



Assessment of Antioxidant and Cytotoxic Activities of Extracts of Some *Ziziphus* Species with Identification of Bioactive Components

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Authors' contributions

This work was carried out in collaboration between both the authors. Authors MBP and SNS designed the study, wrote the protocol, performed the statistical analysis and wrote the first draft of the manuscript. Author SNS managed the analysis of the study and the literature searches. Both the authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate antioxidant activity of crude extracts and cytotoxic activity of partially purified bioactive compounds of *Ziziphus mauritiana* and *Ziziphus oenoplia* extracts.

Study Design: Experimental study.

Background: Breast and lung cancers are known to cause high morbidity and mortality worldwide. *Ziziphus* plants are widely grown species known for their folkloric implications with bioactive phytochemicals believed to be responsible for pharmacological activity.

Materials and Methods: Antioxidant action was determined with crude bark extracts and *in vitro* antiproliferation activities were determined by using partially purified fractions obtained from bark extracts of *Z. mauritiana* and *Z. oenoplia* using DPPH and MTT assay, respectively. Possible active ingredients in the potent fractions were identified by Gas Chromatography – Mass Spectrometry (GC-MS).

Results: Considerable antioxidant activity was demonstrated by *Z. mauritiana* methanol extracts

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(ZMMA); while *Z. mauritiana* alkaloid fraction (ZMA) exhibited highest cytotoxic activity (IC_{50} - 19.35 μ g/ml) against human lung carcinoma A549 cells and *Z. oenoplia* alkaloid fraction (ZOA) against human adenocarcinoma mammary gland MDA-MB-231 cells through MTT assay, its further analysis witnessed reduced MMP expression using gelatin zymography. GC-MS technique employed to identify the bioactive compounds of most potent alkaloid fraction ZMA revealed existence of isoquinoline, morphinan, glaucine and pyrazoline compounds.

Conclusion: Our study concludes occurrence of strong antioxidant, antiproliferative and a possible anti-invasive activity to support robust traditional belief in the medicinal properties of *Ziziphus* species.

Keywords: *Ziziphus mauritiana*; *Ziziphus oenoplia*; antiproliferative activity; antioxidant activity; anti-invasive activity.

1. INTRODUCTION

Plants contain essential components responsible to cure several ailments, serving as natural source of medicine and play an important role in the development of potent therapeutic agents [1].

The genus *Ziziphus* is one such category known to possess ethnomedicinal properties comprising some important species like *Z. jujuba*, *Z. mauritiana*, *Z. spinachristi*, *Z. lotus*, *Z. spinosa* etc. *Ziziphus* is a genus that includes about 40 species of spiny shrubs in the buckthorn family as a fruit tree [2]. There are large numbers of traditional benefits of *Ziziphus* plants since ancient times- leaves, fruits, seeds and barks of these plants have been used medicinally. *Z. mauritiana* (ZM) plant parts traditionally used are: kernels - sedative effect, stop nausea, relieves abdominal pain in pregnancy and wounds healing; seeds - for diarrhea and oral contraceptives; leaves - as an astringent, diaphoretic and prescribed for typhoid in children; bark - as an astringent in gingivitis, for the treatment of diarrhoea and dysentery [3,4]. Bark extracts of ZM are reported to have antiulcer, spermicidal and antimicrobial properties [5]. According to Ayurveda and traditional knowledge, *Z. oenoplia* (ZO) plant parts are also used to cure diseases: root- wound healing, ulcer, ascaris infection, stomachalgia, gastrointestinal disorder; fruit- stomach ache; root- as an astringent, anthelmintic, digestive and antiseptic property [6].

Normal essential metabolic processes within the cell produce free radicals and other reactive species that can attack important macromolecules leading to cell damage. Natural protective mechanisms against the destructive effect of free radicals are antioxidants, a substance capable to neutralize free radicals or

its effects. The term oxidative stress refers to the consequence of imbalance between free radical generation and antioxidant defense. Oxidative stress is assumed to play a crucial role in the aging process and in pathology of age-related diseases. Reactive Oxygen Species (ROS) hampers the normal functioning of a number of biological molecules such as nucleic acids, membrane lipids or enzymes. Hence, plant derived antioxidants might stabilize or neutralize free radicals either by acting as anti-free radical molecules, or by chain-breaking of free radical propagation or by interaction with transition metal ions and show a beneficial role in preventing free radical related diseases [7].

Traditional Chinese Medicine also involves use of *Ziziphus* extract for cancer treatment. The *Ziziphus* extracts have also been reported to possess various therapeutic potential, including cytotoxicity against HCT15, HL-60, Molt-4, Hela and few other cancer cell lines [3,7].

Cancer cells metastasize through the route involving multiple step processes, but the most significant turning point in cancer is the establishment of distant metastasis [8]. Migratory and invasive capabilities help cancer cells invade surrounding tissues and gain direct access into the blood vessels [9]. Motility and invasion of tumor cells into new target tissues results in the formation of a secondary tumor. Degradation of matrix proteins of the basement membrane is essential for the cell to become metastasized. An over-expression of proteolytic enzymes, like zinc-dependent endopeptidases of matrix metalloproteinases (MMPs) family; MMP-2 and MMP-9 (gelatinase A and B) play a crucial role in degrading most extracellular matrix (ECM) components that form the basal membrane [10]. MMP-2 and MMP-9 are associated to tumor invasion and metastasis due to their capacities for tissue remodeling through the extracellular

matrix, basement membrane degradation and induction of angiogenesis. Thus, these gelatinases are essential for tumor cell migration, tumor spreading, tissue invasion of tumor cells, and metastasis [11,12]. Hence by monitoring the level of these two proteins in presence of *Ziziphus* fractions could provide a clue for their mechanism of action.

The objective of present study comprises screening for antioxidant activity of different crude extracts and also the investigation for antiproliferative action along with anti-invasive effects shown by active fractions of different extracts of *ZM* and *ZO*.

2. MATERIALS AND METHODS

2.1 Reagents and Standards

The MTT salt [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide], Dimethylsulphoxide (DMSO), trypan blue, antimycotic antibiotic, all the cultivation media and other additives used for cell culture, also L-ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Himedia (Mumbai, India). All the HPLC grade solvents used were purchased from SRL (Mumbai, India).

2.2 Cell lines and Culture Media

Human adenocarcinoma mammary gland cells MDA-MB-231, human lung carcinoma cells A549 and normal human hepatic cells WRL-68 were purchased from National Centre for Cell Sciences (NCCS), Pune, India. MDA-MB-231 cells were cultured in Leibovitz's L-15 medium with 2 mM L-glutamate (no carbon dioxide), WRL-68 cells were cultured in the same medium as MDA-MB-231 cells (5% carbon dioxide) and A-549 cells were cultured in Ham's F-12 medium with 2 mM L-glutamate (5% carbon dioxide), all supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin.

2.3 Plant Material

Ziziphus mauritiana Lam. and *Ziziphus oenoplia* (L) Mill. Gard. were identified by taxonomist of University Department of Botany, RTM Nagpur University, Nagpur, Maharashtra, India, where a specimen is conserved with the voucher number as RTMNU BD 9138 and 9140, respectively.

2.4 Extract Preparation

Bioactive compounds from the bark of *ZM* and *ZO* were extracted using ethyl acetate and the extract was designated as ZMEA and ZOEa [13]. Another method followed was initial extraction with ethyl acetate followed by methanol (A) extraction of *ZM* (ZMMA) and *ZO* (ZOMA) using soxhlet apparatus [14]. Isolates were also obtained as brownish crude residue with dichloromethane extract of *ZM* (ZMDCM) and *ZO* (ZODCM). This procedure of extraction was adopted as stated by Perez et al. [15]. Extraction method started with methanol containing ascorbic acid at low temperature [16]. This was followed by ethyl acetate and then with methanol (B) extract of *ZM* (ZMMB) and *ZO* (ZOMB). Last extraction method was performed using a mixture of dioxane-water (96:4, v/v) to isolate aqueous-dioxane extract of *ZM* (ZMAD) and *ZO* (ZOAD) as stated by Ramasamy [17]. All these crude extracts were screened for antioxidant activities.

2.5 Partial Purification

Crude extracts were further processed to isolate specific partially purified compounds. ZMEA and ZOEa extracts were column chromatographed over silica gel (60-120 mesh size) and eluted with benzene:ethyl acetate mixture in 2:1 ratio to obtain *ZM* triterpenoids (ZMT) and *ZO* triterpenoids (ZOT) fractions, as mentioned by Kundu et al. [18]. ZMMA and ZOMA extracts were also passed through silica gel 60-120 mesh size column and eluent of ethyl acetate: methanol (1:3) provided *ZM* flavonoids (ZMF) and *ZO* flavonoids (ZOF) as main fractions during partial purification [14]. Similarly, ZMDCM and ZODCM extracts when subjected to silica gel column chromatography and eluents from CH₂Cl₂-MeOH (1:8) furnished *ZM* alkaloids (ZMA) and *ZO* alkaloids (ZOA) fractions, by the method as mentioned by Perez et al. [15]. In the same way, ZMMB and ZOMB extracts were applied on equilibrated Sephadex LH20 in 80% ethanol and eluted in 50% acetone to get partially purified tannin fractions [16] as *ZM* tannins (ZMTn) and *ZO* tannins (ZOTn). Likewise, ZMAD and ZOAD extracts were further processed in distilled water and then finally in absolute ether to get partially purified fractions of *ZM* lignins (ZML) and *ZO* lignins (ZOL), by the method as stated by Ramasamy [17].

Each extract obtained was phytochemically screened for the determination of constituents using standard methods of analysis [19,20].

2.6 Determination of Antioxidant Activity

2.6.1 DPPH radical scavenging assay

The radical scavenging potential was estimated by DPPH method [21]. When the DPPH solution (1, 1-diphenyl-2-picrylhydrazyl) is mixed with a substance capable of donating a hydrogen atom, it produces a reduced form of compound (diphenylpicrylhydrazine) with loss of violet color. The crude solvent extracts [dichloromethane (DCM), methanol (M-A), ethyl acetate (EA), methanol (M-B) and aqueous-dioxane (AD)] of both the *Ziziphus* species were mixed with 1.5 ml freshly prepared DPPH solution (0.05 mM prepared in methanol) at 1, 5 and 10 mg/ml. The change in colour from deep-violet to light-yellow was measured spectrophotometrically at 517 nm after incubation in dark for 30 min at 37°C. DPPH solution was used as negative control and ascorbic acid (250 µM) as reference compound. Tests were carried out in triplicate. The radical scavenging activity was performed to investigate the existence of antioxidant potential of crude *Ziziphus* bark extracts. By comparing the test results with control (without extract) the DPPH percentage inhibition was calculated by using following formula [21],

$$\text{DPPH radical Scavenging activity or \% Inhibition} = \left[\frac{A_c - A_s}{A_c} \right] \times 100$$

Where, A_c is the absorbance of DPPH and A_s is the absorbance of extract

2.7 Evaluation of Cell Viability Assay

2.7.1 Cell proliferation assay

Cytotoxic activity of partially purified *Ziziphus* fractions against WRL-68, MDA-MB-231 and A549 cells was determined by the colorimetric method of MTT reduction [22]. Succinate dehydrogenase present in mitochondria of the cell, reduces tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to insoluble formazan crystals and this activity can be used to estimate functions of viable cells. Five dilutions (10, 25, 50, 75 and 100 µg/ml) of each fraction of both the *Ziziphus* species were tested against the cancer cell lines for 48 h and analyzed for cell

proliferation by MTT assay. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a direct measure of activity of viable cells [23].

2.7.2 Substrate gel analysis

Gelatinolytic activities for the presence or absence of secreted MMPs were analyzed by gelatin substrate gel electrophoresis or zymography. Using this technique both active and latent species can be visualized. Samples (20 µl) were prepared in 2% (w/v) SDS and 10% (v/v) glycerol. Gelatinase zymography was performed on 10% (w/v) polyacrylamide gel copolymerised with 0.2% (w/v) gelatin. Following electrophoresis the gels were washed twice in 50 mM Tris-HCl, pH 8.0, containing 5 mM CaCl_2 , 1 µM ZnCl_2 and 2.5% (w/v) Triton X-100 for 15 min to remove SDS, followed by two washes for 5 min each in 50 mM Tris-HCl, pH 8.0, containing 5 mM CaCl_2 and incubated overnight in the same buffer at 37°C. The gels were stained for 30 min with Coomassie Brilliant Blue R-250 (0.5%) prepared in a mixture of 50% methanol and 10% glacial acetic acid and destained in 7% acetic acid [24].

2.8 Gas Chromatography – Mass Spectrometry

A Varian 4500 GC coupled with Varian MS240 ion trap mass spectrometer (Varian, Walnut Creek, USA) was employed for the determination of analytes using electron ionization (EI) mode. Aliquotes of 1 µl sample were injected using a split programmable temperature injection (STI) Type 1079 kept at 270°C. The ion trap, manifold and the transfer line were kept at 240, 40 and 250°C, respectively. Separations were performed on Varian Chrompack Capillary column WCOT Fused Silica (30 m long, 0.25 mm ID) CP-Sil 8CB and helium (Ultra pure 99.99%) was employed as a carrier gas. Compounds were identified by direct comparison of their MS with data from the NIST library.

2.9 Statistical Analysis

The results were expressed as $\text{Mean} \pm \text{S.E.M.}$ using GraphPad Prism 5.0 (GraphPad, USA). The differences were determined using one-way ANOVA followed by Dunnett's multiple comparison test. IC_{50} was estimated using non-linear regression method (curve fit) with dose-response inhibition of plots for the percentage of

antiproliferation activity against the concentration of tested compounds. Significance level was set at $p=0.05$.

3. RESULTS AND DISCUSSION

Newman and Cragg [25] reported that more than half of the new chemicals approved by World Health Organization were derived directly or indirectly from natural products. Based on this information, ZM and ZO plants traditionally used for treatments of several diseases were examined for their therapeutic activity and presence of bioactive compounds.

3.1 Antioxidant effects of *Ziziphus* Extracts

3.1.1 DPPH radical scavenging assay

Natural antioxidants from medicinal plants are of increasing interest as they prevent deleterious effects of free radicals in biological system, leading to cure numerous ailments and may replace synthetics by natural ones. Results presented in Table 1 indicate the potential free radical scavenging property of crude extracts in a concentration-dependent manner as evaluated by its ability of trapping the unpaired electrons of DPPH. *Ziziphus* extracts were able to reduce the purple-colored radical DPPH to yellow colored radical DPPH-H at par to the standard ascorbic

acid, expressing stronger reducing power. ZMMA extract showed a high antioxidant activity, with an IC_{50} value of 4.11 ± 0.21 mg/ml compared to Ascorbic acid that produced IC_{50} values of 3.94 ± 0.20 mg/ml, while ZOAD (IC_{50} - 5.20 ± 0.36 mg/ml) exhibited the least activity. However, all the crude extracts exhibited significant antioxidant activity.

Based on previous data, it is shown that the powerful antioxidant activity of polar extracts is due to the presence of substances with free hydroxyls [26]. In this context, flavonoids possess an ideal structure for the scavenging of free radicals, since they present a number of hydroxyls acting as hydrogen-donators which makes them important antioxidant agents [27]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, as key role in neutralizing free radicals, quenching singlet and triplet oxygen species, or decomposing peroxides. Results indicate that ZMMA obtained from *Z. mauritiana* bark holds great capability to donate hydrogen therefore represents effective DPPH scavenging activity. Dahiru and Obidoa [28] have shown flavonoid, tannin and phenolic compounds of *Z. mauritiana* with protective effects against oxidative damage induced by chronic alcohol administration in rats. In addition, strong antioxidant property was also demonstrated *in vitro* by ethanolic bark extract of *Z. mucronata* subsp. *mucronata* [21].

Table 1. Radical scavenging activity of *Z. mauritiana* and *Z. oenoplia* bark extracts

<i>Ziziphus</i> species	Concentration			IC_{50} (mg/ml)
	1 mg	5 mg	10 mg	
<i>Z. mauritiana</i>				
ZMDCM	04.41 \pm 0.37	29.83 \pm 0.16	59.17 \pm 0.34	5.02 \pm 0.24
ZMM-A	05.70 \pm 0.18	69.85 \pm 0.72	74.45 \pm 0.51	4.11 \pm 0.21
ZMEA	04.11 \pm 0.29	30.13 \pm 0.60	57.58 \pm 0.36	5.01 \pm 0.27
ZMM-B	04.54 \pm 0.53	59.91 \pm 0.49	67.84 \pm 0.27	4.69 \pm 0.22
ZMAD	02.96 \pm 0.12	06.37 \pm 0.37	21.29 \pm 0.54	5.22 \pm 0.35
<i>Z. oenoplia</i>				
ZODCM	02.99 \pm 0.50	11.03 \pm 0.25	38.01 \pm 0.41	5.17 \pm 0.34
ZOM-A	04.03 \pm 0.33	33.42 \pm 0.51	65.08 \pm 0.23	5.01 \pm 0.20
ZOEA	03.98 \pm 0.18	14.12 \pm 0.36	43.90 \pm 0.07	5.15 \pm 0.37
ZOM-B	03.87 \pm 0.45	28.53 \pm 0.21	61.69 \pm 0.58	5.04 \pm 0.23
ZOAD	02.87 \pm 0.28	08.19 \pm 0.78	29.14 \pm 0.32	5.21 \pm 0.36
Ascorbic acid	09.03 \pm 0.01	84.99 \pm 0.04	87.95 \pm 0.03	3.94 \pm 0.20

Data are presented as Mean \pm S.E.M of three replicates. The abbreviations used denote respective species viz., ZM- *Z. mauritiana* and ZO- *Z. oenoplia*. Abbreviates also denote the solvents used for extraction viz., DCM- dichloromethane; M-A- methanol; EA- ethyl acetate; M-B- methanol containing ascorbic acid and AD- aqueous-dioxane

3.2 Effect of Active Fractions of *Ziziphus* Extract on Viability of Cultured Cells

3.2.1 MTT assay

Partially purified active fractions of *Ziziphus* bark extracts were analyzed for antiproliferative properties against MDA-MB-231 and A549 cells after checking the effects and concentration of *Ziziphus* fractions on WRL-68 cells. Most potent fractions were investigated for anti-invasive effect to find out their mode of action and further analyzed for the presence of promising bioactive components using GC-MS (Fig. 1).

WRL-68 cells were treated with different concentrations of *Ziziphus* fractions (50, 100, 150, 200 and 250 µg/ml) to study the cytotoxic effects, before analyzing its antiproliferative activity on cancer cells. Data clearly reveals that the partially purified *Ziziphus* fractions are less toxic towards WRL-68 cells even at 250 µg/ml concentration (Fig. 2A).

Results obtained with the active fractions of both the *Ziziphus* species showed significant dose-dependent antiproliferative action on the mentioned cancer cell lines (Fig 2). Several reports in the literature exemplify susceptibility of different tumor cell lines to cytotoxic agents [29]. ZM fractions illustrate enhanced antiproliferative activity against A549 cells presenting an order of relative potencies: ZMA > ZMT > ZMF > ZMTn,

while ZML showed no activity (Fig. 2B). Our results are in agreement with literature reporting potent therapeutical effects of *Z. mauritiana* extracts [3,7].

On the contrary, ZO fractions illustrated effectual antiproliferation against MDA-MB-231 cells, as the activity was witnessed in following order: ZOA > ZOT > ZOF > ZOTn, ZOL gave no activity (Fig. 2C). Estimated growth reduction clarifies ZMA (19.35±0.86 µg/ml) and ZOA (25.85±0.47 µg/ml) with highly significant antiproliferation against A549 and MDA-MB-231 cells, respectively (Table 2). A similar previous study had also reported the potent action of extracts of *Z. spina-christi* possessing efficient cytotoxic potency towards Hela and MDA-MB-468 cells [29].

3.3 Identification of Active ZMA Fraction by GC-MS analysis

Most potent bioactive fraction was subjected to compound identification by GC-MS. Based on the mass spectrum comparison with NIST library, ZMA demonstrated the existence of four possible compounds (Fig. 3) as Isoquinolin-6-ol, 7-methoxy-1-methyl- (1), Morphinan, 7,8-didehydro-4,5-epoxy-3,6 dimethoxy-17- methyl-, (5α,6α)- (2), Glaucine (3), 2-Pyrazoline, 5-(1,1-dimethylethyl)-5-hydroxy-3-trifluoromethyl-1-(2-isopropyl-5-methylphenoxyacetyl)- (4).

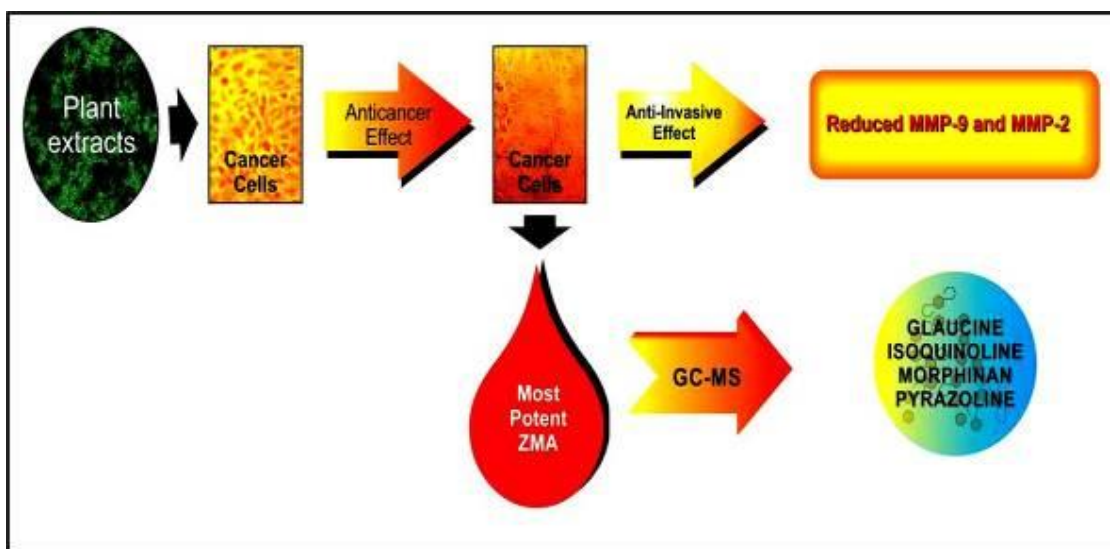


Fig. 1. Schematic representation for the effect of most potent partially purified alkaloid fraction of *Z. mauritiana* (ZMA) bark on A549 cancer cell lines

ZMA - *Z. mauritiana* alkaloid fraction; MMP – matrixmetallo proteinase; GC-MS – Gas Chromatography – Mass Spectrometry

Table 2. Potent fractions of *Z. mauritiana* and *Z. oenoplia* with IC₅₀ values against A549, MDA-MB-231 cancer cell lines and WRL-68 normal cells

Cell lines	<i>Ziziphus</i> active fractions	IC ₅₀ (µg/ml)
A549	ZMA	19.35±0.86
	ZMT	29.96±0.75
	ZMF	34.22±1.91
MDA-MB-231	ZOA	25.85±0.47
	ZOT	37.37±0.16
	ZOF	65.56±0.55
WRL-68	ZMA	73.95±0.93
	ZMT	58.03±1.41
	ZMF	64.31±1.06

IC₅₀ is concentration that reduces cell viability of cancer cell lines to 50%

The data is expressed as Mean±S.E.M from the three triplicate experiments. Range of concentrations: A549 and MDA-MB-231 cancer cells (10, 25, 50, 75, 100 µg/ml), WRL-68 cells (50, 100, 150, 200, 250 µg/ml)

Table 3 and Fig. 3 show the chromatogram and chromatographic data. Peak in the respective spectrum at 184 m/z (100%), 146 m/z (65%), 174 m/z (13%), 147 m/z (10%) seems to be isoquinolin-6-ol 7-methoxy-1-methyl- (1). Another peak at 313 m/z (100%), 59 m/z (40%), 229m/z (26%), 282 m/z (21%) corresponds to morphinan,7,8-didehydro-4,5-epoxy-3,6 dimethoxy-17-methyl-, (5 α ,6 α)- (2) and the peak 355 m/z (100% relative intensity), 340 m/z (45%), 354 m/z (15%), 297 m/z (10%) was consistent with the occurrence of glaucine (3). Peak at 149 m/z (100%), 400 m/z (20%), 57 m/z (20%) seems to be 2-pyrazoline, 5-(1,1-dimethylethyl)-5-hydroxy-3-trifluoromethyl-1-(2-isopropyl-5-methylphenoxyacetyl)-(4).

Potent antioxidant ZMMA extract was also subjected to identification of possible compounds by GC-MS. Results showed presence of known bioactive compounds like stigmasterol, carotene, flavonoids like kaempferol and luteolin with few other compound derivatives already reported to possess several therapeutic properties including antimicrobial effects [30].

The GC-MS chromatogram analysis of the most effective fraction had revealed 4 compounds (Fig. 3). Isoquinolone, morphinan, glaucine and pyrazoline compounds identified through GC-MS analysis of ZMA fraction are already reported with pharmacological activity. Some details about these compounds also mention certain

drawbacks such as, glaucine used as antitussive agent, also with anticonvulsant and antinociceptive properties, acts by blocking calcium channels in smooth muscles like human bronchus but with symptoms of nausea, vomiting and dilated pupils [31]. Morphine and some derivatives employed as an opioid analgesic drug to treat acute and chronic pain, however concerns with a prognostic that constipation, addiction, tolerance and hormone imbalance are other multiple actions causing side effects [32,33]. On the contrary isoquinoline alkaloids like berberine along with traditional usage have been experimented to show anti-inflammatory, antidepressant, neuroprotector, antineoplastic effects and also beneficial effects on HIV, diabetes mellitus and cardiovascular system [34,35]. Pyrazoline derivatives have been reported by Sharma [36] quoting references with anti-inflammatory, analgesic, antimicrobial, antifungal, antidepressant and also anticancer activity. Synergistic activity of these compounds conquering the described side effects cannot be overlooked. This forms a matter of further investigation.

3.4 Effect of Potent *Ziziphus* Fractions on MMP-2 and MMP-9 Level of Cultured Cells

3.4.1 Substrate gel analysis

Cancer cells proliferate in defiance of normal control, but can invade and colonize surrounding tissues giving rise to secondary tumors called metastasis [37]. Invasion and metastasis is dependent on degradation of the extracellular matrix (ECM), by proteases, particularly MMP-2 (gelatinase A; 72 kDa type IV collagenase) and MMP-9 (gelatinase B; 92 kDa type IV collagenase) expressions [24]. Any agent or drug which can interfere with any of these steps can significantly reduce the metastatic potential and can be useful in inhibition of tumor metastasis. To investigate the mechanism of cell death induced by *Ziziphus* fractions in cancer cell lines, zymography analysis was performed to confirm the expression of MMPs as a marker to assess anti-invasive action. The ZMA and ZOA fractions of *Ziziphus* species showing high cytotoxic potential were investigated for anti-invasive activity to diagnose the mode of action by gelatin zymogram analysis. In our study, treatment of A549 and MDA-MB-231 cells with ZMA and ZOA fractions, respectively, were found to reduce the expression of MMP-9 and MMP-2, while ZMF

demonstrated negligible difference in the level of MMPs. Reduction of band intensity and sizes of MMP-9 and MMP-2 compared to the control was observed after developing the gelatin zymograms (Fig. 2D). Previously, we have reported the *in vitro* anticancer activities of ZM

bark fractions with apoptotic effects on MDA-MB-231 and A549 cells [38]. Thus, the alkaloids of ZM and ZO reflect persuasive antiproliferation and anti-invasive effect with their respective cell lines.

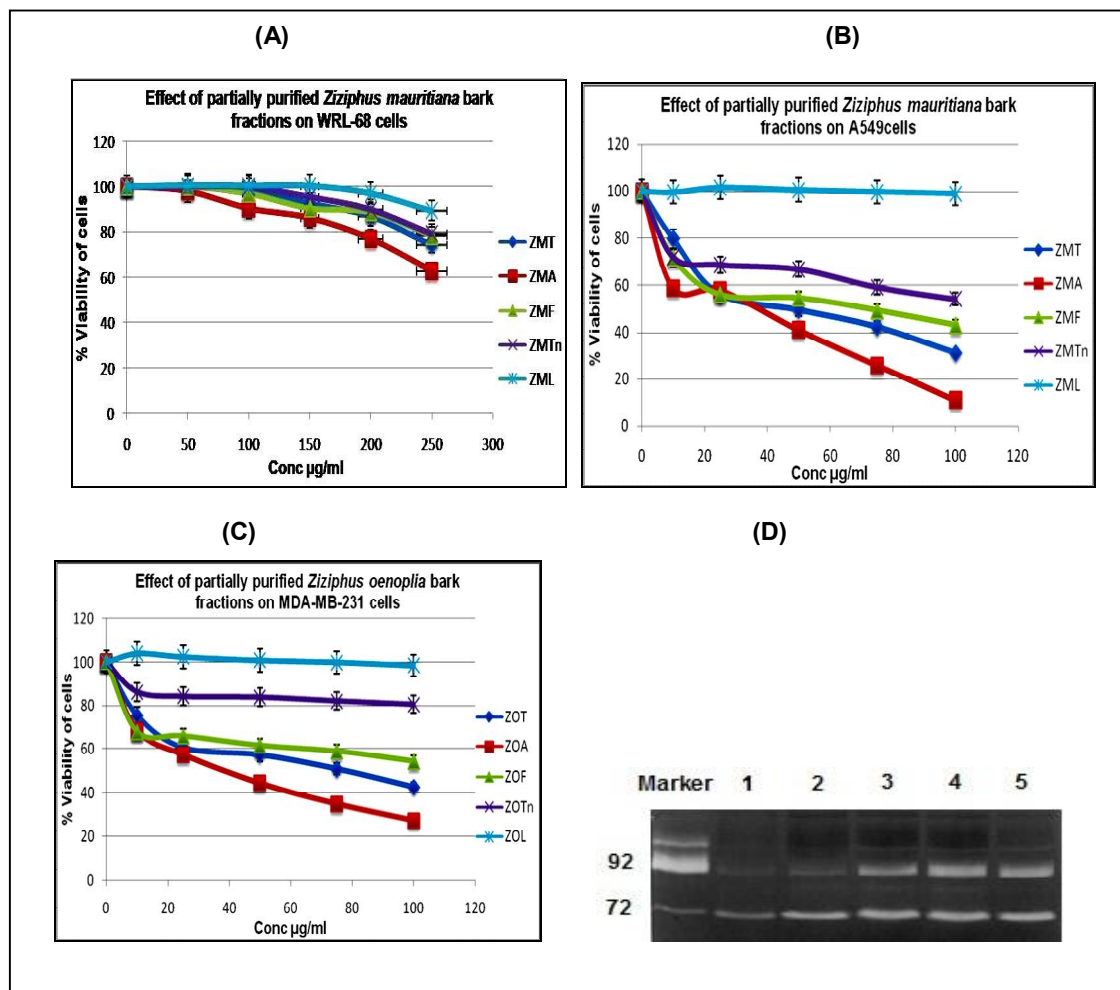


Fig. 2. Proliferation inhibition evaluated for *in vitro* cytotoxicity. Effect of: (A) *Z. mauritiana* fractions on WRL-68 cells, (B) *Z. mauritiana* fractions on A549 cells, (C) *Z. oenoplia* fractions on MDA-MB-231 cells. The results are expressed as percent viability of cells determined relative to untreated control cells, values represent *Mean*±*S.E.M.* (D) Gelatin zymogram analysis for matrix metalloproteinase expression in A549 and MDA-MB-231 cells after respective ZMA and ZOA treatment, showing decrease in the level of MMP-9 (92 KDa) and to a certain extent of MMP-2 (72 KDa) also. The gel shows: Marker; Effect of (1) ZMA on A549 cells; (2) ZOA on MDA-MB-231 cells; (3) ZMT on A 549 cells; (4) Control of A549 cells and (5) Control of MDA-MB-231 cells
Ziziphus mauritiana (ZM), *Ziziphus oenoplia* (ZO): T- triterpenoids, A- alkaloids, F- flavonoids, Tn- tannins and L- lignins

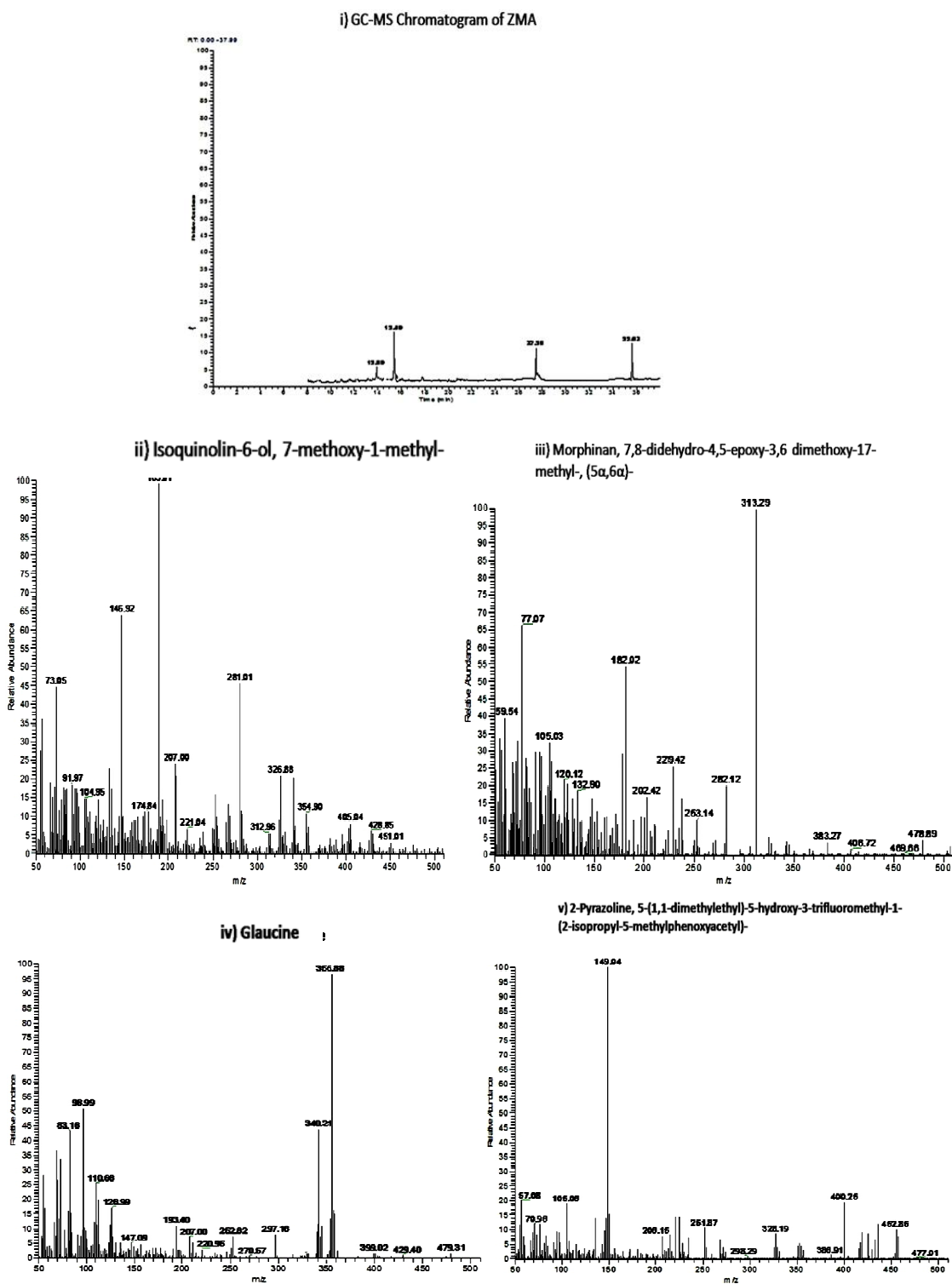
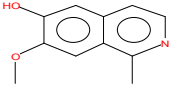
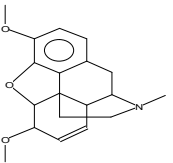
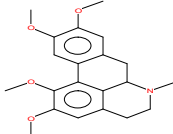
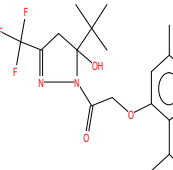


Fig. 3. Gas chromatography – mass spectrometry performed with the most active alkaloid fraction from the bark of *Ziziphus mauritiana*. The compounds identified are shown with its mass spectrum (MS) as isoquinoline, morphinan, glaucine and pyrazoline

Table 3. Identification of chemical constituents of the most effective ZMA fraction by gas chromatography-mass spectrometry (GC-MS)

Compound name	Molecular formula	Molecular weight	Rt (min)	EI-MS m/z (%)	Structure
Isoquinolin-6-ol, 7-methoxy-1-methyl-	C ₁₁ H ₁₁ NO ₂	189	15.40	184(100), 146(65), 174(13), 147(10)	
Morphinan, 7,8-didehydro-4,5-epoxy-3,6 dimethoxy-17-methyl-, (5α,6α)-	C ₁₉ H ₂₃ NO ₃	313	27.39	313 (100), 59(40), 282(21), 229(26)	
Glaucine	C ₂₁ H ₂₅ NO ₄	355	13.90	355 (100), 340 (45), 354(15), 297 (10)	
2-Pyrazoline, 5-(1,1-dimethylethyl)-5-hydroxy-3-trifluoromethyl-1-(2-isopropyl-5-methylphenoxyacetyl)-	C ₂₀ H ₂₇ F ₃ N ₂ O ₃	400	35.62	149 (100), 400(20), 57(20), 105(20)	

Rt= Retention time; EI-MS= Electronic Impact-Mass Spectrometry

4. CONCLUSION

In conclusion, the present study highlights the antioxidant and antiproliferative potential of bark extracts of ZM and ZO. Our results provide promising baseline information for its traditional medicinal property and possibility of *Ziziphus* extracts as a potent agent for development of natural antioxidant and anticancer agents. Purification of active ingredients, their identification and pharmacological, synergistic and toxic activities, if any, certainly needs further investigation.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Present study requires no ethical approval, as it does not deal with any kind of risk. Cancer cell lines usage has already established international ethical considerations.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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