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## Cytotoxic and antioxidant activity of *Zanthoxylum alatum* stem bark and its flavonoid constituents

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### Abstract

*Zanthoxylum alatum* is used in traditional medicinal systems for a number of diseases like diarrhoea, fever, toothache, inflammation, headache, microbial infections, cancer, etc. The aim of the present study was to evaluate the ethyl acetate extract of *Zanthoxylum alatum* stem bark for its cytotoxic and antioxidant potential and to isolate the bioactive constituent(s). Cytotoxicity of ethyl acetate extract was studied on different cancerous cell lines such as pancreatic, lung, breast and colon cancer using MTT assay. *In vitro* antioxidant potential was evaluated using DPPH, Nitric Oxide scavenging assay and ferric reducing power assay. The estimation of total phenolic compounds in the extract was determined by Folin-Ciocalteu's method. Isolation of compounds from ethyl acetate extract was done on silica gel column. Structure elucidation was done by using various spectrophotometric techniques like UV, IR, NMR and MS spectroscopy. All statistical analysis was conducted using Graph Pad Prism software. Ethyl acetate extract of plant has shown significant cytotoxic potential on lung and pancreatic cancer cell lines and also shown antioxidant potential. Flavonoids; apigenin and kaempferol-7-O-glucoside have been isolated from column and has shown significant cytotoxic potential. Flavonoids isolated from ethyl acetate extract were responsible for cytotoxic activity of the extract. Hence the *Zanthoxylum alatum* can be further explored for the development of anticancer drug.

**Keywords:** Apigenin, Cytotoxic, Kaempferol-7-O-glucoside, *Zanthoxylum alatum*

### 1. Introduction

Cancer can be defined as a disease in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division [1]. It is a serious, frightening disease. About a third of humans develop cancer in a lifetime [2]. It is caused by both external factors (tobacco, chemicals, radiation and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions and mutations). Treatment for cancer includes local treatments, such as radiation therapy, surgery and systemic treatments such as chemotherapy and targeted therapy. Despite considerable progress in the management of cancer by conventional synthetic drugs, the search for natural anti-cancer plant products for controlling cancer is very important as synthetic drugs has many side effects [3].

Today much attention has been devoted to natural antioxidant and their association with health benefits. Plants are the potential source of natural antioxidants. Reactive oxygen species (ROS) are generated as byproducts of biological reactions and from exogenous factors [4]. Excess ROS, if not eliminated by antioxidant system, results in high levels of free radicals which causes oxidative stress [5]. Oxidative stress arising from free radicals is the basis of many diseases such as cancer [6, 7]. The curative effects of several medicinal plants are usually due to antioxidant phytochemicals present in it such as polyphenols, flavonoids and phenolic compounds [8].

*Zanthoxylum alatum* Roxb. (ZA) (Syn. *Zanthoxylum armatum*, Rutaceae) is a strong aromatic shrub commonly known as 'Timur' [9]. It is distributed in Himalayas from Kashmir to Bhutan upto 2100 m and in Khasia hills upto 1350 m [10]. The bark of the plant is reported to contain a bitter crystalline principal identical with berberine and it also contains volatile oil, phenolic compounds and resin [11]. The fruit contains about 1.5% of an essential oil consisting chiefly of 1- $\alpha$ -phellandrene with small amounts of linalool. Leaves yields an essential oil which is a carbonyl compound identified as methyl n-nonyl ketone. The roots yields the alkaloids such as dictamine, magnoflorine, fagarine, skimmianine, xanthoplanine [12, 13]. Reported pharmacological activities of ZA in different parts are anti-proliferative [14], hepatoprotective [15, 16], larvicidal [17], antibacterial, antifungal, anthelmintic [18], anti-inflammatory [19], antioxidant [20], antispasmodic, antidiarrhoeal, bronchodilator and in cardiovascular disorders [9], lousicidal potential [21], antidysesthetic [22], piscicide [23], cytotoxic [24]. Phytochemically stem bark

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contains alkaloids, steroids, phenolic compounds and flavonoids.

In continuation of our investigation of *Zanthoxylum alatum* stem bark [25]; the present study was carried out to isolate active compounds from ethyl acetate extract which can be used against cancer.

## Materials and Methods

The stem bark of ZA was collected from the local areas of Tehri (Garwal), Uttrakhand, India and authenticated from NISCAIR, New Delhi (Ref. NISCAIR/RHMD/Consult/2013/2233/14) by Dr. Sunita Garg. Stem bark was shade dried (<40 °C), coarsely powdered and stored in air tight container.

All solvents used were of analytical grade and purchased from Rankem (Ranbaxy, fine chemical ltd.). Silica gel 60 F<sub>254</sub> (E-Merck), silica gel (60-120) used for thin layer chromatography (TLC) and column chromatography was procured from Loba. NMR spectra were recorded on bruker 400 MHz spectrometer using TMS (Tetramethylsilane) as the internal standard and Mass spectra were recorded on ESI-esquire 3000 bruker daltonics instrument. IR spectra were obtained with a Nicolet 380 FTIR spectrometer (Thermo Scientific) in KBr pellets and UV spectra were recorded on a SHIMADZU UV-1700 (double-beam) spectrophotometer.

The cell lines A-549, MIA-PaCa, MCF-7 and CaCo-2 were obtained from National Center for Cell Sciences NCCS, Pune (India). RPMI-1640, Nutrient Mixture F-12 Ham Kaighns Modification and Dulbecco minimum essential eagle medium (DMEM), Foetal bovine serum (FBS), penicillin and streptomycin, MTT reagent, 0.5% Trypan Blue and acridine orange/ethidium bromide were purchased from Himedia.

## Extraction, isolation and purification of compound

The stem bark was shade dried and coarsely powdered with mechanical grinder. The powdered drug (1 kg) was extracted by continuous hot extraction process using soxhlet apparatus with petroleum ether (60-80 °C). The marc obtained was then extracted with ethyl acetate. The total extract was concentrated under reduced pressure to afford a brown semi-solid residue. The dried ethyl acetate extract was subjected to column chromatography in order to isolate bioactive compound(s). Column was packed with silica gel (60-120 mesh size). It was initially eluted with dichloromethane and ethyl acetate and then with ethyl acetate and methanol in gradient manner. Each fraction of 50 ml was collected, concentrated in rota evaporator and left for crystallization. Total number of fractions collected were 420. The fractions having same R<sub>f</sub> values were pooled together. Purification of the isolated compounds was carried out by repeated recrystallization technique. Compound A was isolated from fractions 310-356 (ethyl acetate:methanol, 90:10). Crystals were separated from mobile phase and purification was done by recrystallization using acetone and methanol. Compound B was isolated from fractions 370-395 (ethyl acetate:methanol, 85:15) and purified by successive washing with cold methanol.

The structures of compounds were determined by UV, IR, NMR and MS spectroscopy.

## Cell culture, establishment and maintenance:

All cell lines were maintained in DMEM and nutrient mixture of f-12 Ham's medium supplemented with Penicillin (100 Units/mL), Streptomycin (100 µg/mL) and 10% (v/v) heat inactivated fetal bovine serum (FBS). Cells were maintained in 5% CO<sub>2</sub> humidified incubator at 37 °C. Subculturing was conducted by trypsinization (0.25%) when they were reached 80% confluence. Growth medium was changed every three days.

## Cytotoxicity assay

The prepared extract and isolated compounds has been subjected to cytotoxic assay. The cells of all cell lines were plated 24 h prior to testing in 96 well plates at a density of 3000 cells/well in 100 µL of the medium. After an overnight incubation triplicate wells were treated with varying concentration of extract, docetaxel and compound ranging from (1-150 µg/mL) and incubated for 3 days. After 3 days medium was replaced with 2 µL of MTT solution (5 mg/mL) and cells were incubated for 3 hours. The relative percentage of metabolically active cells compared with untreated controls and then determine on the basis of mitochondrial conversion of 3-(4, 5-dimethylthiazol-2-yl) 2, 5 diphenyltetrazolium bromide (MTT) to Formazan crystals which were dissolved in dimethylsulfoxide (DMSO). Spectrophotometric absorbance of sample was determined by using micro plate reader (BIORAD) at 570/630 nm [26]. Concentrations of sample showing 50% reduction in cell viability (i.e. IC<sub>50</sub>) were then calculated. Control cells (unexposed cells) were taken as 100% viable. The percentage inhibition was calculated by the formula

$$\% \text{ inhibition} = (\text{OD of Control}) - (\text{OD of Treated}) / (\text{OD of Control}) \times 100.$$

OD: Absorbance of each well.

## In vitro antioxidant studies

The ethyl acetate extract was also tested for their free radical scavenging property using different *in vitro* models. All experiments were performed thrice and their results averaged. L-Ascorbic acid was used as standard control in each experiment. Results were expressed in IC<sub>50</sub> values.

## DPPH radical scavenging activity

0.1 mM solution of DPPH in methanol (22.2 mg in 1000 mL) was freshly prepared. Different concentrations of extract and standard (25-400 µg/mL) were added at an equal volume to methanolic solution of DPPH. After 30 min at room temperature, the absorbance was recorded at 517 nm [27]. Radical scavenging activity was calculated by the following formula

$$\% \text{ inhibition} = A_{\text{control}} - A_{\text{test}} / A_{\text{control}} \times 100 \text{ (formula 1)}$$

where, A<sub>control</sub> = Absorbance of control reaction and A<sub>test</sub> = Absorbance of samples of extracts.

## Nitric oxide radical scavenging activity

2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract at various concentrations (25-400 µg/mL) and the mixture was incubated at 25 °C for 150 min. From the incubated mixture 0.5 mL was taken out and added into 1.0 ml sulfanilic acid

reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 mL naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm [28]. The nitric oxide radicals scavenging activity was calculated by the formula 1.

### Reducing power assay

The extract (0.75 mL) at various concentrations (25-400 µg/mL) was mixed with 0.75 mL of phosphate buffer (0.2 M, pH 6.6) and 0.75 mL of potassium ferricyanide (1%, w/v) followed by incubating at 50 °C in a water bath for 20 min. The reaction was stopped by adding 0.75 mL of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10 min. 1.5 mL of the supernatant was mixed with 1.5 mL of distilled water and 0.1 mL of ferric chloride ( $\text{FeCl}_3$ ) solution (0.1% w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power [29].

### Estimation of total phenolic content

The total concentration of phenolic compounds in the extract was determined using a series of gallic acid standard solutions (20-100 µg/mL). Gallic acid solution was prepared by dissolving 10 mg in 10 mL water to make 1 mg/mL solution. The extract (10 mg) was dissolved in 10 mL of water to prepare 1mg/mL solution of extract. Each extract solution (0.1 mL) was mixed with 2 mL of a 2% (w/v) sodium carbonate solution and vortexed vigorously. The same procedure was also applied to

the standard solutions of gallic acid. After 3 min, 0.1 mL of 50% Folin-Ciocalteu's phenol reagent was added and each mixture was vortexed again. The absorbance at 760 nm of each mixture was measured after incubation for 30 min at room temperature and the concentration of the sample solution was determined from the calibration curve. Results were expressed as milligrams of total phenolics content per grams of extract as gallic acid equivalents (GAE) [30].

### Calculation

Total phenolic contents (%) = GAE x V x D x  $10^{-6}$  x 100/W  
Where, GAE- Gallic acid equivalent (µg/ml), V- Total volume of sample (ml), D- Dilution factor, W- Sample weight (g)

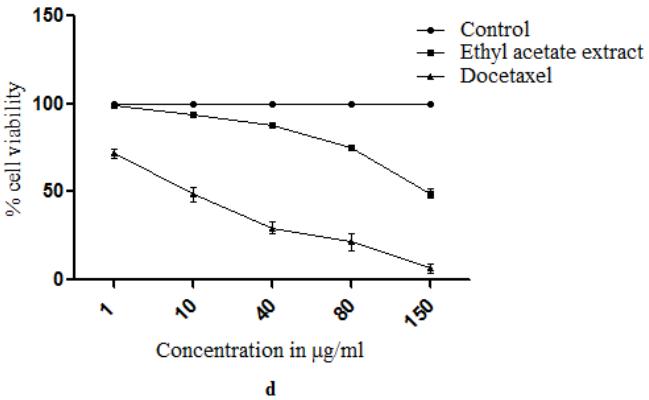
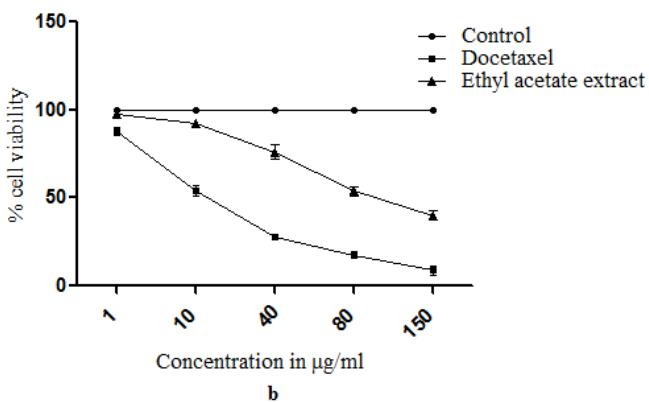
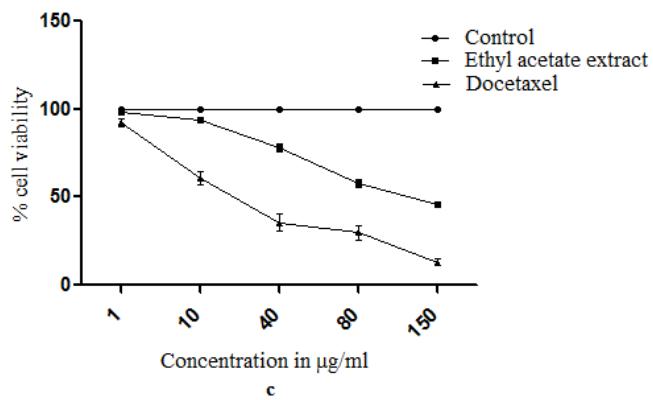
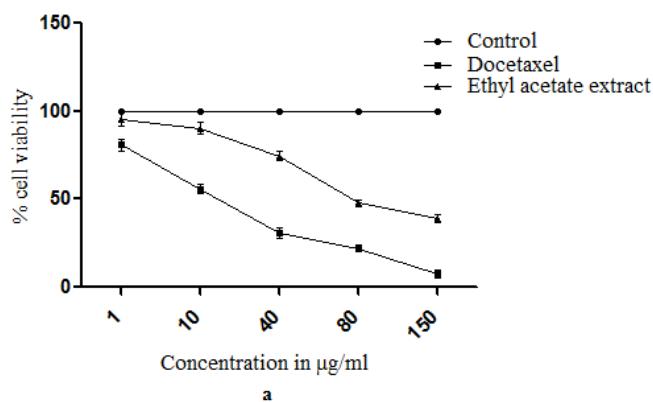
### Statistical analysis

Statistical significance was compared between various treatment groups and controls using t-test and ANOVA. Data were considered statistically significant when P-values were <0.05. All analysis was done in triplicate and results are expressed as mean  $\pm$  S.D. All statistical analysis were conducted using Graph Pad Prism software version 5.

### Results

#### Cytotoxic assay

The ethyl acetate extract (6.5% w/w) of ZA caused significant inhibition of viability in A-549 and MIA-PaCa cancer cell lines (Fig. 1a,b,c,d). Results were expressed in IC<sub>50</sub> values (inhibitory dose inhibited cell growth by 50%).

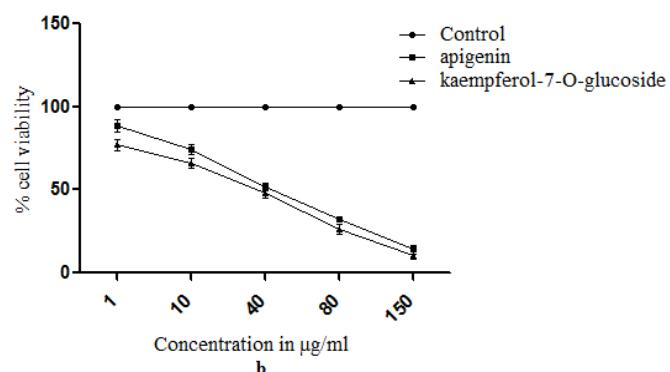
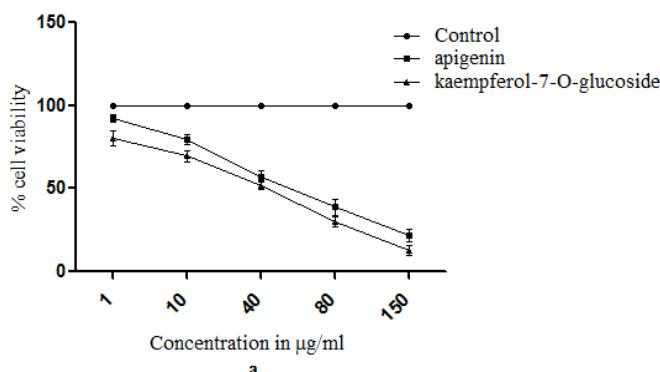


**Fig 1:** Effect of ethyl acetate extract on a) MIA-PaCa, b) A-549, c) MCF-7 and d) CaCo-2 cancer cell line. Values were expressed as mean  $\pm$  SD, (n=3)

The isolated compounds has shown more cytotoxicity than ethyl acetate extract (Fig. 2a, b). In case of A-549 lung cancer cell line the IC<sub>50</sub> value for ethyl acetate extract was found to be 85.33 ± 1.52 µg/mL whereas for apigenin and kaempferol 7-O-glucoside was 53.66 ± 4.72 µg/mL and 35.34± 3.51 µg/mL respectively and in case of MIA-PaCa pancreatic cancer cell

line IC<sub>50</sub> value for ethyl acetate extract was found to be 78.0 ± 1.63 µg/mL whereas for apigenin and kaempferol 7-O-glucoside was 44.33 ± 1.52 µg/mL and 48.43± 2.08 µg/mL respectively (Table 1).

The results of MTT assay revealed that both extract and isolated compounds showed cytotoxic activity.



**Fig 2:** Effect of apigenin and kaempferol-7-O-glucoside on a) Mia PaCa and b) A-549 cancer cell line. Values were expressed as mean ± SD, (n=3)

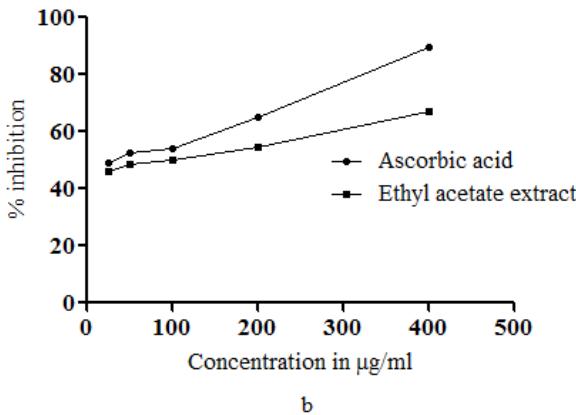
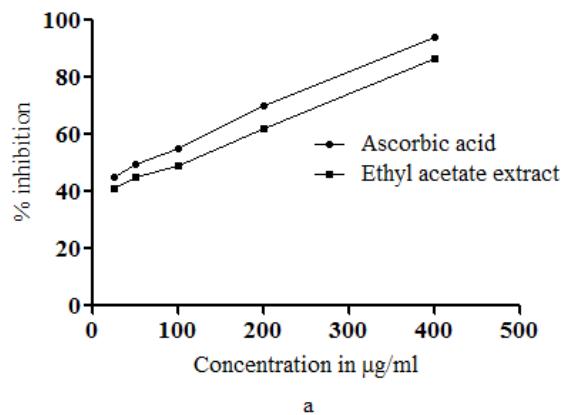
**Table 1:** IC<sub>50</sub> Values of Ethyl Acetate Extract, Apigenin and Kaempferol-7-O-Glucoside on Various Cancer Cell Lines

S. No.	Extract	IC <sub>50</sub> value (µg/ml)			
		MIA-PaCa	A-549	MCF-7	CaCo-2
1.	Ethyl acetate	78.0 ± 1.63	85.33 ± 1.52	113.66 ± 2.08	138.0 ± 2.64
2.	Apigenin	44.33 ± 1.52	53.66 ± 4.72	-	-
3.	Kaempferol-7-O-glucoside	48.43± 2.08	35.34± 3.51	-	-

Each value in the table is expressed as mean ± SD of three determinations

#### Antioxidant activity

The ethyl acetate extract has also shown antioxidant potential. The antioxidant activity increased with increasing concentration in all the models. The percentage inhibition of standard and extract in DPPH and nitric oxide scavenging assay are shown in Fig. 3a,b.



**Fig 3:** a) DPPH radical scavenging effect of Ascorbic acid and ethyl acetate extract and b) NO radical scavenging effect of Ascorbic acid and ethyl acetate extract

IC<sub>50</sub> values of Ascorbic acid and ethyl acetate extracts in DPPH model was found to be 57.30 ± 1.90 and 99.25 ± 2.53 and in case of NO model 42.80 ± 2.61 and 94.81 ± 2.56 respectively. Ethyl acetate extracts has shown reducing power as observed in (Table 2). Higher absorbance indicates more reducing power

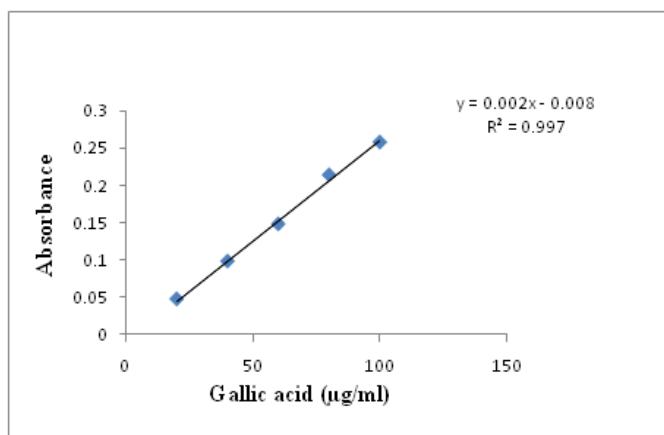
**Table 2:** Reductive Ability of Standard and Various Extracts of *Zanthoxylum Alatum* Stem Bark

Conc. (µg/ml)	Absorbance	
	Ascorbic acid	Ethyl acetate Extract
25	0.127 ± 0.005	0.062 ± 0.007
50	0.212 ± 0.008	0.128 ± 0.008
100	0.467 ± 0.006	0.317 ± 0.008
200	0.897 ± 0.005	0.569 ± 0.009
400	2.121 ± 0.007	1.287 ± 0.009

Each value in the table is expressed as mean ± SD of three determinations

### Total phenolic content

The standard ascorbic acid and ethyl acetate extract were determined for its total phenolic content on the basis of its gallic acid equivalent Folin-Ciocalteu phenol assay. The amount of gallic acid equivalent was determined from the calculation of calibration curve of gallic acid (Fig. 4). The phenolic content found in ethyl acetate extract was 4.36 mg/g GAE.



**Fig 4:** The calibration curve of standard gallic acid.  
Identification of Compounds

Compound A was isolated as yellow colored crystals, 45 mg (0.45% w/w yield);  $R_f$  0.46 (Chloroform: methanol, 90:10); m.p. 342 °C-348 °C. Compound gave intense yellow colour with dil. KOH. UV  $\lambda_{max}$  (methanol): 268 and 332 nm. After addition of  $AlCl_3$   $\lambda_{max}$  (methanol) shifted to 275 and 381 nm. The shift is stable after the addition of HCl; indicating the presence of OH at C-5 position. On addition of sodium acetate it showed the usual shift of benzoate band indicating presence of OH's at C-7 and C-4' positions.

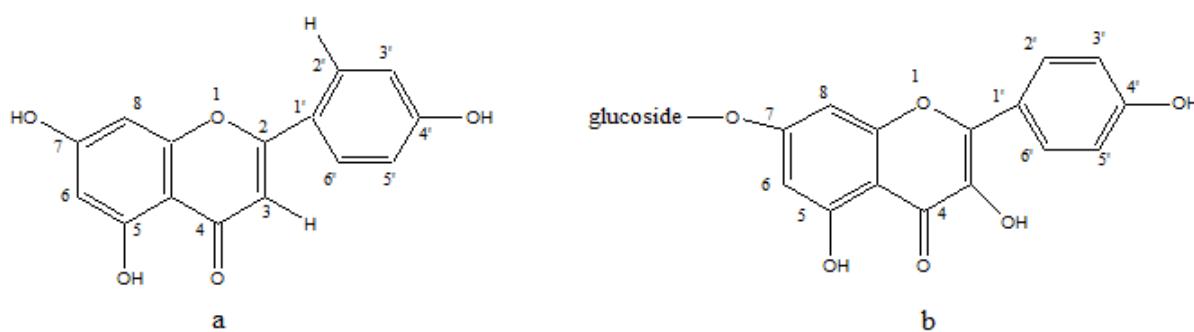
IR (KBr,  $cm^{-1}$ ) spectrum showed stretching for -OH at 3284, for C=O at 1653.74, 1607.56 for aromatic, C-O stretching at 1354, C=C stretching at 1557.56.  $^1H$ NMR ( $CDCl_3$ , 400 MHz,  $\delta$  with TMS=0) showed signals at 7.925 (d, 2H, H-2', H-6',  $J$  = 8.8), 6.8 (s, 1H, H-3), 6.927 (d, H-3', 5',  $J$  = 8.2), 6.489 (d,

1H, H-8,  $J$  = 2.1), 6.199 (d, 1H, H-6,  $J$  = 2.1).  $^{13}CNMR$  displayed signals at C-4, >C=O ( $\delta$  181.72), C-8 ( $\delta$  164.09), C-2 ( $\delta$  163.71), C-5 ( $\delta$  161.41), C-7 ( $\delta$  161.13), C-6 ( $\delta$  157.27), C-2' and 6' ( $\delta$  128.45), C-1' ( $\delta$  121.13), C-3', 5' ( $\delta$  115.92). MS ES-:  $m/z$  270 and  $M^{+}-2$  at 268.

Thus, on the basis of above spectral data compound A was characterized as Apigenin (Fig. 5a) having molecular formula  $C_{15}H_{10}O_5$ .

Compound B was isolated as pale yellow colored crystals, 35 mg (0.35% w/w yield);  $R_f$  0.34 (Chloroform: methanol, 90:10); m.p. 268 °C- 271 °C; UV  $\lambda_{max}$  (methanol): 266 and 351 nm. After addition of  $AlCl_3$   $\lambda_{max}$  (methanol) shifted to 270 and 390 nm. The shift is stable after the addition of HCl; indicating the presence of OH at C-5 position.  $^1H$ NMR (DMSO, 400 MHz,  $\delta$  with TMS=0) showed signals at 8.0 (d, 2H, H2', H6',  $J$  = 8.68 Hz), 7.3 (d, 2H, H5', H3',  $J$  = 8.68 Hz,), 6.1 (s, 1H, H6), 6.3 (s, 1H, H8), 5.3 (s, 1H, H1"); glucose anomeric proton), 4.47-5.22 (m, remaining sugar proton). The sugar was identified after hydrolysis by boiling with 2% HCl followed by basification. Then it was concentrated and run on descending paper chromatography (*n*-butanol: acetic acid: water, 4:1:5) and compared with standard glucose. Thus identified as glucose. Mass spectral analysis of Compound B showed intense peak at 286 of aglycone along with major fragments  $m/z$  at 152 [ $M^{+}-C_7H_4O_4$ ], 134 [ $M^{+}-C_8H_6O_2$ ] and one sugar attached making it monosaccharide i.e.  $[M^{+}+1]$  at  $m/z$  385. Since flavonoid glycoside is fragile molecule, we could not get a molecular ion peak at  $m/z$  448. However, it appears that fragmentation pattern shows the loss of two molecules of water (2x18) and CO (12+16= 28) as indicated by fragment at  $m/z$  385 [ $M^{+}+1$ ].

Thus, on the basis of above spectral data compound B was characterized as Kaempferol-7-O-glucoside (Fig. 5b) having molecular formula  $C_{21}H_{20}O_{11}$ .



**Fig 5:** Structure of a) apigenin and b) kaempferol-7-O-glucoside

### Discussion

The use of natural herbal medicines or dietary agents is being increasingly utilized as an effective way for the management of many cancer treatments. Plants are an excellent source of

chemical compounds with a wide variety of biological activities including anticancer properties. The present study has been designed to isolate the bioactive constituent(s) from ethyl acetate extract of ZA stem bark. Phytochemical

screening of ethyl acetate extract revealed the presence of glycosides, steroids, phenolic compounds and flavonoids. The extract exhibit significant cytotoxic and antioxidant activity. Ethyl acetate extract and isolated compounds in various concentrations (1-150 mg/ml) were screened for its cytotoxic potential by using MTT assay on different cancer cell lines. They caused significant inhibition of viability of cancer cell lines. Results were expressed in IC<sub>50</sub> values. The antioxidant activity was screened by employing established *in vitro* assays such as DPPH, NO, Ferric reducing power. These are the rapid, simple and inexpensive method to measure the antioxidant capacity. It has been considered that there is an inverse relationship between dietary intake of antioxidant rich foods and incidence of human diseases [8].

Column chromatography of ethyl acetate extract lead to the isolation of a two pure flavonoids, apigenin and kaempferol-7-O-glucoside. Flavonoids are the polyphenolic compounds and show a strong antioxidant and free radical scavenging activity [31, 32]. They appears to be associated with reduced risk for certain chronic diseases [33], the prevention of some cardiovascular disorders [34] and certain kinds of cancerous processes [35]. Flavonoids were also reported to inhibit cell growth and proliferation [36] and induce cell toxicity [37] in cancer cells. The ability of flavonoids to exert cytotoxic activity towards cancer cells has generated interest in developing flavonoid based cytostatics for anticancer therapy [38]. Apigenin and kaempferol isolated from other plants has shown anticancer activity in previous study [39-42]. This indicates that our findings seem to be consistent with other previous reports. Apigenin is reported first time from this plant. UV spectrum of methanolic solution of apigenin and Kaempferol-7-O-glucoside supported the flavones structure. The position of hydroxyl particularly if it is located at C-5 position shows  $\lambda_{max}$  bands at their respective position in UV absorption spectroscopy. If hydroxyl is located at C-5 position, the C-5 hydroxyl and C-4 Ketone get strongly chelated on addition of AlCl<sub>3</sub> in methanol with result cinnamate band shifts at least by 50-60 nm towards longer wavelength while band due to benzoylate also shifts to longer wavelength by 10-20 nm. The vicinal hydroxyls at any position also chelates with AlCl<sub>3</sub> but after addition of HCl the chelate bond breaks and the  $\lambda_{max}$  shifts to original positions. In the present case the apigenin showed UV  $\lambda_{max}$  at 332 and 268 nm which shifts to 381 and 275 nm after the addition of AlCl<sub>3</sub> and kaempferol showed UV  $\lambda_{max}$  266 and 351 nm which shifts to 270 and 390 nm after the addition of AlCl<sub>3</sub>. The shift is stable even after the addition of HCl; indicating the presence of OH at C-5 position. On addition of sodium acetate it showed the usual shift of benzoylate band indicating presence of OH's at C-7 and C-4' positions. The UV and NMR data was in good agreement with that of apigenin [43] and Kaempferol-7-O-Glucoside [44, 45]. Therefore isolated principles from ethyl acetate extract of ZA can provide lead to drug discovery for anticancer potentiality.

## Conclusion

Two flavonoids, apigenin and kaempferol-7-O-glucoside were isolated from the ethyl acetate extract of dried stem bark of *Zanthoxylum alatum* Roxb. Apigenin has been isolated first time from the plant. The ethyl acetate extract and both flavonoids has potent cytotoxic activity against pancreatic and

lung cancer cell lines. This proves the uses of the plant in folk medicine. Ethyl acetate extract has a good antioxidant activity supporting the use of the plant as a natural source of phenolic, as antioxidant and anticancer.

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