Phytonutrients and Antimicrobial Activities of Methanolic Extract from Hafr Al-Batin Truffles

Ghassab M. Al-Mazaideh¹ and Farhan K. Al-Swailmi²

¹Department of Pharmaceutical Chemistry, College of Pharmacy, University of Hafr Al-Batin, Hafr Al-Batin, Saudi Arabia.
²Department of Pharmacy Practice, College of Pharmacy, University of Hafr Al-Batin, Hafr Al-Batin, Saudi Arabia.

Authors’ contributions

This work was carried out in collaboration between all authors. Author GMA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author FKA managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The desert truffle is a wild mushroom, also referred to as Kamah or Fagaa. Kamah is a rich source of polysaccharides that have medicinal, antitumoral, antibacterial, and immune-stimulant effects. Studies of hypogeous fungi, especially desert truffles, have recently entered traditional studies of epigeous higher Basidiomycetes. Based on the tasty desert truffle Kamah obtained from Hafr Al-Batin Governorate, Saudi Arabia, as a source of potential antimicrobial agents with both the aim of obtaining novel agents toward bacteria and Fungi of clinical significance. We specifically tested the antibacterial and antifungal efficacy of methanol extracts of Kamah against the Gram-negative bacterial pathogens reference strains E. coli ATCC® 8739, P. Aeruginosa ATCC®9027, S. aureus ATCC®6538, Enterococci NCTC®775 and opportunistic fungus C. albicans ATCC®1231. The extract had MIC (minimum inhibitory concentrations) varying from 100 g/ml to 500 g/ml against the pathogens examined. The LC-QTOF-MS (liquid chromatography coupled to quadrupole time of flight mass spectrometry) phytoconstituents assay chromatogram indicated that the methanol extracts of Kamah comprises 264 with retention periods varying from 1.04 to 18.86, which were

*Corresponding author: E-mail: gmazaideh@uhb.edu.sa;
1. INTRODUCTION

Many of available drugs have been derived directly or indirectly from medicinal plants. These bioactive compounds are mostly isolated from different place of plants such as leaves, stems and roots. Their bioactivity properties like antimicrobial and antioxidant in vitro testing have noticed in many publications in the last decade [1-5]. The antioxidant and antimicrobial activity of bioactive compounds are mainly referred to their ability to chelate metals, redox properties, and reactivity as quenching species of singlet oxygen [6]. Due to these properties, medicinal plants have been used for many years to treat health disorders and prevent diseases. These plants have always been a very good source of many drugs.

Truffles are an exceedingly unusual black mushroom, widely classified as a delicacy and they are one of the most precious of the scents. They are another often known for its fragrance and roots. Their bioactivity properties like antimicrobial and antioxidant in vitro testing have noticed in many publications in the last decade [1-5]. The antioxidant and antimicrobial activity of bioactive compounds are mainly referred to their ability to chelate metals, redox properties, and reactivity as quenching species of singlet oxygen [6]. Due to these properties, medicinal plants have been used for many years to treat health disorders and prevent diseases. These plants have always been a very good source of many drugs.

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Humans have used edible and medicinal mushrooms for thousands of years. Many of the previously known bioactive molecules have been found in different types of mushrooms, including antitumor compounds. Polysaccharides are the most frequently encountered and most strong polysaccharide-derived antitomotics and immunomodulators [10]. In the far East, such as China, where much of the medical understanding and usage of polysaccharides arose. Ganoderma lucidum (Reishi), Inonotus obliquus (Chaga), and many other mushrooms have been gathered and used for centuries in all three of these countries [11].

In Saudi Arabia several places cultivate truffle and have also been an important food for everyday use and income for a long time. The citizens of Saudi Arabia historically live quite well under current natural conditions in the Mediterranean region's dry climate, especially in Hafr Al-Batin, Saudi Arabia, during the springtime, and at truffle festivals [13]. Those aspects of mycological research still form the basis of current medical study, and practice, particularly in the field of stomach, prostatic, and respiratory diseases. Medicinal mushrooms provide a limitless supply of nutritionally, antitumorally, antibacterial and immunostimulatory polysaccharides [14]. The study of hypogynye fungi, in particular so-called desert truffle, has been accompanied recently by traditional studies of epigenous higher Baidonycetees.

Truffle extracts have been investigated extensively for their therapeutic properties. The antibacterial effects of truffle extract, for example, have been well established in the treatment of a variety of diseases, including trachoma [15,16]. Schillaci et al. (2017) investigated the antibacterial activities of the edible desert truffle mushrooms Tirmania pinoyi, Terfezia claveryi, and Picoa juniperi against the strains Staphylococcus aureus (S. aureus) and Pseudomonas aeruginosa (P. aeruginosa) [17]. Paul et al. [18] discovered that Terfezia truffles had antifungal efficacy against plant pathogenic fungi. In other research, Tirmania pinoyi extract was shown to have antimicrobial properties against B. subtilis [18].

Keywords: Kamah; Hafr Al-Batin; antibacterial; antifungal; LC-QTOF-MS.
S. aureus is a significant eye pathogen that can infect the tear duct, eyelid, conjunctiva, cornea, anterior and posterior chambers, and vitreous chamber [19]. The infections that affect the cornea (keratitis) or the inner chambers of the eye (endophthalmitis) are the most dangerous because they can result in a loss of vision or even blindness. Antimicrobial factors are expressed constitutively in each of these ocular sites, and these defences are augmented by a protective host response to the organism. Predisposing factors, such as the use of contact lenses prior to the development of bacterial keratitis or, in the case of endophthalmitis, the trauma caused by cataract surgery or intravitreal injection, are common causes of infection. The bacterial surface’s structural carbohydrates trigger an inflammatory response that reduces bacterial load but also contributes to tissue damage. Several bacterial secreted proteins, such as alpha-toxin, beta-toxin, gamma-toxin, Panton-Valentine leukocidin, and other two-component leukocidins, mediate tissue damage and contribute to the inflammatory response’s induction. Quantitative animal models of keratitis and endophthalmitis have revealed new information about S. aureus virulence and host factors involved in infection control.

The aim of this analysis was to identify phytoneutrants and describe antimicrobial activity of methanolic extract from Hafr Al-Batin truffles LC-QTOF-MS (liquid chromatography coupled to quadrupole time of flight mass spectrometry) method which was used for this research as a phytoconstituents endeavor. This additional aim was to research the biological importance of these metabolites and metabolism of truffles was also completed to establish how they may be used as a therapeutic antibacterial agent. There’s no recent data on Hafr Al-Batin truffles, so this paper was a breakthrough in identifying desert truffle phytoconstituents and their antibacterial function.

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Kamah

Kamah brought from Hafr Al-Batin Governorate, Saudi Arabia during springtime 2021 and they were finding in Hafr Al-Batin truffle festival.

2.1.1 Preparation of methanolic extract

Twenty-five grams of fresh Kamah were soaked in 250 ml of 96% methanol and it was put in the shaker device at 150 rpm, in dark place for five days at room temperature and stored in a refrigerator for one day. The extract was then filtered using a Buchner funnel under vacuum. The filtrate was centrifuged at 3000 rpm for 10 minutes, and then extract concentrated in the rotary evaporator. The crude was left in open vials in the fume hood for two days at room temperature and stored thereafter at 4°C in a glass container until further use [20].

2.1.2 Microorganism and growth conditions

The extraction was tested for growth of bacterial strains and fungus in vitro using disc diffusion method and in agar well diffusion method. This study work was applied two Gram negative bacteria: Escherichia Coli (E. Coli, ATCC® 8739) and Pseudomonas Aeruginosa (P. Aeruginosa, ATCC®9027), two Gram positive bacteria: Staphylococcus aureus (S. aureus, ATCC®6538) and Enterococci (NCTC®775). In addition, C. albicans was tested as opportunistic fungal pathogen of humans (C. albicans, ATCC®1231). Test crude was added to a blank disk where on the following concentration 100 µml, 125 µl, 250 µl, 500 µl, 1000 µl, 1500 µl and 2000 µl. The disk left to absorb the sample for 30 seconds then placed in plate with a culture of CRM (Calicchia resuspension medium) [21]. The media that was used for microorganism: E. Coli with brilliance E. Coli - coliform selective medium, P. Aeruginosa with baird parker agar base, S. aureus with slantze and bartley medium, Enterococcus with Pseudomonas cetrimide agar, and C. Albicans with sabaraud dextrose agar. The samples then placed in incubator as indicated temperature 37°C except for C. albicans at 25°C. The MIC (Minimum inhibitory concentration) were misused after 72hrs and after 5 days for C. Albicans. The sample was tested directly from crude extract prepared for LC/MS/MS analysis.

2.2 Plant Constituents’ Experimental Procedure

Stock solutions were prepared by dissolving of the appropriate amount of substance in Dimethyl sulfoxide-DMSO (analytical grade), then diluted with Acetonitrile then Centrifuged of each sample at 4000 rpm for 2.0 min to be used for identification of exact MS and retention time. All the other reagents, Acetonitrile, methanol, water, and formic acid used were LC/MS grade.

2.2.1 Instrumentation and ms parameters

Bruker Daltonik (Bremen, Germany) Impact II ESI-Q-TOF System equipped with Bruker
Daltonik Elute UPLC system (Bremen, Germany) was used for screening compounds of interest. Standards were used for identification of m/z with high resolution Bruker TOF MS and exact retention time of each analyte after chromatographic separation. This instrument was operated using the Ion Source Apollo II ion Funnel electrospray source. The capillary voltage was 2500 V, the nebulizer gas was 2.0 bar, the dry gas (nitrogen) flow was 8 l/min, and the dry temperature was 200°C. The mass accuracy was < 1 ppm; the mass resolution was 50000 FSR (Full Sensitivity Resolution) and the TOF repetition rate was up to 20 kHz using Elute UHPLC coupled to a Bruker impact II QTOFMS. Chromatographic separation was performed using Bruker Solo C18 2 µm UHPLC column (100 mm x 2.1 mm x 2.0 µm) at a flow rate of 0.51 mL / min and a column temperature of 40°C. Solvents: (A) water with 0.05% formic acid and (B) acetonitrile. The LC gradient was 0 - 27 min linear gradient from 5% - 80% B; 27 - 29 min for 95% B; 29.1 min for 5% B, total analysis time was 35 min with injection volume of 3 µl.

3. RESULTS

3.1 Microbiological Analysis

Table 1 summarizes the MIC (µg/ml) of Kamah extract on the different bacterial strains and one fungus. The MIC values showed antimicrobial effect of methanolic extract of Kamah against four bacterial strains including E. Coli, P. Aeruginosa, S. aureus and Enterococcus. In addition to C. albicans as one of the fungal pathogens of humans.

Table 1 summarizes the MIC (minimum inhibitory concentration) results of Kamah methanolic extract on the different bacterial strains. The MIC values showed that negative Gram bacteria (E. coli and P. Aeruginosa) were inhibited at 100 µl and 125 µl, respectively. The positive Gram bacteria (S. aureus and Enterococcus) were inhibited at 100µl and 125 µl, respectively. Fungi (C. albicans) was inhibited at 500 µl.

3.2 LC-QTOF-MS Analysis

Fig. 1 shows the extracted constituents of methanolic extract of Kamah which they were investigated using LC-QTOF-MS/M. Each constituent in the methanolic extract was quantified and identified by comparing mass fragmentation patterns with standards such as Wiley 9 library spectral data and NIST (Fig. 1).

Table 2 contains the data of identified 21 major compounds (more than 2X10^5 peak area) from methanolic extract of Kamah which includes retention time (RT, min), Area, molecular formula, measured m/z values (precursor mass) and calculated mass error (ppm), and the hit identities from the search of three compound databases, namely PubChem, KEGG Compound and ChemSpider. The raw data of all analytes (264 natural compounds) detected during the LC-QTOF-MS/MS analysis.

4. DISCUSSION

Drug discovery and phytomedicine have recently become hot subjects around the globe in order to discover and cultivate novel antioxidant and antibacterial compounds. Plants are being increasingly important in these fields due to their abundance of secondary metabolites such as alkaloids, polyphenols, terpenoids, hormones, glycosides, as well as other natural ingredients. These constituents demonstrated that herbal medicine has a significant role in clinical studies in infection disorders and in improving health treatment [22]. The capacity of bacteria to intercalate on DNA is related to their action against alkaloids and their derivatives, such as strongly aromatic planar quaternary [23,24]. Several natural phytochemical constituents can be found in Kamah's methanolic extract (Table 2). Antimicrobial and antioxidant activity are believed to be due to the presence of these substances.

| Microorganism | Media | MIC mg/ml |
|---------------|-------|-----------|
| E. Coli       | Brilliance E. Coli /coliform selective medium | 100µl |
| P. Aeruginosa | Baird Parker agar base | 125µl |
| S. aureus     | Slantez and bartley medium | 100µl |
| Enterococcus  | Pseudomonas cetrimide agar | 125µl |
| C. albicans   | Sabaruaud dextrose agar | 500µl |
Fig. 1. Full peak chromatogram of methanolic extract of Kamah using LC-QTOF-MS/M

Table 2. LC-QTOF-MS phytoconstituents analysis of major compounds Kamah methanolic extract

| No. | Component name              | RT (min) | Area - 10^5 | Molecular formula | Precursor mass | Mass error (ppm) |
|-----|----------------------------|----------|-------------|-------------------|----------------|------------------|
| 1.  | Adenosine                  | 5.74     | 11.724      | C_{10}H_{13}N_{5}O_{4} | 268.104        | -0.4             |
| 2.  | Phenylalanine              | 6.12     | 7.711       | C_{9}H_{11}N_{2}O_{3} | 166.086        | 0.8              |
| 3.  | Phenylpropanamate          | 6.12     | 7.711       | C_{9}H_{11}N_{2}O_{3} | 166.086        | 0.8              |
| 4.  | 5-hydroxytryptophan        | 4.75     | 5.627       | C_{11}H_{12}N_{2}O_{3} | 221.092        | 1.9              |
| 5.  | Vitexin                    | 9.13     | 3.570       | C_{21}H_{20}O_{10}   | 433.113        | 2.6              |
| 6.  | Isovitexin                 | 9.13     | 3.570       | C_{21}H_{20}O_{10}   | 433.113        | 2.6              |
| 7.  | Genistin                   | 9.13     | 3.570       | C_{21}H_{20}O_{10}   | 433.113        | 2.6              |
| 8.  | Emodin-8-glucoside         | 9.13     | 3.570       | C_{21}H_{20}O_{10}   | 433.113        | 2.6              |
| 9.  | Ajoflon                    | 9.13     | 3.570       | C_{21}H_{20}O_{10}   | 433.113        | 2.6              |
| 10. | Sophoricoside              | 9.13     | 3.570       | C_{21}H_{20}O_{10}   | 433.113        | 2.6              |
| 11. | Apigenin-7-glucoside       | 9.13     | 3.570       | C_{21}H_{20}O_{10}   | 433.113        | 2.6              |
| 12. | Adenine                    | 2.31     | 3.464       | C_{6}H_{12}N_{5}O_{5} | 136.062        | 0.6              |
| 13. | Salidroseide               | 7.31     | 3.199       | C_{14}H_{20}O_{7}    | 301.155        | -1.4             |
| 14. | Salidoide                  | 7.31     | 3.199       | C_{14}H_{20}O_{7}    | 301.155        | -1.4             |
| 15. | Nicotinamide               | 2.54     | 3.179       | C_{6}H_{12}N_{5}O_{7} | 123.055        | -0.5             |
| 16. | Stachydrine hydrochloride  | 1.51     | 2.897       | C_{7}H_{18}NO_{2}    | 144.102        | 1.3              |
| 17. | p-Coumaric acid            | 3.99     | 2.333       | C_{6}H_{10}O_{3}     | 165.055        | 1.9              |
| 18. | Isoleucine                 | 2.82     | 2.041       | C_{6}H_{12}NO_{2}    | 132.102        | 1.3              |
| 19. | Leucine                    | 2.82     | 2.041       | C_{6}H_{12}NO_{2}    | 132.102        | 1.3              |
| 20. | Schaftoside                | 8.54     | 2.031       | C_{28}H_{32}O_{14}   | 565.155        | 1.9              |
| 21. | Isochaftoside              | 8.54     | 2.031       | C_{28}H_{32}O_{14}   | 565.155        | 1.9              |

Polysaccharides used in medicinal mushrooms have dietary, antitumoral, antibacterial, and immune-stimulating properties. The antimicrobial potential of aqueous extracts of the edible Desert Truffles from Saudi Arabia mushrooms *Tirmania pinoyi*, *Terfezia claveryi*, and *Picoa juniperi* was investigated [17]. The antibacterial function of acid-soluble protein extracts from these three organisms in vitro against the Gram-positive human pathogenic strain *S. aureus* and the Gram-negative strain *P. aeruginosa* demonstrated minimum inhibitory concentrations of 50% against the pathogens studied.

In the third stage of the disease, truffle juice was shown to be very effective against trachoma. Truffle juice was also discovered to have an important inhibitory effect on trachomas fibrosis.
Organic antibiotics, on the other hand, or cortisone-based products, have a host of side effects. As a consequence, we firmly advocate that truffle juice be used in combination with normal eye infections care at all times.

Methanolic extract of Kamah has antimicrobial effects on the growth of *E. coli*, *P. Aeruginosa* as Gram-negative bacteria, *S. aureus* and *Enterococcus* as Gram-positive bacteria and *C. albicans* as fungus. This is the first report on Methanolic extract of Kamah as antimicrobial effects with CRM. Methanolic extract showed potent antibacterial activities against *E. coli*, *P. Aeruginosa*, *S. aureus* and *Enterococcus* with MICs from 100 µl to 25 µl (Table 1). Weak antifungal effect with *C. albicans* was reported with MIC 500 µl. Based on the findings of this study, it can be concluded that Methanolic extract of Kamah is a potent source of antimicrobial agents against Gram-positive and Gram-negative bacterial strains and a weak antifungal agent. So that, it could be used as natural antibacterial agent.

LC-QTOF-MS phytoconstituents analysis chromatogram showed that the methanolic extract of Kamah contains 264 with retention times between 1.04 and 18.86 and they classified as saturated and unsaturated natural products (oxygenated hydrocarbons, carboxylic acids, fatty acids, amino acids, aromatic compounds and vitamins). Major compounds were found to be 21 with peak area more than 2X10<sup>-5</sup> with retention times between 2.3 and 9.13 (Table 2). The highest major identified compounds peaks with retention time were adenosine (11.724), phenylalanine (7.711), phenprobamate (7.711), 5-hydroxytryptophan (5.627). Fig. 2 shows the structure of the main compounds detected with peak area more than 2X10<sup>-5</sup>.

![Adenosine](image)

**Adenosine**  
Retention Time: 5.74 minutes  
Precursor m/z: 268.1040

![Phenylalanine](image)

**Phenylalanine**  
Retention Time: 6.12 minutes  
Precursor m/z: 166.0863
Fig. 2. Structures and Chromatograms of the main compounds (Adenosine, Phenylalanine, Phenoproamate and 5-hydroxytryptophan) detected Kamah methanolic extract

The properties of the described compounds are significant in Kamah because of their antibacterial and antioxidant action, as well as their ability to inhibit lipid oxidation [25]. As a result, Kamah is regarded as one of the most intriguing functional foods. As scavengers of many oxygen molecules, raw kamah have a high antioxidant activity. This result encourages the use of natural Kamah extracts instead of synthetic antioxidants. Despite their intriguing chemical structure and antioxidant function, desert truffles have been shown to have promising antibiotic activity [26]. All of these findings point render desert truffles a fascinating crop to produce and development of a valuable antibiotic substitute to combat antibiotic-resistant pathogens.

5. CONCLUSION

The methanolic extract of Kamah is an active source of antioxidant and antimicrobial agents against Gram-positive and Gram-negative bacterial species, according to the results of this experimental work. As a consequence, it has the ability to be used as a natural antibacterial and antioxidant. Obviously, the extract's antifungal properties were not shown. This may be attributed to the fact that most of the active
ingredients are present in very low concentrations. Our findings may be used to direct prospective in vivo research on the medicinal use of Kamah and its ingredients in treating cancer. As a consequence, it may be used as a natural antibacterial and antioxidant agent in the treatment of a number of diseases, including eye infections.

CONSENT
It is not applicable.

ETHICAL APPROVAL
It is not applicable.

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COMPETING INTERESTS
Authors have declared that no competing interests exist.

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