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Research Paper

Evaluating the Anti Microbial Effects of the Extract from 3 Plants (Thyme, Pennyroyal, Savory) On Spore Growth, Spore Elimination and Growth of Bacillus Cereus

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Abstract : *The increased attention toward replacing chemical antimicrobials and anti oxidants with natural remedies has led to increased studies about plant derivatives and seeking natural plant extracts, in order to find a proper and natural substitute for chemical preservatives. To prove the preservative and anti-microbial characteristics of plant extracts, their effect on infectious bacteria and food decay has to be studied in laboratory and then real settings. In this study the effect of local Thyme, Pennyroyal and Savory in Qom Province on inhibition of Bacillus Cereus in nutrient broth environments with different essence concentrations (0.4, 0.8, 1.5, 2.5, 3, 3.5 and 4.5 µgr/ml) in different time intervals and different temperatures was evaluated. The percentage of grown and inhibited spores of this microorganism was determined by a hemocytometer with specific spore dyeing. Also the bacteria growth and the cell deformation was studied by a optic microscope and photos. The analysis of the three plants (Thyme, Pennyroyal and Savory) in this study was done by GC/MS. The extracts of Thyme and Savory in 37 degrees and in 0.4 and 0.8 µgr/ml concentration had a minimum inhibitory concentration (MIC) equal to 3 µgr/ml in inhibiting the growth of Bacillus Cereus, but had a MIC=3 µgr/ml on the growth and spore production of these bacteria after 48 hours. As can be seen in the extracts studied, the more deterrent summer savory and thyme, and oregano extracts are then.*

Keywords: Spore, Extract, Thyme, Pennyroyal, Savory, Bacillus Cereus, Minimum Inhibitory Concentration (MIC).

Introduction

In the recent two decades there has been a major application of natural food preservatives and flavors in food products, this was after recognizing the harmful effects of using chemical preservatives. It seems like natural preservatives can be used as an efficient way for preserving food for long time periods [1]. Microbial and fungal elimination especially their resistant forms has specific importance in taking care of food products. Due to the increasing limitation of using chemical antimicrobials such as their side effects and inducing resistance in bacteria and the necessity to replace chemicals with natural products from inside the country, we do hope that plant extracts and essences can be studied as replacements for chemicals in food preservation and controlling human and animal diseases. Bacillus cereus is an aerobic and optional anaerobic bacterium and has several vegetative forms and spores. This bacterium can be seen in raw and processed meat, vegetables, rice and dairy products and is one of the factors leading to food intoxication [2-5]. Another study done in 2007 by *Ahmet et al*,[6] the antibacterial effect of the essence and methanol extract of Savory on the bacteria and fungi with a food origin was evaluated. The oily, volatile extracts of this type of Savory was analyzed, this essence was active against 25 bacteria, 8 fungi and one yeast (Candida Albicans) [6]. In 2000, *Haji Akhoondi et al.* conducted biological and chemical studies on the essence of one species of pennyroyal and determined the effect of its volatile oil on 25 species of Anopheles and Artemia Salina Larvae. The rate of mortality after 24 hours was

determined [7]. In another study by *Akhondzadeh et al* in 2005, the effect of the volatile oil from the Shirazi Thyme on the growth of B. cereus in the brain and heart broth was evaluated. According to the results, the logarithm of the growth probability percent of B. Cereus with a small increase in the concentration of the essence decreased and the essence had a significant growth prohibition effect in the small concentration studied and this effect significantly increased when temperature decreased [8]. In the recent study we try to evaluate the extracts of herbal plants such as pennyroyal, thyme, and savory on resistant bacillus cereus and its ability to stop the growth of these bacteria, in order to find a new method to solve the problem of spores in medicine and also to prevent food intoxication in food industries.

Material and Methods

The microorganism species of B, Cereus (PRTCC1015) was ordered from the Razi Serum Institute. The herbal plant of central Iran (Vasf and Anjeeleh in Qom Province) including pennyroyal and thyme were collected in spring. Savory which is an edible plant and is planted by farmers was also provided. The plant identities were confirmed by the Herbarium Institute of the Qom Province Forestry and Natural Resources. Then the plants were dried and grinded by an electrical mill. 50 grams of the dried plant powder with 700 cc of distilled water was poured in a balloon and essence extraction was done for 3 hours in the Clunger Machine with distillation speed of 1cc per minute [6, 9-10]. The isolation of the bacillus cereus spores was done on fortified nutrient agar (FNA). In order to do this the Roux bottles each containing 100cc of FNA were inoculated superficially with 0.5 cc of a 24 hours culture of B. cereus in nutrient broth. After incubating in 32° C for 3 days, more than 90% of spores were produced, were identified by Malachite Green coloring and then the spores were washed by sterile two-times distilled water and then it was densified by 4 times centrifuging through 2500 g rounds per minute for 15 minutes with sterile distillated water. Then it was stored with distillated water in 4°C [11]. The spore suspension was activated in phosphate buffer by warming in a 70° C water bath for 20 minutes. Parts of this activated by heat suspension was diluted with a fresh buffer in a way that its optical density in 610 nm was equal to 1. 100 ml of this solution was transferred to the examining tubes with different densities of the essence (0.4, 0.8, 1.5, 2.5, 3, 3.5 and 4.5 microgram per milliliter) in the basic trypticase soy broth (TSB) environment. The control sample including 100 cc of basic environment and 100 micro liters of activated spore suspension was without extract [12]. The rate of spore growth in the control sample after 8 hours was more than 90% and was studied through Wirtz-Conklin dyeing and microscopic examination. Then each of the test tubes was studied with a certain concentration of extract and the percent of grown spores was counted by a hemacytometer. This test was done in the basic spore producing FNB environment with different extract concentrations in exam tubes and by adding 100 micro liter of the bacteria suspension with an opacity of 1 McFarland. The control exam tube was without extract. Bacteria spore growth was reported after 48,72 and 96 hours microscopically and by counting by a hematocytometer and by spore dyeing.

Results and Discussion

The analysis of the three plants (thyme, pennyroyal and savory) essences in this study was done by the GC/MS system and the molecular weight percent and the prevention time of each combination was determined in the GC/MS system[13-15] and some of these chemicals and their frequencies have been listed in table 1.

Table 1
The percent of chemicals in Thyme, Pennyroyal and Savory

Thyme		Pennyroyal		Savory	
Combination	**Percentage frequency**	**Combination**	**Percentage frequency**	**Combination**	**Percentage frequency**
Thymol	37	polygon	13	Btastyruzul	35
Bozneoul	8	mantole	11	ursolic acid	37
Parasymn	7	de polygon	8	Thymol	7.2
Gamatzynyn	4	linalool	7	Oleanolic acid	4.3
Alpha Pnyn	3	limonene	6.8	Caryophyllene acid	2

Table 2
The growth rate of the B. cereus spores encountered with different doses of 3 plant extracts for 4 hours in the TSB environment

The amount of Essence µg/ml / **Essence sample**	**Temperature** C^0	0.4	0.8	1.5	2.5	3	3.5	4.5
Thyme		90	70	10	5	MIC	-	-

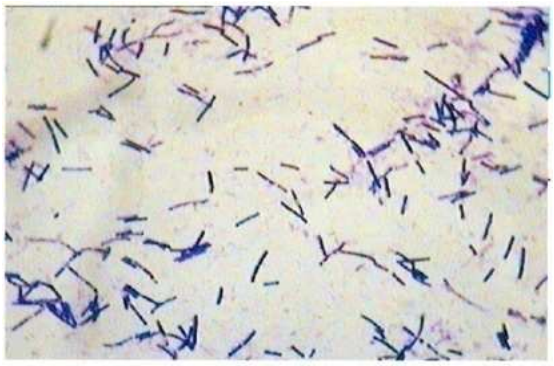
Pennyroyal	37	96	90	85	30	12	2	MIC
Savory		60	50	20	MIC	-	-	-
Thyme	10	64	45	MIC	-	-	-	-
Pennyroyal		72	70	40	5	MIC	-	-
Savory		34	22	MIC	-	-	-	-

Table 3
The percent of spore production and B. cereus growth

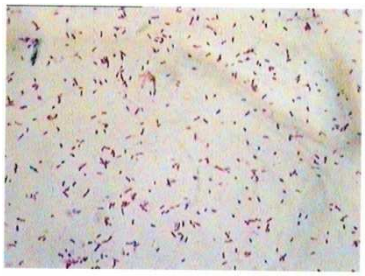
The amount of Essence µg/ml / **Essence sample**	0.4	0.8	1.5	2.5	3.5	4.5	Times h
Thyme	5	0	0	0	MIC	-	48
	7	2	0	0	-	-	72
	7	4	0	0	-	-	96
Pennyroyal	7	4	0	0	0	MIC	48
	10	4	0	0	0	-	72
	10	3	0	0	0	-	96
Savory	5	0	0	0	MIC	-	48
	5	0	0	0	-	-	72
	7	0	0	0	-	-	96

Table 3 shows the percent of spore production and growth of B. cereus bacteria after 48, 72 and 96 hours in the Fortified Nutrient spore producing Broth (FNB) in different concentrations of the plant extract. In the control sample without extract after 48 hours 14% , after 72 hours 30% and after 96 hours more than 50% spore production was seen. All of the exam tubes were observed in 8 hours intervals in certain temperature and pH and by Wirtz-Conklin dyeing the number of spores and grown cells were observed and counted. Microscopic examination of B. cereus colored spores by the Wirtz-Conklin method showed that the spores of this bacillus in water until 24 hours keep their green-blue appearance. In TSB after 4 hours most of these spores grow and create pink bacilli. Figure 1 (a and b) show the growth rate and spore production of the B. cereus cell in different concentrations of thyme extract (0.4 and 0.8 micro gram per milliliter respectively).

Witness



a



B

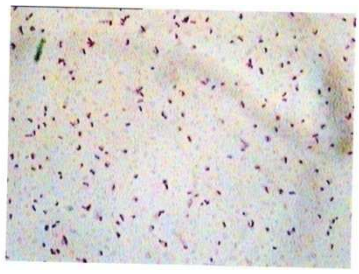


Figure 1: the growth rate and spore production of the B. cereus cell in different concentrations of thyme extract (0.4 and 0.8 micro gram per milliliter respectively)

In the control sample the formation of typical grown bacteria in the FNB environment without extract after 48 hours was seen. In these examinations a direct relation between cell growth and spore production in the cells was observed. Plant extracts from the beginning effected cell growth and cell deformation from chain to mono cellular and short. Eventually by reduced cell

growth, the spore production decreased. This effect was seen in pennyroyal and savory extracts. However in pennyroyal until the concentration of 0.8 microgram per milliliter the chain forms were still seen, but they were eliminated in the concentration of 1.5 microgram per milliliter.

Plant extracts are one of the potential sources of antibacterial compounds, and therefore are very effective and beneficial. In the recent study the effect of different extracts of some regional herbal plants from the central part of Iran, including thyme, pennyroyal and savory on the formation and growth of B. cereus spores in experimental conditions was evaluated. The rate of B. cereus spore production and the morphology of microorganisms in different concentration of three different herbal plants were studied. The spore production of B. cereus in the FNB spore producing environment after 48, 72 and 96 hours along with spore dyeing and their counts were studied. In this test the growth rate of B. cereus in different concentrations of three plant extracts was gradually decreased. But the effect of the extracts of these three plants was different. For example, growth MIC when encountered with Thyme and Savory was 3.5 and for pennyroyal was 4.5 micro grams per milliliter. The rate of spore production gradually decreased in high concentration of all 3 extracts and the form of cells changed from chain to singular, diploid and small sizes.

The percentage of bacteria spore production for all 3 extracts in different concentration after 96 hours has been shown, and after 48 hours in the concentration of 1.5 micro grams per milliliter of all 3 extracts no spore production was seen. However the effect of the Savory and Thyme extracts on spore production was more than Pennyroyal. A similar study by *Milosevic* [16] about the bacteriocidal effect of the Thyme Revolatus essence on gram positive (Staphylococous Aureus) and gram negative (Escherichia Coli) bacteria by the disc perforation method showed the strong bacteriocidal effect of the essence under study due to the high Carvacrol available in the essence, which was similar to other studies. Similar results by *Jensen* were seen when examining the effect of the extract of thyme and Carvacrol and Timol related chemicals on the Shigella Sonnei and Shigella Flexeneri bacteria [17].

In another study by *Akhondzadeh* et al in 2005[8], the effect of the volatile oil from the Shirazi Thyme on the growth of B. cereus in the brain and heart broth was evaluated. According to the results, the logarithm of the growth probability percent of B. Cereus with a small increase in the concentration of the essence decreased and the essence had a significant growth prohibition effect ($p<0.05$) in the small concentration studied and this effect significantly increased when temperature decreased ($p<0.05$). Eventually a complete growth stop (the logarithm of growth probability percent equal to -4.54) after 43 days and in a 0.03 and 0.04 % concentration of essence in 10 °C was observed[8]. Another study done in 2007 by *Ahmet* et al, the antibacterial effect of the essence and methanol extract of Savory on the bacteria and fungi with a food origin was evaluated. The oily, volatile extracts of this type of Savory was analyzed by GC/MS. The 30 extracted chemicals included Timol, Gamma Terpinene, Carvacrol and Rosemin.

This essence was active against 25 bacteria, 8 fungi and one yeast (Candida Albicans). The MIC had a range from 15.62 to 250 micro grams per milliliter. The methanol extract also had a antibacterial effect[6]. In 2000, Haji Akhoondi et al. [7] conducted biological and chemical studies on the essence of one species of pennyroyal and determined the effect of its volatile oil on 25 species of Anopheles and Artemia Salina Larvae. The rate of mortality after 24 hours was determined. The main extracts were Caron, Linalool Limonene, sis and trans dihydro Caron. The LD_{50} for pennyroyal extracts against anopheles and larvae was respectively 9 ± 5 and 9.2 micro grams per milliliter [7]. In the recent study, the three plant extracts had strong prohibiting effects on B. cereus spore growth and production. The presence of Timol 37% in the essence of thyme and beta Sitosterol and Ursolic Acid, 35% and 25% respectively in the essence of Savory and also 11% and 12% of Polgon and Mentol respectively in the essence of Pennyroyal can be the reason for the prohibiting effects of this essence in spore growth and cell growth and these effects increase with decrease in temperature, similar to other studies. However in Savory and Pennyroyal the synergistic effects of the components with the highest dose should not be neglected. In thyme the amount of Timol has a big difference with other compound components such as Bozneol (8%), Parasimen (7%), Carvacrol (2%), gama Tezinin (4%) and alpha Penin (3%). Therefore we think the prohibiting effect is related to Timol. In pennyroyal except Polgon and Menthol in the proportions mentioned, other components such as di-polgon (8%), Linalol (7%), Limonen (6.8%) and Caron (6%) exist, in which despite their low proportions still contribute to the prohibiting effect of these extracts. In savory in addition to beta Sitosterol and Ursolic Acid in the mentioned concentrations above, Timol, Oleanolic Acid and Caryophyllene Oxide are also available by 7.2%, 3.4% and 2% respectively.

However despite the big difference between these components with the two other chemicals which are beta Sitosterol and Ursolic Acid, these chemicals may have no prohibiting effect. According to the results of this study, the essences under study in low concentrations, 0.4, 0.8, 1.5, 2.5 and 3.5 micro grams per milliliter have an important effect on prohibiting spore growth.

Therefore in this study we can conclude that these essences can be used as food preservative against resistant bacteria and food pathogens, and can also be used as a new method for solving the problems related to spores in the food industry and in medicine. Also due to the limitation in

using antibacterial chemicals such as side effects and resistance and the necessity of replacing chemicals products with natural home made products, we do hope that the essence and extracts of these plants, become a basis for further studies about food preservatives and controlling human and animal food intoxication.

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