African Journal of Biotechnology Vol. 10(13), pp. 2514-2519, 28 March, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.768
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Screening of *Zizyphus jujuba* for antibacterial, phytotoxic and haemagglutination activities

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Accepted 23 September, 2010

Plants are very useful, self-generating machines, producing a variety of useful bioactive products. Keeping in view this idea, the crude methanolic extract and various fractions of *Zizyphus jujuba* were screened for antibacterial, phytotoxic and haemagglutination activities. The n-hexane and aqueous fractions showed significant activity of 60 and 66.66% against *Bacillus pumalis* and *Pseudomonas aerugenosa*, respectively. Activity of ethyl acetate fraction was 65.38, 62.96, 62.96 and 72% against *Staphylococcus epidermidis*, *Salmonella typhi*, *P. aerugenosa* and *B. pumalis*, respectively. On the contrary, no activity of this fraction was recorded against *Streptococcus pneumoniae*. The crude, n-hexane, chloroform, ethyl acetate and aqueous fractions showed 41.37, 44.82, 41.37, 55.17 and 44.82% activity against *Enterobacter aerogenes*, respectively. The crude methanolic extract and n-hexane fractions were inactive against *Escherichia coli*, *S. pneumoniae and Klebsella pneumoniae*, respectively. The ethyl acetate fraction was moderately phytotoxic against *Lemna minor* L at 1000 µg/ml. All the other fractions showed low phytotoxic activity at 1000 µg/ml. At 100 µg/ml, all the fractions showed low phytotoxic activity except crude methanolic extract, which was inactive. All the test samples were inactive at 10 µg/ml. All dilutions of the test samples showed no haemagglutination activity against any blood group.

Key words: *Zizyphus jujuba*, antibacterial, phytotoxic, haemagglutination, *Lemna minor*, minimal inhibitory concentration.

INTRODUCTION

About 60% of the world's populations exclusively rely on traditional medicine (plant extracts) for their primary health-care needs (Farnsworth, 1994). Currently, there are increasing incidents of infections due to evolution of new pathogens and resistance of the present pathogens to the existing antibiotics, for example, multi-drug resistant tuberculosis (MDR-TB) is resistant at least to isoniazid and rifampicin, the two most powerful first-line anti-TB drugs (Leimane., 2005). Plants are rich sources of bioactive compounds. Of the world's 25 best selling pharmaceutical agents, 12 are naturally derived products.

Zizyphus jujuba commonly called, Red date, Chinese date or Bera (Pushto), belongs to family *Rhamnaceae*. This family consists of 50 genera and more than 900 species; it is almost cosmopolitan and found mainly in subtropical to tropical areas. The bark, leaves and fruit of several species of *Rhamnaceae* have been used as laxatives, notably *Rhamnaceae cathartica* and *Rhamnaceae frangula*. Many *Ziziphus* species yield edible fruit, among these are: *Z. jujuba* (Chinese jujube) and *Ziziphus mauritiana* (Indian jujube) which are cultivated on a commercial scale (Yilin and Carsten). The roots of *Ziziphus oxyphylla* Edgew and juice of fresh leaves of *Z. mauritiana* L are used for curing jaundice (Gul et al., 2009). A cold suspension of dried roots powder of *Ampelozyziphus amazonicus* is used to prevent malaria (Neto et al., 2008). An infusion of *Rhamnus cathartica* L fruits is used as an antiseptic for wounds (Ivancheva and

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Abbreviations: MIC_{50}, Minimal inhibitory concentration required to inhibit the growth of 50% of organisms; **TB,** tuberculosis; **MDR-TB,** multi-drug resistant tuberculosis; **DMSO,** dimethyl sulfoxide.

Stantcheva, 2000). Traditionally, *Origanum majorana* L is used in asthma, indigestion, headache, rheumatism and protect against hydroquinone induced cytogenesis and histological changes (Jun et al., 2001; Inas et al., 2008).

The methanolic extract of *Zizyphi spinosi* semen, over a concentration range of 0.05–5 µg/ml, prevents N-methyl-D-aspartate (NMDA) induced neuronal cell damage *in vitro* (Jeong et al., 2004).

The seeds of *Z. jujuba* have been used as analgesic, tranquilizer, convulsant and have been prescribed for the treatment of insomnia and anxiety in Asia (Peng and Zhu, 2001). Furthermore, traditionally, jujube is used prophylactically for liver diseases (Khare, 1995). The fruit being mucilaginous is also very soothing to the throat and decoctions of *jujube* have often been used in pharmacy to treat sore throats. *Z. jujuba* extracts exhibited protection against hydroquinone induced cytogenesis (Inas et al., 2008). Theasinensin A, a polyphenol obtained from fruits of *Z. jujuba* suppressed the antibiotic resistance of Methicillin-resistant *Staphylococcus aureus* (Tsutomu et al., 2005). Extracts of *Z. jujuba* fruits and seeds exhibited moderate activity against *Lycoriella ingenua* and *Coboldia fuscipes*, which are important mushroom pests (Jee et al., 2008).

The aim of the present work was to screen the aerial parts of *Z. jujuba in vitro* for possible biological/pharmacological activities, that is, antibacterial, phytotoxic and haemagglutination activities.

MATERIALS AND METHODS

Plant material

The aerial parts of *Z. jujuba* were collected from the native Northern region of Khyber Pukhtunkhawa, Pakistan. The sample was kindly identified by Prof. Dr. Abdur-Rashid, Department of Botany, University of Peshawar, Pakistan.

Extraction

The plant material was shade dried, chopped into small pieces and grounded to powder, using an electric grinder. The powdered material of *Z. jujuba* (7 kg) was soaked in methanol for 15 days, twice, at room temperature, with occasional shaking. Each time, the material was filtered and the filtrate was concentrated at 40°C under vacuum, by rotary evaporator. A blackish crude methanolic extract of *Z. jujuba* (850 g) was obtained.

Fractionation

The crude methanolic extract of *Z. jujuba* (800 g) was suspended in distilled water (500 ml) and partitioned with n-hexane (3 x 500 ml), chloroform (3 x 500 ml) and ethyl acetate (3 x 500 ml), respectively, to yield the n- hexane (200 g), chloroform (160 g), ethyl acetate (110 g) and aqueous (240 g) fractions. 90 g of the crude methanolic extract of *Z. jujuba* was left for biological/pharmacological activities. All the fractions will only contain their particular compounds based on the solubility from the crude extract. For example, the n- hexane fraction will contain only those compounds which are non-polar, and so on.

Antibacterial activity

Determining percent inhibition

Antibacterial activity of the crude methanolic extract and various fractions of *Z. jujuba* were determined against *Escherichia coli*, *Pseudomonas aeruginosa*, *S. aureus*, *Staphylococcus epidermidis*, *Salmonella typhi*, *Bacillus pumalis*, *Klebsella pneumoniae*, *Streptococcus pneumoniae* and *Enterobacter aerogenes* (Ahmad et al., 2009). Eighteen hours old culture of the test organism from the nutrient broth was transferred to sterile nutrient agar plates to make bacterial lawn. After 30 min, using a sterile 6 mm borer, wells were dug in plates. Stock solutions (3 mg/ml) of the test samples were prepared in sterile dimethyl sulfoxide (DMSO, less than 1%). 100 µl of crude methanolic extract and fractions were loaded to their respective wells. Amoxicillin and DMSO (less than 1%) were used as positive and negative controls, respectively. Zone of inhibition was measured (in mm) in comparison with positive control.

Determination of minimum inhibitory concentration (MIC)

After determining the percent inhibition, the MIC_{50} of the test samples at the concentration of 0.9, 1.5, 2.1, 2.7 and 3.2 mg/ml were measured against the test organisms (Banso, 2009). To sterile nutrient broth in the test tubes (4 ml), test samples and test organisms were inoculated, incubated for 24 h at 37°C. Results were recorded after 24 h based on the percent clarity.

Phytotoxic bioassay

The *Lemna* bioassay can be used to detect the effect of heavy metal like cadmium herbicides on the physiology of plants. Herbicides originating from plants' origin are often environment friendly. Therefore search for plants'-origin herbicides, is sensible. Phytotoxic activities of the crude methanolic extract and various fractions of *Z. jujuba* were determined against *Lemna minor* L (Ahmad et al., 2009). Stock solutions (20 mg/ml) were prepared in methanol and different concentrations of the test samples, that is, 1000, 100 and 10 µg/ml were introduced in sterilized flasks, inoculated with test samples and left overnight to evaporate methanol. 20 ml of E-media were then added to the flasks and 16 healthy *L. minor* L plants, with a rosette of three fronds, were introduced into each flask. Parquet was used as standard growth inhibitor. All the flasks were then incubated in growth chamber at 28 ± 1°C for 7 days. The results were noted by counting the number of fronds damaged and growth inhibition was calculated in reference with the negative control.

Haemagglutination

The crude methanolic extract and various fractions of *Z. jujuba* were screened for possible haemagglutination activity, against human erythrocytes of all blood groups (Naqvi et al., 1992). Fresh blood was collected from healthy volunteers, centrifuged and the erythrocytes were separated. 2% erythrocytes suspension was prepared in phosphate buffer (pH 7.4). For activity, stock solution (1 mg/ml) of the test samples were prepared in DMSO and different dilutions (2, 4, 8 and 16) were made from it. From each dilution, 1 ml was added to 2% erythrocytes suspension (1 ml) and incubated at 37°C. Positive and negative results are indicated by rough granules and smooth button formation. Extent of deposition determined the intensity of positive result.

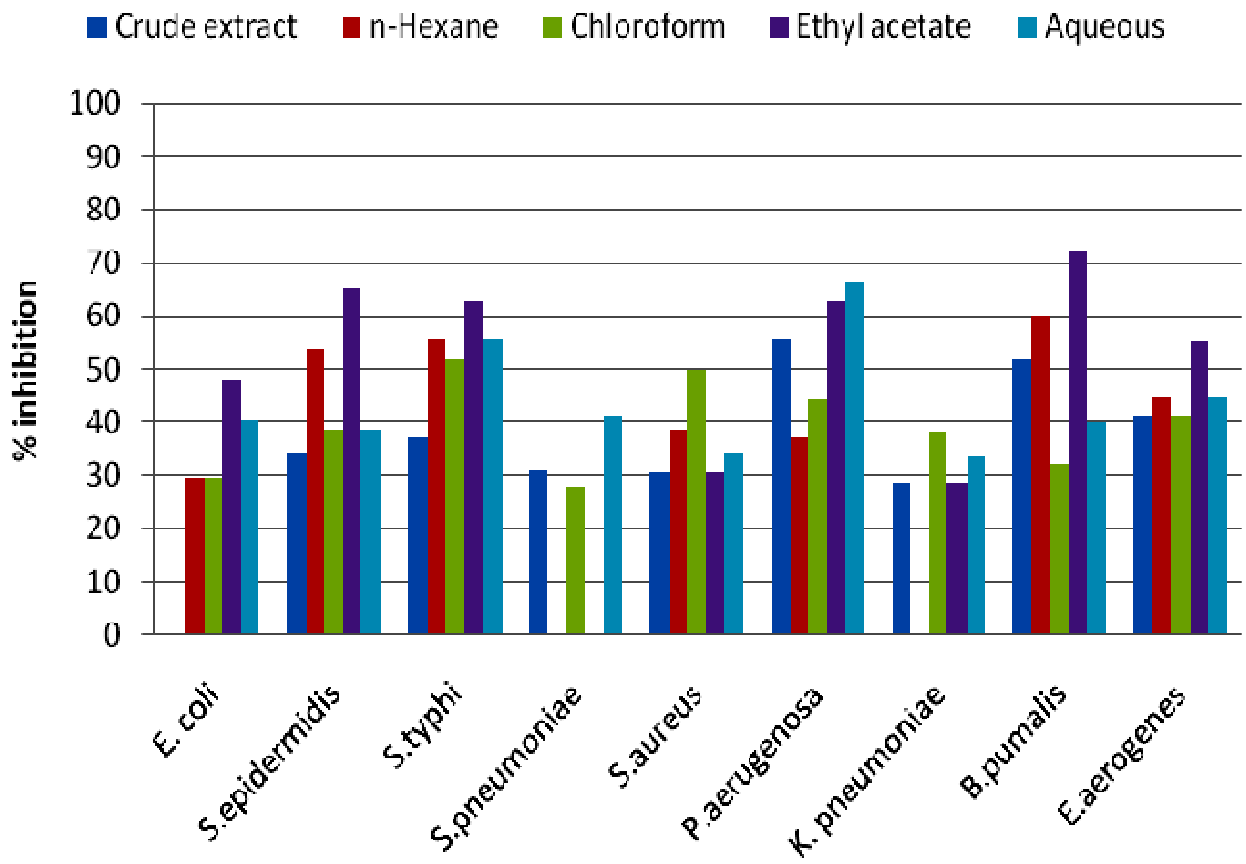

Figure 1. Antibacterial activity of the crude methanolic extract and various fractions of *Z. jujuba*.

RESULTS AND DISCUSSION

The interest regarding the research on medicinal plants has increased over the last few decades due to onset of new infection, in particular, infections by *Enterococcus* and *Staphylococcus* species, which are agents of many intra-hospital infections and antibiotic resistance to available drugs, e.g. *S. aureus* has become resistant to several antibiotics to which it was previously susceptible. Some of the antibiotics to which it is now resistant are pencillin G, macrolides, lincosamides, tetracyclines and gentamicin (Ayliffe, 1997). With the intent of exploring new bioactive compounds from plant origin, we have selected *Z. jujuba*, which is locally used as an analgesic, tranquilizer, convulsant and have been prescribed for the treatment of insomnia and anxiety (Peng and Zhu, 2001). The results of antibacterial activity of the crude methanolic extract and various fractions of *Z. jujuba* are shown in Figure 1. The crude methanolic extract showed moderate activity against *P. aeruginosa, B. pumalis* and *E. aerogens* with 55.55, 52 and 41.37%, low against *S. typhi*, *S. epidermidis*, *S. pneumoniae*, *S. aureus* and *K. pneumoniae* with 37.03, 34.61, 31.03, 30.76 and 28.57% inhibition, respectively. It was inactive against *E. coli.* The n-hexane fraction was significantly active against *B. pumalis* (60%), moderately active against *S. typhi, S. epidermidis* and *E. aerogens* with 55.55, 53.84 and 44.82%, low against *S. aureus*, *P. aeruginosa* and *E. coli* with 38.46, 37.03 and 29.62%, respectively. In contrast, the n-hexane fraction presented no activity against *S. pneumoniae and K. pneumoniae.* The chloroform fraction of the plant was moderately active against *S. typhi*, *S. aureus*, *P. aeruginosa* and *E. aerogens* having percentage inhibition of 51.85, 50, 44.44 and 41.37. Low activity was observed against *S. epidermidis*, *K. pneumoniae*, *B. pumalis*, *E. coli* and *S. pneumoniae* with 38.46, 38.09, 32, 29.62 and 27.58% inhibition, respectively. The EtOAc fraction was significantly active against *B. pumalis*, *S. epidermidis*, *S. typhi and P. aeruginosa* with 72, 65.38, 62.96 and 62.96, moderately active against *E. aerogens*, *E. coli* with 55.17 and 48.14% inhibition. It showed low activity against *S. aureus* and *K. pneumoniae* with 30.76 and 28.57 percentage inhibition and inactive against *S. pneumoniae.* The aqueous fract-ion showed significant activity against *P. aeruginosa* with 66.66% and moderate activity against *S. typhi*, *E. aerogens*, *S. pneumoniae*, *E. coli* and *B. pumalis* with 55.55, 44.82, 41.37, 40.74 and 40% inhibition, respectively. It conferred low activity on *S. epidermidis*, *S. aureus* and *K. pneumoniae* with inhibition percentage of 38.46, 34.61 and 33.33, respectively. MIC_{50}

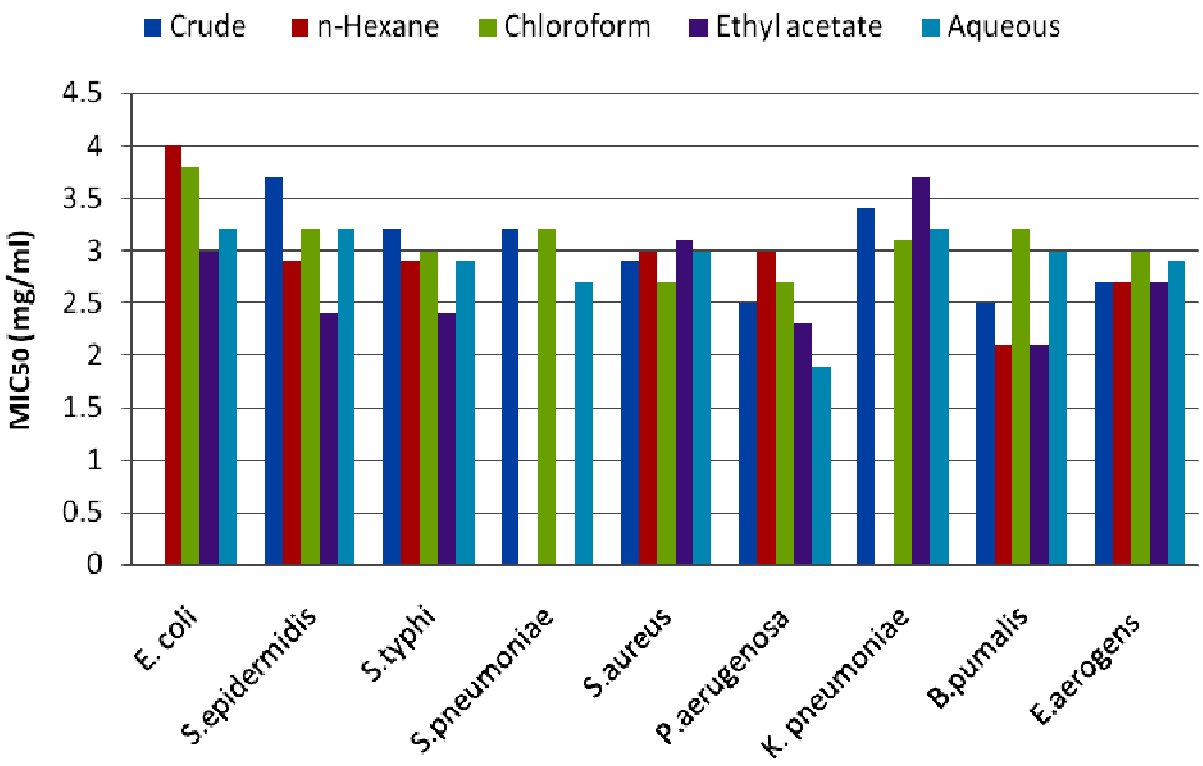

Figure 2. MIC_{50} values of the crude methanolic extract and various fractions of *Z. jujuba*.

of the test samples ranged from 1.9 - 3.8 mg/ml (Figure 2). This work is continuation of our effort on exploring new bioactive compounds. In our previous work, we have utilized similar approach and explored various fractions of Onosma griffithii for antibacterial activity against *E. coli*, *B. subtilis, S. aureus, Shigella flexenari* and *S. typhi* (Ahmad et al., 2009). Same strategy was followed by Ajaiyeoba (2002), in which the n-hexane, ethyl acetate, ethanol and water extract of *Parkia bicolor A. Chev* were tested for antimicrobial activity against *S. aureus, B. cereus, E. coli*, *P. aeruginosa, Aspergillus niger and Candida utilis* (Ajaiyeoba, 2002). The crude methanolic extracts of different plants were tested against gram positive and negative bacteria for new bioactive compounds (Shahidi, 2004). The n-hexane and EtOAc fractions of *Z. jujuba* were significantly active against *B. pumalis*, *S. epidermidis*, *S. typhi and P. aeruginosa* with low values of MIC_{50}.

Lemna plants are miniature aquatic monocotyledonous plants which are very sensitive to bioactive compounds. *Lemna* assay has been used to detect natural anti-tumor and phytotoxic compounds, and may be useful to detect new plant growth stimulants (Rehman, 1991).

Previously, we studied the phytotoxicity of the crude methanolic extracts of *Rumex hastatus*, *Rumex dentatus*, *Rumex nepalensis*, *Rheum australe*, *Polygonum persicaria* and *Polygonum plebejum* (Family *Polygonaceae*) using Lemna bioassay. At the concentration of 1000 µg/ml, all the extracts except *R. hastatus*, were significantly active (Hameed et al., 2009). Phytotoxic activity test of the crude methanolic extract and various fractions of *Z. jujube* was carried out against *L. minor* L (Figure 3). The crude methanolic extract showed 12.5 and 0%; n-hexane, 25 and 6.25%; $CHCl_3$, 18.75 and 12.5%; $EtOA_c$, 43.75 and 18.75%; and aqueous, 31.25 and 12.55% growth inhibition at concentrations of 1000 and 100 µg/ml, respectively. At concentrations of 10 µg/ml, no phytotoxic activity was observed. However this time, the selected species was not phytotoxic in most of the cases. Only ethyl acetate ($EtOA_c$) (43.75%) fraction was moderately active. Aqueous extracts of seven species of freshwater *Eleocharis* spp. were tested for allelopathic activity using common duckweed lemna minor assay. Different species showed different results (Jean et al., 1991). Our results indicate that this species of *Rhamnaceae* is having no potent phytotoxic activity.

The lectins are found ubiquitously in plants and other organisms. Their ability to differentiate different carbohydrates moiety of cell surface and in solution, have promoted speculations on their physiological role. Lectin specificities have been used to study the sugar components on normal and cancerous cell surfaces (Lis and Sharon, 1986), the structural and functional roles of cell surface carbohydrates (Sharon and Lis, 1972), to isolate and characterize glycoconjugates and mutants isolation, resistant to the cytotoxic action of some lectins

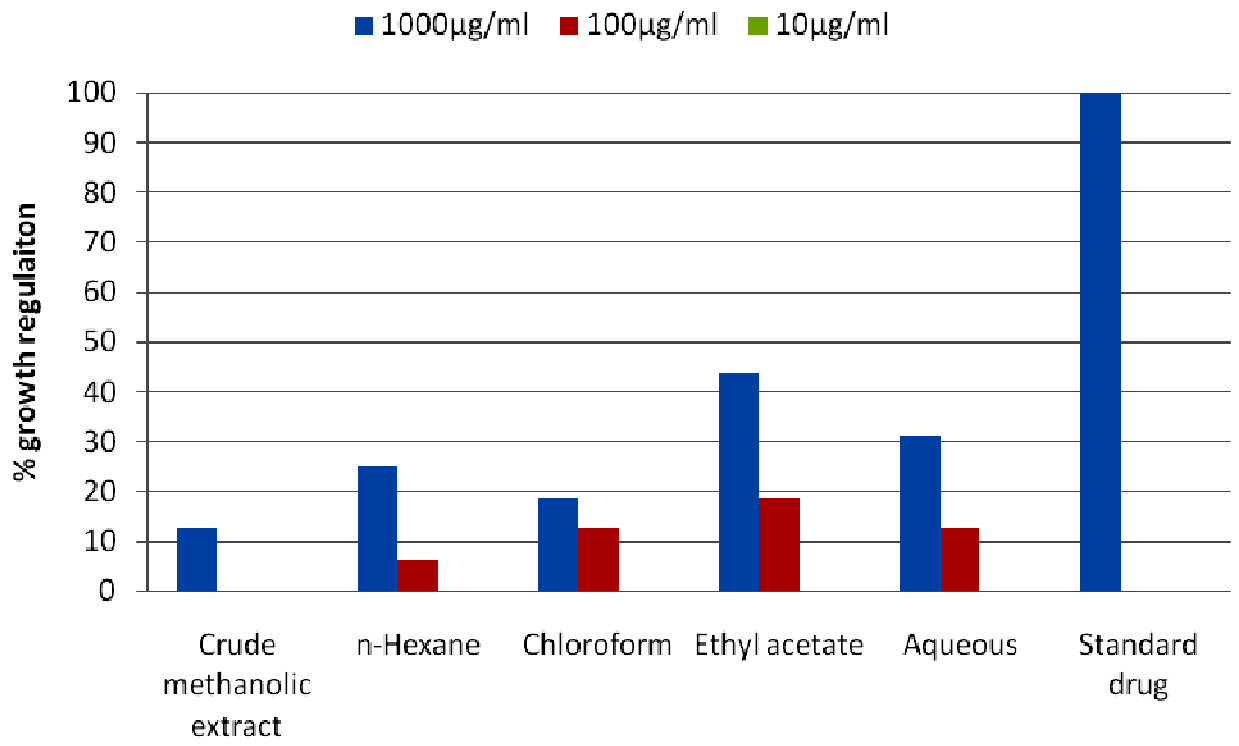

Figure 3. Phytotoxic activity of the crude methanolic extract and various fractions of *Z. jujuba* against *L. minor* L.

(Kuroki et al., 1991), for agglutination of erythrocytes to find the blood type and can be used for estimation of the number of virus particles (Wei and Koh, 1978). Keeping in view the diverse role of lectins, haemagglutination activity of *Z. jujuba* was done against red blood cells (RBCs) of human blood. All dilutions of the test samples (crude methanolic extract and various fractions) showed no haemagglutination activity against any blood group. The species selected in this research was unable to agglutinate RBCs of the human blood, indicating that this species of *Rhamnaceae* lack phytolectins. Many viruses attach to molecules present on the surface of RBCs.

Conclusion

The findings of the present study revealed that *Z. jujuba* (Rhamnaceae) contain potent antimicrobial property. The n-hexane fraction of *Z. jujuba* was significantly active against *B. pumalis* (60%, MIC_{50} =2.1 mg/ml), EtOAc was active against *B. pumalis* (72%, MIC_{50} = 2.1 mg/ml), *S. epidermidis* (65.38%, MIC_{50} = 2.4 mg/ml), *S. typhi* (62.96%, MIC_{50} = 2.4 mg/ml), *P. aerugenosa* (62.96%, MIC_{50} = 2.3 mg/ml) and aqueous fraction against *P. aerugenosa* (66.66%, MIC_{50} = 1.9 mg/ml). So the research should be extended for the isolation of active compounds. This species of *Rhamnaceae* has weak phytotoxic activity. It lacks phytolectin because there was no agglutination of the human RBCs.

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