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In Vitro Antioxidant, Antiinflammatory, Antichelating And Anticancer Activity Of Azima Tetracantha Against Hepg2 Cell Line

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Abstract

Medicinal herbs have long been used to treat a wide range of ailments. In the present study, the antioxidant potency of ethanolic extract was investigated, by determining the secondary metabolites such as flavonoids, tannin and phenolic compounds. The free radical scavenging activity of Ethanolic extract of *Azima tetracantha* (EEAT) was assessed by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide radical (NO), Hydrogen peroxide scavenging assay. Anti-inflammatory property also studied by invitro method such as Inhibition of albumin denaturation and HRBC membrane stabilization. Reducing power also assessed by using Iron chelating property. The Cytotoxicity was investigated against HepG2 cell lines using in vitro techniques such as MTT assay, morphological exams under a light microscope and a fluorescence microscope, DNA fragmentation, and LDH measurement. The results show that the EEAT contains significant antioxidant values. Similar observations were seen in scavenging the free radicals by the EEAT. It exhibits anti-inflammatory and antichelating activity also. *Azima tetracantha* ethanolic extract had significant cytotoxic activity against HepG2 cells, as measured by cellular death, in a dose-dependent manner. The findings of this study add to our understanding of *Azima tetracantha's* anticancer activity against HepG2 cells and support its application in the treatment of liver cancer. Thus, the therapeutic property of the plant *A. tetracantha* can be attributed to its phytoconstituents and its bioactive compounds.

Keywords: Azima tetracantha, Flavanoids, Inflammation, MTT, HepG2 cells.

1.INTRODUCTION:

Medicinal plants are still employed as traditional medicinal systems in many underdeveloped nations to cure numerous infectious diseases with herbal therapies. Medicinal plants form the backbone of Tribal medicines. These tribal medicines continue to play an important role in primary health care in many regions of the world even today, [1]. Plants have been employed as phytomedicine components from time immemorial. Natural products account for approximately 50% of modern medications, and they play a vital role in the pharmaceutical sector, [2]. Secondary metabolites include alkaloids, flavonoids, phenols, glycosides, tannins, resins, carbohydrate, protein, fat, lipid, terpenoid, steroid, xanthoxyllines, coumarin, and so on are produced by all plants. These naturally occurring chemicals have been shown to decrease cancer cell activity by reducing proliferation and triggering apoptotic cell death [3]. Plant derived drugs serve as prototypes to develop more effective and less toxic medicines. Currently, more than 60% of anticancer compounds obtained from herbal, marine, and microbial sources are beneficial to cancer patients. The beneficial effect of plants in cancer treatment has been widely researched and found to be effective [4]. Tribal medicine has received little attention. *Azima tetracantha*, is one of the tribal medicines used in Africa countries and in India. *A. tetracantha*, a rambling shrub is largely known for its medicinal uses in the Indian System of Medicine. *It* belongs to a family Salvadoraceae which consisting of three genera (*Azima* L., *Dobera* Juss., and *Salvadora* L.) with 12 different species. It comprises trees, shrubs, and scramblers distributed in dry, hot regions of Africa, Madagascar, and Asia. In which two genera and three species of *Azima* are represented in the Indian Subcontinent *and* has two species of erect, thorny shrubs distributed in Myanmar and India. In India, only one species, *A. tetracantha* Lam., is distributed [5] and locally known as "Mulchangan". It is specially characterized by the presence of four thorns at right angles to each other to each node,[6]. In medicine, the sap of this plant is administered directly to the teeth to cure dental pain and bleeding gums following tooth extraction, and it also functions as a disinfectant because to its antiseptic properties. The roots of *Azima* has anti-arthritic property which are used in the treatment of rheumatism, dropsy and Abdominal disorders,[7]. The anti-venom activity of *Azima* is used as a treatment for snake bites. The pounded roots and leaves are applied directly to snakebites, whilst an infusion is also taken orally as a treatment for them,[8]. The root extracts are used against many types of cancers such as breast cancer, cervical Cancer, Human peripheral lymphocyte (HPL) etc [9]. The roots have anti-inflammatory property which reduce inflammation by acting on body mechanisms,[10]. It shows hepatoprotective property which protects the liver. *A. tetracantha* Lam which also possesses activities like as stimulant, expectorant, antispasmodic, analgesic, anti-ulcer, anti-diarrhoeal, nephroprotective, hypoglycemic and hyperlipidemic activities,[11].

2.MATERIAL AND METHODS:

2.1. **Plant collection and Extraction:**

The *Azima tetracantha* plant was gathered from the Taramangalam Lakeshore in Salem District, Tamilnadu. To eliminate clinging dust, the leaves were rinsed in fresh water and then dried in the shade. The air dried and powdered leaves were extracted with ethanol in a soxhlet extractor. The successive extracts were saved and analyzed further**.**

2.2Phytochemical analysis:

Qualitative Phytochemical Screening:

The Ethanolic extract of *Azima tetracantha* was tested for the presence of bioactive compounds like protein, carbohydrates, tannins, saponins, flavonoids, glycosides, quinones, phenol, terpenoids, coumarins, anthraquinones, phlobatannin, anthocyanin by using following standard methods [12].

2.3. *In Vitro* Antioxidant activity:

The Ethanolic extract of *Azima tetracantha* was tested for its antioxidant activity by using following methods.

2.3.1. DPPH (1, 1-Diphenyl-2-picrylhydrazyl) radical scavenging activity:

The DPPH method involves transforming a dark blue alcoholic DPPH solution into a non-radical form of yellow diphenyl-picryl hydrazine in the presence of an antioxidant that donates hydrogen. 4 mg of DPPH was dissolved in 100 ml of ethanol and left overnight in a dark environment to produce the DPPH radical. An aliquot of 3ml of 0.004 percent DPPH solution in ethanol was combined with 0.1ml of plant extract at

various doses. After aggressively stirring the mixture, it was left to stand at room temperature for 30 minutes. To determine decolorization, the absorbance of DPPH at 517 nm was measured. In place of the plant extract, 0.1 ml of ascorbic acid was used as a control [13]. The exact ability to scavenge DPPH was estimated using the equation below.

DPPH radical scavenging activity percentage= $[(A0 - A1)/A0] \times 100$

Where; A0 = Absorbance of control, A1 = Absorbance of sample

2.3.2. Hydrogen peroxide scavenging activity:

The extract's capacity to scavenge hydrogen peroxide (H2O2) was assessed by using the Ruch et al., technique [14]. The test tubes were filled with a 0.1 mL aliquot of extracts (25-125 g/mL) and their volume was increased to 0.4 mL with 50 mM phosphate buffer (pH 7.4) before 0.6 mL of H_2O_2 solution was added (2 mM). Prior to measuring the reaction mixture's absorbance at 230 nm, the reaction mixture was incubated for 10 minutes. The positive control was ascorbic acid. Using the following equation, the extract's capacity to scavenge H_2O_2 was calculated:

H_2O_2 scavenging activity percentage= $[(A0 - A1)/A0] \times 100$

Where; A0 = Absorbance of control, A1 = Absorbance of sample

2.3.3. Nitric oxide scavenging activity:

The effectiveness of nitric oxide scavenging was evaluated by spectrophotometric analysis [15]. Various concentrations of diluted ethanol extract were combined with a solution of sodium nitroprusside (5 mmol) in phosphate buffered saline, and the mixture was then incubated at 25°C for 30 minutes. There was a control that contained the same amount of methanol but no test chemical. 1.5 mL of the incubation solution were withdrawn after 30 minutes and diluted with 1.5 mL of Griess reagent (1 percent sulphanilamide, 2 percent phosphoric acid, and 0.1 percent naphthylethylenediamine dihydrochloride). At 546 nm, the absorbance of the chromophore created by nitrite diazotization with sulfanilamide and subsequent coupling with naphthylethylene diamine dihydrochloride was measured. The ability of the extract to scavenge Nitric oxide was calculated using the equation below.

Nitric oxide scavenging activity percentage= $[(A0 - A1)/A0] \times 100$

Where; A0 = Absorbance of control, A1 = Absorbance of sample

2.4. Invitro Anti-inflammatory Activity:

The EEAT was tested for its anti-inflammatory activity by using the following methods.

2.4.1 Inhibition of Albumin Denaturation:

With minimal modifications, an inhibitor of albumin denaturation technique was used in this approach [16]. To prepare the reaction mix, 0.05 mL of distilled water and 0.45 mL of bovine serum albumin (5 % aqueous solution) were combined (0.5 mL; pH 6.3). To reach a pH of 6.3, a small amount of 1 N HCl was utilized. Various plant extract concentrations were added to the reactants and incubated for 20 minutes at 37°C before being heated for 5 minutes at 70°C. 2.5 mL of phosphate buffer saline was added to the Antioxidants samples after they had cooled. Turbidity was determined spectrophotometrically at 600 nm.

Percentage Inhibition (%) = $\frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$

Where: Abs= Absorbance

2.4.2. HRBC Membrane Stabilization Method:

Blood (2 mL) was obtained from healthy volunteers and diluted with an equal amount of sterile Alsever's solution (2 % dextrose, 0.8 % sodium citrate, 0.5 % citric acid, and 0.42 % NaCl in purified water) before centrifugation at 3000 rpm. The loaded cells were rinsed with a saline solution and maintained undisturbed at 4°C in a 10% v/v saline solution. Various concentration of extract (40-200 µg/mL) and aspirin (as a regular and positive control agent, distilled water was used instead of hyposaline to achieve 100 percent hemolysis) were added, then combined with 2 mL of hyposaline, 1 mL of phosphate buffer, and 0.5 mL of 10% HRBC suspension. The blends were incubated for 30 minutes at 37°C, then centrifuged for 20 minutes at 3000 rpm, and the supernatant hemoglobin solution was spectrophotometrically quantified at 560 nm [17].

Stabilization (%) = $\frac{\text{Abs Control - Abs Sample}}{\text{Abs Control}} \times 100$

Where: Abs= Absorbance

2.5. *In Vitro* Anti-chelating Activity:
The EEAT was tested for its anti-chelating activity by using following methods.

2.5.1. Iron chelating activity*:*
The iron chelating property (reducing power) is an important predictor of its potential antioxidant activity. Various concentrations of the extract were combined in 1ml of water with a phosphate buffer (2.5 ml, 0.2 M pH 6.6) and 1 % potassium ferricyanide (2.5 ml). For 20 minutes, the mixture was incubated at 50°C. Trichloroacetic acid (2.5 mL, 10%) aliquots were added to the mixture, which was subsequently centrifuged at 3000rpm for 10 minutes. The solution's upper layer (2.5 ml) was blended with distilled water (2.5 ml) as well as newly made FeCl3 solution (0.5ml, 0.1%). At 700 nm, the absorbance was measured [18].
Reducing power formation (%) =$\frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}}$ X 100.
Where: Abs= Absorbance

2.6. Human Hepatic Cancer Cell Culture.
The human hepatocellular carcinoma (Hep G2) cell line was received from the National Center for Cell Science (NCCS), Department of Biotechnology, Pune, India. The cells were grown in a T25 flask with Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS (Fetal Bovine Serum), 100U/mL penicillin, and 100mg/mL streptomycin. The flask was incubated at 37∘C in a humidified air incubator containing 5% carbon dioxide (CO2).[19]

2.7. MTT assay for cytotoxicity assessments:
The cells' vitality was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay, which is based on mitochondrial dehydrogenase reducing MTT to a purple formazan product in intact cells. Cells were seeded in 96-well microplates (1 ×104 cells/well in 180µl medium) and grown for 24 hours in a humidified incubator (37°C in 5% CO2). EEAT was applied at different concentrations (20, 40, 60, 80, and 100µg/ml) and incubated for 24 hours. The medium was then removed, and 30µl of tetrazolium dye (MTT) solution (5 mg/ml in PBS) was added to each well, which was then incubated for another 4 hours. Following the removal of the untransformed MTT reagent, 100 µl of DMSO was added to dissolve the formazan crystals that had formed. Using an ELISA plate reader, the amount of formazan was measured by measuring the absorbance at 540 nm. [20]

2.8. Morphological analysis:
Under a 20X light microscope, the morphological properties of HepG2 cells after treatment with EEAT at various doses (100-500 g/mL) were studied. After cells were treated with EEAT and cultured for 24 hours, images were acquired and evaluated.

2.9. Acridine Orange and Ethidium Bromide Dual Staining Studies:
Using HepG2 Cell, 25 µL (approx. 1 $\times 10^5$ cells) of treated and untreated cells were taken separately in a micro centrifuge tube and stained with 5 µL of AO-Et Br (acridine orange and ethidium bromide) for about 2 min followed by gentle mixing. Place 10 µL of cell suspension onto a microscopic slide, cover it with a glass cover slip and examine it with a fluorescence microscope using a fluorescein filter. A fluorescence microscope was used to examine the dual staining. [21]

2.10. DNA fragmentation analysis:
HepG2 cells were plated in a 60 mm culture dish at a density of 5×10^5 cells and treated for 24 hours with EEAT (250 and 500 µg/ml). The cells adhered at the bottom were scraped off and centrifuged at 1500 rpm for 5 min at 4ºC with unattached cells. The DNA was prepared from the pelleted cells. The cells were lysed with lysis buffer and extracted with 2ml of phenol (neutralized with Tris-EDTA buffer, pH7.5) followed by extraction with 1 ml of chloroform/ isoamyl alcohol (24:1). The aqueous supernatant was precipitated overnight at -20ºC using 2.5 volumes of ice-cold ethanol and 10% sodium acetate. After centrifugation at 13,000 rpm for 10 min, the pellets were air dried and re-suspended with 50µl of Tris-EDTA buffer. Equal quantities of DNA were electrophoresed in a 1.8% agarose gel containing 0.5 µg/ml of ethidium bromide. After electrophoresis, the gel was photographed under UV light. [22]

2.11. Assay of Lactate Dehydrogenase:
The cells were seeded and treated with various concentrations of EEAT for 24 hours in 37ºC in a 5% CO_2. The cell's suspended media was collected. 0.1 ml of suspended media, 1.0 ml of buffered substrate were added to the tubes and incubated at 37ºC for 15 min. The incubation was continued for another 15 min, after adding 0.2 ml of NAD^+ solution. The reaction was arrested by the addition of 1.0 ml of 2, 4-DNPH reagent and allowed to incubate for 15min at 37ºC. To the blank, 0.1 ml of suspended media was added after arresting the reaction with 2, 4- DNPH. Now 7.0ml of 0.4 N sodium hydroxide solution was added and the color developed was measured at 420 nm in a UV- spectrometer. [23]

2.12. Statistical analysis:
Tests were carried out in triplicate. The findings were analyzed by SD ± mean and they were subjected to Non-linear regression, One-Way ANOVA, in PRISM program version 9.3.1. (Graph Pad Software Inc, USA).

3. RESULT:

3.1. Phytochemical Screening:
The phytochemical analysis of EEAT is shown in Table 1. From the qualitative findings presented in Table 1, it is observed that the EEAT confirmed the presence of phenols, proteins, amino acids, carbohydrates, reducing sugars, alkaloids, flavonoids, quinones, phenols, terpenoids, coumarins and steroids. These are the phytochemicals which are responsible for the antioxidant property either by scavenging free radicals or by preventing their formation [24]. The presence of these bioactive components in *A. tetracantha is* responsible for the obvious anti-inflammatory activities of the extracts of the plant. Absence of saponins, tannins, glycosides, anthraquinone, phylobtannins and anthocyanin was observed in EEAT.

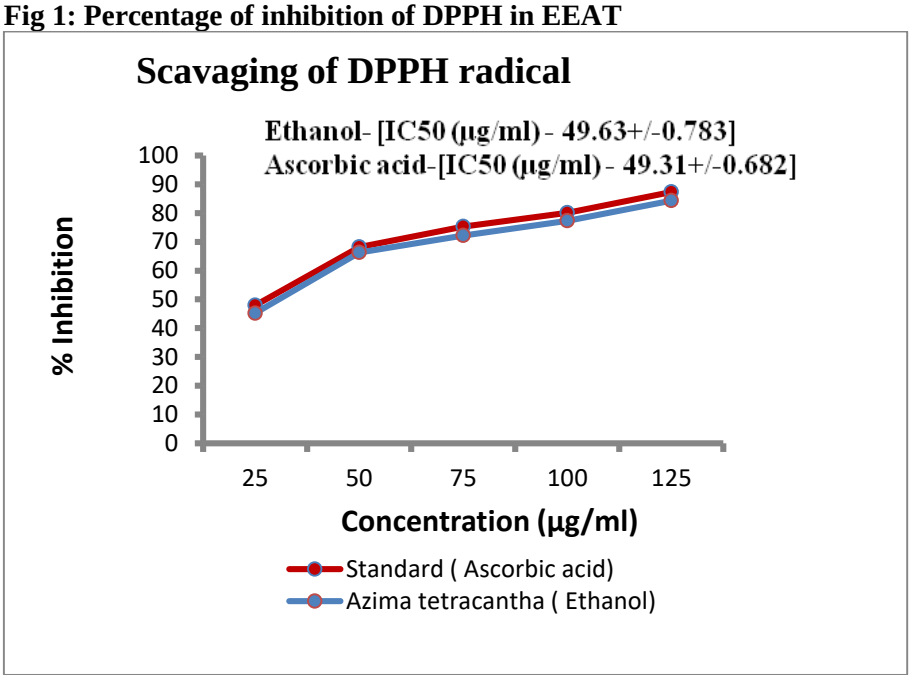
Table1: Quantitative phytochemical analysis of whole plant EEAT studied.

S.No:	Phytochemical constituent	Ethanol extract
1)	Proteins	+
2)	Amino acids	+
3)	Carbohydrates	+
4)	Reducing sugars	+
5)	Tannins	-
6)	Saponins	-
7)	Alkaloids	+
8)	Flavonoids	+
9)	Glycosides	-
10)	Quinones	+
11)	Phenols	+
12)	Terpenoids	+
13)	Coumarins	+
14)	Anthraquinones	-
15)	Steroids	+
16)	Phlobatannins	-
17)	Anthocyanin	-

(+: Positive, −: Negative)

3.2. Invitro Antioxidant Assay:
3.2.1. DPPH (1, 1-Diphenyl-2-picrylhydrazyl) radical scavenging activity:
Azima tetracantha extract showed a dose-dependent scavenging activity and free radical inhibition of DPPH comparable to free radical scavenging activity of ascorbic acid. It shows maximum inhibition of 84.31% at concentration of 125 (µg/ml) **.**The significant difference in percentage inhibition of DPPH of all extracts was compromising in Figure 1 is $P < 0.05$. Moreover, *A.tetracantha* plant extract acquires IC50 49.31 ± 0.783 mg/mL on the scavenging of DPPH. Ascorbic acid used as a standard drug showed an inhibition at 87.31% at 125 (µg/ml) concentration and IC50 value of 49.63±0.682 (µg/ml)**.**

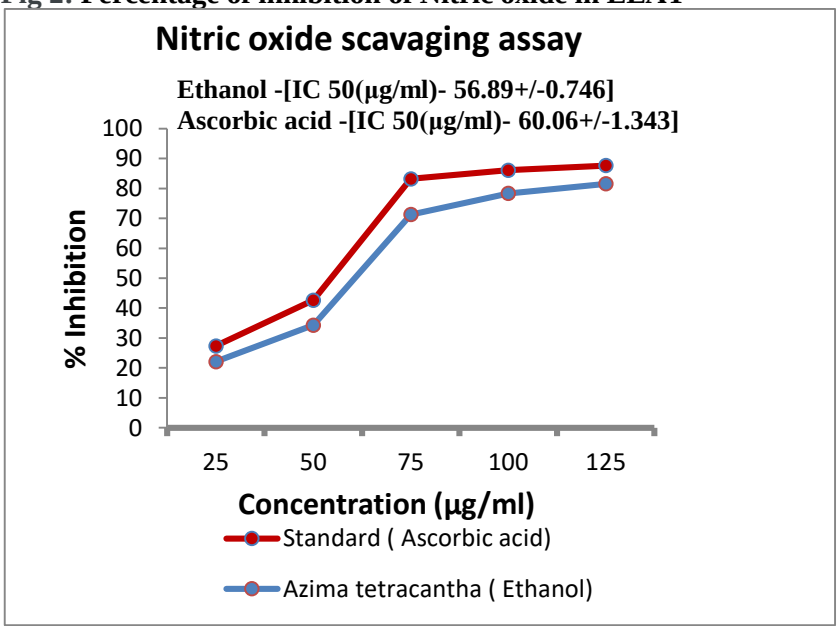
Fig 1: Percentage of inhibition of DPPH in EEAT



3.2.2.Nitric oxide scavenging activity

Azima tetracantha extract showed a dose-dependent scavenging activity and free radical inhibition of NO comparable to free radical scavenging activity of ascorbic acid. There was a minimum suppression of nitric oxide synthesis, with a maximum inhibition of 81.53% at concentration of 125(µg/ml)**.** The significant difference in percentage inhibition of DPPH of all extracts was compromised in Fig 2 is $P < 0.05$. Moreover, *Azima tetracantha* plant extract acquires IC50 56.89 ±0.746 (µg/ml) on the scavenging of NO. Ascorbic acid used as a standard drug showed an inhibition at 87.66% at 125 (µg/ml) concentration and IC50 value of 60.06 ±1.343 (µg/ml)**.**

Fig 2: Percentage of inhibition of Nitric oxide in EEAT

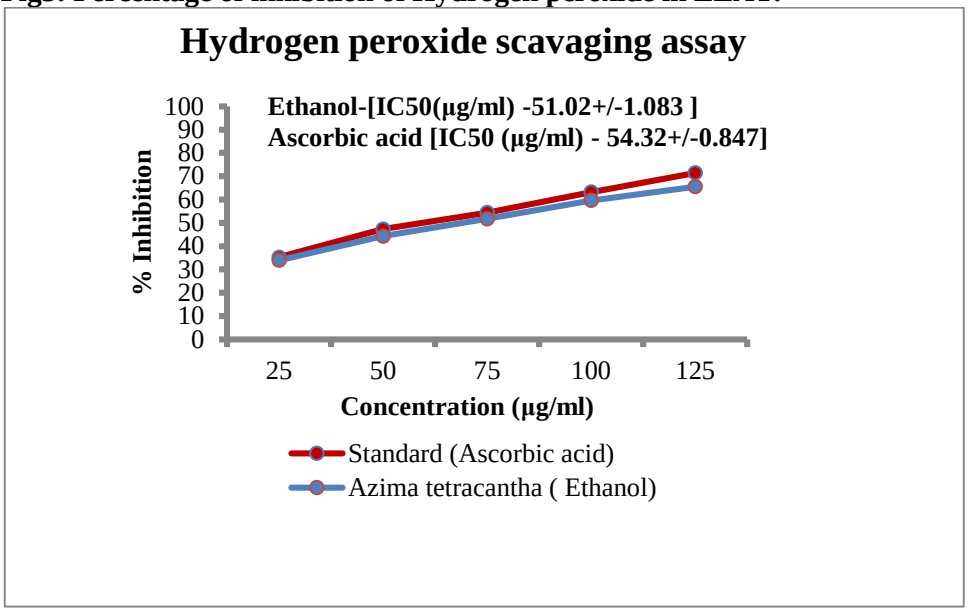


3.2.3. Hydrogen peroxide scavenging activity:

Azima tetracantha extract showed a dose-dependent scavenging activity and free radical inhibition of H_2O_2 comparable to free radical scavenging activity of ascorbic acid. There was a minimum suppression of Hydrogen peroxide scavenging, with a maximum inhibition of 65.52% at concentration of 125(µg/ml)**.** The significant difference in percentage inhibition of DPPH of all extracts was compromising in Fig 3 is $P < 0.05$. Moreover, *A.tetracantha* plant extract acquires IC50 51.02±1.083 (µg/ml)on the scavenging of NO. Ascorbic

acid used as a standard drug showed an inhibition at 71.43% at 125 (µg/ml) concentration and IC50 value of 54.32±0.847 (µg/ml).

Fig3: Percentage of inhibition of Hydrogen peroxide in EEAT.

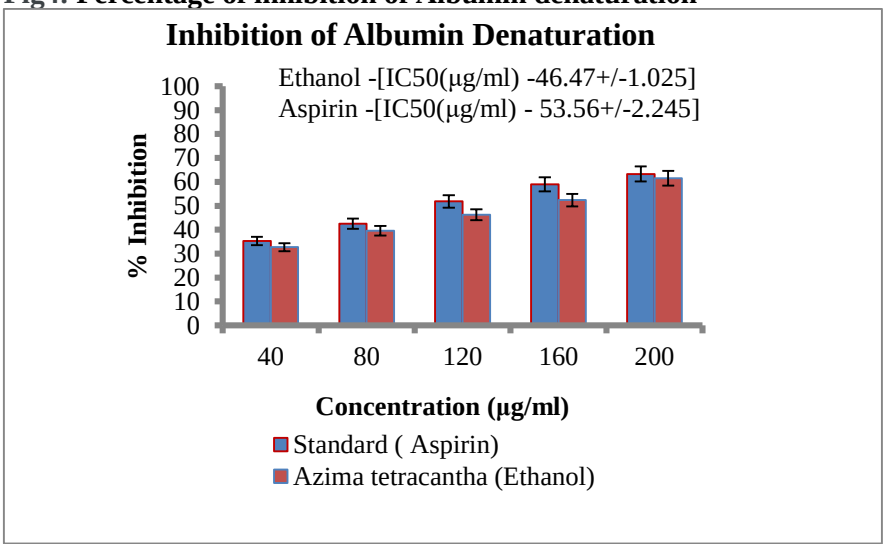


3.3. *In Vitro* Anti-Inflammatory Activity:

3.3.1. Inhibition of Albumin Denaturation:

The effects of anti-inflammatory behavior assessed towards the denaturation of egg albumin are outlined (Fig 4). This figure describes the observations of in vitro anti-inflammatory properties assessed against the denaturation of egg albumin. At a concentration of 200 µg/ml, the highest inhibition obtained was 61.5% and IC50 value of 46.47±1.025µg/ml. Aspirin used as a standard drug showed a 63.3% inhibition at a 200 µg/mL concentration and IC50 value of 53.56±2.245µg/ml. The significant difference in percentage inhibition Albumin Denaturation of Ethanol extract was compromising in Fig 4 $P < 0.05$.

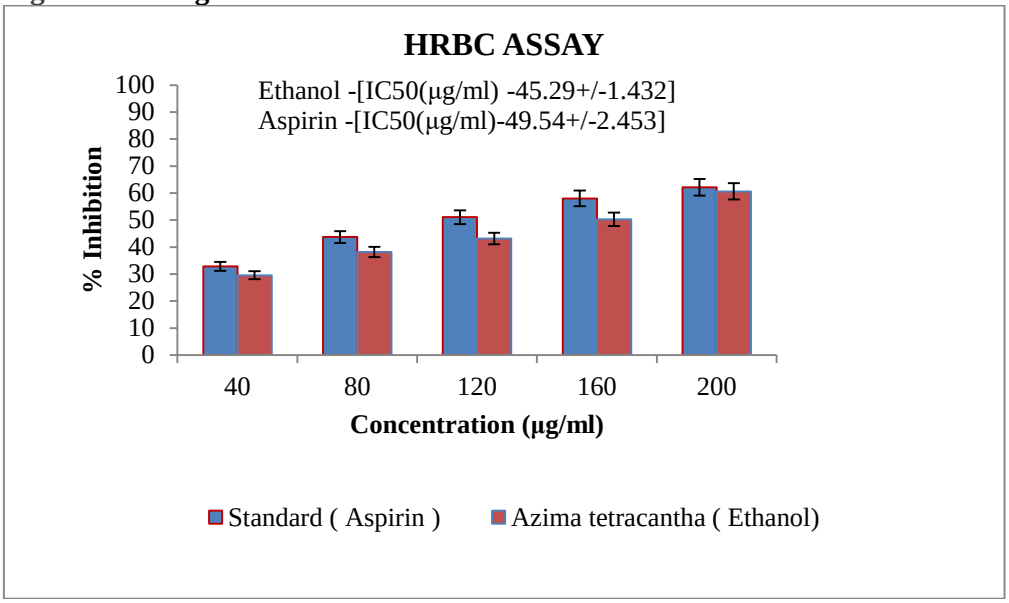
Fig4: Percentage of inhibition of Albumin denaturation



3.3.2. HRBC Membrane Stabilization Method:

In addition, the results of the anti-inflammatory examination for the human red blood cell membrane were revealed. The *Azima tetracantha* ethanol extract produced a 61.21% inhibition of RBC hemolysis at a 200 µg/mL concentration and IC50 value of 45.29+/-1.432 µg/mL, compared with 62.12% produced as standard by the standard drug Aspirin and IC50 value of 49.54+/-2.453 µg/mL (Figure5). The significant difference in percentage inhibition of HRBC Membrane stabilization of EEAT was compromised in Fig 5 is $P < 0.05$.

Fig 5: Percentage of inhibition of HRBC in EEAT in EEAT

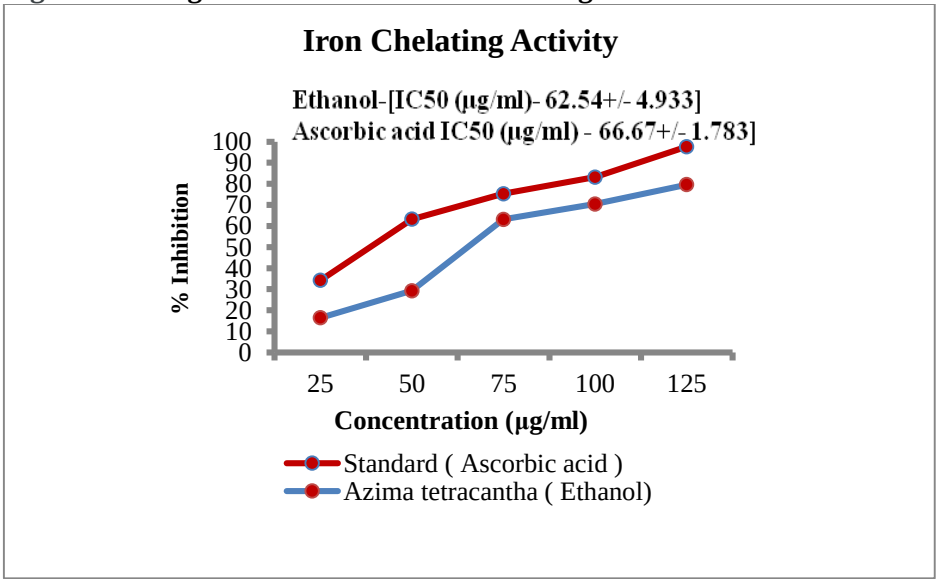


3.4. *In Vitro* Anti Chelating activity:

3.4.1. Iron Chelating Power Assay:

Chelation power assay was carried out to assess the Iron chelation capacity of the crude extracts which illustrated that the crude EEAT possessed remarkable chelation power at 200 μg/ml 79.66% and IC50 value of 62.54±4.933 as compared to standard Ascorbic acid 97.63% and IC50 value of 66.67±1.783. The significant difference in percentage inhibition of Iron chelating of Ethanol extract was compromising in Fig 6 $P < 0.05$ Chelation power of fractions was analyzed and it was observed that the ethanol fraction exhibited significant capacity to chelate ferrous ions (Fig 6).

Fig6: Percentage of inhibition of Iron chelating in EEAT



3.5. MTT assay for cytotoxicity assessments:

The cytotoxicity of treated HepG2 with varying concentrations of EEAT for 24hrs was measured by the MTT assay. The cytotoxic profiles show moderate cell depletion which is displayed in Figures 7. The addition of EEAT to the cell culture medium exhibited a dose dependent cell death. Results are statistically significant ($P < 0.005$). The half maximal inhibitory concentration (IC50) values are presented in Table 2.

Table 2: *In vitro* Cytotoxic activity of EEAT Treated HepG2 cells after 24 h of exposure

	Concentration (µg / ml)	% of Cell Viability
Azima tetracantha (Ethanolic extract)	250	58.75
	500	14.88
IC 50	-	110.6±1.03
R2	-	0.9750

3.6. Morphological study:

The morphological study of the HepG2 cell treated with EEAT showed characteristic features of marked cell death, cell shrinkage whereas control cells observed to be normal. (Fig7)

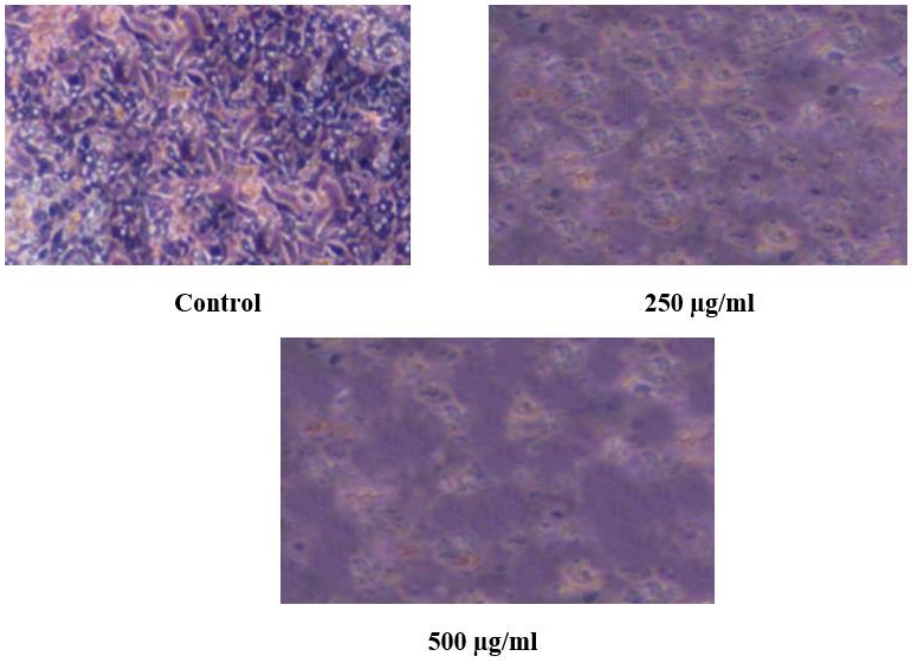


Fig7: *In vitro* Cytotoxic activity of EEAT Treated HepG2 cells after 24 h of exposure

3.7. Acridine Orange and Ethidium Bromide Dual Staining Studies:

The Dual staining of control and EEAT treated HepG2 cells at the concentration of 250µg/ml and 500µg/ml after 24 hrs of exposure are represented in Fig 8. Normal live cells appeared bright green in color whereas EEAT treated groups of apoptotic nuclei of dead cells appeared bright orange. Furthermore, in HepG2 cells, normal nuclei have structured chromatin, whereas apoptotic nuclei have severely condensed chromatin.

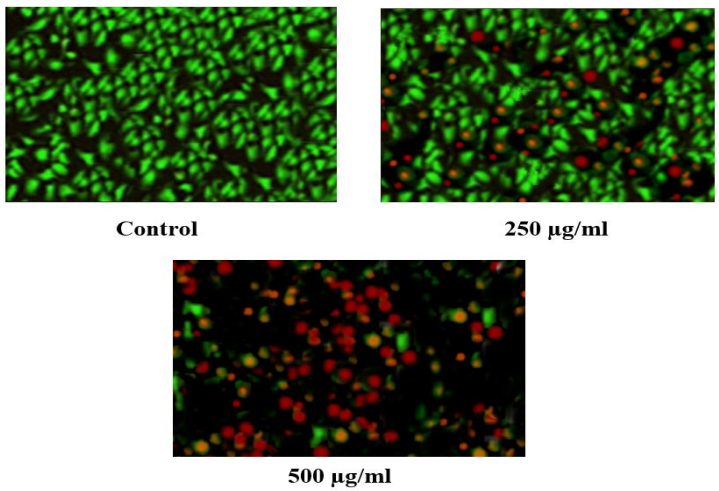
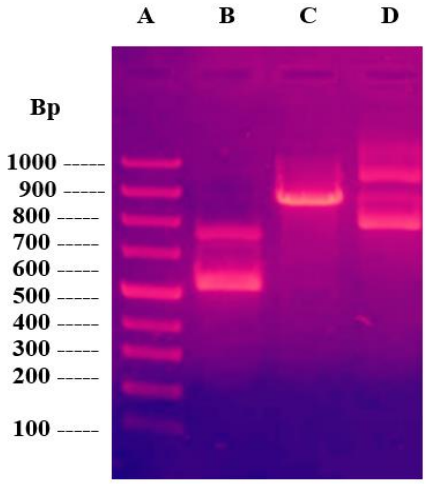


Fig8: Fluorescent microscopic picture of HepG2 cells after 24 hrs of exposure

3.8. DNA Fragmentation

Apoptosis is accompanied by the degradation of chromosomal DNA. Apoptosis is induced by HepG2 after EEAT therapy. The DNA fragmentation results showed that it promoted cell death and caused morphological changes, as illustrated in fig 9.

Fig 9: DNA Fragmentation



*** lane (A) ladder, lane (B) Control, lane (C) 250 µg/ml, lane (D) 500 µg/ml**

3.9. LDH ASSAY :

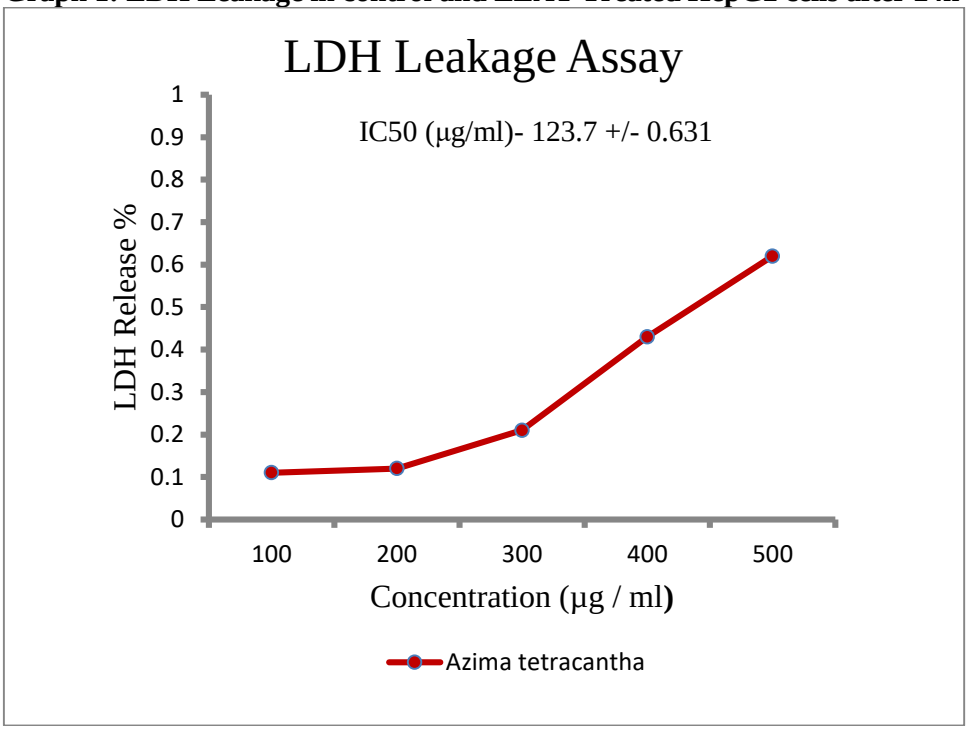
The energy production mechanism was accompanied by an enzyme, Lactate dehydrogenase. The tissue damage represents the leakage of LDH which is measured after the 24 hour. HepG2 cells treated with EEAT are elevated more percentage compared to control cells. The graph 1 indicates the tissue damage takes place at a high level in 24 hours of HepG2 cells. The half maximal inhibitory concentration (IC50) values are represented in Table 3. It also shows significance of $P < 0.005$.

Table3: LDH Leakage in control and EEAT Treated HepG2 cells after 24h of exposure

	Concentration	**LDH activity (µ mol of NADH / per well / min.)**
Control	-	0.09 ± 0.002
DMSO	1% (v/v)	0.10 ± 0.006
***Azima tetracantha* (Ethanolic extract µg / ml)**	100	0.11± 0.006*
	200	0.12 ± 0.01*
	300	0.21 ± 0.03*
	400	0.43 ± 0.03*
	500	0.62 ± 0.03*

Each values represents mean +/- SD
* $P < 0.005$ Vs Control
IC50 (µg/ml)- 123.7 ± 0.631

Graph 1: LDH Leakage in control and EEAT Treated HepG2 cells after 24h of exposure



4.DISCUSSION:

Azima tetracantha is a tribal medicine which is used as a dietary supplement and food item, traditionally in various African countries and Indian traditional systems, [25]. Their therapeutic potentials are associated with their antioxidant potentials which is predicated on its phytochemical content which are present naturally in plants. Now a-days these phytochemicals become more popular because to their countless medicinal uses. It plays an important role against number of diseases like asthma, arthritis, cancer etc. In comparison to pharmaceutical chemicals these phytochemicals don't have any side effects. It's said to be "Man friendly Medicines" because it cures diseases without causing any harm to Humans, [26]. From the result, it shows that the EEAT contains many Phytoconstituents like flavonoids, tannins, phenols, steroids, terpenoids, proteins, carbohydrates and so on. In which Flavonoids are a category of antioxidants that are effective at scavenging DPPH radicals, hydroxyl radicals, and metal-chelating capability, [27]. Flavonoids, Phenols and Tannins are shown to possess a spread of biological actions, including vasodilatory, anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, antiallergic, and antiviral properties, [28]. From our study, it implies that the *A. tetracantha* is rich in flavonoids, Tannins and Phenols. Thus, it shows that the phytoconstituents play an important role in biological activities and is present highly in *A. tetracantha* which makes its therapeutic value higher. These phytoconstituents are liable for antioxidant activity of *A. tetracantha*. They scavenge the reactive oxygen species which are liable for the various diseases and disorders in our body. ROS (reactive oxygen species) are made from both free radicals (O_2 and HO) and non-free radicals (H_2O_2),[29]. However, due to the overproduction of reactive species and/or insufficient antioxidant defence, it leads to acute or ongoing oxidative stress. The detrimental action of free radicals, on the opposite hand, are often inhibited by antioxidant chemicals, which scavenge free radicals and detoxify the organism,[30]. In recent years, there has been a big increase within the use of natural antioxidants, *A. tetracantha* is one among them which is tested for its antioxidant activity against DPPH, Nitric oxide and Hydrogen peroxide. From the results, it postulated that the EEAT have hydrogen donors, which are involved within the scavenging of the radical DPPH by forming the hydrazine. Thus, it is presumed that the ethanol extract acts as a possible therapeutic agent for the control of oxidative damage. *Azima* scavenges the NO by inhibiting the assembly of nitrite due to the presence of flavonoids and tannins in it. Which states that it protects the cells from oxidative damage which prevent cardiovascular diseases, inflammation, diabetes, atherosclerosis, etc [31]. Here, the successive EEAT was tested, where they were found to exhibit a dose dependent hydroxyl scavenging activity. The presence of flavonoid, tannin, phenols, and vitamins accounts for the EEAT radical scavenging power possesses anti-inflammatory activity. The inflammatory response has been linked to the thought of various diseases like arthritis, stroke, and cancer. Protein denaturation has been

linked to the occurrence of the inflammatory response, which leads to a variety of inflammatory disorders, including arthritis, [32]. Tissue harm during life may be due to denaturation of cell protein components or intercellular material. As a result, the capacity of a drug to decrease protein denaturation indicates an obvious potential for anti-inflammatory effect, [33]. The current study depicts the anti-inflammatory activity of *Azima* against Inhibition of albumin denaturation and HRBC membrane stabilization. *Azima tetracantha's* anti-inflammatory capabilities are demonstrated to be promising. This was only preliminary testing to determine concentration-dependent percentages of membrane protection. The study also provides good evidence that *Azima tetracantha* are often used as a possible anti-inflammatory in folkloric and tribal medicine. As a result, the plant could be thought of as a renewable supply of membrane stabilizers, which might be used as an alternate treatment for inflammatory disorders and diseases, [34]. This study also illustrates *Azima tetracantha`s* anti chelating property. Metal ions can cause various anomalies within the body. The iron (II) chelating activity of plant extract is of great significance, because it's been proposed that the transition metal ions contribute to the oxidative damage in neurodegenerative disorders like Alzheimer's and Parkinson's diseases. Also, chelation therapy may be a common practice of neutralizing hemochromatosis within the body especially in cases of treatment of Thalassemia and other anaemia, [35]. The present scenario suggests that the chelation therapy makes use of synthetic compounds which have certain side effects also. Therefore, chelation of metal ions by natural phytochemicals are in consideration. *Azima tetracantha* is a natural anti chelating agent that has an enormous therapeutic importance. It postulates that the *Azima* is used for its therapeutic property against thalassemia, anaemia, Parkinson's disease [36]. The anticancer activity of *Azima tetracantha* was determined by cytotoxicity against human hepatoma cells (HepG2) and induction of apoptosis. Apoptosis has been established as the pharmaco-dynamic target for anticancer treatment in several anticancer activity studies [37]. As a result, this study was conducted to evaluate the in vitro cytotoxicity and apoptotic effects of Ethanolic extract of EEAT against HepG2 cells. The MTT assay, morphological examination, fluorescence microscopy, DNA fragmentation, and LDH measurement were used to evaluate EEAT's anticancer activities. The in vitro pharmacological efficacy is then evaluated using a cytotoxicity assay based on mitochondrial activity. The MTT assay is based on the cellular conversion of tetrazolium salts to brightly coloured formazans. It measures cell respiration as well as formazan production in culture, which is related to the number of live cells present. The amount of formazan formed varies with cell count, indicating the degree of cytotoxicity caused by the drug. [38] According to the findings, EEAT depletes cell growth in a dose-dependent way. As a result, it is stated that the EEAT trigger cell deterioration and apoptosis. The presence of EEAT degrades the cells, causing distinctive alterations such as nuclear membrane degradation, cell shrinkage, and chromatin disorientation. These modifications are the outcome of apoptosis in cells. This explains why EEAT causes apoptosis in malignant cells. The morphological observations revealed the proportion of dead cells and the type of cell death that occurred after treatment. The findings demonstrated that the morphological changes that occurred during cell death were compatible with the hallmarks of cell apoptosis. Early apoptotic cells attract phagocytes without raising inflammation, whereas late apoptotic and necrotic cells emit additional pro-inflammatory danger signals [39]. According to our findings, EEAT-treated HepG2 cells exhibit morphological alterations such as cell shrinkage and death, which are consistent with cell apoptosis. According to this, EEAT causes cell apoptosis in HepG2 cells. To do more research, we employed a fluorescent microscope to identify changes in the nucleus and chromatin material. Acridine Orange (AO) is a fluorescent cationic nucleic acid dye that dyes both living and dead cells. Ethidium Bromide only stains cells with damaged membranes. Early apoptotic cells are stained green with brilliant green patches inside the nucleus as a result of chromatin condensation and nuclear disintegration, but late apoptotic cells incorporate Ethidium Bromide and hence appear orange. [40] According to our findings, EEAT-treated HepG2 cells revealed typical morphological signs of apoptosis, such as nuclear margination and chromatin condensation. When HepG2 is exposed to EEAT, it experiences apoptosis. Apoptosis is marked by chromatin condensation, cell membrane shrinkage, and DNA fragmentation. Apoptosis is defined by blabbing of the plasma membrane, cellular shrinkage, fragmentation of nuclei and chromosomal DNA, and chromatin condensation. Apoptosis was further validated by determining the DNA ladder, which is a result of DNA fragmentation and indicates a late stage of apoptosis. [41]. According to our findings, EEAT-treated HepG2 cells create a DNA ladder, indicating that DNA fragmentation is caused by apoptosis. The LDH leakage assay detects lactate dehydrogenase activity in the extracellular media and is a simple, reliable, and rapid cytotoxicity test. The release of intracellular LDH into the medium is a sign of irreversible cell death caused by cell membrane breakdown. It established the direct role of LDH overexpression in the activation of apoptosis [42]. According to our findings, EEPZ treatment of HepG2 cells increases LDH levels, implying that EEPZ causes cell death via membrane breakdown and induces apoptosis. It concludes that the EEAT has anticancer effect by causing cell degradation and promoting apoptosis [43]. *Azima*

tetracantha (lam) has been used to cure cancer in traditional and folklore medicine. This herb has also been used to treat inflammatory conditions, arthritis, and many forms of toxins [44].

5.Conclusion:

Azima tetracantha is a tribal medicine that has a variety of medicinal characteristics. From the study, we revealed the presence of biologically active constituents in our plant which is responsible for the antioxidant, anti-inflammatory and anti-chelating activity. These biological activities of the plant prove the usage of the plant as a traditional medicine. It also found to have a substantial cytotoxic effect on HepG2 cells, as evaluated by MTT, LDH leakage assay, DNA fragmentation, and morphological examinations. It has the potential to provide powerful agents for the development of anticancer medicines. As a result, *Azima tetracantha* may be explored further for a more comprehensive investigation of its anticancer properties. It can also be used as a treatment for liver cancer. The findings of this study serve as the foundation for future research on this species in order to gain a deeper understanding of its biological activity. It lays the path for the identification of potential therapeutic compounds in *Azima tetracantha* that could be developed into a novel modern drug in future.

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