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Antibacterial Activity of Commiphora molmol (myrrha) against the yeast

Mohammad Hassan Alshehri
Long Island University

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A Master's Thesis Submitted to the Faculty of Richard L. Conolly College,

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In Fulfillment of

The Requirements for the Degree of

Masters of Science

October 19, 2022

MAJOR

Biology

DEPARTMENT

Life Sciences

SPONSORING COMMITTEE

Dr. Mrinal Bhattacharjee, Chairman

Dr. Joseph Morin

Dr. Fatma Abo

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Dr. Joseph Morin

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Abstract

Commiphora molmol (myrrha) is widely used as a traditional medicine around the globe against inflammatory diseases. It is also considered to be effective as anti-fungal, anti-cancer and anti-oxidant agent. The minimum inhibitory concentration, bactericidal activity and heat stability of antibiotic component in myrrha oil extract has been investigated. The MIC was determined in nutrient free buffer and in nutrient rich peptone glucose buffer. The results showed that MIC was 0.6% in nutrient free buffer as no growth was shown by cells, whereas, in peptone glucose media a slight increase in cell count was seen. It can be concluded that it has strong antimicrobial activity on yeast in nutrient free medium as the oil is unable to show antibacterial activity on growing cells. Bactericidal activity of the oil was tested with yeast on both nutrient free phosphate buffer and nutrient rich media. After 15 minutes, 0.6% myrrha has reduced the cell viability by 99% in nutrient free phosphate buffer showing that it specifically kills the non-growing yeast cells compared to growing ones. Heat stability was tested and reported that oil is stable on heating up to 70°C for 90 minutes. Furthermore, the pre-incubation of yeast with myrrha oil extract was studied and results revealed that if pre-incubation take place in nutrient rich medium then there is no effect on viability of cells. Therefore, it can be determined that presence or absence of nutrients is effecting the cell viability. In conclusion, the results of the following study suggest that extract of myrrha can be used as an anti-fungal drug.

I. INTRODUCTION

The traditional lifestyle of human beings has been overruled by the scientific life through the availability of new food products, antibiotics and preventive measures at the doorstep of every individual. Now the pharmaceutical industries are offering new medicines against traditional fungal diseases, whereas, some new strains are a life threat to humans. Similarly, the lifesaving treatments as in case of cancer, are inducing fungal infections in immunosuppressed patients (Pappas et al 2018). At least 3 million species have been reported for fungal lineage including all subcategories of micro-organisms (Heitman 2011).

A human friendly fungus yeast is now considered as an emerging pathogen for microbiologists. Of the five yeast clades of yeast, *Candida albicans* is reported as pathogenic microbe while *Saccharomyces cerevisiae* (baker's or brewer's yeast) is the most studied one. For fungal infection in humans, 70% of the mortality is due to the Candida albicans with increased multi-drug resistance (Schwartz et al. 2018).

Characteristics of Yeast

The unicellular yeast has a similarity index with eukaryotic cells. The fungal organism has a history of use in the well-being of humans. Many new microorganisms are being employed in glucose fermentation and ethanol production, but *S. cerevisiae* yeast is still one of the most useful microorganisms for human beings. *S. cerevisiae* is a unicellular organism and shares some typical characteristics with mammalian cells. It multiplies through budding instead of fission. Somehow,

it is similar to eukaryotic cell by the means of fast growth, formation of smooth cream-colored colonies. Kasemets (2009) have used this model organism for toxicological studies. Ocampo and Barrientos (2008) used this as a disease model and Schenone et.al. (2013) did drug screening by using yeast.

Virulence factors of Yeast

Multiple microbial species interact with other living organisms after forming symbiotic, commensal, or parasitic relations. Similarly, the pathogenic relation of fungal species has been reported for human beings, however, their association with multiple diseases is still under investigation. The human-friendly yeast now needs major attention after its pathogenicity has been claimed by many scientists. Its subgroup *Candida albicans* was known as a human pathogen but causes disease only in facilitated conditions (ven der Does and Rep 2017). The underestimated pathogenicity of baker's yeast was verified when confirmed cases of fungal infections were truly linked with it. Like all other microbial pathogens, yeast needs two processes for pathogenesis: survival within the host cell and damage to host metabolic functions (Casadevall et al. 1999).

Wheeler et al. (2003) studied the virulence genes of two strains, one isolated from decaying fruit and one from a mouse infection model. The laboratory strain was virulent but plant isolate was lethal in mice. It was knockout of SSD1 gene that can alter the cell wall composition of yeast cell leading towards increased virulence of yeast cell in a mouse infection model.

The findings suggested that proinflammatory response in overstimulated cells increases the virulence of the mutant strain. The hypervirulent ssd1Δ/ssd1Δ yeast strain is a more potent elicitor of proinflammatory cytokines from macrophages in vitro. A study revealed the fact that *S.*

cerevisiae also needs the same metabolic genes as C. albicans in order to act as a pathogenic microbe (Silva et al. 2015).

Diseases caused by Yeast

Recent studies have claimed that the rate of diseases due to fungal infections is increasing day by day. Although the mortality rate is higher for bacterial diseases (malaria and tuberculosis), fungal infections are also making new ways (James et al. 2006). Bongomin (2017) have reported the death of 1.6 million people each year due to fungal diseases. The Mycotic Diseases branch of the Centres for Disease Control is concerned about three main classes of fungal infections: opportunistic infections, community-acquired infections, and hospital-acquired infections (McCotter et al 2015). Major fungal pathogens belong to Ascomycota phylum (Heitman 2011). Dean et al (2012) have classified the seven most important plant pathogens and Kim (2016) has classified the three most systematic human pathogens as Ascomycetes. However, out of some of the several available fungal pathogens, only a few are truly capable of parasitizing human beings (Köhler et al. 2015). *Candida albican* is the most common yeast pathogen for human beings causing infections like oral thrush and vaginitis (Denning 2017). Several studies have reported *S. cerevisiae* as a causative agent of fungemia, endocarditis, pneumonia (Aucott et al. 1990, Doyle et al. 1990), peritonitis (Snyder 1992), esophagitis (Konecny 1999), vaginitis (Posteraro et al 1999) asthma (Belchi-Hernandez et al. 1996, cellulitis (Almanza et al. 1998), diarrhea (Candelli et al. 2003), and infections of the skin and urinary tract (Senneville et al. 1996). Enache-Angoulvant and Hennequin (2005) reported more than 90 cases of Saccharomyces invasive infection for which predisposing factor were similar to those of invasive candidiasis. More than 50% of fungemias were linked to the *S. boulardii* (a subtype of *S. cerevisiae*).

Algazq et al. (2017) investigated a patient suffering from laryngeal carcinoma with oral lesions and reported this patient as the first case of *S. cerevisiae* laryngitis. The microscopic examination confirmed the presence of Saccharomyces genus. The possible sources of *S. cerevisiae* infections include grapes and locally brewed beer (Olver et al. 2002).

Till now, fungemia is termed as the most severe clinical syndrome of *Saccharomyces cerevisiae*. Enache-Angoulvant and Hennequin (2005) have reported *S. cerevisiae* fungemia in 31% of immunosuppressed patients and also in healthy persons. A shocking result was obtained when Piarroux et al. (1999) isolated Saccharomyces strains from the blood of a patient who died with sepsis but no other microorganism was found in his blood cultures.

Vaginitis is mainly linked with *C. albicans* but some cases have also been observed for *S. cerevisiae* mimicking symptoms of *C. albicans* (Posteraro et al. 1999). Another study isolated the *S. cerevisiae* from the throat, stool, urine, and perineum samples of 16%, 23%, 10%, and 20% of the cases with hematologic disease (Salonen et al. 2000). It is still a mystery whether *S. cerevisiae* is a persistent commensal or just transiently present after food digestion.

Septicemia is most often caused by bacteria but Eschete and West (1980) have reported the first case of septicemia in a hyperalimented burned man caused by *Saccharomyces cerevisiae*. This was a rare case having origin in a bleeding esophageal lesion, with clinical characteristics including profound neutropenia, thrombocytopenia, hypothermia, and monocytopenia, which was also curable by amphotericin B.

The study helps us to come to a point where oral antimycotic treatment was not sufficient for all *S. cerevisiae* patients. Furthermore, isolation of *S. cerevisiae* from the vaginal tract and other places gave a conclusive statement that Saccharomyces is one of the emerging pathogens for both humans and plants and must be given proper attention before using it as a probiotic.

Treatment of Yeast Infection

Fungal pathogens have more similarities with eukaryotic human beings than prokaryotic bacteria thus putting a great challenge for microbiologists to find effective targeted drugs for fungal infections. The most common pathogen of the human being is bacteria but unfortunately, fungi have more in common with human cells than with bacterial cells. The biggest challenge is to find a drug that only breaks the fungal pathogenic pathway, not the human cells. The available drugs target the cell wall or membrane in case of fungal infections. The antifungal targets currently in use are polyenes, azoles, echinocandins, and allylamines (Bhattacharjee 2016).

The echinocandins containing caspofungin class of drugs works by targeting the catalytic subunit of 1,3 β-glucansynthetase, which in return disrupts the cell wall formation process (Pemán et al. 2009). The important class 'Azole' includes fluconazole and itraconazole that help reduce the chances of yeast infection by interfering with the ergosterol synthesis within the cell wall of yeast (Balkis et al. 2002).

In the case of *S. cerevisiae*, the most common certified drugs available are amphotericin B, caspofungin, and azole derivatives. *S. cerevisiae* has shown susceptibility to amphotericin B (MIC_{90}, 0.5–1 µg/mL) and fluorocytosine (MIC_{90}, 0.25 µg/mL). Rex et al. (1997) have reported a susceptible dose-dependent range of MIC90 for fluconazole and itraconazole for *C. albicans*. This fact may be used to make a new hypothesis that these drugs can play role in the emergence of *S. cerevisiae* infections. Another drug caspofungin has been tested in various cases but its impact on *S. cerevisiae* infection is still doubtful. Fungemia, the most severe syndrome is widely associated with *Saccharomyces cerevisiae*. One of the most successful treatments for *S. cerevisiae* fungemia is the immediate withdrawal of probiotics that should be further facilitated by an antifungal agent or in severe cases withdrawal of central venous catheters is recommended (Piarroux et al. 1999)

Antibiotic resistance of Yeast

Antibiotic resistance is the decrease in the sensitivity level of a microbe towards a controlling active compound. The similarity index of fungal microbes with eukaryotic cells has not only attracted the microbiologists to utilize the fungal microorganisms as a model for mammalian cell study but also has provided a barrier in the control of fungal pathogens (Moen et al. 2009).

Within the mammalian cell, the antibiotic-resistance is linked to the overproduction of the ABC (ATP-binding cassette) transporter (Chang 2003). The ABC proteins are important components of both eukaryotic and fungal cells with membrane transporters that are normally present in all kingdoms. The responsibility of multi-drug resistance in the case of human cancer lies in these ABC proteins, thus leading to tumor development in cancer patients. Recently, multiple cases have been reported for resistance to chemotherapy (Dean et al. 2001). ABC proteins of eukaryotic cells have two homologous halves consisting of a nucleotide-binding domain and a transmembrane domain with six transmembrane spans. Several ABC proteins including ABCB1 (P-glycoprotein, P-gp), ACBG2, and ABCC1 offer resistance to chemotherapeutic agents by pumping cancerous drugs out of these tumor cells (Gottesman et al. 2002) However, in fungal cells, the selection of antibiotic-resistant cells is easy compared to mammalian cells due to the elevated expression of ABC transporter-encoding genes (Sanglard et al. 2009). While ABC transporters offer elevated levels of proteins in mammalian cells, they have been detected as a key issue in all fungal diseases offering a high level of antibiotic resistance. Till now, a detailed picture of antibiotic resistance is available for the yeast *Saccharomyces cerevisiae*. Scientists have utilized this available information of *Saccharomyces cerevisiae* for the detailed study of other pathogenic fungi.

A phenotype known as pleiotropic drug resistance (PDR) involving transcriptional factors and ABC transporters exists in the *Saccharomyces cerevisiae* that has gained scientific sight recently

(Jungwirth and Kuchler 2006). Studies have shown a close relationship between the overexpression of ATP-binding cassettes (ABC) transporters such as Pdr5p, Snq2p, and Yor1p and antibiotic resistance within the yeast cells.

Commiphora molmol (Myrrha)

Commiphora molmol- a genus of the family Burseraceae (De Rapper et al., 2012) has nearly 150-200 species distributed through drier regions of tropical Africa with few species frequently growing in South America. The genus is widely spread from Arabia to India also making its way through Somalia, Kenya, the Middle East, and Ethiopia (Haffor, 2010). Myrrha is the aromatic resins normally collected as exudate, from the bark of Commiphora molmol (Abdul-Ghani et al., 2009). Actually, the Commiphora myrrha produces yellowish or reddish resin (gum) extracted as hardened sap-like material from the tree trunk by making longitudinal cuts (Haffor, 2010).

Commiphora myrrha is a commonly used name of Commiphora molmol (Al-Ruwaili et al, 2012). The composition of myrrha resin includes 2-9% volatile oil, 23-40% resin and the rest is of water-soluble gum with cadinene, elmol, eugenol, cuminaldehyde, commipherol, and commipherin as secondary metabolites. The myrrha-like resins are also known as Indian myrrh, opopanax, balsam, bdellium, or guggul bisabol. The gum contains protein and polysaccharides while sterol, steroids, and terpenes are present in the volatile oil. Its characteristic constituents are furanosesquiterpenes such as furanoelemanes, furanoeudesmanes, and furanogermacranes (Marongiu et al., 2005). These constituents of myrrha are usually present in schizogenous or schizolysigenous ducts. Myrrha has been used as an antiseptic in the treatment of infections and inflammations. For centuries, the resins of Commiphora species have been used in aromatic products like perfumes, incenses, and aromatic wound dressings. At the beginning of the 21st century, the trend from the

clinical medication was moving back toward natural herbs, most commonly in dental medication. The pharmaceutical uses of myrrha include its addition in toothpaste, mouthwashes, and gargles as aromatic antiseptic resin. Several studies have documented its antithrombotic and analgesic activity, the reason behind its use in tooth powders. In the United States, myrrha is added to nearly all kinds of toothpaste and dental powders. If we date back to 1500 BC, the myrrha oil was used with other combinations. Its oldest pharmaceutical uses include the treatment of wounds and skin sores by Ancient Egyptians (Michie & Cooper, 1991). The myrrha is frequently used with frankincense but it can also be combined with other substances like opium and red ochre.

The local habitants of northeastern Africa and Saudi Arabia still use this herb as a remedy against various infections. The oleo gum protects the gastrointestinal lining against the damaging effect of indomethacin and ethanol. Currently, myrrha is being used to help cure abrasions and other skin ailments (El Ashry et al., 2003). Its anti-inflammatory effect is approved by the European Commission as they have allowed its use against oral mucosa and other inflammations. Hanus et al. (2005) have reported its use against cancerous cells in laboratory trials.

Previously, the Chinese Traditional Medicine system relied on natural herbs including myrrha to help treat leprosy, rheumatism, and syphilis. Further, Haffor (2010) has reported myrrha use in stomach acidity in Somalia and Ethiopia. Kimura et al. (2001) have reported its beneficial effect against chronic and acute inflammations. In India, the traditional medicinal system used myrrha to treat edema, cough, constipation, hepatitis, obesity, paralysis, gout, and rheumatism. Myrrha combined with other natural herbs like Panax notoginseng, Safflower petals, Angelica sinensis, Cinnamon and Salvia miltiorrhiza in an alcoholic solution is useful for internal or external purposes (Paraskeva et al., 2008).

An amazing fact about myrrha was revealed when its glucose tolerance ability was checked in a study on rats. This study supported a decrease in blood glucose levels of both normal and diabetic rats under fasting conditions, hence proving it a beneficial agent against non-insulin-dependent diabetes mellitus (Al-Awadi & Gumaa, 1987). To summarize, the anti-inflammatory, anti-fungal, anti-cancer, and antioxidant effects of myrrha resin have been well-documented.

Antibacterial effect of Myrrha

Bacterial inf ections were effectively treated with antibiotics after the discovery of penicillin. Most antibiotics were developed in the early decades after the discovery of the first commercial antibiotics. However, some new technologies have been developed recently and some are in the pipeline. Unfortunately, many antibiotics are not 100% effective treatments because of the development of resistance in bacteria (Bhattacharjee 2016). Now the top-selling effective drugs are either natural products of plant extracts or contain their derivatives (Demain and Sanchez 2009). Myrrha is recently being sighted as an antibacterial agent. Several investigations have reported its antimicrobial activity against staphylococci, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans*. However, its use as an antibiotic was rejected despite several published pieces of evidence. This could be due to the presence of toxic inorganic elements (Ahmad et al., 2017) at low dosage levels. Recently some studies have reported it as a safe antibiotic for humans after even long-term use.

Several studies have reported a weak antibacterial activity of myrrha against multiple bacteria. This could be due to the use of old and traditional testing methods designed to check the antibacterial effect against growing and viable bacteria. (Al-Madi et al., 2019) compared the antibacterial activity of myrrha resins against *Enterococcus faecalis* and *Fusobacterium*

nucleatum, with sodium hypochlorite (NaOCl) and concluded that myrrha extract has strong and notable antibacterial activity against both *F. nucleatum* and *E. faecalis.*

A recent study by Bhattacharjee & Alenezi (2020) reported a strong antibacterial activity of myrrha against non-growing bacteria by using a new method designed specifically to check the inhibitory effect of antibiotics against dormant or non-growing bacteria. The authors also reported that bacteria could not develop resistance against this natural gum. Hurdle et al. (2011) have suggested that one possible mechanism of anti-bacterial activity against dormant bacteria can be by targeting the membrane or bacterial enzymes associated with the membrane. So it may be possible that myrrha gum disrupts the membranous proteins of bacteria as the mechanism of its anti-bacterial activity.

II. MATERIALS

Myrrha Extract:

Commiphora molmol (*myrrha* or *myrrh*) resin was purchased from an herbal store in Riyadh, Saudi Arabia. 4 g of myrrha resin was taken and ground thoroughly These fine pieces were soaked in 5 ml of 95% ethanol for 10 minutes. The mixture was centrifuged for 5 min at 10000 rpm and the supernatant was collected. The extraction process was repeated three more times. The combined ethanol extract was then subjected to centrifugation for 10 minutes at 10000 RPM to collect the supernatant. A rotary evaporator machine (BUCHI R-114) was used to evaporate the ethanol leaving behind 0.97 ml of oil (0.762 g/ml). Since the oil is insoluble in water, a 20% solution of the oil in ethanol was made and used a stock solution for all experiments.

Microbial Strain:

Saccharomyces cerevisiae (baker's yeast, Fleischmann) (stock LIU 288) was used throughout this experiment.

Peptone-glucose Broth:

For peptone-glucose broth, 180 ml of pre-autoclaved deionized water was mixed with 6 grams of dextrose (glucose) and 1.5 grams of peptone. This solution was subjected to microwave at high power for 5 minutes and then cooled to room temperature.

Peptone-glucose Plates:

For yeast growth, the peptone-glucose plates were used. These plates were prepared using the same method mentioned above with minor changes. In this, a double (2X) concentration of peptone-glucose media was prepared with 12 gm of dextrose (glucose) and 3 gm of peptone in 180 ml of pre-autoclaved deionized water. This solution was subjected to microwave at high power for 5 minutes and then cooled to room temperature. Pre-autoclaved solution of 4.5g agar in 150 ml of water was melted by heating at 50% power in a microwave with intermittent mixing. The peptone-glucose solution was added to the heated agar solution. Then, the mixture was cooled and poured to make 12 plates.

III. Results

MIC of myrrha oil in nutrient-rich and nutrient-free media

Yeast was grown overnight in peptone-glucose broth. Then, 5 ml cells were centrifuged, the supernatant was discarded and the cells were re-suspended in 300 µl of peptone-glucose broth. The cells were distributed into six tubes, 50 µl in each tube. Different amounts of myrrha oil solution were added to the tubes to final concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 0.1%. The tubes were

incubated at 30ºC for 1 hour after which serial dilutions from 10^{-2} to 10^{-6} were spread on peptone-glucose plates and incubated for 24 hours at 30ºC. Colonies that grew in each were counted. For MIC determination in phosphate buffer, 5 ml overnight grown yeast culture was centrifuged, the supernatant was discarded and the cells were washed two times in 0.02M phosphate buffer pH 7.0. Finally, the cells were resuspended in 300 µl of 0.02M phosphate buffer pH 7.0. The remaining procedure was same as described above for MIC in peptone-glucose media.

The results in Tables 1 and 2 show that in nutrient-free buffer, the MIC of myrrha oil is 0.6% at which concentration no growth was observed. However, in nutrient rich peptone-glucose media there was one order of magnitude decrease in cell count at 0.6% myrrha but there was no further increase in inhibition above 0.6% myrrh; in fact, there was a slight increase in cell count. Thus, it can be concluded that myrrha exhibits a much stronger antimicrobial activity on yeast in nutrient free medium, in which cells are likely to be not growing than in nutrient-rich medium.

Bactericidal activity of myrrha oil.

In order to demonstrate the bactericidal activity of myrrha oil, a rate of killing experiment was performed with the yeast in nutrient-rich peptone glucose medium as well as in nutrient-free phosphate buffer to mimic growing and non-growing bacteria respectively. First, yeast was grown overnight. Then, two 5 ml aliquots were centrifuged, the supernatants were discarded. The cell pellet in one tube was resuspended in 130 µl fresh peptone glucose medium while the pellet in the second tube was resuspended in 1 ml nutrient-free phosphate buffer were resuspended. The cells in phosphate buffer were centrifuged again and the pellet was finally resuspended in 130 µl of 0.02M phosphate buffer pH 7.0. The cells in each tube were distributed into two microfuge tubes, 65 µl in each. The first tube served as control without myrrh, while myrrha oil was added to the

second tube to a final concentration of 0.6%. The tubes were incubated at 30ºC for various times from 0 to 120 minutes. Then, serial dilutions from 10^{-2} to 10^{-5} were spread on peptone-glucose media plates and incubated for 24 hours at 30ºC. The number of colonies that grew in each was counted. The results for are shown in Table 3. In nutrient-free phosphate buffer, 0.6% myrrha decreased the cell viability by 99% in just 15 min during which time there was only about 50% loss of cell viability in peptone-glucose medium. The control in each case showed very little decrease in cell viability during the 2-hour course of the experiment. Thus, myrrha oil preferentially kills non-growing yeast cells compared to growing cells. Similar results were reported for the effect of myrrha oil on bacteria (Bhattacharjee and Alenezi 2020).

The inhibitory activity of myrrha can be better visualized using a plot of log CFU versus time as shown in Figure 1. The cell viability in peptone-glucose medium decreased by 2 orders of magnitude in 120 min. However, the inhibitory activity in nutrient-free phosphate buffer was much greater showing a decrease of cell viability by at least 4 orders of magnitude in less than 45 min. It is to be noted that a decrease of 4 orders of magnitude was the lower limit of detection because 10^{-2} was the lowest dilution that was spread. In both control tubes in absence of myrrha in growth medium or in phosphate buffer, the cell viability remained almost constant throughout the experiment.

Heat stability of the antibiotic component in myrrha oil

Heat stability of the antibiotic in myrrha oil was determined. Yeast cells were grown overnight. Then, 5 ml of the cell culture was centrifuged, the supernatant was discarded and the cells were washed two times with 300 µl of 0.02M phosphate buffer pH 7.0. Finally, the cells were re-suspended in 300 µl of 0.02M phosphate buffer pH 7.0. At zero time, 10^{-4} and 10^{-5} serial dilutions were spread on two plates as control. The remaining cells were distributed into five tubes A,B,C,D

and E, 50 µl in each tube. In a separate tube 10 µl of 20 % myrrha was taken and heated at 70ºC. After heating for indicated lengths times, the tube was centrifuged for a few seconds to recollect any liquid that had evaporated and condensed on the lid. Then 1.5 µl aliquots of the preheated myrrha was added to each tube at indicated times of preheating: A (0 min), B (15 min), C (30 min), D (60 min), E (90 min). All tubes were incubated for 60 min at 30 ºC and serial dilutions, 10^{-3} and 10^{-2} were spread on two plates. After incubation overnight at 30 ºC, colonies, if any that grew on each plate were counted. The results are shown in Table 4. The results demonstrate that the myrrha oil is stable when heated to 70°C for up to 90 minutes. This is of great importance for long-term storage of the antibiotic.

Effect of myrrha oil on yeast cells emerging from dormancy

Since myrrha oil does not have much inhibitory activity in nutrient rich medium, the question arises about how quickly yeast cells can emerge from dormancy after long-term exposure to nutrient-free buffer.

Yeast was grown overnight in 5 ml peptone-glucose medium to give an A_{600} reading of 0.564. The cells were centrifuged, the supernatant was discarded and the cells were washed three times in 0.02M phosphate buffer pH 7.0. Finally, the cells were resuspended in 500 µl of 0.02M phosphate buffer pH 7.0 and stored at 4 ºC. At indicated times (0, 2 and 24 days) 5 µl aliquots each were removed and used to inoculate 1.0 ml of peptone-glucose media containing either 0% or 0.04% or 0.10% myrrha oil. At the 24-day time point, 5 µl aliquots each were also used to inoculate 1.0 ml of nutrient-free buffer containing either 0% or 0.04% or 0.10% myrrha oil. All tubes were incubated overnight in the shaker at 30 ºC. After overnight (16 hours) growth, serial dilutions of the cells as indicated, were spread on peptone-glucose plates and colonies that grew after overnight incubation were counted. Results are shown in Table 5. As seen in the table, even

after 24 days in nutrient-free buffer, cells immediately emerge from dormancy when transferred to nutrient rich medium and thus are not susceptible to the action of myrrha. However, if myrrha is added to the cells in nutrient-free buffer, all cells are killed.

Nutrient-free buffer needed for initial entry of myrrha into cells

In this experiment, yeast cells were exposed to myrrha for only 10 min either in nutrient-rich medium or in nutrient-free buffer. The myrrha was then removed and the cells were further incubated for one hour in nutrient-free buffer. The purpose of the experiment was to determine if the nutrient-free condition is required only for initial entry of the myrrha into the cell or for further activity of the antibiotic. Yeast cells in 5 mL medium were grown overnight with shaking at 30 ^{o}C. Then, 0.6 ml aliquots of the overnight culture were taken in two Eppendorf tubes, A and B, and centrifuged. The cells in A were resuspended in 60 μl of peptone-glucose medium. Cells in B were washed twice with nutrient-free phosphate buffer and finally resuspended in 60 μl of 0.02M phosphate buffer pH 7.0. From each tube 10 μl aliquots were removed to spread 10^{-5} and 10^{-4} serial dilutions of cells and were considered as zero times. To the remaining 50 μl cells in each tube, 1.5 μl of the 20% myrrha extract was added (final concentration 0.6%). After 10 min at 30 ^{o}C, the two tubes were centrifuged for 1 min, the supernatants were discarded, and the cells in each tube were resuspended in 50 μl phosphate buffer. Both tubes were incubated for 60 min at 30 ^{o}C. Serial dilutions, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2}, of the cells in each tube were spread on the plates. All plates were incubated overnight at 30 ^{o}C and colonies that grew were counted.

The results presented in Table 6 show that preincubation with myrrha in nutrient rich medium, results in very little inhibitory activity against yeast cells even though this was later followed by long incubation in nutrient-free buffer. On the other hand, there was more than 99.9 % decrease in cell viability if the initial preincubation was done in nutrient-free buffer.

Rate of killing of yeast after pre-incubation with myrrha oil extract

The results shown in Table 6 demonstrate that brief preincubation with myrrha oil in nutrient-free medium is essential but does not conclusively prove that further incubation is necessary for more inhibition. A similar experiment was designed to determine the rate of killing after the yeast cells were preincubated with myrrha, which was then subsequently removed. Yeast cells in 5 mL medium were grown overnight with shaking at 30 ^{o}C. Then, 0.6 ml aliquots of the overnight culture were taken in two Eppendorf tubes, A and B, and centrifuged. The cells in A were resuspended in 60 µl of peptone-glucose medium. Cells in B were washed twice with nutrient-free phosphate buffer and finally resuspended in 60 µl of 0.02M phosphate buffer pH 7.0. From each tube 10 µl aliquots were removed to spread 10^{-5} and 10^{-4} serial dilutions of cells and were considered as zero times. To the remaining 50 µl cells in each tube, 1.5 µl of the 20% myrrha extract was added (final concentration 0.6%). After 10 min at 30 ^{o}C, the two tubes were centrifuged for 1 min, the supernatants were discarded, and the cells in each tube were resuspended in 50 µl phosphate buffer. Both tubes were incubated at 30 ^{o}C. Serial dilutions, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2}, of the cell in each tube at indicated times (10, 20, 40, and 60 min) were spread on plates. After overnight incubation at 30 ^{o}C, the colonies that grew were counted.

The results in Table 7 show the effect of preincubation of yeast with myrrha extract. After 10 min of preincubation, the myrrha was removed by centrifugation and the cells were further incubated in nutrient-free phosphate buffer. The results show that it took at least 40 min of further incubation after the 10 min preincubation, both in nutrient-free buffer, to obtain three log units decrease in cell viability. However, if the preincubation was done in nutrient-rich medium, there no further decrease of cell viability upon further incubation in buffer. This suggests that the initial binding of the antibiotic to the cells or its entry into the cells is dependent on the presence or absence of nutrients. Thereafter, the process of killing continues with further incubation.

IV. Discussion

The antibiotics which are in use today were discovered within the first five decades after the discovery of penicillin, the first commercial antibiotic. Many of these antibiotics, which were once considered to be miracle drugs are no longer as effective today. The reason behind this is the inappropriate and over usage of these drugs resulting in the development of mutant bacteria that are resistant to the antibiotics. Another disadvantage of most commercial antibiotics is that they are ineffective against non-growing cells. Inability to kill transiently non-growing bacteria (also known as persisters) is the reason why antibiotics have to be taken for about ten days even though majority of bacteria in an infectioin are killed by the first few doses.

The *Commiphora molmol* is a resinous and aromatic plant naturally grown in India, East Africa and Saudi Arabia and used as a traditional medicine for various problems such as mouth injury, colds and wounds. Earlier study from this lab has shown that myrrha oil has antimicrobial activity on both Gram-positive and Gram-negative bacteria (Bhattacharjee and Alenezi 2020). Myrrha oil has the unique property of preferentially killing non-growing bacteria in nutrient-free buffer. It has weak antibiotic activity in nutrient-rich growth medium. Along with a bacteriostatic antibiotic such as chloramphenicol, myrrha oil is able to kill all cells present in a population even in nutrient-rich growth medium. In this study the antimicrobial activity of myrrha oil against yeast was tested.

The results in Table 1 and 2 show that the MIC of myrrha oil against yeast in nutrient-free buffer is 0.6%, which killed all cells. MIC in nutrient-rich growth medium could not be determined because inhibitory activity is weak. Rate of killing experiment (Table 3 and Figure 1) also showed that myrrha oil is active in nutrient-free buffer, in which all cells were killed in 45 min during which time there was less than one log unit decrease in cell viability in nutrient rich growth medium.

One important property that myrrha oil has is that the active component in it is highly stable to heating at 70 ^{o}C for more than 90 min. Such a property is often found in membrane acting antibiotic. The target site is still unknown but it is believed that the biochemical process taking place in dormant or non-growing cells, may be the bacterial membranes or associated enzymes are involved in the process. Further research is required to study the mechanism of action of myrrha oil. Future studies will also focus on toxicity of myrrha on the host (human) cells. Since myrrha has been widely used for centuries as a traditional medicine suggests that it will not be toxic for the host. Myrrha oil probably contains many compounds. The active component in it is not yet known. More work is needed to identify the active compound in myrrha oil.

Conclusion

The traditional use of the plants for medicinal properties is the basis for deciding which essential oils and plant extracts to investigate further. Previously, many plant oils and extracts, like myrrh, clove, tea tree were in use as topical antiseptics. They were reported to have antimicrobial properties. In this study the extract from myrrha oil was used to study its antibiotic activity both in the presence and absence of nutrients.

The results suggest that the extract from myrrha could be a promising antifungal drug. Its antifungal activity was much higher in nutrient-free buffer than in regular growth medium. This makes it a unique antibiotic that preferentially kills non-growing yeast. In earlier work from this lab it was revealed that myrrha oil preferentially kills non-growing gram-positive or gram-negative bacteria and no resistant mutant of *E. coli* could be obtained even after repeated passages of the bacteria.

For future studies it is necessary to isolate and identify the active ingredient responsible for antifungal activity in myrrha extract. It is possible that there may be more than one active ingredients in the plant extract. Their relative activities and possible synergistic interaction between them can be investigated.

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Table 1: Minimum inhibitory concentration of myrrha oil in peptone-glucose media.

Oil concentration %	Number of viable cells (CFU/ml)				
	(10^3/ml)	(10^4/ml)	(10^5/ml)	(10^6/ml)	Weighted average (10^6 CFU/ml)
0	-	-	178	15	17.5
0.2	-	-	210	-	21.0
0.4	1672	-	156	-	1.8
0.6	1435	-	128	-	1.4
0.8	1717	951	-	-	2.4
1.0	-	652	-	-	6.5

Table 2: Minimum inhibitory concentration of myrrha oil in phosphate buffer.

Oil concentration %	Number of viable cells (CFU/ml)			
	10^3/ml	10^5/ml	10^6/ml	Weighted average (10^6 CFU/ml)
0.0	-	77	14	8.3
0.2	1060	37	-	1.1
0.4	447	7	-	0.45
0.6	0	-	-	0.00
0.8	0	-	-	0.00
1.0	0	-	-	0.00

Table 3. Rate of killing of *Saccharomyces cerevisiae* by 0.6% Myrrha oil. A. In nutrient-rich peptone-glucose medium. B. In nutrient-free phosphate buffer. (*) The lower limit of detection was <100 CFU/ml because 10^{-2} was the lowest dilution that was spread.

A	**Without Myrrha Oil**					**With 0.6% Myrrha Oil**				
Time (min)	**CFU/ml (10^3/ml)**	**CFU/ml (10^5/ml)**	**Weighted Average CFU/ml**	**Log Average CFU/ml**	**Survival (%)**	**CFU/ml (10^3/ml)**	**CFU/ml (10^5/ml)**	**Weighted Average CFU/ml**	**Log Average CFU/ml**	**Survival** (%)
0	1100	76	1164,000	6.07	100	-	-	1164,000	6.07	100
15	1000		1000,000	6.00	86	590	29	613,000	5.79	53
25	960	89	1039,000	6.02	89	163	0	163,000	5.21	14
45	1080	78	1147,000	6.06	99	150	0	150,000	5.18	13
60	800	101	892,000	5.95	77	42	0	42,000	4.62	3.6
120	790	70	851,000	5.93	73	10	0	10,000	4.00	0.9

B	**Without Myrrha Oil**					**With 0.6% Myrrha Oil**				
Time (min)	**CFU/ml (10^3/ml)**	**CFU/ml (10^5/ml)**	**Weighted Average CFU/ml**	**Log Average CFU/ml**	**Survival (%)**	**CFU/ml (10^2/ml)**	**CFU/ml (10^3/ml)**	**Weighted Average CFU/ml**	**Log Average CFU/ml**	**Survival** (%)
0	841	76	908,000	5.96	100	-	-	908,000	5.96	100
15	568	64	626,000	5.80	70	84	0	8,400	4.99	0.9
25	800	82	873,000	5.94	96	7	0	700	2.84	0.08
45	657	112	761,000	5.88	84	0	0	< 100*	<2.0	0.0
60	1180	78	1,245,000	6.10	137	0	0	< 100	<2.0	0.0
120	800	61	852,000	5.93	94	0	0	< 100	<2.0	0.0

Table 4: Heat stability of the antibiotic component in myrrha oil

[Myrrha]	Pre-heating of myrrha at 70 °C (min)	CFU 10^2/ml	CFU 10^3/ml	CFU 10^4/ml	CFU 10^5/ml	Weighted Average CFU/mL	Log Average CFU/ml
0				1101	165	11,510,000	7.06
0.6 %	0	0	0			< 100*	< 2.0
0.6 %	15	0	0			< 100	< 2.0
0.6 %	30	0	0			< 100	< 2.0
0.6 %	60	0	0			< 100	< 2.0
0.6 %	90	0	0			< 100	< 2.0

(*) The lower limit of detection was <100 CFU/ml because 10^{-2} was the lowest dilution that was spread.

Table 5. Effect of myrrha oil on yeast cells emerging from dormancy.

In Buffer at 4 °C	Growth condition at 37°C	Number of viable cells (CFU/ml)			Weighted Average (10^6 CFU/ml)	% Viability
		(10^3/ml)	(10^4/ml)	(10^5/ml)		
0 days	medium + 0.0% Myrrha		740	220	8,727,000	6.94
	medium + 0.04% Myrrha	1260	745		1,823,000	6.26
	medium + 0.01% Myrrha	923	663		1,586,000	6.20
2 days	medium + 0.0% Myrrha		1130	990	19,270,000	7.28
	medium + 0.04% Myrrha	1145	1065		2,009,000	6.30
	medium + 0.01% Myrrha	1215	890		1,914,000	6.28
24 days	medium + 0.0% Myrrha		1280	432	15,560,000	7.19
	medium + 0.04% Myrrha	1423	844		2,061,000	6.31
	medium + 0.01% Myrrha	1194	802		1,815,000	6.26
24 days	buffer + 0.0% Myrrha		73	11	760,000	4.88
	buffer + 0.04% Myrrha	0			< 1000	< 3.00
	buffer + 0.01% Myrrha	0			< 1000	< 3.00

Table 6. Nutrient-free condition is required only for initial entry of the myrrha into yeast cells

Cell Treatment Conditions	Preincubation With Myrrha	Incubation Without Myrrha	CFU 10^2/ml	CFU 10^3/ml	CFU 10^4/ml	CFU 10^5/ml	Weighted Average CFU/ml	Log Average CFU
In nutrient-rich medium	none	none			1832	558	21,730,000	7.34
	10 min in medium	60 min in buffer		1360	880	393	2,372,000	6.38
In nutrient-free buffer	none	none			1405	667	18,840,000	7.28
	10 min in buffer	60 min in buffer	60	9	0	0	6273	3.80

Table 7: Rate of killing of yeast after pre-incubation with myrrha oil extract

Cell Treatment Conditions	Preincubation With Myrrha	Incubation in Buffer Without Myrrha	CFU 10^2/ml	CFU 10^3/ml	CFU 10^4/ml	CFU 10^5/ml	Weighted Average CFU/ml	Log Average CFU
In nutrient-rich medium	none	none			1940	737	24,336,000	7.4
	10 min in medium	10 min		1722	985	692	3,062,000	6.5
		20 min		1985	1105	602	3,326,000	6.5
		40 min		1736	1233	588	3,204,000	6.5
		60 min		1315	840	358	2,264,000	6.4
In nutrient-free buffer	none	none			1565	650	20,136,000	7.3
	10 min in buffer	10 min	552	126	83	0	68,600	4.8
		20 min	189	62	23	0	24,700	4.4
		40 min	92	19	0	0	10,100	4.0
		60 min	75	8	0	0	7,550	3.9

Fig. 1. Rate of killing of *Saccharomyces cerevisiae* in presence (filled symbols) or absence (open symbols) of 0.6% Myrrha oil in nutrient rich peptone-glucose medium (squares) or nutrient-free phosphate buffer (circles).