THE EFFECT OF ENVIRONMENTAL CONDITIONS ON THE ANTIBACTERIAL PROPERTIES OF *CALENDULA OFFICINALIS*

1. ABSTRACT

A health practitioner is concerned about the quality of the medication prescribed as it must remain unaltered throughout the treatment period. *Calendula officinalis* is a well-researched herb, known for its antimicrobial efficacy and was therefore used in this study to establish whether its antibacterial properties changed when exposed to different environmental conditions. An herbal extract and a homeopathic mother tincture were used in this study. Although both samples were prepared from *Calendula officinalis*, they were extracted differently. The stock samples and dilutions of the samples were exposed to different environmental conditions (Control – UJ Dispensary, Direct Sunlight, Kitchen Scenario, Office Scenario and Decreased Temperature) and the antibacterial properties were evaluated with the Kirkby Bauer Disc Diffusion Method and the broth dilution minimum inhibitory concentration test. Some samples were also separated and analysed with Gas Chromatography Mass Spectrometry. Statistical analysis of the Kirkby Bauer Disc Diffusion results was done and compared accordingly with the Mann Whitney U-Test and the kurtosis and skewness values. A comparison of the results for the broth dilution minimum inhibitory concentration test and the Gas Chromatography Mass Spectrometry was also done and reported on. The test results showed that the samples exposed to the different environmental conditions changed in their activity as well as their composition. The testing on the diluted samples were halted due to the inconsistency of the results and a suggestion was made to allow further research on this topic. Amber glass bottles showed overall better protection against environmental conditions than blue glass bottles. The samples in the control environment, the UJ Dispensary, with temperature constant at an average of 21ºC and with minimal exposure to sunlight, external lighting and electromagnetic waves and radiation showed stability and would be proposed as the best storage environment for herbal extracts and homeopathic mother tinctures.

2. INTRODUCTION

Calendula officinalis (*C. officinalis*) is a member of the Asteraceae family (Efstratiou *et* al.,2012) that is identified by its orange-yellow florets and broadly lance-shaped leaves (Czygan *et al*., 2004). *C. officinalis* has various active compounds including carotenoids, phenolic compounds, saponins and in lesser concentrations, proteins, amino acids, saturated hydrocarbons, vitamin C, mineral substances and soluble poly-carbohydrates that play a vital role in the healing of tissues (Efstratiou *et al.*, 2012; Butnariu and Coradini, 2012). When the *C. officinalis* flowers are picked, a sticky substance is present due to the resinous bracts that form the base of the flower head. Resin

is an important compound for the healing and antimicrobial properties of *C. officinalis.* (Blankespoor, 2012).

Triterpenic alcohols and polyunsaturated fatty acids such as calendic acid (which is a polyunsaturated fatty acid) is also contained in *C. officinalis.* These specifically allow for the anti-inflammatory properties of *C. officinalis.* The soluble poly-carbohydrates play a vital role in the healing of tissues (Butnariu and Coradini, 2012).

The actions of *C. officinalis* are documented to range from healing, anti-inflammatory, haemostatic to an antimicrobial, antiviral and antifungal (Bone, 2003). Murphy (2010) refers to *C. officinalis* as the "great herbal anti-septic" as it protects wounds from putrefaction. It does not cause any irritation or sensitivity to the wound or surrounding skin and assists with local pain management (Bone, 2003). The possible mode of action to assist with wound healing is to increase the blood flow to the area, allowing higher oxygen supply to the affected tissues (Ehrlich, 2013).

Van Wyk and Wink (2004) define a liquid herbal extract as a mixture of chemical compounds extracted from plant material by using an organic solvent such as ethanol or water. The extract used in this study was a 1:2 dilution of the plant material, extracted by using a cold percolation method with 90% ethanol. Homeopathic practitioners usually make use of the mother tincture of a plant instead of an herbal extract. Depending on the company manufacturing these, it is mainly extracted according to the method defined in the German Homeopathic Pharmacopoeia or the French Homeopathic Pharmacopoeia. For the purpose of this study, the mother tincture was prepared according to the German Homeopathic Pharmacopoeia, HAB method 3A. In this method, the plant materials are minced and added to 62% ethanol. It is then stored in airtight containers for 10 days while swirling from time to time. The temperature during this period may not exceed 20ºC. The mixture is then filtered, and the liquid portion is termed the mother tincture of the plant.

The aim of the Kirby-Bauer Disc Diffusion method is to determine the sensitivity of bacteria to a substance. The microorganism is inoculated on a Mueller-Hinton agar plate in the presence of the testing substance impregnated filter paper discs. A positive result for this test is a rounded area of nonbacterial growth around the impregnated discs showing antimicrobial activity; this area is referred to as the zone of inhibition (Hudzicki, 2009). MIC (Minimum Inhibition Concentration) is the lowest concentration of a substance that will show antimicrobial action by showing the inhibition of visible growth of an organism after it has been incubated overnight. This method has an important use to determine the susceptibility of organisms to antimicrobial substances. It is also

used to compare the susceptibility testing of the performance of other tests. In medicinal laboratories, it is also used to distinguish between unusual resistances to a substance or to test drug-resistant bacteria (Andrews, 2001). A study done by Roopashree *et al.* in 2008 showed that a *C. officinalis* extract had to have a minimum inhibition concentration of 32mg/ml to show antibacterial efficacy. In the study, *C. officinalis* flowers were extracted with ethanol and tested on *S. aureus*. In another study done by Chakraborthy in 2008, a minimum inhibition concentration of 13mg/ml was found when extracting dried leaves of *C. officinalis* with ethanol and testing it on *S. aureus.* The *C. officinalis* extract that is used during this experimental study has a concentration of 500mg/ml and is extracted from the flowers of the plant with 90% ethanol (Mediherb, 2013).

There are two methods in determining MIC: the first being agar dilution and the second broth dilution. With agar dilution, different concentrations of an antimicrobial substance is added to nutrient agar followed by the application of a standardized number of organisms to the surface of an agar plate. With a broth dilution, as done in this test, bacteria is inoculated into a liquid medium nutrient broth and added to different concentrations of the antimicrobial agent. Growth is allowed overnight and the MIC value is noted. The broth dilution is often done in a 96-well microtiter plate and is only suitable for aerobic bacteria; in this study, *S. aureus* is used (Wiegand *et al.*, 2008).

Gas chromatography (GC) is a very accurate and precise technique that is widely used. Usually the sample is introduced as a vapour onto the column. The solubility is dependent on the vapour pressure of the component, which would in turn determine the affinity between the stationary phase and the compound. Due to the differences in the vapour pressures, the molecules continuously move between the mobile gas phase and the stationary phase. As soon as a molecule enters the gas phase, it is moved to the detector, therefore different molecules with different physical and chemical properties will arrive at the detector at different times. These different times are referred to as residence times (Prichard, 2003).

Mass Spectrometry (MS) electrically charges molecules, then send them through a magnetic field where the molecules are broken into charged particles with different charges. It then identifies the substances accordingly. Therefore, the GC separates the particles and the MS identifies them, making the combination of the two instruments of great scientific value (Douglas, 2010).

New, well cleansed or sterilised, colourless, neutral flint glass containers should be used to store extracts, mother tinctures and dilutions in a cool dry place. Mother tinctures may not undergo extreme temperature changes as this may cause sediment to form. The same applies to

homeopathic remedies (Mandel and Mandel, 2002). Preservation of different homeopathic remedies is set out in the different Homeopathic Pharmacopoeias according to the reactivity of the remedies, but general rules have been set to standardise the preservation of homeopathic remedies. Remedies should be stored in well stoppered bottles, which are kept in boxes or drawers, protecting them from direct light. Remedies should not be exposed to extreme temperatures, and should be stored in a cool dry place. Remedies should not be kept close to anything that can affect the purity of the remedies, such as dust, strong odours, smoke, moisture, radiation, strong light or sunlight (Banerjee, 2011).

3. MATERIALS AND METHODS

This research study utilised an experimental quantitative design. The initial planned method for the study included the Kirkby Bauer Disc Diffusion test with different dilutions of the mother tincture and herbal extract. After the collection of the first set of results obtained from the diluted samples with different ethanol concentrations, MIC and GC/MS was added to support the results obtained.

3.1 Sample Preparation

The homeopathic mother tincture and the herbal extract were diluted with distilled water, 20% ethanol and 62% ethanol and placed in duplicate in blue and amber glass bottles. Undiluted samples were also used. Two different types of dilutions were used; one that is according to the German Homeopathic Pharmacopoeia (3:7) and one that is used in practise by some homeopaths (1:10).

3.2 Exposure Factors

The *C. officinalis* samples were exposed to five different environmental conditions. These included: Control (University of Johannesburg Homeopathic Dispensary), Direct Sunlight, Kitchen Scenario, Office Scenario and Decreased Temperature.

The Control environment UJ Dispensary) was chosen as the temperature is kept constant at an average of 21℃ and placed in a drawer away from any strong odours and direct light; exposure to radiation is also minimal in this environment. The samples remained in the same location for seven days without being moved. The direct sunlight group samples were placed in direct sunlight for seven consecutive days. Daily exposure time was done from 8:00 to 17:00. A thermometer was placed in the same position as the samples and the temperatures of the day were noted as well as the date of the exposure (as reference to seasonal factors). The samples that were placed in the kitchen scenario were placed next to a microwave oven that was intermittently active for one hour

a day (between 8:00 and 17:00). The exposure times were noted. The samples in the office scenario group were placed next to a Wi-Fi router that established constant connections with three laptops and two portable devices. The one computer screen also emitted light onto the sample during this time. No sunlight exposure or external synthetic lighting was present. The exposure was done for seven consecutive days for eight hours on each day. The temperature of the office was measured hourly and noted. After the exposure time for the day, the samples were stored in a polystyrene cooler box to ensure minimum exposure to other sources. For the decreased temperature group, the samples were placed in a household fridge for seven consecutive days and the temperature was measured hourly during the day. Samples remained in the fridge overnight. No strong odours were present in the fridge. The average temperature in the fridge was 4ºC. After the exposure time for the day, the samples were stored in a polystyrene cooler box to ensure minimum exposure to other sources.

3.3 Kirkby Bauer Disc Diffusion Test

The prepared samples were tested with the Kirkby Bauer Disc Diffusion test before exposure (BE) and after exposure (AE) to the environmental conditions for comparison.

A mixture of nutrient agar and nutrient broth was prepared as per the instructions set out by the manufacturers. 25ml of the mixture was pipetted into individual McCartney flasks and autoclaved for sterilisation. After autoclaving, the agar-broth mix in each flask was decanted into individual petri dishes. This was done to ensure that the depth of the agar was the same in all petri dishes, as inconsistency could cause deviation in results. Agar plates were left to cool and solidify, and stored in a cool dry place for no longer than 24 hours.

100µl of the liquid bacteria (0.5 McFarland standard concentration) was pipetted onto each agar plate and spread using sterile techniques. Six plates were done at a time. Paper discs were placed on the bacteria-spread plates by using sterile techniques (five paper discs per petri dish). 7.5µl of the *C. officinalis* sample was impregnated on each individual paper disc, starting with petri dish one and ending with the sixth (therefore 30 discs). Each paper disc was left for 3 minutes, and then another 7.5µl was impregnated again on each disc. The petri dishes was then sealed with polyfilm and incubated for 24 hours at 37ºC. After the incubation period, the agar plates were removed and placed on a dark surface. The diameters of the zones of inhibition were measured by taking a horizontal and vertical measurement of each paper disc.

When the measurements were done for the samples that were diluted with distilled water and lower concentration ethanol than the stock solution of the mother tincture and herbal extract; it was found

that the zones of inhibition was milky in colour and not completely clear, therefore only the stock solutions of the mother tincture and herbal extracts were included in the results.

A microscope slide was prepared and the organism was identified after each new batch of *S. aureus* to ensure that there was no contamination by other organisms. Samples from the bacteria stock solution as well as bacteria from the agar plates that were incubated, were tested.

3.4 MIC (Minimum Inhibition Concentration)

All the mother tincture and herbal extract samples that were exposed to environmental conditions were used. As a control, one sample of the herbal extract and mother tincture which had not been exposed to any environmental conditions, was used for comparison.

Each sample was diluted five times from the crude sample. Each dilution was done 1:1 with Mueller Hinton broth, thereby concluding six concentrations for each sample. The Mueller Hinton broth and the McCartney flasks used for the dilutions, were autoclaved to ensure sterility. The dilutions and filling of the microtiter plates were done in a sterile laminar flow cabinet.

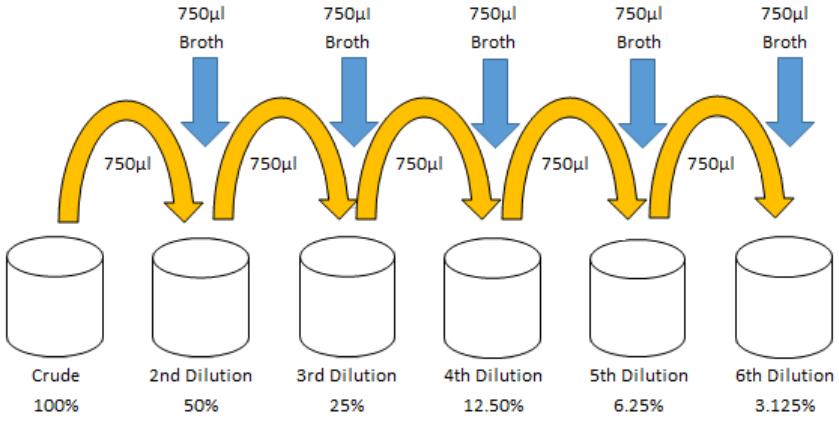

Figure 3.1: Dilutions of samples (1:1) with Mueller Hinton Nutrient Broth

The wells were loaded with the samples in a five-fold replication with the different dilutions as in Figure above. 100µl of the sample was added to 100µl of *S. aureus* in Mueller Hinton broth (0.5 McFarland standard concentration). This changed the dilutions as follows: the crude solution in the first row changed to 50%, the 50% dilution to a 25% dilution, the 25% dilution to a 12.50% dilution, the 12.50% dilution to a 6.25% dilution, the 6.25% dilution to a 3.125% dilution and then the 3.125% dilution to a 1.5625% dilution.

After all the wells were filled, the microtiter plates were covered and incubated at 37ºC overnight. The next day the microtiter plates were taken out of the incubator and 10µl of a Resazurin sodium

salt solution was added to each well, excluding those filled with distilled water. The plates were covered again and incubated for two hours at 37ºC. After two hours the plates were removed and colour changes were noted and photographed.

The mother tincture's concentration was not determined by the suppliers, therefore results was set as a percentage with the crude sample starting at 100% (50% after the bacteria was added). The Mediherb herbal extract was certified to have a concentration of 500mg/ml (Mediherb; 2013), therefore the first dilution in the microtiter plate was 250mg/ml.

3.5 Gas Chromatography Mass Spectrometry (GC/MS)

Due to cost and time constraints, only a few samples were used for the GC/MS to establish whether there is a change in the active ingredients when samples are diluted with polar and non-polar solvents and whether there is a difference in active ingredients when exposed to different environmental conditions. The differences between the mother tincture and herbal extract's active ingredients were also established. The following samples were tested: unexposed herbal extract, unexposed mother tincture, extract 3:7 dilution with 90% ethanol, extract 3:7 dilution with 20% ethanol, extract 3:7 dilution with DMSO, extract exposed in the UJ Dispensary in a blue bottle, extract exposed to direct sunlight in a blue bottle, extract exposed in the microwave scenario, extract exposed in the office scenario and extract exposed to decreased temperature.

For the GC/MS, 100µl of each sample was placed in individual vials; samples were dried and 1ml of 99% ethanol was added to the dried samples. Residue from the drying process were dissolved in the ethanol by rotation to have the vials ready for the GC/MS separation and analysis. The GC/MS instrument used for the separation of the different *C. officinalis* samples was a GC-TOFMS system (Pegasus 4D, Leco Corp., St. Joseph, MI) and the extraction was done with chloroform (analytical grade). The GC/MS was equipped with an Rxi-5Sil (1,4-bis(dimethylsiloxy)phenylene dimethyl polysiloxane; 30m x 200µm Restek (Bellefonte, PA) column with film thickness of 0.18µm. The oven program started off with a temperature of 50ºC for 30 seconds and then increased to 310ºC in increments of 10ºC per minute, and finally held at 300ºC for two minutes. The injection port's temperature was set at 225ºC and the detector at 280ºC. A pulse split injection mode was used with a 10:1 split at 30 seconds. The carrier gas flow rate was $1ml.min^{-1}$. The signal-to-noise (S/N) was set at 100 and a baseline was obtained just above the level. The peaks obtained in the study were identified by comparison with the NIST 08 Mass Spectral Library. Peaks below 700 (as set out on the ChromaTOF program), was noted as unknown. The identification of the compounds were done by matching them with the MS library

and they have not been confirmed by co-injection with authentic standards. A scan range of *m/z* 25 to 500 at the rate of 200Hz was used to confirm the mass fragment of the derivatives. The mass fragmentation patterns were identified by using ChromaTOF software, Version 4.22 (LECO Corp.). The GC/MS procedure was done in triplicate for all samples and the data noted was the average from the three independent tests.

4. RESULTS

4.1 Kirkby Bauer Disc Diffusion

In **Figures 4.1**, **4.2**, **4.3** and **4.4** below, the difference in measurements of the zones of inhibition can be viewed for the herbal extracts and the mother tinctures in both blue and amber glass bottles.

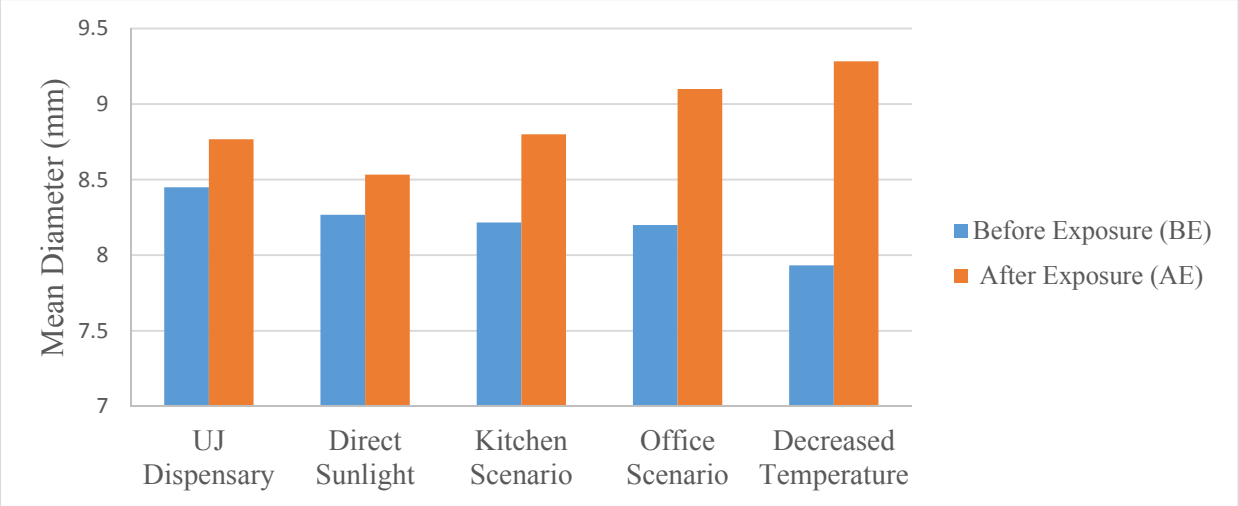

Figure 4.1: Mean inhibition diameters for the extract samples (blue bottles)

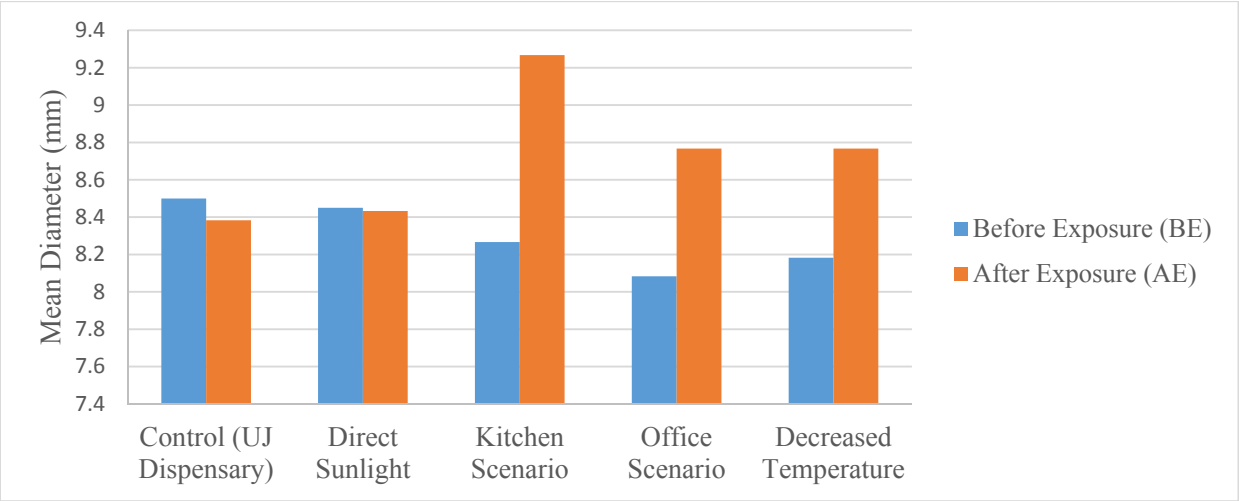

Figure 4.2: Mean inhibition diameters for each extract sample (amber bottles)

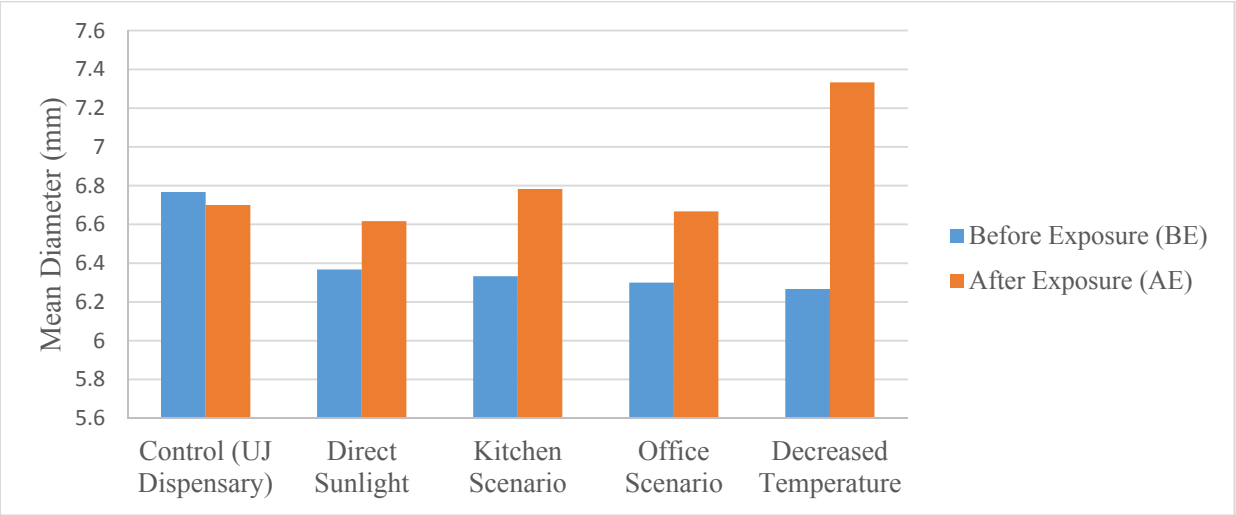

Figure 4.3: Mean inhibition diameters for the mother tincture samples (blue bottles)

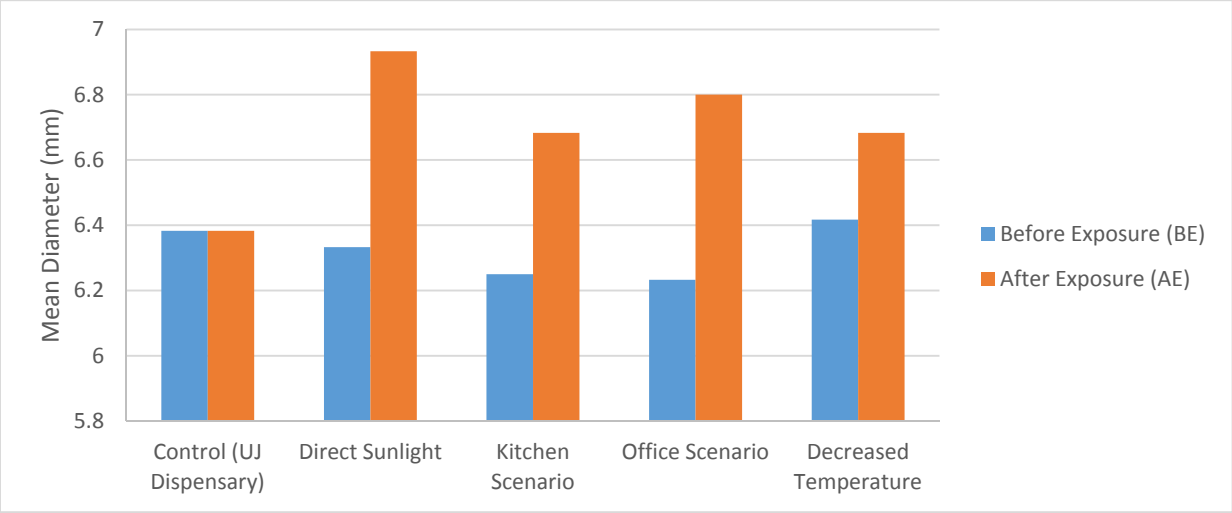

Figure 4.4: Mean inhibition diameters for the mother tincture samples (amber bottles)

4.2 Minimum Inhibition Concentration (MIC) and Gas Chromatography Mass Spectrometry (GC/MS)

The MIC and GC/MS tests were decided upon when the planned tests were not sufficient to explain certain results. The MIC tests were done on all the AE samples and as a control, unexposed samples were used. The GC/MS tests were used to establish whether possible changes can be established between the herbal extract and the mother tincture as well as between different dilutions solvents used.

Table 4.1: MIC values for samples in the blue and amber glass bottles

Plate	Side	Contents	Exposure	Bottle	MIC %	MIC Value
1	Left	Extract	None (Control)	Stock	6.25	31.25mg/ml
1	Right	Mother tincture	None (Control)	Stock	12.50	Unknown
2	Left	Extract	UJ Dispensary	Amber	6.25	31.25mg/ml
2	Right	Extract	Direct Sunlight	Amber	12.50	62.50mg/ml
3	Left	Extract	Kitchen	Amber	12.50	62.50mg/ml
3	Right	Extract	Office	Amber	6.25	31.25mg/ml
4	Left	Extract	Decreased Temp	Amber	12.50	62.50mg/ml
4	Right	Extract	UJ Dispensary	Blue	6.25	31.25mg/ml
5	Left	Extract	Direct Sunlight	Blue	12.50	62.50mg/ml
5	Right	Extract	Kitchen	Blue	6.25	31.25mg/ml
6	Left	Extract	Office	Blue	12.50	62.50mg/ml
6	Right	Extract	Decreased Temp	Blue	12.50	62.50mg/ml
7	Left	Mother tincture	UJ Dispensary	Amber	12.50	Unknown
7	Right	Mother tincture	Direct Sunlight	Amber	12.50	Unknown
8	Left	Mother tincture	Kitchen	Amber	12.50	Unknown
8	Right	Mother tincture	Office	Amber	12.50	Unknown
9	Left	Mother tincture	Decreased Temp	Amber	12.50	Unknown
9	Right	Mother tincture	UJ Dispensary	Blue	12.50	Unknown
10	Left	Mother tincture	Direct Sunlight	Blue	12.50	Unknown
10	Right	Mother tincture	Kitchen	Blue	25.00	Unknown
11	Left	Mother tincture	Office	Blue	12.50	Unknown
11	Right	Mother tincture	Decreased Temp	Blue	25.00	Unknown

5. DISCUSSION

5.1 Zones of Inhibition of Herbal Extract in blue bottles

The measured zones of inhibition is an illustration of the antibacterial properties the sample exhibits against *S. aureus.* The samples in the UJ Dispensary showed an increase in the antibacterial measurement, but results were not statistically significant. The samples exposed to Direct Sunlight in blue glass showed no statistical significant change. The samples exposed to the Kitchen Scenario, Office Scenario and Decreased Temperature, showed a statistical relevant increase in the antibacterial measurement.

5.2 Zones of Inhibition of Herbal Extract in amber bottles

Statistically, there was a difference in the groups exposed to the Kitchen Scenario, Office Scenario and to Decreased Temperature. The groups exposed in the UJ Dispensary and in the direct sunlight showed no statistically significant change. When referring to **Figure 4.2**, it can be visualised that there is a substantial change from the BE to the AE measurements for the groups exposed to the Kitchen Scenario, Office Scenario and the Decreased Temperature, whereas in the groups exposed to the UJ Dispensary and to Direct Sunlight, a very small, insignificant change can be seen. The BE values for the three groups that changed were more or less constant, with the highest difference in the AE group for the group exposed to the Kitchen Scenario, then the Office Scenario and then the group exposed to Decreased Temperature.

5.3 Zones of Inhibition of Mother Tincture in blue bottles

According to **Figure 4.3**, the increase in antibacterial measurements can be seen for all the groups except for the group exposed in the UJ Dispensary. For the four groups that had an increase, it can be seen that the BE measurements for each group had an average range of 6.2mm and 6.4mm. It then shows that the biggest difference was with the samples exposed to the Decreased Temperature followed by the samples exposed in the Kitchen Scenario, then by the samples in the Office Scenario and finally the ones exposed to Direct Sunlight. The samples in the UJ Dispensary group, remained the same.

5.4 Zones of Inhibition of Mother Tincture in amber bottles

There was not any statistical differences between the BE and AE for the samples exposed in the UJ Dispensary, but there was, however, a change in the antibacterial measurements for the samples exposed to Direct Sunlight, Decreased Temperature and in the Kitchen and Office Scenarios.

5.5 Dilutions and further testing

The original protocol for this study was to dilute each sample with different ethanol concentrations including 20% ethanol, 62% ethanol and distilled water. The proposed dilutions were a 1:10 dilution (general dilution) and a 3:7 dilution (as per the German Homeopathic Pharmacopoeia). In practise, 20% ethanol is often used as this is what is usually available in a homeopathic dispensary. The groups of samples exposed in the UJ Dispensary and to the Direct Sunlight were tested BE and AE. Due to the inconsistency of the diluted samples as well as the cloudiness of their zones of inhibition, it was decided to stop with the testing of the dilutions. This brought about the decision to experiment with a non-polar solvent such as DMSO (Dimethyl sulphoxide). Discs were prepared with autoclaved DMSO and no zones of inhibition were seen. When DMSO was used as

a solvent to dilute the *C. officinalis*, zones of inhibition were noted. These zones were clear whereas the diluted samples with the polar solvents (ethanol and distilled water) were cloudy. Due to the cloudiness of the zones of inhibition when the samples were diluted with a polar solvent, a diluted sample was tested on GC/MS to establish any change in active ingredients.

C. officinalis contains resin that is important for the healing and antimicrobial properties of the plant. The resin is found at the base of the flower heads and mainly protects the plant from insects and invading organisms (Blankespoor, 2012). Resins are generally not soluble in water and readily soluble in ethanol due to the consistency of the resin (Coppen, 1995). This might lead to the explanation for the dilutions with water that turns to a milky consistency delivering inconsistent zones of inhibition. Further research in this field is necessary.

This can bring difficulties for those mixing different herbal extracts and/or mother tinctures with each other, as the efficacy might be compromised. This can also pose a challenge when administering a herbal extract or mother tincture to a patient, as it is usually suggested to add a few drops of the extract or mother tincture to water before taken by the patient. This might be different for other plant extracts, therefore further research needs to be done.

5.6 Minimum Inhibitory Concentration (MIC)

Due to the different extraction methods used as well as the different plant parts that were used with the herbal extract and mother tincture, it can be expected that the two samples would not have the exact same properties and strengths. The Mediherb herbal extract was certified as having a concentration of 500mg/ml, but the concentration of the homeopathic mother tincture was not known, therefore the MIC can only be compared as percentages.

The two control samples were not exposed to any environmental conditions. The MIC for the control herbal extract was 6.25% and for the mother tincture it was 12.5%. This means that a minimum of only 6.25% of the herbal extract was needed to elicit an antibacterial (*S. aureus*) inhibitory response and a higher concentration of 12.5% of the mother tincture was needed to show the same response. Therefore it shows that the herbal extract has a stronger antibacterial inhibitory effect towards *S. aureus* than the mother tincture.

Roopashree *et al.* (2003) did a study on the antibacterial properties of a few herbal extracts including *C. officinalis*, also extracted with different solvents. The MIC was tested for all their samples, and it was reported that the *C. officinalis* extracted with ethanol had a MIC of 32-

64mg/ml. The dried flowers of the *C. officinalis* plant was used during the study. According to the results of the MIC on the control herbal extract, a 6.25% concentration is needed. When converting this to the actual concentration (stock concentration being 500mg/ml), it shows that the extract had a MIC of 31.25mg/ml, being well in the same region as the study done by Roopashree *et al.* (2003).

The herbal extract samples exposed to the UJ Dispensary (control environment) showed the same MIC than the unexposed samples, showing no difference in the samples after exposure to the control environment (concentration remained at 6.25%). The samples exposed to the Direct Sunlight needed an increased concentration to show MIC (concentration of 12.5% needed). The herbal extract sample in the blue bottle exposed to the Kitchen Scenario had the same MIC as the control sample. The sample in the amber bottle, however, needed a higher concentration of the extract to elicit the same response. Therefore the MIC increased to 12.5%, where the control and the sample in the blue bottle had a MIC of 6.25%. This shows that the blue bottle showed more protection against electromagnetic waves from a microwave than the amber bottle. Some suggestions have been made that amber glass prevents shorter wavelengths of light to pass through, including ultraviolet light. It is suggested that other colours of glass do not to offer the same protection (Wrap, 2015). It is also dependent on what is stored in the glass container as some compounds would absorb light and others wouldn't.

The herbal extract samples exposed to the Office Scenario also had a difference. The amber glass bottle showed more protection against the electromagnetic waves and radiation elicited from the Wi-Fi Router cell phones and computer devices. The sample in the blue bottle had a higher MIC (12.5%) than the sample in the amber bottle and the control (6.25%).Both herbal extract samples (in the blue and amber glass bottles) that were exposed to Decreased Temperature averaging at 4ºC had a higher MIC (12.5%) than the control (6.25%). This shows that the antibacterial properties of the samples exposed to a low temperature decreased, possibly due to degradation of the extract in the colder environment.

The control homeopathic mother tincture had a MIC of 12.5%. The samples that were exposed in amber glass bottles all had a MIC of 12.5% AE in the UJ Dispensary, Direct Sunlight, Kitchen Scenario, Office Scenario, as well as the Decreased Temperature. This shows an overall stability of the MIC of the mother tincture when exposed to different environmental conditions in amber glass bottles. The mother tincture samples exposed to different environmental conditions in blue glass bottles showed different results when compared to the samples in the amber glass bottles. The samples exposed to the UJ Dispensary, Direct Sunlight and to the Office Scenario remained

the same as the control mother tincture sample (MIC 12.5%). The samples exposed to the Kitchen Scenario and Decreased Temperature (averaging at 4ºC), however, had an increased MIC of 25%, showing that a higher concentration of the mother tincture was needed to elicit the same antibacterial action.

5.7 Gas Chromatography Mass Spectrometry (GC/MS)

The main aim of the GC/MS separation test was to establish a possible reason why the antibacterial measurements increased after exposure to different environmental conditions. Extract samples from the stock solution that were not exposed was used as a control for comparison purposes. An unexposed mother tincture sample was also tested. This allowed for the herbal extract and mother tincture to be compared, as both samples were the same species of *C. officinalis,* but extracted using a very different extraction method, showing that the extraction method is very important as is the parts of the plants that are used.

At the start of the study, when dilutions with 20% ethanol, 62% ethanol and distilled water were performed, it was found that the samples became milky and it was decided to exclude these samples from the Kirkby Bauer testing. With the GC/MS test, an extract sample (from the stock solution) was diluted with 90% ethanol (concentration of ethanol by which extraction took place), 20% ethanol and DMSO (non-polar solvent). The dilution was a 3:7 dilution, and the samples were separated on the GC/MS. The diluted samples were compared with each other and with the unexposed extract sample (stock solution - control). More separated compounds corresponded between the control sample and the sample that was diluted with the 90% ethanol than with the 20% and 62% ethanol.

The last comparison was between the exposed extract samples that were exposed to the different environmental conditions in blue glass bottles. Different compounds were identified between the samples exposed to different environments. Further research should be done, however, in the separation and analysis of compounds when exposed to environmental conditions.

6. CONCLUSION

It can be concluded from the Kirkby Bauer tests and the MIC tests that an environment with a constant temperature around 20º C away from direct sunlight and artificial lighting as well as any electromagnetic waves is a good environment to store herbal extracts and mother tinctures.

With the Kirkby Bauer tests it was proposed before the study that the antibacterial properties of the samples should either remain the same (no effect from environmental conditions) or become

less (degrading). With initial analysis it was thought that the antibacterial properties were enhanced but, when looking at the further testing, it was actually determined that the samples became unstable, rather than enhanced.

The herbal extract and mother tincture samples that were stored in the amber glass bottles showed better resistance to the Direct Sunlight than the blue glass bottles. The herbal extract samples in both the blue and amber glass bottles, however, showed better stability than the mother tincture samples. Therefore it can also be concluded that different herbal materials will react slightly differently towards environmental conditions – a possible reason for this would be the active compounds in the extract.

In the Kitchen and Office Scenarios definite changes took place regardless of bottle colour. It can be concluded that an increased temperature above an average of 21ºC with electromagnetic waves present degrades herbal extracts and mother tinctures. A decreased temperature of average 4ºC also has a degrading effect on herbal extracts and mother tinctures. An overall conclusion can be made that amber glass bottles offer more protection than blue glass bottles against various environmental conditions.

It can also be concluded that when one would like to dilute a herbal extract or mother tincture, the same solvent that was used during the extraction, should be used as this can also have an impact on the efficacy of the herbal extract or mother tincture.

It is suggested that more research should be done on different herbal extracts and mother tinctures when diluting them with different solvents. This would aid to establish whether it is safe for practitioners to mix herbal preparations, dilute them or for administration to their patients. More samples should be separated on GC/MS or other separation techniques after exposure to environmental conditions to establish whether new compounds are formed when exposed to different environmental conditions. A study to research the light passing through different colour glass and thickness of glass should be done to provide more insight.

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