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Evaluation of antifungal, antioxidant and phytochemical screening of *Ziziphus jujuba* Miller leaves from the northwestern region of Algeria

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Abstract

Zizyphus jujuba is used in Algeria in various herbal remedies. Unfortunately this species is little known phytochemically. In order to valorize medicinal plants grown in northwestern Algeria, we were interested in evaluating the antioxidative and antifungal activities of *Zizyphus jujuba*, and to determine the main phytochemical classes involved in these activities to improve the therapeutic knowledge of this species. Phytochemical screening was performed using standard methods. Quantification of total polyphenols, flavonoids and condensed tannins was performed by colorimetric techniques using a UV-visible spectrophotometer. The extract was further evaluated for their antifungal activity against seven strains of pathogenic fungi, by well microdilution method, and the free radical scavenging activity of extract was measured by using 2,2-diphenyl-1-picrylhydrazyl (DPPH). The results of phytochemical screenings showed the presence of flavonoids, alkaloids, saponins, gallic tannins, leucoanthocyanins and cardiac glycosides. Quantitative analysis of phenolic compounds shows that total polyphenols, flavonoids and condensed tannins amounts were 240.78 (mg GAE/g DE); 113.6 (mg EC/g DE) and 78.02 (mg EC/g DE), respectively. The evaluation of the antioxidative activity, show that the extract of *Zizyphus jujuba* leaf has an IC_{50} of 0.38 mg/ml and at a dose of 1 mg/ml reduces 91.78% of oxidized DPPH in a very short time. Among the seven fungal pathogenic strains used for the test of antifungal activity, the most potent activity was against *Candida albicans* with a minimum inhibitory concentration (MIC) of 0.0975 mg/ml. *Ziziphus jujuba* leaves contain different biologically active molecules which justify their use in phytotherapy.

Key words: *Zizyphus jujube*, Phytochemical, Antifungal, DPPH

Full length article **Corresponding Author*, e-mail: zoubir_31@yahoo.fr

1. Introduction

Ziziphus jujuba locally called Sfisef in northwestern Algeria, belongs to the family Rhamnaceae, and is a thorny tree 4 to 10 m long. The leaves are 4 to 8 cm long, ovate to elliptic in shape with finely toothed edges, bright green in color, but yellow in autumn before falling. Small yellow flowers develop in the axils of the leaves in early summer. The fruit is an edible drupe, round to elongated, smooth and green before maturity, it folds and becomes mahogany or reddish-brown at the final stage of maturity, carries a single seed.

In traditional medicine, Jujube is introduced as a blood purifier, sedative, expectorant, cough suppressant, anti-asthmatic, laxative, wound healer, and anaphrodisiac. It also contributes to the treatment of rectal and intestinal ulcers/diseases as well as liver diseases. Ripe jujube fruit has laxative properties, but unripe jujube heals diarrhea [1]. Their fruits and seeds are usually applied in traditional Chinese medicine for the treatment of various diseases, such as anorexia, lassitude, insomnia, anxiety [2]. The leaves have been also used as a folk medicine to treat children suffering from typhoid fever, furuncle and eczema in China [3].

Currently, several laboratory works *in vivo* or *in vitro* confirms that this species has interesting biological activities such as anti-inflammatory [4], anti-diarrhoeal [5], sedative [6], anticancer [7], antibacterial [8], antiobesity [9], antidiabetic [10] and hepatic protective [11-13].

In Algeria *Zizyphus jujuba* is very little known chemically, so no work is done on the antifungal activity on

this species. As part of the valuation of medicinal species, the present study was conducted to identify molecules of secondary metabolism by chemical screening, quantify polyphenols, flavonoids and tannins, and to evaluate the antioxidative and antifungal activities of this species growing in northwestern Algeria.

2. Materials and methods

2.1 Plant material

The leaves of *Zizyphus jujuba* were collected from the region of Sfisef, this region takes the vernacular name of the species studied because of their abundance. It is located in the province of Sidi Bel Abbes in northwestern Algeria. The species has been identified by the forest protection directorate of the region. The leaves were dried out of light and moisture at room temperature and then reduced to a fine powder.

2.2 Phytochemical screening

Phytochemical analysis of the extracts was performed according to standard methods with some modifications [14-16]. Leaves of *Zizyphus jujuba* were screened for the following phytoconstituents: alkaloids, tannins, saponins, terpenoids, flavonoids, cardiac glycosides, quinones, anthraquinones, anthocyanins and leucoanthocyanins.

2.2.1 Test for flavonoids (cyanidin reaction)

To 5 ml of infusion added 5 ml of hydrochloric alcohol, 0.5 g of magnesium chips and 1 ml of isoamylic alcohol which produced red or orange or violet color due to presence of flavonoids.

2.2.2 Test for leucoanthocyanins

The above referred procedure was also followed for testing the presence of cyanidin except addition of magnesium turnings. After 15 min of heating on a water bath, the appearance of red or cherry-red or brown or purplish red color indicated the presence of leucoanthocyanin.

2.2.3 Test for alkaloids

About 25 mg of extract was dissolved in 5 ml of 5% aqueous HCl and filtered. These filtrates were divided into three equal parts. Drops of Wagner, Mayer and Dragendorff reagents were added to each. A red-brown precipitate (Wagner), yellowish-white precipitate (Mayer) and red-orange precipitate (Dragendorff) indicated the presence of alkaloids.

2.2.4 Saponins (foam test)

Exactly 1 ml solution of extract was diluted with distilled water to 20 ml and shaken vigorously and kept for 15 min. Development of stable foam suggests the presence of saponins.

2.2.4.1 Determination of foaming index

For determination of foaming index, 1 g of aerial parts was taken in a 250 ml conical flask containing 100 ml of boiling water. The moderate boiling was maintained for 30 min. It was cooled and filtered into a 100 ml volumetric flask and the volume was readjusted to 100 ml with distilled water. In 10 stoppered test tubes (height 16 cm, diameter 16 mm), the solution was taken in successive portion of 1, 2, 3, up to 10 ml. The volume of the solution in each test tube was adjusted with distilled water to 10 ml. The tubes were corked and shaken in a lengthwise motion for 15 sec, two shakes per second was maintained in each tube. The test tubes were allowed to stand for 15 min and height of the foam was measured. Foaming index =1000\a; Where, a= the volume in ml of the decoction used for preparing dilution in the tube where foaming to a height of 1 cm is observed.

2.2.5. Test for tannins

Approximately, 2–3 drops of $FeCl_3$ (1%) solution were added to 5 ml of infusion in water. A blackish-blue color indicated the presence of Gallic tannins, while a greenish-black color indicated the presence of catechol tannins.

2.2.6. Test for cardiac glycoside (keller-killiani test)

Each extract (5 ml) was treated with 2 ml of glacial acetic acid containing a few drops of ferric chloride and 1 ml of H_2SO_4 along the side of the test tube. The formation of brown ring at the interface gives positive indication for cardiac glycoside and a violet ring may appear below the brown ring.

2.2.7. Test for quinones

Concentrated sulphuric acid (1 ml) was added to 1 ml of plant extract. The formation of red color indicates the presence of quinones.

2.2.8. Test for anthraquinone (borntrager test)

Powdered drug is mixed with chloroform or any water immiscible solvent which was filtered and to the filtrate, added ammonia. The ammoniacal layer showed after shaking pink red or violet color due to presence of anthraquinone.

2.2.9. *Test for anthocyanins*

Anthocyanins were found by adding 5 ml of 5% infused with 5 ml of H_2SO_4 (10 %) and 5 ml of NH_4OH (50 %). The color of the infused is accentuated by acidification, then turns blue in basic medium in the presence of anthocyanins.

2.3 Preparation of the hydroethanolic extract

About 10 g of powder was extracted with ethanol (70 %) at a ratio of 1:10 (w/v) using the maceration method for 12 h at ambient temperature thrice. The extracts were collected and concentrated using a rotary vacuum evaporator at 40 ˚C and the solid crude extracts were kept in the refrigerator (4˚C) till further analysis.

2.4 Quantification of phenolic compounds

2.4.1 Determination of total polyphenol content

The total phenolic contents were determined by the Folin-Ciocalteu method [17]. 20 µl of extract (1 mg/ml) was mixed with 1.58 ml water, and then added 100 µl of the Folin-Ciocalteu (1/10) and 300 µl of Na_2CO_3 (7.5%). After 90 min at room temperature, the absorbance was measured at 765 nm. The total polyphenol content was determined from extrapolation of calibration curve which was made by preparing gallic acid solution (10 to 100 mg/ml). The estimation of the phenolic compounds was carried out in triplicate. The total phenolic contents were expressed as milligrams of gallic acid equivalents (GAE) per g of dried extract (DE).

2.4.2 Determination of total flavonoids content

The total flavonoids content of leaves extract was estimated by the method that uses aluminium chloride [18]. 1 ml of sample containing 1 mg/ml of dry extract was mixed with 5 ml of distilled water and subsequently with 0.3 ml of a $NaNO_2$ solution (5 %) (Merck). After 6 min, 0.3 ml of 10 % $AlCl_3.6H_2O$ (Merck) was added and mixture was allowed to stand for 6 min, then 2 ml of 1 mol/l NaOH was added to the mixture. Immediately, water was added to bring the final volume to 10 ml and the mixture was thoroughly mixed and allowed to stand for another 15 min. The absorbance of the mixture was then determined at 510 nm. Flavonoid contents were calculated using a standard calibration curve developed by using the known concentrations of catechin (10–100 mg/ml). The results were expressed as mg of catechin equivalent (EC) per g of dried extract (DE).

2.4.3 Determination of condensed tannin content

The condensed tannin contents were determined by a modification of the vanillin/HCl method [19]. 100 µl of extract (1 mg/ml) was pipetted into a test tube and 5 ml vanillin/HCl reagent was added. The reaction was carried out in the dark at room temperature for 20 min, and then absorbance was measured at 500 nm.

Condensed tannin contents were calculated using a standard calibration curve developed by using the known concentrations of catechin (10–100 mg/ml). The results were expressed as mg of catechin equivalent (EC) per g of dried extract (DE).

2.5 Study of antioxidant activity

2.5.1 Scavenging activity against 1,1-diphenyl-2-picryl hydrazyl radical

The antioxidant activity of the samples and standards was determined by way of the radical scavenging activity method using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). 0.1 ml of the sample at different concentrations (0.05–10 mg/ml) was added to 3.9 ml of DPPH methanolic solution (60 µmol/l). The blank sample consisted of 0.1 ml of methanol added to 3.9 ml of DPPH. After 30 min of incubation period at room temperature in the dark, the absorbance was measured at 517 nm [20].

$$\% \textbf{ Scavenging Activity} = [(\mathbf{A_0} - \mathbf{A_1})/\mathbf{A_0}] \times \mathbf{100}$$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of the extract. IC_{50} value and the percent inhibition of DPPH are calculated from the graph. Ascorbic acid was used for comparison.

2.5.2 Kinetic behaviour of the DPPH radical-scavenging activity

In this study, we examined the kinetics of the chemical reaction between the DPPH and the extract at a concentration of 1 mg/ml every 5 min for 30 min, compared to a blank by replacing the extract with methanol. Ascorbic acid at a concentration of 1 mg/ml is used for comparison.

2.6 Antifungal activity

2.6.1 Fungal strain

Seven pathogenic strains of fungal isolates were provided by the laboratory of mycology and parasitology from the university hospital center of Sidi Bel Abbes (Algeria), all isolates were identified using growth and colony characteristics, morphology, germ tube formation and the sugar fermentation, according to standard methods. They were maintained in culture on Sabouraud glucose (4%) agar medium. Fungal isolates and their origins are grouped in Table 1.

2.6.2 Minimum inhibitory concentration against different fungal strains

The minimum inhibitory concentration (MIC) was determined in sterile 96-well microplates using the broth microdilution method, by the technique described previously [21] with some modifications.

Aliquot of the crude hydroethanolic leaves extract was dissolved in DMSO 10 % at concentrations of 100 mg/mL and sterilized by filtration.

Fungal culture was suspended in 5 ml sterile 0.85 % NaCl. Turbidity was adjusted to Mc Farland 0.5 scale, equivalent to 1.5×10^6 (CFU/mL).

Each well contained 50 µL of serial double dilutions of the extract (in a Sabouraud broth medium with concentrations that range from 100 mg/ml to 0.0487 mg/ml) is inoculated aseptically with 50 µl of inoculum suspension. The control well containing broth Sabouraud medium without extract was inoculated only with fungal suspension. The plates were incubated at 28 ˚C for 3 days and read visually.

The MIC: It is the lowest concentration of the sample at which the tested microorganisms do not demonstrate any visible growth after incubation.

2.7 Statistical analysis

All determinations were made in the triplicate and the values were averaged and reported along with the standard deviation (±). Statistical analysis was carried out by using Microsoft Excel 2010 software.

3. Results and Discussion

3.1 Phytochemical screening

The results of the phytochemical screening of *Zizyphus jujube* are summarized in Table 2.

Phytochemical tests carried out on *Zizyphus jujuba* leaves show the presence of flavonoids, alkaloids, saponins, gallic tannins, leucoanthocyanins and cardiac glycosides, and absence of quinones, anthraquinones and anthocyanins.

The phytochemicals compounds detected are known to be of medicinal importance and can protect or lower the risk against several chronic diseases [22-23]. We found that the saponin foam index is high, which means that the leaves of this species are a very important source of these molecules. Saponins have been found to possess significant anti-cancer, antimicrobial, hypocholesterolemic, hypoglycemic, immuno-stimulant and anti-inflammatory properties [24-28]. This species is known as a sedative. This activity results from the hypnotic effect of saponins such as jujubosides which may be a good source of main compounds for new hypnotics. The serotonergic system may involve in the hypnotic effect of jujubosides [29]. Cardiac glycosides are used for congestive heart failure and cardiac arrhythmias treatment; however, *Zizyphus jujuba* has been used in phytotherapy to treat asthma [1]. Flavonoids, phenolic acids and caffeic acid were isolated and identified by NMR and mass spectrometry in *Ziziphus oxyphylla*; a species of the same genus of jujuba [30].

3.2 Total phenolic, flavonoids and tannin contents

The results of quantitative analysis are represented as a histogram in Figure 1 and summarized in the table 3.The total phenolics, flavonoids and tannin contents of hydro-ethanolic extracts were 240.78 (mg GAE/g DE); 113.6 (mg EC/g DE) and 78.02 (mg EC/g DE), respectively. As compared to previous works on the same specie [31-32], different values for these phytochemicals were recorded. This difference results from several factors: the geographical location, the powder/solvent ration, the extraction technique, the method used for the quantification and the standard used. Flavonoids are believed to be the major bioactive components in jujube leaves, which have been shown to be responsible for cardio-protective, anticancer, antidiabetic, anti-aging and neuro-protective effects [33-34]. And the best sampling time is June so that it is a better new resource for health promotion based on flavonoids of *Ziziphus jujuba* leaves [3].

3.3 Study of antioxidant activity

The DPPH radical scavenging ability of the hydro-ethanolic extract of *Zizyphus jujuba* leaves in comparison with that of ascorbic acid is shown in Table 3.

The ability to trap DPPH with the hydro-ethanolic extract of *Zizyphus jujuba* leaf was slightly lower than that of ascorbic acid at doses ranging from 0.1 to 1 mg / mL. Therefore, IC_{50} of *Zizyphus jujuba* leaf extract was slightly higher (0.38 mg/ml) than that of ascorbic acid (0.31 mg/ml). At a dose of 1 mg/ml, *Zizyphus jujuba* leaf extract reduced 91.78% of oxidized DPPH (Figure 2).

Other studies show that, the DPPH inhibition capacity varies between 90 and 98% for raw leaf extracts of *Zizyphus jujuba* from Oman, obtained using different solvents [35], and it was between 70.69 % and 93.93 % in fruit extract from different regions of Iran [32], and it was between 33.6 and 98.6 % in different Chinese jujube obtained from different areas [36].

The kinetics study of the hydroethanolic extract of the leaves of *Zizyphus jujuba* at a concentration of 1 mg/ml against the DPPH, shows that the reaction was fast and almost instantaneous. The color change expresses that DPPH• (radical form) changes to DPPH-H (reduced form) in the first five minutes to attain equilibrium state and complete reduction (Figure 3).

Ziziphus jujuba leaf infusion contains rich sedative flavonoids and antioxidants. These findings suggest that this infusion has good radical scavenging ability by reducing the risk of oxidative damages and it could be a very healthy beverage for the public [33]. Moreover, it was found [37] that *Ziziphus jujuba* contains antioxidant molecules that protect the body from oxidative stress induced by H_2O_2 and from neurodegeneration. The recent previous study showed the presence of eight flavonoids including kaempferol and quercetin glycosides, which were responsible for stronger DPPH and ABTS radical-scavenging activities in a much shorter time [34]. *Ziziphus jujube* also exhibited anti-proliferative activities [38] due to presence of flavnoids.

3.4 Antifungal activity

The results of the antifungal activity are shown in figure 4, and the minimum concentrations of inhibition on each fungal species are grouped in the table 4. The hydro-ethanolic leaves extract showed antifungal activity against all fungi tested but with different concentrations. The leaf extract of *Zizyphus jujuba* showed antifungal activity against yeast *Candida albicans* and filamentous fungi such as *Aspergillus* sp. and *Rhizopus* sp., and dermatophytes like *Trycophyton* sp. and *Microsporum.* The maximum activities have been found against *Candidas* (0.0975 mg/ml), *Aspergillus niger* (0.39 mg/ml) and *Microsporum canis* (3.12 mg/ml).

Both *Alternaria* sp. and *Penicillium* sp. have been eliminated with the same concentration of the extract 12.5 mg/ml. However, the lowest activity is determined against *Rhizopus* sp. with minimum inhibitory concentration of 50 mg/ml. In comparison with other studies on the same specie, the ethanolic extract of *Zizyphus jujuba* fruit showed MIC of 2.35 mg/ml against *Candida albicans* and 2.86 mg/ml against *Aspergillus fumigatus* [39]. Methanolic extract of *Zizyphus jujuba* leaves has MICs of 15 mg/ml against *Aspergillus flavus*, 20 mg/ml against *Tricophyton rubrum* and 35 mg/ml against *Candida albicans* [40]. The phytochemical analysis of the leaf extracts showed the presence of saponnins, tanins, flavonoids and alkaloids, which might be responsible for antifungal activity of the plant. Plants produce phytoconstituents to protect themselves against pathogenic microorganisms. The antimicrobial potency of the plant is attributed to the chemical structure and concentration of their active constituents.

Many polyphenols, such as simple phenols, flavonoids and tannins have antifungal activity, which depends in part on the number and position of the hydroxyl groups. They can inhibit the enzymes of microorganisms by reacting with sulfhydryl groups or form complexes with extracellular and soluble proteins. They could also disrupt cell membranes [41-42]. The previous studies confirmed the toxicity of tannins to filamentous fungi and yeasts. Even low tannin concentrations altered the morphology of *Crinipellis perniciosa* germ tubes [43-44]. In contrast, a previous study conducted in Pakistan indicated that *Zizyphus jujuba* lack potent antifungal activity and had no activity against *Aspergillus flavus* and a moderate antifungal activity against *Aspergillus niger* [45]. Saponins are stored in plant cells as inactive precursors that can be readily converted into biologically active antibiotics by enzymes in response to pathogen attack. They appear to act by disrupting the membrane integrity of fungal cells [41]. The alkaloids can exhibit antimicrobial activity by several mechanisms including DNA intercalation, targeting RNA polymerase, gyrase and topoisomerase IV, and inhibition of cell division [46-48], perturbations in the biosynthesis or metabolism of heme [49], and inhibiting enzyme activity [50].

Table 1: List of fungal strains used and their origins

Clinical fungal strains	Source
Candida sp.	Oral mycosis
Aspergillus niger	Ear discharge
Rhizopus sp	Hospital air sample
Alternaria sp	Hospital air sample
Penicillium sp	Tinea unguium
Trichophyton rubrum	Tinea corporis
Microsporum canis	Tinea capitis

Table2: Qualitative phytochemical analysis of *Zizyphus jujba* leaves

Flavonoïds	+
Alkaloïds	+
Saponins Foam Index	+ 1000
Tannin ($FeCl_3$)	+ Gallic
Leucoanthocyanins	+
Cardiac Glycosides	+
Quinones	-
Anthraquinones	-
Anthocyanins	-

Table 3: IC50 values and the percentage inhibition of DPPH of hydro-ethanolic leaf extracts of *Zizyphus jujuba* and ascorbic acid

Sample	IC_{50} (mg/ml)	% DPPH radical scavenging activity at 1mg/ml
Hydro-ethanolic extract of *Zizyphus jujba* leaves	0.38	91, 78
Ascorbic acid	0.31	99.98

Table 4: Minimum inhibitory concentrations values of hydro-ethanolic leaves extract of *Zizyphus jujuba* against clinical fungal isolates

Clinical Fungal Strains	MIC (mg/ml)
Candidas	0.0975
Aspergillus niger	0.39
Rhizopus sp	50
Alternaria sp	12.5
Penicillium sp	12.5
Trichophyton rubrum	6.25
Microsporum canis	3.12

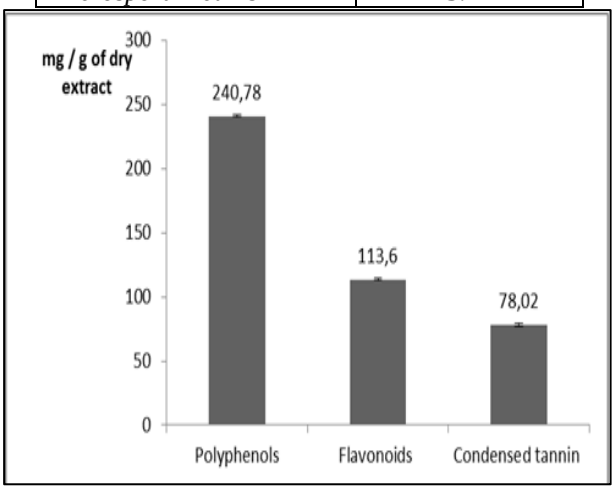

Figure 1: Total phenolics, flavonoids, and condensed tannins contents of hydro-ethanolic extract of *Zizyphus jujuba* leaves. Total phenolics were expressed as mg of GAE/g of DE. Total flavonoids and condensed tannins contents were expressed as mg of CE/g of De

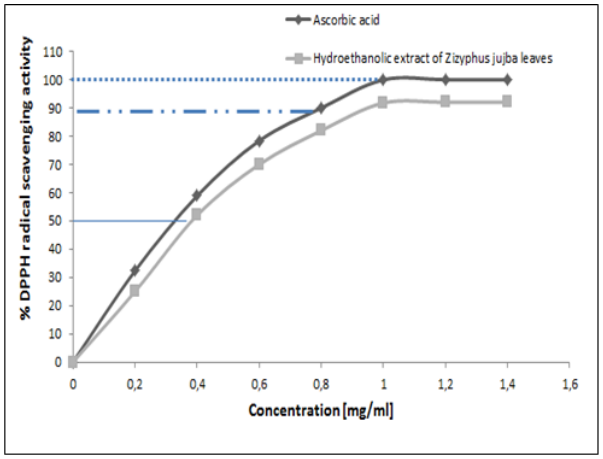

Figure 2: Radical scavenging activity for leaves extracts of *Zizyphus jujuba* and ascorbic acid at different concentrations. The figure shows the percentage inhibition of DPPH and the IC50.

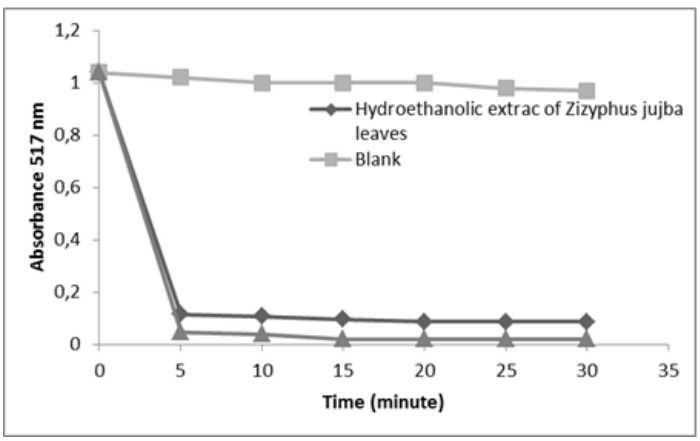

Figure 3: Kinetic behavior of antioxidant activity of hydro-ethanolic extract of *Zizyphus jujuba* leaves against DPPH, compared with that of ascorbic acid and blank

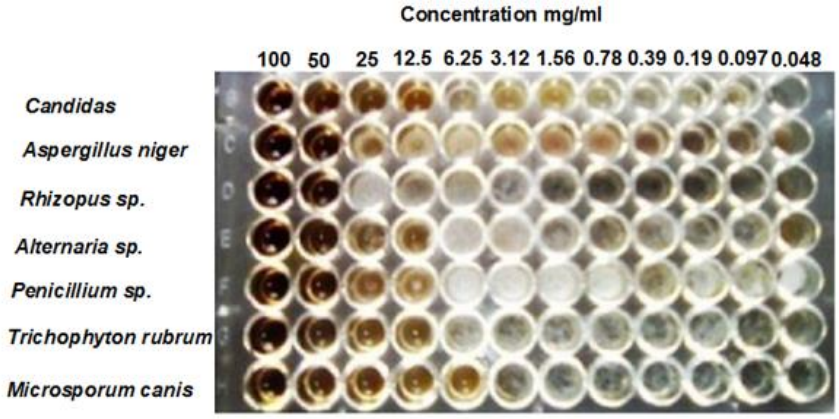

Figure 4: Micro-dilution plates representing the minimum inhibitory concentrations (MIC) of hydro-ethanolic leaves extract of *Zizyphus jujuba* by Broth microdilution method, against different isolated pathogenic fungal strains

4. Conclusion

It can be concluded that *Zizyphus jujuba* contains various important bioactive compounds belonging to the different phytochemical classes, which justifies its use as a medicinal plant. This species can be used against pathogenic fungi and oxidative damage. Further studies are needed to isolate active biomolecules from leaf extracts to elucidate mechanism of action.

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