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In vitro anti-*Candida* activity of *Quercus infectoria* gall extract-based vaginal cream and its local tissue effects *in vivo*

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ABSTRACT

Aims: Aqueous extract of *Quercus infectoria* (QI) galls has been reported to possess anti-fungal and anti-inflammatory activities. Hence, this study aimed to determine *in vitro* antimicrobial activity of formulated QI gall extract-based vaginal cream against *Candida albicans* and to evaluate the possible side effects on the cervicovaginal epithelium of healthy rats.
Methodology and results: Three different cream formulations containing 10%, 20%, and 30% of QI gall extract respectively were tested for their antimicrobial activity against *C. albicans* (ATCC 10231) by using disc diffusion test. Microbroth serial dilution method was performed in determining the minimum inhibitory concentration (MIC) and fungicidal concentration (MFC). The 30% formulated extract cream (FEC) was applied topically on the cervicovaginal surface of healthy Sprague Dawley (SD) rats and examined for local tissue effects histologically. The mean scores of inhibition zone diameter were compared by one-way ANOVA and post-hoc test using PRISM software. All extract cream formulations displayed a relatively good anti-*Candida* activity. The MIC values exhibited by 10%, 20%, and 30% FEC against *C. albicans* were 1.094 mg/mL, 0.547 mg/mL, and 0.068 mg/mL, respectively. The 10% and 20% FECs showed a significant difference (P=0.0254) in the mean of inhibition zone diameter. The lowest MFC value (0.068 mg/mL) was shown by 30% FEC. There were no abnormal changes seen at the vagina and cervical mucosa after 2 weeks application of 30% FEC.
Conclusion, significance and impact of study: QI gall extract formulated in the cream base has an anti-*Candida* activity *in vitro* and the present finding suggests that this herbal cream formulation is potentially useful in preventing vaginal candidiasis without causing any unwanted local side effects.

Keywords: Vulvovaginal candidiasis, *Quercus infectoria*, formulated cream, anti-*Candida* activity, toxicity

INTRODUCTION

Candida is the most common cause of fungal infections worldwide, ranging from superficial mucosal infections to life-threatening invasive diseases. The most common species isolated is *Candida albicans* (Zaidah *et al*., 2008; Datta *et al*., 2018). *Candida* normally lives on the skin and in the mucosal membranes of the urinary tract as well as genital organs of humans without causing infection. However, overgrowth of the organism can cause symptoms to develop, depending on the infected area. Oral candidiasis, napkin dermatitis, and vaginal candidiasis are some of the common superficial types of *Candida* infections.

According to the Centers for Disease Control and Prevention, CDC (2015), overgrowth of *Candida* species in vagina may occur when there are changes in normal vaginal acidity and hormonal balance, leading to vaginal candidiasis. It is an infection of female genital tract, characterized by whitish or yellowish discharge from vagina. Surveys conducted all over the world indicated that 75% of women had at least one episode of vaginal candidiasis (Achkar and Fries, 2010), in which pregnancy was the most common predisposing factor. There are other risk factors which include surgery, burns, and the use of in-situ catheter. The microorganism can spread into the blood stream, causing invasive candidiasis or candidemia especially among immunocompromised patients with high mortality rate of 50% to 60% (Mikulska *et al*., 2012).

Vaginal candidiasis is commonly treated by using antifungal agents of azole group such as fluconazole, clotrimazole, and miconazole. The medication is usually in the forms of vaginal cream, ointment, or tablet.

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However, these drugs can cause side effects (Yonashiro Marcelino *et al*., 2018). A topical anti-fungal agent might cause itchiness, mild burning sensation, rash, and blistering in the area of use. Patients who are using fluconazole are particularly at risk of developing infections due to fluconazole-resistant *C. albicans* (Marchaim *et al*., 2012). This fungal resistance often results in treatment failure leading to serious consequences and can cause death especially among immunocompromised or critically ill patients. These worrying situations have driven the necessity for researchers to search for newer anti-*fungal* agent which can devoid the side effects and at the same time retain its therapeutic efficacy. Nowadays, people have increasingly opted for an alternative medicine rather than conventional medicine (Paithankar, 2010) because of its efficiency, safety, and economic feasibility.

Quercus infectoria (QI), which is also known as 'Manjakani' in Malaysia and 'Majupal' in India, has been used traditionally for treatment of many ailments. The gall extract of this plant was previously reported to possess antidiabetic, anti-inflammatory, antibacterial, and antifungal properties (Basri and Fan, 2005; Basri *et al*., 2012). Methanol and aqueous extracts of QI galls displayed *in vitro* anti-*Candida* activity against all tested *Candida* species (Baharuddin *et al*., 2015). However, this study was prompted due to the lack of studies in the microbiological efficacy and local toxicity of the formulated creams containing QI gall extract for topical use at the affected area. This cream may serve as an alternative treatment for vaginal candidiasis. Therefore, this study was conducted to determine the antifungal activity of formulated vaginal creams containing QI gall extract against *C. albicans in vitro* and to evaluate its effect on cervicovaginal mucosal tissue of Sprague Dawley (SD) rats.

MATERIALS AND METHODS

Plant extraction

QI galls purchased from a local herbal supplier in Kota Bharu, Kelantan, Malaysia were identified based on their physical appearance as described by Shrestha *et al*. (2014). The galls were washed with distilled water and left to dry at room temperature. Once dried, they were crushed and grinded to produce fine powder. The aqueous extract of QI gall powder was prepared based on the previous study conducted by Baharuddin *et al*. (2015). QI gall powder (100 g) was immersed in 500 mL of sterile distilled water (ratio of 1:5) in 50 °C water bath for 72 h. The mixture was filtered using fine filter followed by Whatmann filter paper No. 1. The filtrates were concentrated using rotary evaporator at 80 °C before getting freeze-dried at -50 °C for a few days until fine crystal-like crude extracts were obtained. The crude extracts were weighed out and the percentage yield was calculated. Finally, they were stored in the airtight jars at -20 °C.

Microorganism culture

Candida albicans (ATCC 10231) was used in this study and grown on the Sabouraud dextrose agar (SDA) at 35 °C for 24 h prior to the inoculum preparation. For disk diffusion test and microbroth dilution test, yeast inoculum concentration of 1×10^6 CFU/mL (equivalent to 0.5 McFarland) was freshly prepared by suspending few colonies from the overnight growth plate in brain heart infusion (BHI) broth. Further inoculum dilution was done by diluting the organism suspension with saline water (1:50) followed by dilution with the Roswell Park Memorial Institute (RPMI) medium (1:20) to obtain the final concentration of 1×10^3 CFU/mL for the broth microdilution method.

Cream formulation

Creams containing three different extract concentrations (10%, 20%, and 30%) were formulated based on studies conducted by Paithankar (2010) and Bhide and Nitava (2015) with slight modifications. The composition percentage of each formulation is shown in Table 1. Oil phase composition containing emulsifier (stearic acid) and water phase material were mixed and homogenized at 70 °C in a separate container simultaneously. After heating, the water phase mixture was slowly added into the oil phase mixture with constant manual stirring at 70 °C. The homogenized mixture was stirred at room temperature until smooth semisolid cream was formed. Sterility testing of the formulated cream was performed according to the standard microbiological procedure (World Health Organization, 2012).

Table 1: Composition of creams.

Phase	Ingredients	Percentage			
Oil	Stearic acid	7%			
	Cetyl alcohol	2%			
	Mineral oil	10%			
		Cream base	A	B	C
Water	Glycerin	2%	4%	8%	12%
	Triethanol amine	2%	4%	8%	12%
	QI gall extract	-	10%	20%	30%
	Distilled water	77%	63%	45%	27%

Carboxymethyl cellulose (CMC) (0.5 g) and distilled water mixture (which has been dissolved in water bath at 60 °C) were slowly added into the formulated cream (3.5 g) until liquid form obtained. Final concentration of 70 mg/mL was produced for each formulated cream prior to disk diffusion and broth microdilution tests.

Anti-*Candida* activity

Disk diffusion test was performed in order to investigate the susceptibility of formulated extract cream (FEC) against *C. albicans* by following the guidelines of clinical and laboratory standards institute (CLSI, 2012) with slight

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modification. Blank discs were impregnated with 100 µL (7 mg/disc) of three different FECs (10%, 20%, and 30%), 100 µL of amphotericin B (70 µg/mL) as a positive control, and 100 µL of negative control (containing the cream base, diluted CMC, and saline water. The discs were left to dry for 10-15 min. The susceptibility of *C. albicans* towards FEC was evaluated based on the diameter of inhibition zone (mm) produced after 24 h of incubation.

The minimum inhibitory concentration (MIC) of each FEC against *C. albicans* was determined by using two-fold serial microdilution assay (RPMI broth and diluted FEC in CMC mixture with concentration ranging from 35 mg/mL to 0.068 mg/mL). All the procedures were prepared in accordance to the guidelines in the CLSI document M27-A3 (CLSI, 2008). In the 96-well microtiter plate, 100 µL of diluted *C. albicans* (1×10^3 CFU/mL) was added into the wells containing 100 µL of serially diluted FECs and positive control (without FEC) well. Positive control well was used to control the adequacy of the broth for bacterial growth while the FEC in the broth was used as a negative control to ensure medium sterility. Turbidity in the wells were inspected visually and compared with the controls after 24 h of incubation to obtain the MIC value (lowest concentration of FEC with clear solution).

The test wells that showed complete fungal growth inhibition (without turbidity) as well as the negative control well were sub-cultured onto SDA plate and incubated at 35 °C for 24 h to determine the minimal fungicidal concentration (MFC) values. The MFC was defined as the lowest FEC which grew fewer than three colonies on the agar surface after incubation. All tests were performed in triplicates.

In vivo study of local tissue effect

Twelve-weeks-old female SD rats weighing approximately 200-250 g were supplied by Animal Research and Service Centre (ARASC), Universiti Sains Malaysia (USM) Health Campus, Kelantan. The animals were grouped and marked at least five to seven days before initiation of the extract dosing to provide individual identification and were acclimatized to the condition of the laboratory.

The procedure on the lower reproductive tract of a rat was performed following Debata *et al*. (2013) with slight modification. The intravaginal administration and dosing of FEC were done according to Gendron and Mansell (2015). Twelve healthy female rats were equally divided into three groups. Groups 1 and 2 were subjected for intravaginal applications with phosphate buffer saline (PBS-treated control) and base cream (BC-treated control) respectively while 30% FEC was used in group 3 (FEC-treated group).

The vaginal cytology was done in all groups to determine the stages of estrous cycle. The vaginal secretion was collected by using glass Pasteur pipette filled with a small amount of normal saline (NaCl 0.9%). The tip of the pipette was pushed gently 2-5 mm in depth into the vagina and the fluid was flushed. The bulb of the pipette was gently squeezed and released two or three times to flush the cells from the vaginal lining. Two drops of resulting cells suspension were expelled onto a labeled glass slide. Glass Pasteur pipette with rubber teats and rounded tips were washed repeatedly for the cleaning between animals in order to prevent sample contamination. The unstained materials were then observed under a light microscope with 10× and 40× objective lenses.

Then, 100 µL of PBS, diluted BC and 30% FEC (70 mg/kg) colloidal solutions were slowly infused by micropipetting directly into the vagina of the rats in group 1, 2, and 3 respectively. The applications were administered once daily for two weeks. Following the treatment, all rats were then euthanized using pentobarbitone (100 mg/kg) after which the body weight of each rat was determined. Dissected cervical and vaginal tissue samples were subjected to routine histological procedure. The hematoxylin and eosin (H&E) stained tissues were examined under light microscope with low and high magnification (100× and 200×).

Phytochemical analysis

Detection of tannin, saponin, flavonoid, and alkaloid in 30% FEC was carried out using standard screening procedures in accordance to the previous studies (Manjamalai *et al*., 2010; Alebiosu and Yusuf, 2015).

Statistical analysis

Data entry and statistical analysis were performed using PRISM software. One-way ANOVA and post-hoc tests were used to compare the mean inhibition zone diameters of FECs containing three different extract concentrations (10%, 20%, and 30%). P-value of less than 0.05 was considered as significant.

Animal ethics approval

This study was approved by the Animal Research Ethics Committee of USM (JEHUSM) with certificate number USM/Animal Ethic Approval/2015/(652).

RESULTS

Percentage yield of extract

The percentage yield of QI galls water extract was calculated based on the formula: Percentage yield of extract = Weight of extract / Weight of QI gall powder x 100. Thus, the extraction performed in this study has yielded 41.35 % of QI gall extract.

Anti-*Candida* activity

The antimicrobial activities of different FECs (10%, 20%, and 30%) against *C. albicans* (ATCC 10231) by disc diffusion method are shown in Table 2. The mean inhibitory zone diameter value was calculated from three plate readings. Positive control (amphotericin B) has

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exhibited the largest inhibition zone diameter followed by 20%, 30% and 10% FECs. The 10% and 20% FECs showed a significant difference (P=0.0254) in the mean of inhibition zone diameter (Table 2). FEC containing 30% of extract has displayed the lowest MIC and MFC values (0.068 mg/mL). The MFC/MIC ratios of all tested creams were below 4, indicating their fungicidal activity (Table 3).

Table 2: Mean inhibition zone diameter of different cream extract formulations against *Candida albicans* (ATCC 10231).

Formulation	Mean ± SEM (mm)	**P*-value
10% FEC	14.33 ± 0.33	
20% FEC	18.00 ± 1.00	[a]0.0315
30% FEC	16.33 ± 0.67	
10% FEC	14.33 ± 0.33	[b]0.0254
20% FEC	18.00 ± 1.00	
10% FEC	14.33 ± 0.33	[b]0.0550
30% FEC	16.33 ± 0.67	
20% FEC	18.00 ± 1.00	[b]0.2378
30% FEC	16.33 ± 0.67	
Positive control	27.00 ± 1.00	
Negative control	-	

ATCC: American type culture collection; SEM: standard error of mean; FEC: formulated extract cream.
**P*-value was determined using [a]One-way ANOVA and [b]post-hoct test (Tukey's Test), P < 0.05 was considered as significant.
(-) no inhibition zone.

Table 3: MIC and MFC values of different cream extract formulations against *Candida albicans* (ATCC 10231).

Formulation	MIC (mg/mL)	MFC (mg/mL)	MFC/MIC ratio
10% FEC	1.094	1.094	1
20% FEC	0.547	1.094	2
30% FEC	0.068	0.068	1

MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration; ATCC: American Type Culture Collection; FEC: formulated extract cream.

Histopathology examination

The photomicrographs of tissues dissected from cervix and vagina show that 30% FEC-treated group (Group 3) showed no evidence of increased inflammation similarly seen in the PBS-treated control group (Group 1) and BC-treated control group (Group 2). The vaginal epithelium of rats did not show any ulceration, areas of necrosis, or inflammatory cells infiltration. Histopathological examination of the cervix has demonstrated that it was covered by stratified squamous epithelium and the layer below the epithelium was composed of normal connective tissue (Figure 1). These findings have demonstrated that daily application of extract vaginal cream for 2 weeks did not affect the lower reproductive tract tissue in FEC-treated rat model.

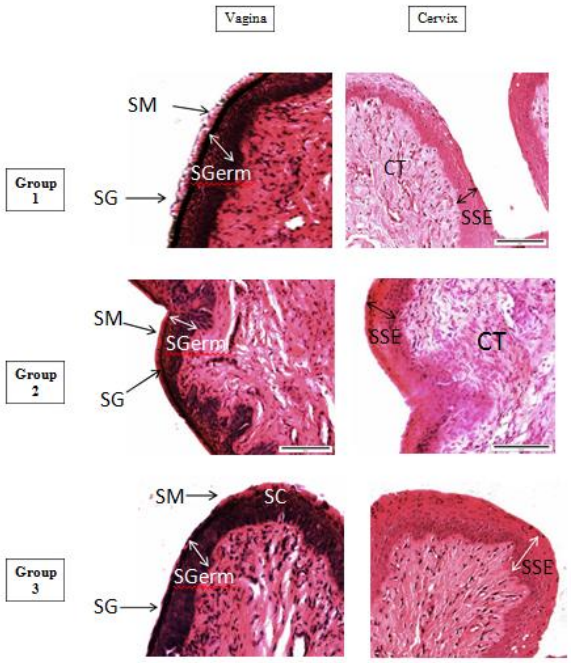

Figure 1: Histopathological examination of cervix and vagina with H&E staining. The images from each group were shown with 200× magnification. Group 1: PBS-treated control group, Group 2: BC-treated control group, Group 3: FEC-treated group. Stratum mucification (SM), stratum granulosum (SG), stratum corneum (SC), stratum germinativum (SGerm), squamous epithelium (SSE) and connective tissue (CT) are indicated by the arrows.

Qualitative phytochemical analysis

Phytochemical tests performed to screen for phytochemical compounds in 30% FEC showed presence of only tannin while others such as flavonoid, saponin, and alkaloid were not detected.

DISCUSSION

Topical drugs preparation has minimum side effects compared to oral treatments (Jorge *et al*., 2010). Most of topical medications like creams, gels, lotions, and ointments are epicutaneous. There are many studies which has incorporated herbal plants into creams containing oil and water emulsion (Bhide *et al*., 2015; Gupta *et al.* 2015). In this study, QI gall water extract was formulated in oil and water emulsion-based cream with several possible combinations between excipients. The selection of excipients was based on the previous studies (Paithankar, 2010; Bhide *et al*., 2015). The final combination has yielded the best FECs in semisolid form with brownish colour, slightly oily, and producing herb odour. In these formulations, each cream contained the same percentage of oil phase excipients but different water phase content. The efficacy of formulated creams is

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directly related to the quality of the added ingredients and their concentration (Pindari Herb Farm, n.d.). Furthermore, the effectiveness of cream absorption on the skin surface is depending on the ability of oil phase or water phase in order to transfer the ingredients. The best formulation cream consists of oil phase components between 15% and 30% of total volume of the cream. It plays an important role when it comes to the texture of the cream as well as its quality. Oil phase content in all formulated creams in this study was 19%. Thus, the best texture obtained is easy to apply and can be washed away with water, resulting in increased compliance.

Various forms of intravaginal drugs have been developed due to the increased prevalence of vaginal candidiasis worldwide. The intravaginal route or vaginal drug delivery presents several advantages over conventional drug delivery which include ease of administration, lower risk of side effects, reduced gastrointestinal irritation, and provision of optimum pharmacokinetic profiles (Choudhury *et al*., 2011; Gendron and Mansell, 2015). Currently, metronidazole and clindamycin vaginal creams used for the treatment of bacterial vaginosis have already been proven as efficacious as oral delivery (Choudhury *et al.*, 2011). Therefore, the present study seeks to elucidate the effect of a formulated cream for intravaginal administration with a hope to enhance drug delivery and minimize potential side effects.

QI gall extract has been reported to possess antifungal properties (Hassan, 2011; Najwa, 2014; Baharuddin *et al*., 2015) due to the high content of phytochemicals such as tannins and gallic acid. Selection of *C. albicans* in this study was done based on the previous report which had stated that historically this organism in particular has been the predominant species causing *Candida* infections (Almirante *et al*., 2005). The resistance of this organism to azoles represents a major challenge for empirical, therapeutic, and prophylactic strategies especially in immunocompromised and severely ill-patients. This study showed all formulated creams with different percentage of QI extract concentrations exhibited an inhibitory activity against *C. albicans*. The inhibition zone size produced in disc diffusion test was independent with the concentration of extract used in the cream. There are several reasons which lead to this observation including the absorption rate of substances into the blank disc which influences the diffusion rate thus affects the inhibitory zone diameter. The 30% and 20% FEC might have different viscosity due to different extract concentration contained. Statistically however, there are no significant differences in the inhibition zone sizes between 20% and 30% FECs.

In this study, the MIC values were low in all tested formulations against *C. albicans* indicating their significant anti-*Candida* potentials. Meanwhile, the MFC values ranged from 0.068 mg/mL to 1.094 mg/mL and the MFC/MIC ratios were 2 and less. Thus, all formulated creams containing QI aqueous extract might be regarded as fungicidal against *C. albicans* as indicated by Pankey and Sabath (2004) in which the microbiological definition of fungicidal activity has been taken arbitrarily as MFC to MIC ratio of less than or equal to 4. This finding also aligns with the previous study done by Baharuddin *et al*. (2015) whereby both methanol and aqueous QI gall extracts exhibited fungicidal activity against ATCC isolates of *C. albicans*. In addition, the highest percentage of QI gall extract in the formulation (30% FEC) displayed the lowest MIC and MFC values (0.068 mg/mL), denoting an effective growth inhibition and killing of the microorganism. Therefore, the results of this study suggest the potential use of QI gall extract in vaginal cream as one of the potential phytotherapeutic agents in the treatment of vaginal candidiasis.

In addition to a wide availability of laboratory rats in our institution, the use of rats was preferable in order to employ a more convenient procedure related to extract application intravaginally. The reproductive cycle or commonly known as estrous cycle of a rat comprises of four different phases which are proestrus, estrus, metestrus, and diestrus which occur within four to five days (Weswood, 2008). Each phase is characterized by morphological changes in ovaries, uterus, and vagina. During proestrus stage, the vaginal epithelium consists of four layers which are stratum mucification, stratum corneum, stratum granulosum, and stratum germinativum. (Paccola *et al*., 2013). The morphological changes during each stage might lead to errors in interpretation and reporting of results if this estrous cycle was not monitored. In this study, all rats were sacrificed during proestrus stage which was determined by vaginal cytology taken every morning to minimize inflammation variation which might interfere with the result analysis. Based on the photomicrograph of tissues treated with the extract cream (Figure 1), part of the stratum mucification was not intact. Some of the superficial mucoid layer might have lost during late proestrus stage (Westwood, 2008). Besides, the late proestrus stage in Group 3 was indicated by the stratum corneum which might have appeared during mid to late proestrus stage.

Cell morphological and tissue structural changes were observed to evaluate the local tissue effect produced after the application of formulated cream into the rat's vaginal or cervical tissue. It may occur due to the biological response of body tissues to harmful stimuli such as pathogens or irritants. In this study, daily application of vaginal FEC with 30% QI gall extract for two weeks did not cause any sign of inflammation in the mucosa of vaginal and cervical tissues of the healthy rat as there were no ulceration, areas of necrosis, or inflammatory cells infiltration observed. These findings were similar to the previous study done to investigate *in vivo* local toxicity effects of curcumin in formulation of vaginal cream (Debata *et al*., 2013). Thus, with regard to these preliminary findings, it is suggested that QI vaginal cream is feasible and safe to use.

Preliminary phytochemical screening has identified the presence of tannins in the formulated QI gall extract cream. This finding correlated well with previous study done by Baharuddin *et al*. (2015) in which pyrogallol (hydrolysable tannin) was detected in the water extract of

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QI gall. Tannins are water soluble with molecular weights ranging between 500 and 3000 Da (Lim *et al*., 2006; Jesus *et al*., 2012). The hydrolysable tannins consist of gallic acid esters and ellagic acid glycosides, formed from shikimate, in which the hydroxyl sugar groups are esterified with phenolic acids. According to Lim *et al*. (2006), hydrolysable tannins were found to possess better antibacterial and antifungal activities compared to mixed and condensed tannins. In addition, previous study done by Basri *et al*. (2012) also proposed that tannins exert major antibacterial and antifungal effects in both methanol and aqueous extracts of QI galls. Therefore, tannins found in the formulated cream might have played an important role towards anti-*Candida* activity in this study. Tannins can interact with the protein contained in the yeast membrane and form hydrogen bonds. This formation could cause conformational changes in the protein molecule which can potentially result in cell death. The ability of tannins to influence the integrity of yeast cell membrane represents an ideal strategy to prevent prolonged infections (Mailoa *et al*., 2014).

As *in vitro* test results may not always correlate with *in vivo* efficacy, more in-depth studies are warranted in the coming researches in order to evaluate the *in vivo* anti-*Candida* properties of QI gall extract vaginal cream using infected female rats as well as to measure their effectiveness or toxicity. Quantitative phytochemical test is also necessary to investigate the major compound in the cream formulated. Besides, further cream base evaluations are to be done which include pH determination, homogeneity, and spreadibility tests to make it suitable and applicable on the human skin.

CONCLUSION

In conclusion, the findings indicate that QI gall extract formulated in vaginal cream can inhibit *C. albicans* growth *in vitro* without causing any local adverse effect as observed *in vivo*. Therefore, the significant antifungal activity of the cream formulation suggests its potential use as one of the effective phytotherapeutic agents in the treatment of candidiasis especially those caused by *C. albicans*.

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