Potential Effect of Natural Musk and Probiotic on Some Pathogens Strain

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This study investigates the isolation and identification of beneficial bacterial strains from Musk. Also, the antagonistic effect of different natural animal Musk concentrations and the filtrates produced from the growth of bacterial which isolated from it on some pathogenic microorganisms was studied. Five isolates were selected and identified, namely *Lactobacillus acidophilus* (a_1), *Lactobacillus acidophilus* (a_2), *Lactococcus lactis sub sp lactis* (b_1), *Lactococcus lactis sub sp lactis* (b_2) and *Streptococcus thermophilus*. Results also indicated that Musk extract and filtrates of its strains growth has an inhibition and bactericidal effects on the growth of some pathogenic microorganisms such as *Staphylococcus aureus* and *Penicillium puberulum* fungus. Musk itself is more effective on the growth of pathogens than the effect of strains isolated from it. Antagonistic effect of *P.puberulum* showed more pronounced inhibitory effect than *S. aureus*. Results also showed that the filtrates have the ability to inhibit the growth of tested microorganisms.

**Keywords:** Natural animal Musk, Antagonism, Pathogens, bactericidal, inhibition.

**INTRODUCTION**

Animal Musk is one of the natural substances. Many investigations were carried out to study the use of natural sources to inhibit the growth of many pathogenic microorganisms for human, animals and plants and to consolidate the idea of “return to natureMusk effectiveness as antibiotic for some pathogenic fungi as *Aspergillus niger*, *Microsporum conis* and *Trichophyton rubrum* as well as its effect on *Candida albicans* is demonstrated (saddiq, 2004). In her applied study to determine the effect of natural Musk on human lung cells cancer, Saddiq (2008) reported that Musk extract has an effective role in destroying a high level of cancer infected tumor cells using tissue culture tests. She also indicated that treatment with Musk extract and Seder is highly effective in growth inhibition and reducing the biomass of *Aspergillus flavus* pathogenic fungus, that produce Aflatoxin resulting various hazards for bio tissues as liver toxicity. Musk also proved more effective against the tested fungi more than Nystatin antibiotic (Saddiq and El-Eliany, 2009).

*Staphylococcus aureus* is one of the dangerous pathogenic bacteria that lived mainly in nasal membranes and skins of human or animals causing inflammations, blisters, sources as well as complication of acute wounds and burns resulting in inflammation of urinary tract, ear, eye and blood sepsis (Chateau, 2004).

*Pseudomonas aerogenosa*, is one of the opportunistic pathogens to human and cause infections at hospitals. It is also responsible for chronic pulmonary disease in individuals suffering from cystic fibrosis, this bacterium resist antibiotics (Jander *et al*, 2000)Individuals are also exposed to Aflatoxins either directly or indirectly as a result of growth of toxin – producing fungi. Most of carcinogenic mycotoxins are B_1, B_2, G_1 and G_2 which produced by *A. flavus*, *A. parasiticus* and *Penicillium puberulum* that grow on wheat grains, peanuts, favabeans and other different food stuffs (Abdel-Hamid , 2000).Some of yeasts also cause skin lesions such as *Candida albicans* which live and found in gut, mouth and vagina and cause diseases in case of natural immune deficiency. It also infects nails and changes its color to yellowish-green or dark (Reynolds *et al*, 1996)Chemical treatment of these diseases by antibiotics have its side effects on human health, in addition of possible development of resistant microbial strains which may cause various problems (Black, 1996). Elewski (2000)
studied the side effects of Griseoflavin, Ketocanazol and Itraconazole drugs which used for diseased skin treatment. These drugs cause headache, nausea, vomiting and other side effects on muscular, nervous, digestive and reproductive systems. The aim of this study is to investigate the antagonistic effect of Musk and its filtrates, as a natural source treatment. Also, this study investigates the isolation and identification of beneficial bacterial strains from Musk and investigates inhibition effect on the growth of pathogenic bacteria.

MATERIALS AND METHODS

Materials

Musk

Natural Musk (animal Musk) used in this study was extracted from navel. (Ghazal) as a powder as described by Saddiq, (2004).

Microorganism strains

Pathogenic bacteria

Staphylococcus aureus ATCC 6538, Pseudomonas aeruginosa ATCC 9027 and Bacillus subtilis ATCC 6633 were obtained from Microbiology laboratory, National Research Center, Cairo, Egypt.

Pathogenic Fungi

Penicillium puberulum and Candida albicans was obtained from Cairo MIRCEN, Faculty of Agriculture, Ain Shams university, Egypt.

Media

Ready to use media, i.e. Nutrient agar for enrichment of pathogenic bacteria; MRS (Oxoid) agar. Anaerobic conditions were adapted using anaerobic Jar and anaerobic gas generating kits from Oxoid, Hampshire, England. Pre-prepared Sabouraud Dextrose agar (SDA) was also obtained from Sigma Co. (Oxoid CM41 media) for enrichment of Fungi.

Methods

Physical examination of natural Musk extracts:

Aqueous animal Musk extracted from natural animal Musk powder were examined for color, appearance, Texture and smell. Aqueous Musk extract was prepared from the natural animal black Musk powder at 1.0% concentration. After 24 hrs, extract was filtered and stored in dark glass bottles at 5°C until use (Saddiq, 2004).

Isolation and Identification of bacteria from Musk extract

Isolation of bacteria by pour and streak plates was applied according to Collee et al, (1989). 1 ml sample was taken aseptically from natural Musk. It was transferred to 9 ml of sterile buffered peptone water (BPW). Five 10-fold dilutions of the homogenates were then prepared and these were inoculated on plates of Nutrient media, MRS agar (Oxoid) (De Man et al., 1960) and MRS supplemented with 0.05% L-cysteine HC1 (Sigma Chemical Co., St. Louis, Mo), acidified with glacial acetic acid to pH 5.7 or M17 and incubated aerobically and an aerobically using Gen Kits in Oxoid jar for 48 h at 37°C. Colonies with typical characteristics were randomly selected from plates and tested for Gram stain, cell morphology, and catalase reaction (Harrigan and McCance, 1990). During test the cultures were kept in MRS agar plus 0.05% L-cysteine HC1 at refrigerator temperature. Identification was done according to Bergy’s Manual of Determinative Bacteriology (Holt et al, 1994) and fermentation processes for different sugars for isolates using API-50CH strips and API-20 CH strips (Bio. Merieux).

Preparation of broth filtrates from the growth of bacteria isolated from Musk

Fresh culture of strains isolated from Musk were enriched in specific media broth for each strain and incubated at aerobic or anaerobic condition at 37± 2°C for 24 hrs. After incubation, a cell-free solution was obtained by centrifuging (6000 x g for 15 min) the culture, followed by filtration of the supernatant through a 0.2 µm pore size (Schleicher & Schuell, Germany) cellulose acetate filter. Some supernatants were neutralized with 1 N NaOH to pH 6.5, and the inhibitory effect of the hydrogen peroxide was eliminated by the addition of catalase (5 mg/ml). Un-neutralized (general inhibitory effect) and neutralized (bacteriocin and bacteriocin-like metabolites) supernatants of the strains were checked for antibacterial activity against pathogenic bacteria in inoculated nutrient agar (Schillinger & Lucke 1989 and Reinheimer et al. 1990). Once solidified, the dishes were stored for 2 hrs in a refrigerator.
Antagonistic tests

**Antagonistic effect of Musk concentrations on bacteria and Fungi:**

Antagonistic effect of Musk concentrations on the growth of some pathogenic microorganisms was carried out. Diffusion in solid media method according to Collins (1989) was used for *Staphylococcus aureus*. O.D was measured colorimetry using spectrophotometer (Spectro, labomed, Inc) at 620 mm. Sabouraud Dextrose agar was used to study the Antagonistic effect on *Penicillium puberulum*. Inhibition zones were measured compared to a control sample.

**Antagonistic effect of the isolated strains from Musk:**

Five isolates from bacterial strains isolated from Musk were selected to study its antagonistic effect, depending on its ability to inhibit the tested pathogen, *S. aureus*. Antagonistic experiments were carried out using filtrates of isolates of some pathogens as: *S.aureus* ATCC 6538, *Pseudomonas aeroginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633 and *Candia albicans* yeast. Antagonistic effect was determined according to Savadogo *et al* (2004) and Girum *et al*, (2005) using disc diffusion filter papers. Results recorded by measuring growth inhibition zone for tested bacteria using Varnier Caliper unit. These isolates were identified at microbiology lab, National Research Centre, Cairo, Egypt.

**Statistical study**

To test the effect of different treatments, statistical program "Spsspc" was used to apply T-test (Abu-zeid, 2003).

**RESULTS**

**Physical examination of natural Musk extract:**

Laboratory examination of Musk extract showed that it is found to be black to brown color, odorless, viscous and tends to be almost free of apparent impurities.

**Isolation of microorganisms from Musk extract using pour-plate and streak-plate methods.**

Results of microorganisms isolation experiments on Nutrient media by pour and streak plate methods revealed that natural Musk contain a great number of bacterial strains depending on media types. It was found that MRS agar is the best media for enrichment of greater number of bacterial strains compared to Nutrient agar. The number of colonies reached 350 isolates differs in morphological characteristics. Five isolates were selected and were identified at Microbiology Lab., National Research center as follows:

1. *Lactobacillus acidophilus* (a<sub>1</sub>).
2. *Lactobacillus acidophilus* (a<sub>2</sub>).
3. *Lactococcus lactis sub sp lactis* (b<sub>1</sub>).
4. *Lactococcus lactis sub sp lactis* (b<sub>2</sub>).
5. *Streptococcus thermophilus*.

**Morphological and biochemical tests for isolated bacterial strains:**

Table (1) shows results of some morphological and biochemical tests for selected bacterial isolates. Data for all isolates was found to be gram positive. *Lactobacillus acidophilus* appeared as rods in individuals or pairs.

Both of *Lactococcus lactis sub sp lactis* (b<sub>1</sub>) and *Lactococcus lactis sub sp lactis* (b<sub>2</sub>) appeared as cocci single or in pairs *Streptococcus thermophilus* was Cocci single or in pairs. Identification was done according to Bergy’s Manual of Determinative Bacteriology (Holt *et al*., 1994) and fermentation processes for different sugars for isolates using API-50CH strips and API-20 CH strips (Bio. Merieux).

**Antagonistic effect of Musk concentrations on *Staphylococcus aureus* and *Penicillium puberulum*:**

Antagonistic effect of *Penicillium puberulum* showed more pronounced inhibitory effect, than *S. aureus*. Clear halos were formed around the hole containing Musk extract. Inhibitory zone diameter was 22.76 mm compared to zero for control sample, after 5 days of growth at the same concentration as shown in (Table 2 & Figure 2).

**Antagonistic activity media of filtrates from the growth of bacteria isolated from Musk on some pathogens.**

The purpose of this experiment is to study the ability of isolated strains from Musk to produce anti-growth substances in their growth media that may inhibit the growth of the test pathogens distributed on solid media as each of *S. aureus* ATCC 6538, *Pseudomonas aeroginosa* ATCC 9027 and *Bacillus subtilis* ATCC 6633 as well as *Candia albicans* yeast. Disks of filter papers saturated with filtrates from isolated strains were added. Results showed that the filtrates have the ability to inhibit the growth of tested microorganisms. Inhibition clear zones were observed around the disks in different areas according to isolated strain type from Musk.
Table (1): Identification, morphological, physiological and biochemical properties of strains isolated from natural musk

<table>
<thead>
<tr>
<th>Identification</th>
<th>Cellmorphology</th>
<th>Gram stain</th>
<th>Catalasea ctivi</th>
<th>Methyl Red</th>
<th>Movemen t</th>
<th>Gelatine test</th>
<th>Ureates t</th>
<th>Oxidase test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus acidophilus (a₁)</td>
<td>Rods</td>
<td>+</td>
<td>_   +</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Lactobacillus acidophilus (a₂)</td>
<td>single / in pairs</td>
<td>+</td>
<td>_   +</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Lactococcus lactis sub sp. lactis (b₁)</td>
<td>Cocci single / in pairs</td>
<td>+</td>
<td>_   _</td>
<td>_</td>
<td>+</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Lactococcus lactis sub sp. Lactis (b₂)</td>
<td>+</td>
<td>_   _</td>
<td>_</td>
<td>+</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Streptococcus thermophilus</td>
<td>Cocci single / in pairs</td>
<td>+</td>
<td>_   _</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
</tbody>
</table>

+ : positive reaction, - : negative reaction

Identification according to their morphological, cultural, physiological and biochemical characteristics as described in Bergy's Manual of Determinative Bacteriology (1994), fermentation of different sugars, done by the identified isolates, was determined by API-50 CH strips and API-20 CH strips (BioMerieux)

Table (2): Effect of different concentrations of musk on the growth of Staphylococcus aureus and Penicillum puberulum on MRS agar

<table>
<thead>
<tr>
<th>Concentrations %</th>
<th>Inhibition zone diameter ( mm ), SE ( Average )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>0.32**±1.21</td>
</tr>
<tr>
<td>10</td>
<td>0.40**±2.02</td>
</tr>
<tr>
<td>15</td>
<td>0.32 **±2.95</td>
</tr>
<tr>
<td>20</td>
<td>1.00±4.18</td>
</tr>
<tr>
<td>25</td>
<td>0.50**±6.07</td>
</tr>
</tbody>
</table>

significant at 1% ** significant at 5%

Figure (1): Effect of different concentrations of musk on the growth of Staphylococcus aureus and Penicillum puberulum on MRS agar
Fig. (2): Antagonistic effect of filtrates from strains of bacteria isolated from natural Musk on some pathogenic microorganisms on MRS agar.

Table (3): Antagonistic effect of filtrates from strains of bacteria isolated from natural Musk on some pathogenic microorganisms on MRS agar.

<table>
<thead>
<tr>
<th>Filtrates from isolated strains</th>
<th>Inhibition zones diameter (mm) for pathogens (Average ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>Lactobacillus acidophilus(a1)</td>
<td>0.141±2.50</td>
</tr>
<tr>
<td>Lactobacillus acidophilus(a2)</td>
<td>0.252±1.73</td>
</tr>
<tr>
<td>Lactococcus lactis sub sp. lactis(b1)</td>
<td>0.153±6.81</td>
</tr>
<tr>
<td>Lactococcus lactis sub sp. lactis(b2)</td>
<td>0.152±4.95</td>
</tr>
<tr>
<td>Streptococcus thermophilus</td>
<td>0.310±3.13</td>
</tr>
</tbody>
</table>

Table (3) shows that the greatest area of inhibition was observed for S. aureus pathogen when using the filtrates from the growth of Lactobacillus acidophilus (b1) and Lactobacillus acidophilus (b2). Also, the least inhibition zone diameter was observed for Lactococcus lactis sub sp lactis (a1). However, the greatest inhibition zone for P. aeruginosa was observed when using filtrate from the growth of Lactococcus lactis sub sp lactis (a1), being 5.53 mm compared to the other strains. For B. subtilis, it could be observed that Lactococcus lactis sub sp. Lactis (b2) showed an inhibition zone of 5.23 mm. The effect of Lactobacillus acidophilus (a2) was observed in inhibition the growth of Candida albicans, values being 6.11 mm compared to 0.987 mm for Str. thermophilus as shown in fig. (2) Photo(1).

**DISCUSSION**

This study is carried out to enhance the inhibition ability of natural Musk and evaluate the antagonistic effect of different natural animal Musk concentrations and the filtrates produced from the growth of bacterial isolates on some pathogenic microorganisms.

Several chemical compounds where manufactured as Musk alternatives have Musk like smell but differ in chemical formula of Musk. Musk xylene, Musk ambrette and Musk ketone, used in aromatic industries, considered the most similar to natural Musk. However, Musk xylene is used in large scale in pharmaceutical products, dyes, pesticides, household products and personal care products (Mottaleb et al, 2004), Lois et al .,(1999) studied...
the harmful effect of the chemical compounds of Musk artificial alternatives as Musk xylene and Musk ketone on liver enzymes of rats (Cytochrome p. 4.50 enzymes) using oral (mouth) injection of the two compounds in different doses (10-200 mg/kg) for 7 days. This treatment led to activation of CYP1A, 3 A and 2B Values amounted 2-4 folds of control sample with a small increase in immune proteins. Also, Lois et al., (2002) noticed that the high dose of Musk xylene (200 mg/kg) resulted in an increase of rat liver weight by 65% and an increase in the secretion of P-450 enzyme by two folds as well as an increase in protein content compared to the control sample. This indicates to the importance of using natural sources with the same composition and from its origin source to avoid any side effects resulting from artificial chemical compounds, this is supported by alternative medicine.

Table (2) shows the inhibitory effect of Musk extract on the growth of S. aureus and P. pseudomonas aeruginosa ATCC9027 pathogens. Results indicated that Musk extract is more effective on the tested fungus as the diameter of inhibition zone reached 22.76 mm compared to 6.07 mm for S. aureus at 25% Musk extract concentration. The inhibitory effect of natural Musk is due to its content of compounds and metabolic products such as alkaloids, flavonoids, sterols and antibiotics. These compounds may affect bacterial and fungal cells through increasing the permeability of cell membranes resulting in leakage of cell important contents led to cells analyzing and death. Also, Musk extract compounds may inhibit the microorganisms through inhibiting the synthesis of nucleic acids resulting in formation of abnormal proteins (Kobayashi and Meddof, 1977). However, its inhibitory effect may be due to the presence of volatile oils. In addition, this study stated that Musk contains beneficial microorganisms such as Lactobacillus acidophilus (a1,a2), L. lactis subsp. lactis (b1 & b2) and Str. thermophilus that have a antagonistic effect of these filtrates may be due to Bacteriociens. Bacteriociens are proteins or peptides or lipopeptides or carbohydrate proteins produced by lactic acid bacteria that destroy other microorganisms (Farkas-Himsley, 1980; Konisky, 1982; Klaenhammer, 1988; Devicent et al, 1990, and Ohmomo et al., 2000). Bacteriociens are also produced in animal intestine and have an inhibiting effect against gaseous spors (Corret et al., 2007). They reported also that L. lactis Sub sp. lactis have the possibility of producing Niacin, recognized as food preservative.

Isolates obtained during this study has shown its ability to inhibit the growth of pathogenic S. aureus. However, inhibition of bacteria and yeast was determined using diffusion method (Arici et al, 2004). Results were in agreement to Rays and Mehanna (2007) that the inhibitory effect may be due to the produced substance such as acetic acid, lactic acid and little formic acid through reducing the media pH which inhibit the growth of pathogenic microorganisms (Rasic, 1980). Our results were in agreement with Hawley (2006) who studied the effectiveness of L. acidophilus against the growth of Enterobacter sakazakii in infant milk, inhibition zone reached 20-30 mm. El-Sadik et al (2007) reported that lactic acid bacteria strains (Str. thermophilus, L.acidophilus and L.casei) have the efficiency to inhibit pseudomonas aeruginosa contaminated some dairy products. Also in accordance to Sheikh-Youssef et al, (2008) who indicated the capability of Bifidobacterium to produce Bacteriocienes that compete with pathogens and prevent its sticking on epithelial cells of intestinal surface. Lactic acid bacteria is widely used in food preservation through its secretion of inhibitory substances such as lactic acid, acetic acid, hydrogen peroxide, ethanol, diacetyl, Bacteriociene-like compounds and antibiotic-like substances as well as Bacteriociens that inhibit pathogenic microorganisms (Alhashidi and Al-Zenki, 2008).

Finally, it could recommend inviting scientists to carry out further studies on the chromosomal abnormalities assay (CA) to know the antagonistic effect of Musk microorganisms and its preventive effect against genotoxicity of chemicals.
ACKNOWLEDGMENT

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