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CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES OF LEAVES OF *ZIZIPHUS MAURITIANA* L. NATIVE TO PAKISTAN

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Abstract

Ziziphus mauritiana L., is a fruit tree well known for its nutritional and medicinal benefits. The aim of the current study was to investigate the chemical composition as well as biological (antioxidant, antimicrobial, antitumor and anticancer) attributes of different solvent extracts from the leaves of *Ziziphus mauritiana*. It was established by colorimetric method that chloroform extract had greater amount of total phenolics (84.69 ± 0.92 µg GAE/mg of extract), while methanol extract contained higher content of total flavonoids (46.94±1.55 µg QE/mg of extract). Meanwhile, methanol extract exhibited higher DPPH free radical scavenging potential (IC_{50} = 0.11 mg/mL) and antimicrobial (antibacterial and antifungal) activity among others. Overall, *E. coli* was noted to be the most resistant microbial strain against all the tested extracts. Chloroform extract showed strongest antitumor (IC_{50} = 70.74µg/mL) and anticancer activity (IC_{50} values of 27.78 and 18.32 µg/mL against human cancer cell lines U937 and HCT-116, respectively) and significantly inhibited the viability of these cell lines.According to GC-MS analysis methyl stearate (15.59%), plamitic acid (38.55%) and α-linolenic acid (26.45%) were identified as the major components of methanol, chloroform and hexane extracts, respectively in addition to presence of several other bioactives. The results of this study conclude that *Z. mauritiana* leaves extract with efficient biological activities can be explored for potential uses as antioxidant, antitumor and anticancer agents for pharmaceutical industry.

Key words: Solvent extracts; DPPH radical scavengers; Flavonoids; Phenolics; GC-MS; Human cancer cell lines.

Introduction

Antioxidants are effective compounds that can delay, interrupt or inhibit the process of oxidative reactions by neutralizing free radicals via donation of hydrogen atom or electron, quenching singlet and triplet oxygen and chelating metals and thus play a proactive role towards improving shelf-life of food products as well as reducing the incidence of different ailments such as cancer, aging and inflammation (Dalleau *et al*., 2013; Lobo *et al*., 2010; Khadri *et al*., 2010). Currently, owing to the perceived toxic effects of synthetic antioxidants and multiple microbial drug resistance (MDR), the use of plants-derived natural antioxidants and bioactives, is therefore focus of current scientific research (Alnajar *et al.,* 2012).Plants based phenolic antioxidants, due to their unique structural features and multiple biological actions, play a promising role in the treatment of certain cancer and infectious diseases (Prasad *et al*., 2012; He *et al*., 2011; Miyasaki *et al.,* 2013; Bhalodia & Shukla, 2011). Due to the revival of interest in the use of plants as a source of food and medicine, currently the researchers are largely engaged in exploring and studying the bioactives composition and biological principles of more and more plants (Elless *et al*., 2000; Lucock, 2004; Shahidi, 2009; Liu *et al*., 2008).

Ziziphus mauritiana (*Z. mauritiana*), a member from the family *Rhamnaceae* with local name Ber, is a fruit tree which grows in tropical and sub-tropical regions of the world. Different parts of this plant have been used in the traditional medicine for the treatment of different ailments such as asthma, allergies, depression and ulcers (Marwat *et al*., 2009). Nevertheless, some studies so far have also investigated the phenolics composition of the fruit of *Z. mauritiana* and illustrated the scientific basis for the uses of different parts of this plant for the treatment of diabetes, ulcer and inflammation (Bhatia & Mishra, 2010; Cisse *et al*., 2000; Gupta *et al*., 2012; Memon *et al*., 2012a; Siddharth *et al*., 2010).

Z. mauritiana is a potential plant both for food and phyto-medicine applications, but until now there are no comprehensive study reported on the detailed phytochemicals and biological attributes of different solvent extracts from the leaves of this plant. The present study therefore mainly attempts to investigate and compare the bioactives composition and biological (antioxidant, antimicrobial, antitumor and anticancer) activities among different solvent (methanol, chloroform and hexane) extracts produced from the leaves of locally grown *Z. mauritiana* so as to explore their potential uses as ingredient for the development of natural drugs and nutraceuticals.

Material and Methods

Chemicals and reagents: Aluminum chloride hexahydrate (Fluka chemicals, GmbH), agar powder (DAEJUNG Chemicals and metals, Korea), nutrient

broth, Sabouraud dextrose broth (Lab M limited, Topely house, UK) were used. Besides, 2,2′- diphenyl-1-picrylhydrazyl (DPPH), gallic acid (GA), quercetin, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Sodium dodecyl sulfate and N,N-Dimethylformamide were provided by Fisher Scientific, USA. RPMI 1640with L-glutamine was purchased from Mediatech Inc. Manassas, VA, USA. Methanol, hexane and chloroform used for extraction purpose were purchased from Merck KGaA (Darmstadt, Germany). Folin-Ciocalteu's phenol reagent, sodium carbonate, nutrient agar, potato dextrose agar were acquired from Applichem, GmbH, Darmstadt, Germany.

Plant material: The leaves of *Z. mauritiana* were collected from botanical garden, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan. The specimens were identified by Dr. Mansoor Hameed (Botanist, Department of Botany, University of Agriculture, Faisalabad, Pakistan) using standard herbarium technique.

Preparation of bioactive extract: Fresh leaves of *Z. mauritiana* were rinsed with distilled water to remove any dust and particulate matter. Leaves were spread on a filter paper sheet in well ventilated room. The dried leaves were ground with the help of food processor (Singer, FP-500) into a fine powder. The powder was passed through sieve (0.25 mm). Sieved powdered material was stored in tightly packed polyethylene bags. Extraction was carried out as described previously (Sultana *et al*., 2007) with slight modification. Briefly, 10g of leaf powder was extracted with 100 mL methanol on an orbital shaker at 350 rpm for 6 hrs. The excess solvent from the filtrate was evaporated under vacuum using a rotary evaporator. The crude concentrated extract was transferred to brown colored sample vial and stored at -4°C for further uses. Similar extraction procedure was repeated with hexane and chloroform. The extracts thus produced by three different extraction solvents were analyzed independently for different assays.

Total phenolic contents: Folin-Ciocalteu method was used to estimate total phenolic contents (Jagadish *et al*., 2009; Slinkard & Singleton, 1997). The extract solution (1 mL) was mixed with 7.5mL of double deionized water, 500µL of Folin-ciocalteu reagent and one mL of 5% Na_2CO_3. Mixture was incubated for 90 minutes at room temperature. Absorbance was measured at 760nm by using UV-Vis spectrophotometer (Lambda EZ 201, Perkin Elmer, USA). The content of phenolics was expressed as µg gallic acid equivalents (GAEs) per mg of extract. A standard calibration curve for gallic acid was prepared by running a series of standard solutions.

Total flavonoid contents: A previously described method was followed to assess total flavonoid contents of the extracts (Lamaison & Carnat, 1990; Quettier *et al*., 2000).In this experiment, 2 mL of each extract (methanol, chloroform and hexane) solution was mixed with 2 mL of aqueous $AlCl_3.6H_2O$ (0.1mol/L). The mixture was incubated at room temperature for 10 minutes. Absorbance of the final reaction mixture was measured at 417nm. The content of flavonoids was expressed as µg quercetin equivalents (QEs) using standard calibration curve.

Free radical (DPPH) scavenging assay: DPPH free radical scavenging activity of the subject extracts was determined according to a previous method (Ozturk *et al*., 2011). The reaction was carried out in capped glass test tubes which were tightly wrapped with aluminum foil from top to bottom. The DPPH radical stock solution used was freshly prepared. Briefly, 4 mL of methanol solution of DPPH (0.1mM) was mixed with 1mL of each of the extract (methanol, chloroform and hexane) solution. Reaction mixture was placed in darkness for 30 minutes. Then the absorbance was taken at 515nm with UV-Vis spectrophotometer (Lambda EZ 201, Perkin Elmer, USA). The inhibition percentage of DPPH radicals was calculated as:

$$\text{Inhibition (\%) of DPPH radicals} = Ac - As / Ac \times 100$$

where Ac is absorbance of control (reaction in which all reagents participated except plant extract) and As is absorbance of sample (plant extract).

Antimicrobial activity

Disc diffusion assay: The antimicrobial activity of methanol, chloroform and hexane extracts of *Z. mauritiana* leaves against tested bacterial and fungal strains was measured by agar disc diffusion method (Anon., 1997). The bacterial and fungal strains were obtained from National Institute for Biotechnology and Engineering, Faisalabad, Pakistan. Sterile Nutrient agar was inoculated with 100µL suspension of tested bacteria while sterile Potato dextrose agar was inoculated with 100µL of tested fungi. The inoculated nutrient agar and potato dextrose agar were then poured into sterilized petri plates individually. Sterile filter discs impregnated with 50µL of sample solution were placed in inoculated petri plates with the help of sterile forceps. Rifampicin and Terbinafine were used as a positive control in bacterial and fungal inoculated plates, respectively. The plates were incubated at 37°C for 24 hrs and at 27°C for 48 hrs for maximum bacterial and fungal growth, respectively. Antibacterial and antifungal activities were evaluated by measuring diameter (millimeter) of inhibition zones with the help of zone reader.

Estimation of minimum inhibitory concentration (MIC) values: The antimicrobial activity of different solvents (methanol, chloroform and hexane) extractof *Z. mauritiana* leaves was estimated by measuring minimum inhibitory concentration (MIC). Broth micro-dilution method (Anon., 1999) was followed for determination of MIC values. Briefly, dilution series of each extract solution (100 µL) were prepared from 0.01 to 15.0 mg/mL in a 96-well microtiter plate, 50 µL of nutrient broth (NB) and Sabouraud dextrose broth (SDB) were added for bacterial and fungal strains, respectively, onto microtitreplates. Then two fold serial dilutions were done

using a micropipette so that each well carried 50µL of plant extract solution in a serially decreasing concentration. Thereafter, 10µL inoculum of tested microbial strain was added to each well. Dimethyl sulfoxide in NB and SDB was used as a negative control, while NB containing Rifampicin and SDB containing Terbinafine were used as positive control for bacterial and fungal strains, respectively. The plates were incubated at 37°C for 24 hrs and at 27°C for 48 hrs for bacteria and fungi, respectively. Triplicate measurements were made for each microbial strain.

Antitumor assay: Antitumor activity of the extract was examined according to procedure described previously (Inayatullah *et al*., 2007). The *Agrobacterium tumefaciens* strain used in this assay was generously provided by Dr. MazharIqbal, Principal Scientist, Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan.

Anticancer assay: U937 (Human leukemic monocyte lymphoma cells) and HCT-116 (Human colon carcinoma cells) cells were obtained from American Type Culture Collection (Manassas, VA, USA). The human leukemic monocyte lymphoma cells (U937) and human colon carcinoma cells (HCT-116) were maintained in RPMI-1640 and DMEM (Dulbeco's modified Eagle medium), respectively, that were supplemented with 10% fetal bovine serum (Atlanta Biologicals), and antibiotic (10,000 I.U/mL). Cultures were maintained in flasks in humidified (95% air) incubator at 37ºC with 5% CO_2. Briefly, 5,000 cells of U937 and HCT-116 were seeded individually in Biolite 96-well (Thermo Scientific, Korea) plates and were incubated (95% humidified air/5% CO_2 at 37ºC) with methanol, chloroform and hexane extracts of *Z. mauritiana*leaves for 72 hrs (Prasad *et al*., 2010). Then 20µL of 5 mg/mL MTT was added to each well. After 2 h incubation, each well was supplemented with 100µL of lysis buffer. Cells were further incubated for 5 hrs and optical density values were measured at 570nm by ELISA Plate Reader (Dynex Technologies, USA).

Gas chromatography-mass spectrometry (GC-MS) analysis and identification of compounds: The GC/MS analysis of methanol, chloroform and hexane extracts was performed using a Phenomenex, Torrance, GC-MS equipped with ZB-5mSi-fused silica capillary column (30m X 0.25mm, 0.50 µm). GC-MS had electron energy of 70Ev, ion source temperature of 230ºC and electron emission of 34.6 µA. The temperature of analyzer was maintained at 150ºC. Helium was used as carrier gas at flow rate of 1mL/min. The injector and interface temperature was set at 290ºC and 360ºC, respectively. The oven temperature was programmed as 50ºC (1min) to 310ºC (20min), at increasing rate of 6ºC/min. Compounds were identified on the basis of comparison of their relative retention time and mass spectra with those of the NIST library data of GC/MS system (Ezhilan & Neelamegam, 2012).

Statistical analysis: Minitab software version 16 was applied to perform analysis of variance (ANOVA) and to determine significant differences ($p<0.05$).

Results and Discussions

Total phenolic and total flavonoid contents: Plants based phenolic acids and flavonoids are valued as a group of potent natural antioxidants (Duthie & Morrice, 2012).These compounds possess wide spectrum of biological activities and are helpful in reducing the chances of occurrence of oxidative stress related disorders and infectious diseases (Huang *et al*., 2012; Pandey & Rizvi, 2009).

Total phenolics content (TPC) and total flavonoids content (TFC) of methanol, chloroform and hexane extracts of *Z. mauritiana* leaves as depicted in Fig. 1, revealed a significant ($p<0.05$) difference in relation to extraction solvents. Chloroform extract had the highest content of total phenolics (84.69 ± 0.92 µg GAE/mg of extract) followed by methanol extract (77.88 ± 1.10 µg GAE/mg of extract) and then hexane extract (56.22 ± 1.46 µg GAE/mg ofextract). Our results are in accordance with a previous investigation (Shahriar *et al*., 2013) on *Wathiniasomnifera* root,wherein, maximum amount of phenolic was examined in chloroform extract followed by methanol and hexane extracts.Another research group (Yusri *et al*., 2012), also observed similar trends as in the present study regarding extraction efficacy of solvents (chloroform > methanol > hexane) towards recovering phenolic compounds from *Hibiscus cannabinus* L. seeds. Meanwhile, the maximum amount of total flavonoids (46.94±1.55 µg QE/mg of extract) was observed in methanol extract followed by chloroform (40.13 ± 3.95 µg QE/mg of extract) and then hexane extract (18.07 ± 0.79 µg QE/mg of extract).

Difference in the amounts of phenolics and flavonoids in different extracts may be attributed to nature of extracting solvent as well as the chemical nature and availability of the compounds extracted (Bae *et al*., 2012). The phenolic contents determined in *Z. mauritiana* leaves extracts in our study were found to be higher than those previously reported for *Z. mauritiana* fruit extract (Memon *et al*., 2012a) and *Z. mauritiana* seed extract (San *et al*., 2013). In comparison to present data of leaves, Lamien-Meda *et al.* (2008) reported a higher phenolic and flavonoid contents in *Z. mauritiana* fruit extracts. Such variation in phenolic and flavonoid contents within different parts of *Z. mauritiana* might be linked to the chemical nature, solubility and availability of the compounds being extracted (Capocasa *et al*., 2008).

Free radical (DPPH) scavenging activity: Free radicals, formed as result of oxidation, are one of the major causes of degenerative diseases (Phamhuy *et al.,* 2008). Pharmacological evaluation of plant extracts is incomplete without assessment of their free radical scavenging activity. Therefore, in the present study, DPPH free radicals scavenging potential of different solvents (methanol, chloroform and hexane) extract of *Z. mauritiana* leaves was apprised. DPPH free radical scavenging activity of the extracts was noted to be increased in a dose dependent manner over a concentration range of 0.025 to 0.8 mg/mL

(Fig. 2).This is in accordance with a previous investigation wherethe percentage of free radicals scavenging was demonstrated to be concentration dependent (Heo *et al*., 2007). In our study, methanol extract (IC_{50} = 0.11 mg/mL) exhibited significantly higher antioxidant activity in the DPPH system compared with chloroform (IC_{50} = 0.63 mg/mL) and hexane (IC_{50} = > 1.0 mg/mL) extracts. The present IC_{50} value of *Z. mauritiana* methanol extract (0.11 mg/mL) is in agreement with a previous study (Shreedhara *et al*., 2011), which reported an IC_{50} value of 0.10 mg/mL for the ethanol extract of *Z. mauritiana* leaves. The overall order of free radical scavenging activity of three solvent extracts was as follows: methanol > chloroform > hexane (Fig. 2). Our results are in consistence with previous studies (Mohdaly *et al*., 2010; Hayouni *et al*., 2007) which revealed a strong influence of extracting solvents on the antioxidant activity of the plant extracts.

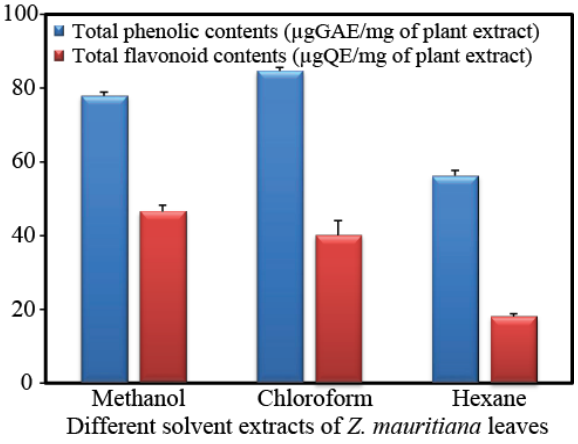

Fig. 1. Total phenolic and total flavonoid contents of *Z. mauritiana* leaves extracts (methanol, chloroform and hexane). Values are Mean ± SD of triplicate determinations. Total phenolic contents are expressed as microgram gallic acid equivalents per milligram of plant extract (µg GAE/ mg of plant extract). Total flavonoid contents are expressed as microgram quercetin equivalents per milligram of plant extract (µg QE/mg of plant extract).

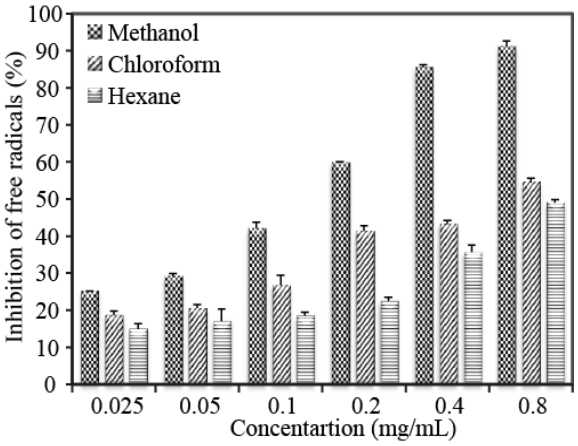

Fig. 2. DPPH free radical scavenging activity (%) of methanol, chloroform and hexane extracts of *Z. mauritiana* leaves. Values are mean ± SD of triplicate determinations (p<0.05).

Antimicrobial activity: Methanol, chloroform and hexane extracts obtained from the leaves of *Z. mauritiana* were tested against a set of six pathogenic microorganisms (Tables 1, 2). Analysis of variance showed that the extracts exhibited significantly (p<0.05) varying antimicrobial potential against the tested microbial strains. The difference in antimicrobial activity in relation to three extraction solvents might be attributed to difference of the polarity of the solvents as well as chemical nature of the extracted components/bioactives. An earlier investigation revealed that variation in chemical composition of plant extracts directly influenced the biological activities (Esmaeili *et al*., 2012). Overall, maximum antimicrobial activity was observed for methanolic extract which could be predicted by relatively a larger diameter of inhibition zones (Table 1) and smaller minimum inhibitory concentration (MIC) values (Table 2). Whereas, a poor antimicrobial activity was recorded for *n*-hexane extract with the smallest inhibition zones (9.0 to 13.3 mm) and the largest MIC values (0.94 to 7.53 mg/mL).

The recorded diameter of the inhibition zones in the present antimicrobial assays ranged from 9.0mm to 23.3mm. Among the bacterial strains largest inhibition zone (21.6mm) was observed against *B. subtilis*. However, in another study Nagumanthri *et al*. (2012) examined relatively smaller inhibition zone against *B. subtilis*by the methanol extract of Indian *Z. Mauritianai* leaves. In contrast to our results, Abalaka *et al*. (2010), reported a larger zone of inhibition by *Z. mauritiana* leaves extract against *E. coli*. These differences might have been due to the different extraction techniques used to prepare the extracts as well as unique chemical nature of the extracted compounds (Edoga *et al*., 2005; Turkmen *et al*., 2007).Our results showed that *E. coli* was the most resistant microorganism among the selected bacterial strains. This may be attributed to the fact that cell wall of gram negative bacteria is bestowed with an outer membrane and distinct periplasmic space that were absent in gram positive bacteria (Ceylan & Fung, 2004; Staszewski *et al*., 2011). Furthermore, for the fungal strains, we observed that methanol extract of *Z. mauritianai* leaves had relatively a larger zone of inhibition (23.3mm) against *A. niger*as compared to *F. solani* (16.6 mm). This trend is in line with a previous study in which selected plant extracts exhibited a greater inhibition activity against *A. niger* than *F. solani* (Leeja & Thoppil, 2007). Rifampicin and terbinafine as positive controls possessed relatively a better antimicrobial activity compared with the tested extracts.

Antitumor activity: A bench top potato disc assay was used to assess the antitumor potential of methanol, chloroform and hexane extracts of *Z. mauritiana* leaves against a plant pathogen namely *Agrobacterium tumefaciens*. This pathogen is involved in the induction of neoplastic disease (Crown gall tumor) in dicotyledonous plants. Kanwal *et al*. (2011) reported that potato disc assay is inexpensive, animal-sparing, rapid, statistically reliable and safer technique for preliminary screening of antitumor agents. Many researchers used this technique for screening of antitumor potential of different plants (Islam *et al*., 2013; Bibi *et al.,* 2011).

Table 1. Antimicrobial activity of methanol, chloroform and hexane extracts of *Z. mauritiana* leaves estimated by disc diffusion method.

Solvent extract	Diameter of inhibition zones (mm)					
	Bacterial strains				Fungal strains	
	Escherichia coli	*Bacillus subtilis*	*Pasteurellam ultocida*	*Staphylococcus aureus*	*Aspergillusniger*	*Fusariumsolani*
Methanol	12.3 ±0.1[b]	21.7±0.3[d]	----	14.6 ±1.2[a]	23.4 ±1.3[e]	16.7±0.1[b]
Chloroform	----	----	10.8 ±0.2[b]	17.2 ± 0.3[c]	19.5 ±1.0[d]	----
Hexane	9.0 ±0.0[c]	13.3 ±1.6[e]	----	11.4 ± 0.7[b]	----	----
*Rifampicin	21.7 ±1.4[e]	24.7 ±0.5[f]	23.3 ±1.7[d]	28.1 ± 1.0[e]	NT	NT
**Terbinafine	NT	NT	NT	NT	25.7 ±1.7[a]	24.0 ±0.8[c]

(----) indicates no antimicrobial activity. NT = Not tested. Data is represented as mean ± SD of triplicate determinations. Mean with different superscript letter within the same column indicate significant difference (p<0.05) among extraction solvents employed. *Standard antibiotic for bacteria; * *Standard antibiotic for fungi.

Table 2. Antimicrobial activity of methanol, chloroform and hexane extracts of *Z. mauritiana* leaves estimated by minimum inhibitory concentration (MIC) assay.

Solvent extract	MIC (mg/mL)					
	Bacterial strains				Fungal strains	
	Escherichia coli	*Bacillus subtilis*	*Pasteurellam ultocida*	*Staphylococcus Aureus*	*Aspergillus niger*	*Fusariumsolani*
Methanol	1.26 ±0.90[a]	0.15±0.40[c]	----	0.88 ± 0.0[f]	0.10 ±0.03[d]	0.61±0.0[c]
Chloroform	----	----	3.7 ±1.4[c]	0.45 ± 0.2[b]	0.23 ±0.12[a]	----
Hexane	7.53 ±1.1[d]	0.94±0.70[b]	----	1.62 ± 0.5[a]	----	----
*Rifampicin	0.11 ±0.0[b]	0.04±0.0[a]	0.09 ±0.10[e]	0.07 ± 0.0[d]	NT	NT
**Terbinafine	NT	NT	NT	NT	0.02 ±0.0[b]	0.04 ±0.01[f]

(----) indicates no antimicrobial activity. NT = Not tested. Data is represented as mean ± SD of triplicate determinations. Mean with different superscript letter within the same column indicate significant difference (p<0.05) among extraction solvents employed. *Standard antibiotic for bacteria; * *Standard antibiotic for fungi.

Table 3. Inhibition (%) of tumors by methanol, chloroform and hexane extracts of *Z. mauritiana* leaves.

Solvent extract	Inhibition (%) of tumors at various doses (mg/mL)					
	0.025	0.05	0.1	0.2	0.4	0.8
Methanol	12.60±0.56[a]	14.85±0.86[b]	21.83 ± 0.43[d]	22.66±5.06[f]	43.53±0.71[c]	63.37 ± 1.12[e]
Chloroform	22.24 ± 1.82[c]	37.34 ± 3.56[a]	72.63 ± 0.38[b]	79.40±1.20[bd]	80.43 ± 1.66[e]	86.28 ± 2.38[f]
Hexane	16.82±3.66[a]	24.75±1.41[f]	36.92±2.26[b]	59.27±1.39[e]	63.25±0.57[d]	69.46 ± 0.86[c]

Data is represented as mean ± S.D of triplicate determinations. Mean with different superscript letters within the same row indicate significant difference (p<0.01) among tested doses.

It can be depicted from the results of Table 3 that the tested solvent extracts notably decreased the growth of *A. tumefaciens*. Tumor inhibition by the tested extracts was observed to be followed in a dose dependent mode (0.025 to 0.8 mg/mL). We observed a minimum tumor inhibition (Table 3) and maximum number of tumors (Fig. 3) in methanol, chloroform and hexane extracts at concentration of 0.025 mg/mL, while maximum tumor inhibition (%) inmethanol (63.37%), chloroform (86.28%) and hexane (69.46%) extracts was examinedat concentration of 0.8 mg/mL.

Our findings were in agreement with a previous study (Mahmood *et al*., 2011) in which increase in crown gall tumor inhibition was observed to be followed with the increase in dose of plant extracts. Overall, the order of antitumor activity of the tested three solvent extracts was followed as: chloroform (IC_{50} = 70.74 µg/mL) > hexane (IC_{50} = 188.5 µg/mL) > methanol (IC_{50} = 596.4 µg/mL). Variation in antitumor potential of the tested extracts may be linked to thediffering chemical nature ofthe compounds extracted by different extraction solvents (Kuete *et al*., 2008).

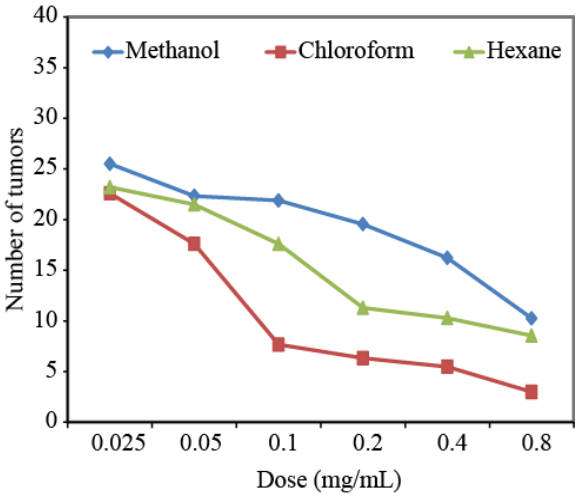

Fig. 3. Number of tumors for different solvent extracts of *Z. mauritiana*leaves examined in potato disc assay.

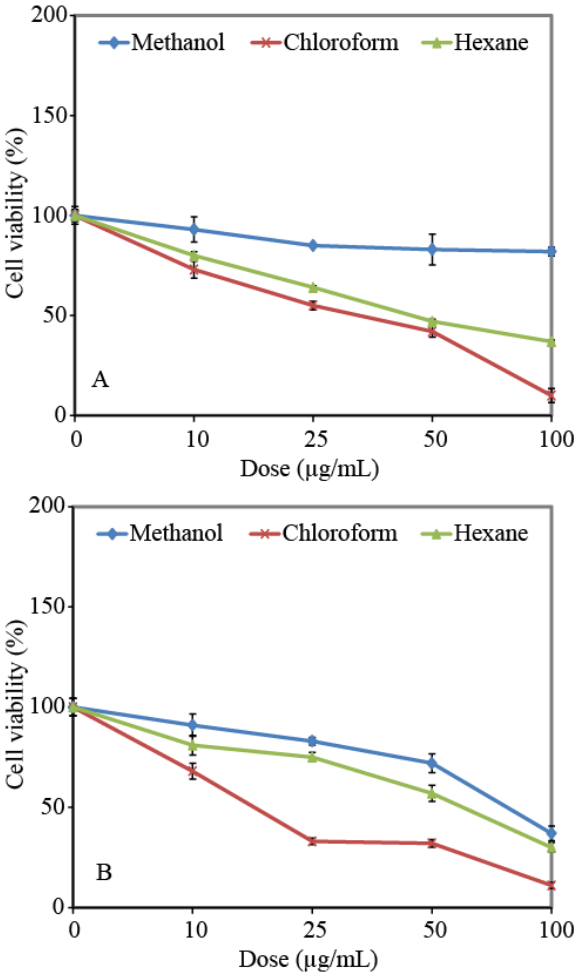

Fig. 4. Anticancer activity of methanol, chloroform and hexane extracts of *Z. mauritiana* leaves at various doses (10, 25, 50, 100 µg/mL) against (A) U937 (human leukemic monocyte lymphoma) and (B) HCT-116(human colon carcinoma) cell lines. Data are represented as mean ± SD of quadruplicate determinations.

Anticancer activity: Selected plant derived bioactives have gained considerable attention for the treatment of cancer due to their safety and sustainability (Edoga *et al*., 2005). Recent reports indicate the use of plant extracts in phase II clinical trials as a supplemental treatment (Edoga *et al*., 2005).In the current study we investigated anticancer activity of methanol, chloroform and hexane extracts of *Z. mauritiana* leaves at different doses (10, 25, 50 and 100 µg/mL) against two different human cancer cell lines (U937, leukemic monocyte lymphoma and HCT-116, colon carcinoma), as shown in Fig. 4 A and B. It was observed that growth of both the cell lines (U937 and HCT-116) decreased for 72 hrs as result of exposure to *Z.mauritiana*leaves extracts.Chloroform extract of *Z.mauritiana* leaves was found to be the most potent anticancer agent against U937 (IC_{50} = 27.78 µg/mL) and HCT-116 (IC_{50} = 18.32 µg/mL) cells. The IC_{50} values of hexane extract were 48.91µg/mL and 55.09 µg/mL against cell proliferation of U937 and HCT-116 cells, respectively.

The methanol extract was comparatively less effective to inhibit the cell growth of both the cell lines with IC_{50} values of around 100 µg/mL against U937 and 84.89 µg/mL against HCT-116 cell lines. Similar results for polar extract of *Z. mauritiana* were investigated previously against HL-60 (Mishra *et al*., 2011). According to criterion of American National Cancer Institute, IC_{50}< 30 µg/mL represents potentially active anticancer extracts. Potent anticancer activity of chloroform extract in the present study might be attributed to high concentration of phenolic constituentsin this extract.According to investigations phenolic compounds exhibit anticancer activity by apoptosis induction and anti-angiogenesis (Wang *et al*., 2012), topoisomerase inhibition (De Mejia *et al*., 2006), upregulation of p53 (Chan *et al*., 2010) and cell cycle arrest pathways.

Chemical composition of *Z. mauritiana* leaves: The data for GC-MS analysis is shown in Table 4. It can be seen that methyl stearate (15.59%) and α-linolenic acid (26.45%) were the major components of methanol and hexane extracts, respectively. Other, important components identified in methanol extract were: palmitic acid (13.57%), squalene (12.09%), phytol (9.78%), methyl palmitate (7.81%), linoleic acid methyl ester (5.98%) and vitamin E (2.35%). On the other hand, a considerable quantity of palmitic acid (16.26%), squalene (12.83%), α-tochopherol (3.92%), γ-sitosterol (2.72%), phytol (2.52%), *trans*-geranylgeraniol (2.34%), octacosane (2.04%), methyl palmitate (1.01%) and myristic acid (0.73%)was detected in hexane extract.Similarly, a considerable amountof α-tochopherol (10.01%), stearic acid (5.82%), vitamin E (5.41%), uneicosane (4.79%), α- nonadecylene (3.77%), bacchotricuneatin C (3.48%), myristic acid (2.80%) and lauric acid (1.66%) was noted to be present in chloroform extract.

Rarely reports on the chemical composition of *Z. mauirtiana* leavesare available in the literature. Previously, palmitic (0.1%), linoleic (0.1%) and stearic (0.1%) acids were detected in head space volatiles from flower of *Z. mauritiana* (Alves *et al*., 2005). The presence of a notable amount of an essential fatty acid (α-linolenic acid, ω-3) in

methanol (14.21%) and hexane (26.45%) extracts of *Z. mauritiana* leaves in the present study can be supported by the occurrence of this acid in the leaves of some related species such as *Ziziphus spinachrisiti* (20%) and *Ziziphus lotus* (9.5%) (Benammar *et al.,* 2010; Nazif, 2002). In line with α-linolenic acidcontent of methanol extract in the present analysis, in an earlier study, methanol extract of leaves of *Nothapodytesnimmoniana* also contained considerably high amount (17.40%) of α-linolenic acid (Uma & Balasubramaniam, 2012). Meanwhile, Chebouat *et al*. (2013), investigated quite different chemical components in crude alkaloidal extract of *Z. mauritiana* plant. Moreover, in the present analysis, squalene was also detected at a considerable level (12.09-12.83%) in methanol and hexane extracts of the tested leaves. Squalene is known for its chemo preventive effects against various human carcinoma cell lines (Ronco & Stefani, 2013).

A recent study conducted by Nyanga *et al*. (2013) investigated a notable amount of vitamin E in the fruit sample of *Z. mauritiana*.In the present analysis, distribution of vitamin E in methanol and chloroform extracts of *Z. mauritiana* leaves was also examined (Table 4). Anticancer effect of vitamin E analogues has been established *In vivo* and *In vitro* trials (Constantinou *et al*., 2008). Saturated isomer of vitamin E can suppress proliferation in a wide range of carcinoma cells including colon, breast, lung, liver, stomach, prostate and pancreas (Comitato *et al*., 2010; Sakai *et al*., 2006).

Table 4. Composition of chemical components of methanol, chloroform and hexane extracts of leaves from *Z. mauritiana* analyzed by Gas chromatography mass spectroscopy (GC-MS).

[a]Components	[b]RT	Methanol extract	Chloroform extract	Hexane extract
		Composition (%)		
Diglycerol	8.68	0.30 ± 0.01^{b}	----	----
2,3 dihydrobenzofuran	13.31	0.60 ± 0.12^{c}	----	----
1,2 diacetate glycerol	13.81	1.44 ± 0.09^{ab}	----	----
Uneicosane	18.76	----	4.79 ± 0.34^{d}	----
Lauric acid	19.00	----	1.66 ± 0.05^{a}	----
Myristic acid	22.35	----	2.80 ± 0.13^{ad}	0.73 ± 0.51^{ef}
E-15-Heptadecenal	23.13	----	12.31 ± 0.01^{a}	----
Phytol acetate	23.34	----	----	1.02 ± 0.16^{bc}
Methyl palmitate	26.55	7.81 ± 0.11^{a}	2.83 ± 0.25^{bf}	1.01 ± 0.43^{ce}
Palmitic acid	27.28	13.57 ± 0.02^{c}	38.55 ± 0.08^{d}	16.26 ± 0.01^{b}
Hentriaconate	27.57	----	3.25 ± 0.14^{ae}	----
Linoleic acid, methyl ester	30.08	5.98 ± 0.40^{cf}	----	0.45 ± 0.38^{b}
Phytol	30.14	9.78 ± 0.19^{d}	----	2.52 ± 0.15^{ef}
Methyl stearate	30.19	15.59 ± 0.01^{b}	2.31 ± 0.07^{ac}	0.53 ± 0.06^{de}
Linoleic acid	30.73	4.75 ± 0.08^{e}	----	1.37 ± 0.04^{b}
α-Linolenic acid	30.87	14.21 ± 0.32^{ab}	----	26.45 ± 0.01^{a}
Stearic acid	31.21	1.94 ± 0.45^{a}	5.82 ± 0.28^{bd}	----
α-Nonadecylene	31.73	----	3.77 ± 0.04^{cf}	----
Archidic acid methyl ester	33.65	1.60 ± 0.02^{de}	----	----
Carbromal	33.78	0.76 ± 0.05^{bc}	----	----
Bacchotricuneatin C	34.14	----	3.48 ± 0.16^{df}	----
3-methyl piperidine	34.21	0.48 ± 0.07^{c}	----	----
o-methyl delta-tochopherol	36.71	----	----	0.47 ± 0.09^{bc}
Octacosane	38.33	----	----	2.04 ± 0.08^{d}
Cyclobarbital	38.95	0.61 ± 0.13^{ef}	----	----
Squalene	40.23	12.09 ± 0.35^{d}	----	12.83 ± 0.26^{d}
Trans-Geranylgeraniol	41.74	----	----	2.34 ± 0.35^{ae}
2,4- Dimethyl Benzoquinoline	42.47	----	----	2.28 ± 0.03^{a}
α-tochopherol	44.16	----	10.01 ± 0.16^{a}	3.92 ± 0.01^{b}
Vitamin E	44.17	2.35 ± 0.09^{ad}	5.41 ± 0.22^{bc}	----
4-Chloro-2-trifluoromethylbenzoquinoline	45.93	----	----	1.74 ± 0.01^{e}
Thymol TMS	47.51	1.26 ± 0.03^{ef}	----	----
γ-sitosterol	47.65	----	----	2.72 ± 0.48^{ac}
17-Hydroprogesterone	54.12	----	----	3.42 ± 0.02^{de}
Total		**95.12± 0.38**	**96.99± 0.10**	**83.1± 0.53**

(----) = not detected. [a]Compounds are identified on the basis of comparison of retention time and mass spectra in NIST data base. Values are Mean ± SD of triplicate determinations. Mean with different superscript letters within the same row indicate significant difference ($p<0.05$) among extraction solvents employed. [b] Retention time in minutes

Conclusion

The current study concluded that chloroform extract from the leaves of *Z. mauritiana* had the highest amount of phenolics as well as exhibited stronger antitumor and anticancer activities. Nevertheless, methanol extract with relatively greater content of flavonoids depicted a higher DPPH scavenging and antimicrobial activity. Such variations of the antioxidant and biological attributes within different extracts of *Z. mauritiana* leaves can be attributed to the polarity and nature of extraction solvent as well as the chemical constituents being extracted. The presence of a notable amount of α-linolenic acid in methanol and hexane extracts of the leaves of *Z. mauritiana*is fascinating towards exploring the potential uses of its leaves as a new dietary source of essential fatty acids. The findings of thiswork also support the isolation of anticancer/antitumor components from the leaves of *Z.mauritiana*for the development of natural pharmaceutics to be useful in the treatment of cancer and microbial infections.

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