Evaluation of the Antifungal effects of Glycyrrhiza glabra L.,Morus nigra L., and Urtica urens L. extracts against some pathogenic fungi in vitro

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Abstract

From patients at Al-Yarmouk Teaching Hospital who were suspected of having different fungal illnesses, fifty specimens were taken. Samples were collected from different parts of the body (lining of the mouth and nose, vagina, between the thighs) for patients from the age of two months to the age of 68 years. The samples were transferred to the laboratory and cultivated on Sabouraud dextrose agar (SDA) medium to ensure obtaining pure samples.Three replicates were made for each sample and kept at a temperature between 28 and 37 degrees Celsius for a period between 48 hours and 14 days; after that, it was time to conduct visual and microscopic diagnostic tests. where 42 (84%) samples showed fungal growth and 8 (16%) samples did not show fungal growth, as the samples were: Candida albicans 16 (32%); Candida tropicalis 7 (14%); Candida kruzei 10 (20%); Candida parasilosis 5 (10%); Cryptococcus neoformans 2 (4%); Trichophyton rubrum 1 (2%); and Aspergillus fumigatus 1 (2%).Alcoholic extracts of Urtica urens L., Morus nigra L., and Glycyrrhizia glabra L. were prepared, and tests were conducted to detect some of the active substances of these extracts (alkaloids, phenols, and terpenes). These extracts showed that they contain these substances, but in varying concentrations, as they were present in the licorice extract in higher concentrations than in the other two plants, and in the blackberry, extract were less than the previous two extracts.Following that, the three plant extracts were tested for their ability to inhibit the growth of some yeasts and fungi, and their effect against fungi was compared to the antifungal Clotrimazole. where it appeared on the inhibitory effect of Glycyrrhiza glabra extract at a concentration of 2 mg/ml, where it inhibited the growth of all yeasts and fungi tested compared to other extracts, while the least inhibition was of Morus nigra extract at a concentration of 1.5 mg/ml. inhibitory effect of most yeasts and fungi tested. Trichophyton rubrum was the most sensitive to plant extracts, while Candida albicans was the least sensitive to plant extracts.

Keywords: Clotrimazole, Evaluation, Glycyrrhiza glabra L.,Morus nigra L., and Urtica urens, extracts, pathogenic fungi, Candida,Cryptococcus, Trichophyton, Aspergillus

Introduction

The discovery of new chemotherapy agents can benefit from the structures that may be found in plants. The bioactive compounds found in plants, including terpenoids, flavonoids, alkaloids, and tannins, have been enhanced in vitro to exhibit antibacterial effects. (Sakalani and Chandra 2012; Abdullah, et al.,2015). The current global trend is methodical, strategic research and development of novel pharmaceuticals derived from natural resources. Over 30% of the chemotherapeutic drugs currently recommended in clinics are natural product-derived medicines. (Al-Tekreetiet al. 2017).

Human pathogens are becoming increasingly resistant to routinely used antibiotics, prompting researchers to search for new antimicrobial compounds from various sources, such as plants. (Erdogrul, 2002).Urtica urens L., also known as dwarf nettle, is an annual herb that belongs to the Urticaceae family. It is native to Eurasia and is thought to be interchangeable in terms of medicine. It prefers wet, rich soil and tends to grow in large patches. Its leaves are fleshy, dropping, serrated, and roughly heart-shaped. Stinging hairs are present on the stem and leaves. Formic acid, acetic acid, leukotrienes, histamine, 5-hydroxytryptamine, acetylcholine, and butyric acid are all present in the stinging hairs on the leaves (Nouri, et al., 2015). The leaf extract of Urtica urens L. contains appreciable levels of polyphenols that are considered antibacterial compounds (Jimoh, et al., 2010; Al-Abbasi, et al.,2022).

Because it contains substances including organic acids, phenolic acid, carotenoids, amino acids, fatty acids, and flavonoids, the tall species of stinging nettle, Urtica dioica L., demonstrates bactericidal and fungicidal effects (Devkota, et al., 2022).While it has been discovered that the dwarf species Urtica urens L. has an antibacterial action on several types of bacteria, including Shigella sonnei, Salmonella, and Shigella flexeneri, (Rahman, et al., 2010). A good substitute for topical antibiotics that are used to treat skin wound healing and to control Pseudomonas aeruginosa and contaminated wounds is a plant extract from Urtica urens L. extract, 25% ointment. (Hussain, et al., 2017).

The Moraceae family, which includes the black mulberry (Morus nigra L.), has a sizable population in North and South America, Asia, Africa, and Europe. It is a 13-meter-tall deciduous tree with a heavy head of limbs that emerges from a rough stem. The upper surface of the leaves is covered in short, stiff hairs and is thick, blunt-toothed, and lobed. It is a significant plant from an economic standpoint that is utilized in sericulture as food for the tamed silkworm.(Awasthi, 2004). The leaf decoction has diuretic, fever-reducing, and blood-purifying effects. Tap worms might be ejected from the body using the bark of Morus nigra

L., and its extracts have been shown to have antifungal and antibacterial properties. Fruit extracts were said to protect biomembranes and biomolecular structures from peroxidative damage. (Dheeb, et al., 2014). Mulberry fruits have a wide range of biochemical activities, including anti-cancer, anti-hyperlipidemia, and antioxidant qualities, according to several studies.This is because they are naturally rich in phenolic compounds, such as anthocyanins, flavonols, and phenolic acids. (Dheeb,et al. 2015).

All sections of this plant contain phenolic chemicals, many of which have anti-bacterial and anti-oxidant characteristics. (Minhas, et al., 2016).

Liquorice is the popular name for the plant Glycyrrhizia glabra L, which belongs to the Leguminoceae family and has a mild natural sweetness. The liquorice plant is a legume that is indigenous to regions of southern Europe, Asia, and India. It is a perennial herb with pinnate leaves that are from 7 to 15 cm long and grows to a length of one meter. The odor derives from a complex and varying collection of chemicals, of which anethol is only a tiny part, and the roots are stoloniferous. Glycyrrhizin, which has a sweet taste 30–50 times sweeter than sugar, accounts for a large portion of the sweetness in liquorice. Glycyrrhizin, a substance in liquorice, has antiviral and antibacterial properties (Hussein, et al. 2019). Liquorice roots contain phytoestrogens called isoflavone glabrene and isoflavone glabridine. (Somjen, 2004). Glabridin is resistant to Candida albicans and effective against yeasts and fungus. (Alfauomy, et al., 2020). Due to its extensive medicinal characteristics, liquorice has been utilized as an herbal medicine for millennia. The plant's antifungal, anti - bacterial, and antioxidant properties have been used in medicinal settings. (Kalaiaras and Pugalend, 2011;Dheeb,et al.,2019).

In this study, three plant extracts from Urtica urens L., Morus nigra L., and Glycyrrhizia glabra L plants were tested under laboratory conditions to determine the effect of these extracts on the growth of the fungus.

Candida is a type of yeast that is the most common cause of infection. Among the fungi, there are about 20 different species of Candida.Candida infections can be systemic or superficial. Oropharynx candidiasis (affecting the oral mucosa, palate, and tongue), esophagitis, conjunctivitis, vaginitis, or gastrointestinal candidiasis are examples of superficial infections of the cutaneous or mucocutaneous tissues. Meningitis, pyelonephritis, esophagitis, endocarditis, and disseminated candidiasis (Candida septicemia) are examples of systemic infections that can be lethal and affect numerous organs. (Hussain, 2011).

Cryptococcus neoformans is an opportunistic fungus that is widespread in the world and infects humans and most animals (Sidrimet al., 2010). Meningitis, which is most frequently caused by yeast spread, is a condition caused by C. neoformans that has a significant propensity to infect the brain's central nervous system more often than other locations in the body(Park et al., 2009).C. neoformans occurs in people with weakened immune systems (Jarvis et al., 2013). Another factor that contributes to infection is the excessive and indiscriminate use of antibiotics (rassinet al., 2013).

Trichophyton rubrumIt is one of the most common skin fungi, as it largely infects all parts of the body except for the scalp. (Abdelal, et al., 2013). T. rubrum is one of the most important dermatophyte fungi that causes a superficial infection called ringworm. It spreads globally, causing chronic skin infections and other skin infections in humans, with infection rates ranging between 4% and 41% (Mahmood, et al., 2019).

One of the most significant human pathogenic filamentous fungi, Aspergillus fumigatus, infects persons with compromised immune systems and causes a number of illnesses, such as allergic pulmonary aspergillosis and chronic pulmonary aspergillosis (Steinbach et al., 2012;Ibrahim,et al.,2017; Hussain,et al.,2018).

Clotrimazole It is widely used as an antifungal against many types of fungi that are pathogenic to humans (Kumar, et al., 2021). The antibiotic Clotrimazole effectively stops the growth of Candida by preventing the formation of ergosterol, which is really necessary for the development of the Candida biological membranes. (Hussain, 2011). The antibiotic clotrimazole is not used in the treatment of systemic fungal infections as it is considered toxic (Khater and Khan, 2022).

Material and methods

Collection and Isolation the samples:

The study group consisted of patients attending Yarmouk Teaching Hospital, where 50 samples were collected, who were suspected of having various fungal diseases, by the specialist doctor, where samples were collected from different areas of the body (from the lining of the mouth and nose, the vagina, and between the thighs). For patients of different ages between 2 months - 68 years.

The samples were transferred to the laboratory, and cultured on Sabouraud dextrose agar (SDA) medium, to ensure obtaining pure samples (3 replications were made for each sample and kept at 32 °C for a period of 7-14 days).

Identification of the samples:

The fungal cells are transferred to Petri dishes and cultivated on (SDA) media with chloramphenicol 0.05 g to stop bacterial development after the fungal growth begins. To avoid the growth of contaminated fungus, 30% ammonium hydroxide was applied to the pure culture in place of cycloheximide. The edge of the culture dishes was added with one drop of 30% ammonium hydroxide. For each concentration of extracts, anti-fungal, and fungi type, 3 replicates were made and swirled in a circular motion for 20 minutes prior to growing. The dishes were left incubating for 7–14 days at a temp. of 32 degrees Celsius. (Bander et al., 2015).

Yeast species determined:

The Vitek2 test was used to identify the species of yeast. Prepared pipes for the device were placed in a special mold called a Cassete. Those tubes were loaded with 3 cc of the device's saline solution and placed in a section of the yeast colonies to be analyzed at an age of 1 to 2 days. The colonies were transferred to the tubes with a sterile needle, and the tubes were thoroughly agitated by the vibrator to absorb the solution.Each card's serial number was inputted using a barcode, and a laser beam was then focused at each card's serial number as it was introduced into the computer because yeast has a density of between 1.8 and 2.2. Only ten isolates may be hidden in each template's special list; a number was then written for each isolation, and even the data was saved. The airless filler door was then opened, and the template was placed within. The door is shut and the button is fastened. The process lasts for 70 seconds.After applying heat to the capillary tube linking each card and sealing it, the mechanism raises the cards so that operations can be performed on them inside the device. The next step is loading, which indicates the machine word. The device needs to be taken out so that the mould on the door can be cleaned off and the specimens can be incubated there until the next day, when the finished results are delivered in the form of a report.) (Badr and Abaas, 2017).

Mold determined:

A - Morphology Examination of the colonies

The phenotypic examination is one of the important means that must be taken into account to differentiate between fungi, and it includes several things that must be taken into account, including the number of days that it takes for the fungus to start growing, and after the appearance of growth on the surface of the culture medium, the colony’s apparent shape, color, and texture (Powdery, Cottony, Villous) and the color of the colony is examined from the opposite side, and once the growth has ceased, the colony's diameter is measured. In this

type of diagnosis, the following sources were relied upon: (Dahham,et al., 2019), (Dheeb, et al. ,2015), (Kwon – chung, and Bennett 1992) and (Abed, et al. ,2020).

B - Microscopical Examination of the colonies

A drop of Lactophenol cotton blue dye, was situated on a clean glass slide using a sterile inoculation needle, part of the fungal filaments was transferred from the area between the fungal growth center and its edge, to a glass slide, and mixed with the dye, then a slide cover was placed on it and pressed gently by the base of the loop, for the purpose of brushing the sample on this drop.

A low power 10X examination was performed on the prepared slide before a high power 40X examination, to note the fungal hyphae, their shapes and branches, and conidia of different shapes and sizes, and the way they were arranged on the fungal hyphae. Reliance was made on the same references used in diagnosing the phenotype of the colonies.

Preparation of plant extracts

For the preparation, leaves from Morus nigra L., and Urtica urens L. plants as well as rhizomes from the Glycyrrhiza glabra L., were gathered from various parts of Baghdad.

Table 1: The plant used in the study

Plant	The scientific name	The plant parts
Dwarf nettle	**Urtica urens L.**	**Leaves**
Black mulberry	**Morus nigra L.**	**Leaves**
Licorice	**Glycyrrhizia glabra L.**	**Roots**

These plant components were cleaned with tap water and allowed to air dry for 5 days at room temp. To make a powder, the dried plant components were placed in a blender. Each sample contained 30 g of Glycyrrhiza glabra L. roots, Morus nigra L. leaves, and Urtica urens L. leaves. Furthermore, 300 mL of 99% ethanol solvent were added to a conical flask, which was thoroughly mixed, and it was then set in a heat shaker. for 48 hours at 30 °C and 150 rpm.Using "Whatman No. 1 filter paper," the were filtered, and the filtrate was spun at 3000 rpm for ten mins to separate it. The precipitate was thrown away, and the supernatant was used to concentrate in a 40°C oven for four hours, yielding a viscous semi-liquid that was then stored below zero degrees. (El-Hilali, et al. ,2016).

Chemical detection of some active substances in plants.

1 - Detection of alkaloids: Following the procedure used by Bandar and his team in 2009, 10 g of plant samples were boiled in 50 ml of distilled water that had been acidified with 40%

hydrochloric acid, after which the solution was filtered and allowed to cool. Finally, 0.5 ml of the filtrate was checked in a test tube with 0.5 mL of Wagner reagent. The presence of a brown color indicates successful detection.

2 - Detection of phenols: The approach taken by Deeb (2009) was used. 2 ml of a 1% ferric chloride solution were combined with 3 ml of plant extract. A bluish-green hue showing up is indicative of successful detection.

3 - Detection of terpenes: The procedure described by Deeb (2009) was utilized, in which 1 g of the plant extract was dissolved in 1-2 ml of chloroform, followed by the addition of a drop of anhydrous acetic acid and a drop of sulfuric acid. The presence of a brown hue is proof that terpenes are present.

Preparation of concentrations

Concentrations of 1.5 g and 2 g were prepared for the previously prepared plant extracts, and concentrations of 0.0001 g and 0.005 g of the antifungal Clotrimazole, that were dissolved in the organic matter were prepared dimethyl sulfoxide (DMSO).

Effect of plant extracts and antifungal on (Trichophyton, Aspergillus)

The SDA medium free of antifungal and antibacterial drugs was mixed with the dried extracts at a concentration of (1.5, 2) mg per ml, and after hardening of the culture medium, it was placed in the middle of a petri dish. This is the approach adopted by Bandar and his group (2009). The diameter of the colonies was measured as they grew (an average of 2 orthogonal diameters) per day. A disc of fungal inoculum with a diameter of 5 mm was cut from the edge of a seven-day-old fungal colony, and this disc was placed in a hole of the same diameter made in the culture medium. Then the plates were incubated at 25 °C for 12 days. The same methodology was applied to clotrimazole, but with concentrations of (0.0001, 0.005) mg/ml, with three replications.

Activity of the plant extracts and antifungal against Candida sp.

The method followed by Abdullah and his group (2013) was used, where after culturing Candida on SDA medium, holes were drilled with a diameter of 5 mm in which concentrations (1.5, 2) mg/ml of the plant extract were placed, and concentrations (0.0001, 0.005) mg/ml of the antifungal, The inhibitory zone's diameter was then measured in millimeters after being incubated at 37 °C for 48 hours. There were 3 replicates for each concentration.

Results and discussion

Through culture traits and fungal growth of the collected samples, it was found that 42 (84%) samples were positive, 8 (16%) did not show fungal growth, the possibility that some patients had taken doses of antibiotics before taking the sample.

Table 2: The percentage of positive samples is shown.

Samples	Number	Percentage %
Candida albicans	16	32%
Candida tropicalis	7	14 %
Candida kruzei	10	20 %
Candida parasilosis	5	10 %
Cryptococcusneoformans	2	4 %
Trichophyton rubrum	1	2 %
Aspergillus fumigatus	1	2 %
Total	42	84 %

Table No. (2) shows that infection with yeast is higher than infection with filamentous fungi. This is consistent with the study by Garcia Rubio and his group (2020). due to the way that the yeast cell wall's constituent parts are created, which has a higher proportion of chitin. While the amount of chitin in the walls of Paracoccidioides brasiliense and Blastomyces dermatitidis during their fungal phases is only 25–30% of that in the yeast form, it is 3 times more. in the walls of C. albicans.

Glycoproteins, which make up 20–30% of the weight basis of the wall of filamentous fungi and 30–50% of the dry weight of the fungi in yeast, are also present between the components of the cell wall. It performs a variety of tasks, including cellular shape maintenance, adhesion mechanisms, cellular defense against diverse chemicals, molecule absorption, signal transmission, and synthesis and rearrangement of wall constituents. It is a crucial component of a fungus cell's pathogenicity. (Garcia Rubio et al.,2020 ;Abdulbaqiet al.,2018).

Table 3: Medicinal active ingredients found in plant extracts

Active substances / plant extract	Alkaloids	Phenols	Terpenes
Urtica urernsLeaves	+	+	+
Morus nigraLeaves	Weak	Weak	Weak
Glycyrrhiza glabra Rhizomes	+	+	+

Secondary metabolites are the active ingredients in plants in general, which are antioxidant and antimicrobial compounds used by plants to defend themselves against microbial and insect infestations (Alfauomy, et al., 2020).

The outcome of the chemical exposure of some of the active substances of the plants showed that the burning nettle and licorice plant had positive results for the tests of alkaloids, phenols and terpenes, where the licorice plant showed a very clear change in the color of the detector compared to the burning nettle plant and black berries. Black mulberry contained these components, but in weak concentrations compared to the previous two plants.

These results were compared with the findings of; Gaafar and his group (2020) Through their study on the dwarf nettle plant; Wang and his group (2022) when they studied the chemical components of the black mulberry plant; Alfauomy and his group (2020) studied licorice plants, where they all used HPLC analysis to find out the effective chemical compounds present in these plants, which plants use to protect themselves from pathogens.

These studies showed that the concentration of active compounds in licorice is higher than what is found in burning nettle and black mulberry, and that their concentration in burning nettle is higher than what is found in black mulberry plant. These outcomes concurred with those of our investigation, which are displayed in Table 4.

Table 4: The result of Antifungal impact of *Glycyrrhiza glabra* L., *Morus nigra* L., and *Urtica urens* L. extracts contra some pathogenic fungi in vitro.

Plant extract	Plant extract concentration mg/ml	*Candida albicans*	*Candida tropicalis*	*Candida kruzei*	*Candida parasilosis*	*Cryptococcusneoformans*	*Trichophyton rubrum*	*Aspergillus fumigatus*
Urtica urerns Leaves	1,5	12 ± 0,00	11 ± 0,7	9 ± 0,10	7 ± 0,00	12 ± 0,13	60 ± 0,52	50 ± 0,90
	2	14 ± 0,00	13 ± 0,14	14 ± 0,20	12 ± 0,00	17 ± 0,22	79 ± 0,44	64 ± 0,87
Morus nigra Leaves	1,5	5 ± 0,17	9 ± 0,50	8 ± 0,30	5 ± 0.00	9 ± 0,17	23 ± 0,13	10 ± 0,22
	2	7 ± 0,13	10 ± 0,12	13 ± 0.21	8 ± 0,11	14 ± 0,72	36 ± 0,17	24 ± 0,43
Glycyrrhiza glabra Rhizomes	1,5	9 ± 0,22	8 ± 0,14	10 ± 0,11	10 ± 0,12	13 ± 0,80	77 ± 0,33	70 ± 0,71
	2	17 ± 0,27	14 ± 0,27	17 ± 0,17	12 ± 0,12	20 ± 0,70	89 ± 0,20	84 ± 0,88
Control Clotrimazole	0,0001	45 ± 0,33	45 ± 0,00	45 ± 0,02	25 ± 0,00	45 ± 0,00	100 ± 0,00	100 ± 0,00
	0,005	45 ± 0,00	45 ± 0,00	45 ± 0,00	45 ± 0,00	45 ± 0,00	100 ± 0,00	100 ± 0,00
	Inhibition Zone (mm) ± Standard Error (SE)							

Table (4) illustrates a study of the effect of three plant extracts (Urtica urens leaves, Morus nigra leaves, and Glycyrrhiza glabra rhizomes) at different concentrations and their effect on

inhibiting the growth of some yeasts and fungi. The inhibitory effect of these extracts was also compared with the inhibitory effect of the antifungal Clotrimazole.

The results in the table show that the Glycyrrhiza glabraL. extract had the highest ability to inhibit fungal species compared to the other types of plant extracts used in the study, the fungus T. rubrum 89 mm showed the greatest inhibition at a rate of 2 mg/ml, while C. tropicalis 8 mm showed the least inhibition at a dose of 1.5 mg/ml.

This is because the licorice plant contains a high concentration of secondary metabolite compounds as shown in Table (3), as well as a result of the study of Alfauomy and his group 2020.

followed by an extract of Urtica urens L.The fungus T. rubrum 79 mm had the strongest inhibition in concentricityof 2 mg/ml, whereas C. parasilosis 7 mm had the lowest inhibition at a value of 1.5 mg/ml.This is because the burning nettle plant contains concentrations of effective compounds, as shown in Table (3), that inhibit the growth of fungi, but their concentration is lower than that of the licorice plant, as shown in the study of Gaafar and his group 2020.

Finally, an extract of Morus nigra L.the lowest inhibition was for C. albicans and C. parasilosis 5 mm at a concentration of 1.5 mg/ml, while the highest inhibition was for the fungus T. rubrum 36 mm at a dose of two mg/ml. where secondary metabolite chemicals are concentrated in the black mulberry plant, as shown in Table (3), it works to inhibit the growth of fungi less effectively than the two previous plants, but as we noticed in the study of Wang and his group 2022.

It was also noted that fungi (Trichophyton rubrum,Aspergillus fumigatus**)** were more affected by plant extracts compared to yeasts, and that yeast Cryptococcusneoformans was more affected by extracts, compared to Candida species, whose results were close.

This is due to the presence of glycoproteins, which account for 30–50% of the dry weight of the yeast cell wall in Candida albicans yeast as opposed to 20–30% in filamentous fungus, and a focus of chitin that is three whets higher in this yeast than in the other yeasts.Where glycoproteins work to increase the process of yeast adhesion to the host's body and the occurrence of infection, as well as these proteins work to protect yeast cells from the components in the surrounding environment, including the active substances of plant extracts, and the chemical compounds of antifungals, compared to filamentous fungi(Awad and Dheeb, 2020).

Conclusions

The importance of secondary metabolites found in plants and the possibility of using them in the treatment of various microbial infections. The antibiotic clotrimazole is considered toxic when used in systemic fungal infections, so it is preferable to use useful, safe, non-toxic and natural plant extracts.

The cell wall is the first virulence element for microbes in general, as it helps them adhere to the host and cause infection, protects them and makes them more resistant to antibiotics. Fungi differ in their pathogenicity due to the various parts of their cell walls, as well as in their resistance to antifungals.

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