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Comparative Analysis of the Anti-bacterial Activity of Four Plant Extracts

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A B S T R A C T

Plants and their secondary metabolites, serve as potential benefits due to the production of several pharmaceutical compounds. They possess several biological activities and properties affected by several external factors. This study aims to investigate the antibacterial activity of four plant extracts, cloves (*Eugenia caryophyllata*), mint (*Mentha piperita*), rosemary (*Rosemarinus officinalis*), and cherry (*Prunus avium*), against *Bacillus subtilis*, a gram positive bacteria, by broth microdilution method; as well as to examine the quantity and efficiency of these extracts with the change in both the extraction and storage temperatures. The extracts were extracted using two different temperatures (100°C and 80°C) and their antibacterial activity was assessed at the extraction day and after one month storage at 4°C and 30°C. Qualitative tests and phytochemical screening were done as well to test the presence of terpenoids, flavonoids and phenolics. By the end of the experiments, it was realized that cloves posses the highest antibacterial activity due to the presence of terpenoids, flavonoids and phenolics, followed by cherry then rosemary, whereas mint possessed the least; furthermore, the higher extraction temperature favors greater yield as well as better quality and activity. However, the storage temperature's effect depends on the plant material, its constituents and extraction temperature, but still 30°C storage temperature lowered the antibacterial activity.

Introduction

Plants are good sources of pharmaceutical compounds. Natural products could be potential drugs for humans or livestock species, and act as intermediates for synthesis of useful drugs(Makkar *et al.*, 2009). The bioactivities of plants are generally ascribed to the presence of plant secondary metabolites (Makkar *et al.*, 2009), which produce definite physiological actions on human body (Mohammedi and Atik, 2011).

The extracts can be categorized into several classes among are terpenoids, flavonoids and phenolics known to be active against bacteria, viruses and protozoa (Murphy Cowan, 1999). The quantities and phytochemical composition of the plant

extracts are highly affected by the extraction method used, its time and temperature; the nature, polarity and concentration of the solvent; along with the nature of the plant material (Handa *et al.*, 2008; Saeed and Tariq, 2008; Jalal *et al.*, 2009; Dai and Mumper, 2010; Islam *et al.*, 2010; Tiwar *et al.*, 2011). As a result, the physiological properties and biological capacities, antibacterial of these extracts are influenced.

Lebanon and the Mediterranean region are rich in floral biota traditionally used as spices, food additives and preservatives. Among which are, Cloves (*Eugenia caryophyllata)*, Mint (*Mentha piperita*), Rosemary (*Rosmarinus officinalis*), Cherry (*Prunus avium*) where they have wide range of medicinal properties and activities (Ababutain, 2011; Tavassoli and Djomeh, 2011; Abdel-Massih *et al.*, 2011). This study aims to compare the antibacterial activity of the aqueous plant extracts of the aforementioned plants against *Bacillus subtilis*, a gram positive bacterium; and to study the influence of the extraction temperature on the yield and the antibacterial activity of the tested extracts. Furthermore, to find the effect of the storage temperature, one month storage, on the properties of these extracts.

Materials and Methods

Collection of the Plant Material

Dried cloves buds (*Eugenia cayophyllata*), cherry peduncle (*Prunus avium*), mint (*Mentha piperita*) and rosemary (*Rosmarinus officinalis*) leaves were collected from the commercial Lebanese market.

Extraction of the Plant Extract

Ten grams of the plant material were ground in order to reduce the plant's size, rupture the organs, tissues, and cell structures, increasing the access between the medicinal ingredients and the extraction solvent (Handa *et al.*, 2008). Hundred milliliters of distilled water, maintained at two different temperatures, boiling and 80°C, where added to the plant material. They were left for 30 minutes in a boiling and 80°C water bath, respectively. Later on, they were allowed to cool at room temperature along with a continuous shaking. The content was filtered using whatmann no. 1 filter paper, and the filtrate was lyophilized using alpha 1 - 4 LD plus Christ freeze dryer.

Extracts' Storage

The powders obtained from the freeze dryer were dissolved in water and divided into three sets:

Set 1: Analyzed directly at day 0
Set 2: Stored at 4°C for further studies after one month
Set 3: Stored at 30 °C for further studies after one month

Phytochemical Screening

Qualitative analysis was done to study the presence of certain active compounds. It was done at the extraction day for set 1 and after one month for set 2 and 3, to study the storage temperature effect with time on the compounds' presence.

Terpenoids Test

5 ml of each extract was mixed with 2 ml of chloroform. Then 3ml of concentrated H_2SO_4 was carefully added. Formation of a layer of reddish brown coloration at the interface indicated the presence of terpenoids.

Flavonoids Test

4 ml of the extract was treated with 1.5 ml of 50% methanol solution and warmed. To the warmed solution, metal magnesium and 5-6 drops of concentrated HCl was added. Red color was the indicator for flavonoids.

Phenolics Test

A fraction of the extract was treated with 5% $FeCl_3$ reagent. Formation of deep blue black color revealed the presence of phenolics.

Antibacterial Assay

The antibacterial test was done for the three sets mentioned earlier.

Collection of the Bacterial Strains

A gram positive bacterium, *Bacillus subtilis OMG 168* was used in order to test the antibacterial activity, minimum inhibitory concentration (MIC), of the plants. This was provided by the Laboratory of Microbiology in the School of Doctoral Sciences and Technology (EDST) at the Lebanese University.

MIC Determination

MIC of the plants was determined by the broth microdilution method using microplates of 96 wells each. 120 µl of the broth was added to the wells of the plate except those of the first column. To the first well of the first column 240 µl of the plant extract was added. Then serial dilution of the extracts starts by taking 120 µl of the first well content to the second, mixed well, and so on. Later on, 120 µl of the bacterial suspension was added to each well. Microplates containing the cultures were incubated for 24 hours at 37°C.

Results and Discussion

Nowadays, consumers tend to be more suspicious of chemical additives and preservatives; thus the exploration of naturally occurring antimicrobials, for food preservations and other medicinal uses, receives increasing attention (Das *et al.*, 2010) and interest owing to their versatile applications (Pandey and Singh, 2011). A variety of bioactive compounds, present in different parts of a plant, has spurred a renewed interest in developing an alternate therapy (Pramila *et al.*, 2012). Several factors affect the quality of these compounds thus their physiological properties.

Extracts' Yield

As mentioned above, several extraction conditions affect the quantity of the extracts obtained. In our study, the extraction temperature was the parameter, where the extracts' yield, at the two temperatures 100°C and 80°C, was calculated using the following formula:

$$\% \text{ yield} = \frac{\text{weight of the extract after lyophilization}}{\text{weight of the powdered plant material}} \times 100$$

The graph highly shows that 3% more, cloves, rosemary, cherry, and mint, extract respectively, was yielded as the extraction temperature increased from 80°C to 100°C. The results are consistent with several reviews which proved that an increase in the extraction temperature can promote higher analyte solubility, by increasing both the solubility and the mass transfer rate. In addition, the viscosity and the surface tension of the solvents are decreased at higher temperature, which helps the solvents reach the sample matrices, improving the extraction rate and extract efficiency (Dai and Mumper, 2010).

Active Compounds' Presence

Plants are capable of synthesizing aromatic secondary metabolites, most of which are phenols or their oxygen-substituted derivatives (Pandey and Singh, 2011) that show antimicrobial effect and serve as plant defense mechanisms against pathogenic microorganisms (Pandey and Singh, 2011).

Active Compounds' Presence and the Extraction Temperature Effect on their Availability

Table 1 indicates the presence of the three active compounds, terpenoids, flavonoids and phenolics in cloves. Hema R. *et al.*, 2010 and others, detected Eugenol in cloves (Ababutain, 2011) one of the most powerful antimicrobial phenolics (Songsong Li, 2011). Several studies also indicated the presence of flavonoids in cloves (Ababutain, 2011). Furthermore, certain reviews stated that cloves yield a terpenoid known as petalostemumol with excellent activities against *Bacillus subtilis* and other bacteria (Murphy Cowan, 1999).
The table shows that the tested compounds were present in rosemary. Various reports reported the presence of flavonoids, phenols, volatile oil and terpenoids in rosemary (Waggas and Balawi, 2008). Moreover, others showed several terpenoids and monoterpenes (isocarnosol, camphor, etc...) present in the rosemary extracts (Genena *et al.*, 2008).

Few studies were done to investigate the chemical composition of cherry. Our study shows that one of the major constituents of cherry are phenolics, similar to (Melichacova *et al.*, 2009) who found that *Prunus avium* contain a considerable quantity of phenolics and polyphenols. Several studies indicated that menthol is one of the mint's major components along with other chemicals (Gardiner, 2000), however, the experiments haven't detect the presence of the tested compounds; this is due to that the solution tested is highly diluted where the compounds are of low undetectable concentrations.

With respect to the extraction temperature effect, the phytochemical analysis showed that the temperature has no effect on the presence or absence of these compounds, except terpenoids present in rosemary. This is due to the abundance of these chemicals, since as mentioned earlier the higher the temperature, the higher the yield, as a result more active compounds will be extracted. In addition, this condition varies based on the type of compounds, as well as the plants used.

Effect of the Storage Temperature on the Presence of Phyto-chemical Compounds

The high storage temperature lead to the absence of several chemicals in some of the tested plants mainly terpenoids and flavonoids. Flavonoids content decreased at high temperature of storage (Klimczak, 2006) so they cannot be detected by simple qualitative tests. Moreover, some of the natural products may volatilize or undergo physical and chemical changes.

Antibacterial Assay Result

Antibacterial Activity of the Plant Extracts

The antibacterial activity of the plants under study is assessed by evaluating their MIC values, shown in the graphs below. The first corresponds to those extracted at 100°C and the second for those extracted at 80°C.

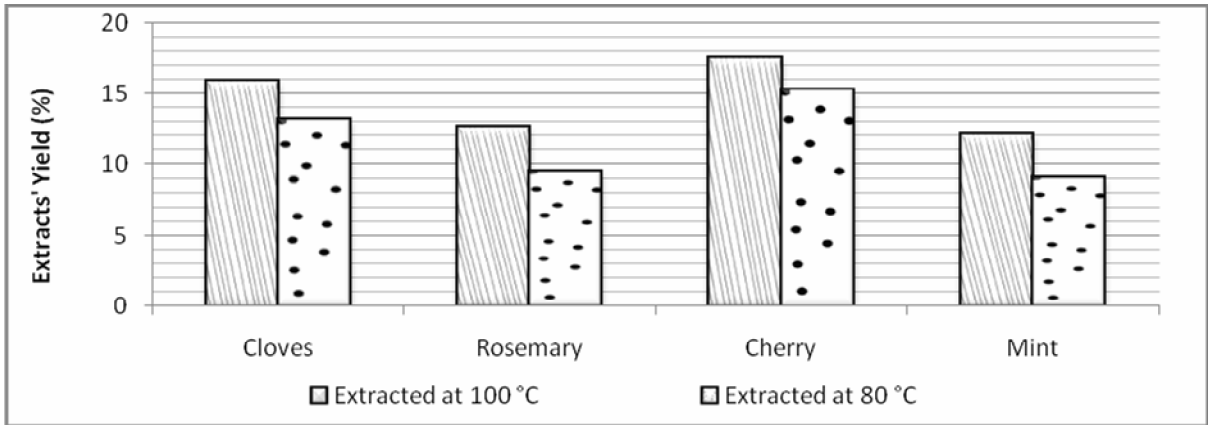


Figure 1: The graph reveals the extracts' yield (%) as a function of the extraction temperature in the plants studied; where extracting the extract at 100°C and 80°C increased and decreased the yield of the extracts obtained respectively.

Table.1 Presence of active compounds extracted at two different temperatures

		Terpenoids	Flavonoids	Phenolics
Cloves	Extracted at 100 °C	+	+	+
	Extracted at 80 °C	+	+	+
Rosemary	Extracted at 100 °C	+	+	+
	Extracted at 80 °C	-	+	+
Cherry	Extracted at 100 °C	-	-	+
	Extracted at 80 °C	-	-	+
Mint	Extracted at 100 °C	-	-	-
	Extracted at 80 °C	-	-	-

(+) = presence (-) = absence

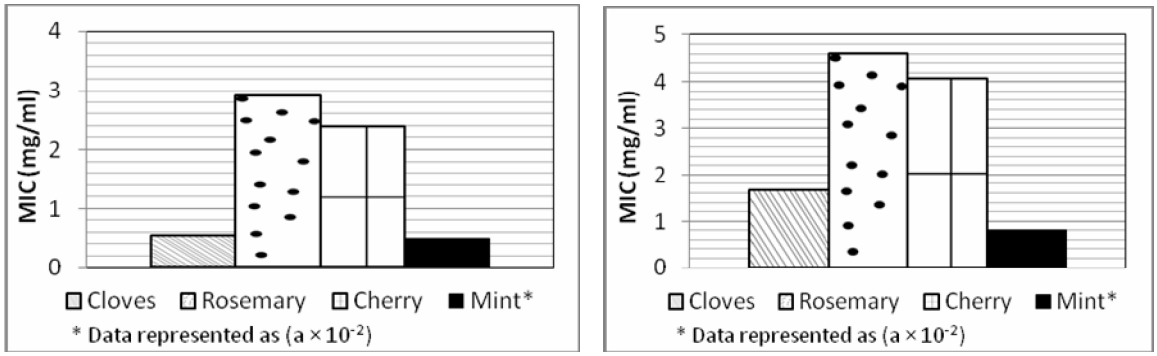


Figure.2 MIC (mg/ml) of the extracts extracted at 100°C (Right) and 80°C (Left) against *B. subtilis*; where cloves showed the highest activity and mint the lowest at both extraction temperatures, where the higher the temperature the higher the possessed activity

Table.2 Availability of active compounds after one month storage at 4°C and 30°C

			Terpenoids	Flavonoids	Phenolics
Cloves	Extracted at 100 °C	0 day	+	+	+
		After 1 month storage at 4 °C	+	+	+
		After 1 month storage at 30 °C	-	-	+
	Extracted at 80 °C	0 day	+	+	+
		After 1 month storage at 4 °C	-	+	+
		After 1 month storage at 30 °C	-	-	+
Rosemary	Extracted at 100 °C	0 day	+	+	+
		After 1 month storage at 4 °C	+	+	+
		After 1 month storage at 30 °C	-	-	+
	Extracted at 80 °C	0 day	-	+	+
		After 1 month storage at 4 °C	-	-	+
		After 1 month storage at 30 °C	-	-	+
Cherry	Extracted at 100 °C	0 day	-	-	+
		After 1 month storage at 4 °C	-	-	+
		After 1 month storage at 30 °C	-	-	+
	Extracted at 80 °C	0 day	-	-	+
		After 1 month storage at 4 °C	-	-	+
		After 1 month storage at 30 °C	-	-	+
Mint	Extracted at 100 °C	0 day	-	-	-
		After 1 month storage at 4 °C	-	-	-
		After 1 month storage at 30 °C	-	-	-
	Extracted at 80 °C	0 day	-	-	-
		After 1 month storage at 4 °C	-	-	-
		After 1 month storage at 30 °C	-	-	-

(+) = presence (-) = absence

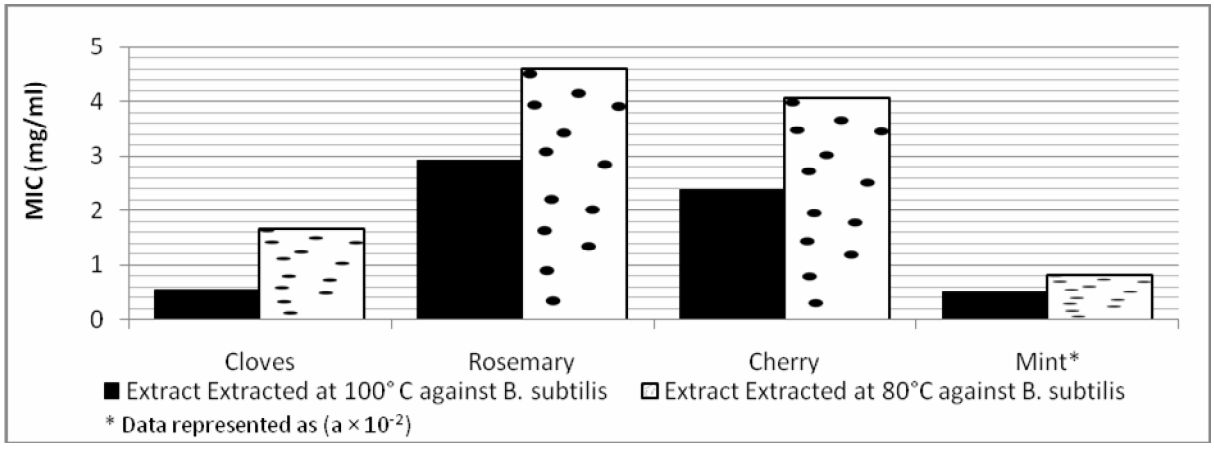
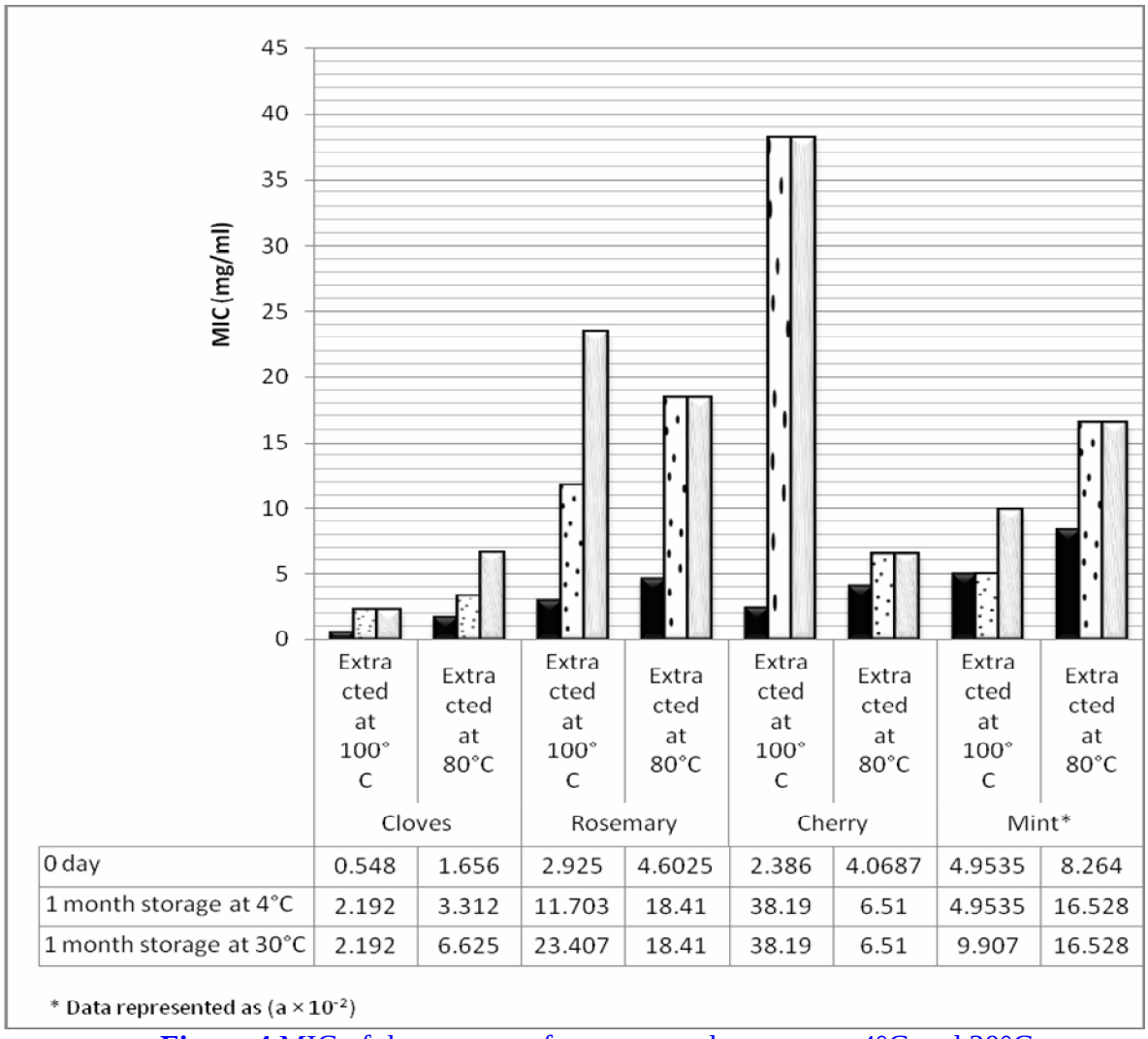


Figure.3 MIC of the extracts extracted at 100°C and 80°C; the high extraction temperature enhanced the antibacterial activity of the for studied plant



	Cloves		Rosemary		Cherry		Mint*	
	Extracted at 100° C	Extracted at 80°C	Extracted at 100° C	Extracted at 80°C	Extracted at 100° C	Extracted at 80°C	Extracted at 100° C	Extracted at 80°C
0 day	0.548	1.656	2.925	4.6025	2.386	4.0687	4.9535	8.264
1 month storage at 4°C	2.192	3.312	11.703	18.41	38.19	6.51	4.9535	16.528
1 month storage at 30°C	2.192	6.625	23.407	18.41	38.19	6.51	9.907	16.528

* Data represented as ($a \times 10^{-2}$)

Figure.4 MIC of the extracts after one month storage at 4°C and 30°C

MIC is defined as the lowest concentration able to inhibit the growth of the tested bacteria. As a result, the lower the MIC, the higher the anti-bacterial activity of the extract against *B. subtilis* will be. According to this concept, the graphs show that cloves possess the highest antibacterial activity, followed by cherry which has slightly higher antibacterial activity than rosemary; however mint showed the least activity.

Cloves was considered by several studies one of the most biologically active plants. Several studies showed that eugenol present in cloves had a broad antibacterial activity (Ababutain, 2011), and proved that cloves extract possess high activity against B. subtilis (Abou Shanab et al., 2004). In addition, (Nascimento et al., 2000) reported that cloves extract inhibited 64.2% of the tested bacteria.

A number of studies showed the significant antibacterial activity of rosemary against several gram negative and gram positive bacteria among which is B. subtilis (Moghtader and Afzali et al., 2009; Stojanović-Radić et al., 2010). This activity can be referred to the presence of several terpenoids and phenolics, mainly isocarnosol and other minor compounds.

This was proved by (Horiuchi et al., 2007) who showed that the addition of carnosol to a certain medium lowered the MIC values. Others also attributed the antibacterial activity of rosemary to carnosic acid and carnosol and indicated that apolar phenolic compounds from rosemary extracts may be responsible for their antibacterial activity (Proestos et al., 2005; Tavassoli and Djomeh, 2011).

Cherry (prunus avium), as revealed by the graph, possessed high antibacterial activity against B. subtilis, where it was the second among the tested plants. Few studies are present; one of these was (Ordogh et al., 2010) who reported the antibacterial activity of cherry against S. pyogenes and P. acne but not against B. subtilis. This study indicated that the reason behind this activity is still unknown. However, the results of the qualitative test done above along with the total phenol content found by (Melichacove et al., 2009) justify the inhibitory effect of cherry, by the presence of phenolic compounds.

According to the results obtained, mint showed the least antibacterial activity, this highly agrees with several other studies, which either showed a slight antibacterial activity of mint, or stated that mint cannot be considered one of the bactericidal plants. A study revealed low antibacterial activity of mint on B. subtilis however it was mentioned that mint's activity is both strain and dose dependent (Bupesh et al., 2007). This activity is referred to the presence of menthol that possesses high antibacterial activity when present at high concentrations, which is not the case in this study.

The results mentioned earlier, proved that every plant is characterized by its own antibacterial activity, due to the secretion of several major and minor compounds, terpenoids, flavonoids and phenolics. The mode of action of terpenoids is still unknown but is referred to the membrane disruption by the lipophilic character of these chemicals, where some studies found that increasing the hydrophilicity of kaurene diterpenoids by addition of a methyl group drastically reduced their antimicrobial activity (Kiarostami et al., 2010).

Flavonoids have a broad antibacterial activity (Al-Zubaydi et al., 2009), due to the ability to complex with extracellular and soluble proteins; and to precipitate proteins on the bacterial cell wall, forming complex with it (Kiarostami et al., 2010). It might be also, that the phenol groups present tend to form hydrogen bonds with cell wall protein and hence, destroy the cell membranes (Al-Zubaydi et al., 2009).

The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, characterized by their high affinity to bind with the enzyme active sites (Al-Zubaydi et al., 2009), possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Tavassoli and Djomeh, 2011), resulting in denaturing of the enzyme and loss of its function (Al-Zubaydi et al., 2009).

Effect of the Extraction Temperature

Several parameters affect the quantity, along with quality of the extract obtained. Temperature affects the abundance of the active compounds present in the extract, as

a result influences their bioactivities. The figure below shows the antibacterial activity of the extracts extracted at 100°C and 80°C where the faded bars corresponding to the latter. As indicated, the higher the extraction temperature the lower the MIC will be. This is attributed to the increase in the terpenoids, flavonoids and phenolic content.

Sathishkumar et al., 2008, showed that at higher temperatures, flavonoids diffused more quickly from the cell to the extracting solvent. (Naeem et al., 2012) stated that temperature has a significant influence on the phenolic content, where the highest content was reported at the highest temperature this is due to the increase in both the solubility of the solute and the diffusion coefficient.

Few studies proved the influence of temperature on the antibacterial activity of the extract. Among these, a study clearly showed that using cloves decoction (extracted at boiling temperature) is better than using its infusion (extracted at room temperature), since it increased the antibacterial activity of the extract against the bacteria used (Saeed and Tariq, 2008).

Moreover, other studies indicated that hot aqueous extract of A.vera showed a pronounced antibacterial activity; however the cold didn't (Al-Zubaydi et al., 2009). This was referred by (Wang et al., 1998) to the presence of higher quantity of phenols. (Abubakar, 2009) indicated that an increase in the temperature increased the antibacterial activity of the extract.

Effect of the Storage Temperature

The factors affecting the quality and properties of the plant extracts can be, those related to the extraction method and those related to the storage process. The graph below illustrates the effect of the storage temperature on the antibacterial activity of the extracts obtained.

After one month storage the antibacterial activity was lowered. Moreover, some extracts were more negatively affected at higher temperature, 30°C, than others. These are cloves extract extracted at 80°C and rosemary and mint extract extracted at 100°C. This decrease in the antibacterial activity can be justified, by the decrease of both the flavonoid and phenolic content as the storage temperature increased. Klimczak et al. (2006) showed in his study that the higher the storage temperature the lower the total flavonoids and total phenol will be (Klimczak et al., 2006). As a result, the storage of plant extracts affects its activity negatively and depends on the type of the plant material understudy, the extraction and the storage temperature.

In the study done, cloves, rosemary, cherry and mint antibacterial activity was conducted, where cloves possessed the highest whereas mint showed the least. Furthermore, the activity of these plants and the availability of the active compounds are affected by the extraction and the storage temperature. Eventually, further studies must be done to extract and characterize the active compounds behind this activity and assess it against gram negative strains, along with comparison with the commercially used antibiotics.

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