THE CANADA SCIENCE FAIR JOURNAL

ARTICLE

Biology

Inhibition of bacteria growth with different concentrations of Canadian honey from honeybees (Apis mellifera) in distilled water, and implications on wound healing.

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Honey has long been used in traditional medicine as a topical treatment for wounds in countries across the world, as it is known to accelerate healing (Bucekova et al., 2017), help reduce odour, edema (swelling), exudate, (fluids from wound) and scarring (Blair et al., 2009). This is due to properties such as high viscosity, low pH, and high sugar content (Hossain et al., 2022). Although the composition of honey varies considerably based on the habitat of the honeybees (*Apis mellifera*) and the pollen used, it is typically composed of around 80% carbohydrates, 17% water, and 3% other compounds such as proteins and enzymes (Strelec et al., 2018). For example, an antibacterial compound – which causes what is known as non-peroxide activity - is methylglyoxal. This is found in high concentrations in honey from Australia and New Zealand (Mandal & Mandal, 2011).

Honey inhibits the growth of microorganisms by exerting osmotic pressure on bacteria which causes dehydration. The high osmolarity also helps absorb inflammatory exudates, thereby speeding up the healing process (Molan & Rhodes, 2015). Although the low water content of honey allows it to be a useful topically, it is more notably used for its antibacterial qualities because it contains hydrogen peroxide (Brudzynski et al., 2011); one of the products of the process of glucose oxidation. However, because glucose oxidation requires honey to first be diluted in order to be activated (Alshareef et al., 2022), the correlation between the water content in honey and the effectiveness of its antibacterial properties has become a point of discussion.

Honey is highly acidic and usually has a pH between 3.2 and 4.5 (Molan, 1992). When diluted to below 50%, the acidity decreases (Molan, 1992). This allows the enzyme glucose oxidase to activate, which has an optimal pH range at around 4-6 (Molan, 1992). Hydrogen peroxide and gluconic acid are then produced out of glucose by using oxygen molecules and vitamin B2 as cofactors; this process is known as glucose oxidation. The natural preservative hydrogen peroxide, stops bacteria growth and cell division by inflicting oxidative damage on the cell wall, DNA, and proteins. (Brudzynski & Lannigan, 2012) Hydrogen peroxide by itself is not typically used for open wound treatment as it is rapidly degraded by the enzyme catalase and also causes damage to healthy cells. However, honey can be used instead since it allows for a slow release at much lower concentrations (International, n.d.).

Due to being the third most common cause of bacterial infection in wounds (Puca et al., 2021), the experiment will be using *Escherichia coli*. Multidrug resistance in *Escherichia coli* has become an increasing threat because of overuse and improper prescriptions (Mohsen et al., 2020). This is a problem as it can lead to serious infections and in more severe cases, death (*Antibiotic resistance, 2020*). *Escherichia coli* also represents more than half of gram-negative bacteria (classification of bacteria that do not become violet in color with Gram staining) infections that occur in the bloodstream, such as surgical wound infections and increases the difficulty in treating them. (*NHS England » Preventing Healthcare Associated Gram-Negative Bloodstream Infections (GNBSI)*, n.d.) Since antibiotic resistant *Escherichia coli* infections have been reported to be sensitive to the antibacterial activity of honey (Saeed et al., 2009), further research should be done in integrating honey into future applications surrounding bacterial infections in wound care.

Previous papers suggest that the dilution level of honey is important in determining its antibacterial strength. Albaridi (2019), found that the ideal concentration against bacteria was between 30%-50% concentration of honey. The minimum inhibition concentration for *Escherichia coli* of honey was also observed to be around 6%- 8% (Molan & Rhodes, 2015) although when below 30%, there was a low affinity between glucose and glucose oxidase (Lehmann et al., 2019). However, Molan (1992) found that increasing dilutions positively correlated with increased potency of honey.

MATERIALS AND METHODS:

Materials

Paper towels, Tape, [8] 50ml beaker, Incubator and Hot plate, [30g] Unpasteurized Billy Bee Honey, Micropipette and disposable heads, Sealable and

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disposable bag, Marker , Tweezer, [6] nutrient agar plates (diameter = 10cm), [200 ml] Distilled water, Waste beaker, [10ml] tube of MG1655, *Escherichia coli*, [50 ml] 70% ethanol, Scoopula and stirring rod, Bunsen Burner and match, [30] Paper disks, Test tube rack, Apron and Goggles, 200 ml beaker, Scale, Disposable gloves, Metal L shaped cell spreader, Saran wrap

Manipulated Variable:

Concentration of honey in distilled water, in weight to volume percent: 25%, 30%, 35%, 40%, 45%, 50%.

Responding Variable:

Diameter of zone of inhibition (mm ± 0.5 mm) of *Escherichia coli* bacteria on agar plates, that are created by paper disks submerged in different honey concentrations.

Controlled Variables:

Table 1: Controlled variables for experiment, method and importance.

Methods

Distilled water should be sterilized before diluting honey (Molan, 1992). This is to prevent contamination and other bacteria from growing. When not in use, nutrient agar plates and liquid bacteria culture were stored overnight in the fridge at 4°C. This is to prevent dehydration of the agar and rapid bacterial growth (Thiel, 1999). Agar plates should be placed upside down when incubating to prevent moisture from gathering onto the surface of the agar plate which causes mixing with the honey and bacteria (Symington & Scholar, 2015). The incubation temperature should be at 30.0°C +/- 0.1°C, which is within the optimal *Escherichia coli* growing temperature of 21°C to 49°C (Ferrer et. al, 2003).

The range of concentrations used were based on previous experiments, as stated above. No

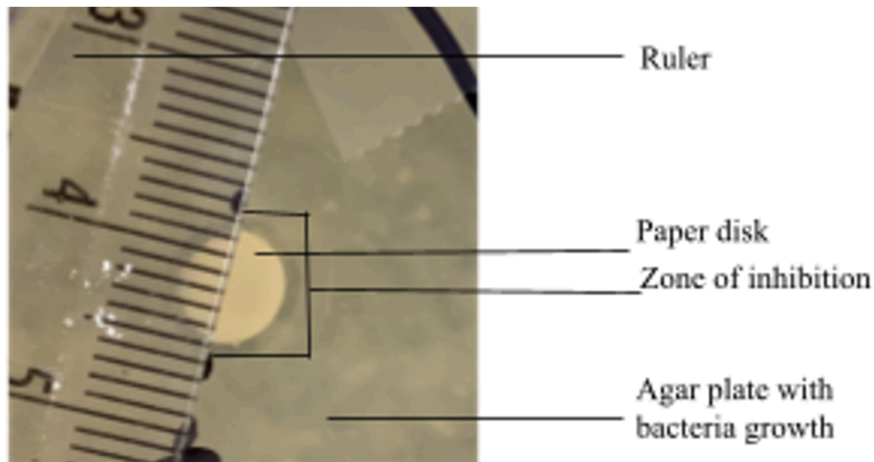

Figure 1: How diameter of zone of inhibition of E.coli is measured using ruler, for the 4th trial of the 50% concentration, after incubation. Photo by me. 1/27/23.

Figure 2: 12.00pm in school. The set up of beakers at the lab station. Photo by me. 1/24/23.

concentration higher than 50% is used to reflect the natural dilution of honey with bodily fluids when applied to wounds (Molan & Rhodes, 2015).

The disk diffusion method (Hossain et al., 2022) will be used in the experiment. It is used as a simple method to measure the effectiveness of an antibacterial compound. 6.0 mm sized paper disks are thoroughly dipped in different concentrations of honey and will be placed onto an agar plate with growing bacteria. As the honey diffuses through the agar plate, it will cause the surrounding area to inhibit bacterial growth. The diameter without bacterial growth will be measured in mm; the stronger the solution is and more susceptible the bacteria is to the solution, the larger the diameter (also called the zone of inhibition or ZOI) will be. Areas with faded and partial bacterial growth are not measured as part of the ZOI (Albaridi, 2019).

For statistical analysis, the mean of diameter will be found for each concentration and graphed.

One way ANOVA (a type of statistical test) will be calculated to determine if there is a statistical difference between the groups. Standard deviation will be used to evaluate reliability of data.

Procedure

Washed hands with soapy water. Ensured the area is safe: wore apron, safety goggles, and disposable gloves. Tied back long hair and tucked in loose clothes if necessary. Made sure the working area did not have any hazards such as spilled substances. Disinfected table and all tools with 70% ethanol and paper towel. Identified fire safety tools such as extinguishers and fire blankets. Took eight 50 ml and one 200ml beaker and wiped thoroughly with 70% ethanol and a paper towel to sterilize.

First boiled 200 ml of distilled water in a 200ml beaker labeled with distilled water, on a hot plate. After 3 minutes of visible boiling, a sheet of tin foil was placed over the beaker to let it cool. I then set up a station using 8 sterile 50 ml beakers with tape labeled each concentration of honey (25%, 30%, 35%, 40%, 45%, 50%). Labeled one with 70% ethanol and the other with honey. Squeezed out honey in the honey beaker. Pour 25 ml ± 0.5ml of 70% ethanol into a 50 ml beaker labeled with “70% ethanol”. Dipped the scoopula and stirring rod into the 70% ethanol and wiped clean. Put the 25% labeled beaker onto a scale and measured tare. Scooped 2.50g ± 0.01g of honey into the beaker of water on the scale using scoopula. Poured 10.00ml± 0.5ml of distilled water into the beaker and stirred well with the rod. Repeated this for each beaker and the respective concentration (eg. 3.50 ± 0.01g of honey and 10.00 ml ± 0.5ml of distilled water to create the 35% concentration, refer to table 3) until all the beakers were filled. Refer to figure 2 for set-up.

Labelled 6 agar plates with 25%, 30%, 35%, 40%, 45%, 50%. Turned to the back side. Used a marker and divided the agar plate into five equal sections. Labelled

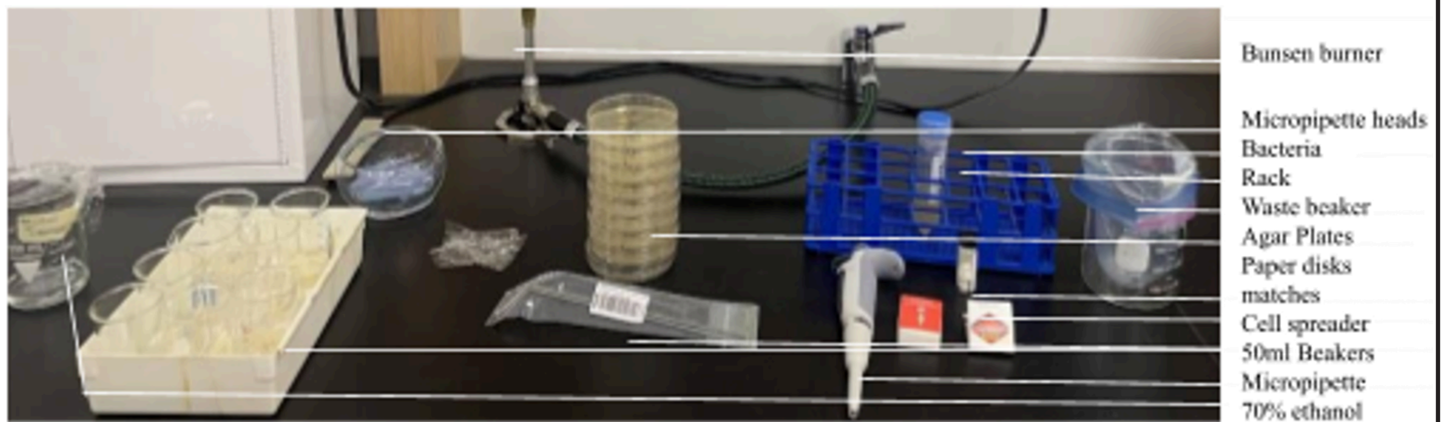

Figure 3: 1:00 pm at school. Set up for agar plate preparation. Photo by me. 1/24/23

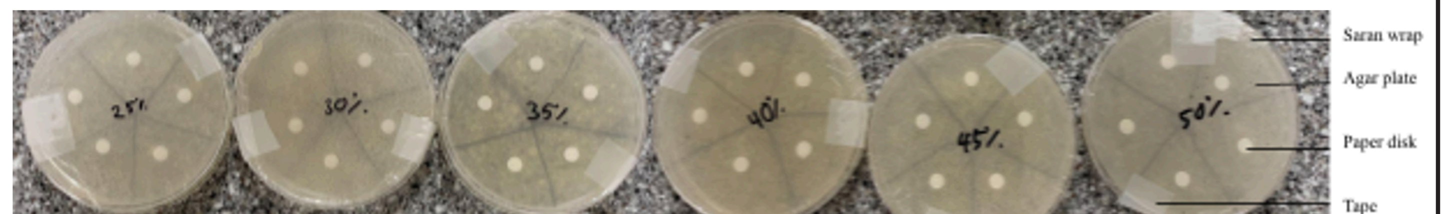

Figure 4: Agar plates with bacterial growth after 44 hours ± 0.3 hours. Photo by me.1/27/23

each section with numbers 1-5 to represent the different trials. Sanitized the table again and opened the Bunsen burner using matches. Refer to Figure 3 for the set up of agar preparation. Set tube of liquid culture of bacteria on rack and uncapped lid. Used a mechanical pipette and set it to 0.1250ml +/- 0.0001ml. Inserted bacteria onto an agar plate. Disposed of the pipette tip into a bag. Took a metal L shaped cell spreader and sterilized (dipped into the 70% ethanol and heat over flame until it extinguishes). Spread over the bacteria agar plate using the cell spreader in a circular motion, making sure to cover the entire surface. Rotated the plate in a circle while spreading it. Then lightly used the corner of the spreader and go back and forth from the top to the bottom of the plate. Rotated 90 degrees and repeat. Sterilized the spreader. Repeated for each agar plate.

Turned on the Bunsen burner for a small flame to sterilize metal tweezers (dipped in 70% ethanol and heat over flame). Then picked up one filter paper disk and dip it into the 25% honey solution with tweezers. Placed the disk in the middle of a section of the agar plate. Ensured that it was in the middle of the drawn section. Resanitized the tweezer. Repeated until there was one paper disk in each of the sections of the agar plate. Then continued for each solution and the respective agar plate. Placed lid on agar plate, sealed with saran wrap and taped shut. Turned over the agar plates and incubated at 30.0 °C +/- 0.1°C. After 44 hours ± 0.3 hours, removed all 6 plates from the incubator. Refer to figure 4 for recorded qualitative observations. Used a ruler to measure the diameter of the zone of inhibition for all disks at the bottom of the plate. Refer to Figure 1 for recorded values in the observation table. Sealed agar plates in a plastic bag and safely disposed of all materials in trash. Resanitized any tools and surfaces. Washed all beakers.

Results

Table 1: Mass of honey (g ± 0.01g) and water (ml ± 0.5ml) used to create different % concentration of honey. Refer to Figure 5 for sample calculations.

Percent concentration created with calculated error propagation (w/v)	25% ± 1.35%	30% ± 1.60%	35% ± 1.85%	40% ± 2.10 %	45% ± 2.35%	50% ± 2.60%
Mass of honey added (g ± 0.01g)	2.5	3	3.5	4	4.5	5
Water added (ml ± 0.5ml)	10	10	10	10	10	10

Table 2: Diameter (mm ± 0.5mm) of ZOI of Escherichia coli growth, after 44 ± 0.3 hours of incubation at 30 °C +/- 0.1°C.

	Percent concentration with calculated error propagation (w/v)					
Trial	25% ± 1.35%	30% ± 1.60%	35% ± 1.85%	40% ± 2.10 %	45% ± 2.35%	50% ± 2.60%
1	6	7	7.2	7	7	7.5
2	7	6.8	7.9	6.9	7	7.9
3	6.1	7	7.1	6.3	7	7.6
4	6	7	6.6	6.7	7	8.3
5	6.5	7.5	7.7	6.7	7.4	7.4

the zone of inhibition diameters include the 6.0mm sized paper disks, (w/v) refers to weight (g)/volume (ml)

Table 3: Average diameter (mm ± 0.5mm) of ZOI of Escherichia coli growth, after 44 hours ± 0.3 hours of incubation at 30.0 °C +/- 0.1°C and standard deviation.

	Percent concentration with calculated error propagation (w/v)					
	25% ± 1.35%	30% ± 1.60%	35% ± 1.85%	40% ± 2.10 %	45% ± 2.35%	50% ± 2.60%
Avg. diameter of inhibition (mm ± 0.5 mm)	6.3	7.1	7.3	6.7	7.1	7.7
Standard deviation (mm)	0.43	0.26	0.51	0.27	0.18	0.36

*all values are shown rounded to the nearest tenth, but exact numbers are used in calculations

$$\% \text{ concentration} = \frac{2.50\ g \pm 0.01\ g}{10.00\ ml \pm 0.5\ ml} \times 100\%$$

$$\% \text{ concentration} = \frac{2.50\ g \pm 0.4\%}{10.00 ml \pm 5.0\%} \times 100\% = (0.250 \pm 5.4\%) \times 100\% = (0.250 \pm 0.0135) \times 100\% =$$

25.0% ± 1.35%

Figure 5: Sample calculation for percent concentration (w/v) and error propagation for 25%.

$$Sx = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$$

$$Sx = \sqrt{\frac{(6.0-6.3)^2 + (7.0-6.3)^2 + (6.1-6.3)^2 + (6.0-6.3)^2 + (6.5-6.3)^2}{5-1}}$$

$$Sx = \sqrt{\frac{0.75}{4}} = \mathbf{0.43\ mm}$$

Calculate the F critical value

First find the variance for each concentration:

$$SS_{25\%} = \Sigma(x-\bar{x})^2$$

$$= (6.0-6.3)^2 + (7.0-6.3)^2 + (6.1-6.3)^2 + (6.0-6.3)^2 + (6.5-6.3)^2$$

$$= 0.3^2 + 0.7^2 + 0.2^2 + 0.3^2 + 0.2^2 = \mathbf{0.7}$$

Then add the variance for each concentration together to get the error sum of squares

$$SSE = \text{sum of variances} = \Sigma(\Sigma(X-\bar{X})^2) = SS_{25\%} + SS_{30\%} + SS_{35\%} + SS_{40\%} + SS_{45\%} + SS_{50\%} =$$

$$0.7 + 0.3 + 1.1 + 0.3 + 0.1 + 0.5 = \mathbf{3.0}$$

Calculate regression sum of squares

$SSR = \Sigma(n(X_i - \bar{X})^2)$ where X_i = mean of concentration $\bar{X}$ = mean of the means of each concentration

$$SSR = 5(6.3-7.0)^2 + 5(7.1-7.0)^2 + 5(7.3-7.0)^2 + 5(6.7-7.0)^2 + 5(7.1-7.0)^2 + 5(7.7-7.0)^2 = \mathbf{5.9}$$

Calculate total sum of squares

$SST = SSR + SSE = 5.9 + 3.0 = \mathbf{8.9}$

Calculate degrees of freedom

n = total values k = number of groups

$df_{regression} = k-1 = 6-1 = \mathbf{5}$

$df_{error} = n-k = 30-6 = \mathbf{24}$

$df_{total} = n-1 = 30-1 = \mathbf{29}$

Calculate mean squared errors

$MS_{regression} = SSR/df_{regression} = 5.9/5 = \mathbf{1.2}$

$MS_{error} = SSE/df_{error} = 3.0/24 = \mathbf{0.1}$

Calculate ratio of mean of squares (F-value)

$F = MS_{regression} / MS_{error} = \mathbf{9.3}$

Figure 6: Calculations to find standard deviation, variance, sum of squares, total sum of squares, degree of freedom, mean squared error and mean of squares for 25% concentration of honey

Table 4: Table describing the one-way ANOVA test comparing the mean zone of inhibition of bacteria and concentration of honey.

Source	*SS*	*df*	*MS*	F Value	F Critical	Acceptance/rejection of H0 and HA
regression	5.9	5	1.2	9.36	2.62*	H0: rejected HA: accepted
error	3	24	0.1	-	-	-

**To find the F critical value use ANOVA F Distribution Table with a = 0.05*

H_0: There is no statistical difference between the means of ZOI diameter for each concentration

H_A: There is a statistical difference between the means of ZOI diameter for each concentration.

Different concentrations of unpasteurized Canadian honey (25%, 30%, 35%, 40%, 45%, 50%) and distilled water, versus the diameter of inhibition of *Escherichia coli* growth in agar plates. 6.0 mm in diameter represents that there was no bacteria growth inhibition.

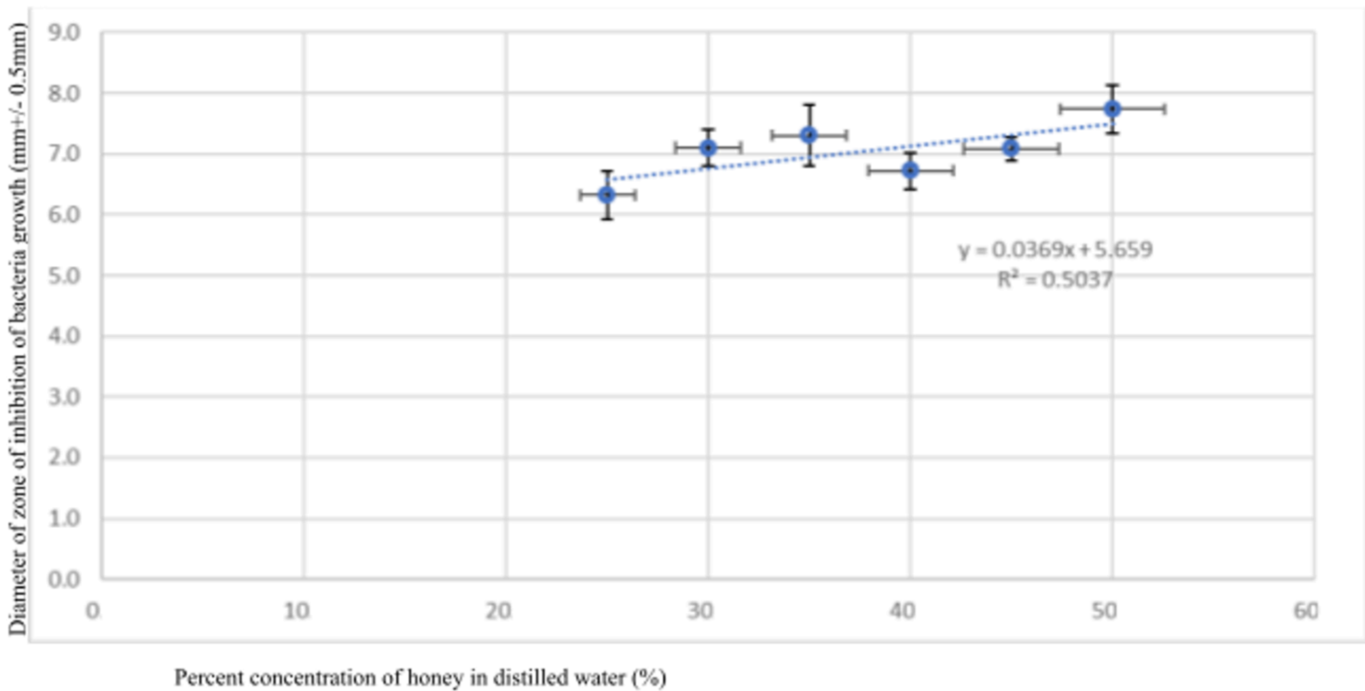

Figure 7: Scatter plot diagram displaying the average diameter of zone of inhibition of Escherichia coli in agar plates after 44 ± 0.3 hours of incubation, of different concentrations of honey. Data was collected at Sir Winston Churchill High School on 1/27/23 at around 10.00am. Linear regression line shows line of best fit produced by Excel. Vertical error bars show the standard deviation of the diameter of ZOI. The horizontal error bars show error propagation of percent concentration of honey.

Discussion

The percentages for honey were calculated as well as the average ZOI diameter for each concentration. The data was then plotted on a graph (See Figure 7).

Error propagation was used for the horizontal error bars. These were calculated by combining the measurement error of the tools to find the mass of honey and volume of water to create an error propagation for the final percent concentrations. These values for the concentrations 25%, 30%, 35%, 40%, 45%, 50% were 1.35%, 1.60%, 1.85%, 2.10%, 2.35%, 2.60% respectively.

The vertical error bars in the graph are the calculated standard deviations. The standard deviations were measured to look at the spread of the data. I calculated the standard deviations for the concentrations of 25%, 30%, 35%, 40%, 45%, 50% to be 0.43mm, 0.26mm, 0.51mm, 0.27mm, 0.18mm, 0.36mm respectively. The smallest standard deviation was 0.18mm for the 45% concentration of honey, which was 7.1mm. This makes up around 2.5 % of the original value. The largest standard deviation was 0.51mm for the 30% concentration of honey, which was 7.3mm. This made up around 7.0% of the original value. The standard deviations are very small percentages of the original data, which indicates high precision in the data. However, I noticed that there was overlapping between the vertical error bars when the data was plotted. Thus, a one way ANOVA test was performed to see if there is a statistical difference between the mean value for each concentration. This was done by finding the F statistic, which was F = 9.36. The F Critical value (Fcritical = 2.62) was found at a significance level of 0.05 (indicating a confidence interval of 95%) using the F table. Since the F value was greater than the F critical value, the null hypothesis (there is no statistical significance amongst the data) can be rejected, and the alternative hypothesis (there is a statistical significance between the means) can be accepted.

The linear regression was created with Excel, with the equation being $y = 0.0369x + 5.659$ with an R-value of 0.5037. Based on the figure, the line of best fit, and the calculations, it can be observed that there is a moderate correlation between the percent concentration of honey in distilled water, and the diameter of the inhibition zone; as the concentration increases, the diameter of the inhibition zone of the bacteria also increases. These results indicate that in the experiment, 50% concentration honey has the highest antibacterial properties of honey available. For example, the average zone of inhibition size for 25% ± 1.35% honey was at 6.3mm ± 0.5 mm compared to the average ZOI diameter for 50% ± 2.60% which was at 7.7mm ± 0.5 mm.

These results correlate with the findings of Mandal & Mandal (2011), which investigated Manuka and Ulma honey instead of Canadian honey. The experiment revealed that despite diluting the honey to encourage

glucose oxidation, the antibacterial qualities still decreased. This suggests 1) that the presence of non-peroxide compounds play a vital role in the antibacterial qualities of honey and 2) diluting the honey too much reduces the amount of glucose oxidase needed for glucose oxidation. Several studies suggest that even in the presence of added catalase (present in pollen and breaks down hydrogen peroxide), honey is still antibacterial (Brudzynski, 2011)(Mandal & Mandal, 2011). Ultimately, this may indicate that the inclusion of compounds that make up the non-peroxide activity, are just as important as the inclusion of hydrogen peroxide for honey to be effective at killing bacteria.

These results also imply that when using honey as a topical on wounds, there will still be antibacterial strength if the concentration of honey does not go below 25% due to dilution occurring with wound exudates (Ngan, 2015). Preventing this dilution however is crucial in maximizing antibacterial strength.

In the experiment, it was assumed that hydrogen peroxide activity would increase once the honey was diluted, and that it played a crucial role in inhibiting bacterial growth. This is reasonable because it has been observed and is widely known (Molan, 1992), however it should be recorded next time to ensure that it is directly correlated with concentration.

An obvious limitation of the experiment was the permeability of the agar plate to the honey. The disk diffusion method fails to account for the viscosity of honey and can create large ZOI diameters not due to its antibacterial abilities, but rather by its ability to diffuse across the agar plate. This limitation could have reduced the size of ZOI diameters created at higher concentrations because of the slower diffusion of non-peroxide compounds such as defensin-1 and glucose oxidase, which are heavier in molecular weight. (Szweda, 2017). Next time, instead of using the disk diffusion method as an in-vitro method of testing its antibacterial properties, the broth microdilution assay can be used. This method is not affected by the viscosity and ability for the antibacterial solution to diffuse, because a liquid medium is used (Henrick, 2021).

Another limitation was through the usage of unpasteurized honey. Pasteurization is used by food industries and includes the process of heating food to kill bacteria, increase shell life and attractiveness (McCormick, n.d.). The process of unpasteurized honey does not include heating, however, it does filter it. Although I originally thought that it would be beneficial to use a filtered honey to reduce the number of unwanted microorganisms, pieces of pollen, propolis and wax in the honey, it could have also reduced the antimicrobial potency of the honey itself by removing important beneficial compounds as well. (McCormick, n.d.) For example, phenolic acid has been studied for its antibacterial qualities, and originates from pollen (Kahraman et al., 2022). Next time, raw honey can be used instead as it may increase the effectiveness at inhibiting bacterial growth.

A random error in the experiment that could have reduced the accuracy of the experiment could have been from light exposure. Glucose oxidase is known to be sensitive to light and can be destroyed with photo-oxidation (White & Subers, 1964). Throughout the experiment, the honey was stored in a clear container. In order to minimize this random error to prevent glucose oxidase from degrading and hindering the creation of hydrogen peroxide, the honey should be stored in a light protected container.

A strength of the experiment were the precautions taken to minimize errors relating to contamination. For example, only sterilized distilled water was used, to ensure that unwanted pathogens or microorganisms would be incubated. A non-pathogenic strain was used so that just in case it was spread, it would not cause harm. Furthermore, all tools were either sterilized with ethanol before and after use, or sealed and disposed of. These precautions also ensured the safety of the environment around the experiment.

CONCLUSION:

In conclusion, the experiment shows that there is a positive correlation between the concentration of unpasteurized Canadian honey and antibacterial potency. It also implies that as a topical, it is recommended for honey that is 50% or higher in concentration is used in order to exhibit the maximum amount of antibacterial properties and encourages further testing in in-vivo conditions. Zainol et al. (2013) found similar findings, helping ensure accuracy of the data while the precision is reflected in the standard deviation calculations. Moving forward with these results, it would be interesting to actually remove all hydrogen peroxide activity from honey via. catalase, and how that affects the antibacterial properties of raw honey using the broth microdilution method.

ACKNOWLEDGEMENTS:

I would like to thank my teachers at Sir Winston Churchill and the International Baccalaureate program for this opportunity. I would also like to thank Dr. Rebekah DeVinney from University of Calgary for giving me the supplies that made the experiment possible. Also, thank you to The Canadian Science Fair Journal for allowing me to publish my work.

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Hello, my name is Maria Huang! I am a high school student passionate about using STEM to help others around me. This is in large part due to the pandemic, which inspired me to use my love for science to help make a positive difference in the community. As an avid hiker and camper, I am also interested in researching sustainability/earth related topics to satisfy my curiosities about nature. My hobbies outside of school include figure skating, running and cooking.