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Comparative Study on the Quality and Antibacterial Activity of Spearmint (*Mentha spicata*) Leaves and Infusion

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Abstract: *In the present study, a comparative investigation between the quality of spearmint leaves and infusion was performed and antibacterial activity against common pathogenic bacteria. Samples (300) represented loose, packed and fresh leaves in addition to their infusions were examined for the microbiological profile and quantitative analyzed for total aflatoxin. Antibacterial activity of spearmint essential oil was determined by disk diffusion method against pathogenic bacteria. Also, moisture content was evaluated. Considering the results obtained, the moisture content was determined as 85, 8.45 and 7.37% in fresh, loose and packed leaves, respectively. Overall, levels of microbial loads were generally exceeded the acceptable levels according to the Egyptian Standards in spearmint leaves, while no detectable microbial counts were found in infusion samples. Although, loose leaves contained the highest microbial load, the fresh leaves contained the lowest with Total Aerobic Viable Bacteria (TAVB) ($5.7x10^4$~$5.5x10^7$ cfu/g), molds and yeasts count ($3.5x10^2$~$8.7x10^3$ cfu/g), spore-forming bacteria ($9x10^2$~$4.7x10^4$ cfu/g), coliform group in MPN ($3.2x10^1$~$2.7x10^2$ cells/g), Escherichia coli (of positive samples 18~28%) and Bacillus cereus ($3.6x10^1$~$1.1x10^2$ cfu/g), while Salmonella spp, Shigella spp, Closteridium perfringens and Staphylococcus aureus were not detected. Total aflatoxins (37.32~48.73µg/kg) was detected in loose and packed leaves while was not detected (<5 µg/kg) in fresh leaves and infusion samples. The Gram-positive bacterial count seems to be more susceptible to the investigated essential oil, comparing to the Gram-negative ones. Finally, the study pointed out that spearmint leaves consider hazards which urges the need to provide a health control system, and the importance of spearmint essential oil, as an antimicrobial agent.*

Keywords: *Spearmint, Microbiology, Aflatoxins, Antibacterial activity.*

I. Introduction

Spearmint (*Mentha spicata*) is specie of mint native to North Africa, Egypt and Morocco. It belongs to the genus *Mentha* in the family Labiateae (Lamiaceae). It is widely used in commercially manufactured product, cooking and medicine for its aromatic and flavorsome qualities **(Sulieman *et al*., 2011 and Mandana *et al*., 2011)**. Leaves of mint plant are frequently used in herbal tea and for culinary purpose to add flavour and aroma. The distinctive smell and flavour, a characteristic feature of *Mintha spp.* is due to the naturally occurring cyclic terpene alcohol called menthol **(Parmila *et al.*, 2012)**.

The plant genus *Mentha* contains 25-30 different species and known for its antimicrobial, antiviral and insecticidal activity. Essential oils of mint are extensively used in toiletry, food and pharmaceutical industries because of its aromatic, stimulate and carminative nature **(Karicheri and Antony, 2016).** There is a relationship between the chemical structure of the most abundant compounds in the essential oil and the antimicrobial activity **(Helal *et al.*, 2006).**Despite the beneficial effects of herbs, some microorganisms and their metabolites can be found which could lead to serious health problems. Among the microorganisms that may be present, moulds are the most relevant, mainly due to their mycotoxins production capacity. Although the presence of pathogenic microorganisms is relatively rare, there are some exceptions, such as, *Bacillus cereus*, *Clostridum perfringens*, *Campylobacter jejuni*, *Escherichia coli*, *Escherichia vulneris*, *Enterobacter aerogenes*, *Enterobacer cloacae*, and *Pseudomonas aeruginosa.* In this kind of commodities production conditions may lead to an increasing risk of microbial contamination. Thus microbial spoilage of the raw herbal material is one of the factors governing the global market of herbs **(Santos *et al*., 2013).**

Microbial contamination of plants influenced by environmental factors such as temperature, humidity, extent of rainfall during the pre-harvesting, harvesting, and post-harvesting periods, handling practices and storage conditions of crude and processed medicinal plants materials **(Chotchoungchatchai *et al*., 2012)**. Aflatoxins (Afs) are difuranocoumarin derivatives synthesized primarily by the fungi *Aspergillus flavus* , *Aspergillus parasiticus* and, to a lesser extent, by the *Aspergillus nomius* **(Santini *et al*., 2015)**.

The growth of fungi that produced Afs in stored commodities is strongly influenced by environmental conditions: in fact high moisture content can increase the Afs amount of 10 fold in 3-day period (**Hell *et al*., 2008)**. The application of hot water extraction (herbal infusion) usually compensates for microbiological contamination, since it can be expected that boiling water markedly reduces the viable counts by several log units and also inactivates possible pathogens (**Mukundi, 2015)**.

The Egyptian Standard (ES: 2367/2006 and ES: 7136/2010) has proposed to set tolerance levels of dried mint at 10^4 cfu/g for the total aerobic viable count and 10^2 cfu/g for molds and yeasts count and coliform group count and should be free from pathogenic bacteria. Also total aflatoxins should not exceed 10 µg/kg. A general poor microbiological quality of spices and herbs especially those sold as loose in the open air markets. Spices and herbs owe their safety to their low moisture content, but once they get in contact with water-rich food products, microbial populations could develop quickly due to the increasing in water activity (**Debs-Louka *et al*., 2013).** The aim of this study was to investigate the microbial, total aflatoxins and moisture comparison between spearmint leaves and infusions. Furthermore, study the effect of its essential oil against some pathogenic bacteria.

II. Materials & Methods

2.1. Sampling

A grand total of 300 random samples represented by spearmint leaves included fresh, dry loose and packed forms and there infusions (50 samples of each) were collected from retile markets in Cairo governorate, Egypt. Each sample was kept in a separate sterile plastic bag and preserved in an ice box then transferred to the laboratory under complete aseptic conditions without undue delay and examined as quickly as possible. The infusions were prepared by soaking 1.5 g of the samples in 200 ml boiled water **(Al-Sohaibani *et al.*, 2011).** The samples were subjected to moisture content, microbiological and aflatoxin analysis as well as the antibacterial property of its essential oils.

2.2. Moisture content

Moisture content of peppermint leaves determined by drying the samples in air forced draft oven (Delab, Mod.: HST-5062, Germany) at 105±5ºC till constant weight achieved according to **AOAC (2006)**.

2.3. Microbiological analysis

Twenty-five grams of the sample were aseptically weighed in sterile stomacher bags, diluted with 225 ml peptone water, homogenized in a stomacher for 2 min (10^{-1} dilution) and serially diluted in 9 ml of peptone water **(Soriano *et al.*, 2002)**. Microbiological analysis was performed according to the procedures recommended by the International Commission on Microbiological Specification for Foods **(ICMSF, 1978 and 1996), (Harrigan, 1998) and (ISO, 2013).** Samples were examined to determine Total viable bacterial cout, molds and yeasts count, *B. cereus, Staph. aureus,* coliform group, *E. coli, L. monocytogenes, Salmonella spp., Shigella spp.*, spore-forming bacterial count and *Cl. perfringens*. The respective media were used in dehydrated forms (Oxoid, Difco and LAB-M) in which preparation of media were performed and incubated following the individual instructions: Plate Count Agar; Sabouraud Dextrose Agar; MacConky Broth; Brilliant Green Lactose Broth 2%; Lactose Broth, EMB, Tryptone water, MRVP Medium and Simmon Citrate Agar; PPEMBA; Baired-Parker's Medium and Brain Heart Infusion; Selenite Cystine Broth, Tetrathionate Brilliant Green Broth, Bismuth Sulphate Agar, Brilliant Green Agar, TSI, LIA, SS Agar and XLD Agar; Listeria Enrichment Broth and Oxford Medium, in addition to the serological kits of Bacto Salmonella O antiserum.

2.4. Total aflatoxin analysis

Twenty five grams of ground samples were prepared and subjected to Veratox kit for total aflatoxin (no. 225902, Neogen, UK), a Competitive Direct-Enzyme Linked Immuno-Sorbant Assay (CD-ELISA) in a microwell format. That allows obtaining exact concentrations in µg/kg following its individual instructions. The test was read in a microwell reader (Dynatech Laboratories, UK) with Software Version 1.2 to yield optical densities **(Lupo *et al*., 2010)**.

2.5. Antimicrobial assay

2.5.1. Microbial culture

Four strains of bacteria, i.e., *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 10876, *Escherichiae coli* ATCC 10536 and *Salmonella typhimaurium* ATCC 14028, wee used as test microorganisms.

2.5.2. Extract of spearmint oil

The fresh leaves samples were grinded and subjected to hydrodistillation by Clevenger type apparatus at 100ºC/4hrs according to **Sulieman *et al.* (2011)**. The yellow essential oil was separated and dried by anhydrous sodium disulfate (0.5 g), filtered through a 0.22µm filter (Millipore, USA), kept in a dark vail at 4ºC prior further analysis.

2.5.3. Disk diffusion assay

The antibacterial activity of *M. spicata* essential oil was examined by disk diffusion assay according to **Shahbazi (2015)**. Separate sets of the filter paper discs (diameter 6 mm; Whatman no. 1) were prepared by impregnated with 10, 20 and 30 µl of the essential oil and placed on the surface of Mueller Hinton Agar medium inoculated with the given microorganisms. The plates were incubated 37ºC overnight and examined for the zone of inhibition. Positive (tetracycline) and negative Dimethyl Sulfoxide Controls were considered in the present test. All experamints were repeated in triplicate.

2.6. Statistical analysis

The individual microbial observations were analyzed and expressed in terms of Mean±Standard Deviation (SD). T-test for paired comparison was carried out to detect the significant difference ($p<0.05$) between the means of spearmint leaves and infusion samples using statistical software (Windows software version 19, 2010, SPSS, Inc, Chicago, IL). The descriptive statistics were carried out to characterize the distribution of the evaluation of the samples **(Levin *et al*., 2013).**

III. Results And Discussion

The results of microbial analysis including TAVB, molds and yeasts count, spore-forming bacterial count, coliform group, *E. coli, B. cereus, Staph. aureus, Salmonella spp., Shigella spp.* and *Cl. perfringens* from 300 samples of spearmint leaves (fresh, loose and packed) and their infusions are described in **Table (1)**. These results show statistical significant microbial difference ($p<0.05$) were found between spearmint leaves and infusions. Also, *Staph. aureus, Salmonella spp, Shigella spp* and *Cl. perfringens* were not detected in any of the samples, either spearmint leaves or infusions. It was clearly notice that loose samples are exposed to outer atmosphere contained the highest load of TAVB i.e. $5.5x10^7 \pm 1.4x10^7$ cfu/g, while was <10cfu/g in infusion samples. Packed samples come second with TAVB average $2.7x10^6 \pm 1.8x10^6$ cfu/g. The fresh samples were the lowest count i.e., $5.7x10^4 \pm 2.2x10^4$ cfu/g. The spore-forming bacterial count ranged within $9x10^2 \pm 8.7x10^2 \sim 4.7x10^4 \pm 2.5x10^4$ cfu/g.The loose samples seem to have more molds and yeasts count ($8.7x10^3 \pm 7.3x10^3$ cfu/g) than packed ($6.2x10^2 \pm 5x10^2$ cfu/g) and fresh ($3.5x10^2 \pm 2.7x10^2$ cfu/g). All of the leaves contained the molds and yeasts count above the tolerable limit 10^2 cfu/g, which is hazardous.The loose samples showed coliform group counts ($2.7x10^2 \pm 6.0x10^1$ cfu/g) exceeded the acceptable levels. On the other hand, fresh and packed samples were within the acceptable levels. The presence of pathogenic bacteria including *E. coli* (18 ~ 28 %) and *B. cereus* ($3.6x10^1 \pm 0.4x10^1 \sim 1.1x10^2 \pm 8.3x10^1$ cfu/g) was also observed. Contamination with *E. coli* may be a result of either the habitats (proximity of settlements and animals that could contaminate the herbs with urine and feces) or the poor hygiene of the workers **(Stevic *et al.*, 2012)**.

Table 1. The microbiological profile (cfu/g) of spearmint leaves forms and infusions

Forms	Type of data	TAVB	Molds & Yeasts	Coliform group (MPN)[1]	*E. coli*[2]	*B. cereus*	Spore-forming bacteria
Fresh leaves	L	$5.7x10^4 \pm 2.2x10^4$	$3.5x10^2 \pm 2.7x10^2$	$3.2x10^1 \pm 2.2x10^1$	18	$3.6x10^1 \pm 0.4x10^1$	$9x10^2 \pm 8.7x10^2$
	I	<10	<10	<3	ND	ND	<10
	p	0	0	0	0.003	0	0
Loose leaves	L	$5.5x10^7 \pm 1.4x10^7$	$8.7x10^3 \pm 7.3x10^3$	$2.7x10^2 \pm 6.0x10^1$	28	$1.1x10^2 \pm 8.3x10^1$	$4.7x10^4 \pm 2.5x10^4$
	I	<10	<10	<3	ND	ND	<10
	p	0	0	0	0	0	0
packed leaves	L	$2.7x10^6 \pm 1.8x10^6$	$6.2x10^2 \pm 5x10^2$	$7.8x10^1 \pm 1.3x10^1$	22	$7.6x10^1 \pm 3.1x10^1$	$6.0x10^3 \pm 4.5x10^3$
	I	<10	<10	<3	ND	ND	<10
	p	0	0	0	0	0	0

L: Mean±SD of leaves samples TAVB: Total Aerobic Viable Bacteria
I: Mean±SD of infusion samples ND: Not Detected
1: count in cells/g 2: count in percent
P: Significant (2-tailed) -: t cannot be computed because the standard error of the difference is 0
* No detectable counts of *Stap. aureus, Salmonella spp., Shigella spp.* and *Cl. perfingens* were found in any of the samples.

Concerning the **Egyptian Standards (ES:2367/2006)** require that TAVB should not exceed 10^4 cfu/g but anaerobic bacteria, molds and yeasts count, and coliform group below 10^2 and should be free from *E. coli, Salmonella spp, Shigella spp* and *Clostridium spp.* In the same context, **Debs-Louka *et al.* (2013)** mentioned that samples which sold as loose and exposed to outer atmosphere (sold in an open-air marketplace), are for the majority microbiologically rejected. Handling and packing after drying may result in contamination of processed leaves with microorganisms. Moreover, environmental dust settling on different parts of the plant can potentially carry bacterial and mould spores **(Abd El-Aty *et al*., 2014)**.

Fig. (1), gives the percentage estimation of the moisture content of the leaves samples under investigation. The moisture content of fresh samples (85%) is higher than the dry leave samples either loose (8.45%) or packed (7.37%).

During this time, if the moisture of the product were to increase to levels allowing spore germination, significant mold growth and possibly mycotoxin production could occur **(Stević *et al.*, 2012).** The aw of the final product will not support growth of pathogenic bacteria but may enable survival **(EFSA, 2013)**.

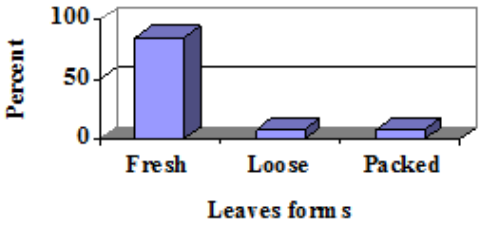

Fig. (1). The moisture content of spearmint leaves forms.

The diameters of the growth inhibition zones are shown in **Table (2).** The results indicated that spearmint oil has apparent antimicrobial activity against tested pathogenic bacteria. The essential oil (10, 20 and 30 µl) exhibited moderate level of antibacterial activity against all test microorganisms. The spearmint oil showed potent antibacterial activity against *Staph. aureus, B. cereus, S.typhimurium* and *E. coli,* where the inhibition zones ranged (6.3±0.6 ~ 14.7±0.6 mm), (7 ~ 16 mm), (4.3±0.6 ~ 14.3±0.6 mm) and (4±1 ~ 11.7±0.6 mm), respectively. The highest sensitivity showed by *B.cereus* (7, 13 and 16 mm) while *E.coli* showed the lowest (4±1, 8.7±0.6 and 11.7±0.6 mm) against the previous mentioned doses, respectively.

Table 2. Antibacterial effect of *M. spicata* essential oil against pathogenic bacteria

Pathogenic bacteria	**Dose of *M. spicata* essential oil**		
	10 µl	**20 µl**	**30 µl**
Staph. aureus	6.3±0.6	11.3±0.6	14.7±0.6
B. cereus	7	13	16
S. typhimurium	4.3±0.6	9	14.3±0.6
E. coli	4±1	8.7±0.6	11.7±0.6

This finding agreed with **Salim *et al*. (2015)** in which, microbiological activities of spearmint against *Staph. aureus* and *E. coli* resulted in high inhibition. Menthol is the main constituent of mint oil, which is bactericidal against *Staph. aureus* and *E. coli* **(Balakrishnan, 2015)**. **Znini *et al*. (2011)** suggested that, the antibacterial activity of *M. spicata* essential oil could be attributed to the presence of carvone and limonene. It has been reported that carvone is one of the most efficient antimicrobial agents of various plants. There were complied with that found by **Sulieman *et al*. (2011)**. They also concluded that spearmint and spearmint oil can be used as antibacterial, so that they can be used in food preservation.The current results show that Gram positive bacteria were higher in sensitivity response than gram negative bacteria against all the spearmint oil doses. The low susceptibility of Gram-negative bacteria could be attributed to the presence of hydrophobic lipopolysaccharide in their outer membrane which provides protection against different agents **(Shahbazi, 2015)**.

It is evident from the results illustrated in **Fig. (2)**, relative high levels of total aflatoxins were also found in loose (48.73 µg/kg) and packed (37.32 µg/kg) dry samples, while no mean detectable levels were observed in fresh

leave samples (<5 µg/kg). Furthermore, no detectable levels of total aflatoxins were found in any of the infusion samples (<5 µg/kg). Only 20% of aflatoxins were transferred to hot water. Therefore, the levels of afltoxins in medicinal herbs were considered to safe especially considering the aflatoxin transfer ratio **(Lee *et al*., 2010)**.

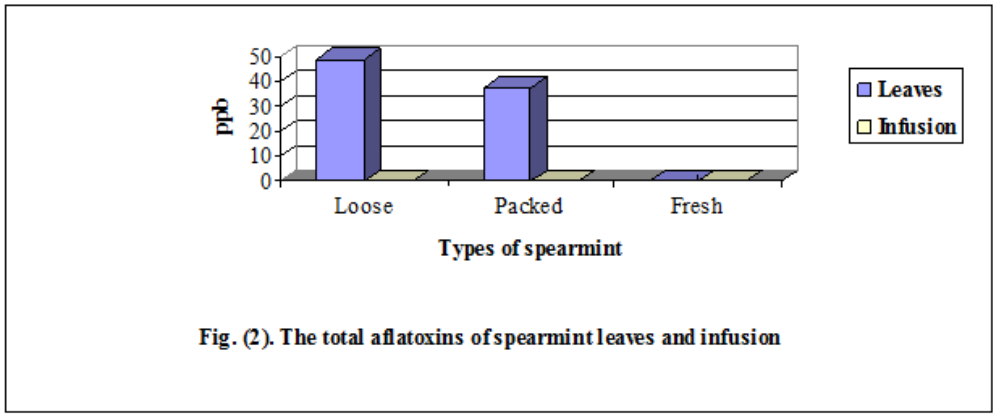

Fig. (2). The total aflatoxins of spearmint leaves and infusion

The increase in the consumption of herb medicines has made their use a public health problem due to the potential fungal contamination and the risk of the presence of mycotoxins **(Lee *et al*., 2011).** According to **Egyptian Standards** (**ES: 7136/2010)** and **Commission Regulation (EC: 1881/2006)**, changes of the concentration of the contaminant caused by dilution process. Liquid samples of the herbal extracts did not register contamination with aflatoxins. On the other hand, 24.0% of the solid samples were contaminated by aflatoxins **(Mukundi, 2015)**.

IV. Conclusion

The results of this study indicated high contamination level in dry spearmint leaves rather than fresh leaves and infusions. The maximum microbiological load and total aflatoxin content in dry leaves set by the Egyptian Standards were exceeded in loose and packed samples, while all of the infusion samples were within the acceptable limits. It is therefore important for regulatory monitoring the contaminants in spearmint leaves available in the markets. In addition, post-harvest procedures (drying and storing) should be controlled to minimize fungal growth and thus prevent aflatoxin contamination. The essential oil has remarkable antibacterial activity against common food-borne pathogenic bacteria associated with outbreaks.

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دراسه مقارنه على جودة أوراق ومشروب النعناع وتأثيره المضاد للبكتريا

عماد عاطف حلمى جرجس

قسم صحة الطعام - المعهد القومى للتغذيه - الهيئه العامه للمستشفيات و المعاهد التعليميه - جمهورية مصر العربيه

الملخص العربى: *تهدف هذه الدراسه إلى مقارنة السلامة الميكروبيه لأوراق ومشروب النعناع وتأثيرها المضاد لبعض البكتريا الممرضه. تمثل 300 عينه 3 مجموعات من أوراق النعناع تشمل الأوراق الجافه غير المعبأه والمعبأه فى أكياس والأوراق الخضراء بالإضافه إلى المشروب الناتج عنها ودراستها من حيث السلامه الميكروبيه وتقدير الأفلاتوكسينات الكليه كميا. ودراسة تأثير الزيوت الطياره للنعناع المضاد للبكتريا باستخدام طريقة الإنتشار القرصى. وأيضا دراسة نسبة الرطوبه فى عينات الأوراق. وقد أظهرت النتائج أن نسبة الرطوبه كانت 85 و8.45 و7.37٪ فى عينات الأوراق الخضراء و الغير معبأه والمعبأه فى أكياس على الترتيب. وعموما فقد، تخطى الحمل الميكروبى لأوراق النعناع الحدود المسموح بها فى المواصفات القياسيه المصريه، بينما كانت عينات المشروب خاليه من أى أحمال ميكروبيه. برغم أن عينات الأوراق الجافه الغير معبأه كانت تحتوى على أعلى أعداد ميكروبيه و كانت الأوراق الخضراء كانت تحتوى على الأقل، بحيث كان العد الكلى للبكتريا الهوائيه ($5.5x10^7 \sim 5.7x10^4$ وحدة تكوين مستعمره/جرام)، الفطريات والخمائر ($3.5x10^2 \sim 8.7x10^3$ وحدة تكوين مستعمره/جرام)، البكتريا المكونه للجراثيم ($9x10^2 \sim 4.7x10^4$ وحدة تكوين مستعمره/جرام)، مجموعة القولون (بالعد الأكثر إحتمالا، $3.2x10^1 \sim 2.7x10^2$ خليه/جرام)، E.coli (العينات الموجبه، 28~18٪) و B.cereus ($1.1x10^2 \sim 3.6x10^1$ وحدة تكوين مستعمره/جرام)، بينما خلت كل العينات من Salmonella spp. و Shigella spp. و Cl.perfringens و Staph.aureus. وتراوحت الأفلاتوكسينات الكليه بين (37.32 ~ 48.73 ميكروجرام/كجم) فى عينات الأوراق الجافه الغير معبأه والمعبأه فى حين لم توجد فى عينات الأوراق الخضراء وعينات المشروب (< 5 ميكروجرام/كجم). كانت البكتريا الموجبه لجرام هى الأكثر تأثرا من السالبه لجرام للزيوت الطياره للنعناع. وكمحصله للدراسه فإن عينات أوراق النعناع تحتوى على مخاطر، مما يتطلب عمل برنامج للتحكم فيها، كما أن الزيوت الطياره فى النعناع لها تأثير مضاد للبكتيريا.*

الكلمات المفتاحيه *- النعناع، ميكروبيولوجى، أفلاتوكسينات، تأثير مضاد للبكتريا.*