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Azima tetracantha Leaf Methanol Extract Inhibits Gastric Cancer Cell Proliferation through Induction of Redox Imbalance and Cytochrome C Release

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Featured Application: The antiproliferative effects of *Azima tetracantha* support its traditional uses; additionally, it may emerge as a possible source of promising anticancer biomolecules in the future.

Abstract: *Azima tetracantha* is a traditionally used medicinal plant in the Ayurvedic system and folk medicines. The plant has been used for various purposes including inflammatory ailments, arthritis, and various types of toxicities. There are no available reports on its anticancer activity; hence, the study aimed to evaluate its anti-proliferative potential in gastric cancer cells (AGS and KATO III). We observed a dose-dependent decrease in cell proliferation in both the gastric cancer cells; furthermore, a concomitant reduction in the cellular antioxidant status was observed. Pre-treatment with *A. tetracantha* methanol extract showed a significant reduction in intracellular glutathione levels, and subsequently raised thiobarbituric acid reactive substances. Together with this, a significant increase in the cytochrome c release was noted in *A. tetracantha* treated cells, alongwith an increase in the expression of pro-apoptotic genes such as BAX, CASP3, CASP7 and APAF1. Furthermore, RTqPCR analysis indicated an increased expression of the anti-apoptotic gene BCL2 in a dose-dependent manner. In addition, to confirm the role of reactive oxygen species in the proliferation inhibition, DCFH-DA-based analysis was carried out, where a dose-dependent increase in ROS levels was observed in these cells. Overall, the study confirms the anticancer efficacy of *A. tetracantha* leaf methanol extract mediated through the induction of redox imbalance and cytochrome c release.

Keywords: *Azima tetracantha*; gastric cancer; apoptosis; redox imbalance; cytochrome c

1. Introduction

Gastric cancer or stomach cancer is one of the leading cancers in males which accounts for considerable mortality globally. Genetic factors are an important regulator of the carcinogenic responses in the stomach; among these, the most prominent is the mutations of a tumor suppressor gene, CDH1, that encodes the E-cadherin-class proteins [1]. Apart from these factors, infections of *Helicobacter pylori* and certain human papillomaviruses are also reported to have a significant association with gastric carcinoma [2,3]. Dietary habits and other infections are also known to be associated with the incidence of the onset

and advancement of gastric neoplasms [4]. Increased consumption of fatty foods [5], and diets rich in sugars [6] and salts [7] are reported to increase the incidence of gastric cancer. In addition, several oncogenic signaling pathways are also found to be upregulated in gastric cancers; these include the TGF-beta signaling family [8], receptor tyrosine kinase family proteins [9], especially epidermal growth factor receptor [10], and various growth factors [11]. According to recent reports from the USA, gastric cancer is the fifth most common cancer with an annual occurrence of over 1 lakh new cases per year and accounts for about 7 lakh deaths annually [12,13]. The overall five-year survival rates of gastric cancer are also estimated to be lower with an average of 31% for metastatic cases and less than 67% for non-metastatic cases [14]. Hence, the treatment of gastric cancer is of greater importance and therefore various therapeutic strategies are being employed for this tumor [15]. Among the forerunners of cost-effective anticancer drugs, the natural products isolated from plants are the most important compounds.

Various plants are being utilized for cancer prevention and therapy by traditional practitioners in Ayurvedic and Chinese medicines; however, the main limitation of their application is the reduced scientific support for their efficacy. The Western Ghats are an important source of such medicinal plants and the present study evaluated the possible application of a widely used anti-inflammatory medicinal plant *Azima tetracantha* for its possible application in gastric cancer control. The plant is widely used in Ayurvedic medicines and folk medicinal systems as a dietary supplement [16]; however, limitted studies have analyzed their application as a dietary ingredient. The plant is a rich source of a bioactive compound called friedelin; the antioxidant and anti-inflammatory properties of the isolated friedelin from *A. tetracantha* have also been reported [16,17]. The gastro-protective effect of this plant, whereby it inhibits gastric ulceration and inflammatory damages in animal models, has also been reported [18]. However, there are no further reports available on the plant for further anticancer properties or other biological efficacies. It is noteworthy that the plant, being a dietary supplement and gastro-protective agent, may be useful, and it may emerge as an anticancer agent against gastric cancer.

2. Materials and Methods

2.1. Laboratory Reagents, Chemicals, and Other Supplies

All chemicals used for routine laboratory biochemical assays were purchased from SRL Pvt. Ltd. (Mumbai, Maharashtra, India) and were of reagent-grade quality. The reagents and culture media components for cell culture were obtained from Sigma Aldrich (St. Louis, MI, USA) and Thermo Scientific (Waltham, MA, USA); all these reagents were compatible with cell culture or had a grade of molecular biology.

Gastric cancer cells Kato-III and AGS, as well as non-cancerous human cell line (HEK-293), were provided by the Department of Biotechnology, National Centre for Cell Science, Pune, India. The cells were maintained in quarantine for 1 week and observed for any signs of contamination or other infections. The cells were maintained in standard conditions.

2.2. Extraction of Azima Tetracantha and Phytochemical Analysis

Authenticated specimens of *Azima tetracantha* leaves were obtained from Dr. Anisha Sathyan M., Malabar Christian College, Calicut, Kerala, India. The leaves were cleaned and dried before powdering and were extracted using the Soxhlet apparatus and serially extracted using different solvents such as petroleum ether, chloroform, acetone, and methanol for a period of 6 h. All the solvents used were of HPLC-grade quality to ensure accuracy in LC/MS analysis. The phenolic content [19] and total flavonoids [20] in the extracts were quantified using the already mentioned procedures.

Liquid chromatography/mass spectroscopy studies on the phytochemical composition of the extract were conducted according to the methods described in our previously published article [21].

The extracted materials were suitably dissolved in solvents such as dimethyl sulfoxide (DMSO) and for each assay, a DMSO control was maintained. The final value was calculated

by normalizing with this value. Furthermore, wherever it is necessary, the solvent blank was applied to normalize the issue.

2.3. Efficacy of A. tetracantha Leaf Methanol Extract on the Neoplastic Cell Lines

The noncancerous human cell line HEK293 and neoplastic cell lines of gastric origin were selected (AGS and Kato-III cells). The cells were cultivated in the complete DMEM media supplemented under defined atmospheric conditions. The cells upon attachment were exposed to different doses of *A. tetracantha* extract. The toxicity was determined as percentage cell death by comparison with the untreated HEK293/Kato-III/AGS cells using the MTT assay [22] using the standard formula;

$$\% \, Cell \, death = \frac{Absorbance \, of \, Control - Absorbance \, of \, Sample}{Absorbance \, of \, Control} \times 100$$

In order to provide more clarity on the role of reactive oxygen species on the cytotoxicity, the cells were pre-treated with 0.125 mM reduced glutathione or 1 mM buthionine sulfoximine (BSO) to observe the changes in cell viability.

2.4. RTqPCR Analysis

The different gastric cancer cells were treated with the IC50 dose and sub-lethal doses of *A. tetracantha* for 24 h. The cells were collected by mechanical cell scrapers and they were directly used for the synthesis of cDNA using standard kits available by strictly adhering to the producer's directions (CellAmp™ cell to cDNA kit, Takara Bio, Bangaluru, India). The gene expression profiling was carried out using qPCR (Applied Biosystem 7500, Applied Biosystems) with selected primers (Table 1). The fold change in the expression of individual genes was calculated from the CT values using the $2^{-\Delta\Delta CT}$ method with respect to the untreated control cells; the beta actin gene was used as a housekeeping gene as per previously published methods [23].

Table 1. The primer sequences of various genes involved in apoptosis and anti-apoptotic genes; ACTB (β-actin) was used as a housekeeping gene.

Gene	Direction	Sequence
CASPASE-3	Forward Reverse	5′-GCTGGATGCCGTCTAGAGTC-3′ 5′-ATGTGTGGATGATGCTGCCA-3′
CASPASE-7	Forward Reverse	5′-GGGCCCATCAATGACACAGA-3′ 5′-GTCTTTTCCGTGCTCCTCCA-3′
APAF-1	Forward Reverse	5′-TCTTCCAGTGGTAAAGATTCAGTT-3′ 5′-TTGCGAAGCATCAGAATGCG-3′
BAX	Forward Reverse	5′-GAGCTAGGGTCAGAGGGTCA-3′ 5′-CCCCGATTCATCTACCCTGC-3′
BCL2	Forward Reverse	5′-ACCTACCCAGCCTCCGTTAT-3′ 5′-GAACTGGGGGAGGATTGTGG-3′
β-ACTIN	Forward Reverse	5′-ACTACCTCATGAAGATCCTC-3′ 5′-TAGAAGCATTTGCGGTGGACGATGG-3′

2.5. Estimation of Reactive Oxygen Species by DCFH and Cytochrome-C Release

The intracellular reactive oxygen species levels were determined by 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Thermo Fisher Scientific, Waltham, MA, USA) as described by the producer's manual. DCFH-DA fluorescence was determined using a fluorescence spectrophotometer at Ex/Ew wavelength of 485/520 nm. Cytochrome c release was estimated using standard kits according to the manufacturer's instructions (GeneTex, India) and the results are represented in relative fluorescence units.

2.6. Statistical Analysis

Results of the anticancer study and qPCR analysis are represented as Mean ± SD of six independent observations. The test of significance was carried out using a one-way analysis of variance, followed by a Tukey post hoc test (GraphPad Prism version 7.0, La Jolla, CA, USA).

3. Results

3.1. Quantitative and LCMS-Based Screening of Phytoconstituents

The quantitative determination of phenol products in the different extracts of *A. tetracantha* was determined spectrophotometrically. Among these, methanol extract had higher phenol derivatives (277.35 ± 10.4 mg GAE/g) followed by acetone extract (175.34 ± 8.8 mg GAE/g). In the petroleum ether extract, the lowest recording of the phenol compounds was observed. Corroborating this, the flavonoid content was also higher in the methanol and acetone extracts (Table 2). The liquid chromatographic analysis coupled with the mass spectra indicated the presence of various phenolic acids and also complex flavonoid compounds in the methanol extract (Supplementary Material S1).

Table 2. The quantitative analysis of the predominant phytoconstituents of the different extracts of *A. tetracantha* by spectrophometric methods.

Parameters	Petroleum Ether	Chloroform Extract	Acetone Extract	Methanol Extract
Total phenols (TPC)	72.35 ± 4.3	92.22 ± 4.1 [a]	175.34 ± 8.8 [a,b]	277.35 ± 10.4 [a,b,c]
Total flavonoids (TF)	11.05 ± 1.5	13.19 ± 2.6 [a]	29.67 ± 3.4 [a,b]	48.06 ± 3.85 [a,b,c]

[a]—indicates significant variation with petroleum ether, [b]—indicates significant variation with chloroform, [c]—indicates significant variation with acetone extracts.

3.2. Anti-Proliferative Activity of A. tetracantha Methanol Extract and Changes in the Cellular Redox Status

Figure 1a indicates the anti-proliferative potential of the extract in both Kato-III and AGS cells in a dose-dependent manner over 48 h ($p < 0.01$). However, the toxicity was lower in the non-cancerous human embryonic kidney cells, possibly indicating its selective anticancer properties (Figure 1a). The IC50 values of the extract in Kato-III cells was found to be 44.69 ± 1.09 µg/mL and the same in AGS cells was 58.04 ± 1.32 µg/mL; however, the cytotoxicity was significantly lower ($p < 0.001$) in non-cancerous HEK293 cells with an IC50 value of 109.75 ± 1.85 µg/mL. Furthermore, the addition of reduced glutathione to the cells along with *A. tetracantha* extracts induced a reduction in the toxic effect of the extract; furthermore, the addition of BSO, an inhibitor of glutathione biosynthesis, enhanced the cytotoxicity of the plant extract in cells (Supplementary Material S2a,b).

Additionally, when the *A. tetracantha* is exposed to the HEK293 cells at dose of 45 and 60 µg/mL (close to the IC50 doses in Kato-III and AGS cells), the observed % cell death was nearly 18% and 25%, respectively; this in turn confirms the significantly lower toxicity of *A. tetracantha* in the cancerous cells (Supplementary Material S3). As indicated in Table 3, the cellular antioxidant levels and lipid peroxidation indicators in the cells (AGS and Kato-III) exposed to different concentrations of *A. tetracantha* methanol extract were altered. The catalase activity was significantly increased in the different doses of *A. tetracantha* methanol extract ($p < 0.05$); on the contrary, we observed a significant and dose-dependent reduction in reduced glutathione (GSH). Similarly, a corresponding increase in the lipid peroxidation products (TBARS and conjugated dienes) was observed after the different doses of *A. tetracantha* methanol extract.

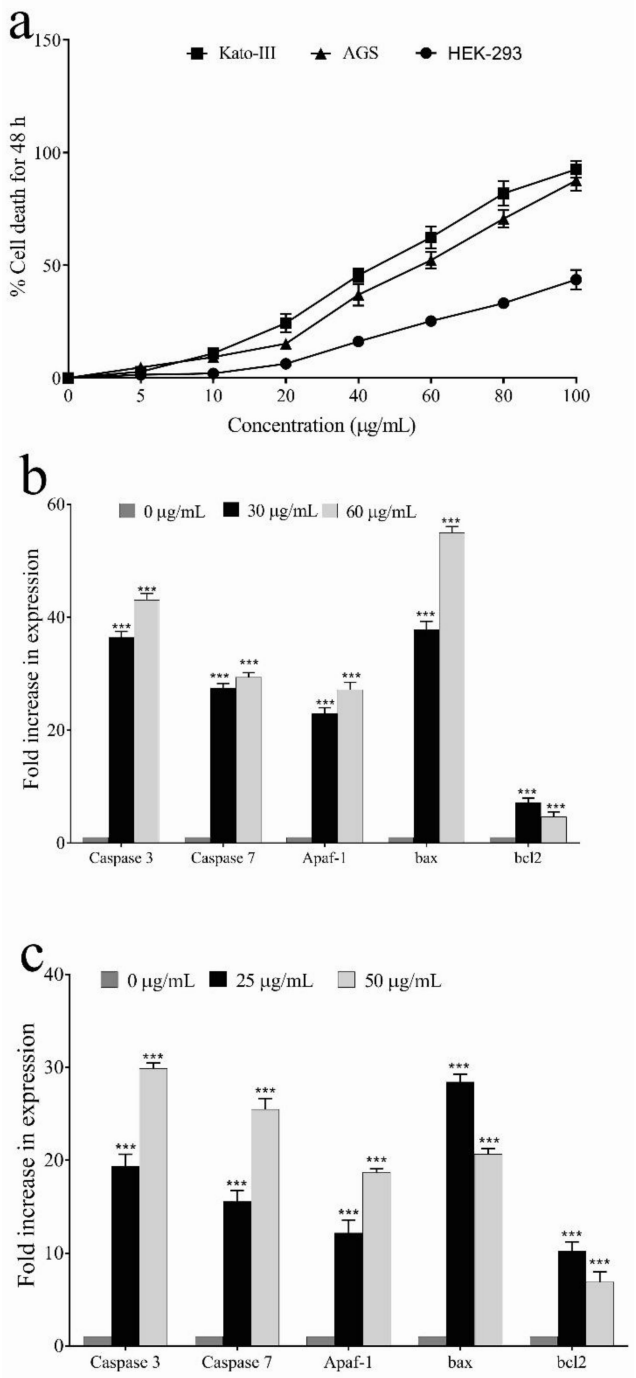

Figure 1. Inhibitory potential of *A. tetracantha* on the proliferation of neoplastic cells of human gastric origin (**a**). qPCR-based detection of apoptotic gene expression in the different gastric neoplastic cells: Kato III (**b**) and AGS cells (**c**). The fold change in expression of each gene was calculated by the $2^{-\Delta\Delta CT}$ method in comparison with the untreated control cells. (*** indicate $p < 0.001$).

Table 3. Cellular level of non-enzymatic redox markers and radical scavenging enzyme activities in gastric neoplastic cells treated with varying doses of methanol extract of *Azima tetracantha*. The * indicates the significance variation by $p < 0.05$, ** indicates the variation of $p < 0.01$ and *** represents variation of $p < 0.001$.

Cell	Dosage	Catalase (U/mg Protein)	Reduced Glutathione (μmoles/mg Protein)	Thiobarbic Acid Reactive Substances (nmoles/mg Protein)	Conjugated Dienes (nmoles/mg Protein)
Kato-III	Untreated	21.6 ± 1.03	3.77 ± 0.19	1.35 ± 0.11	22.5 ± 2.4
	25 μg/mL	34.2 ± 1.19 *	3.08 ± 0.22 *	3.21 ± 0.41 *	66.3 ± 2.7 *
	50 μg/mL	51.7 ± 2.04 **	2.62 ± 0.18 ***	4.64 ± 0.34 **	82.8 ± 3.1 ***
AGS	Untreated	44.1 ± 2.82	2.51 ± 0.26	2.46 ± 0.20	36.1 ± 2.9
	30 μg/mL	68.3 ± 3.55 *	2.11 ± 0.19 *	3.65 ± 0.16 *	55.7 ± 2.6 *
	60 μg/mL	94.5 ± 3.9 ***	1.69 ± 0.33 **	4.51 ± 0.12 **	79.3 ± 4.5 ***

3.3. RTqPCR, Cytochrome C Release, Reactive Oxygen Species Evaluation

The molecular mechanisms modulated by *Azima tetracantha* natural extract were estimated utilizing qPCR-mediated analysis (Figure 1b), reactive oxygen species, and cytochrome c release. Our results show a significant increase in the CASP3, CASP7, APAF1 and BAX genes in *A. tetracantha* ($p < 0.05$) methanol-extract-treated AGS and Kato-III cells (Figure 1b,c). Notably, we observed only a slight increase in the expression levels of the anti-apoptotic gene BCL2 after the *A. tetracantha* methanol extract treatment. The release of mitochondrial cytochrome c was found to be increased in the cells treated with *A. tetracantha*-extract-treated cells (Figure 2). Together with this, the intracellular level of reactive oxygen species was also increased dose dependently ($p < 0.05$) in both the cells (Figure 2).

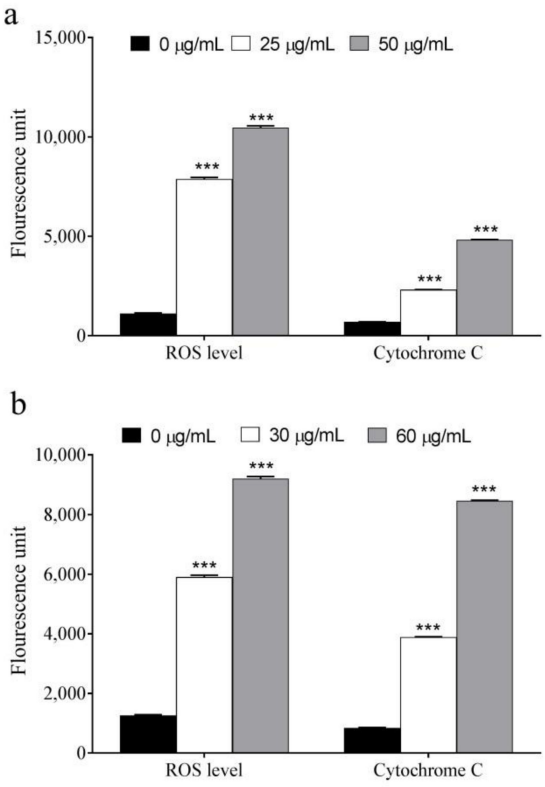

Figure 2. Changes in the reactive oxygen species levels and cytochrome C levels of Kato-III (**a**) and AGS cells (**b**) after *A. tetracantha* leaf methanol extract treatment. (*** indicate $p < 0.001$).

4. Discussion

The plant *Azima tetracantha* is widely used in traditional medicinal systems including Ayurvedic and Chinese medicines.In addition, the plant decoctions are used as dietary supplements by various tribes in India. However, few studies indicate the scientific evidence of the biological and pharmacological properties of the plant. The present study evaluates the potential antiproliferative role of *A. tetracantha* extract against different gastric cancer cells and its possible mechanism of action.

Among the different solvents, the methanol extract showed the highest levels of polyphenols and flavonoids. In addition, the LCMS profiling indicated the presence of different polyphenols comprising simple phenolic acids and complex dietary polyphenols. Previous results by Bennett et al. [24] indicate the presence of flavonoid compounds and glucosinolates in *A. tetracantha* tissues. Notably, the extract showed significant anticancer potential against the two gastric cancer cell lines, AGS and Kato-III. To provide the mechanistic basis of action, the expression of apoptotic genes including CASP3, CASP7, APAF1 and BAX was evaluated. These genes are known to be associated with apoptotic cell death in various cell types, and the changes in their expression levels modulate the mitochondrial-mediated apoptosis in cells [25,26]. Furthermore, the proteins Apaf-1 and cytochrome c, together with Caspase 9, are involved in the formation of the apoptosome complex via the intrinsic pathway [27]. Interestingly, our data show that the *A. tetracantha*-extract-mediated increase in BAX expression levels in both Kato-III and AGS cancer cells was higher than the increase obtained for BCL2 mRNA levels, leading to an increase in the BAX/BCL2 mRNA ratio, which has already been used to explain the anticancer effects of flavanols on breast and prostate cancer cells [28]. The cytochrome c release from damaged mitochondrial membrane was also found to be increased in those cancer cells exposed to different doses of *A. tetracantha* methanol extract; in addition, the cellular reactive oxygen species levels were also found to be upregulated in these cells. It is thus possible that the ROS-mediated mitochondrial damage performed by *A. tetracantha* extract could explain the anti-proliferative activity of the plant.

A. tetracantha is known to possess antioxidant potentials [16] and to inhibit inflammatory signaling [29] and cancer cell proliferation [30]; hence, together with these antioxidant and anti-inflammatory properties, the anticancer property may enhance the functional value of the plant.

5. Conclusions

The results indicate the anti-neoplastic applications of the *A. tetracantha* extracts in gastric cells. The action is mainly mediated through the induction of oxidative radicals and redox imbalance in these cells.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12010120/s1, Supplementary Material S1: Composition of the polyphenols in methanolic extract of *Azima tetracantha* leaves analyzed by LC-MS. Supplementary Material S2: The effect of *Azima tetracantha* extract alone treatment (a), *A. tetracantha* co-treated with glutathione biosynthesis inhibitor- buthionine sulfoximine (BSO) on the cell death induced by *Azima tetracantha* extract (b). Supplementary Material S3. The comparison of % cell death at individual doses of *Azima tetracantha* extract treated cells; the comparison was made with the non-cancerous cell (HEK293) to the AGS and Kato-III cells. Supplementary Material S4. The effect of *Azima tetracantha* extract alone treatment and the BSO co-treatment on the cell death of Kato-III and AGS cells.

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