The Effectiveness of Topical Propolis Administration in the Treatment of Vulvovaginal Candidiasis in Mouse Model Experiments

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Abstract
Objective : This study is an analytical study with an experimental design, namely the *Post Test Only Control Group Design*, which assesses the effectiveness of administering Propolis to mice infected with vulvovaginal candidiasis
Methods : This research was conducted at the *Animal House of* USU's FK and Microbiology Laboratory at USU Hospital. This research was conducted from 27 November to 7 December 2019. The study population was 36 experimental mice infected with vulvovaginalis candidiasis . The Collony Forming Unit (CFU) was examined on 36 rats divided into 2 groups, namely control and treatment and then examined by CFU every day from Day 0 to Day 4. The data obtained was assessed by the *Mann Withney* hypothesis test
Results : D ari 36 samples were divided into 2 groups found in group A, which did not receive Propolis, found the number of *CFU* from day zero until the fourth day was 92.22 *CFU* , 128.89 *CFU* , 78.67 *CFU* , 56.61 *CFU* , and 51.61 *CFU* . Whereas in Group B that received *Propolis* , there was a mean *Colony Forming Unit (CFU*) that declined from zero to fourth day, namely 97.56 *CFU* , 15.06 CFU, 2.61 *CFU*, 0.5 *CFU* , and on last day obtained 0 *CFU* . Topical propolis is effective in treating Vulvovaginal Candidiasis (KVV) in experimental model mice where the administration of Propolis can reduce the amount of *CFU in the* rat's vagina from day to day.
Conclusion: Topical propolis is effective to reduce the number of *CFUs* in rats infected with vulvovaginal candidiasis (KVV)

Keywords: Propolis, Topical, Vulvovaginal Candidiasis (KVV), *Colony Forming Unit (CFU)*.

Introduction

Vulvovaginal candidiasis (KVV) is an infection caused by Candida species especially Candida albicans although there are other non- Candida albicans Candida (NCAC) species such as Candida glabrata. Although KVV is the second most common vaginal infection, it is not included in sexually transmitted diseases and because candida is considered part of the normal vaginal flora. However, research has confirmed the transmission of candida organisms by vaginal intercourse and other forms of sexual activity . *C.albicans* species are part of the normal flora of the vagina but there are many factors that can cause excessive growth leading to candidiasis . How Candida species can cause infection is mediated by a number of virulence factors which include adhesion, biofilm formation, extracellular hydrolytic enzyme production, hyphal formation and phenotypic transition . The diagnosis of KKV requires pelvic examination. The combination of thick white vaginal discharge and vulvar pruritis is not sensitive and is not specific itself for diagnosis. Erythema and edema of the vulva and vaginal tissue, together with thick, white vaginal discharge, support the diagnosis. KVV vaginal secretions have a pH <4.5, and budding yeast and pseudohyphae can be seen on a wet mount. Negative *Whiff* tests and gram staining can reveal *polymorphonuclear* cells *(PMN)* and *pseudohyphae* . When there is complicated CVV evidence, collecting vaginal fluid for *yeast* culture and speciation can help with direct therapy . In this study, *Colony Forming Unit (CFU)* examination was used as a parameter for the success of CVV treatment with topical Propolis. For the quantification of fungal loads, a series of 10-fold serial dilutions was carried out using sterile PBS and plated to the *CHROMagar* ™ plate . *CFU is* calculated after incubation at 35 ° C for 24 hours and expressed as CFU / 100 *µl lavage* fluid or also called *CFU / plate* or *CFU* per plate agar . The most prominent and general aspect that can be relied on with mouse models is that these rodent models and human infections are very dependent on estrogen . Estrogen uses the multifunctional permissive role for vaginal candidiasis through a number of effects both *host* and *Candida* . Mice must be placed in a stable pseudoestrus condition to establish an experimental vaginal infection. In mice this is usually achieved by ovariectomy followed by estrogen treatment although recent research shows that a special estrogen regimen is sufficient to allow *C.albicans* infection . Of note, ovariectomy has been shown to cause a decrease in the number of *lactobacillus* components of the vaginal microbiota . In mice, administration of estrogen is a standard treatment by considering the variable susceptibility of different animal strains to estrogen. In both rodents, pseudoestrus induces changes that are highly dependent on the stage in the vaginal epithelium, with a surface of keratinization where the fungal cells are very attached and multiply with hyphal growth and eventually form biofilms. In addition to these structural modification properties, other estrogen-induced biological activities appear to have special relevance for CVC / CVC. Specifically, estradiol has been reported to inhibit the differentiation of Th17 cells, a

subset of CD4 cells that should be involved in the mucosal antifungal defense, with strong evidence, however, in the mouth and intestine rather than vaginal infections. Overall, there is evidence that the response to estrogen has similarities in rodents and humans. The basic protocol using mice for KVV research was established in 2014 by the *NIH (National Institute of Health)* , namely:

1. Female CBA / J mice were injected subcutaneously in the lower abdomen (UNIT 1.6) with 0.1-0.5 estradiol valerate in 20-100 µl *sesame* oil 3-6 days before vaginal inoculation and repeated every week until the study was completed.
2. Prepare a solution of 2.5x10 ^{6} C. *albicans* blastospora cells for culture in 0.02 ml of sterile *PBS (phosphate-buffered saline)* .
3. Inoculate a 0.02 solution of fungal cells or sterile *PBS* (for negative control mice) in the vagina using a micro pipette and a sterile tip. Insert the tip until it runs out into the vaginal lumen, near the cervix. Do it under anesthesia or quickly to minimize stress mice. Anesthetics use *ketamine* (100 mg / kg rat) and *xylazine* (10 mg / kg rat) *intraperitoneal* (UNIT 1.6) in each rat several minutes before inoculation.
4. Perform this vaginal smear on anesthetized mouse (serial measurement on days 4,7 and 10 after inoculation) and on the last calculation of the rat in euthanasia. The trick, wash the vagina by spraying 100 µL *PBS* forwards and backs several times. Avoid trauma to the lumen wall when taking serial measurements.
5. Prepare homogeneous serial solution 1:10 there is a sterile PBS. Prepare 3 plates of *YPD* (*Yeast-Extract Peptone Dextrose*) with each solution of 25-50 µL. Incubation for 24-48 hours. Count the number of colonies as *CFU* .

KVV can be induced in mice. Preliminary observations show that the optimal time for induction of vaginal infection is during the estrous stage in mice. The estrous stage is characterized by the appearance of epithelial cells in the vaginal exudate. The estrous-cycle stage of mice is 3-4 days in duration. Mice that are infected during the estrous stage will develop the infection, which can disappear spontaneously afterwards. Therefore, most researchers used a mouse model in a constant state of estrus by inoculating female mice with *estradiol benzoate* 3-4 days before inoculation with *C. albicans* . The infection is induced by intravaginal inoculation of 10 ^{7} *C.albicans* yeast cells and can be maintained by repeated weekly inoculation of *estradiol benzoate*

Candida inoculation in rat vagina. [26]

A) Rats are confined for inoculation. Mice are placed on wire inserts and held by the base of the tail, slightly upward to lift the legs and open the vaginal opening. The rat's hips can be stabilized with the same hand when trying to hold the tail restraint.

B) Exposure of the inoculum into the vaginal lumen. The tip of the pipette is gently inserted about 5 mm into the vaginal lumen. The suspension inoculum is then stored.

Propolis is a mixture of complex compounds also called bee glue is a natural resin product collected by bees from several plants and mixed with beeswax and bee saliva that contains enzymes (gl - glucosidase). Bees use propolis in their hives as a protection against predators and microorganisms, to repair damage, as a thermal insulator, and as an aseptic defense to prevent microbial infections in larvae . Propolis is a lipophilic material that is hard and easily breaks when cold but soft, flexible, and very sticky when warm, has an aromatic odor and different colors, including brown, green, and red. In terms of chemical composition consists of 50% resin, 30% wax, 10% essential oil, 5% pollen, and 5% other substances which include minerals and organic compounds such as phenolic acids (cinnamic and caffeic acids) or estern flavonoids (flavones, flavanons, flavonols, and dihydroflavonols chalcones), terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenes, and β- steroids . Propolis *in vivo* suppresses the *lipoxygenase pathway* from arachidonic acid metabolism during inflammation. Anti-inflammatory activity of propolis is by inhibiting *PGE2* and reducing cytokines. Other mechanisms of propolis that are reported are affecting the activity of inflammatory cells (cell migration, *macrophage* activation), inhibiting *tumor necrosis factor (TNF)* , reducing nitric oxide synthesis and reducing enzymatic activity during the healing process. From *in vitro studies* using topical propolis extract it was concluded that both propolis-based gels (G2 and G3) and cream (E3) were partially able to control KVV in mouse models. These results indicate that propolis is a promising and potential alternative therapy for controlling CVC. Considering the fact that propolis is a complex substance with several possible antifungal active compounds, it may be more difficult for resistance to evolve in *C. albicans* , because it will require several concurrent mutations. In addition, it is possible to increase the therapeutic value by combining propolis with other antifungals such as *azoles* or *caspofungin* agents . Another

promising aspect of this combination therapy is the fact that propolis inhibits the yeast transition to hyphae which contributes to the overall *virulence* of *C. albicans* and may even be a target for the development of antifungal drugs.

Method

This study used an experimental design namely the Post Test Only Control Group Design in November-December 2019 by using 36 experimental rats infected with vulvuvaginalis candidiasis that had been bred in the Department of Mathematics and Natural Sciences Unit of the *Animal Lab* Unit of North Sumatra University (USU) and *CFU* examination at the Department of Microbiology i Hospital North Sumater University . Then 36 mice were divided into two groups: group 1 was a control group that was not given treatment and group 2 is a group that was given per lakuan be Propolis topical. The results of this study are presented in the frequency distribution table. To assess the effectiveness of topical propolis administration in mice with vulvovaginal candidiasis, inferential statistical analysis was performed using *independent T-Tests* . *Test* if the data is normally distributed. If the data are not normally distributed, the *Mann Withney* hypothesis test will be used with a confidence level of 95%.

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Results

This study analyzed data from the 36 mice that had been infected with Jamus *Candida albicans* de ngan Propolis Topical administration that includes the inclusion and exclusion criteria, Table 1 gain characteristics of the study subjects a total of 36 samples were obtained 25 mice (59.4%) with a pH of 7.0 and 11 mice (30.6%) with a pH of 8.0. Whereas for body weight, 23 rats (63.9%) with body weight of 90-150 grams and 13 rats (36.1) with body weight of 150-250 grams.

Table 1 Characteristics of research subjects

Characteristics	N	%
Vaginal pH		
7.0	25	69.4
8.0	11	30.6
Body weight (grams)		
90 - 150	23	63.9
150-250	13	36.1

Table 2 Average CFU in group A (Control) and group B (Treatment)

Day	CFU / Plate	
	Group A	Group B
0	92.22	97.56
1	128.89	15.06
2	78.67	2.61
3	56.61	0.5
4	51.61	0

Table 2 shows that group A, who did not get Propolis, obtained the number of *CFUs* from zero to fourth day was 92.22 *CFU* , 128.89 *CFU* , 78.67 *CFU* , 56.61 *CFU* , and 51.61 *CFU* . Whereas in the group that received *Propolis* , there was a mean *Colony Forming Unit (CFU*) which declined from the first day to the fifth day. On zero day, the average *CFU* of 18 samples was 97.56 *CFU* , on the first day 15.06 CFU, on the second day 2.61 *CFU*, on the third day 0.5 *CFU* , and on the fifth day 0 *CFU were* obtained . Tables 3 show that the tests conducted with control comparisons are used to assess the effectiveness of antifungal power statistically. On day zero the value of p = 0.499 was obtained, it was concluded that it had no meaning while on day 1,2,3,4 the value of p <0,001 was obtained.

Table 3. Differences Effectiveness Granting Propoli *s* Topical in infected mice KVV Based on Value p

Day	Average CFU (Mean)		Value of p
	Control	Propolis	
Day 0	92.22	97.56	0.499
Day-1	128.89	15.06	<0.001 *
Day 2	78.67	2.61	<0.001 *
3rd day	56.61	0.5	<0.001 *
4th day	51.61	0	<0.001 *

* *Mann-Whitney Test* (p <0.05)

Discussion

From the research, 36 subjects were obtained. Based on the characteristics of vaginal pH, the majority of study subjects had a vaginal pH of 7.0, as many as 25 subjects (69.4%). Based on research conducted by Vacca (2017), acidic pH can interfere with Candida growth. Because, at low pH, structural changes occur to the cell wall that makes the *host* immune system more easily recognize Candida. As a result, the body's immune reaction occurs in acidic conditions, while in alkaline conditions, Candida relatively grows better without an immune reaction from the body.

In this study, it was found that the mean CFU was different between the two groups, that is between group A who did not get *Propolis* tended to increase CFU and group B who got *Propolis* decreased CFU starting the first day of *Propolis* administration . Meanwhile, groups that did not get *Propolis* tended to experience an increase in CFU. In a study conducted by Capoci (2015), it was found that in a comparative study between two groups, namely the group receiving *Propolis* extract and the group not receiving *Propolis* extract (control) , there was a decrease in CFU in all all isolate groups receiving *Propolis* extract . This is because *Propolis* has the ability to inhibit *biofilms* which is one of the strong factors of attachment between *Candida albicans* and vaginal mucosa.

In a study conducted by Wagh (2013) shows that propolis in vivo suppresses the lipoxygenase pathway from arachidonic acid metabolism during inflammation. Anti- inflammatory activity of propolis is by inhibiting PGE2 and reducing cytokines, other mechanisms by influencing inflammatory cells (cell migration, macrophage activation), inhibiting TNF, reducing nitric oxide synthesis and reducing enzymatic activity during the healing process.

The results of McLennan SV (2007) show that propolis inhibits neutrophil and macrophage infiltration and inhibits the activity of the myeloperoxidase enzyme, thus accelerating wound healing. KVV is also an inflammatory process wherein the inoculation of *C. albicans* in mice also causes a vulvovaginal wound in mice. If prolonged inflammation can cause tissue necrosis resulting in further tissue damage and inhibit wound healing. Propolis accelerates wound healing through its antioxidant and antimicrobial activity. Propolis can also get rid of free radicals that inhibit cell regeneration so that tissue regeneration can take place normally.

This study uses propolis extract from New Zealand with a concentration of ± 0.16% extract per drop. As explained earlier that Propolis is a resin from bees and this depends on the geographical conditions of each place. Study the effectiveness of anti-candida of propolis has been studied by Siti Farida (2019), which examined the in vivo effectiveness of propolis *wax* derived from bees *Tetragonula sp* living in Sulawesi-Indonesia and obtained *C.albicans* can be eliminated by administering it because Propolis Propolis from Sulawesi this contains a polyphenol component that has antioxidant activity, it was also found that the flavonoid content of Indonesian propolis was higher than propolis originating from Romania, Taiwan, Brazil and China. The bioactive components of polyphenols, terpenoid acids, flavonoids have anti-inflammatory, anti-microbial, antioxidant and anti-viral activities.

Mahadewi (2017) also reported that propolis from Indonesia contains anti-fungal marker components such as: *thymol, cucumene, tetraline, and Ep-coumaric acid* which are potential as anti-candida.

Conclusion

In conclusion, the characteristics of the study subjects were found in all rats with vaginal pH 7-8, all mice used in this study were positively infected with KVV, Estradiol valerate could induce pseudoestrus conditions in Wistar strain mice used in the study. As well as the effectiveness of administration of propolis in mice infected with CVV on day 1,2,3,4 compared to controls found a significant difference with the value of $p < 0.001$ so that it can be concluded that propolis shows a promising therapeutic effect on KKV.

References

[1.] Cassone A. Vulvovagina Candida albicans infections: pathogenesis, immunity, and vaccine prospects. Royal college of obstetricians andgynaecologists; 2014: 785-94.
[2.] Murtiastutik D. Sexually transmitted infections. First edition. Surabaya: Airlangga University Press; 2008. h. 56-63.
[3.] Centers for disease control and prevention (CDC). Vulvovaginal candidiasis. Sexually transmitted diseases, treatment guidelines 2015. Morbidity and mortality weekly report (MMWR). Recommendation and reports vol 64 (3): 25-6.
[4.] Carvalho RS. Propolis: A Complex Natural Product with a Plethora of Biological Activities That Can Be Explored for Drug Development. Hindawi Publishing Corporation 2015.
[5.] Rusda M, Siregar M Fidel Ganis, Lelo Aznan, et al. The Therapeutic Effect of Nigella Sativa Extract on Female Wistar Rats vaginal Candidiasis Model, Proceeding Book of the 3rd International Conference on Community Research and Science Engagement (IC2RSCE), 2019.
[6.] Cassone A, Sobel JD. Experimental Models of Vaginal Candidiasis and Their Relevance to Human Candidiasis. *Infect Immun* 2016; 84: 1255–1261.
[7.] Khoirotunnisa Uswatun Hasanah. Antifungal Propolis Test Against Candida Albicans and Pityrosporum Ovale. Faculty of Medicine, Universit as Muhammadiyah Surakarta 2012.
[8.] Dota KFD, et al. Antifungal Activity of Brazilian Propolis Microparticles against Yeast Isolated from Vulvovaginal Candidiasis. Hindawi Publishing Corporation 2010.

[9.] Moghim H, Taghipoor I, Shahinfard N, et al. Antifungal effects of Zataria multiflora and Nigella sativa extracts against Candida albicans. *J HerbMed Pharmacol JJ HerbMed Pharmacol* 2015; 4: 138–141.
[10.] Cleff MB, Wendisch I, Cabana AL, et al. Experimental vaginal candidiasis: Assessment of Origanum vulgare for its treatment. *African J Microbiol Res* 2011; 5: 4207–4211.
[11.] Fard FA, Zahrani ST, Bagheban AA, et al. Therapeutic effects of Nigella Sativa Linn (Black Cumin) on Candida albicans vaginitis. *Arch Clin Infect Dis* 2015; 10: 1–5.6. Salem ML. Immunomodulatory and therapeutic properties of the Nigella sativa L. seed. *Int Immunopharmacol* 2005; 5: 1749-1770.
[12.] Nabhan AF. Vulvovaginal candidiasis. *ASJOG* 2006; 3: 73–79.
[13.] Gonçalves B, Ferreira C, Alves CT, et al. Vulvovaginal candidiasis: Epidemiology, microbiology and risk factors. *Crit Rev Microbiol* 2016; 42: 905–927.
[14.] Bitew A, Abebaw Y. Vulvovaginal candidiasis: Species distribution of Candida and their antifungal susceptibility patterns. *BMC Womens Health* 2018; 18: 1–10.
[15.] Maurissen W, Van Meensel B, Verguts J, et al. Vulvovaginal candidiasis. *Tijdschr Geneeskd* 2009; 65: 1105-1110.
[16.] Wozniak KL, Wormley FL, Fidel PL. Candida-specific antibodies during experimental vaginal candidiasis in mice. *Infect Immun* 2002; 70: 5790–5799.
[17.] Yano J, Kolls JK, Happel KI, et al. The Acute Neutrophil Response Mediated by S100 Alarmins during Vaginal Candida Infections Is Independent of the Th17-Pathway. *PLoS One* 2012; 7: 3–10.
[18.] Mayer FL, Wilson D, Hube B. *Candida albicans* pathogenicity mechanisms. *Virulence* 2013; 4: 119–28.
[19.] van Schalkwyk J, Yudin MH, Allen V, et al. Vulvovaginitis: Screening for and Management of Trichomoniasis, Vulvovaginal Candidiasis, and Bacterial Vaginosis. *J Obstet Gynaecol Canada* 2015; 37: 266-274.
[20.] Hainer BL, Gibson M V. Vaginitis: Diagnosis and treatment. *Am Fam Physician* 2011; 83: 807–815.
[21.] Al-ahmadey ZZ, Mohamed SA. Vulvovaginal candidiasis: Agents and its virulence factors. *Microbiol Res Int* 2010; 2: 28–37.
[22.] Nash EE, Peters BM, Lilly EA, et al. A murine model of Candida glabrata vaginitis shows no evidence of an inflammatory immunopathogenic response. *PLoS One* 2016; 11: 1–14.
[23.] Conti HR, Huppler AR, Whibley N, Gaffen SL. Animal Models for Candidiasis. NIH Public Access. 2014 Protoc Immunol Curr; 105: 19.6.1-19.6.17.
[24.] Segal E, Frenkel M. Experimental In Vivo Models of Candidiasis. *J Fungi* 2018; 4: 21.
[25.] Calderon L, Williams R, Martinez M, et al. Genetic susceptibility to vaginal candidiasis. *Med Mycol* 2003; 41: 143–147.
[26.] Yano J, Fidel, Jr. PL. Protocols for Vaginal Inoculation and Sample Collection in the Experimental Mouse Model of & lt; em & gt; Candida & lt; / em & gt; vaginitis. *J Vis Exp* . Epub ahead of print 2011. DOI: 10.3791 / 3382.
[27.] Carrara MA et al. A New Model of Vaginal Infection by Candida albicans in Rats. Mycopathologia (2010) 170: 331-338.
[28.] Ong V. Bartizal K. Hughes D. Miesel L. CD101 Gel Formulation is Highly Efficientacious Against Azole-Resistant Candida albicans in a Rat Model of Vulvovaginal Candidiasis. 2016-IDSOG-CD101-rat-azole-R-VVC-Poster-Final.pdf
[29.] Monzote L. In vitro antimicrobial assessment of Cuban propolis extracts. Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. 107 (8): 978-984, December 2012
[30.] Castro PA. The identification of the cell targets is important for propolis-induced cell death in Candida albicans. Elsevier 2013
[31.] Iswanto H. Kuswandari S. Mahendra PKW. Effect of Topical Application of Propolis 10% on Healing Wounds Post Tooth Extraction Persistent Deciduous Disease (Study in Children Ages 6-10 years). Journal of Dentistry, Vol. 7, No. 2, April 2016: 80-85.
[32.] Vacca I. 2017. Fungal physiology: Acidic pH interferes with Candida persistence. Nature Reviews Microbiology 15 (7), 382-382.
[33.] Capoci IRG, Bonfim-Mendonca PS, Arita GS, *et al* . 2015. Propolis is an efficient and fungicide and inhibitor of biofilm production by Vaginal *Candida albicans* . Evidence Based Complementary and Alternative Medicine.
[34.] Wagh, VD, 2013, Propolis: A Wonder Bees product and Its Pharmacological Potentials, Advances in Pharmacological Sciences, Hindawi Publishing Corporation, Brazil, 1-6.
[35.] Ramos AFN, Miranda JL. Propolis: A Review of Its Anti-Inflamatory and Healing Properties. Journal of Venomous Animals and Toxins including Tropical Diseases. 2007; 13 (4): p. 706
[36.] Farida S, Sahlan M, Rohmatin E, Adawiyah R. 2019. The beneficial effect of Indonesian propolis wax from Tetragonula sp. as a therapy in limited vaginal candidiasis patients. Saudi Journal of Biological Sciences. Elsevier
[37.] Mahadewi AG, Christina D, Hermansyah H, Wijanarko A, Farida S, Adawiyah R et al., 2017. Selection of discrimination markers from various propolis for mapping and identifying anti Candida albicans activity. AIP Conf