Biology Exteded Essay

Investigating the antibacterial activities of methanol, water and hexane extracts of *Hypericum bityhnicum* on *Streptococcus pyogenes*

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Abstract

In the present study methanol (-*MeOH*), water (-H_2O), Hexane (*Hx*) extracts of *H. bithynicum*, are screened for their in vitro antibacterial activity.

The research question of the experiment is: "Is there a statistically significant difference among the Minimal Inhibitory Concentration (MIC) values of methanol, hexane and water extracts of *H. bithynicum* with regards to values on *Streptococcus pyogenes* in laboratory conditions*?"*

In this respect, the aim of this investigation is to scrutinize the bactericidal properties of the extract of *Hypericum Bithynicum* plant prepared with methanol, water and hexane solvents on the bacterium.

The hypothesis is that; there would be a statistically significant difference with respect to obtained Minimal Inhibitory Concentration values between the groups as; hexane and water extracts will bear greater antibacterial activity towards *S. pyogenes* when compared to methanol extract of *H. bithynicum.*

In order to examine the hypothesis and to find an answer to the research question, the Broth Dilution Method was used. For growing and diluting the bacterial suspensions, Mueller Hinton Broth (MHB; Difco) and Mueller Hinton Agar (MHA; Oxoid) were assigned, along with the utilization of microdilution method. After incubation of impregnated bacteria and extracts for 18 hours, the lowest concentration of the extracts that completely inhibited the macroscopic growth of bacteria (MIC) was indicated.

The data obtained showed that average MIC values of methanol extract is 63.28 μg/ml, hexane extract is 32.20 μg/ml and water extract is 30.06 μg/ml Consequentially, it was revealed that the antibacterial activity of water extract is stronger than that of hexane which displayed more effective antibacterial properties when compared to the methanol extract (since the group that had the lowest MIC values had greater antibacterial activity). ANOVA results were parallel with the

hypothesis of the experiment as, the difference between the extracts methanol and water was not statistically significant; whereas the pairs: methanol-water and methanol-hexane displayed significant mean difference in terms of their MIC values.

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I. Introduction

Plants and plant extracts have been used in skin disorder treatments for centuries among the public in many different forms, such as; drinking as tea, eating as paste, taking as pill or dissolving in oil. Each day, bacteria are becoming resistant to more antibiotics. Hence the use of plant extracts as antiseptics and antimicrobial agents captures more attention. There is increasing interest in using natural plants on resident oral bacteria and as antibiotic for eradication of pathogenic bacteria.

Being the most frequently used herbs in folk medicine, Hypericum belongs to the family *Hypericaceae*, and is a genus of approximately 400 species of flowering plants. All members of the genus are commonly referred to as St. John's wort. In folk medicine, St. John's wort, which is known to display strong antibacterial and antiviral properties, is used in the treatment of burns and wounds.[1]

"The little holes whereof the leaves of Saint John's wort are full, do resemble all the pores of the skin and therefore it is profitable for all hurts and wounds that can happen thereunto." ***William Coles (1626–1662)*** [2]

Among Hypericum genus, *Hypericum bithynicum* has not been projected to any practical regarding antimicrobial activities. However, like all other species of Hypericum genus, it is commonly involved in folk medicine especially for treatment of burns and wounds.

In Turkey, *H. Bithynicum*, is consumed in two ways: First one is; accompanied with olive oil in such a way that people dissolve the beneficial portion of the plant itself in olive oil, keep it in the oil for some time and apply it onto the wounds and burns. Secondly, it is usually taken as tea, hence in its dissolved form in water.

1. "Hypericum" Wikipedia, The Free Encyclopedia. 10.11.2011, 22:30 http://en.wikipedia.org/wiki/Hypericum
2. "Hypericum" 11.11.2011, 14:51 http://www.hypericum.com/hyp10.htm

One of the most ubiquitous pathogens that cause skin disorders is *Streptococcus pyogenes*, which is a Gram-positive bacterium. It is known to cause group A streptococcal and acute *Streptococcus pyogenes* infections in addition to some infections such as impetigo and cellulitis. Impetigo is a major infection observed in the superficial layers of the skin whereas cellulitis is the infection of the deeper layers of the skin. [3] Even though *S. pyogenes* fails to resist against penicillin, the recent increase in the number of strains that are resistant to current antibacterial agents, remarks that new antibiotic agents should be improved.

Severe forms of the aforementioned diseases have been detected around the world. As for Turkey, sequels of *S. pyogenes* infections are still an important problem and an increase in various invasive infections due to *S. pyogenes* is recently observed. Henceforward, the treatment of infections due to *S. pyogenes* is very significant in Turkish medicine.

Taking this information into consideration, three different solvents of *H. bithynicum* are prepared and compared in terms of their in vitro antibacterial activity so as to provide substantially impregnable antibacterial substance for the designated bacteria*: S. pyogenes*. These solvents were determined since; in scientific processes, methanol extract of the selected substances are prepared to test their antimicrobial effect on microorganisms. Water solvent is used due to its common use as tea. Additionally, hexane is used as a solvent with regards to the manner in which, public consumes *H. bityhnicum* in daily life as dissolved in olive oil. Since hexane is known as an good dissolver of oily substances, it is used to represent the form of *H. bithynicum* in olive oil.[4]

On the whole, it can be stated that this study is concerned with investigating whether the usage of the H. *bityhnicum* plant which is believed to have healing effects on wounds and burns among the public, is correct in the sense that it includes substances that are able to display greater activity and that are dissolved to higher amounts in water and in oily compounds. Therefore, the research of the

3. "Streptococcus pyogenes" 03.12.2011, 23:05
http://textbookofbacteriology.net/streptococcus.html
4. "Hexane Oil Resemblance" Wikipedia, The Free Encyclopedia. 07.12.2011, 21:04
http://en.wikipedia.org/wiki/Hexane

experiment is: “Does a statistically significant difference among the Minimal Inhibitory Concentration (MIC) values of methanol, hexane and water extracts of *H. bithynicum* exist with regards to values on *Streptococcus pyogenes* in laboratory conditions*?”*

Hence, in this study methanol (-*MeOH*), water (-H_2O), Hexane (*Hx*) extracts of *H. bithynicum*, will be screened for their in vitro antibacterial activity against standard strain of *Streptococcus pyogenes*.

II. Hypothesis

Even though St. John's-worts exhibit distinctive chemical properties, a detailed research concerning their content has not been carried out. Two main compounds of interest; hyperforin and hypericin, are proved to be found in all species under Hypericum genus and were subjected to multifarious studies. However, there are no further scientific studies related to any other components that *H. bithynicum* characteristically embodies. Its properties are caused by a wide range of interacting factors. For this reason, the hypothesis of this experiment will be established via the properties of *H. perforatum*, which bears plentiful resemblance to *H. bithynicum* in terms of both its healing effects and the ways in which it is used by the public (as tea and oily form).

H. perforatum may be taken as pills or as tea in its effective form. Few standardized preparations are available, and alcoholic extracts have been studied in the concerned researches. A common use of *H. perforatum* is oily extract as it is reported to be strongly antibiotic alongside assisting healing of wounds, concussions and first-degree burns. Both hypericin and hyperforin, that are chemical compounds of St. John's wort, are known to display antibiotic properties. Consequentially, due to their wide range of usage among the public; water and hexane can be predicted to be better solvents for *H. bithynicum* when compared to methanol. As William Coles have stated in 17th century, Hypericum is known to display brilliant antibacterial properties when dissolved in oil-like substances and water. [5]

In this respect, the hypothesis of this study is that there will be a significant difference in terms of antibacterial activity between the methanol and water, hexane extracts of *H. bithynicum* on *S. pyogenes*. The extracts prepared with water and hexane will display greater antibacterial activity when compared to the methanol extract. However, supposedly it will not be possible to attain a statistically significant difference between the antibacterial activities of water and hexane extracts.

5. "Hypericum" 06.12.2011, 21:30
http://www.hypericum.com/

III. Method Development and Planning

This study is concerned with the comparison between the *in vitro* antibacterial activities, of H. *bithynicum* that are attained from three different solvents: methanol *(-MeOH*), water *(-H_2O*) and hexane (*Hx*). In vitro activity is associated with the experiments using components of any organism that have been isolated in order to allow for a more detailed and precise analysis.

When deciding on the plant species, the fact that *H. bithynicum* is a plant which hasn't been subjected to many studies before is taken into consideration. Also, its frequent utilization among the public, was significant in terms of interrogating whether the way of using this plant in healing processes of wounds and burns is correct.

S. pyogenes, which is determined as the testing microorganism, is a Gram positive bacterium. Plant extracts are generally more active against Gram positive bacteria rather than Gram negative bacteria.[6] Additional to this fact, *S. Pyogenes* is chosen with regards to its concurrency with the healing effects of *H. bityhnicum* (burns and wounds).

In this respect, Broth dilution is used in determining the antibacterial effects of the different extracts on the bacteria S. *pyogenes*. The minimum inhibitory concentrations (MIC) of the extracts that are applied on the bacteria are calculated and compared.

In Broth dilution, generally determined via using the 96-well microtiter plate process, bacteria are inoculated into a liquid growth medium where it is exposed to different concentrations of an antibacterial agent. For a certain period of time varying between 16 to 20 hours after incubation, bacterial growth is determined and finally the MIC value is read. [7]

6. "Gram Poisitive Bacteria", 05.12.2011, 21:05
http://journals.tubitak.gov.tr/biology/issues/biy-05-29-4/biy-29-4-3-0506-4.pdf
7. "Broth Dilution", 08.12.2011, 10:45
http://www.ncbi.nlm.nih.gov/pubmed/18274517

Minimum inhibitory concentrations (MICs) are widely used in researches concerning the *in vitro* activity of new antimicrobials, and in some cases, are used by diagnostic laboratories to confirm resistance. MIC value can be defined as the lowest concentration of an antimicrobial agent that is observed to inhibit the visible growth of a microorganism after overnight incubation. [8]

As a result, since the MIC value denotes the minimal concentration that is enough for inhibiting the microorganism's activity, the smaller the MIC value of an extract is, the more effective the extract in that solvent turns out. Hence, the dependent variable in this experiment is the calculated MIC value of each extract while the independent variable is the type of solvent used.

I have achieved settlement on this method regarding several issues. First one is the statistical data analysis provision. Since quantitative data that is applicable for statistical analysis permits concluding whether there is a statistically significant difference between different extracts of *H. bityhnicum*, the data obtained via this method is significant for this study.

However, there are two other methods that are used in several studies in order to determine antibacterial activity which also allow for statistical analyses: First one is Agar Dilution method which involves the incorporation of different concentrations of the antimicrobial substances into a nutrient agar medium followed by the application of a standardized number of cells to the surface of the agar plate. Second one is Epilsometer test (Etest) which basically works as creating circular microbial inhibition zones when applied to inoculated agar plates, thus constitutes a predefined and continuous concentration gradient of different antimicrobial agents.[9]

8. "Minimum Inhibitory Concentration", 05.12.2011, 20:38
http://www.abbiodisk.com/pdf/pi/75002206.pdf
9. 05.12.2011, 22:42
Antimicrobial Chemother. (2001) 48 (supply 1): 5-16. This article appears in: Antimicrobial Susceptibility Testing: BSAC Working Party Report

Among these methods, I have chosen Broth Dilution method based on two issues: Firstly, the other methods are applied to substances with known antibiotic properties. Since antibiotic activity of H. bityhnicum is not proven before, these methods are invalid for this study. Secondly, according to the Clinical and Laboratory Standards Institute (CLSI), Broth dilution is the most reliable method in the sense that substances with different molecular weights, solubility, or chemical structures can be precisely used. Since miscellaneous plant extracts possess dissimilar molecular structures, Agar Diffusion brings about the possibility that some of the substances may not diffuse, thus yield inaccurate results.

Another issue that has to be taken into consideration while conducting this study, is the type of bacterial medium that will be used. Müller-Hinton Agar and Mueller Hinton Broth which are microbiological media that are widely utilized to determine the sensitivity of several pathogens towards antibiotics, are used during antibacterial activity tests. [10]

The bacterial suspensions used for inoculation (Mueller Hinton Broth and Mueller Hinton Agar), are prepared at 10^5 CFU (colony forming unite/ml) by diluting fresh cultures at McFarland 0.5 density (10^8CFU ml^{-1}), which is the standard procedure. [11]

Note that it is important to indicate that the bacteria that are used throughout the experimentation process should not be resistant against any bactericidal agents concerning this study.

Volumes of culture suspensions and plant extracts are equally inoculated into all the wells of the micro plate (10µl). The micro dilution method is also employed identically so that the concentrations of each extract decreased equivalently.

10. “Mueller-Hinton Agar” 05.12.2011, 22:30
http://www.mikrobiyoloji.org/genelpdf/920020264.pdf
11. The standard procedures are taken from: “A study of cytotoxicity of novel chlorokojic acid derivatives with their antimicrobial and antiviral activities” – Aydemir MD and Özçelik 2011, 11.09.2011

Two major variables that should be kept constant during the study are; the temperature of the laboratory (environmental temperature) and the inoculation time. Keeping these variables constant is important in the sense that they both affect the growth of bacteria, which is apparently directly proportional to the dependent variable of the experiment, the MIC values. Bacteria have varying requirements in terms of the range of temperatures in which they will grow. However, most pathogens, which are known as mesophiles, grow at temperatures between 5°C and 63°C, commonly referred to as the growth or "danger" zone and have an optimum temperature for growth of about 37°C. Most pathogens become inactive (dormant) at low temperatures and as the temperature rises, they start to multiply more rapidly. S. pyogenes is classified under the mesophilic microorganisms therefore; it can grow within the temperature range 5°C and 63°C, with an optimum of 35ºC-37ºC. Also, in ideal conditions (i.e. in moist foods at 37°C) bacteria will grow and multiply by dividing into two every 20 minutes. After 6 hours, in ideal conditions, one bacterial cell could become 131.072 bacteria. Hence, during antibacterial activity tests, inoculation time could be adjusted within the range of 18-24 hours. [12]

In this respect, in order to standardize these conditions, the bacteria are kept in the incubator during inoculation time. The conditions in the incubator must be kept constant for all trials so as to prevent confronting any factor other than independent variable. In this respect, for all groups and trials, the sealed micro plates are kept in the incubator with identical conditions; 35ºC, for equal time intervals; 18 hours.

While working on this research, to provide the aforementioned lab conditions and to be able to work with bacteria, I studied in the laboratories of Gazi University Pharmacy Faculty and received support on account of treating the bacteria and using the lab tools such as Micropipettes and Rotavapor.

12. "Optimum Temperature for Bacterial Growth" 22.02.2012, 22:23
http://archive.food.gov.uk/hea/teachers/plainenglish/part2.html

Materials that are used in the experiment:

- 200 mL MeOH
- 200 mL Distilled Water
- 200 mL Hexane
- 6 x Standard sized filter papers
- Hypericum bithynicum plant (15 grams in total)
- Rotavapor
- Mueller Hinton Broth (MHB; Difco)
- Mueller Hinton Agar (MHA; Oxoid)
- Pure strain non-antibiotic resistant *Streptococcus pyogenes*
- 96-Well Standard Micro Plate
- Micropipette
- Heraeus series 6000 Incubator

IV. Method

Preparation of the Extracts

- For hexane and methanol extracts; 100 mL of solvent is poured over 5 grams of squeezed *H. bithynicum*. After 24 hours, the solution is filtered and via adding 100 mL of the solvents to the remainders, the action is repeated once more.
- Obtained residues are collected respectively in labeled conical flasks. Under low pressure and at 45 ^{o}C, the filtrates are subsequently evaporated via using Rotavapor, which is a laboratory instrument that is used in the evaporation of solvents under vacuum, whether at a single-stage or as straight distillation. [13]
- For the water extract, since it requires more time to evaporate same amounts of solutions with water, 75 mL distilled water is added to 5 g squeezed plant material twice similar to the other extracts.
- The filtrate is evaporated through lyophilization which is a process that extracts the water from any substance so that the substance remains stable on its own at room temperature. [14]

Preparation of Microorganism Culture

- Antibacterial activity tests are carried out against standard (ATCC; American type culture collection). As standards; gram-positive strains of *Streptococcus pyogenes* (ATCC 13615) are used for the determination of antibacterial activity.
- For growing and diluting of the bacteria suspensions, Mueller Hinton Broth (MHB; Difco) and Mueller Hinton Agar (MHA; Oxoid) are utilized. The bacterial suspensions used for inoculation, are prepared at 10^5 CFU by diluting fresh cultures at McFarland 0.5 density (10^8CFU ml^{-1}). [15]
- Suspensions of bacteria are added in each well of the diluted extracts, density of 10^5cfu/ml.

13. "Rotavapor" 07.12.2011, 21.40
http://www.erowid.org/archive/rhodium/pdf/rotary.evaporator.pdf
14. "Lyophilization" 07.12.2011, 21.50
http://www.lyo-san.ca/english/lyophilisation.html
15. The standard procedures are taken from: "A study of cytotoxicity of novel chlorokojic acid derivatives with their antimicrobial and antiviral activities" – Aydemir MD and Özçelik 2011, 11.09.2011

- The micro dilution method is employed for antibacterial activity test. Media are placed into each 96 wells of the micro plates. Extract solutions at 512 µg ml^{-1} are added into first rows of micro plates and two fold dilutions of the compounds (256-0.125 µg/ml) are made by dispensing the solutions to the remaining wells. 10µl culture suspensions are inoculated into all the wells. [16]
- All organisms are projected to five trials throughout the study. The sealed micro plates are incubated at 35ºC for 18 hours. Methanol, hexane, H_2O, pure microorganisms and pure media were used as control wells.
- The lowest concentration of the extracts that completely inhibits the macroscopic growth is determined and the minimum inhibitory concentrations (MICs) are reported. The determined MIC values are further studied in upper and lower doses which range from 1 to 4µg/ml.

16. The standard procedure of microdilution are taken from the article: "A study of cytotoxicity of novel chlorokojic acid derivatives with their antimicrobial and antiviral activities" – Aydemir MD and Özçelik 2011

V. Results

- The table below denotes the antibacterial activity of each extract (methanol, water and hexane) of *H. bithynicum* by means of the obtained Minimum Inhibitory Concentration values of each group.

Table 1: The values of Minimum Inhibitory Concentration (MIC) obtained for each extract after 18 hours of incubation of S. pyogenes

MIC values of Each H. Bithynicum extracts (µg/ml).			
Extracts / Trials	**-MeOH**	**-H_2O**	**-Hexane**
n1	64.0	32.0	32.0
n2	66.0	28.9	36.4
n3	58.2	32.8	30.4
n4	64.2	28.0	30.0
n5	64.0	28.6	32.2

VI. Data Analysis

The formulas below are used to determine the statistical values of obtained data.[17]

Mean:

$$\bar{x} = \frac{1}{n} \cdot \sum_{i=1}^{n} x_i$$

where;

n is the trial number (in this case, it is 5)

x_i is the MIC value for the trial number i.

Standard Deviation:

SD= $$\sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \overline{x})^2} = \sqrt{\frac{1}{N}\left(\sum_{i=1}^{N} x_i^2\right) - \overline{x}^2}.$$

where,

n is the trial number (in this study, it is 5)

x_i is the MIC value for trial number i

is the mean for the selected group

Standard Error:

$$SD_{\bar{x}} = \frac{\sigma}{\sqrt{n}}$$

where;

n is the trial number (in this study, it is 5)

ẍ is the mean for the selected group

ϭ is the standard deviation calculated above for the corresponding group

Variance:

V(x) = $$\sigma^2 = \frac{1}{N}\sum_{i=1}^{N}(x_i - \mu)^2$$

where;

n is the trial number (in this case, it is 5)

17. The shematic demonstrations of the formulas are taken from:
http://en.wikipedia.org/wiki

μ is the mean of the related group

x_i is the MIC value for trial number i

σ is the standard deviation

Table 2: Calculated sum, mean, standard deviation, variance and standard error values along with the count of trials given for each type of extract

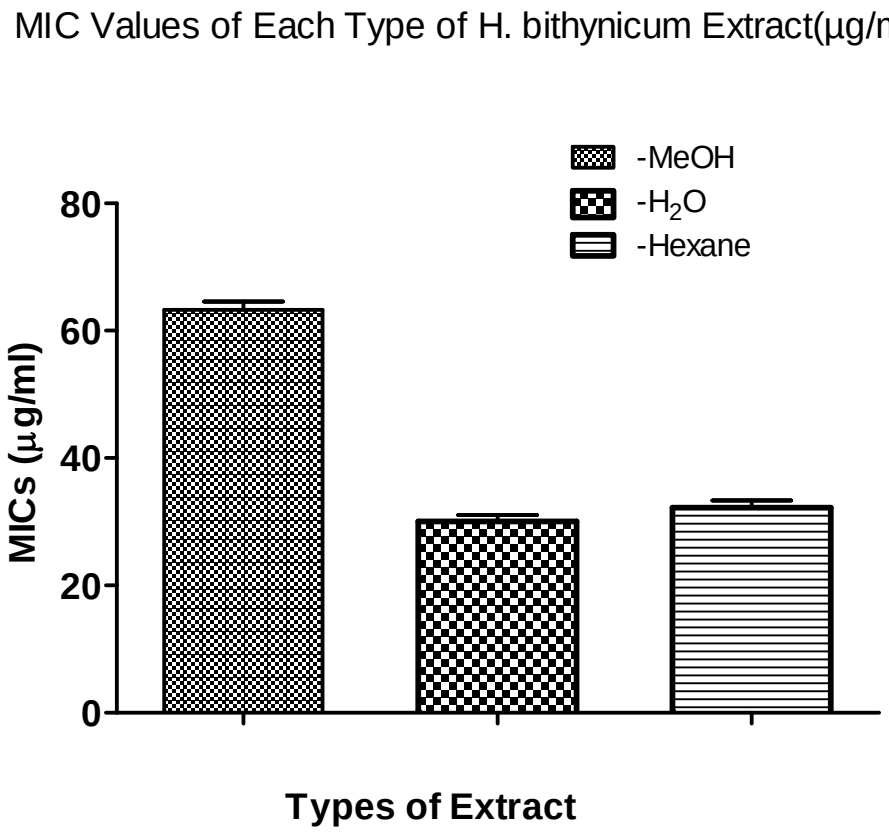
Extracts	***Count***	***Sum***	***Average MIC Value* (μg/ml).**	***Variance***	***SD***	***SE***
-MeOH	5	316.40	63.28	8.76	2.96	1.32
-H_2O	5	150.30	30.06	4.75	2.18	0.97
-Hexane	5	161.00	32.20	6.45	2.54	1.13

Table 3: Single Factor Analysis of Variance (ANOVA) results given for each extract

Source of Variation	***SS***	***df***	***MS***	***F***	***Number of Groups***	***p-value***	***Existence of Significant Difference (p < 0.05)***	***R squared***
Between Groups	3457		1728	259.7	3	P<0.0001	Yes	0.9774
Within Groups	79.85	12	6.654					

Table 4: Newman-Keuls: Multiple Comparion Test results given for each pair of extracts. The data below is obtained via using GraphPad Prism

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?
-H_2O vs –MeOH	-33.22	28.80	Yes
-H_2O vs –Hexane	-2.140	1.855	No
-Hexane vs –MeOH	-31.08	26.94	Yes



Graph 1: The comparison between the MIC values of each type of extract; methanol, water and hexane. The error bars are arranged based on the standard errors of each group

VII. Evaluation

The aim of this study was to evince whether there is a statistically significant difference in terms of antibacterial activity between the methanol, water and hexane extracts of *H. bityhnicum* on *S. Pyogenes* in standard lab conditions. Within the frame of this aim, the hypothesis has been established as; there would be a significant mean difference between the pairs: methanol and hexane, and methanol and water; whereas water and hexane extracts would not diverge in terms of their mean values. Hence, the antibacterial activity of water and hexane extracts against *Streptococcus pyogenes,* would be considerably greater than that of methanol extract, in light of the colloquial utilization of H. *bityhnicum* in public.

The results obtained supported the hypothesis as water and hexane extracts displayed stronger antibacterial effects compared to the methanol extract. The MIC values of methanol extract ranged between 58.2 and 64.2, with a mean value of 63.3; water extract between 28.0 and 32.0, having a mean of 30.1; last but not least hexane ranged between 30.0 and 36.4, with a mean of 32.2. Note that these ranges are typical of Broth Dilution Method.

My null hypothesis was that there will not be any statistically significant mean difference between the methanol, water and hexane extracts with respect to their Minimal Inhibitory Concentrations (MIC values) that are obtained through applying Broth Dilution Method on *S. pyogenes*. My alternative hypothesis was that there would be a statistically significant difference between the extracts with respect MIC values. Regarding the results obtained in Table 3, p-value that is computed via One-way ANOVA, was smaller than 0.05. Hence, the null hypothesis is rejected. Apparently, a statistically significant difference exists between the methanol, water and hexane extracts with regards to their MIC values.

As for making comparison within the groups, Newman-Keuls: Multiple Comparion Test, which demonstrates difference of mean values in pairs among the group, was carried out and the results were congruent with the hypothesis of the study. (See Table 4) P-values obtained from the comparison between methanol-hexane and methanol-water, were smaller than 0.05. Nevertheless, the p-value of the pair hexane-water was larger than 0.05. Consequentilally, the hypothesis of this experiment, that is stated above, is supported by exprimental evidence.

Even though the hypothesis is confirmed, one asset of the results seems to be unexpected. Considering the mean values calculated and demonstrated in Table 2, the antibacterial activity of the extracts can be juxtaposed as; Antibacterial effect of H. bityhnicum in water solvent is larger than that of hexane, and the least activity is observed in the methanol extract. Therefore, regarding a comparison between water and hexane extracts; the results obtained indicate that water extract has higher antibacterial properties than hexane extract.

Although there major errors were not confronted in the process of the experiment, as in all experiments, there were several factors that have possibly affected the results. These issues, along with corresponding suggestions are given below:

1. The resemblance between the skin and the nutritional agar used. The Mueller Hinton nutritional agar that is used throughout the antibacterial activity tests imitate the human skin, which exhibits the medium for bacteria to settle and replicate in practical. The utilization of Mueller-Hinton nutritional agar is necessary for this experiment; however it does not expose the conditions that bacteria are confronted with in daily life. Via applying in vivo studies, the healing effect of *H. bithynicum* in different solvents could be detected

through regarding multifarious parameters. For instance, in case of existence of epitelizav-sikatrizanite activity (which is present in Bephanten), it is revealed that the treatment of wounds and burns could be carried out much faster.

2. The extent of filter papers' impregnation by the solutions. Even though unlikely, during the extraction method, one solution may be absorbed to higher extent, which would affect the results as some portion of the antibacterial agent might have left in the filter paper, which will result in attaining lower antibacterial activity. Further filtration materials such as Belt filter, Rotary vacuum-drum filter, Cross-flow filters, Screen filter could be used instead of filter papers. [18]
3. Variety of testing bacteria. This study is only valid for the bacteria Streptococcus pyogenes. In order to extend the scope of the experiment, the three extracts could be implanted on different bacteria types which cause skin disorders. Since antibacterial activity of *H. bityhnicum* is observed against S. pyogenes, the possibility of observing activities and comparing the antibacterial properties of plant extracts with different solvents is apparently very high. *S. pyogenes* is a Gram positive bacterium. Additional to more samples of gram positive bacteria, gram negative bacteria types could be used in the experiments. Some examples for the bacteria that may be used to extend this study would be; *Escherichia coli* and *Pseudomonas aeroginosa,* which are gram-negative bacteria, *Staphylococcus aureus, Staphylococcus epidermitis and Streptococcus pneumoniae,* which are gram-positive bacteria.
4. Variety of pathogen type. Since *H. bityhnicum* is related to skin disorder treatments, some microorganisms other than bacteria could be used in the experiments. For instance, some fungus that can be used in antifungal tests would be; *Candida albicans, C. parapsilosis*, and *C. krusei*. Furthermore, these extracts can be applied to dermatophites. [19]

18."Methods of Filtering Solutions" 15.02.2012, 00:20

http://en.wikipedia.org/wiki/Filtration#Methods

19. "Fungus" 08.12.2011, 20:50

http://www.mikrobiyoloji.org/TR/Genel/BelgeGoster.aspx?F6E10F8892433CFFA79D6F5E6C1B43FFF266E5F7BA2C37A9

VIII. Conclusion

The research question of this study was: "Is there a significant mean difference among the methanol, hexane and water extracts of *H. bithynicum* with regards to Minimal Inhibitory Concentration values on *Streptococcus pyogenes* in laboratory conditions*?"*

Through obtaining data via Broth Dilution method, determining the minimal inhibitory concentrations of the extract in each solvent, and applying statistical data analyses (ANOVA) to this data, the settlement of conclusion to this investigation topic is achieved despite the fact that the experiment can be repeated for several times to provide more accurate results.

This study was significant in the sense that it investigated whether the utilization of *H. bithynicum* among the public was accurate. In light of the information attained from this study, via the supply of further antibacterial testing methods, the plant *H. bithynicum*, which is widely involved in folk medicine especially in Turkey, could be proven to be an antibacterial substance, and converted into a pharmacological compound, either in the form of pomades and ointment, or pills. This utilization of *H. bityhnicum* would even be more significant as nowadays, bacteria are developing resistance to antibiotics. Hence, each day it is getting harder to find suitable treatments for bacterial infections and these kinds of plant extracts are becoming new interest as antimicrobial agents.

In this respect, supplementary studies can be carried out in the plant extract in which higher antibacterial activity are observed. Via preparing one or two main extracts that represent the plant extracts in which antibacterial activity has been observed, the antibacterial agent within the plant extract can be identified. The antibacterial factors can be fractioned using column chromatography. Several acquired subfractions could be purified and higher active fractions can be further fractioned with solvents having different polarities until a thoroughly purified substance is obtained. After obtaining the purified component of the plant extract, through applying several other tests concerning the side effects and possible risks that these agents may lead into, the substance can be

put forward, if still appropriate, ready to bring about an augmentation of a solution, pomades or ointment. [20]

20. 08.12.2011, 22:20
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