Formulation of Antifungal Vaginal Suppositories from Oregano Oil (*Plectranthus aromaticus*)

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Abstract: The antifungal activity of essential oils from plants, including Oregano, has already been acknowledged in the past. Oregano oil contains high phenolic compounds such as carvacrol and thymol. Studies show that these components have antifungal activity.

Candida albicans, the causative agent of Candidiasis, is a fungus that causes mouth, throat, and vagina infections. Candidiasis can be acquired during pregnancy by hormonal contraceptives, excessive use of certain antibiotics and corticosteroids, and uncontrolled diabetes. In this research, different procedures were done to prove that oregano oil is a potent active ingredient in the formulation of vaginal suppositories.

Oregano oil was extracted from *Plectranthus aromaticus*, an oregano species found in San Manuel Isabela, and isolated through steam distillation. It undergoes different pre-formulation studies such as organoleptic, stain, and identification tests such as phenol content determination and specific gravity. A compatibility test was conducted to select what excipients must be used without causing instability in oregano oil. The fusion method was done in the manufacturing of the vaginal suppositories. Pharmaceutical product evaluations were done after the manufacturing process. The computed average weight of the suppositories was 0.5343g. The microbial screening was done in two trials and a triplicate test. Results showed a complete inhibition with oregano oil which has 16.32mm on the first trial and 16.45mm on the second trial. The nystatin disc, the positive control, gave 17.44mm on the first trial and 16.98mm on the second trial. The suppository base, the negative control, has no inhibition in all trials. The formulated suppository has 15.33mm on its first trial and 16.17mm on the second. The *in-vitro* research study results demonstrate evidence that oregano oil is effective in suppressing the growth of *C. Albicans* and can be used as an active ingredient in the formulation of an antifungal vaginal suppository.

Keywords: Candida albicans; antifungal; vaginal suppositories; Oregano oil

1. Introduction

1.1 Rationale

Vaginal thrush or Candidiasis is a yeast infection commonly caused by *Candida albicans*. It is characterized by discomfort and irritation around the vagina, usually experienced by women. This infection is typically treated with an antifungal vaginal suppository.

Oregano (*Plectranthus aromaticus*) is from the family of Lamiaceae. It is an herbaceous plant widely cultivated in Africa, India, and Asia. It contains amounts of carvacrol and thymol that act synergistically in killing the *Candida albicans* for Candidiasis (Manohar, 2001). The chromatographic analysis revealed its primary constituents: 4-terpineol, carvacrol,y- terpinene, and thymol. The phenolic monoterpenes in the essential oil have an antifungal effect on *C. Albicans* (Cleff, et

al., 2013).

However, there is no current study on using the essential oil from Oregano to treat Candidiasis. This study aimed to formulate an antifungal suppository using oregano oil as an active ingredient.

1.2 Research Objectives

The general focus of this research is to formulate an antifungal vaginal suppository using oregano oil as the active ingredient. Specific objectives are the following:

1. To extract the essential oil from oregano leaves;
2. To conduct pre-formulation and formulation studies of oregano oil; and
3. To evaluate the formulated form of oregano oil as an antifungal vaginal suppository.

1.3 Research Questions

The following questions were answered by the end of the study:

1. What is the percentage yield of Oregano oil?
2. What are the physicochemical properties of Oregano oil?
3. What are the excipients compatible with Oregano oil?
4. What are the characteristics of formulated antifungal vaginal suppository?
5. Is there a significant difference between the antifungal activities of the Nystatin disc, suppository, and negative control?

2. METHODOLOGY

2.1 Preparation of Plant Sample

The collection of plant samples containing the oregano leaves was collected from San Manuel, Isabela, and was botanically identified and authenticated at the Bureau of Plant Industry.

2.2 Isolation of Oregano Oil

The fresh leaves were subjected to steam distillation to collect oils and their active constituents. Two hundred grams of oregano leaves were cut into small pieces and placed in a round bottom flask. A sufficient amount of water was added to submerge all oregano leaves. The materials were heated for 2-4 hours until no more volatile oil was produced. The oregano oil volume was collected in a graduated Clevenger tube and drained and collected by passing it through a small amount of anhydrous sodium sulfate. Results were recorded, and the percent yield was computed using the succeeding formula (Eq. 1).

$$\%Y = (V_f / W_o)100 \qquad \text{(Eq.1)}$$

where:
$\%Y$ = Percentage yield
V_f = Volume of Oregano oil
W_o = Weight of Oregano leaves

2.3 Pre-Formulation Studies

Organoleptic test. The organ senses were used to determine the oregano oil properties, such as color, odor, appearance, and texture. Theoretically, oregano oil is a pale yellow to amber liquid with a pungent, spicy aroma. It must not possess a rancid odor.

Stain test. The researchers weighed two grams of oregano oil, added five mL of hexane, and then heated it until it boiled. A few drops of extract were placed on a white paper and observed. The absence of stain on white paper signifies an essential oil.

Phenol test. The researchers measured the volume of oregano oil in a cassia flask shaken vigorously with caustic soda. An additional amount of alkali was added to make the undissolved portion to the calibration mark of the flask. The percentage of phenol was calculated using the formula below (Eq2):

$$\%Ph = \frac{V_o - V_L}{V_o} \, x \, 100 \qquad \text{(Eq.2)}$$

where:
$\%Ph$ = Percentage of phenol
V_o = Volume of oil
V_L = Volume of residual oily layer

Specific gravity. The sample's mass was determined using an analytical balance. The sample was placed inside the Pycnometer and was filled with water. The researchers waited for the temperature to reach 25ºC; there must be no bubbles forming when the stopper was placed. Mass was recorded. The specific gravity of the sample was calculated using the formula below (Eq3). Theoretically, its specific gravity is 0.9300-9800.

$$SG = \frac{\rho_{sx}}{\rho_{H_2O}} \, x \, 100 \qquad \text{(Eq.3)}$$

where:
SG = *S*pecific gravity
ρ_{sx} = Density of the substance
ρ_{H_2O} = Density of the water

Refractive index. Using the Refractometer, a few oregano oil drops were added to form a film and were placed between the prisms. The color, turbidity, and thinness of the film of oregano oil made it more comfortable. ABBE refractometer (Atago digital refractometer) was used to determine its index refraction. Its theoretical value is 1.510-1.520.

Compatibility test. Oregano oil and other excipients blends were kept at 25℃ room temperature in a stability chamber for ten days. Caking, liquefication, discoloration, odor, and gel formation were all detected for physical changes. The Shimadzu IRTracer-100 was used to generate the infrared transmission spectra.

Table 1. Compatibility Test

Materials	**Functions**
Oregano Oil	Active ingredient
Polyethylene glycol (PEG)	Base
Glycerinated Gelatin	Base
White Beeswax	Stiffening agent
Yellow Beeswax	Stiffening agent

Miscibility. In an empty vial, one mL of oregano oil was mixed with different solvents such as water, benzene, n-hexane, chloroform, and ethanol.

Forced degradation study. Oregano oil was subjected to 50 ± 2ºC temperature and 75 ± 5% relative humidity inside the stability chamber. The sample was evaluated on the 10th day in the stability chamber (Brummer, 2011). Different stress conditions are applied over drug substances, giving different results on product degradation; this is mainly used to determine product stability under accelerated conditions.

2.4 Formulation Studies

Method of manufacturing. Using the Fusion molding technique, the base was loaded into the evaporating dish and dissolved—the rest of the excipients to the mixer containing the base. Mix until complete solubilization and cool down. Lastly, the oregano oil was added slowly into the mixture with purified while stirring. It was immediately transferred into the molder and refrigerated until firm.

W*eight variation.* The researchers weighed 20 suppositories individually and as a whole. The average weight was calculated (Limitation: NMT 2 suppositories differed from the average weight by more than 5%). Not more than two of the suppositories differed from the average weight by more than the % error listed. If more than two suppositories are different from the average weight by 5%,

calculate double the percent error as follows:

$$UL = \overline{X_w} + \frac{(5)(\overline{X_w})}{100} \quad \text{(Eq.4)}$$

where:
UL = Upper limit
$\overline{X_w}$ = Average weight

$$LL = \overline{X_w} - \frac{(5)(\overline{X_w})}{100} \quad \text{(Eq.5)}$$

where:
LL = Lower limit
$\overline{X_w}$ = Average weight

2.5 Antifungal Activity Test

Preparation of inoculum. A 20 mL of water was inoculated with the *Candida albicans*. The culture was incubated at 25°C for 24 hours. For the inoculation, 0.2 mL of inoculum was pipetted into a small petri dish, then 15 mL of melted Sabouraud Dextrose Agar was combined into the petri dish and was mixed until the uniform distribution of the inoculum was achieved. The researchers allowed the sample to solidify.

***Microbial testing*.** The effectiveness of the essential oil was determined using the disc diffusion method antifungal susceptibility test. A strain sample of *Candida albicans* was inoculated on a media Sabouraud Dextrose Agar with 4mm thickness. The filter paper disc was positioned on the medium surface. The formulated suppository, nystatin disc as a positive control, and polyethylene glycol (PEG) as negative control were added. For 24 hours, the plates were kept at 37°C.

Data analysis. The experimental design was validated using variance (ANOVA) analysis. The experimental design was used in this study to differentiate any significant differences in the said variable.

3. RESULTS AND DISCUSSION

3.1 Isolation of Oregano Oil

The percentage yield of oregano oil is 7.17%, as shown in Table 2. According to the studies of Solyu, Yigitbas, Solyu, & Kurt (2007), the average yield of Oregano was about 6-9%(v/w).

Table 2. Average Yield of Oregano Oil from *Plectranthus aromaticus*

	1st Extraction	2nd Extraction	3rd Extraction
Weight of Oregano leaves	200 grams	200 grams	200 grams
Volume of Oregano Oil	15 mL	14 mL	14 mL

Percentage Yield	7.5%	7%	7%
Average Percentage Yield		7.17%	

3.3Pre-Formulation Studies

Organoleptic test. Table 3 presents the organoleptic test or physical evaluation of oregano leaf extract conducted and yielded the following results: the color was yellow, the odor was mint, the taste was bitter, liquid in appearance, and the texture was greasy. According to Parchem (2017), the appearance is liquid. The color of the oil is a pale yellow to amber liquid. The odor is a robust penetrating odor of Oregano and has a pungent taste.

Table 3. Results Organoleptic Studies

Organoleptic Parameters	**Actual Results**
Color	Yellow
Odor	Mint
Appearance	Liquid
Texture	Greasy

Stain test. There was an absence of stain in the paper for the three trials. The stain test determines the difference between fixed oils to essential oils. If the oil disappears spontaneously, it is an essential oil. This test showed that oregano oil does not leave any stains on the paper, which signifies that oregano oil is necessary.

Phenol test. For the first and second trials, the sample has a %phenol of 70% and 60%, respectively; this indicates that oregano oil is composed of phenolic compounds. According to Han et al. (2017), the chemical makeup of leaf-flower oils is predominantly made up of terpenoid and phenolic molecules, which account for more than 60% of the overall content.

Specific gravity. The specific gravity of the oregano oil was 0.9892. According to (Jenkins 1977), the theoretical specific gravity of oregano oil is 0.82-1.2 at 25°C.

Refractive index. At 20ºC, the refractive index of the sample was 1.51. According to Attokaran (2017), the refractive index of the essential oil from Oregano is 1.506-1.512 at 20°C.

Compatibility test. There were no interactions between oregano oil and the other commonly used excipients in a suppository formulation. The spectra confirmed no interactions between the oregano oil and the excipients.

Miscibility. Table 4 shows that oregano oil is miscible to benzene, n-hexane, chloroform, and ethanol while immiscible to water. According to Parchem

(2017), oregano oil is immiscible in a solvent such as water due to its polarity and strong hydrogen bonding, which is miscible in alcohols and oils.

Table 4. Miscibility Test Results

Solvent	Trial 1	Trial 2	Trial 3	Results
Water	(-)	(-)	(-)	Immiscible
Ethanol	(+)	(+)	(+)	Miscible
Chloroform	(+)	(+)	(+)	Miscible
N-hexane	(+)	(+)	(+)	Miscible
Benzene	(+)	(+)	(+)	Miscible
Ether	(+)	(+)	(+)	Miscible

Note: (+) Miscible and (-) Immiscible

Forced degradation study. There are no significant changes in the physicochemical characteristics (organoleptic, stain test, phenol test, specific gravity, IR, refractive index) of the oregano oil detected before and after the evaluation.

3.4 Formulation Studies

Table 5 shows the characteristics of the formulated antifungal vaginal suppositories.

Table 5. Product Evaluation

Parameters	Actual Results
Color	Yellow
Odor	Mint
Shape	Bullet
Weight Variation	Passed

3.5 Antifungal Activity Test

Figure 1: Antifungal Screening

Table 6 and Figure 1 show the oregano oil's effectiveness as an active ingredient in formulating an antifungal vaginal suppository.

Table 6. Zone of inhibition in millimeters (mm)

	Trial 1	**Trial 2**	**Reactivity**	**Remarks**
Oregano oil	16.32 mm	16.45 mm	(+)	Complete inhibition
Suppository	15.33 mm	16.17 mm	(+)	Complete inhibition
Nystatin disc	17.44 mm	16.98 mm	(+)	Complete inhibition
PEG	0	0	(-)	No inhibition

Note: (+) Complete inhibition and (-) No inhibition

Table 7. Data Summary (Zone of inhibition *Candida albicans* (mm)

Groups	**N**	**Mean**	**Std. Dev.**	**Std. Error**
Oregano Oil	2	16.385	0.0919	0.065
Suppository	2	15.75	0.594	0.42
Nystatin Disc	2	17. 21	0.3253	0.23
PEG	2	0	0	0

Table 8. ANOVA Results Between Nystatin Disc, PEG, and Suppository

Source	**Degrees of Freedom (df)**	**Sum of Squares (ss)**	**Mean Square**	**F-Stat**	**P-value**
Between Groups	2	1726.375468	863.187734	1620.976548	0.00000
Within Groups	15	7.987664	0.532511		
Total:	17	1734.363132	102.021361		

Table 8 shows the ANOVA results between nystatin disc (positive control), PEG (negative control), and suppository. The null hypothesis (H0) is rejected since the p-value is less than 0.05. The p-value is 0.00000, indicating that the likelihood of a type 1 error (rejecting a correct H0) is low (0.0 percent). The rejection of the null hypothesis using p-value is supported by the f-statistic test having a result equal to 1620.976548; this does not fall below the acceptable critical value range of 95 percent [-∞: 3.6823]. Based on the statistics, the averages of nystatin disc (positive control), PEG (negative control), and suppository are not equal and statistically big enough to be significantly different.

Table 9. Tukey Kramer Test In Between Groups

Pair	Difference	SE	Q	Lower CI	Upper CI	Critical Mean ($\alpha = 0.05$)	p-value
X1-X2	17.4444	0.29791	79.66546	16.3501	18.53878	1.09434	0.00000
X1-X3	16.3222	0.29791	49.98112	15.227877	17.41656	1.09434	0.00000
X2-X3	15.3333	0.29791	29.68433	14.238985	16.42767	1.09434	$4.82e^{-12}$

Note: X1 is Nystatin Disc (positive control), X2 is PEG (negative control), X3 is Suppository, α is significance level

Since all the pairs have a p-value < 0.05, all the following pairs are significantly different.

Table 10. Summary of differences

Group	Nystatin Disc	PEG	Suppository
Nystatin Disc	0.0	17.44	16.32
PEG	17.44	0.0	15.33
Suppository	16.32	15.33	0.0

Table 10 summarizes the absolute difference between the means of Nystatin Disc (positive control), PEG (negative control), and Suppository, where each group was subtracted from each group, including their value.

4. CONCLUSIONS

In this research, the oregano extract obtained through steam distillation from oregano leaves (*Plectranthus aromaticus*) signifies that it can be used as an active pharmaceutical ingredient in formulating an antifungal vaginal suppository.

5. ACKNOWLEDGMENTS

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6. REFERENCES

Attokaran, M. (2017). Natural Food Flavorants and Colorants, Second Edition. John Wiley & Sons Ltd.

Cleff, M.C., Madrid, I., Meinerz, A. R., Maireles, M. C., Mello, J. R., Rodrigues, M. R., &Escareno, J. J. (2013). Essential oils against Candida spp: in vitro antifungal activity of Origanum vulgare. African Journal of Microbiology Research, 2245-2250.

Brummer, H. (2011). How to Approach a Forced Degradation Study. Life Science.

Han, F., Ma, G.-q., Yang, M., Yan, L., Xiong, W., Shu, J.-c., . . . Xu, H.-l. (2017). Chemical composition and antioxidant activities of essential oils from

different parts of Oregano. Journal of Zhejiang University-Science.

Jenkins, G. L. (1977). Jenkins' Quantitative Pharmaceutical Chemistry. Retrieved from The Good Scent Company Information System: http://www.thegoodscentscompany.com/data/es1101441.html

Manohar, V., Ingram, C., Gray, J., Talpur, N., Echard, B., Bagchi, D., & Preuss, H. (2001). Antifungal activities of origanum oil against Candida albicans. Molecular and Cellular Biochemistry.

Parchem. (2017, July 19). Safety Data Sheet (Oregano oil). Retrieved from Parchem: https://www.parchem.com/siteimages/Attachment/GHS%20Oregano%20Oil%20MSDS.pdf

Solyu, S., Yigitbas, H., Solyu, E., & Kurt, S. (2007). Antifungal effects of essential oils from Oregano and fennel on Sclerotinia sclerotiorum. Journal of Applied Microbiology.