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The Effects of *Fagus Sylvatica* Leaf Extract on the Growth Rate of *Tetrahymena*: A Concentration-Dependent Analysis

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Abstract

This study investigates the effects of various concentrations of *Fagus sylvatica* leaf extract on the growth rate of *Tetrahymena*. Four concentrations of leaf extract (0 mg/mL, 0.6 mg/mL, 1.2 mg/mL, and 1.8 mg/mL) were tested, which were achieved by adding 5 mL of leaf extract at the specified concentrations and 5 mL of diluted *Tetrahymena* stock, giving a final volume of 10 mL for each treatment. Each concentration had three replicates to ensure reliability. A hemocytometer was used to count cells every three hours for three days, and the average change in cell concentration per day was calculated. The results indicated a decreasing trend in the *Tetrahymena* growth rate as the concentration of leaf extract increased, with average changes per day of 1.5155 for 0 mg/mL, 1.5084 for 0.6 mg/mL, 1.4815 for 1.2 mg/mL, and 1.4495 for 1.8 mg/mL. Statistical analysis revealed significant differences in growth rates among the concentrations (ANOVA, $p = 0.0271$). Pairwise comparisons using the Tukey HSD test identified a significant difference between the 0 mg/mL and 1.8 mg/mL concentrations ($p = 0.0298$). These findings demonstrate a concentration-dependent inhibitory effect of the leaf extract, with higher concentrations resulting in reduced growth rates. Further research is needed to confirm these results and explore the effects of higher extract concentrations and different experimental conditions to better understand the potential mechanisms underlying this inhibitory effect.

I. Introduction

Studies on plant phenolic compounds and their bioactivity have recently gained attention due to their wide-ranging effects. These effects have applicability in a variety of fields, particularly health and environmental biology and notably include antimicrobial, antioxidant, and growth inhibitory impacts on microorganisms (Cheremnykh et al., 2022). In the natural context, phenolic compounds play an essential role in plant defense mechanisms and have been shown to inhibit microbial growth (Kanjana et al., 2024). Despite these findings, it has also been determined that this compound group contributes differently to bioactivity and antimicrobial activity. *Fagus sylvatica* (European beech) is a plant species known for its rich phenolic content, including flavonoids, saponins, tannins, alkaloids, steroids, and terpenoids (Formato et al., 2021).

In this study, we aim to evaluate the effects of different *Fagus sylvatica* leaf extract on the growth rate of *Tetrahymena*, a model organism frequently used for biological and ecological research due to its rapid growth rate (Cassidy-Hanley et al., 2012). Using *Tetrahymena* in our study will exhibit greater changes in the data due to its ability to reproduce rapidly, allowing us to plot our data and visually notice the trends of the microorganism growth rate. According to Kanjana et al. (2024), plant extract can affect microbial organisms by inhibiting their growth rate due to the phenolic compounds in the leaves. Due to this, we expect to see the number of *Tetrahymena* cell growth increase with a higher leaf extract concentration. However, the rate of cell growth would decrease as the concentration of *Fagus sylvatica* leaf extract increases.

II. Methods

Preparation of *Fagus sylvatica* Leaf Extract:

Approximately 35 wet leaves of *Fagus sylvatica* were collected and were dried in the oven at 30°C for 24 hours. Once the leaves had dried, they were crushed in a zip lock bag until it appeared as a fine powder. 100 mL of 100% ethanol was measured using a 25 mL pipette and transferred to a 250 mL Erlenmeyer flask with the crushed leaves. The solution was mixed with a magnetic stir plate and stir bar for one hour. Then, the solution was centrifuged for 10 minutes in a glass tube. In this experiment, we used the Fisher scientific model CL centrifuge. Without interrupting the leaf pellets, the liquid was carefully extracted from the centrifuged solution using a 25 mL pipette. The extract was poured onto the petri dishes to allow the ethanol to evaporate overnight. Using a petri dish on a balance, 0.183 g of the leaf extraction was weighed. The extract was added to a 250 mL Erlenmeyer flask along with 50 mL of *Tetrahymena* media. Then, the solution was mixed with a magnetic stir plate and stir bar for 10 minutes, resulting in 50 mL of leaf extract at a concentration of 3.6 mg/mL in the media. Using a vacuum filter, the media was reconstituted and filter sterilized.

Preparation of *Tetrahymena* Original Stock Dilution:

A hemocytometer was used to determine the concentration of the *Tetrahymena* original stock. Using a micropipette, 10 uL of *Tetrahymena* fixative and 100 uL of the *Tetrahymena* original stock was measured and added into a counting tube. To ensure everything is mixed, the mixture was resuspended with a pipette. Using a micropipette, 20 uL of the sample was transferred into the hemocytometer with a glass slip on it. Under a microscope, the cells in the counting chamber were counted and the data was recorded. With a 10 mL and 25 mL pipette, 62.5 mL of *Tetrahymena* and 37.5 mL of medium was added to a 250 mL Erlenmeyer flask, which yielded 100 mL of *Tetrahymena* media with a concentration of 2×10^4 cells/mL.

Preparation of Test Tube Concentrations:

Using a 10mL pipette, 5 mL of the working stock was transferred to each of the 12 test tubes. For each test tube, varying amounts of the leaf extract were added to achieve concentrations of 0 mg/mL, 0.6 mg/mL, 1.2 mg/mL, and 1.8 mg/mL, ensuring a final volume of 10 mL in each test tube. From now on, the *Tetrahymena* stock that has been added with varying amounts of the leaf extract will use the term 'Concentrated Stock'.

	0 mg/mL	0.6 mg/mL	1.2 mg/mL	1.8 mg/mL
Leaf extract (mL of 3.6 mg/mL of leaf extract concentration)	0	1.67	3.34	5
Tetrahymena media (mL)	5	3.34	1.67	0
Working stock (mL of 2 x 10^4 cells/mL)	5	5	5	5
Final volume (mL)	10	10	10	10

Table 2.1 *Preparation of varying concentrations in the test tubes with 3 replications.*

***Tetrahymena* Cell Counting:**

Using a micropipette, 10 uL of *Tetrahymena* fixative and 100 uL of the concentrated stock was added into a labelled-counting tube. Then, it was resuspended with a pipette. 20 uL of the sample was measured and transferred into the hemocytometer with a glass slip on it using a micropipette. Under a microscope, the cells in the counting chamber were counted and the data was recorded. The cells counted with each concentration had three replicates, and were counted three times a day, for three days.

III. Results

The weight of the leaves was measured as 0.3715 g per leaf. After the extraction process, 0.183 g of extract was obtained. This weight information can be utilized to adjust the concentration of the extract to match the requirements for a specified volume of *Tetrahymena* media, ensuring precise preparation for experimental use.

Trial run	Researcher 1	Researcher 2	Researcher 3	Concentration (cells/mL)
1st count	99	94	95	3.3 x 10^4
2nd count	77	80	76	2.6 x 10^4
3rd count	110	107	103	3.6 x 10^4
Average				3.2 x 10^4

Table 3.1 *Calculation of concentration of Tetrahymena original stock under microscope*

The concentration of the original stock is calculated by taking the average of the counts of number of cells/square (Blue) x dilution factor of hemocytometer square (3.125 x 10^4) x correction for fixative (1.1).

Calculation of volume of original stock and *Tetrahymena* media needed for dilution:

$$C_1V_1 = C_2V_2$$

Where:

- C_1 = concentration of the original stock (3.2×10^4 cells/mL),
- V_1 = volume of the original stock (to be calculated),
- C_2 = desired concentration (2×10^4 cells/mL),
- V_2 = final volume (100 mL)

Substituting the values:

$$(3.2 \times 10^4 \text{ cells/mL})V_1 = (2 \times 10^4 \text{ cells/mL})(100 \text{ mL})$$

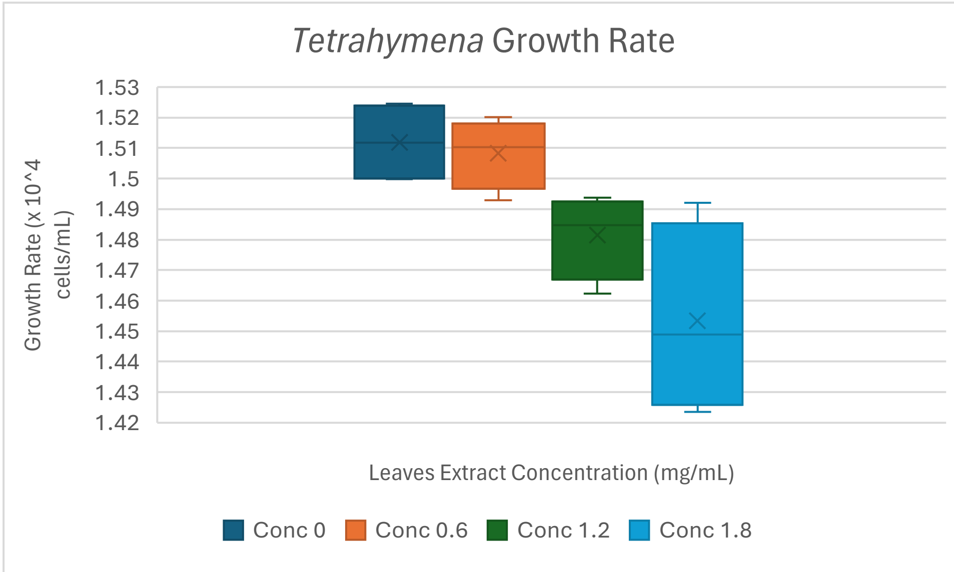
Solving for V_1:

$$V_1 = \frac{(2 \times 10^4 \text{ cells/mL})(100 \text{ mL})}{(3.2 \times 10^4 \text{ cells/mL})}$$

$$V_1 = 62.5 \text{ mL}$$

Thus, the volume of original stock needed is 62.5 mL.

The experiment involves four concentrations of leaf extract: 0 mg/mL, 0.6 mg/mL, 1.2 mg/mL, and 1.8 mg/mL. The dataset includes measurements taken three times per day with three replications for each concentration across three days. This results in a total of 288 data points, which are used to plot the growth rate slopes for each concentration.



Graph 3.1 *Boxplot of Tetrahymena growth rate with different concentrations of leaf extract*

To account for these differences, we further analyzed the data using ANOVA and Tukey HSD to determine if the differences in cell concentration changes across the leaf extract concentrations are statistically significant.

Hypotheses:

- Null Hypothesis (H_0): There is no significant difference in the average daily change in cell concentration across the different concentrations of leaf extract.
- Alternative Hypothesis (H_1): There is a significant difference in the average daily change in cell concentration across the different concentrations of leaf extract.

Data Summary				
Groups	**N**	**Mean**	**Std. Dev.**	**Std. Error**
Group 1	3	1.5155	0.0137	0.0079
Group 2	3	1.5084	0.014	0.0081
Group 3	3	1.4815	0.0169	0.0097
Group 4	3	1.4495	0.0372	0.0215

ANOVA Summary					
Source	**Degrees of Freedom DF**	**Sum of Squares SS**	**Mean Square MS**	**F-Stat**	**P-Value**
Between Groups	3	0.0081	0.0027	5.2501	0.0271
Within Groups	8	0.0041	0.0005		
Total:	11	0.0122			

Table 3.2 *Table of One-Way ANOVA*

treatments pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inferfence
A vs B	0.5478	0.8999947	insignificant
A vs C	2.5991	0.3243084	insignificant
A vs D	5.0504	0.0298877	* p<0.05
B vs C	2.0513	0.5057256	insignificant
B vs D	4.5026	0.0513499	insignificant
C vs D	2.4513	0.3688579	insignificant

Table 3.3 *Table of Tukey HSD*

IV. Discussion

Tetrahymena cells were counted with three replicates, three times a day, for three days using a hemocytometer. By Day 2 of counting cells, we were already able to infer that the growth of *Tetrahymena* cells was dependent on the *Fagus sylvatica* leaf extract concentration.

The average change in cell concentration per day, as calculated from the slope of the linear best-fit line, varies across the different concentrations of leaf extract. For 0 mg/mL, the average change is 1.5155 per day, while for 0.6 mg/mL, it decreases to 1.5084 per day. At 1.2 mg/mL, the average change is 1.4815 per day, and for 1.8 mg/mL, it further decreases to 1.4495 per day. These results indicate a declining trend in the slope with increasing concentrations of leaf extract, suggesting that higher concentrations of leaf extract are associated with a reduced rate of change in cell concentration over time.

Since the p-value is less than the threshold of 0.05 (p-value = 0.0271), we reject the null hypothesis (H_0) and accept the alternative hypothesis (H_1). This indicates that there is a significant difference in the average daily change in cell concentration across the different concentrations of leaf extract. The pairwise comparison shows a significant difference in the average daily change in cell concentration between 0 mg/mL and 1.8 mg/mL (p-value = 0.0298). This specific result further supports the overall finding that the concentration of the leaf extract significantly influences the growth rate of *Tetrahymena* cells.

This trend is consistent with prior research on the bioactivity of plant phenolic compounds, which are known for their antimicrobial and growth-inhibitory properties. The inhibitory effect is likely due to the phenolic compounds present in *Fagus sylvatica* leaves, such as flavonoids, tannins, and terpenoids, which are well-documented for their antimicrobial and growth-suppressing properties (Formato et al., 2021). These compounds can interfere with microbial cell functions, such as disrupting membrane integrity, inhibiting enzyme activity, or affecting nutrient uptake, leading to reduced cell proliferation.

Phenolic compounds are also known for their pro-oxidant properties under certain conditions, which can lead to the production of reactive oxygen species (ROS). Elevated

ROS levels can induce oxidative stress in microbial cells, damaging cellular components such as DNA, proteins, and lipids, ultimately suppressing growth (Kruk et al., 2022). The significant decline in growth at higher concentrations (e.g., 1.8 mg/mL) suggests that the phenolic compounds or other bioactive substances in the extract reach a threshold where their inhibitory effects become more pronounced. Toxic effects at higher concentrations may exacerbate the decline in growth rate, as observed in the Tukey HSD results.

Some possible sources of uncertainty and variability that could have contributed to some data were errors in the concentration preparation by inaccurately diluting the *Fagus sylvatica* leaf extract, leading to incorrect concentration levels. A way this error could be minimized is using precise pipetting techniques and calibrating the pipettes before use, as well as preparing dilutions in bulk to ensure uniformity. Another source may be counting *Tetrahymena* cells. Variations in cell counts when using a hemocytometer could arise due to inconsistent counting methods or operator differences of the group members. To avoid this issue, we can standardize counting protocols, including how many squares to count and how to distinguish live cells from dead cells, as well as having multiple individual counts and taking the average of these counts to ensure lower error and variation. The variability in leaf extract composition could also impact our results. Natural variations in the chemical composition of *Fagus sylvatica* leaves can affect the bioactivity of the extract. To minimize this issue, leaves from the same tree were collected and were processed under the same conditions, leading to less fluctuation in the bioactivity of *Fagus sylvatica* leaf extraction.

V. Conclusion

The results of our analysis indicate that while the concentration of *Fagus sylvatica* leaf extract appears to be associated with a decline in the growth rate of *Tetrahymena*, the differences in cell concentration changes across the different concentrations of leaf extract were found to be statistically significant.

Thus, although there is a general decline in the growth rate of *Tetrahymena* as the concentration of *Fagus sylvatica* leaf extract increases, further research is needed to confirm these findings. Future studies could explore using higher extraction concentrations, optimizing extraction methods such as using multiphase extraction, or testing different durations of exposure to better understand the potential effects of *Fagus sylvatica* leaf extract on *Tetrahymena* growth.

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