Antimicrobial efficacy of extracts of Saudi Arabian desert Terfezia claveryi truffles

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abstract

Background: Terfezia claveryi truffles are known for their nutritional value and have been considered among traditional treatments for ophthalmic infections and ailments.

Objectives: We sought to investigate the in vitro antimicrobial efficacy of several T. claveryi extracts from Saudi Arabia. Certain pathogenic fungi and gram-negative and gram-positive bacteria were included.

Methods: Dry extracts were prepared using methanol, ethyl acetate, and distilled water, while the latter was used for preparing fresh extracts. The extracts were microbiologically evaluated through the disc-diffusion agar method; the zones of inhibition of microbial growth were measured post-incubation. The minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) were determined in Müller-Hinton Broth through the microdilution susceptibility method. Anti-biofilm activity was assessed for potent extracts.

Results: Dry extracts showed potent activity (>16-mm inhibition zones) against gram-positive (Bacillus subtilis IFO3007 and Staphylococcus aureus IFO3060) and gram-negative (Pseudomonas aeruginosa IFO3448 and Escherichia coli IFO3301) bacteria. The activity against fungi was moderate (12–16-mm inhibition zones) for both Aspergillus oryzae IFO4177 and Candida albicans IFO0583; there was no activity against Aspergillus niger growth. Methanolic extract had the lowest MIC and MBC, exhibiting remarkable activity against S. aureus growth. Fresh extract showed moderate activity against bacterial growth and inactivity against fungal growth. Methanolic extract showed potent anti-biofilm activity (IC50, 2.0 ± 0.18 mg/mL) against S. aureus.

Conclusions: T. claveryi extracts showed antibacterial effects potentially suitable for clinical application, which warrants further in-depth analysis of their individual isolated compounds.

1. Introduction

In the Arabian region, the desert truffle's common name is Al-Fag'a, while Al-Kam'ah is the classic Arabic name, which means “hidden”. Generally, Arabian desert truffles belong to two genera: Tirmania and Terfezia (Bokhary, 1987). Tirmania nivea (white-colored truffle) is called Zubadi or Zubaidi, as a common name, in some Arabian countries. On the other hand, the Terfezia sp. is called Ikhlasi or Kholasi and includes Terfezia boudieri (black-colored truffles) and Terfezia claveryi (brown-colored truffles) (Al-Rawi and Taha, 2010; Bokhary, 1987; Mandeel and Al-Laith, 2007). The Arabian Peninsula represents a well-known source for the latter dark, brown-colored truffles (Al-Rahmah, 2001).

Desert truffles are pale sandy brown, grey or white in color, irregularly spherical, have a slightly spongy texture, have sizes ranging from 1 to 7 cm, and lack a particular smell (Iddison, 2021). They are usually harvested after the rainy season (February...
Essential constituents found in truffles.

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Desert truffles, which are edible mycorrhizal fungi, constitute a traditional food in several cultures because of their medicinal and nutritional properties (Wang and Marcone, 2011). Truffles are considered as very old food that was used instead of meats, and have been highly consumed because of their pleasant aroma and taste (Dundar et al., 2012; Mandeel and Al-Laith, 2007). Their distinct nutritional value appears to be secondary to their rich content of proteins, minerals, vitamins, and unsaturated fatty acids (Patel, 2012). They have been also used for treating ophthalmic infections in traditional medicine (Janakat and Nassar, 2009).

Many compounds have been found in different truffles and some of which have been shown to exhibit different antimicrobial effects (Table 1).

The nutritional value of truffles has been extensively studied (Al-Delaimy, 1977). Unsaturated fatty acids (19.6 %) with a large content of linoleic acid comprise T. claveryi (Saddiq and Danial, 2012). T. claveryi species truffles of the isolated from Iraq comprise the following: 62 % linoleic acid and 17.6 % protein (Al-Kaisey et al., 1996). A study from Saudi Arabia on T. claveryi reported the following composition: 28 % carbohydrate, 16 % protein, and 78 % total moisture (Bokhary and Parvez, 1993). It is noteworthy that similar truffle species of different regions may not have similar chemical compositions. These differences of the chemical profiles could be the result of several factors related to the environment including the climatic changes, the amount of rainfall, season, and soil types (Hussain and Al-Ruqai, 1999).

In addition to the truffles’ nutritional value, flavor, and aroma, they are largely underutilized sources of therapeutic compounds. Antimicrobial, anti-cancer, anti-inflammatory, immunosuppressive, and antioxidant effects of truffles have already been reported (Dundar et al., 2012; Hannan et al., 1989; Mandeel and Al-Laith, 2007). T. claveryi species was reported useful in the treatment of ophthalmic ailments. Ocular infections are highly linked with bacterial biofilms, as increasing evidence has been reported. A biofilm is a characteristic of bacterial growth when bacteria attach to each other and/or to a surface (Zegans et al., 2002). Hence, ocular infections may be mediated by bacterial biofilms, making bacteria persistent on abiotic surfaces that they attach to, either when implanted eyes or through direct biofilm formation on the ophthalmic biotic surfaces (Saraswathi and Beuerman, 2015; Hou et al., 2012). A boiled water extract of truffle was claimed as an effective remedy for trachoma by the Bedouins; the extracts showed growth inhibition of the etiological agent of trachoma, Chlamydia trachomatis (Mandeel and Al-Laith, 2007). Additionally, the antimicrobial activity of several T. claveryi extracts (methylalkyl, aqueous, and partially purified protein) were in vitro tested against Staphylococcus aureus and Pseudomonas aeruginosa (Janakat et al., 2004, 2005). A potent proteinaceous antimicrobial agent against P. aeruginosa, that may be useful for treating ophthalmic infections, was reported to exist in the aqueous extract (Janakat et al., 2005).

This research aimed to in-vitro study the antimicrobial efficacy of certain extracts of T. claveryi, collected from Northern Saudi Arabia, against selected strains of pathogenic fungi and gram-negative and gram-positive bacteria. These microbial strains were not used in previous studies related to the antimicrobial efficacy of truffle extracts.

### 2. Materials and methods

#### 2.1. Sample preparation

Truffles were purchased from the seasonal truffle market of the northern part of Saudi Arabia and were kept in clean polythene bags during transportation. These fruiting bodies of typical desert T. claveryi, locally called Ikhlasi or Kholasi were dark brown in color, small in size, and round in shape (Bokhary, 1987). Distilled water was used for washing a sample of 2 kg thoroughly for 10 times to remove adherent soil. Kitchen sucking papers were then used for blotting the sample that was later cut into small slices.

#### 2.2. Preparation of extracts

Fine pulverized powder of parts of the sample was obtained by drying the parts in a hot oven set at 35–40 °C, until constant weight was achieved after 3 days, followed by milling. The powder was then stored capped at room temperature (25–27 °C). Three parts of the powder, each of approximately 200 g, were used for the extraction through the hot maceration method at 40 °C. Distilled water, ethyl acetate, and methanol were used, in 500 mL each, for the extraction of each part of the powder, respectively. A rotary evaporator was used to filter and concentrate the extracts (Buch, 2012).
USA). The plant extract residues (5 mg each) were re-dissolved in one mL of sterilized distilled water and were stored at −20 °C until use (Saddiq and Danial, 2012).

Another 200 g sample of the fresh slices was homogenized with distilled water (1:3 w/v), at room temperature, using a full-speed household blender for 3 min. The homogenate was kept in a refrigerator overnight, filtered through cheesecloth, and then centrifuged for 15 min at 4,000 rpm. The supernatant was then dried using a rotary evaporator. The dried matter of the aqueous extracts was re-suspended using distilled water and kept at −20 °C until use (Janakat et al., 2004; Viksø-Nielsen et al., 1997).

2.3. Microorganism strains

A panel of standard strains of microorganisms was used for testing the antimicrobial activity of T. claveryi extracts. These included two gram-negative bacteria (P. aeruginosa IFO3448 and Escherichia coli IFO3031), three gram-positive bacteria (Micrococcus luteus IFO3232, Bacillus subtilis IFO3007, and S. aureus IFO3060), and three pathogenic fungi (Aspergillus niger IFO4414, Candida albicans IFO0583, and Aspergillus oryzae IFO4177). The source of these strains was the Institute for Fermentation, (IFO, Osaka, Japan). The microorganism suspensions were adjusted at 10^6 colony-forming units (CFU/mL).

2.4. The in vitro determination of antimicrobial efficacy

The agar disc-diffusion method was employed to perform primary screening (Clinical and Laboratory Standards Institute, 2005). Separate inoculation of the Müller-Hinton agar medium plates with the studied microorganisms was performed. The measurement of the diameters of inhibition zones was then carried out after incubating the inoculated plates at 37 °C for 24 h. The incubation of the plates of the fungi was performed at 25 °C for 48 h. The antifungal fluconazole and the antibacterial ampicillin were used as positive control (10 μg/disc), while a blank disc, immersed in distilled water, was used as negative control.

The Müller-Hinton broth microdilution susceptibility method was used for the evaluation of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts with high activity against the same microbes from the primary screening (Murray et al., 1995; Wiegand et al., 2008). The extracts then underwent twofold derail dilutions. The lowest concentration that killed the microorganism determined the MBC values, while the lowest concentration that inhibited the growth of the microorganism determined the MIC values (confirmed by subcultures onto sterile Tryptone soya agar plates to verify negative growth). In addition, the extracts with potent efficacy were further challenged for biofilm activity against S. aureus IFO3060, which can form a biofilm, in comparison with the antibacterial erythromycin as a reference antibiotic drug.

Negative control wells were filled with broth only, while 200 mL of bacterial suspension were used to fill sterile 96-well plates. The aerobic incubation of covered plates was then carried out at 37 °C for 24 h. Each well’s content was then aspirated, and 250 mL of sterile physiological saline was used to wash the well three times. All non-adherent bacteria were removed by vigorously shaking the plates. The remaining bacteria that were still attached were fixed with methanol, and after 15 min each plate was emptied and left for dryness. Staining of the plates was performed with Gram staining's crystal violet (0.2 mL, 2 %) for 5 min. Following that, running tap water was used to rinse off the excess stain from the plates. After air drying the plates, the adherent cell-bound dye was resolubilized by glacial acetic acid (33 % v/v). Finally, each well’s optical density (OD) measurement was carried out at 570 nm (Stepanovic et al., 2000).

3. Results

The preliminary outcomes of the prepared extracts’ antimicrobial testing are depicted in Table 2. Most of the prepared extracts showed different inhibition degrees against the tested bacteria. The activity was weak against the pathogenic fungus C. albicans and there was no activity against the other fungi. Ethyl acetate, methanolic, and hot aqueous water extracts (dry extract) showed potent activity against the gram-negative E. coli and P. aeruginosa and the gram-positive S. aureus and B. subtilis bacteria.

As seen in Table 3, the microbiologically most active extracts in the primary screening were tested for the MIC and MBC against the same microorganisms. The activity demonstrated by the methanolic extract against B. subtilis (gram-positive bacterium) was good. Table 4 shows that the same extract has exhibited a more potent anti-biofilm activity (IC_{50}) than the ethyl acetate extract (2.0 ± 0.18 mg/mL and 3.8 ± 0.20 mg/mL, respectively), compared to the IC_{50} of the reference drug erythromycin against the standard strain of the gram-positive S. aureus IFO3060 (0.45 ± 0.15 μg/mL).

4. Discussion

Traditionally, most of the studied biological effects of truffles have been related to the antimicrobial activities of desert truffles. For example, the agar-well diffusion assays have demonstrated the antimicrobial activity of T. bouliieri (Fidan et al., 2022; Hamza et al., 2016). It was suggested that these truffles may have some benefits in the treatment of ophthalmic and dermatologic diseases. In addition, antimicrobial activity was reported for some extracts of T. claveryi against P. aeruginosa (Gargano et al., 2017).

Truffle extract antimicrobial activity has been also studied based on the solvents used (Dib-Bellahouel and Fortas, 2019; Gargano et al., 2017). Such an activity was reported for methanolic extracts of Terfezia sp. against gram-positive bacteria, such as S. aureus and B. subtilis (Dib-Bellahouel and Fortas, 2019). In addition, methanolic and aqueous extracts of T. claveryi, plus aqueous extracts of proteins from the same species, have demonstrated antimicrobial activity against S. aureus (Gargano et al., 2017). Furthermore, an inhibition of 40.9 % of P. aeruginosa growth was reported by aqueous T. claveryi extract, while no activity was reported for methanolic extracts (Dib-Bellahouel and Fortas, 2019).

This research studied the antimicrobial efficacy of extracts of T. claveryi obtained by different solvents. The results show that the methanolic, hot aqueous, and ethyl acetate extracts of the dried truffle powder have antimicrobial activity against gram-positive and gram-negative bacteria and fungi (C. albicans). The most effective of which was the methanolic extract while the ethyl acetate one may have promising activity on the same microorganisms, in addition to A. oryzae. The hot aqueous extract was less active than the methanolic extract. On the other hand, the cold aqueous extract of the dried truffle powder and the cold aqueous extract of fresh truffles have shown the least activity with no effect on P. aeruginosa and all the studied fungi.

The methanolic extract in the current study has shown the strongest activity against the gram-positive bacterium B. subtilis as reflected by its least MIC and MBC compared with other extracts. Moreover, it exhibited a more potent anti-biofilm activity than the ethyl acetate extract against the gram-positive standard strain of S. aureus IFO3060.

This study has also revealed that there could be different antimicrobial compounds in different extracts of T. claveryi. The methanolic extract generally contains polar and non-polar active constituents, the ethyl acetate extract contains mainly nonpolar and semipolar compounds, and the aqueous extract contains polar compounds. The precise mechanisms of antimicrobial activities of...
Antibiofilm activity of selected extracts against standard strains of gram-positive and gram-negative bacteria.

| Extracts                      | Gram-positive bacteria | Gram-negative bacteria | Fungi            |
|-------------------------------|------------------------|------------------------|------------------|
| Ethyl acetate (dried truffle powder) | S. aureus (22) B. subtilis (25) M. luteus (15) | E. coli (20) P. aeruginosa (18) | C. albicans (16) A. oryzae (12) A. niger (–) |
| Methanolic (dried truffle powder) | S. aureus (28) B. subtilis (30) M. luteus (16) | E. coli (22) P. aeruginosa (20) | C. albicans (12) A. oryzae (–) A. niger (–) |
| Hot distilled water (dried truffle powder) | S. aureus (19) B. subtilis (20) M. luteus (14) | E. coli (17) P. aeruginosa (13) | C. albicans (–) A. oryzae (–) A. niger (–) |
| Cold distilled water (Fresh Truffle) | S. aureus (12) B. subtilis (11) M. luteus (8) | E. coli (9) P. aeruginosa (–) | C. albicans (–) A. oryzae (–) A. niger (–) |
| Positive controls            |                        |                        |                  |
| Ampicillin                   | NT                     | NT                     | NT               |
| Fluconazole                  | NT                     | NT                     | NT               |

NT, not tested.

Mean activity values of triplicate results: > 16 mm = strong, 12–16 mm = moderate, 8 to < 12 mm = weak, and < 8 mm = no activity.

### Table 3

MBCs and MICs of selected extracts against standard strains of gram-positive and gram-negative bacteria.

| Extracts                      | MIC (µg/mL) | MBC (µg/mL) |
|-------------------------------|-------------|-------------|
| Ethyl acetate (dried truffle powder) | 3.5         | 1.0         |
| Methanolic (dried truffle powder) | 6.5         | 1.0         |
| Hot distilled water (dried truffle powder) | 4.2         | 1.0         |
| Positive control              |             |             |
| Ampicillin                   | 1.0         | 0.5         |

MBC, minimum bactericidal concentration (µg/mL for ampicillin and mg/mL for extracts); MIC, minimum inhibitory concentration (µg/mL for ampicillin and mg/mL for extracts); NT, not tested.

Mean values of triplicate results.

### Table 4

Anti-biofilm activity of selected extracts against Staphylococcus aureus IFO3060.

| Extracts                      | Anti-biofilm activity (IC50) |
|-------------------------------|------------------------------|
| Ethyl acetate (dried truffle powder) | 3.8 ± 0.20                  |
| Methanolic (dried truffle powder) | 2.0 ± 0.18                  |
| Reference drug                | Ethyrlorocyn                  | 0.45 ± 0.15                |

IC50, half maximal inhibitory concentration (µg/mL for erythromycin and mg/mL for extracts).

Mean values ± standard deviation of triplicate results.

The compounds found in truffles have not been reported previously. However, it is proposed that polysaccharides, laccases, lectins, terpenes, phenolic compounds, and anandamide (endocannabinoid) may play the inhibitory role. Bacterial exopolysaccharides can be recognized and eliminated by lectins (Passos da Silva et al., 2019). Moreover, the oxidation of phenolic compounds may be catalyzed by laccases to liberate hydrogen peroxide and superoxide anion radicals, which are both suggested to inhibit pathogenic bacteria (Nadim et al., 2015).

Anandamide, an endocannabinoid, affects quorum sensing and motility, in addition to other specific functions of prokaryotic organisms (Pacioni et al., 2015). The quorum sensing system plays a regulatory role on the expression of many bacterial physiological and virulence factors (Friedman et al., 2019; Lima et al., 2019; Passos da Silva et al., 2017; Xu et al., 2019). Quorum sensing also regulates the formation of biofilms such as the biofilm structure formation, dispersal of biofilm cells, and accumulation of biofilm biomass. It has also been suggested that auto-aggregation in the bacterial system may be promoted by quorum sensing (Deng et al., 2020; Sionov and Steinberg, 2022). Hence, the possible presence of anandamide in the T. claveryi methanolic and ethyl acetate extracts may explain the potent activity of these extracts on the biofilm of S. aureus IFO3060 (Mukherjee and Bassler, 2019).

On the other hand, biofilms increase the resistance to antimicrobial agents, posing a challenge to human health care (Sharma et al., 2019). Infections associated with biofilms are real therapeutic challenges for microbiologists and clinicians, and the development of novel antimicrobial strategies is an urgent requirement to counteract the resistance to antibiotics in infectious diseases (Saraswathi and Beuerman, 2015). However, more studies are needed because the molecular structures of the proposed bioactive molecules have not been identified by researches that investigated plant extracts with anti-biofilm activity. Studies on natural anti-infective therapy with specific anti-biofilm agents are currently between phase-I and phase-IV clinical trials (Lu et al., 2019).

The antibacterial activity of truffles could also be related to their phenolic compounds. Several studies have shown that polyphenolic compounds are significantly active against S. aureus, B. subtilis, E. coli, Bacillus cereus, and Acetobacter aceti (Qin et al., 2019). Therefore, the antibacterial efficacy of the methanolic and ethyl acetate truffle extracts might be related to the effects of polyphenols such as p-hydroxy benzoic acid, apigenin, rutin, gentisic acid, ferulic acid, catechin p-coumaric acid, protocatechuic acid, and cinnamic acid (Lima et al., 2019).

The difference between the mechanisms of action by which phenolic compounds affect gram-positive and gram-negative bacteria is not clear. The overall data from earlier studies have demonstrated that phenolic-rich extracts and individual phenolic compounds have an inhibitory effect on the growth of different pathogenic bacteria (Lima et al., 2019; Metsämuuronen and Sirén, 2019).

Terpenoids such as p-cymene, carveol, limonene, guaiane, and cumene hydroperoxide, have also shown selective activity against some gram-positive and gram-negative bacterial and yeast species.
These compounds may interfere with the biofilm development and microbial adhesion on cellular and inert substrates and inhibit the microbial ability to adhere to the inert substrates (Alfonzo et al., 2017; Ceresa et al., 2020; Valdivieso-Ugarte et al., 2019). Therefore, terpenoids may interfere with the first stage of the infectious process.

4.1. Limitations of the study

We were unable to obtain enough truffles for further investigation because they are very expensive: the market price of a kilogram of truffles ranges between €600 and €6000, according to the species (Üstün et al., 2018). In addition, growing truffles is very difficult and often does not bring results as expected. Moreover, finding and collecting truffles naturally is not an easy process (Hall et al., 2008; Üstün et al., 2018). This is because the mycelia of truffles grow underground, and they do not have a stalk or gills (Hall et al., 2008).

5. Conclusions

This study revealed that various extracts of T. claveryi have promising antibacterial, antifungal, and antibiotic effects. Therefore, these species of truffles, in addition to others, could be a rich natural source of anti-inflammatory compounds for medical applications.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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