NOTE TO USERS

ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF TWO DESERT TRUFFLES, *TIRMANIA* AND *TERFEZIA*

BY

SALEH A ALI

DEPARTMENT OF FOOD SCIENCE AND AGRICULTURAL CHEMISTRY

MACDONALD CAMPUS OF MCGILL UNIVERSTY

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ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF TWO DESERT TRUFFLES

With great pride I dedicate this thesis to my parents for their love, sacrifice and help extended to me.

ABSTRACT

Two species of desert truffles, *Tirmania* and *Terfezia* were collected from the Northern desert region in Saudi Arabia for antimicrobial and antioxidant activity testing. Both species were extracted with four types of extraction solutions, methanol, ethanol, ethyl acetate and water to test for antimicrobial activity and with three extraction solutions. methanol, ethanol and water for antioxidant activity. Using disc diffusion method, the extracts were subjected to twenty three different microorganisms to observe the antimicrobial activity by measuring clear zones. Methanol extract from *Tirmania* was the most effective, followed by those extracted with ethanol, water and ethyl acetate respectively. In *Terfezia*, ethanol extract was better than methanol extract in effectiveness. Ethyl acetate extracts were the least effective. The results indicate that truffles possess antimicrobial activity with broad spectrum effects against Gram positive, Gram negative, aerobic and anaerobic bacteria as well as *Saccharomyces*, while no effect was recorded with fungi. The results on antioxidant activity showed that truffles have very strong antioxidant property with 99.9% with ethanol extracts of *Tirmania* species and 95.5% with ethanol extract of *Terfezia* species using β-carotene bleaching method and antioxidant property with 96.1% with ethanol extracts of *Tirmania* species and 95.3% with methanol extract of *Terfezia* species using DPPH free radical method.

RÉSUMÉ

Des truffes de deux espèces, *Tirmania et Terfezia,* furent cueillies dans la région désertique du Nord de l'Arabie Saoudite afin de tester leur activité antimicrobienne et antioxydante. Les deux espèces furent soumises à quatre types de solutions d'extraction (méthanol, éthanol, acétate d'éthyl et eau) pour l'activité antimicrobienne et à 3 trois types d'extraction (méthanol, éthanol, eau) pour l'activité anitoxydantes. Utilisant la méthode des disques, les extraits furent soumis à 23 microorganismes différents afin d'observer l'activité antimicrobienne en mesurant les zones claires. Les extraits au méthanol provenant de l'espèce *Tirmania* procurèrent le meilleur rendement, suivie respectivement des extrait à l'éthanol, à l'eau et à l'acétate d'éthyl. Pour l'espèce *Terfezia,* l'extrait à l'éthanol procura un meilleur rendement que l'extrait au méthanol. Les extraits à l'acétate d'éthyl furent les moins efficaces. Les résultats indiquent que les truffes possèdent une activité antimicrobienne de large étendue contre les bactéries gram-positives, gram-négatives, aérobiques et anaérobiques ainsi que contre les *Saccharomyces*, mais n'a en revanche aucun effet contre les champignons. Les résultats concernant l'activité antioxydante ont démontré que les truffes possèdent de très fortes propriétés antioxydantes avec 99,9% pour les extraits de l'espèce Tirmania à l'éthanol et 95,5% pour les extraits de l'espèce Terfezia à l'éthanol en employant la méthode de blanchissement de β-carotène et propriétés antioxydantes avec 96,1% pour les extraits de l'espèce Tirmania à l'éthanol et 95,3% pour les extraits de l'espèce Terfezia à le méthanol en employant la méthode de libre radical de DPPH.

CONTRIBUTION OF AUTHORS

This thesis was written by Ali, S.A., and B.H. Lee. 2006. Antimicrobial and antioxidant activity of two desert truffles, *Tirmania* and Terfezia. It has been revised and will be submitted to Journal of Food Science

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LIST OF ABBREVIATIONS

BHA: Butylated hydroxyanisole.

BHT: Butylated hydroxytoluene.

LDL: Low density lipoprotein.

MIC: Minimal inhibitory concentration.

MRSA: Methicillin resistant *Staphylococcus aureus.*

ROS: Reactive oxygen species

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FOREWORD

This thesis is submitted in the form of original papers suitable for journal publication. The first chapter is a general introduction and literature review presenting the theory and background information on this topic. The next two chapters represent the body of the thesis, each being a complete manuscript. The last section is a summary of the major conclusions. The format of this thesis has been approved by the Graduate and postdoctoral Studies office at McGill University, and follows the conditions outlined in the Guidelines concerning Thesis Presentation, which are as follows:

"Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis."

If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

INTRODUCTION

Many antibiotics in clinical use were developed from fungal and *Actinomycetes* metabolites. During the last decades several pathogenic microorganisms developed resistance to the available antibiotics. Infections by multidrug resistant isolates of *Candida* sp., *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus* spp., *Enterococcus* sp. and *Escherichia coli*, among others, became more and more frequent stimulating the search for new antibiotics with novel mechanisms of action (Thomson and Moland, 2000). The first investigations on the potential of *Basidiomycetes* as sources of antibiotics were performed by Anchel, Hervey, and Wilkins in 1941 (Sandven, 2000), while the truffle fungi (*Ascomycetes*) have not been investigated yet.

Truffles are considered to be one of the oldest foods known for their nutritional value, especially when compared with meat and fish (Al-Delaimy, 1977). Truffle extract is used as a nourishing and invigorating preparation for convalescents in Mediterranean countries (Singer, 1961). Furthermore, truffle aqueous extract is used as a folk medicine in Iraq, Saudi Arabia and Eastern Jordan to ameliorate eye ailments (Janakat et al., 2004).

On the other hand, the reactive oxygen species (ROS), like superoxide anion radical O, hydroxyl radical ($^{\cdot}OH$) and peroxyl radical ($ROO^{\cdot}$), are constantly generated *in vivo* by two processes, aerobic metabolism and exogenous sources such as UV radiation, environmental pollution as well as the diet. The formation of ROS can cause

The objectives of this study were:

i) To investigate the antimicrobial activity of desert truffles, *Tirmania* and *Terfezia.*

ii) To investigate the antioxidant activity of desert truffles, *Tirmania* and *Terfezia.*

Chapter 1

LITERATURE REVIEW

ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF TRUFFLES

This chapter was summarized as a publication entitled "Antimicrobial and antioxidant activity of two desert truffles, *Tirmania* and *Terfezia*", which was co-authored by Saleh Ali and Byong H. Lee. This paper was written by Saleh Ali and supervised by Dr. Byong H. Lee, who acted in an editorial capacity, evaluating the manuscript. This chapter serves as an introduction to the thesis, showing the background for the research that was done and the significance of this project. One manuscript will be submitted to *Journal of Food Science.*

1.1. ABSTRACT

Truffles are hypogeous fungi that grow underground or near the roots of some plants. Truffles can be divided into two types: tuber and desert truffles. They are highly appreciated for their nutritional and economical values. Many conditions play role for the growth and reproduction of truffles, those are soil type, rainfall and association with vascular plants. Due to high price and low quantity (harvest) of truffles, scientists tried to cultivate truffles. There are few signs for success with tuber species but not with desert truffles. Truffles are rich in proteins, carbohydrates and they appear to be a good source of vitamins C, B and β-carotene.

The conservation of truffles is another important subject. Truffles are canned and preserved for long time, but canning reduce flavour and taste, consumers usually prefer to preserve them "raw" in refrigerator. Concerns on finding new antibiotics or antimicrobial agents bring the subject of antimicrobial activity of truffles to attention since its extract is used in folk medicine to treat eyes infections. This subject is still a new that needs to be investigated in details. Antioxidant activity is another important subject in field of food preservation and human health in that only few studies have been done on antioxidant activity of truffles.

1.2. Introduction

Truffles are defined in The American Heritage® Dictionary of English Language as "any of various fleshy, ascomycetous, edible fungi, chiefly of the genus *Tuber,* that grows underground on or near the roots of trees and are valued as a delicacy" (Pickett, 2000).

According to mycologists, truffle is the reproductive structure of a hypogeous mycorrhiza fungus (Maciarello and Tuker, 1994). It is reserved for the sporocarps of *Ascomycetes* – the most famous being the fruiting bodies of Tuberaceae fungi, which are aromatic products of the European forest (Giovannetti et al., 1994).

Economically speaking, truffles are said to be the most expensive, delicate, and odd food ever known to man. It is a food that is linked to aristocracy and limited to the rich. It is also known for being a treasure of the profound biome (Maciarello and Tuker, 1994).

Truffles are historically known fruits and they were eaten by different people three thousands years ago (Bokhary et al., 1990). Interestingly enough, truffles were eaten by ancient Egyptians and by Europeans as far back as the sixth century BC. (Giovannetti et al., 1994). Truffle is mentioned in the Holy Quran and in Bible as an edible fruit in the story of Jacob (third patriarch of the Jews, son of Isaac and Rebecca, who lived some time around 1600 B.C.). Arabs used truffle as edible fungus and as medicine for eye infections in the early 600 A.D (Musselman, 2000) .

Truffles are generally regarded as uncommon, they may be more common than estimated, yet they are generally difficult to find. The reason why they're difficult to find is that they usually grow under different kinds of trees and underground (McIlvaine and Macadam, 1973). They are also more common in sandy soil and near the roots of trees (Cooke, 1895). Besides, truffles are often found in association with vascular plant, with which they establish a mycorrhizal relationship (Moore-Landecker, 1996).

As for the physical characteristics, truffles have a diameter of about two inches, they are either smooth or wrinkled below, from the outside appearance, they are rather smooth with a pale reddish-brown color; while inside there is a compact texture, resembling potatoes but softer, with a marble-white colour. (McIlvaine and Macadam, 1973).

Truffles are also distinguished as odourous fruits, they have strong smell and flavour, which makes them very attractive for animals, who follow the smell and dig until they find the fruits (Webster, 1970). These characteristics of strong smell and flavour are attributed to the volatile components, rather than their biosynthetic pathways (Maciarello and Tuker, 1994).

Truffles are estimated to have species exceeding one hundred and twenty, of which fifty belong to the genus *Tuber*. The *Tubers* mostly grow in Europe and are often forest truffles. Though most of these species belongs to Europe, they are widely distributed all over the world. However, they are rarely found in North America (Cooke,

1895). The other known species are *Tirmania* and *Terfezia* which are commonly referred as desert truffle. They grow in Kingdom of Saudi Arabia, Kuwait, Iraq and other neighbour countries (Bokhary and Parvez, 1993).

Truffles are mainly important as a vital source of food for forest animals, but they are important gourmets in many countries, especially in food industry, restaurants, and markets (Roman and Boa, 2004).

Truffles are not food for the poor and the prices vary according to the species, and even vary from one season to another. For example, the price of fresh desert truffles (*Tirmania* and *Terfezia*) may be as low as U.S. $ 7.00 per kg during a good season but could go as high as U.S $ 20 or 40 (Awamah and Alsheikh, 1978). While in 1994, black truffles (*Tubers*) sold for $350 to $500 per pound (Fogel, 1998).

1.3. Morphology and Taxonomy

Morphological characteristics of truffles like shape, size and diameter may vary from one species to another. Yet, there are other common features that most of them share.

Tuber melanosporum is an example of a typical tuber species, which belongs to the division of *Ascomycetes* just like all other genuine truffles in taxonomy (*Ascomycotina*, *Discomycetes*, *Pezizales*, *Tuberaceae*). It is a hypogenous fungus, which grows in a facultative mycorrhizal association with species of oak, rarely with other tree

species. The fruiting bodies (receptacula) are firm and 30-200 mm in size, round to irregularly tuberous. The outer surface (peridium) is covered with thick dark brown to black lumps which are many-sided and 3-6 mm in size, whereas the inner surface (gleba) varies from white color when the plant is still young, to a violet black color at maturity, containing 2-6 spores (Maciarello and Tuker, 1994).

On the other hand, *Tirmania* is an example of desert truffle with taxonomical features similar to those of *Ascomycotina, Discomycetes, Pezizales, Terfeziaceae*. Unlike tubers, this species is hypogenous, subglobose, turbinate, pyriform, sometimes irregularly shaped. It weighs from few grams to 1,000 grams, the outer surface is white to reddish - white and smooth to slightly wrinkled, but the inner surface is soft and fleshy, containing 8 spores (Awamah and Alsheikh, 1978). Table 1.1 summarizes the morphological characteristics of truffles.

1.4. Biology of the Truffle

1.4.1. Lifecycle of truffles

Truffle has a very complex life cycle, requiring many steps: starting by spreading and germination of spores, followed by mycelium production, contact with the host root, formation of the fungal mantle and the Hartig net, and development of extraradical mycelia and fruit bodies. The production of fruit bodies depends on the development of mycorrhizae, mutualistic symbioses between the truffle mycelium and the roots of gymnosperms and angiosperms (Rossi et al., 2000).

The first step in the lifecycle of typical *Tuber* species is the germination of monocaryotic hyphae after ascospores are released by the truffle. Then comes the stage of development of hyphae to produce mycelium, followed by growth of mycelium around roots to form mycorrhize. It is habitat for fungus when two hyphae from different mycelia come into contact, cytoplasmic fusion takes place to form anastomoses. This will give dicaryotic cell that will form dicaryotic mycelia as end product of the first stage.

The second step is known as symbiosis. The dicaryotic mycelium, which is capable of producing ectomycorrhizae, first throws a mycelial sheath or mantle over the young root tips. From the mantle, the hyphae radiates into the intercellular spaces within the root tips. Mycorrhitic root tips thicken progressively without elongating. The intercellular hyphae transforms into short, swollen cells surrounding the root cells, the so-called "Hartig net". These are the cells through which exchange of nutrients takes place between fungus and plant (Giovannetti et al., 1994). The mycelium acts as a transfer tube transferring water and mineral directly to the roots, and takes the carbohydrates from the host. After the mycorrhiza has been established, the hyphae continue to colonize new root tips, and so the mycelium spreads. This kind of mycorrhitic association is known as an ectomycorrhiza, since the cells of the fungus do not penetrate the cells of the host (Giovannetti et al., 1994). In this stage many other things will take place and form: formation of the fruiting bodies (carpophores) begins, pseudoparenchyma (that will give the peridium later on) appears as a result of hyphal aggregation, sterile cells, and fertile cells will be distinguished.

Saprophytic stage is the third step in the proposed *Tuber* life cycle. It is the stage in which carpophores are able to nourish on dead or decaying organic matter in a saprophytic form. In this stage, new hyphae grown in the small carpophores will replace the old ones and do their job in nourishing the whole growing organism.

The last vital step in this lifecycle is the enrolment of bacteria and other microbes in the feeding or alimentary chain and the production of aroma that is important in the development of the hyphae. Figure 1.1 shows the whole lifecycle of *Tuber* sp.

1.4.2. Life cycle of the desert truffles

The stages of mycorrhizal development in dessert truffle species, i.e., *Tirmania* and *Terfezia* differ from the corresponding stages in *Tuber* in many aspects. However, it has been shown that unlike *Tuber*, both monocaryotic mycelia (haploid, monosporic) and mycelia stemming from germination and co-culture of many spores are the most probably dicaryotic which may produce mycorrhizae. The differences between *Tuber* and desert truffles are thus:

1- The ectomycorrhizae roots lack a true mantle.

2- Species of *Terfezia* and *Tirmania* have been found to be endomycorrhitic.

3- The hyphae penetrating into the cortical host cells, where they establish a dense clump (Giovannetti et al., 1994).

Different germination stages of *Terfezia boudieri* are shown in Figure 1.2.

1.4.3. Conditions favoring truffle productions

For each living organism, there are suitable conditions that best favor its growth and reproduction. For truffles, these conditions are mainly climatic, where truffles live and grow, in addition to conditions of their behaviour and habit.

Two factors are mainly responsible for specifying the limitation in the production of the fungi. These are rainfall (quantity and season time) and associated vascular plants (density and distribution). Other important condition that may play important roles in the production of truffles is soil type, which is the most important one (Awameh and Alsheikh., 1980).

In Italy, few studies showed some physical evidences proving that the mass density of the soil in which the truffle is planted is an important factor of carpophore production (Giovannetti et al., 1994). Truffles seem to grow better in sandy soil, near the roots of trees (Cooke, 1895). Desert truffle lives in soil types that are either gypsiferous desert, gravely saline desert or gravely gypsiferous saline desert (Awamah et al., 1979). Table 1.2 represents porosity of aggregates from productive topsoil and underlying calcareous rock in France and Italy.

Rainfall is another important factor affecting growth of truffles. The growth, quantity and quality of truffle depend mainly on the quantity of rain that falls in the beginning of winter. It is noticeable that as the total rainfall during the desert truffle growing season varies from one year to another, the amount of the truffle follows it very

closely (Awamah and Alsheikh, 1978). Table 1.3 represents the amount of rainfall during the growing season for 3 years (1975-1978) in Kuwait and the effect of it on the amount of truffle produced.

Truffles are often associated with vascular plants, with which they establish a mycorrhizal relationship (Moore et al., 2001). Each species of truffle has a different tendency, as the type of plant that is associated with. *Tuber* fungi differ in their associated plant from desert plant. The specificity of the host is sometimes low, where the symbiotic relationship could be more than one or two plant species, whereas on some cases they are highly specific. Here are some examples:

a- *Tuber melanosporum*: Perigord truffle (*T. melanosporum*) is associated with the roots of *Quercus* ssp. (Webster, 1970) like *Q. pubescens*.

b- *Tuber aestivum*: usually associated with *Q. pedunculata* and other species. (Giovanetti, 1994).

c- *Terfezia boudieri*: is associated with *Helianthemum salicifolium*.(Awameh and Alsheikh, 1979).

Research has shown that the abundance of such plant has a positive effect on the quantity of truffles.

1.5. Other factors that affect the production of truffles

a. Low pH has a negative effect on truffles in the wild, as it leads to reduction of calcium content of the soil

b. Acidification of rain and pollution might have a negative effect on the quantity of the crops.

c. Intensive collection without agricultural management is another factor that can harm the yield.

d. Pastoralism and migration to newly established suburbs aggravate the process of truffle landscape degradation (Giovanetti et al., 1994).

1.6. Truffle cultivation

1.6.1. Aspects of truffle cultivation

Truffles which have been consumed by Pharos 3000 years ago are still an important dish in many countries, usually linked to the rich due to its high or very expensive price (Bokhary,1987). That is why attempts have been made to cultivate truffles, in order to increase their quantity, and consequently reduce prices. However, despite the fact that truffle cultivation has been discussed and experimented randomly for so many years, yet the process has never been successful (Perez-Gilabert et al., 2005). There were noticeable efforts and attempts to spread truffles, or produce a profitable seed, but the results were not satisfactory. Nevertheless, an attempt was made by a nobleman of Southern France to raise truffles in woods by sprinkling the soil with water in which the parings of truffles had been massaged, protecting the ground, and this trial accomplished a reasonable success (Cooke, 1895).

Although systems for the successful cultivation of truffles are still secretive and proprietary, Singer (1961) revealed complete procedural instructions for cultivation of *T. melanosporum* and other species of *Tuber*. It is worth mentioning that the cultivated truffles have been confirmed by many experts to have the same taste and quality as those growing wild. This might be an encouraging point for exerting more efforts for cultivating truffles.

There are numerous commercial schemes to produce truffles with several common factors:

- First host trees are chosen; likely a species of *Quercus* is used.
- Then comes the seedling stage for host trees in a nursery situation.
- Before introducing *Tuber* sp. inoculum, host trees are started in calcareous soil, after being sterilized to remove likely competitive organisms.
- Trees are then established, mycorrhizae are planted in the field, then follows a regime of regular pruning.
- Either root pruning or soil aeration is used to encourage lateral root growth in the host tree (Maciarello and Tuker, 1994).

An example of the commercial schemes to produce truffles can be seen in "New World Truffieres", a North American Company that produces truffle tree seedlings (oaks, hazelnuts and other species) inoculated with *Tuber melanosporum* and other truffle species.

The cultivation cycle of truffles is rather unique, for truffles begin to appear several years after planting the inoculated seedlings, whereas production can continue for decades. However, the species of the host tree is a rather critical determinant of the commencement and duration of production. Another unique feature in truffles cultivation is that yields vary dramatically, while some farms produce as much as 150 pounds per acre each year; others produce little. However, yields in Europe typically range between 25 and 35 pounds per acre each year. Nevertheless, some farms are achieving yields in excess of 100 pounds per acre as a result of methods improvement (New World Truffieres, 2003).

1.6.2. Cultivation of *Tuber melanosporum*

A. Production of the Truffle-Host Plants:

This is the very important initiative step in which spores inoculated and contacted with mycorrhized roots followed by mycelial inoculation.

B. Selection of Suitable Location:

Choosing the site of inoculation and growing is a critical factor for truffle productions. Several factors are to be considered regarding the suitability of the site, including moisture, organic matter, climatic conditions and other conditions required for the growth of *T. melanosporum*.

C. Plantation Preparation:

The first step is choosing suitable field and depth, *T. melanosporum* needs to be embedded in 40-50 cm, in clean soil with no other plants except the host plant required for the mycorrhizal relationship which is *Quercus* sp.

D. Allowing mycorrhizal and roots development. This is done by:

(a) Controlling growth of root system and shape of the plant.

(b) Monitoring all physical and chemical parameters of the soil during the first three years of truffle cultivation.

(c) Protecting the plant, controlling disease and wild plants.

(d) Controlling moisture and irrigation (Giovanetti et al., 1994).

E. Production of Truffle: Only three years after planting, first yields of truffles will be obtained, these will be about 35 - 100 grams per seedling. The average life of truffles plantations is about 30 years, and the yield increases year by year, provided suitable conditions are ensured. Consequently, all the required costs for proceeding this investment will be contained in purchasing the truffle-infected seedlings; in addition to the small annual costs for maintenance. (Dr. Khanaqa Research Institute, 2002).

1.6.3. Desert truffle cultivation

So far, desert truffle has never been managed to grow under cultivation. However, it seems possible to cultivate them on an extensive scale (Giovanetti et al., 1994). Nevertheless, the procedures described above may be applicable for cultivating this species of truffles, paying the due consideration for the production condition of desert truffles (Feeney, 2002).

Desert truffles should be adapted to their habitat conditions, including being supplied with the basic elements, essential for their growth, along with settling the conditions to the natural environment while developing equipments that can be used in

later stages. However, the procedure is still under laboratory investigations with many attempts and experiments, which might result in successful outcomes soon, making desert truffles available on the market and surprisingly enough, at reasonable prices (Perez-Gilabert et al., 2005; Awamah and Alsheikh, 1978).

1.7. Nutritional value of truffle

Truffles are highly appreciated for being rich in proteins; their protein content is higher than that of most vegetables (Ahmed et al., 1981) and (Murcia et al., 2002). They also comprise an important quantity of essential amino acids, comparable to that of animal proteins, in addition to mineral salts, particularly phosphorus and potassium. They are also rich in carbohydrates (Bokhary and Pervez, 1993).

Besides, they appear to be a good source of vitamins C & B complex and β-carotene, all of which have protective effects because of their antioxidant and anti-radical properties. *Terfezia claveryi* and *Picoa juniperi* contain large quantities of unsaturated fatty acids, among which linoleic acid predominates. Truffles and mushrooms also contain many phenols, which are very efficient scavengers of peroxyl radicals (Murcia et al., 2002).

Even more important fact is that instead of being that rich in vegetable proteins and carbohydrates, truffles are low in calories and fats, which makes them very healthy foods. Moreover, no toxic compounds were detected in desert truffles and thus they are very safe to eat (Ahmed et al., 1981).

In comparison with other edible fungi, desert truffles represent higher carbohydrates than others. The edible mushroom *Pleurotus pulmonarius* showed similarity in proteins, fat and ash percentages in the chemical compositions, but the difference is that the percentage of fiber is much higher and the percentage of carbohydrate is much less than that of truffles (Silva et al., 2002). *P. ostreatus* possess high protein content like most other mushrooms while the fiber of this mushroom species are similar to that of truffles (Manu-Tawiah and Martin, 1987).

Chemical compositions of *Terfezia caveryi*, *Terfezia boudieri*, *Pleurotus pulmonarius* and *Pleurotus ostreatus* is shown in Tables 1.4, 1.5, 1.6, and 1.7.

1.8. Truffle conservations

Truffle conservations are also an important issue. The European Council for the Conservation of Fungi focuses attention on threats and conservation problems of fungi (Moore et al., 2001). This is shown through "The Red Data Lists", which were recorded via different studies for the hypogeous fungi. It is estimated that 14 genera of *Ascomycetes* and 17 *Basidiomycetes* genera exist. A total of 118 species are recorded, among which 56 are *Ascomycetes* and 62 *Basidiomycetes* on the lists, in one or other threat category. This means that about 50% of all existing hypogeous fungi are threatened in these parts of Europe (Moore et al., 2001).

Unfortunately, very little attention for the types of storage and microflora that can spoil truffles has been paid. Consumers usually buy truffles and store them in

refrigerators for long periods. Spoilage can be a problem that reduces quality and quantity of truffles. Bokhary et al. (1990) stated that 23 species of fungi were isolated from spoiled truffles. In general, common mold flora like *Aspergillus*, *Mucor*, *Penicillium* and *Rhizopus* were found to be dominant among the other mycoflora found on truffles; *Aspergillus* being the predominant species, followed by *Mucor*. Multiple genera and species were sometimes isolated from one cube, whereas in other cases one species was found on many cubes. There is no obvious record for bacterial infection. Thus, considering type of storage and validity of truffles are critical points before and after collecting the yields.

The most effective method for preserving truffles for a long time (almost a year) is found to be through cutting them, boiling them and storing them in freezer. However, this method of storage may result in partial loss of the taste or nutritional value of truffles (Bokhary et al., 1990).

1.9. Antimicrobial activity of truffles

1.9.1. Food antimicrobials

While some antibiotics are produced by some bacteria like those from the *Streptomyces* species (Jay et al., 2005), most antibiotics that are used in the modern world were originally isolated from fungi (Rosa et al., 2003). With the development of the first antibiotics, microbial resistance became a topic of major concern. This has led to the increasing demand for new antibiotics.

Antibiotics are classified based on their mode of action. The most widely used antibiotics are those that interfere with the synthesis of bacteria cell walls like penicillin, vancomycin and bacitracin. Table 1.8 represents some mechanisms of antibacterial drug action (Prescott et al., 2002).

Antibiotics were the answer in treating patients who were affected by different pathogenic microorganisms. Studies on antibiotic resistance gained popularity with misuse, overuse and the natural phenomena of bacterial mutation (NARMS, 2003).

Antibiotic resistance spreads fast. While 0.02 per cent of *Pneumococcus* strains were penicillin resistant according to a survey conducted by the National Center for Disease Control and Prevention between 1979 and 1987, today this number has increased to 6.6 per cent (Lewis, 1995). Scientists are working hard to discover new antibiotics to replace the old ones.

Each year, food producers lose a major portion of their products owing to pathogenic microorganisms that attack and destroy their food. Food preservatives are introduced to minimize losses, although customers may not be happy with this practice. People who are aware of the increased probability of antibiotic resistance will try to avoid the use of antibiotics. Jay et al., (2005) laid out certain conditions to control the use of antibiotics. They may be summarized as follows:

1. The antibiotic agent should kill, not inhibit, the flora and should ideally decompose into innocuous products or be destroyed on cooking of products that require cooking.

2. The antibiotic should not be inactivated by food components or products of microbial metabolism.

3. The antibiotic should not readily stimulate the appearance of resistant strains.

4. The antibiotic should not be used in foods if used therapeutically or as an animal feed additive (Jay et al., 2005), Table 1.9.

The quality of a food product decreases from the time of harvest or slaughter until it is consumed. Quality loss may be due to microbiological, enzymatic, chemical, or physical changes. The consequences of quality loss caused by microorganisms include consumer hazards, due to the presence of microbial toxins or pathogenic microorganisms, and economic losses due to spoilage (Raghavan and Orsat, 1995). Many food preservation technologies, some in use since ancient times, protect foods from the effects of microorganisms and inherent deterioration. Inhibition effect in microorganisms is caused by chilling, freezing, water activity reduction, nutrient restriction, modification of packaging atmosphere, or non-thermal treatments (e.g., high-pressure processing) or through addition of antimicrobial compounds (Rodgers, 2005).

Food antimicrobial agents are chemical compounds added to or present in foods that retard microbial growth or kill microorganisms, thereby resisting deterioration in safety or quality (Doyle et al., 2001) . The major targets for antimicrobials are food poisoning microorganisms (infective agents and toxin producers) and spoilage microorganisms whose metabolic end products or enzymes cause off-odours, off-flavours, texture problems, discoloration, slime, and haze. Food antimicrobials are sometimes referred to as preservatives (Torrence and Isaacson, 2003). However, food

preservatives include food additives that are antimicrobial agents, antibrowning agents (e.g., citric acid), and antioxidants (e.g., butylated hydroxy anisole, BHA). The most widely used traditional preservatives are propionates, sorbates, and benzoates (Jay et al., 2005).

Most food antimicrobial agents are only bacteriostatic or fungistatic and not bactericidal or fungicidal. Because food antimicrobials are generally bacteriostatic or fungistatic, they will not preserve a food indefinitely (Doyle et al., 2001). While synthetic antibiotics allow us to control pathogenic microorganisms, antimicrobial agents help us to control many food and cosmetic spoilage. In spite of the presence of synthetic antibiotics and antimicrobial agents, the need for new substances that possess antimicrobial activity still exists (Torquato et al., 2004).

1.9.2. Truffle extract as antimicrobial agent

Truffles are well known as edible fungus but have not been known to be used as antimicrobial agents. This is a rather new topic, which has not been widely investigated and thus only few references are available. Although some studies have discussed different aspects related to truffles as far as the production, cultivation, nutritional value, biology & chemical analysis, preservation & storage, only few has paid attention to the antimicrobial activity of truffles.

Truffles have been used for a long time and have been popular in folk medicine in Middle East countries, especially for curing eye and skin infections (Chellal and

Lukasova, 1995). Old medical books, which belong to early Islamic eras, explained different ways of using the extracts of truffles as a medicine, which suggests that truffles have antimicrobial activity. Chellal and Lukasova (1995) have performed some experiments to ascertain the antimicrobial activity of the *Terfezia* and *Tirmania* truffles on certain species of pathogenic bacteria. They showed some positive results concerning bacteriostatic activity and inhibition of microbial growth of some bacteria. Their results showed the existence of truffle's activity against some Gram positive bacteria but not Gram negative bacteria (Table 1.10).

Al-dagr mentioned in his book (Rawae' men Alteb Al-Islami) that a research by (Almu'tz Bellah) has been carried out to test the activity of truffle's extract in treating eyes with trachoma, the leading cause of human blindness caused by *Chlamydia trachomatis*. The disease is characterized by inflammation of the conjunctiva with follicle formation (Newman and Nisengard, 1988), Almu'tz Bellah extracted the truffle juice by grinding and centrifuging the samples to get the aqueous layer and then used few drops of the truffle extracts, chloramephenicol and tramycin (antibiotics) 5 times a day for one month on the infected eyes. The results were positive in curing the eyes at early stages of the disease and preventing complications at middle stages. Almu'tz Bellah were unable to duplicate the results in laboratory.

Studies conducted in testing the antimicrobial activity of fungi, for instance, mushrooms can provide us with guidelines in order to examine the antimicrobial activity in desert truffles.

Rosa et al. (2003) screened 103 Brazilian *Basidyomycetes* for antimicrobial activity using Minimal Inhibitory Concentration (MIC) test. Fifteen strains out of 103 presented significant activity against one or more of the target microorganisms (both pathogenic and non pathogenic bacteria in addition to fungus). Table 1.11 represents these results.

1.10. Antioxidant activity of truffles

Antioxidant is a substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate (Packer et al., 2002). Natural antioxidants from dietary sources include phenolic and polyphenolic compounds, chelators, antioxidant vitamins (especially C & E) and enzymes, as well as carotenoids and carnosine. While some common sources of antioxidants are dark berries, pomegranate and noni, the main source for antioxidants are garlic, broccoli, cabbage, onions, cauliflower, red beets and some other food (Grajek et al., 2005). Coffee and green tea are also known to have high concentration of these antioxidants (Yen et al., 2005).

Antioxidants in foods may originate from compounds that occur naturally in the food stuff or from substances formed during processing (Shahidi and Wanasundara, 1992). During the normal process of oxidation, antioxidants form highly reactive free radicals. They also form some such species from ionizing radiations, thereby playing a significant role in our lives. These can readily react with and damage other molecules: in some cases the body uses this to fight infection. In other cases, the damage may be to the

body's own cells. The presence of extremely easily oxidisable compounds in the system can "mop up" free radicals before they damage other essential molecules. Antioxidant activity is a fundamental property important to life. It has been demonstrated that many of the biological functions, including antimutagenicity, anticarcinogenicity, and antiaging arise from this property. Furthermore, many natural antioxidants exhibit a wide range of biological effects such as antibacterial, antiviral, antiinflammatory, antiallergic, antithrombic, and vasodilatory (Liyana-Pathirana and Shahidi, 2005).

Mode of action of antioxidants can be described by the following four routes:

1. Chain breaking reactions, e.g. alpha-tocopherol which acts in lipid phase to trap reactive oxygen species "ROS" radical.

2. Reducing the concentration of reactive oxygen species such as glutathione.

3. Scavenging initiating radicals such as superoxide dismutase which acts in aqueous phase to trap superoxide free radicals.

4. Chelating the transition metal catalysts where a group of compounds serves an antioxidant function by sequestration of transition metals that are well-established pro-oxidants. In this way, transferrin, lactoferrin, and ferritin function to keep iron induced oxidant stress in check and ceruloplasmin and albumin as copper sequestrants.

1.10.1. Classification of antioxidants

Antioxidant defense system against oxidative stress is composed of several phases lines, and the antioxidants are classified into four categories based on their function (Noguchi et al., 2000).

1. First line of defense is the preventive antioxidants, which suppress the formation of free radical (enzymes: glutathione peroxidase, catalase; selenoprotein, transferrin, ferritin, lactoferrin, carotenoids, etc.).
2. Second line of defense is the radical scavenging antioxidants, suppressing chain initiation and/or breaking chain propagation reactions: radical scavenging antioxidants.
3. Third category: repair and de novo antioxidant (some proteolytic enzymes, repair enzymes of DNA, etc.).
4. A fourth line is an adaptation where the signal for the production and reactions of free radicals induce formation and transport of the appropriate antioxidant to the right site.

Table 1.12 presents important enzymatic and non enzymatic physiological antioxidants.

Several food additives, including pectin, ascorbic acid (vitamin C) and tocopherol-derived compounds are used as antioxidants to help guard against food deterioration and to maintain their nutritional value (Halliwell, 1999). However, the consumer is becoming increasingly concerned about the use of traditional synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) because they are capable of increasing the storage life of fatty food materials (Zia-ur Rehman, 2006). Some concerns are also based on the possibility of these compounds

being carcinogenic (Chen et al., 1992). Thus, natural sources of antioxidants are becoming important.

Natural sources of antioxidants provide researchers with a variety of options. More than 20 such assured sources have been mentioned by Shahidi (1997) while many more are waiting to be discovered.

Many preservatives are added to foods as antioxidants or antimicrobial agents. Some of these additives like phenolic compounds are known to possess both of these properties (Shahidi and Naczk, 2004). Phenolic compounds, namely, BHT and BHA are antioxidants having antimicrobial activity and are thus called indirect antimicrobials. Phenolic compounds possess antimicrobial activity against a wide range of microorganisms such as viruses, mycoplasmas and protozoa. Table 1.13 lists some antioxidants that possess antimicrobial activity (Jay et al., 2005).

1.11. CONCLUSION

Truffle is an edible fungus that grows underground and associated with vascular plant. Tuber "black truffle" grows in Europe, while desert truffle grows in Southern Europe and Arab Peninsula. Truffle is very expensive food due to the limited amount of harvest and distribution. Climatic conditions play very important role in the production of truffles. Active research is underway to cultivate and harvest this fungus successfully. Truffles can be preserved by canning or refrigeration for long period of time, but the disadvantage of preserving by this method is that it losses the freshness and flavour. Only

few studies presented the antimicrobial importance of desert truffle and thus it is still unknown area of research. Because truffles contain many vitamins and β-carotene, etc. as well, studies on antioxidants activity of truffles are important but very little work has been carried out so far.

Table 1.1. Summary of morphological characteristics of truffles. Adopted from (Maciarello and Tuker, 1994) and (Awamah, 1979).

Species	Receptacula	Peridium	Gleba	No. of spores
Tuber melanosporum	Firm 30-200 nm, round	Brown to black color, polygonal, 3-6 mm	Whitish when young, violet black at maturity	2-6
Termania	Subglobose, turbinate, pyriform, sometimes irregularly shaped	White to reddish white and smooth to slightly wrinkled	Soft and fleshy	8
Tuber aestivum	Irregular, very small	Colorless to yellowish, elliptical to rounded, covered with ridges	Whitish when young, dark hazel at maturity	2-6
Terfezia	Subglobose, napiform to turbinate and lobed	Pallid, light to very dark brown	Solid, fleshy and light to dark brown	3,5 or 8 spores

Table 1.2. Porosity of aggregates from productive topsoils and underlying calcareous rock in France and Italy (Giovanetti et al, 1994).

				Bulk density	Total porosity	Mean pore radius		Pore size distribution (%)				
Country	Area	Material sampled	Tuber species	(g/cm^3)	(%)	macro- (20 -10 µm)	micro- (< 10 µm)	80-10 µm	10 -1 µm	1 -0.1 µm	0.1-0.01 µm	<0.01 µm
France												
	Cahors	Topsoil	*T. melanosporum*	1.27	14.3	37.6	0.42	21.3	10.3	39.3	17.8	11.3
		Bedrock	*T. melanosporum*	2.07	37.6	37.6	6.68	44.2	21.4	23.4	8.8	2.2
	Montpellier	Topsoil-1	*T. melanosporum*	1.67	24.3	22.0	0.60	18.7	26.9	46.2	6.0	2.0
		Topsoil-2	*T. melanosporum*	1.21	18.5	18.8	6.60	28.1	22.5	29.7	13.1	6.5
		Bedrock	*T. melanosporum*	2.10	43.2	9.4	0.15	18.2	16.8	36.3	24.6	2.3

Table 1.2. Porosity of aggregates from productive topsoils and underlying calcareous rock in France and Italy (Continued).

				Bulk density	Total porosity	Mean pore radius		Pore size distribution (%)				
Country	Area	Material sampled	Tuber species	(g/cm^3)	(%)	macro- (20 -10 µm)	micro- (< 10 µm)	80-10 µm	10 -1 µm	1 -0.1 µm	0.1 -0.01 µm	<0.01 µm
Italy												
	Ascoli P.	Topsoil	*T. melanosporum*	1.45	18.7	26.6	0.01	29.6	10.9	24.7	22.6	12.2
		Bedrock	*T. melanosporum*	3.13	25.8	13.3	0.13	18.9	16.3	17.1	36.4	11.4
	Asti	Topsoil	*T. magnatum*	1.65	20.0	20.0	0.21	2.4	0.9	48.3	36.6	11.8
		Bedrock	*T. magnatum*	1.47	37.6	37.6	0.84	38.9	14.7	34.2	9.7	2.5
	Cuneo	Topsoil	*T. magnatum*	1.01	26.3	37.6	6.68	45.4	19.9	23.2	8.4	3.2
		Bedrock	*T. magnatum*	1.99	44.9	12.0	0.42	7.7	4.9	66.4	16.8	4.2

Table 1.3. Total rainfall (in mm) during three successive truffle seasons and its effect on productivity (Awamah and Alsheikh, 1979).

Month/ Season	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	Total	Amount of Kamah
75-76	0.0	3.0	25.6	40.1	95.9	18.9	61.8	245.3	Good
76-77	0.6	1.2	9.0	20.9	T	7.0	2.7	41.4	Negligible
77-78	56.6	2.2	44.8	44.0	3.1	29.3	0.6	180.6	Good

Table 1.4. Chemical composition of *Terfezia caveryi* ascocarp (Bokhary and Parvez, 1993).

General Analysis	% of dry weight
Total ash	4.0 ± 0.3*
Total protein (N X 6.25)	16 ± 0.1
Total carbohydrate	28 ± 0.1
Total crude fiber	4.0 ± 0.1
Total crude fat	2.0 ± 0.2
Undetermined†	46.0

* ± standard deviation

† no detail from the reference.

Table 1.5. Chemical composition of *Terfezia boudieri* (Ahmed et al., 1981).

Constituent	Amount %
Moisture	77.70
Crude fat	6.40[a]
Crude protein	17.19[a]
Carbohydrates	59.73[b]
Crude fiber	3.80[a]
Ash	12.88[a]

[a] Moisture-free basis " the sum of moisture- free bases equal 100 %"

[b] Estimated by difference

Table 1.6. Chemical composition of *P. pulmonarius* (Silva et al, 2002).

General Analysis	% of dry weight
Total ash	6.85 ± 0.15*
Total protein (N X 6.25)	10.63 ± 0.05
Total carbohydrate	0.80 ± 0.08
Total crude fiber	49.02 ± 0.22
Total crude fat	4.17 ± 0.13
Undetermined†	28.53

* standard devi

† no detail from the reference.

Table 1.7. Chemical composition of *P. ostreatus* biomass (Manu-Tawiah and Martin, 1987).

General Analysis	% of dry weight
Total ash	7.9 ± 0.6*
Total protein (N X 6.25)	40.1 ± 1.8
Total crude fiber	5.9 ± 0.5
Total crude fat	3.7 ± 0.4
Undetermined†	42.4

* standard deviation

† no detail from the reference.

Table1.8. Mechanisms of antibacterial drug action (Prescott et al, 2002).

Drug	Mechanism of action
Cell wall synthesis inhibition: Penicillin, Ampicillin, Methicillin, Vancomycin.	Inhibit transpeptidation enzymes involved in the cross linking of the polysaccharide chains of the bacterial cell wall peptidoglycan. Activate cell wall lytic enzymes
Protein synthesis inhibition: Streptomycin, Gentamycin,	Binds with the 30S subunit of the bacterial ribosome to inhibit protein synthesis and causes misreading of mRNA
Nucleic acid synthesis inhibition: Quinolones	Inhibit bacterial DNA gyrase and thus interfere with DNA replication, transcription and other activities involving DNA
Cell membrane disruption: Polymyxin B	Binds to the plasma membrane and disrupts its structure and permeability properties
Metabolic Antagonism: Sulfonamides	Inhibit folic acid synthesis by competition with *p*-amino benzoic acid

Table 1.9. Properties of some antibiotics (Jay et al, 1983).

Property	Tetracycline	Subtilin	Tylosin	Nisin	Natamycin
Widely used in food	No	No	No	Yes	Yes
First food use	1950	1950	1961	1951	1956
Chemical nature	Tetracycline	Polypeptide	Macrolide	Polypeptide	Polyene
Used as heat adjunct	No	Yes	Yes	Yes	No
Heat stability	Sensitive	Stable	Stable	Stable	Stable
Microbial spectrum	G + , G -	G +	G +	G +	Fungi
Used medicinally	Yes	No	Yes*	No	Yes†
Used in feeds	Yes	No	Yes	No	No

* In treating poultry diseases
† Limited, not limited

Table 1.10. Inhibition of some bacteria and yeast by the extracts of *Terfezia* or *Tirmania* fresh weight (Chellal and Lukasova, 1995).

Micro organisms	Inhibition zone diameter (mm)									
	1	2	3	4	5	6	7	8	P	B*
Bacillus subtillus rec+	0	24	10	0	10	0	15	12	48	40
Bacillus subtillus rec -	0	22	11	0	10	0	13	12	47	36
Staphylococcus aureus (pathogenic)	19	20	0	0	11	11	13	11	52	42
Staphylococcus aureus (non pathogenic)	18	20	0	0	0	0	0	0	56	47
Streptococcus pyrogens	0	19	0	0	0	0	0	0	46	42
Pseudomonas aeruginosa	0	0	0	0	0	0	0	0	0	0
Kluyveromyces fragilis	0	0	0	0	0	0	0	0	0	0

*1-3: methanolic extract from *Terfezia* prepared respectively at 25°, 45° and 65 °C; 4-6: the same extracts from *Tirmania*; 7: ethyl acetate extract from *Terfezia* prepared at 65°C; 8: the same extract from *Tirmania*; P: Pristinamycine, 81.5 µg/disc. B: Bacitracine, 10 µg/disc.

- Amount of solvents used in extraction is not given.

Table 1.11. Antimicrobial activity of *Basidiomycetes* extracts (Rosa et al, 2003).

		Fungi										Bacteria						
Species	Isolates	ALB	GLA	KRU	PAR	TRO	CER	SUB	FAE	COL	MON	ERA	TYP	AUR	API	SAP	PNE	PYO
Agarius cf. nigrecentulus	UFMGCB31															+		
Agrocybe perfecta	CCB161			+														
Basidiomycetes	Bm															+		
Climadacon pulcherrimus	CCB191			+														
Gloeoporus thelephoroides	Bm						+											
Hexagonia hydnoides	Bm						+											
Irpex lacteus	CCB196	+	+		+		+			+			+	+				
Leucoagaricus cf. cinereus	UFMGCB40									+								
Marasmius cf. bellus	UFMGCB43									+								
Marasmius sp.	UFMGCB45									+								
Nothopanus hygrophanus	CCB216										+			+				
Oudemansiella canarii	CCB179+	+	+	+		+												
Pycnoporus sanguineus	CCB277			+							+			+				
Phellinus sp.	Bm						+											
Tyromyces duracinus	UFMGCB49															+		

+: Inhibition halos larger than 12 mm diameter

Abbreviations:

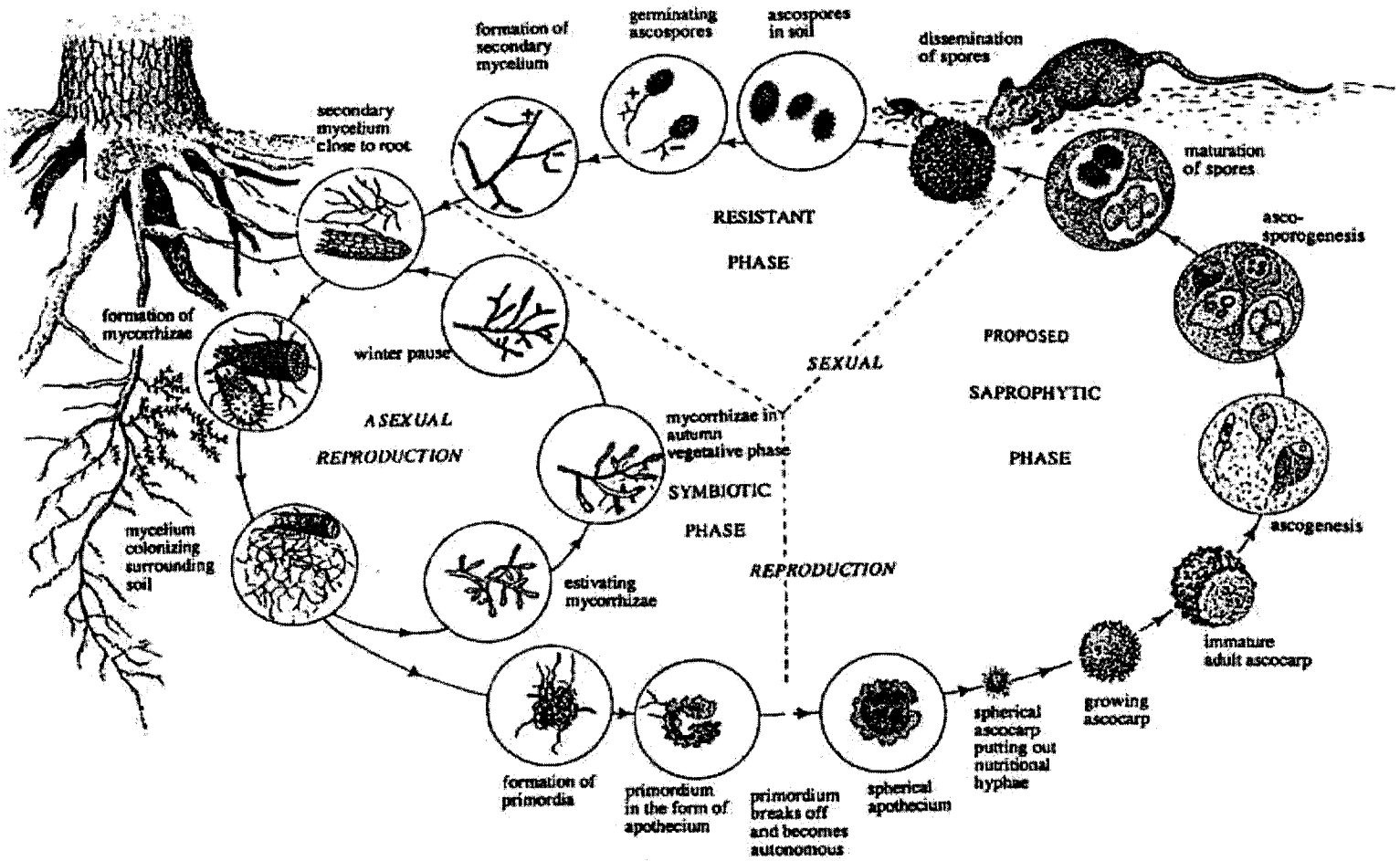
CCB:	Basidiomycetes Culture Collection of the Instituto de Botanica, Sao Paulo, SO, Brazil
UFMGCB:	Basidiomycetes Cuture Collection of the Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil
ALB:	*Candida albicans*
GLA:	*C. glabrata*
KRU:	*C. krusei*
PAR:	*C. parapsilosis*
TRO:	*C. tropicalis*
CER :	*Bacillus cerens*
SUB :	*B. subtilis*
FAE:	*Enterococcus faecalis*
COL:	*Escherichia coli*
MOND:	*L. monocytogenes*
ERA:	*Pseudomonas aeruginosa*
TYP:	*Salmonella typhimurium*
AUR :	*Staphylococcus aureus*
EPI :	*S. epidermis*
SAP :	*S. Saprophyticus*
PNE :	*Streptococcus pneumoniae*
PYO :	*S. pyogenes*

Table 1.12. Important enzymatic and nonenzymatic physiological antioxidants (Noguchi, et al, 2000).

Enzymatic antioxidants	Location	Properties
Superoxide dismutase (SOD)	Mitochondria, cytosol	Dismutase superoxide radicals
Glutathione peroxidase (GSH)	Mitochondria and cytosol	Removes hydrogen peroxide and organic hydroperoxide
Catalase (CAT)	Mitochondria and cytosol	Removes hydrogen peroxide
Nonenzymatic antioxidants	Location	Properties
Vitamin C	Aqueous phase of cell	Acts as free radical scavenger and recycles vitamin E
Vitamin E	Cell membrane	Major chain-breaking antioxidant in cell membrane
Uric acid	Product of purine metabolism	Scavenger of OH radicals
Glutathione	Nonprotein thiol in cell	Serves multiple roles in the cellular antioxidant defense
Alpha-lipoic acid	Endogenous thiol	Effective in recycling vitamin C, may also be an effective glutathione substitute
Carotenoids	Lipid soluble antioxidants, located in membrane tissue	Scavengers of reactive oxygen species, singlet oxygen quencher
Bilirubin	Product of heme metabolism in blood	Extracellular antioxidant
Ubiquinones	Mitochondria	Reduced form are efficient antioxidants
Metals ions sequestration: transferrin, ferritin, lactoferrin,		Chelating of metals ions, responsible for Fenton reactions
Nitric oxide		Free radical scavenger, inhibitor of LP

Table 1.13. Some FDA approved antimicrobial chemicals used in foods (Jay et al, 2005).

Compound	Primary Use	Most Susceptible Organisms
Butylated hydroxyanisole (BHA)	Antioxidant	Bacteria, some fungi
Butylated hydroxytoluene (BHT)	Antioxidant	Bacteria, viruses, fungi
t-Butylhydroxyquinoline (TBHQ)	Antioxidant	Bacteria, fungi
Propyl gallate (PG)	Antioxidant	Bacteria
Nordihydroguaiaretic acid	Antioxidant	Bacteria
Ethylenediaminetetraacetic acid (EDTA)	Sequestrant/stabilizer	Bacteria
Sodium citrate	Buffer/sequestrant	Bacteria
Lauric acid	Defoaming agent	Gram-positive bacteria
Monolaurin	Emulsifier	Gram-positive bacteria, yeasts
Diacetyl	Flavoring	Gram-negative bacteria, fungi
Phenylacetaldehyde	Flavoring	Fungi, Gram-positive bacteria
Menthol	Flavoring	Bacteria, fungi
Vanillin, ethyl vanillin	Flavoring	Fungi
Phosphates	H_2O binding, flavoring	Bacteria
Spices/spice oils	Flavoring	Bacteria, fungi



Life cycle of *Tuber* spp. (drawn by E. Rebaudengo)

Figure 1.1. Lifecycle of *tuber* species (modified from Giovanetti et al, 1994).

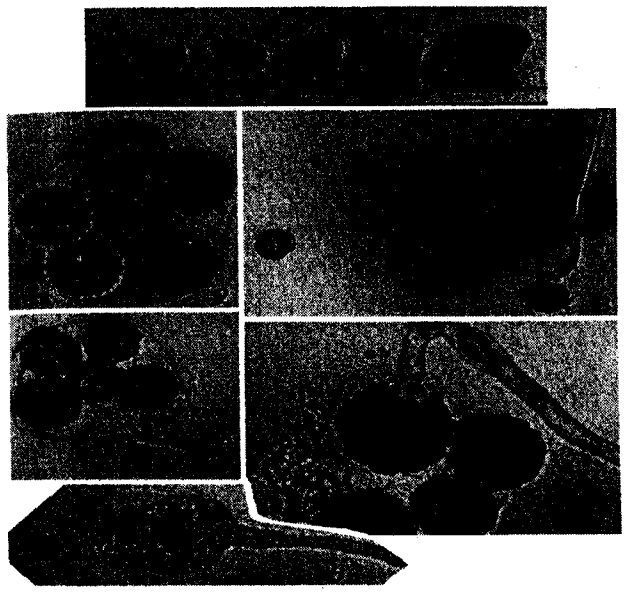


Figure 1.2. Different germination stages of *Terfezia boudieri* (Adapted from Awama and Alsheikh., 1980).

Chapter 2

ANTIMICROBIAL ACTIVITY OF *TIRMANIA* AND *TERFEZIA* TRUFFLES

In order to verify if *Terfezia* and *Tirmania* species of truffles possess antimicrobial activity or agents, truffles were finely ground and different extraction solutions were used to extract the proposed active compound and tested against 22 species of bacteria, *Saccharomyces* and fungi.

The major results of this study are summarized as a manuscript suitable for journal publication in the Journal of Food Science. The manuscript is entitled "Antimicrobial activity of *Terfezia* and *Tirmania* species of truffles" was co-authored by Saleh A. Ali and Byong H. Lee. The project was supervised by Dr. Byong H. Lee, while the actual experimental work and the manuscript writing were done by Saleh A. Ali.

2.1. Abstract

Eight kilograms of *Terfezia* and *Tirmania* species were collected from Saudi Arabia desert in February 2005, and both species were tested and identified in Kuwait. After washing and cutting truffles aseptically and verifying for the purity, the ground truffles (400 g) of each species were extracted with methanol, ethanol, water and ethyl acetate with 100 g for each solvent. The extracts were transferred to filter paper discs for antibiotic tests using different volumes of 10, 25 and 50 μL and were tested against 22 different microbial species. Very positive results were found with Gram positive, Gram negative, aerobic, anaerobic, pathogenic and nonpathogenic bacteria as well as *Saccharomyces*, but no results were shown with fungi. The active compound showed the broad spectrum activity against bacteria, and clear zone ranged from 10 cm up to 29 cm using 25 μL of extracts.

Key words: *Terfezia*, *Tirmania*, antimicrobial activity

2.2. Introduction

Antibiotics are secondary metabolites produced by microorganisms that inhibit or kill a wide spectrum of other microorganisms and they are classified by their chemical structure, mode of action and according to the bacterial strains they affect. Their ability to fight pathogenic microorganisms and infectious diseases makes them very important for our lives. Most of these antibiotics are produced by fungi and few from bacteria, but the research to find new antibiotics is of primary concern due to an increase in antibiotic resistant bacteria.

Truffle is a fungus which possesses high nutritional value that has been used for years in folk medicine. As the antimicrobial activity of truffles remains uninvestigated, and only a few references are available for studies on truffles, this study is considered to be a very original one.

In folk medicine, the extract of truffles is used to treat infected eyes, mostly trachoma disease with no side affects. The common pathogenic microorganisms are *Staphylococcus, Streptococcus* and *Meningococcus* besides some other Gram negative bacteria (Krohn et al., 1993). This is the first intensive studies to investigate the antimicrobial activity that may be present in truffles.

2.3. Materials and Methods

2.3.1. Chemicals and growth media

All chemicals and microbial media were obtained from: methanol (CH_3OH), ethanol (CH_3CH_2OH), ethyl acetate ($CH_3COOC_2H_5$) (Fisher Scientific), Tween 80 ($C_{64}H_{124}O_{26}$) (Sigma), nutrient agar (Difco), Mueller Hinton agar (Difco), brain heart infusion agar (BBL), LB broth (Fisher Scientific), nutrient broth (Difco), trypticase soy agar (TSA) (Difco), YEPD (Difco), reinforced clostridial medium (Oxoid CM149), MRS broth (Difco), potato dextrose agar (Sigma). Filter paper discs size: ¼ and ½ Inch (6.5 and 13.0 mm) (S&S # 740-E), and Petri dishes were obtained from Whatman and Fisher, respectively.

2.3.2. Truffle samples

Eight kilograms of each *Terfezia* species and *Tirmania* species, were collected from Saudi Arabia desert and identified by morphological and taxonomical characteristics (Awamah and Alsheikh, 1980) in Mycology laboratories of the Department of Microbiology, Faculty of Science, Kuwait University.

2.3.3. Microorganisms

In bacteria, a total of 18 strains were tested:

Staphylococcus aureus, Streptococcus pyrogens (ATCC 14289), *Clostridium perfringens* (ATCC 13124), *Escherichia coli O157:H7, Salmonella typhimurium, Salmonella*

enteritidis, Shigella sonnie (ATCC 29930), *Listeria monocytogens, Bacillus subtilis, Bacillus cereus*, 5 *Lactobacillus* species (*L.acidophilus* ATCC 11975 (L1), *L.plantarum* ATCC 14917 (L8), *L.casei* ATCC 4646 (L9), *L.acidophilus* LAMA (L19) and *L.casei* LC10 (L23)) and 3 *Bifidiobacterium* species (*B.animalis* ATCC 25527 (B3), *B. infantis* ATCC 15697 (B13) and *B. breve* ATCC 15700 (B21).

In *Saccharomyces*, one *Saccharomyces cerevisiae* was examined

In fungi, *Rhizopus oryzae* (ATCC 34612), *Penicillium aurantiogriseum* (ATCC 34613) and *Mucor circinelloides* (ATCC 15242) were studied.

2.3.4 Media preparations

Media were prepared aseptically following the instructions indicated on the media container, the required amount were measured, pH were adjusted and then media were autoclaved, left to cool down and poured in Petri dishes for further use.

Mueller Hinton agar (Difco) was used as standard media for antimicrobial test for all bacteria, yeast and fungi, according to (NCCLS, 2000; NCCLS, 2002; NCCLS, 2003). Nutrient agar (Difco) was used for *Staphylococcus aureus, Salmonella typhimurium, Salmonella enteritidis, Shigella sonnie* (ATTC 29930) and *Bacillus cereus*. Reinforced clostridial medium (Oxoid CM149) was used for *Clostridium perfringens* (ATCC 13124). Brain heart infusion agar (BBL) was used for *Listeria monocytogenes*. LB agar (Fisher Scientific) was used for *Escherichia coli O157:H.* Trypticase soy agar (Difco) was used for *Streptococcus pyrogens* (ATTC 14289) and *Bacillus subtilis.* YEPD (Difco) was used for *Saccharomyces cerevisiae*, potato dextrose agar (Sigma) was used for

Rhizopus oryzae (ATCC 34612), *Penicillium aurantiogriseum* (ATCC 34613) and *Mucor circinelloides* (ATCC 15242).

2.3.5. Sample preparation and extraction

Brown and white Saudi truffles (*Terfezia* and *Tirmania*) were purchased from the local market in February 2005.

Eight hundred grams of fresh truffle ascocarps of *Tirmania* and *Terfezia* (400 g each) were cut into small pieces and portions, (100 g) were soaked in 300 mL of distilled water (1:3) for 24 h to wash them and remove the debris (Janakat, 2004). Truffles were freeze-dried in freeze dryer (Labconco, USA) for 48 h. 140 g of *Tirmaina* and 160 g of *Terfezia* were obtained as dried truffles. They were ground in a household blender (Philips) for 1 min at high speed. 140 g and 160 g of truffles prepared were divided into 4 equal batches (each of them). Each sample was extracted with extraction solutions (methanol, ethanol, water and ethyl acetate) (1:1) which means 40 g of sample with 40 mL of the solvents and mixed in shaker (Forma Scientific, USA) for 2 h at 37 °C. The extracts were then centrifuged at 8,000 rpm at -4 °C for 15 min using Beckman centrifuge J2-21 (USA). Subsequently, methanol and ethanol evaporated by rotary evaporator (BUCHI R-210/R215, Switzerland) at 40°C, dissolved with least amount of solvent (10 mL of methanol for 10 g of *Tirmania* and *Terfezia*. 10 mL of ethanol for 10 g of *Tirmania* and *Terfezia* and kept at – 20 °C for later use. Ethyl acetate was evaporated by nitrogen gas, 3 g of *Tirmania* and 2 g of *Terfezia* dissolved in 3 mL of and 2 mL of

solvent. Water was evaporated by freeze dryer and each 10 g was dissolved in 10 mL of water. The stock solutions of the extracts (100 µL/mL) was prepared and kept at – 20 °C for later use.

2.3.6. Inoculum preparation

With a sterile loop, the tops of four to five colonies of target bacteria from pure culture were picked up. The colonies were suspended in 5 mL of sterile saline solution. The inoculum turbidity was standardized to equivalent of a 0.5 McFarland standard. While for fungi, stock inoculum suspensions were prepared from 7-day-old cultures grown on potato dextrose agar (Difco) according to the NCCLS guidelines (NCCLS, 2002).

2.3.7. Disc diffusion method

The entire surface of a Mueller-Hinton agar plate was inoculated using a sterile swab. Discs (¼ inch) containing 10 µL, 25 µL and 50 µL (in concentration of 100 µL/mL of the solvent) of each extract were placed after allowing them to dry for six hours with the control containing pure methanol, ethanol and ethyl acetate in order to make sure that complete evaporation of extraction solvents was done. Using sterile forceps the discs were placed onto the agar surface and gently pressed down to ensure contact. Plates were incubated at 35°C or 37°C for 18 and 24 h, depending on the growth conditions of bacteria. Subsequently, the diameter of inhibition zone around each disc was measured.

This procedure conforms to the National Committee for Clinical Laboratory Standards (NCCLS) documents M31-A2 and M2-A7 (NCCLS, 2000). Same procedure was applied with the changes regarding *Saccharomyces* and fungi from NCCLS guidelines (NCCLS, 2003).

2.4. RESULTS AND DISSCUSION

2.4.1. Extraction efficiency and inhibition studies

Overall, 8 extracts studied showed the significant activity against one or more of the target microorganisms, among which 6 out of the 8 extracts (*Tirmania* methanol, ethanol, water extracts and *Terfezia* methanol, ethanol and water extracts) showed the inhibition zones larger than 16 mm diameter using 50 µl extract and larger than 10 mm diameter with 10 and 25 µl extracts (Tables 2.1-2.13). Furthermore, all these 6 extracts which presented wide antimicrobial spectrum were active against Gram positive and Gram negative bacteria, and *Saccharomyces*, while none of the extracts exhibited activity against fungi.

Among the four solvents used (methanol, ethanol, water and ethyl acetate), ethanol extract was the most active one for *Terfezia*. Ethanol was able to inhibit the growth of 10 pathogenic bacteria (*Staphylococcus aureus, Streptococcus pyrogens, Clostridium perfringens, Escherichia coli* O157:H7, *Salmonella typhimurium, Salmonella enteritidis, Shigella sonnie, Listeria monocytogenes, Bacillus subtilis, Bacillus cereus)*

tested as well as the yeast, *Saccharomyces cerevisiae*. (Tables 2.1-2.13) However, the ethanol extract was not effective against beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* studied (the results were negative for 7 species out of 8 studied).

Methanol extracts of *Terfezia* showed significant inhibitions that were similar to those of *Tirmania*. The extracts were also able to inhibit 8 pathogenic bacteria *Staphylococcus aureus, Streptococcus pyrogens, Clostridium perfringens, Salmonella typhimurium, Salmonella enteritidis, Shigella sonnie, Bacillus subtilis, Bacillus cereus* as well as *Saccharomyces cerevisiae.* The ethanol and methanol extracts also inhibited the beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* that may not be useful.

For *Tirmania* species, methanol extract was the most active one among the four solvents (methanol, ethanol, water and ethyl acetate) tested. The methanol extract inhibited 8 pathogenic bacteria, *Staphylococcus aureus, Streptococcus pyrogens, Clostridium perfringens, Escherichia coli* O157:H7, *Salmonella enteritidis, Shigella sonnie, Bacillus subtilis, Bacillus cereus* and the yeast, *Saccharomyces cerevisiae*.

Water extract of *Tirmania* presented positive results against some micro organisms, such as *Staphylococcus aureus, Shigilla sonnie, Salmonella enteritidis, Listeria monocytogenes, Bacillus subtilis* and *Clostridium perfrengens.* While ethyl acetate in the case of *Tirmania* presented very low or no activity against tested microorganisms.

The water extract of *Terfezia* also showed the inhibition to many of the tested microbes, some of which are: *Escherichia coli* O157:H7, *Staphylococcus aureus, Shigilla sonnie, Bacillus cereus, Bacillus subtilis, Salmonella typhymurium, Clostridium perfringens* and the yeast, *Saccharomyces cerevisia.* While ethyl acetate extract showed some inhibition for 4 bacteria, namely, *Staphylococcus aureus, Shigilla sonnie, Bacillus cereus* and *Clostridium perfringens,* in addition to the yeast, *Saccharomyces cerevisiae.*

Differences in the *Tirmania* and *Terfezia* results indicated that *Terfezia* in general possessed a higher inhibition activity than *Tirmania*, and larger inhibition zones were recorded. On the other hand, both *Tirmania* and *Terfezia* extracts presented broad spectrum activity for inhibition of Gram positive bacteria, *Staphylococcus aureus* and *Listeria monocytogenes* and Gram negative bacteria, *Escherichia coli* O157:H7 and the yeast, *Saccharomyces cerevisiae.* It was also found that extracts of both truffles were effective against aerobic (*Salmonella typhimurium, Salmonella enteritidis* and *Shigella sonnie*) and anaerobic bacteria (*Clostridium perfringens*). The results also showed that none the *Tirmania* and *Terfezia* extracts represented any activity against fungi.

Control test was held with all solvents and concentrations using the solvent (methanol, ethanol, water and ethyl acetate) alone in the filter paper discs after allowing them to dry for 4 and 6 hours (double test) and placed on the surface with the microorganisms being tested, the results showed no inhibition zones at all.

Figure 2.1 shows the maximum inhibition zone reached by the extract in each sample on Mueller Hinton agar which is the standard agar for antimicrobial activity test. Figure 2.2 shows the maximum inhibition zone reached by the extract in each sample on their specific media. Antimicrobial activities in all extracts were less clear when they were tested for antimicrobial activity on Mueller Hinton agar than the one with the specific media of every microbes. Figures 2.3.1, 2.3.2 and 2.3.3 show examples of controls while 2.3.4, 2.3.5 and 2.3.6 show some examples of inhibition zones in the experiment against different types of microorganisms.

The standard zones of inhibition are divided into three levels, namely, (1) susceptible (2) intermediate, and (3) resistant. Susceptible or sensitive means that the bacteria are killed or inhibited by the antibiotics or active compounds while intermediate means some inhibition occurs. However, it may not be sufficient to stop or remove all bacteria using this antimicrobial agent and resistant refers to bacteria that are not affected by antimicrobial agent.

National Committee for Clinical Laboratory Standards (NCCLS, 2000) determined the susceptibility of a drug against many known bacteria, and these show that drugs have different measurements of inhibition zone and susceptibility level, and many variables play significant roles in specifying the susceptibility such as the amount of antimicrobial substance used, clear zone measurement, type of bacteria and other such variables.

In order to determine the susceptibility of this active compound, the isolation and purification are important to determine the exact amount of the drug for MIC and exact concentration.

In comparison with other fungi like *Basidiomycetes* for their antimicrobial activity, desert truffles showed higher activity. For instance, 35 species of the Shiitake mushroom, *Lentinula edodes* was screened for antimicrobial activity using 10 µl at concentration 100 µl/mL (Ishikawa et al., 2001). The results showed that *Lentinula edodes* inhibited Gram positive bacteria but not Gram negative ones. The inhibition zones of truffles extract in our experiments was larger than those of the Shiitake mushroom. For example, the inhibition zones with *Lentinula edodes* extracts of shiitake were only 12, 4, 13, 0, 0 mm for *Bacillus cereus, Listeria monocytogenes*, *Staphylococcus aureus, Escherichia coli*, *Shigella sonnei,* respectively (Ishikawa et al., 2001).

In a study of the artist conk, *Ganoderma applanatum* (mushroom), the results demonstrated antimicrobial activity against Gram-positive *Bacillus cereus, S. aureus,* but were less active against the Gram-negative, *E. coli*, and *P. aeruginosa* (Smania, 2001). While desert truffles showed high activity against both Gram positive and Gram negative bacteria. Similar studies have been performed with *Lycoperdon pusilum* and *Lycoperdon giganteum* mushroom (Table 2.14). The table shows the inhibition zone and bacteria used in the experiments that give another evidence for the strength of antimicrobial activity of desert truffles in comparison with mushrooms (Jonathan and Fasidi, 2003).

It is interesting to know that mushrooms not only possess antimicrobial activities against bacteria and multi-resistant bacteria but also possess antiviral activities (Lindequist et al., 2005). As a corollary desert truffles may also have antiviral activity as well, but it needs to be investigated.

2.5. CONCLUSION

This study shows interesting antimicrobial activities by *Tirmania* and *Terfezia* truffles which may contain active compound(s) or bioactive secondary metabolite(s) that are effective against different types of bacteria in low concentrations. The results showed that the extracts of *Terfezia* are more effective than those of *Tirmania*. Among different extracts studied, ethanol extracts of *Terfezia* were more effective than other extracts prepared by four solvents, while methanol extract of *Tirmania* was most effective among other extracts. The results also showed that the antimicrobial activity was effective against bacteria and yeast, but not against fungi.

The isolation and identification of the active compounds from the most promising extracts are currently under investigation. To our knowledge, this work is the first of its field in the scientific community to investigate the potential antimicrobial activity of desert truffles, *Tirmania* and *Terfezia* species, and will certainly stimulate further investigation of this rich source of bioactive secondary metabolites.

Table 2.1. Inhibition of *Staphylococcus aureus* by the extracts of *Tirmania* and *Terfezia*.

Truffle Type	*Tirmania*												*Terfezia*											
Extraction solvents	Methanol			Ethanol			Water			Ethyl acetate			Methanol			Ethanol			Water			Ethyl acetate		
Concentration (µl)	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10
Regular media	Clear Zone Measurement (mm)																							
1*	34	28	23	25	19	15	25	19	12	21	12	11	35	29	25	28	23	18	30	24	19	21	20	12
2	33	25	21	24	20	15	20	15	12	19	12	11	32	27	22	23	17	11	27	21	18	21	19	11
3	33	26	21	25	20	16	22	16	9	20	13	11	33	27	24	23	18	13	29	24	18	22	20	12
M.H. Media	Clear Zone Measurement (mm)																							
1*	23	20	17	18	14	11	15	11	10	14	7	-†	18	14	10	20	15	12	16	10	8	14	8	-†
2	22	20	18	18	14	11	15	11	10	15	8	-	18	14	11	20	15	10	14	9	8	14	9	-
3	23	21	18	17	13	11	14	12	10	-	8	-	17	13	10	21	16	12	14	10	7	15	9	-

* Triplicate test was performed
† (-) means that no inhibition zone obtained
Note: Controls showed negative results

Table 2.2. Inhibition of *Salmonella typhymurium* by the extracts of *Tirmania* and *Terfezia*.

Truffle Type	*Tirmania*												*Terfezia*											
Extraction solvents	Methanol			Ethanol			Water			Ethyl acetate			Methanol			Ethanol			Water			Ethyl acetate		
Concentration (µl)	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10
Regular media	Clear Zone Measurement (mm)																							
1*	17	14	7	16	11	9	-	10	-	14	10	9	19	14	10	24	19	14	19	15	11	16	9	-
2	14	10	-	15	11	10	-	8	-	14	10	7	19	13	10	26	19	15	19	13	8	14	9	7
3	15	12	7	16	12	9	-	8	-	-	9	7	21	16	10	26	22	15	18	14	9	15	10	8
M.H. Media	Clear Zone Measurement (mm)																							
1*	14	8	-	14	8	8	-	8	-†	-	-	-	14	8	-	21	15	14	16	12	9	-	8	-†
2	-	8	-	-	8	7	-	-	-	-	8	-	15	9	7	22	16	14	15	9	8	14	8	-
3	14	8	-	14	8	7	-	8	-	-	-	-	14	8	-	22	16	14	15	11	9	-	8	-

* Triplicate test was performed
† (-) means that no inhibition zone obtained

Note: Controls showed negative results

Table 2.3. Inhibition of *Escherichia coli* O157:H7 by the extracts of *Tirmania* and *Terfezia*.

Truffle Type	*Tirmania*												*Terfezia*											
Extraction solvents	Methanol			Ethanol			Water			Ethyl acetate			Methanol			Ethanol			Water			Ethyl acetate		
Concentration (µl)	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10
Regular media	Clear Zone Measurement (mm)																							
1*	21	15	12	-	8	-	14	8	8	-	8	-	22	16	13	20	15	11	16	12	8	-	8	-
2	20	15	11	-	-	-	14	8	-	-	8	7	17	11	9	22	17	12	14	10	7	-	8	-
3	21	15	12	-	8	-	14	7	-	-	-	-	16	10	7	23	16	15	14	10	-	-	7	-
M.H. Media	Clear Zone Measurement (mm)																							
1*	16	10	8	-	-	-	-†	8	-	-	-	-	14	8	-	18	14	12	14	8	-	-	-	-†
2	15	9	7	-	-	-	-	8	-	-	7	-	14	8	-	19	14	12	14	8	-	-	-	-
3	14	9	8	-	-	-	-	7	-	-	-	-	14	8	7	18	14	12	14	8	-	-	-	-

* Triplicate test was performed
† (-) means that no inhibition zone obtained
Note: Controls showed negative results

Table 2.4. Inhibition of *Salmonella enteritidis* by the extracts of *Tirmania* and *Terfezia.*

Truffle Type	*Tirmania*												*Terfezia*											
Extraction solvents	Methanol			Ethanol			Water			Ethyl acetate			Methanol			Ethanol			Water			Ethyl acetate		
Concentration (µl)	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10
Regular media	Clear Zone Measurement																							
1*	19	13	10	14	9	7	16	11	8	14	10	-	22	16	11	25	15	12	17	11	7	-	11	-
2	18	13	10	14	9	-	15	11	7	14	10	-	21	16	10	28	17	14	15	10	7	-	10	-
3	18	14	10	-	8	-	15	12	8	14	9	-	22	15	11	25	15	12	15	11	7	-	10	-
M.H. Media	Clear Zone Measurement																							
1*	14	11	8	-	-	-†	14	9	8	-	8	-	16	13	9	20	14	11	14	8	-	-†	8	-
2	14	12	8	-	8	-	14	9	7	-	8	-	16	12	9	20	15	12	-	8	-	-	-	-
3	15	12	8	-	-	-	14	9	8	-	9	-	17	12	9	20	15	12	14	8	-	-	8	-

* Triplicate test was performed
† (-) means that no inhibition zone obtained
Note: Controls showed negative results

Table 2.5. Inhibition of *Listeria monocytogenes* by the extracts of *Tirmania* and *Terfezia.*

Truffle Type	*Tirmania*												*Terfezia*											
Extraction solvents	Methanol			Ethanol			Water			Ethyl acetate			Methanol			Ethanol			Water			Ethyl acetate		
Concentration (µl)	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10
Regular media	Clear Zone Measurement (mm)																							
1*	15	11	8	-	8	-	-	7	-	-	8	-	14	9	7	16	12	8	-	7	-	-	8	-
2	14	10	8	-	8	-	-	7	-	-	7	-	14	9	-	17	11	8	-	7	-	-	-	-
3	15	11	9	-	7	-	-	-	-	-	-	-	-	9	-	16	11	8	-	7	-	-	7	-
M.H. Media	Clear Zone Measurement (mm)																							
1*	15	9	7	-	-	-	14	8	-†	-	-	-	-	8	-	18	13	9	14	8	-	-	-	-†
2	14	8	-	-	-	-	14	7	-	-	-	-	14	8	-	19	14	10	14	8	-	-	-	-
3	14	9	8	-	-	-	14	7	-	-	-	-	-	8	-	18	13	10	14	8	-	-	-	-

* Triplicate test was performed
† (-) means that no inhibition zone obtained
Note: Controls showed negative results

Table 2.6. Inhibition of *Bacillus subtilus* by the extracts of *Tirmania* and *Terfezia.*

Truffle Type	*Tirmania*												*Terfezia*											
Extraction solvents	Methanol			Ethanol			Water			Ethyl acetate			Methanol			Ethanol			Water			Ethyl acetate		
Concentration (µl)	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10
Regular media	Clear Zone Measurement (mm)																							
1*	18	15	11	19	14	11	14	-	-	-	9	-	19	14	10	18	13	10	16	12	10	15	10	8
2	18	14	10	19	14	11	14	8	-	-	8	-	19	14	10	19	15	10	16	11	9	14	10	8
3	17	15	11	18	13	10	14	-	-	-	9	-	18	14	10	18	13	10	15	12	9	15	10	7
M.H. Media	Clear Zone Measurement (mm)																							
1*	14	8	-	16	13	10	14	8	7	-†	-	-	15	11	9	17	13	10	14	11	8	-	8	-†
2	14	9	-	16	12	10	14	7	-	-	8	-	16	13	10	17	12	10	15	12	9	14	9	-
3	15	9	-	15	12	9	14	7	-	-	-	-	16	11	10	17	12	10	14	12	8	-	9	-

* Triplicate test was performed
† (-) means that no inhibition zone obtained
Note: Controls showed negative results

Table 2.7. Inhibition of *Bacillus cereus* by the extracts of *Tirmania* and *Terfezia.*

Truffle Type	*Tirmania*												*Terfezia*											
Extraction solvents	Methanol			Ethanol			Water			Ethyl acetate			Methanol			Ethanol			Water			Ethyl acetate		
Concentration (µl)	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10
Regular media	Clear Zone Measurement																							
1*	24	19	13	19	14	11	15	10	8	-	-	-	24	16	14	28	18	14	21	15	11	18	9	-
2	24	20	13	19	14	11	15	9	7	-	-	-	24	15	13	28	18	13	20	14	11	17	10	-
3	24	19	12	19	13	11	16	10	8	-	-	-	23	15	12	27	18	14	21	14	10	17	9	-
M.H. Media	Clear Zone Measurement																							
1*	21	16	10	15	11	9	14	8	-†	-	-	-	16	11	7	20	13	9	15	11	9	14	8	-†
2	20	15	10	16	13	10	-	8	-	-	-	-	16	11	7	19	14	11	15	11	9	14	7	-
3	20	15	10	16	12	9	14	8	-	-	-	-	16	11	7	20	13	10	16	12	9	14	7	-

* Triplicate test was performed
† (-) means that no inhibition zone obtained
Note: Controls showed negative results

Table 2.8. Inhibition of *Shigella sonnie* by solvents extracts of *Tirmania* and *Terfezia.*

Truffle Type	*Tirmania*												*Terfezia*											
Extraction solvents	Methanol			Ethanol			Water			Ethyl acetate			Methanol			Ethanol			Water			Ethyl acetate		
Concentration (µl)	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10
Regular media	Clear Zone Measurement (mm)																							
1*	20	15	14	-	8	-	17	11	10	14	8	-	22	17	12	27	22	19	16	12	8	22	15	10
2	20	14	12	-	-	-	19	13	10	14	8	-	23	18	12	28	23	19	16	12	11	20	13	10
3	21	15	14	-	8	-	17	11	10	14	-	-	22	18	13	28	22	19	18	11	10	19	13	10
M.H. Media	Clear Zone Measurement (mm)																							
1*	18	13	10	-	8	-†	17	13	9	-	8	8	21	13	9	25	20	15	14	8	8	16	8	8
2	18	13	9	-	8	-	16	12	8	-	8	-†	20	13	9	25	19	15	14	8	7	16	8	8
3	18	14	10	-	8	-	16	12	9	-	8	7	21	14	10	24	20	14	14	8	8	15	7	7

* Triplicate test was performed
† (-) means that no inhibition zone obtained
Note: Controls showed negative results

Table 2.9. Inhibition of *Clostridium perfringens* by the extracts of *Tirmania* and *Terfezia.*

Truffle Type	*Tirmania*												*Terfezia*											
Extraction solvents	Methanol			Ethanol			Water			Ethyl acetate			Methanol			Ethanol			Water			Ethyl acetate		
Concentration (µl)	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10
Regular media	Clear Zone Measurement (mm)																							
1*	30	21	20	23	20	15	20	12	10	20	15	10	34	25	22	25	15	10	23	17	13	30	20	19
2	30	22	21	22	19	15	19	11	10	18	14	8	34	25	22	24	16	10	23	17	13	30	21	19
3	30	23	20	23	20	14	20	12	9	18	14	8	33	25	22	25	15	11	23	18	13	31	21	20
M.H. Media	Clear Zone Measurement (mm)																							
1*	21	15	15	14	10	8	18	16	9	15	10	8	24	17	12	16	9	-†	17	12	8	18	14	10
2	21	15	14	14	10	8	18	15	9	14	8	-	23	17	13	16	8	-	17	12	8	18	13	10
3	22	16	15	14	11	9	18	14	9	14	9	7	24	28	12	17	9	7	17	13	8	17	14	10

* Triplicate test was performed
† (-) means that no inhibition zone obtained
Note: Controls showed negative results

Table 2.10. Inhibition of *Streptococcus pyrogens* by the extracts of *Tirmania* and *Terfezia.*

Truffle Type	*Tirmania*												*Terfezia*											
Extraction solvents	Methanol			Ethanol			Water			Ethyl acetate			Methanol			Ethanol			Water			Ethyl acetate		
Concentration (µl)	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10
Regular media	Clear Zone Measurement (mm)																							
1*	15	10	8	-	8	-	-	-	-	-	-	-	20	13	8	20	15	13	14	8	-	17	11	9
2	14	10	-	-	-	-	-	-	-	-	-	-	20	13	9	21	16	12	14	8	-	16	11	8
3	14	9	-	-	-	-	-	-	-	-	-	-	21	14	9	20	16	12	14	8	-	16	12	8
M.H. Media	Clear Zone Measurement (mm)																							
1*	15	12	8	-	-	-	-†	-	-	-	-	-	17	12	8	16	12	10	-	-	-†	-	9	-
2	14	10	8	-	-	-	-	-	-	-	-	-	16	10	7	16	11	9	-	-	-	-	8	-
3	14	9	8	-	-	-	-	-	-	-	-	-	16	11	8	16	11	10	-	-	-	14	8	-

* Triplicate test was performed
† (-) means that no inhibition zone obtained
Note: Controls showed negative results

Table 2.11. Inhibition of *Saccharomyces cerevisiae* by the extracts of *Tirmania* and *Terfezia.*

Truffle Type	*Tirmania*												*Terfezia*											
Extraction solvents	Methanol			Ethanol			Water			Ethyl acetate			Methanol			Ethanol			Water			Ethyl acetate		
Concentration (µl)	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10
Regular media	Clear Zone Measurement (mm)																							
1*	16	12	8	14	8	-	14	9	8	-	-	-	17	13	9	25	17	13	17	12	8	18	13	8
2	16	11	8	14	7	-	16	11	8	-	-	-	17	13	8	25	15	12	14	10	7	17	13	8
3	15	11	7	14	7	-	14	9	7	-	-	-	16	14	9	25	16	12	14	11	8	17	12	8
M.H. Media	Clear Zone Measurement (mm)																							
1*	14	10	8	-	-	-	-	8	-†	-	-	-	14	10	7	23	14	11	14	8	-†	14	8	-
2	14	10	8	-	8	-	-	7	-	-	-	-	15	11	7	21	16	14	14	8	-	14	8	-
3	15	9	7	-	-	-	-	8	-	-	-	-	15	10	8	21	14	12	14	7	-	15	9	-

* Triplicate test was performed
† (-) means that no inhibition zone obtained
Note: Controls showed negative results

Table 2.12. Inhibition of fungi by the extracts of *Tirmania* and *Terfezia.*

Truffle Type	*Tirmania*												*Terfezia*											
Extraction solvents	Methanol			Ethanol			Water			Ethyl acetate			Methanol			Ethanol			Water			Ethyl acetate		
Concentration (µl)	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10
Regular media	Clear Zone Measurement (mm)																							
Mucor	-	-	-†	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhizopus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M.H. Media	Clear Zone Measurement (mm)																							
Mucor	-	-	-	-	-	-	-	-	-	-	-†	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhizopus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Triplicate test was performed
† (-) means that no inhibition zone obtained
Note: Controls showed negative results

Table 2.13. Inhibition of *Bifideobacterium* and *Lactobacillus* species by the extracts of *Tirmania* and *Terfezia.*

Truffle Type	*Tirmania*												*Terfezia*											
Extraction solvents	Methanol			Ethanol			Water			Ethyl acetate			Methanol			Ethanol			Water			Ethyl acetate		
Concentration (µl)	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10	50	25	10
M.H. media	Clear Zone Measurement (mm)																							
Bifidio. B3	14	9	9	14	9	8	14	9	8	-	8	-	-	-	-	-	-	-	-	-	-	-	8	-
Bifidio. B13	14	9	8	14	9	8	14	8	8	14	11	8	1	8	1	14	8	8	14	9	8	-	10	9
Bifidio. B21	17	9	8	14	8	-	-	-	-	-	-	-	25	17	-	-	-	-	-	-	-	-	-	-
Lacto. L1	18	11	8	-†	-	-	-	-	-	-	9	8	16	8	8	-	-†	-	18	12	-	15	10	10
Lacto. L8	-	11	-	-	-	-	-	-	-	-	10	8	14	8	-	-	-	-	-	-	-	-	10	9
Lacto. L9	14	9	8	-	-	-	-	-	-	-	11	10	14	9	-	-	8	-	-	-	-	-	10	8
Lacto. L19	-	8	-	-	-	-	-	-	-	15	10	8	14	9	-	-	8	-	-	8	-	16	10	9
Lacto. L23	21	16	13	-	-	-	-	8	-	15	9	8	18	14	-	-	-	-	15	10	8	15	11	8

* Triplicate test was performed
† (-) means that no inhibition zone obtained
Note: Controls showed negative results

Table 2.14. Antibacterial activities of *L. pusilum* and *L. giganteum* extracts using filter paper disc method (Jonathan and Fasidi, 2003)

Test Bacteria	Aqueous Extract		Methanol Extract		Ethanol Extract	
	LyP *	LyG *	LyP	LyG	LyP	LyG
	Zone of inhibition (mm). in triplicates					
B. cereus	9.0a	5.0a	10.0b	9.0a	13.0b	12.0b
E. coli	12.0a	8.0a	15.0a	11.0a	19.0a	17.0a
K. pneumoniae	-	7.0a	11.0b	9.0a	14.0b	11.0b
P. vulgaris	4.0b	9.0a	8.0b	11.0a	10.0bc	12.0b
P. aeruginosa	-	-	-	7.0a	13.0b	13.0b
S. aureus	5.0b	8.0a	10.0b	7.0a	14.0b	9.0b

* LyP = *L. pusilum*, LyG = *L. giganteum*, (-) = Inactive, values followed by the same letter along each vertical column are not significantly different by Duncan 's multiple range test ($P<0.05$).

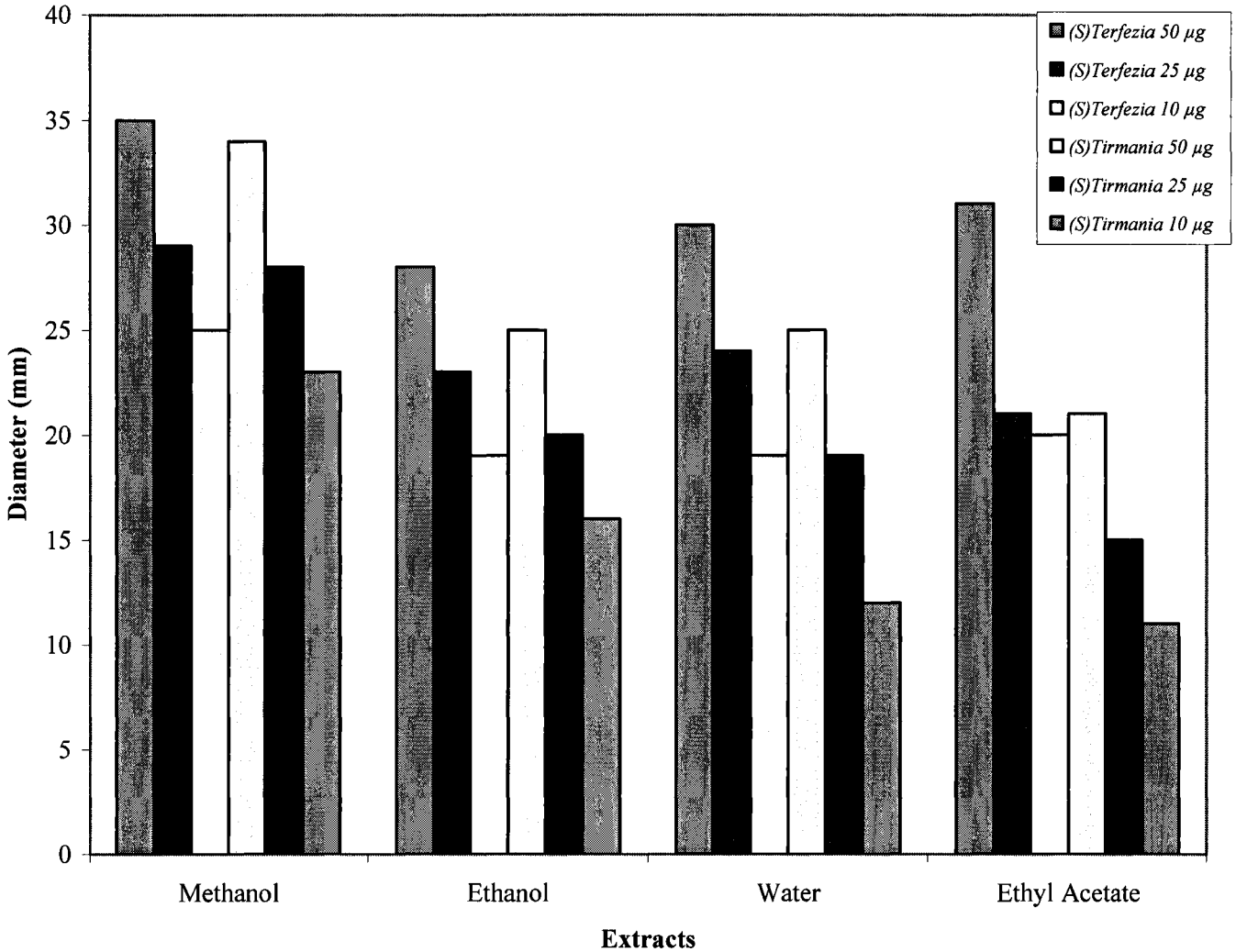


Figure 2.1. Extraction results with methanol, ethanol, water and ethyl acetate for 10, 25 and 50 µg of *Termania* and *Terfezia* truffles using regular media (recommended for every microbes).

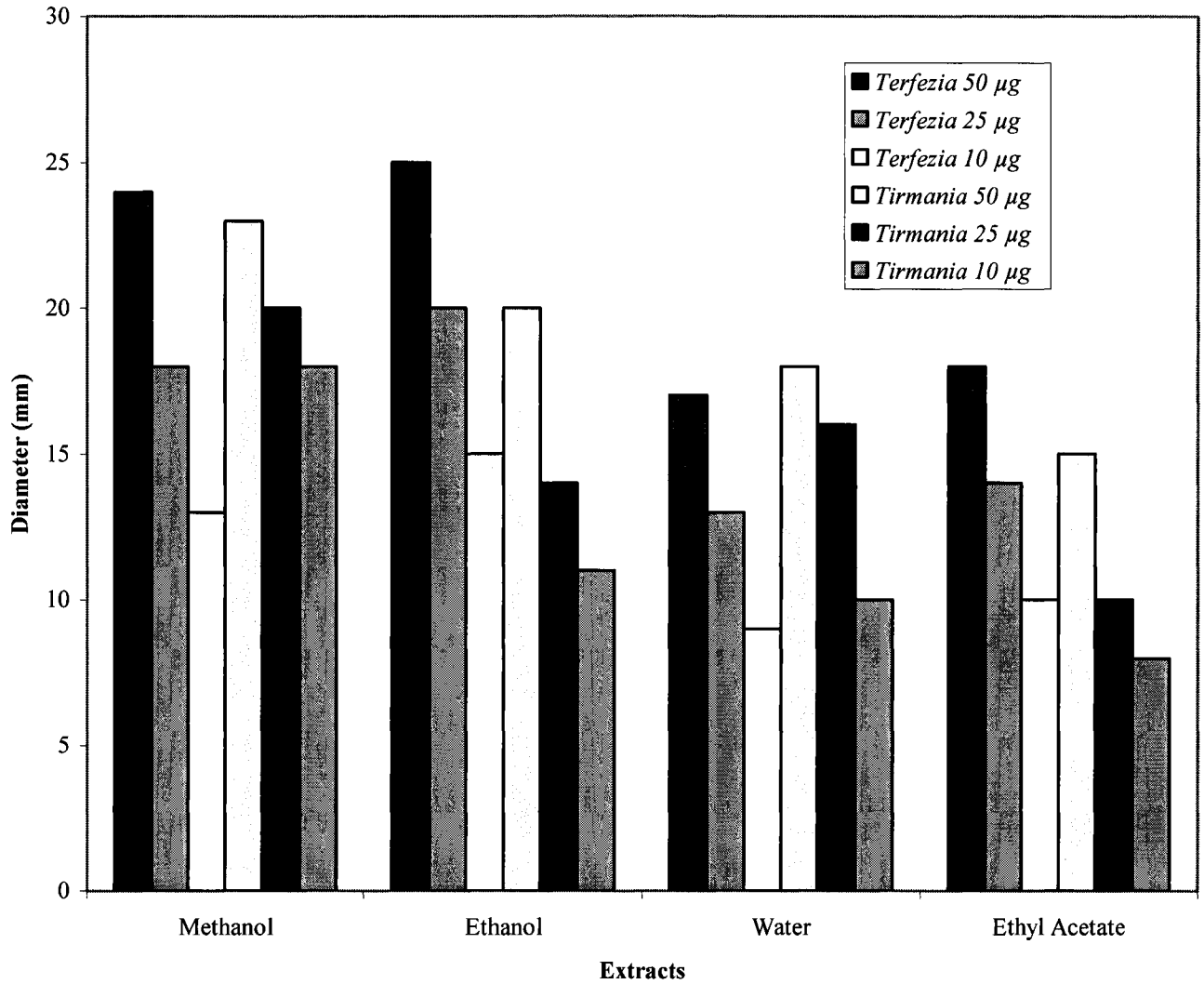


Figure 2.2. Extraction results with methanol, ethanol, water and ethyl acetate for 10, 25 and 50 µg of *Termania* and *Terfezia* desert truffles using Mulleur Hinton agar.

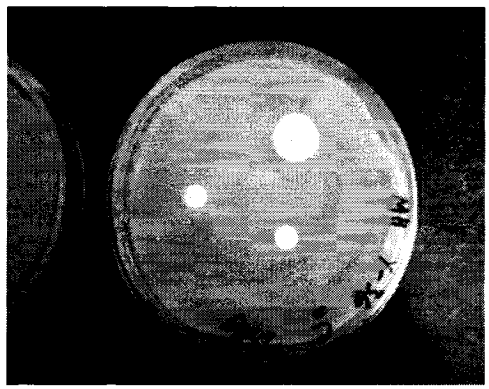


Figure 2.3.1. Control sample of *Saccharomyces cerevisiae.*

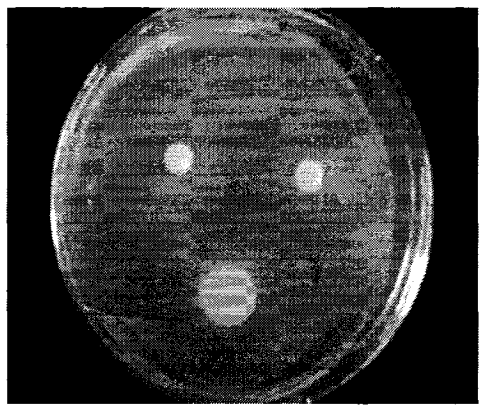


Figure 2.3.2. Control sample of *Streptococcus aurues.*

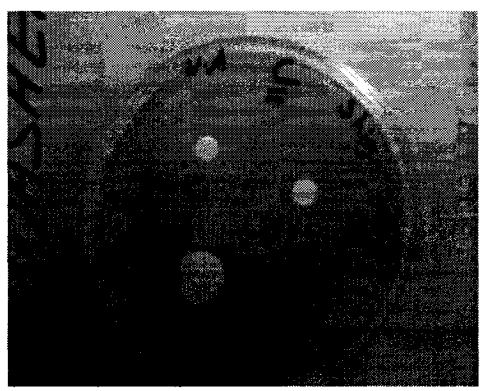


Figure 2.3.3. Control sample of *Shigella sonnie.*

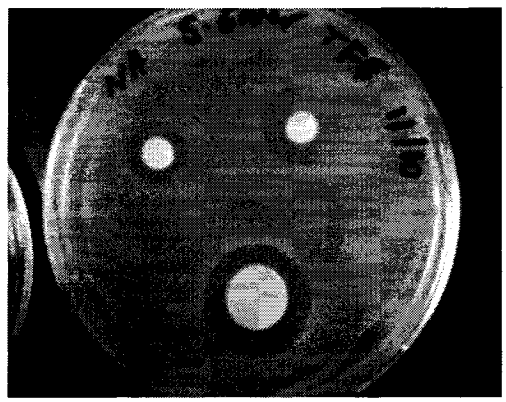


Figure 2.3.4. Inhibition zone of *Salmonella enteritidis.*

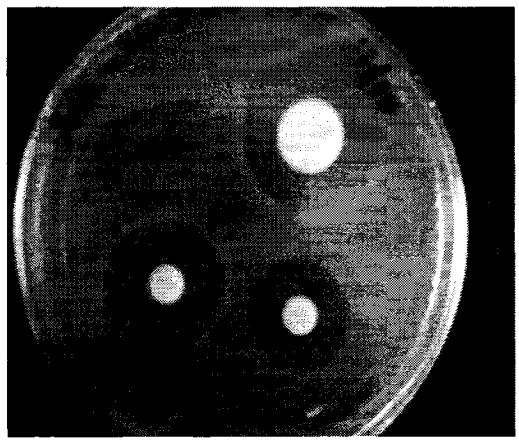


Figure 2.3.4. Inhibition zone of *Staphylococcus aureus.*

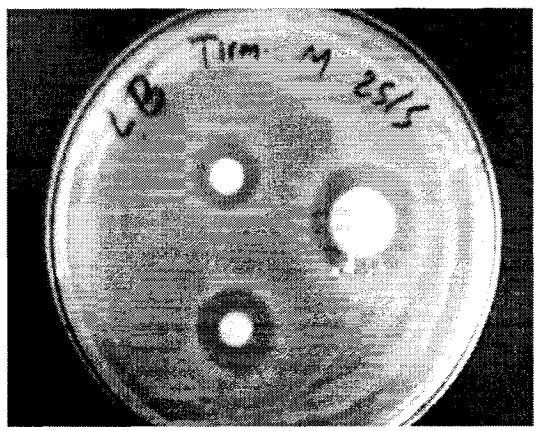


Figure 2.3.5. Inhibition zone of *Lesteria monocytogens.*

Chapter 3

ANTIOXIDANT ACTIVITY OF *TIRMANIA* AND *TERFEZIA* TRUFFLES

In order to verify if *Terfezia* and *Tirmania* species of truffles possess any antioxidant activity or agents, freeze-dried truffles were finely ground and different solvents were used to extract the potential active compounds and tested for the antioxidant activity.

The major findings of this study have been summarized as a manuscript suitable for the publication in the Journal of Food Science. The manuscript entitled "Antioxidant activity of *Terfezia* and *Tirmania* species of truffles" was co-authored by Saleh A. Ali and Byong H. Lee. The project was supervised by Dr. Byong H. Lee, while the actual experimental work and the manuscript writing were done by Saleh A. Ali.

3.1. Abstract

The antioxidant activities of water, ethanol and methanol extracts of *Tirmania* and *Terfezia* of truffles were determined by the ability of a compound to minimize the coupled oxidation of linoliec acid and β-carotene in an emulsified aqueous system and by using DPPH as stable free radical. In both methods the antioxidant activity by ethanol extracts of *Tirmania* was the most effective, showing 99.8 % activity (in β-carotene bleaching method) and 95.5 % activity (in the DPPH free radical method) among the three extracts tested. While antioxidant activity of water extract was the least, methanol extract was almost as effective as ethanol. *Terfezia* species was less effective than *Tirmania*, but the ethanol extracts of *Terfezia* showed 95.5 % and 92.1 % activity. Methanol extracts of *Tirmania* and *Terfezia* also showed significant activities of 98.3 % and 94.2 % in β-carotene bleaching method and 95.4 % and 95.3 % in DPPH free radical method .

The results obtained in the present study indicate that *Tirmania* and *Terfezia* truffles are a potential source of natural antioxidants.

Key Words: Antioxidant activity, reducing power, *Terfezia*, *Tirmania*

3.2. Introduction

Reactive oxygen species (ROS) or as commonly called active oxygen species, are various forms of activated oxygen, that include free radicals such as hydroxyl radicals ($^{\bullet}OH$) and superoxide ions ($O^{\bullet}_2$-), as well as non-free-radical species like hydrogen peroxide (H_2O_2) (Squadriato and Pelor, 1998). ROS formation in living organisms may take place in different ways, including some of these are by products of normal aerobic respiration, stimulated polymorphonuclear leukocytes, macrophages, and peroxisomes. These radicals appear to be the main endogenous sources of most of the oxidants produced by cells, while exogenous sources of free radicals include tobacco smoke, certain pollutants, organic solvents, ionizing radiation and pesticides (Davies, 1994). The presence of free radicals may lead to lipid peoxidation which, in turn, may lead to the deterioration of food quality (Sasaki et al., 1996). In addition, reactive oxygen species are believed to be involved in more than 100 diseases, including malaria, acquired immunodeficiency syndrome (AIDS), heart disease, stroke, diabetes, and cancer (Alho and Leinonen, 1999). Nevertheless, all aerobic organisms, including human beings, have antioxidant defense mechanism and numerous damage removal and repair enzymes that protects them against serious oxidative damages and remove or repair damaged molecules (Sun et al., 1998). It has been observed that the natural antioxidant mechanism may become inefficient and under these circumstances, it has to be supplemented by dietary intake of antioxidants (Halliwel, 1994).

Some synthetic antioxidant compounds, such as propyl gallate, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) which are commonly used in processed foods support the antioxidant defense mechanism of aerobic living organisms. However, it has been suggested that these compounds have some side effects. In addition, an inverse relationship between dietary intake of antioxidant rich foods and the incidence of human disease has been suggested (Yildirim et al., 2001). Therefore, search for natural antioxidants is gaining importance.

Truffles are healthy food that is low in calories and fat but rich in vegetable proteins. Their protein content is higher than that of most vegetables, with amino acid composition having comparable amount to that of animal proteins (Danell and Easker, 1992). They appear to be a good source of vitamins B, C, and minerals (Murcia et al., 2002). Among the important constituents of truffles are vitamins A, C, and β-carotene in which their antioxidant and antiradical properties grant them the property to have protective effects against free radicals. Truffles and mushrooms also contain many phenols, which are very efficient scavengers of peroxyl radicals (Gazzani et al., 1998).

The aim of the present study was to investigate the antioxidant activity of two desert truffles *Tirmania* and *Terfezia* using β-carotene bleaching method and DPPH free radical method.

3.3. Materials and Methods

3.3.1. Materials

All chemicals were of chromatography grade quality. Methanol, ethanol, propyl gallate, BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) were obtained from Fisher. Linoleic acid (cis-9, cis-12-octadecadienoic acid; 99%), β-carotene (approx. 95%) and 2-diphenyl-l-picrylhydrazyl (DPPH) (Sigma) were from Sigma, and nitrogen gas (MEGS), and hexane (Fisher) were of high grade of purity. Two types of desert truffles *Tirmania* and *Terfezia* were tested.

3.3.2. Determination of the antioxidant activity with β-carotene bleaching method

Antioxidant activity was measured by the ability of a compound to minimize the coupled oxidation of linoleic acid and β-carotene in an emulsified aqueous system, which loses its orange color when reacting with radical (Parejo et al., 2002). This experiment was carried out by the method of Emmons et al. (1999). 5 mg of β-carotene was dissolved in 50 mL of chloroform, and 3 mL filtered β-carotene solution was added to a volumetric flask, together with a 50 mL of linoleic acid and 400 mg of Tween 20. Under a stream of nitrogen gas, chloroform was removed, 100 mL of distilled water was added and then the mixture was vigorously shaken and saturated with oxygen. Aliquots (3 mL) of β-carotene/linoleic acid emulsion were mixed gently in 100 µl (100 µl/mL) of dry sample dissolved in different solvents (methanol, ethanol and water) and 100 µg/g for common antioxidants dissolved in water. Absorbance was measured at 0 time and then incubated

in a water bath at 50 °C for 60 min to check the ability of an antioxidant to minimize the coupled oxidation by difference in absorbance reading. Oxidation of the emulsion was monitored spectrophotometerically (Ultrospec 100 UV/Vis; Biochrom Ltd.) by measuring absorbance at 470 nm over a 60 min period. Control samples contained 100 μL of solvent in place of the extract and water was used as a blank. The antioxidant activity is expressed as percent inhibition relative to the control after 60 min incubation using the following equation:

$AA\ \% = 100\ (DR_c - DR_s)/DR_c$

Where AA% is the antioxidant activity, DR_c is the degradation rate of the control = ℓn (A/B)/60, DR_s is the degradation rate in the presence of the sample = ℓn (A/B)/60, A is initial absorbance at time zero, and B is the absorbance after 60 min. All tests were mean of triplicates.

3.3.3. Determination of antioxidant activity with the DPPH free radical method

In this method, antioxidant activity was measured using Beta et al. (2005) method that involves the use of the free radical 2,2-diphenyl-l-picrylhydrazyl (DPPH), where antioxidants are allowed to react with the stable radical in a methanol solution. Antioxidant activity are determined by measurement of the decrease in absorbance of DPPH at 515 nm. As a result of a color change the absorbance decreased when the DPPH radical was reduced by an antioxidant through donation of hydrogen to form a stable DPPH-H molecule. Freeze dried truffle samples (0.1g) were extracted with methanol, ethanol and water (1 mL) for 2 h in shaker (Forma Scientific) and then centrifuged at

3,000 rpm for 10 min in Safety centrifuge (Fisher Scientific, USA). The extract (0.1 mL) was reacted with 3.9 mL of 6×10^{-5} mol/L of DPPH solution (2.4 mg of DPPH in 100 mL of methanol). Absorbance (A) at 515 nm was determined at 0 and 30 min. Extraction solvents (methanol, ethanol and water) were the blanks.

Antioxidant activity was calculated as % of discoloration

= (1 – [A of samplet=30/A of controlt=0]) × 100. All tests were mean of triplicates.

3.3.4 Statistical analysis

Data were analyzed by analysis of variance ($P < 0.05$), and means separated by Duncan's multiple range test (SAS Institute Inc., 1985)

3.4. RESULTS AND DISCUSSION

3.4.1. Determination of the antioxidant activity with β-carotene bleaching method

Antioxidant activity was determined by the linoleic acid oxidation system method in which the amount of peroxides formed in an emulsion during incubation were determined spectrophotometerically by measuring absorbance at 470 nm. High absorbance is an indication of high concentrations of peroxides formed.

The antioxidant activities of ethanol extracts derived from both *Tirmania* and *Terfezia* samples were the most effective, but methanol extracts also showed strong activity with higher percentage in both samples. The water extract in both samples exhibited intermediate level of activity.

The linoleic acid oxidation assay used for determining antioxidant activity measures the inhibition of linoleic acid autoxidation. Tables 3.1 and 3.2 demonstrate the absorbance values obtained during the autoxidation of linoleic acid in the presence of *Tirmania* and *Terfezia* truffle extracts during 60 min incubation in water bath. On the basis of these results, the substances were divided into two groups according to the linoleic acid autooxidation inhibition percentages.

The first group included the substances with high antioxidant activity: ethanol extract of *Tirmania* (99.8 % inhibition), methanol extract of *Tirmania* (98.3%), ethanol extract of *Terfezia* (95.5%), and methanol extract of *Terfezia* (94.2%). These substances showed very high levels of antioxidant activity after 60 min incubation at 50°C. Under similar conditions, a second group of substance, water extracts of *Tirmania* (76.8%) and water extract of *Terfezia* (70.2%) exhibited medium levels of antioxidant activity after 60 min of incubation at 50°C. In this experiment all samples presented antioxidant activity of at least 70.2%. Therefore, these results show that both *Tirmania* and *Terfezia* truffles exhibited high antioxidant activity.

The similar experiments were carried out to compare the antioxidant activities of food preservatives such as propyl gallate, BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene). They exhibited 99.8%, 96%, and 88.5% of antioxidant activity, respectively (Table 3.3).

Figure 3.1 represents the differences in antioxidant activities between *Tirmania* and *Terfezia*. *Tirmania* possessed higher antioxidant activity than *Terfezia*. The standard deviation for *Tirmania* ranged from 0.2 to 1.0, while the standard deviation for *Terfezia* ranged from 0.1 to 3.0.

Figure 3.2 shows antioxidant activities (in percentage) of 6 extracts and 3 common chemical antioxidants. The results showed that the methanol and ethanol extracts of *Tirmania* and *Terfezia* are very strong antioxidant, as compared with those of the common antioxidants, while water extracts showed a medium level of activity.

3.4.2. Determination of antioxidant activity with the DPPH free radical method

The antioxidant potential of truffle extracts was evaluated using the stable DPPH radical. The free radical scavenging activity of truffle extracts was determined using 2,2-diphinyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$). This system allowed truffle extracts to react with $DPPH^{\cdot}$ in a methanol solution. The reduction of $DPPH^{\cdot}$, indicating a positive antiradical activity of truffle extracts, was followed by monitoring the decrease in its absorbance at 515 nm during the reaction. Tables 3.4, 3.5 and 3.6 shows the reaction of DPPH with *Tirmania* and *Terfezia* truffles and with common food antioxidants (propyl gallate, BHT and BHA). The order of antioxidant activity of truffles in this method was similar to that of linoleic acid oxidation system except that methanol extract of *Terfezia* represents higher antioxidant activity than that of ethanol. These samples were, in decreasing order, propyl gallate (97.4%) > ethanol extract of *Tirmania* (95.5%) >

methanol extract of *Tirmania* (95.4%) > methanol extract of *Terfezia* (95.3%) > BHA (93.0%) > ethanol extract of *Terfezia* (92.5%) > BHT (86.2%) > water extract of *Tirmania* (79.3%) > water extract of *Terfezia* (76.3%). According to the DPPH tests, truffle extracts acted as a direct free radical scavenger, and its potency was similar to that of common antioxidants and similar to the results obtained from linoleic acid oxidation system, indicating that truffle extracts showed a good antiradical activity.

The results also represent two group of extracts, the first group with very high antioxidant activity (ethanol and methanol extracts of *Tirmania* and *Terfezia* truffles as well as proply gallate and BHA) and a second group of moderate antioxidant activity (water extract of *Tirmania* and *Terfezia* truffles and BHT).

Figure 3.3 represents the differences in antioxidant activities between *Tirmania* and *Terfezia*. *Tirmania* possessed higher antioxidant activity than *Terfezia*. The standard deviation for *Tirmania* ranged from 0.3 to 0.4, while the standard deviation for *Terfezia* ranged from 0.3 to 0.5.

Figure 3.4 shows antioxidant activities (in percentage) of 6 extracts and 3 common chemical antioxidants. The results showed that the methanol and ethanol extracts of *Tirmania* and *Terfezia* are very strong antioxidants, as compared with those of the common antioxidants, while water extracts showed a medium level of activity.

Statistical analysis showed that extraction of β-carotene was significantly affected by truffle samples (*Tirmania* and *Terfezia*) and extraction solvents ($P< 0.01$), without any interaction (Table 3.7). Extraction of β-carotene yield was significantly ($P< 0.01$) higher

for *Trimania* than *Terfezia* samples. There was no significant difference in methanol or ethanol extracts of β-carotene bleaching method in *Tirmania* and *Terfezia*, with water yielding lowest ($P< 0.01$). There was significant ($P< 0.01$) interaction between truffle samples and extraction solvents for DPPH extraction. For *Trimania*, extraction with water yielded significantly the lowest ($P< 0.01$) concentration of DPPH, but there was no significant difference between methanol or ethanol extracts. However for Terfezia, there was a significant difference ($P< 0.01$) between the three extraction solvents. methanol extraction yielded highest concentration of DPPH, with lower yield for ethanol extract, and water was the lowest.

In a comparative study, raw, frozen and canned *Terfezia* samples were tested for antioxidant activity (Murcia et al., 2002). Samples were extracted with ethanol. Murcia et al. (2002) showed that the antioxidant activity of raw, frozen and canned *Terfezia* were 95.7%, 94.1% and 72.9%, respectively. Their results of raw and frozen sample were similar to our results, while the results of canned samples indicates that antioxidant activity of *Terfezia* might be lost during canning process (Murcia et al., 2002).

3.5. Conclusion

The desert truffles, *Tirmania* and *Terfezia* possessed high antioxidant activity that was as high as the common chemical antioxidants. *Tirmania* exhibited higher antioxidant activity (99.8%) than *Terfezia* (95.5%) in β-carotene method and in DPPH free radical method as well with (96.1%) for *Tirmania* and (95.3%) for *Terfezia*. Ethanol extracts

showed the highest antioxidant activity in both *Tirmania* and *Terfezia*. The results conclude that the desert truffles, *Tirmania* and *Terfezia* are an excellent source of natural antioxidants that can be used as food preservatives and for human consumption. Thus, these results open new horizons for replacing the artificial antioxidants that are dangerous to health and are carcinogenic with natural compounds. Identification and chemical characterizations of the natural antioxidants are under investigations to determine the stability of antioxidation activity during storage under various temperatures and other factors that govern its efficiency.

Table 3.1. Antioxidant activity of *Tirmania* extracts (β-carotene bleaching method).

Antioxidants Activity (%)*					
Solvetns / triplicates	1	2	3	Mean	SD ±
Methanol	98.0 %	98.8 %	98.2 %	98.3 %	0.4
Ethanol	99.9 %	99.5 %	99.9 %	99.8 %	0.2
Water	77.1 %	75.7 %	77.6 %	76.8 %	1.0

*Antioxidant activity was determined by the method of Emmons et al. (1999).

Table 3.2. Antioxidant activity of *Terfezia* extracts (β-carotene bleaching method).

Antioxidants Activity (%)*					
Solvents / triplicates	1	2	3	Mean	SD ±
Methanol	93.4 %	94.8 %	94.4 %	94.2 %	0.7
Ethanol	95.5 %	98.4 %	92.6 %	95.5 %	2.9
Water	70.1 %	70.1 %	70.3 %	70.2 %	0.1

*Antioxidant activity was determined by the method of Emmons et al. (1999).

Table 3.3. Antioxidant activity of common antioxidants (β-carotene bleaching method).

Antioxidants Activity as (%)*					
Antioxidants / triplicates	1	2	3	Mean	SD ±
Propyl gallate	99.9 %	99.7 %	99.9 %	99.8 %	0.1
BHA	95.8 %	96.2 %	96.0 %	96.0 %	0.2
BHT	88.6 %	88.6 %	88.3 %	88.5 %	0.2

*Antioxidant activity was determined by the method of Emmons et al. (1999).

Table 3.4. Antioxidant activity of *Tirmania* extracts (DPPH free radical method).

Antioxidants Activity (%)*					
Solvetns / triplicates	1	2	3	Mean	SD ±
Methanol	95.0%	95.8%	95.4%	95.4 %	0.4
Ethanol	96.5%	95.8%	96.1%	96.1 %	0.4
Water	79.0%	79.4%	79.5%	79.3 %	0.3

*Antioxidant activity was determined by the method of Beta et al. (2005).

Table 3.5. Antioxidant activity of *Terfezia* extracts (DPPH free radical method).

Antioxidants Activity (%)*					
Solvents / triplicates	1	2	3	Mean	SD ±
Methanol	94.8%	95.5%	95.7%	95.3 %	0.5
Ethanol	92.2%	92.9%	92.4%	92.5 %	0.3
Water	76.4%	76.7%	75.9%	76.3 %	0.4

*Antioxidant activity was determined by the method of Beta et al. (2005).

Table 3.6. Antioxidant activity of common antioxidants (DPPH free radical method).

Antioxidants Activity as (%)*					
Antioxidants / triplicates	1	2	3	Mean	SD ±
Propyl gallate	97.8 %	96.9 %	97.5 %	97.4 %	0.5
BHA	92.6 %	93.6 %	92.8 %	93.0 %	0.5
BHT	86.4 %	86.2 %	85.9 %	86.2 %	0.3

*Antioxidant activity was determined by the method of Beta et al. (2005).

3.7. Statistical analysis of antioxidant activity of *Tirmania* and *Terdezia* by β- carotene bleaching method and DPPH free radical method.

	Trimania			*Terfezia*					Significance(*P* value)	
	Methanol	Ethanol	Water	Methanol	Ethanol	Water	SEM	Cultivar	Method	Interaction
β- carotene	98.3a	99.8a	76.8c	94.2b	95.5b	70.2d	0.75	< 0.01	< 0.01	0.21
DPPH	95.4a	96.1a	79.3c	95.3a	92.5b	76.3d	0.21	< 0.01	< 0.01	< 0.01

Statistical differences were analyzed by SAS ($P < 0.05$).
Values with the same letter are not significantly different.
SEM : Standard error of mean
Cultivar : Truffles sample: *Tirmania* and *Terfezia*
Method : Extraction solvents : methanol, ethanol and water.
Interaction : the interaction between cultivar and method

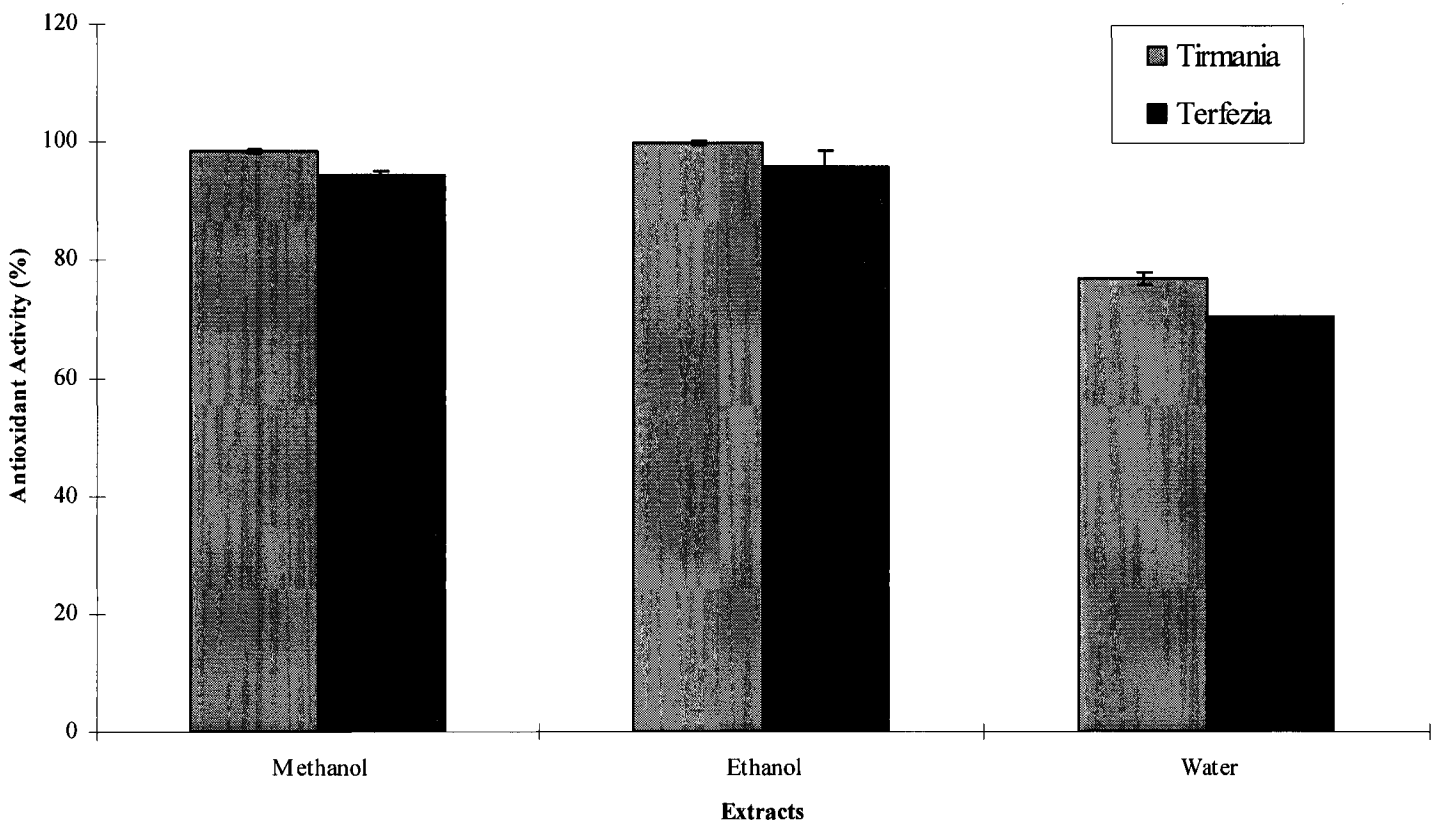


Figure 3.1. Antioxidant activity with methanol, ethanol and water extracts of *Termania* and *Terfezia* truffles (β-carotene bleaching method).

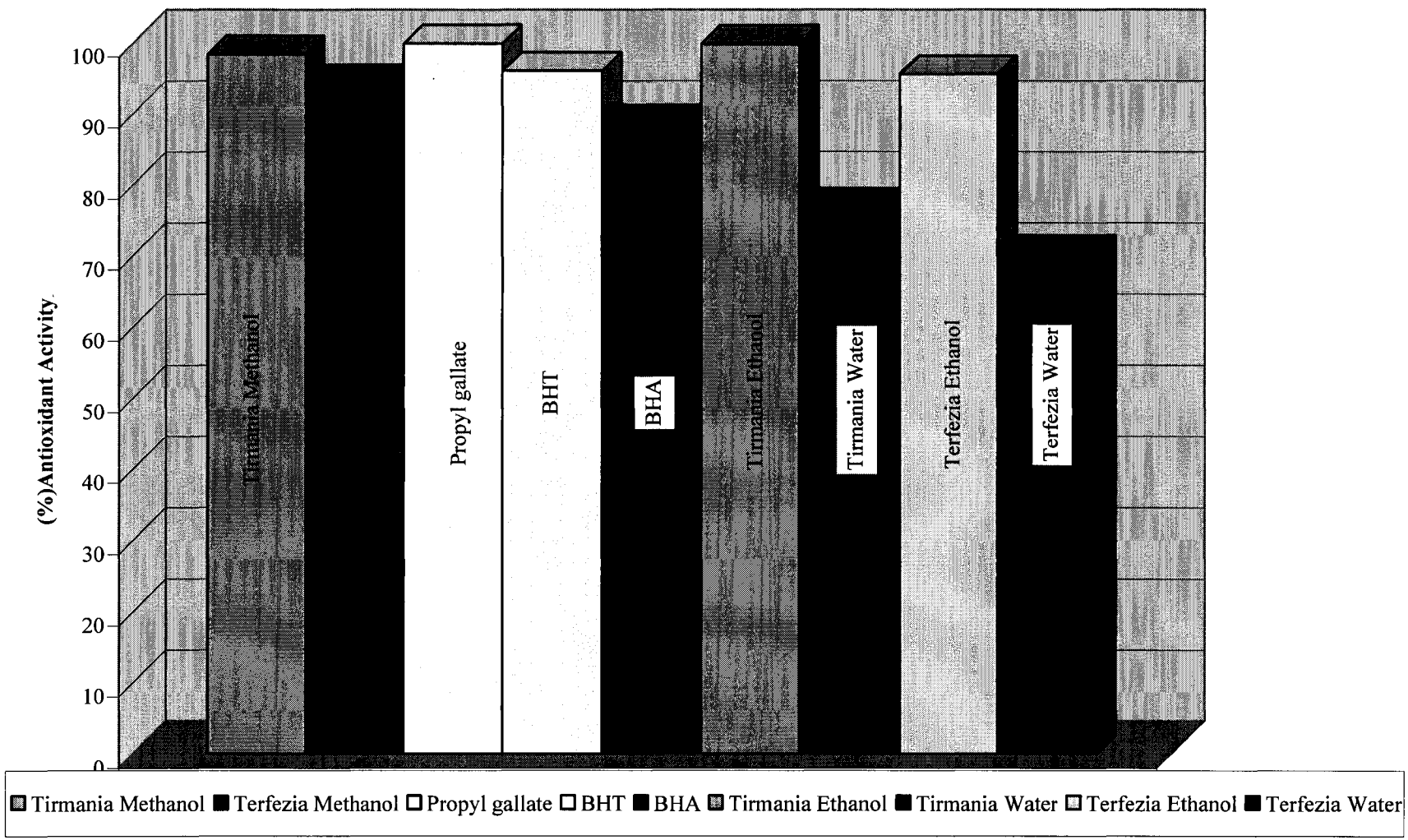


Figure 3.2. Comparison of artificial and natural antioxidants (β-carotene bleaching method).

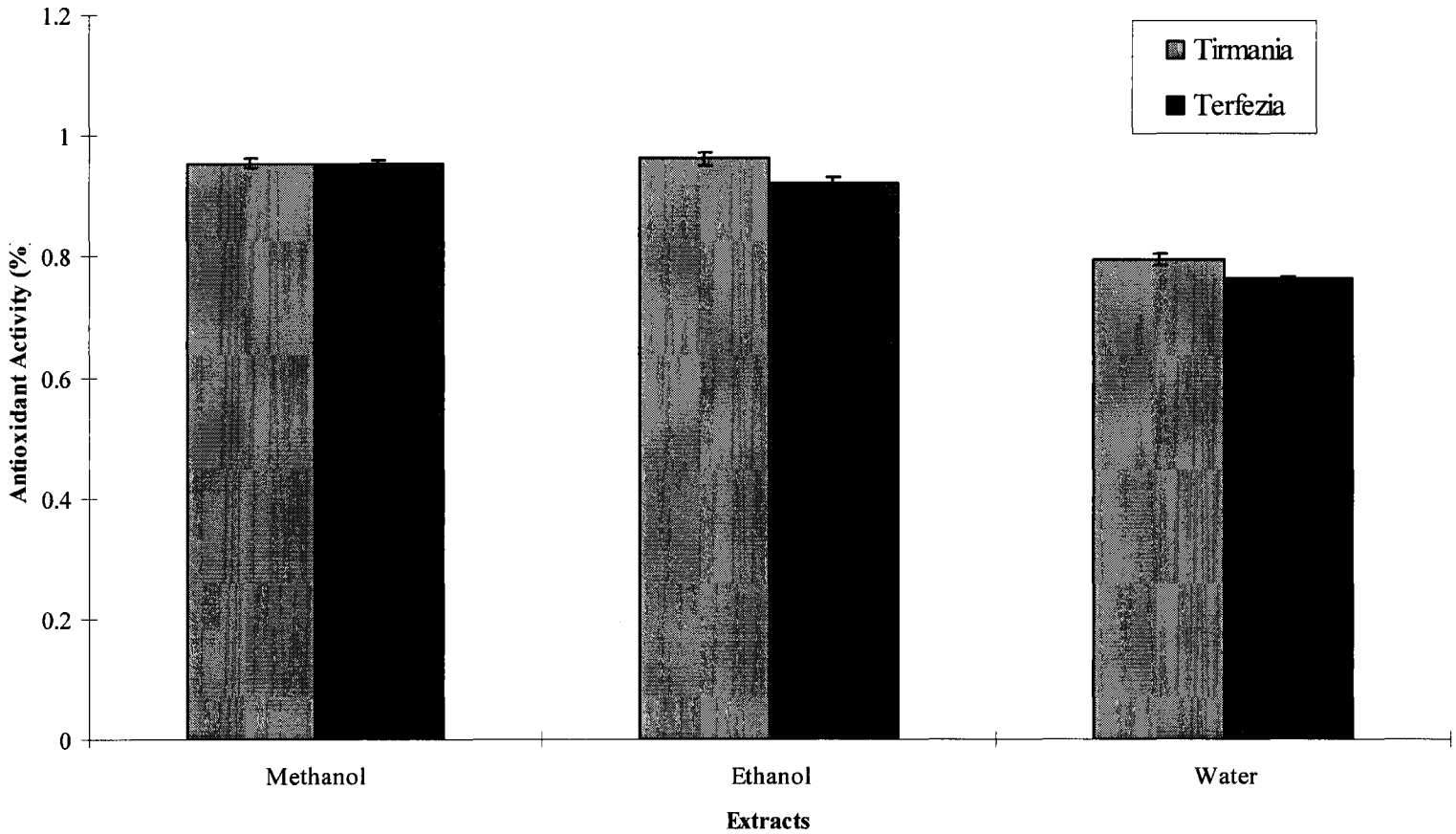


Figure 3.3. Antioxidant activity with methanol, ethanol and water extracts of *Termania* and *Terfezia* truffles (DPPH free radical method).

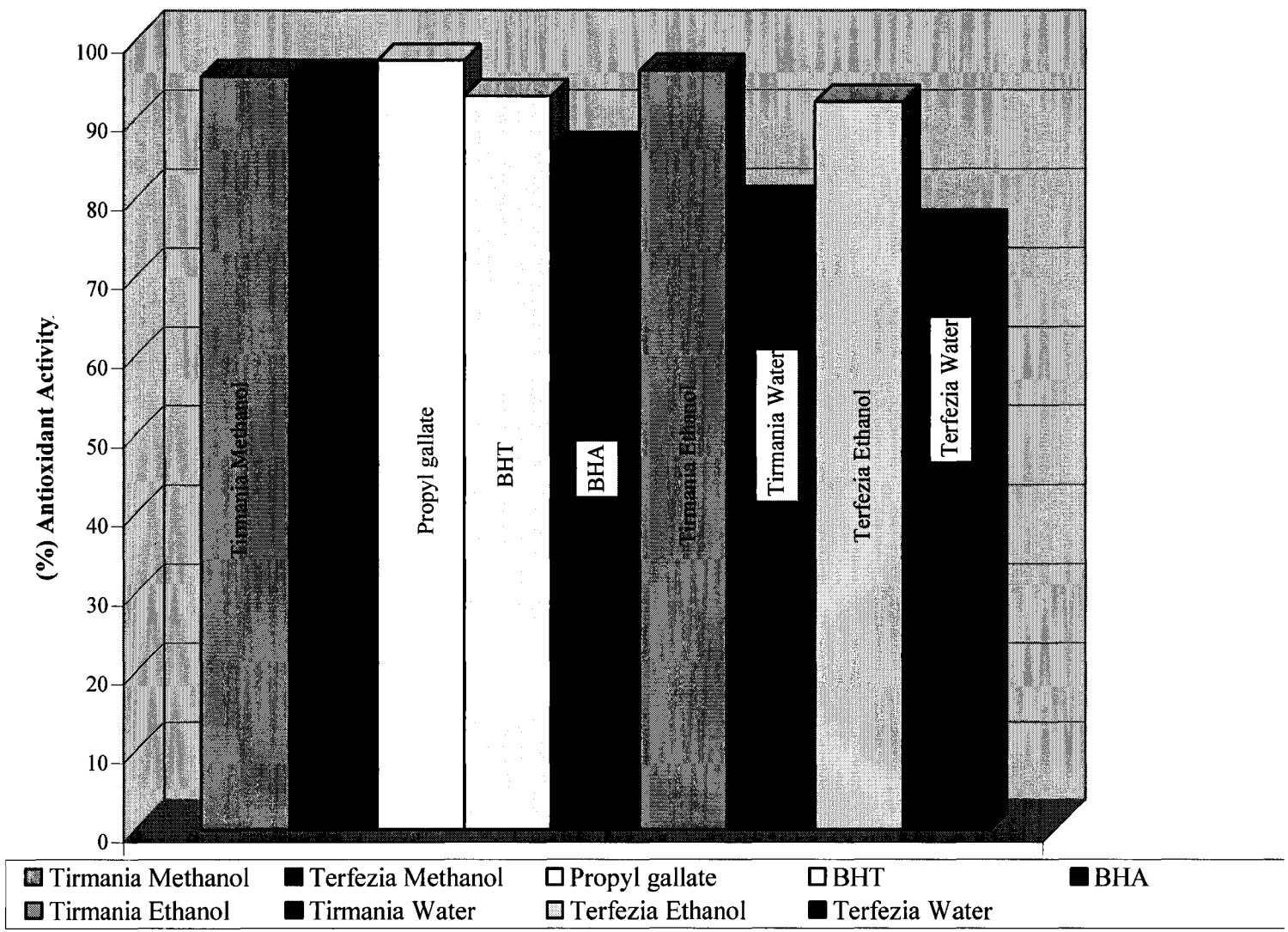


Figure 3.4. Comparison of artificial and natural antioxidants (DPPH free radical method).

GENERAL CONCLUSIONS

Both *Tirmania* and *Terfezia* truffles possessed high antimicrobial activity against a wide variety of microorganisms, among which many are food-borne pathogens that can cause serious diseases. *Terfezia* extracts showed higher antimicrobial activity than those of *Tirmania.* Methanol was the most efficient solvent used to extract the active compounds. Ethanol was also a good solvent and both extracts were effective against bacteria and *Saccharomyces,* but not against fungi. Ability of inhibiting bacteria was measured by inhibition zone up to 25 mm in diameter on Mueller Hinton agar and up to 35 mm in diameter on nutrient agar media. The active compound(s) are still unidentified, but showed broad spectrum effect, and were found to be effective in low concentrations.

Both types of truffles showed high antioxidant activities, which are very important in the field of food safety since they are from a natural source, as more restrictions are being made against artificial ones. Both ethanol and methanol extracts showed very high antioxidant activities up to 99.9% inhibition using β-carotene bleaching method and up to 96.1% inhibition using DPPH free radical method, indicating that truffles are a great source of natural antioxidants.

Further investigation should be continued to isolate and identify these active compounds to evaluate their properties, chemical characteristics and uses of these active compounds.

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