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Original Article

Pharmacological evaluation of crude and partially purified fractions of *Z. jujuba* Mill. Gard. bark

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ABSTRACT

Traditional background and subsequent research investigations has already confirmed *Ziziphus jujuba* possessing bioactive compounds with therapeutic properties. The present study has been aimed to elucidate and compare the capabilities of different extracts of *Z. jujuba* bark for antioxidant and antiproliferative activities. Free radical scavenging activity was analyzed by DPPH assay. CPA47 endothelial cells were assessed with *Z. jujuba* bark partially purified fraction for *in vitro* antiproliferative potential by MTT assay and wound healing ability was evaluated by cell migration assay. Composition of potent fraction for the existence of specific class of compounds was subsequently detected using Gas Chromatography – Mass Spectrometry (GC-MS). *Z. jujuba* methanol extract (ZJM-A) effectively exhibited antioxidant activity with IC_{50} value 4.211 ± 0.22 mg/ml. Active partially purified *Z. jujuba* triterpenoid (ZJT) fraction significantly inhibited proliferation of CPA47 cells in a dose dependent manner with IC_{50} value 35.67 ± 0.14 µg/ml (R^2=0.97). ZJT fraction with significantly high cytotoxic activity against CPA47 cells exhibited attenuation of endothelial cell migration. GC-MS results revealed existence of bioactive compounds in most effective partially purified ZJT fraction as lupeol, betulin, Urs-12-en-28-oic acid, 3β-hydroxy-, methyl ester and 2-Hydroxy-10-isopropenyl-3,3,5a,5b,12b-pentamethyloctadecahydrodicyclopenta[a,i]phenanthrene-1,7a(1H)-dicarboxylic acid. Results of our research work indicate that Z. *jujuba* bark contains bioactive phytochemicals with potent antioxidant, antiproliferative and antiangiogenic properties.

Keywords: *Ziziphus jujuba*, Antiproliferative activity, Wound healing assay, GC-MS, DPPH

INTRODUCTION

Free radicals accumulated during several biochemical reactions in natural aging process have been reported to damage cellular components such as proteins, lipids and DNA causing diseases and toxicity leading to cellular death. Aerobic organisms protect their cells from oxidative damage due to oxidants, through an antioxidant mechanism involving superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), glutathione, ascorbic acid and tocopherol. These main antioxidant agents help in cellular protection by eliminating free radicals, such as reactive oxygen species (ROS). Nowadays, fruits, vegetables and medicinal plants are receiving considerable attention due to the presence of active components capable of preventing oxidative damage [1]. Medicinal plant based phytochemicals are gathering significant attention as cancer chemopreventive agents. Research data illustrated antitumor activity in several extracts of herbs. Phytochemicals have been demonstrated to promote DNA repair or antioxidant action to mediate anticancer effects and/or to inhibit cancer activating enzymes [2].

Angiogenesis refers to the growth of new capillaries from pre-existing capillaries. Solid cancer critically relies on neo-angiogenesis to grow and metastasize, hence major research and developmental efforts are required in angio-therapy modalities. Angiogenesis related disorders can be targeted by exploring potential drugs as angiogenesis inhibitors and/or promoters [3].

Natural or semi-synthetic drugs obtained from natural sources correspond to around 78% of new drugs approved by FDA, from 1983-1994 [4]. The genus *Ziziphus* is one such category known to

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acquire ethnomedicinal properties comprising of some important species like *Z. jujuba*, *Z. mauritiana*, *Z. spinachristi*, *Z. lotus*, *Z. spinosa* etc. Besides its traditional benefits, *Ziziphus* plants have also been reported to possess numerous therapeutic potential, including cytotoxicity against HCT15, HL-60, Molt-4 and Hela cancer cell lines also [5-8]. *Ziziphus jujuba* has also been reported to have neuronal stabilization activity, anti-stress, menopausal insomnia, sedative properties, also nerve control of men and women of all ages. In particular, flavonoids, saponins, and polysaccharides extracted from *Z. jujuba* had sedative, hypnotic effects and enhance pentobarbital induced sleeping behaviors [9-10]. Adequate experimental evidence and ethno-medical data has also been accrued pertaining to therapeutic activities including anticancer properties of *Z. jujuba* plant [5, 10].

Traditional information concerning effect of *Ziziphus* extracts on wound healing has already been investigated through bioassay on human skin cells [11], but no evidence has yet been reported with antiproliferative effects of other secondary metabolites on growth of endothelial cells and its underlying mechanism. Continuation to our screening programme on pharmaceutical phytochemicals of *Ziziphus* plants [12, 13], the present study has been taken up to investigate antioxidant, *in vitro* antiproliferative assay of crude extracts and partially purified fractions of *Z. jujuba* along with the mode of action.

MATERIALS AND METHODS

Chemicals: The antimycotic antibiotic, trypsin, trypan blue, cultivation media and other additives used for cell culture, DPPH were purchased from Himedia (Mumbai, India). All the HPLC grade solvents were purchased from SRL (Mumbai, India). Horse serum and Ham's F-12 medium were purchased from Gibco BRL (UK).

***Cell lines and Culture media*:** The Bovine pulmonary endothelial cell CPA47 were purchased from National Centre for Cell Sciences (NCCS) Pune and cultured in Ham's F-12 medium with 2 mM L-glutamate, supplemented with 10% (v/v) horse serum, 100 U/ml penicillin and 100 mg/ml streptomycin, at 37 ºC temperature with 5% carbon dioxide atmosphere.

Plant material and extract preparation: Plants of Rhamnaceae family were identified as *Ziziphus jujuba* Mill. Gard. from the Department of Botany, RTM Nagpur University, Nagpur, Maharashtra, India, where a specimen has been conserved with the voucher number as RTMNU BD 9141. Bioactive compounds from the bark of *Z. jujuba* were extracted in ethyl acetate solvent (ZJEA) [14]. Another method followed constituted initial extraction with ethyl acetate and finally with methanol (A) (ZJM-A) using soxhlet apparatus [15]. Secondary metabolites were also obtained from *Z. jujuba* bark as brownish crude residue with dichloromethane solvent (ZJDCM) [16]. Extraction method to obtain tannins from *Z. jujuba* bark was started with methanol containing ascorbic acid at low temperature [17] followed by ethyl acetate and then with methanol (B) (ZJM-B). Last extraction method used was the solvent mixture of dioxane-water (96:4, v/v) to isolate aqueous-dioxane soluble compounds of *Z. jujuba* (ZJAD) as stated by Ramasamy [18].

Partial purification of *Z. jujuba* crude bark extracts: All the solvent extracts were processed to procure specific classes or group of compounds in its purified form. ZJEA extract was column chromatographed over silica gel (60-120 mesh size) and eluted with Benzene:Ethyl acetate solvent mixture to obtain *Z. jujuba* triterpenoids (ZJT) in 2:1 solvent mixture ratio, as mentioned by Kundu *et al*. (1989) [19]. ZJM-A extract was also passed through silica gel 60-120 mesh size column and eluted with ethyl acetate:methanol (1:3), to isolate *Z. jujuba* flavonoids (ZJF) as main components during partial purification [15].

Similarly, ZJDCM extract when subjected to silica gel column chromatography and eluted with CH_2Cl_2-MeOH (1:8) furnished *Z. jujuba* alkaloids (ZJA) [16]. In the same way, ZJM-B extract was applied on equilibrated Sephadex LH20 in 80% ethanol and eluted by 50% acetone [17] to get *Z. jujuba* tannins (ZJTn) in partially purified form. Likewise, ZJAD extract was further processed in distilled water and then finally with absolute ether to get a fraction of *Z. jujuba* lignins (ZJL) [18]. All these partially purified fractions of *Z. jujuba* were monitored for their *in vitro* antiproliferative competence against CPA47 cells using MTT assay [23].

***Phytochemical screening*:** Phytochemical screening of *Z. jujuba* bark crude extracts and partially purified fractions was performed by standard methods [20, 21].

***DPPH radical scavenging assay*:** All the *Z. jujuba* bark crude extracts were screened for antioxidant activity by estimating its free radical scavenging potential using 2, 2-diphenyl-2-picrylhydrazyl hydrate (DPPH) method [22]. When DPPH solution was mixed with a substance, that can donate a hydrogen atom, it gives rise to the reduced form (diphenylpicryl hydrazine), with the loss of

the violet color. Each of crude extracts (1, 5 and 10 mg/ml) of *Z. jujuba* was mixed with 1.5 ml freshly prepared DPPH solution (0.05 mM) in methanol. Change in color from deep-violet to light-yellow was measured at 517 nm after incubation in dark for 30 min at 37 ºC. DPPH solution in methanol was used as negative control and ascorbic acid (250 µM) was used as a reference compound. All these assays were carried out in triplicate.

***Cell proliferation assay*:** Cytotoxic activity of partially purified *Ziziphus* fractions against CPA47 cells was determined by the colorimetric method of MTT reduction [23]. 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, which is estimated for activity of viable cells. Five dilutions of each *Z. jujuba* fractions in culture media (25, 50, 75, 100, 125 µg/ml) were assessed against CPA47 cell proliferation for 48 h by MTT assay. Since reduction of MTT can only occur in metabolically active cells, the level of activity is thus considered as a measure of viability of the cells [24].

***Wound healing assay*:** The wound-healing assay is simple, economical, and one of the earliest developed methods to study directional cell migration *in vitro* [25]. This method mimics the cell migration during wound healing occurring *in vivo*. The fundamental principle of the assay is that, a "wound gap" in a cell monolayer created by scratch, is followed by monitoring the "healing" of the gap by cell migration towards the center, thereby filling up the "gap". Factors that alter the motility and/or growth of the cell can increase or decrease the rate of "healing" of the gap. For this assay, the cells were grown as a confluent cell monolayer.

A small area was then disrupted by scratching a line through the layer. Cells were rinsed very gently with PBS and replaced with 1.5 ml media containing partially purified fractions of *Z. jujuba* bark (25, 50, 75, 100 and 125 µg/ml) and grown for 24 h. The open gap was inspected microscopically over time to observe the migration of cells to fill the disrupted area.

Results were estimated with micrometer measurements under a light microscope in order to monitor the closing or healing of the wound in a defined surface area [26]. The percent closure rate was determined as mentioned below:

1. To determine the surface area of the defined wound area
   Total Surface Area = 0.9mm x length
2. To determine the surface area of the migrated cells in to the wound area.
   Migrated Cell Surface Area = length of cell migration (mm) x 2 x length
3. Percent Closure (%) = Migrated Cell Surface Area / Total Surface Area x 100

***Gas Chromatography – Mass Spectrometry*:** A Varian 4500 GC coupled with Varian MS240 ion trap mass spectrometer (Varian, Walnut Creek, USA) was employed for determination of analytes in most potent partially purified *Z. jujuba* fraction (ZJT) using electron ionization (EI) mode. Split less injections of 1 µl volume were carried out with a split programmable temperature injection (STI) Type 1079 kept at 270 ºC. The ion trap, manifold and the transfer line were kept at 240, 40 and 250 ºC, respectively. Separations were performed on Varian Chrompack Capillary column WCOT Fused Silica (30 m long, 0.25 mm ID) CP-Sil 8CB, 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.

***Statistical Analysis*:** Results are expressed as mean ± S.E.M. using GraphPad Prism 5.0 (GraphPad, USA). The differences have been 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 percent of anticancer activity against the concentration of tested compounds using GraphPad Prism. Significance level was set at $p<0.05$.

RESULTS AND DISCUSSION

Tremendous traditional medicinal usage to cure several disorders and ailments during historic time was not truly based on the knowledge of its chemical constituents [27]. Later, literature reports divulged the occurrence of bioactive compounds called secondary metabolites like alkaloids, terpenoids, flavonoids, glycosides, waxes and fatty acids in medicinal plants, recognized to be responsible for their medicinal and pharmacological actions [28].

The possibility of such herbal compounds in cancer treatment involved targeting of many biomolecules. This demands assessment of medicinal plants for the level of toxicity in herbal preparations to determine its relevant prospect as pharmacological drug [29]. Promising antioxidant properties demonstrated by crude extracts (Table 1) assures participation of biologically active phytochemicals in free radical scavenging process. Selective partially purified *Z. jujuba* fractions (ZJT, ZJF and

ZJA) were employed to inhibit growth/ proliferation of CPA47 cells (Fig 1) subsequently, the most effective ZJT fraction exhibited reduction in wound healing effect on CPA47 endothelial cells revealing its antiangiogenic effect.

***Phytochemical studies*:** Preliminary phytochemical screening carried out for the detection of phytoconstituents in crude *Z. jujuba* extracts demonstrated presence of alkaloids, tannins, saponins, flavonoids, triterpenoids and lignins in its respective solvent extracts. Phytochemical screening of partially purified *Z. jujuba* fractions revealed the presence of triterpenoids, flavonoids, alkaloids, tannins and lignins in ZJT, ZJF, ZJA, ZJTn and ZJL fractions, respectively, and hence accordingly designated as per the specific class of compounds.

***DPPH radical scavenging activity*:** Results of DPPH radical scavenging activity of each crude *Z. jujuba* extracts revealed concentration dependent antioxidant effects with most potent being ZJM-A extract (Table 1). Inhibitory concentration calculated as IC_{50} values illustrates ZJM-A extract possessing maximum activity with 4.211 ± 0.22 mg/ml, ZJM-B with 5.054 ± 0.28 mg/ml, compared to standard value 3.789 ± 0.2 mg/ml (ascorbic acid). Best antioxidant effects were obtained with crude *Z. jujuba* methanolic extracts in a dose-dependent manner. These results have been supported by data documented by Kumari [27] revealing methanol extract of *Nyctanthes arbor-tristis* with maximum activity at 1000 mg/ml and moderate activity with 100 mg/ml.

Table 1: Free radical scavenging activity of different *Z. jujuba* crude extracts

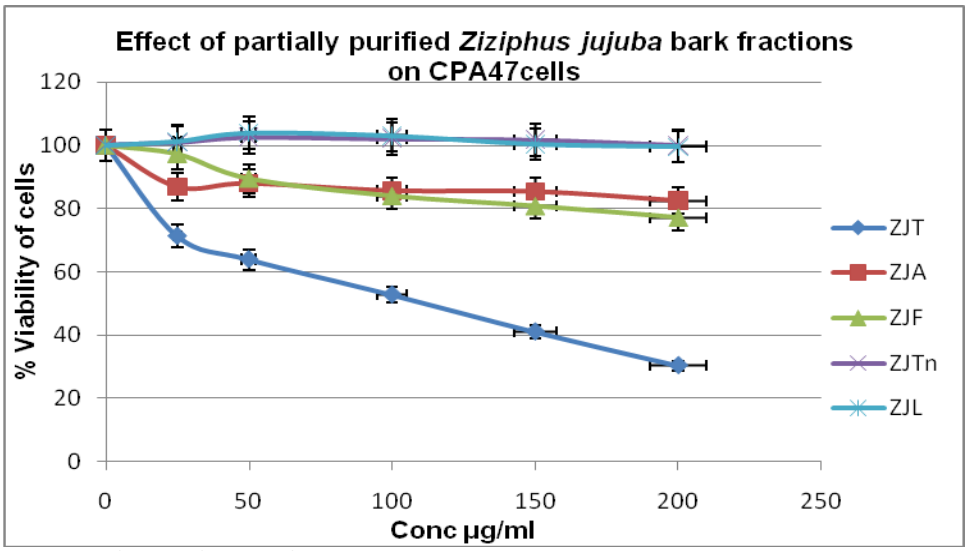
***Ziziphus* Extracts**	**Antioxidant activity at different concentrations**			
	1 mg/ml	5 mg/ml	10 mg/ml	IC_{50}
ZJDCM	04.09 ± 0.17	16.95 ± 0.32	50.29 ± 0.18	5.137 ± 0.30
ZJM-A	07.72 ± 0.25	69.87 ± 0.40	74.06 ± 0.09	4.211 ± 0.22
ZJEA	06.61 ± 0.41	28.09 ± 0.75	63.37 ± 0.26	5.071 ± 0.36
ZJM-B	04.97 ± 0.18	31.66 ± 0.53	70.91 ± 0.30	5.054 ± 0.28
ZJAD	01.93 ± 0.35	04.12 ± 0.47	21.07 ± 0.51	5.297 ± 0.31
Ascorbic acid	09.03 ± 0.01	79.99 ± 0.04	87.75 ± 0.03	3.789 ± 0.20

Data are presented as means ± standard error of three replicates. Solvent extracts of *ZJ- Z. jujuba* as DCM- dichloromethane, M-A- methanol, EA- ethyl acetate, M-B- methanol containing ascorbic acid, AD- aqueous-dioxane

Research data analyzing the antioxidant properties of various plant extracts could be beneficial in academia and food industry, exploring its capability as natural additives to replace the synthetic ones [1]. Results indicate that ZJM-A extract possesses maximum capacity to donate hydrogen; hence presumed to carry efficient phytochemical compounds with highest DPPH scavenging potential. The outcome of our research work is in agreement with recent investigation showing antioxidant effects of *Z. jujube* fruit extract in experiments with rat testis [30].

***Analysis of antiproliferative effect*:** The impact of different concentration of partially purified *Z. jujuba* bark fractions on CPA47 cells was observed with significant antiproliferative properties. Further, the effect of most promising ZJT fraction was assessed to determine its effect on spreading and migration of endothelial cells by scratch or wound healing assay.

***Antiproliferative effect of partially purified fractions of Z. jujuba bark on CPA47 cells*:** Partially purified *Z. jujuba* fractions showed a strong inhibition of cell proliferation in a dose response manner. The proliferation activity is inversely related to an increase in concentration. The inhibitory effect was initiated at low concentration of ZJT fraction as compared to other fractions (IC_{50}=35.67 µg/ml, R^2=0.97). On the contrary, ZJTn and ZJL fractions showed no inhibition of proliferation even at maximum concentration (Fig 1).



Partially purified bark fractions of *ZJ- Z. jujuba* as T- Triterpenoids, A- Alkaloids, F- Flavonoids, Tn- Tannins and L- Lignins, designated as per the specific class of compounds

Figure 1: Effect of partially purified fractions of *Ziziphus jujuba* on proliferation of CPA47 cells.

***Effect of ZJT fraction of Z. jujuba bark on CPA47 endothelial cell migration*:** Every bioactive component acts in a specific way to execute remedial property and trailed to track further for the mode of action of highly effective *Z. jujuba* fraction. To elucidate effect of ZJT fraction on migration of culture cells, an *in vitro* assay that mimics the wounding of endothelial cells was carried out by scrapping endothelial monolayer, leaving an area devoid of cells. Results revealed that endothelial migration was attenuated after ZJT treatment for 24 h (Fig. 2).

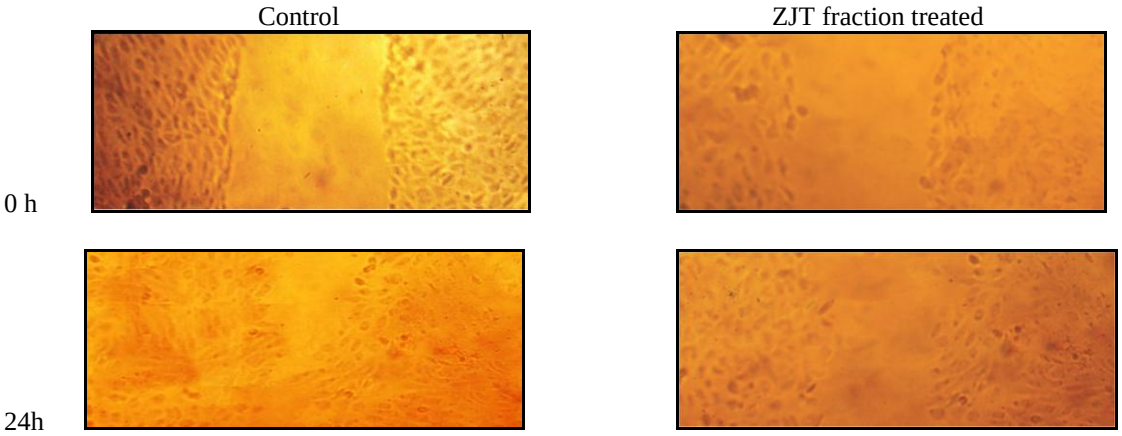
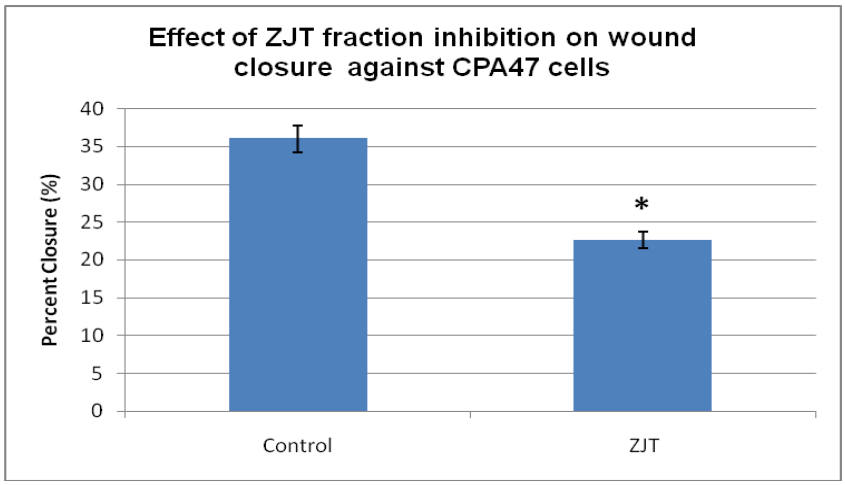


Figure 2: Influence of ZJT fraction on endothelial cell (CPA47) migration in wound healing assay. Control group (growth medium treated cells) wound closure for 0h and 24h. ZJT fraction treated (50 µg/ml) wound closure for 0h and 24h. Control endothelial monolayer showed normal growth to heal the wounded area while ZJT fraction demonstrated restricted growth rate of endothelial cells.

Comparison of cell migration in control and ZJT fraction treated cells exhibited that control cells migrate to cover up the space created by scratch; whereas ZJT treated cells did not migrate. Pattern of cell migration as depicted portrays a clear reduction in cell migration with treated cells. The ZJT fraction administration created 13.39 % difference in wound closure rate between treated and control group (Fig. 3).



All values are in mean ± SEM *p<0.05

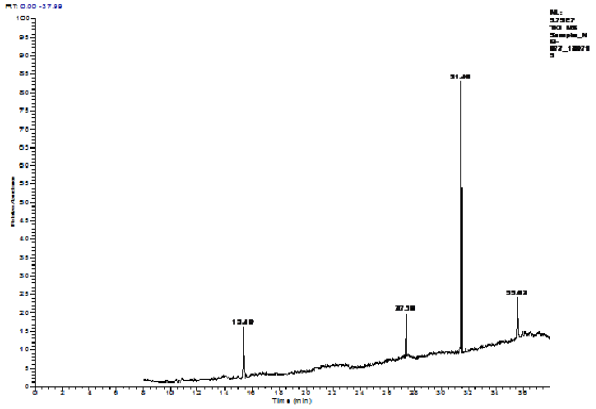
Figure 3- Difference in migration of CPA47 cells in absence and presence of triterpenoid fraction of *Z. jujuba* bark (50 µg/ml) in 24 hours

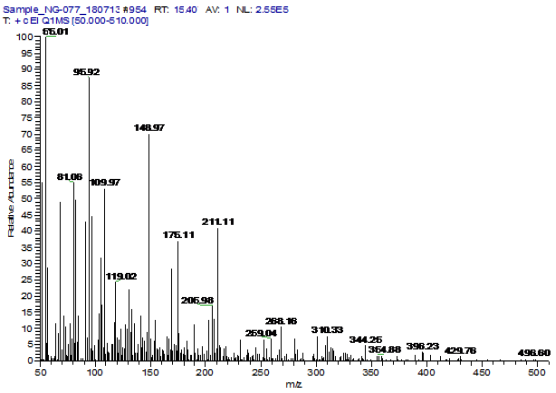
The ZJT fraction concentration (50 µg/ml) applied for *in vitro* analysis showed efficient growth inhibition of endothelial cells with 22.61% wound closure compared to 36% in the control after 24 h.
An approach towards anti-angiogenesis would always target to inhibit one or more of the check point in the pathway [31]. Reports in this direction of research explore numerous phytochemicals accomplished to inhibit angiogenesis [28, 32].

Proliferation of endothelial cells is essential for angiogenesis. Study of research reports suggested that angiogenesis inhibitors designed to inhibit tumor growth are able to reduce adipose tissue mass by cutting off the blood supply [3, 28]. Angiogenesis is an essential step in wound-healing process, while in embryonic development it refers to the formation of new capillaries from pre-existing vascular network. Many diseases, however, are driven by continual unregulated angiogenesis. Furthermore, as tumor generation is similar to wound healing, increased knowledge of wound repair may lead to unanticipated advances in tumor therapy. Chemotactic migration or motility of endothelial cells is assayed by the wound-healing assay. Wound healing involves complex series of interactions between different cell types, cytokine mediators and the extracellular matrix [33]. Researches done in this area relay a direct connection of impaired angiogenesis with delayed wound healing and retarded tumor growth [34]. Screening of *Ziziphus* extracts for anticancer potential on CPA47 revealed moderately less cytotoxic activity. These endothelial cells were examined with potent ZJT treatment for antiangiogenic effects by wound healing assay (Fig. 1).

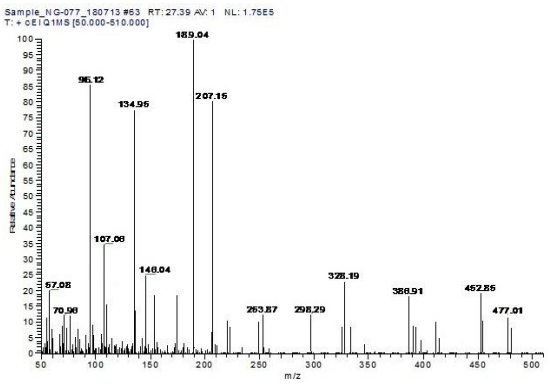
***Identification of Active partially purified ZJT fraction by GC-MS analysis*:** GC-MS analysis conducted on the effective ZJT fraction of *Z. jujuba* bark revealed to identify four chemical compounds (Table 2), disclosing presence of compounds belonging to triterpenoid group. The leading peak was lupeol in the GC-MS profile (Fig – 4), followed by betulin.
2-Hydroxy-10-isopropenyl-3,3,5a,5b,12b-penta methyl octadecahydro dicyclopenta [a,i] phenanthrene-1,7a(1H)-dicarboxylic acid was the most prolific volatile chemical, followed by urs-12-en-28-oic acid, 3β-hydroxy-, methyl ester.

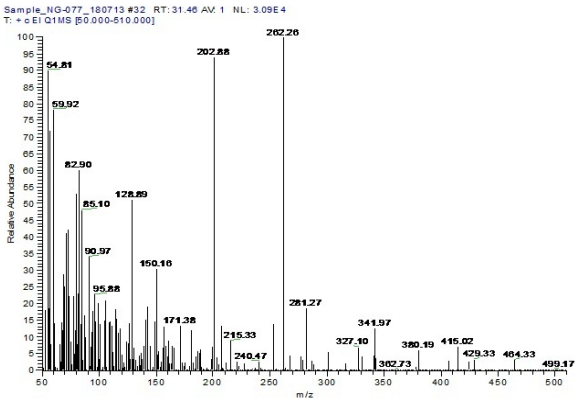
i) GC-MS of ZMA **ii) MS of Lupeol**





iii) MS of Betulin **iv) MS of Urs-12-en-28-oic acid, 3β-hydroxy-, methyl ester**





v) 2-Hydroxy-10-isopropenyl-3,3,5a,5b,12b-pentamethyloctadecahydrodicyclopenta[a,i]phenanthrene-1,7a(1H)-dicarboxylic acid

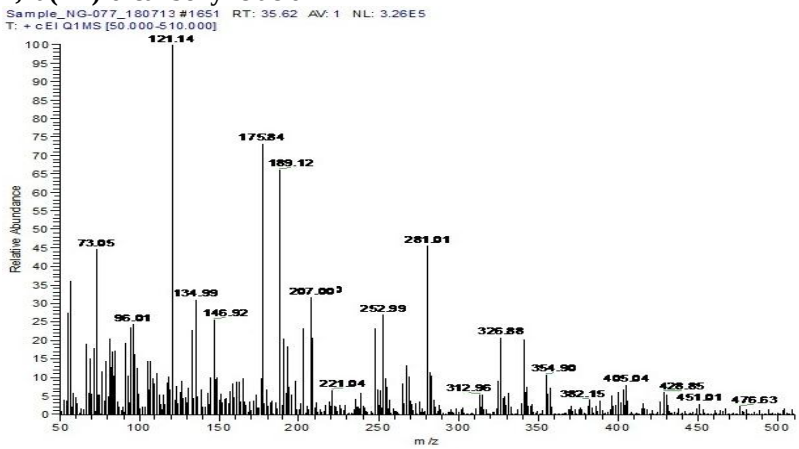
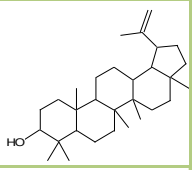
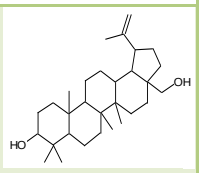
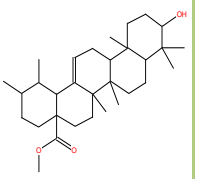
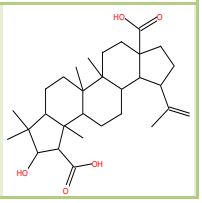


Figure 4- Gas chromatography – mass spectrometry profile of most active triterpenoid fraction from the bark of *Ziziphus jujuba* (ZJT). The compounds identified are shown with its mass spectrum (MS) as lupeol, betulin, 2-Hydroxy-10-isopropenyl-3,3,5a,5b,12b-pentamethyloctadecahydrodicyclopenta[a,i]phenanthrene-1,7a(1H)-dicarboxylic acid and Urs-12-en-28-oic acid, 3β-hydroxy-, methyl ester.

Table 2- Identification of chemical constituents of the most effective partially purified ZJT fraction by Gas Chromatography - Mass Spectrometry (GC-MS)

Compound name	Molecular formula	Molecular weight	Rt (min)	EI-MS m/z (%)	Structure
Lupeol	$C_{30}H_{50}O$	426	15.40	55 (100), 81 (55), 95 (87)	
Betulin	$C_{30}H_{50}O_2$	442	27.39	189 (100), 95 (85), 207 (84)	
Urs-12-en-28-oic acid, 3β-hydroxy-, methyl ester	$C_{31}H_{50}O_3$	470	35.62		
2-Hydroxy-10-isopropenyl-3,3,5a,5b,12b-pentamethyloctadecahydrodicyclopenta[a,i]phenanthrene-1,7a(1H)-dicarboxylic acid	$C_{30}H_{46}O_5$	486	31.46	121 (100), 175 (74), 189 (67)	

***Bioactive composition of ZJT fraction*:** In recent years, researchers focused on the identification of novel anticancer agents from medicinal plants. Phytochemicals have been proved to perform a significant role as anticancer agent, amongst them triterpenoids due to the broad range of exceptional bioactive capabilities mostly receive great attention. Reports have already been declared with triterpenoids from medicinal plants possessing enormous anticancer activity, with ability to induce apoptosis and also capable of inhibiting tumor angiogenesis [35]. GC-MS chromatogram of ZJT fraction has identified four triterpenoids responsible for its bioactive properties. The identified triterpenoids of ZJT fraction have already been reported with pharmaceutical activities. Betulin, a triterpenoid has already been evaluated with anticancer and antiangiogenic property by chorioallantoic membrane (CAM) and animal model [36]. Proliferation-inhibition, apoptosis-induction on multi-drug resistant cancer cells, immunomodulatory effect for tuberculosis treatment and suppression of TNF on endothelial cells has been reported with ursolic acid [37]. Lupeol is another promising bioactive agent that has been reported to exhibit antitumor, anti-inflammatory and antiangiogenic potential [38]. Valuable effects of the medicinal plants are based on the drug category under investigation and the bioassay method followed, to classify the plant extracts as active or inactive along with its mechanism of action [29]. Based on this concept, few researchers have shown the angiomodulator effect of natural compounds extracted from different species of Rhamnaceae family [39, 40] including reports found mentioning the antiangiogenic potential of *Z. jujuba* [9]. Present report on ZJT fraction also endorses the antiangiogenic potential of *Z. jujuba*.

CONCLUSION

The present study reports that the traditional medicinal plant *Z. jujuba* possesses antioxidant activity, varying with contents of bioactive components obtained in different solvent extracts. Also, the results depicted that the triterpenoid fraction (ZJT) has potential cytotoxic properties acting via impaired angiogenesis. Further studies in experimental model would clarify its exact mechanism of action.

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