGGAGCACACAAATGAAGAAGC-3′
β-actin	Forward	5′-AAGATCCTGACCGAGCGTGG-3′
	Reverse	5′-CAGCACTGTGTTGGCATAGAGG-3′

Table 2
The total polyphenol and total flavonoid contents of hexane, chloroform, acetone, and methanol extracts of *Azima tetracantha* leaves and expressed per gram of leaf extract.

	Hexane extract	Chloroform extract	Acetone extract	Methanol extract
Total phenols (TPC)	84.21 ± 5.7	89.56 ± 6.1	142.60 ± 12.4	254.81 ± 17.1
Total flavonoids (TF)	13.23 ± 2.6	16.42 ± 3.4	26.52 ± 3.8	44.11 ± 4.03

TPC is estimated as mg equivalent of gallic acid/g and TF is estimated as mg equivalent of quercetin/ g.

3. Results

3.1. Phytochemical characterization of Azima tetracantha

The results showed a total polyphenol content of 254.81 ± 17. 1 mg GAE/g extract in the methanol leaf extract of *A. tetracantha* extract; however, other solvent extracts were having a lower polyphenol quantity (Table 2); the flavonoid content (44.11 ± 4.0 3 mg QE/g), was also found to be high in the methanolic extract of the plant. Further, characterization using LC/MS, the presence of various flavonoids and phenolic acid, especially the presence of quercetin and cinnamic acid derivatives, are also identified (Table 3).

3.2. A. tetracantha anti-radical potential and inflammation inhibition abilities

The anti-radical and reduction ability of the various solvent extractions from *A. tetracantha* leaves was evident from the experimental setup. Compared to other extracts, the methanol extract had significantly higher scavenging potential (34.14 ± 2.19 and 44.96 ± 1.77 μg/mL). The metal reduction abilities (FRAP) was also higher in methanol extract over other solvents (IC50 = 58.24 ± 6. 98 μg/mL) (Table 4).

Together with the antioxidant effects, *A. tetracantha* leaf extracts exhibited lipoxygenase inhibitory properties; the phenol-rich methanol extract was having the highest activity (71. 42 ± 6.36 μg/mL). The nitric oxide radicals that are produced by nitric oxide synthase in cells; the ability of methanol extract from the *A. tetracantha* leaves in neutralizing the nitric oxide radicals was also found to be high in the methanol extract of (IC50 of 94. 23 ± 8.11 μg/mL) (Table 4).

Table 3
Phytochemical composition of the methanol extract of *Azima tetracantha* leaves analyzed by LC-MS using gradient elution method.

Sl. No.	Retention time (mins)	Compounds identified
1	2.6	Ferulic acid
2	4.2	p-Coumaric acid
3	5.5	Hydroxycinnamic acid derivative
4	6.7	4-Hydroxycinnamic acid
5	8.9	7-O-methylquercetin
6	10.2	Methyl-O-quercetin glucoside
7	11.6	Quercetin 3-O-glucoside
8	16.3	Myricetin-3-O-rutinoside
9	19.7	Quercetin
10	24.2	Isorhamnetin 3-O-glucoside

Unidentified and unexpected compounds have been omitted from final list.

3.3. In vitro anti-proliferative and apoptotic activities of A. tetracantha

The active extract (methanol) of *A. tetracantha* leaves was used to analyze the anti-proliferative potential against human breast cancer cells. The highest activity was observed against MCF-7 cells (IC_{50} value of 37.62 ± 2.94 μg/mL); however, the antiproliferative potential of the extract was reduced against MDAMB231 cells (IC_{50} value 69.11 ± 5.02 μg/mL), a triple-negative breast cancer cell (Fig. 1a).

Providing further insights into the mechanistic basis, the apoptotic effect of *A. tetracantha* extract has been observed (Fig. 1b); treatment of MCF-7 cells with 10, 20, and 30 μg/mL extract dose-dependently increased the expression of apoptotic genes including Apaf-1 and other executioner caspases 3 and 7.

4. Discussion

Azima tetracantha is a dietary supplement and food item traditionally used in various African countries and Indian traditional systems. Despite their ethnic and traditional usage, the plant is less explored for its biopharmaceutical potentials. The present study thus analyzed the quantitative polyphenol/flavonoid content of the *A. tetracantha* leaves and their radical scavenging and reducing potentials. Further, the active extract was characterized by LCMS and anti-proliferative potential was evaluated. The results pointed out the existence of polyphenolic antioxidants in the multiple solvent extractions; among these the methanol extract has highest level of these chain-breaking bioactives. There observed the presence of various chain-breaking antioxidants such as polyphenols and flavonoids in the bioactive constituents using LCMS. Our results are well-corroborated with the reports of Bennett et al. (2004), where the presence of similar glucosinolates, flavonoids, and alkaloids in the various tissues of *A. tetracantha*.

Further, there observed significant radical scavenging potential and reducing power for the extract. Though there is no literature on the antioxidant properties of the crude extract, it has been identified that the bioactive compound, friedelin, from *A. tetracantha*, exhibits strong antioxidant potentials (Sunil et al., 2013). Besides, when comparing the IC50 values of the present study with that of the fridelin (Sunil et al., 2013), the crude extract was indicated to have a higher antioxidant potential. The additive or synergistic effect of the individual bioactive compounds in the plant may have contributed to this effect. Apart from the antioxidant potential, the

Table 4
The half-maximum inhibition (IC50) concentration of the different antioxidant and anti-inflammatory assays of hexane, chloroform, acetone, and methanol extracts of Azima tetracantha leaves.

	Hexane extract	Chloroform extract	Acetone extract	Methanol extract
DPPH	188.40 ± 12.50	133.50 ± 10.30	83.10 ± 7.40	34.14 ± 2.19*
H_2O_2 scavenging	126.90 ± 18.30	102.50 ± 17.40	67.30 ± 6.20	44.96 ± 1.77*
FRAP	102.30 ± 11.40	92.90 ± 13.50	83.00 ± 4.60	58.24 ± 6.98*
Lipoxygenase inhibition	173.90 ± 21.40	145.20 ± 17.30	104.60 ± 9.30	71.42 ± 6.36*
Nitric oxide scavenging	149.30 ± 15.60	120.60 ± 10.40	129.40 ± 12.10	94.23 ± 8.11*

* Indicates significant difference at $p < 0.05$ with all other extracts.

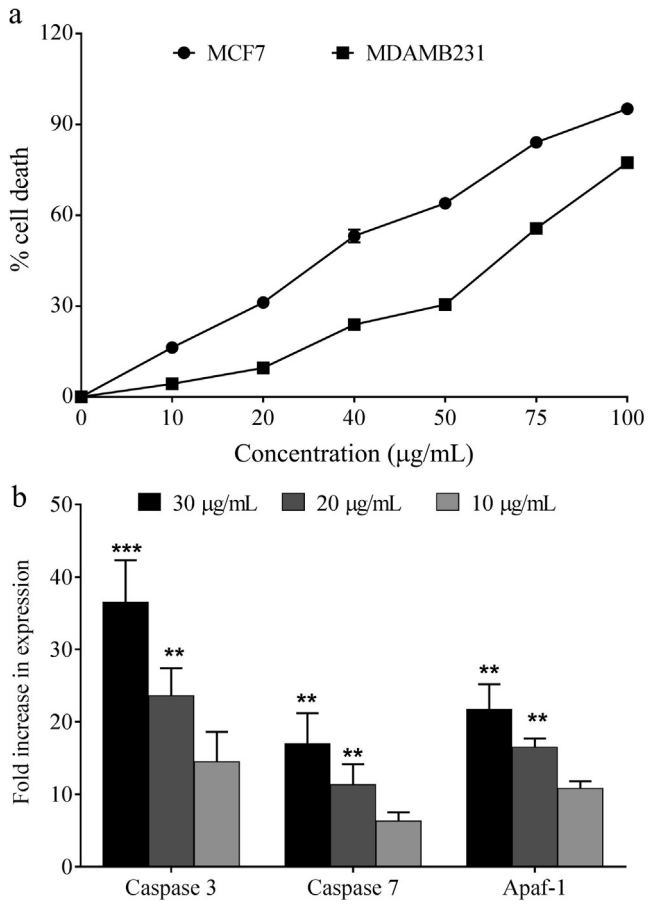

Fig. 1. Anti-proliferative activities of the *A. tetracantha* leaf methanol extract in MCF-7 and MDAMB231 cells (a) Further, the mechanism of cytotoxicity was evaluated in terms of the changes in expression of apoptotic genes, in MCF-7 cells (b).

anti-inflammatory properties of the plant have been also analyzed as the ability of inhibition of lipoxygenase activity as well as scavenging of nitric oxide radicals. The Lipoxygenase enzyme as well as nitric oxide are important inflammatory mediators in the body and have been involved in the commencement and advancement of innumerable inflammatory diseases (Ayola-Serrano et al., 2021; Kulkarni et al., 2021). It is also reported many therapeutic drugs modulate the expression of these enzymes and exert their anti-inflammatory activity (Shahid et al., 2021). The results observed significant inhibition of lipoxygenase enzyme by the *A. tetracantha* extract and thereby contributed to the anti-inflammatory properties of the extract. The individual flavonoid compounds identified in *A. tetracantha* have been previously reported to inhibit the inflammatory signaling pathways (Joseph et al., 2016). It is thus possible that the anti-inflammatory potential of *A. tetracantha* may be mediated, through these flavonoid compounds.

Further, the anti-proliferative potential of the extract was high against the MCF-7 cells, which is considered as an estrogen receptor-positive cell line (Altharawi et al., 2020); on contrary, the cytotoxicity towards MDAMB231 cells, a triple-negative breast cancer cell (Hero et al., 2019), is much lower. At present it is difficult to explain the disparity; however, it is expected that the presence of compounds with estrogenic potential usually known as phytoestrogens such as quercetin may have contributed to this phenomenon (Tanwar et al., 2021). Several of these phytoestrogens are known for their anti-proliferative activities at their dose-dependent nature. Besides, there observed a dose-dependent upsurge in the expression of Apaf-1 and caspase 3/7 genes in *A. tetracantha* leaf extract-treated cells. Apaf-1 and caspase 3/7 are involved in apoptotic cell death by activating the mitochondrial-mediated intrinsic pathway (Yadav et al., 2021). Previous studies have indicated that the individual flavonoid compounds reported in the LCMS analysis of *A. tetracantha* independently induce apoptosis in various cancer cells including those of mammary in origin (Ávila-Gálvez et al., 2021; Malayil et al., 2020). The mechanistic basis of their anticancer property is also reported to be mediated through apoptosis or autophagic cell death. Hence, it is possible that, the anti-proliferative activity of *A. tetracantha* leaf extract may be mediated through its bioactive flavonoid compounds via intrinsic pathway-mediated apoptosis.

5. Conclusion

Overall, the methanol extract of *A. tetracantha* leaves have higher polyphenol content and also shown to have promising antioxidant and anti-inflammatory activity. Apart from that, the anti-proliferative activities of the extract have also been observed in breast cancer cells; the intrinsic pathway-mediated apoptosis seems to be the possible mechanism of anti-proliferative activity by *A. tetracantha*. The bioactive flavonoid compounds present in the extract may be responsible for its biological/ pharmacological properties and therefore may be developed as future drug candidates.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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