

## DEVELOPMENT AND STABILITY EVALUATION OF VAGINAL SUPPOSITORY CONTAINING *GLYCYRRHIZA GLABRA* L. FOR THE TREATMENT OF *CANDIDA ALBICANS* INFECTION

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### ABSTRACT

**Objective:** The present study assessed the antioxidant activity, phytochemical content, and anti-*Candida albicans* property of ethanolic extract of *Glycyrrhiza glabra* L. (licorice). In addition, suppository formula (SF) was developed with licorice extract (LE), and the stability of SF was evaluated.

**Methods:** The total phenolic and flavonoid content was measured by colorimetric methods. 2, 2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), ferric reducing antioxidant power (FRAP), inhibition of lipid peroxidation (LPO), nitric oxide (NO), and superoxide (SO) radical scavenging assays were performed to evaluate antioxidant property. Antimicrobial activity was determined by plating method. The active principle was determined by high-performance liquid chromatography method.

**Results:** The licorice sample was extracted with 95% ethanol, and 26.91±1.35% of yield was observed. The LE contains phenolic acids (167.70±3.18 mg gallic acid equivalent/g extract), flavonoids (162.53±9.95 mg quercetin equivalent/g extract), and glabridin (3.90±0.05 mg/g extract). LE exhibited the scavenging activity in terms of 810.53±25.37 µM of Trolox equivalent/mg of extract, 165.04±5.10 µM of FeSO<sub>4</sub> equivalent/mg of extract, and 3750.35±1.25, 68.25±0.07, and 511±0.80 µM of Trolox equivalent/mg of extract in ABTS, FRAP, LPO, NO, and SO assays, respectively. About ≥62.50, ≥125, and ≥250 µg/mL of LE was recorded as minimal inhibitory concentration against *C. albicans*, *Lactobacillus casei*, and *Lactobacillus acidophilus*, respectively. The SF was formulated and the stability was evaluated. The activity and color of SFs did not differ on storage. Moreover, no spot formation was observed.

**Conclusion:** The SF with LE could be the safe therapeutic strategy for the treatment of candida infection in vaginal region.

**Keywords:** Antimicrobial activity, *Candida albicans*, *Glycyrrhiza glabra* L., Suppository formula, Licorice, Ethanolic extract.

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### INTRODUCTION

Microbial species are ubiquitous and extensively spread all over the surroundings, especially in human body parts. The association of microbial community and the human body is native, and most of the microbes are nonpathogenic, and being considered as normal flora or commensal flora. Nevertheless, some microbial species becomes lethal to the host system because of the extended biomass of particular species, especially fungal species [1,2]. The fungal infections are the fourth leading cause of microbial infections, particularly by *Candida* species such as *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida lusitanae*, *Candida parapsilosis*, and *Candida tropicalis* [3,4].

Women are the frequent victim of *Candida* infection in the form of vaginal candidiasis (VC), and VC is the second most common vaginal infection in developed countries like the USA, and *C. albicans* is the leading cause of VC, whereas non-*albicans Candida* infection also has been increased gradually [5]. The symptoms of fungal infections are not always noticeable; the severity and incubation period of infection varies based on an individual's immunity. Some of the common cause of fungal infections is a sedentary lifestyle, drugs, improper diet, stress, etc. [6]. The VC is associated with strong itching, swelling, vulval erythema, vulval edema, irritation, and white discharges.

Citronella has significant antifungal activity against *C. tropicalis*, a non-*albicans Candida* species that cause vulvovaginal candidiasis (VVC)

[7]. The leaf extracts of *Pimenta dioica* showed anti-*C. albicans* activity [8]. The modified flavonoids from *Citrus limetta* exhibited increased antifungal activity against *C. albicans* [9].

Although several studies reported the bioactive compounds, the drug delivery is the precious step in the treatment of any diseases; in particular, the treatment of VC is done by oral and suppository supplementation of drugs. The suppository treatments are relatively effective since the drugs are delivered in proximity to the target tissues. Vito *et al.* [10] reported the antimicrobial activity of vaginal suppository formulation (SF) with tea tree oil against *Candida* spp. The plant-based SFs for the treatment of VC are found to be not promising, and it requires in-depth research on finding new active compounds from plant materials against *C. albicans*.

Licorice is one of the ancient, still widely used therapeutic herb, and it is used either alone or in combination with another phytomedicine. Licorice and its bioactive compounds are reported for anti-inflammatory, antiviral, antihepatotoxic, antibacterial activity, and also act as an expectorant and emollient in cosmetics [11,12]. Glabridin, a well-studied prenylated isoflavone of *G. glabra* L. and reported for many biological activities such as estrogen-like activity, inhibition of cytochrome, binding with proliferator-activated receptor gamma, and melanogenesis [13]. The antidiabetic nature and weakening of fat accumulation properties of the glabridin have been reported using

diabetes and obese mice models [14,15]. The recent study reported the anti-*Candida* property of *G. glabra* L. against several *Candida* species and strains, and also explained the anti-*Candida* biofilm activity against *C. glabrata* and *C. tropicalis* strains [16].

Thus, the present study evaluated the anti-*C. albicans* property of ethanolic extract of licorice. Antioxidant activity, and phytochemical content of the selected active extract has also been evaluated. The study also deals with the development of the SF with active extract, assessment of the physicochemical content and stability of the SF.

## METHODS

### Sample collection and extraction

*G. glabra* L. (licorice) were collected from the local farmers of Chiang Mai, Thailand, and the species was confirmed with herbarium specimens of Faculty of Pharmacy, Chiang Mai University, Thailand. The samples were dried at 60°C for 24 h and milled to a fine powder. Then, 1 kg of the sample was macerated in 2 L of 95% ethanol for 48 h and filtrated through Whatman No. 1 filter paper before evaporation at 50°C using vacuum evaporator (Buchi Rotavapor RE-120). The percentage of yield was calculated (Equation 1) and stored at -20°C until use.

$$\text{Yield (\%)} = (\text{weight of the extract (g)}/\text{weight of the licorice powder (g)}) \times 100 \quad (1)$$

### Estimation of phytochemical content

#### Total phenolic and flavonoid content

The total phenolic and flavonoid content of licorice extract (LE) was determined as described previously by Pengkumsri *et al.* [17]. The total phenolic and flavonoid content was denoted as mg of gallic acid equivalent (mg GAE) per g of extract, and mg of quercetin equivalent (mg QE) per g of extract, respectively.

#### Determination of free radical scavenging property of extracts

The antioxidant property of extracts was assessed by 2, 2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), ferric reducing antioxidant power (FRAP), inhibition of lipid peroxidation (LPO), nitric oxide (NO), and superoxide (SO<sup>-</sup>) radical scavenging assays as described by Pengkumsri *et al.* [17].

#### *C. albicans* inhibition assays

The agar dilution assay was performed to assess the anti-*Candida* activity of extracts. In brief, *C. albicans* was cultured in Sabouraud dextrose broth (SDB) at 37°C for 24 h and diluted to achieve  $1.5 \times 10^5$  cells/ml of broth. Then, 100  $\mu$ L of cell suspension was plated by mixing with 10 ml of Sabouraud dextrose soft agar. About 6 mm sized, agar wells were created, and 100  $\mu$ L of LE was introduced. The inhibition zone was measured after incubation at 37°C for 24–48 h. 100  $\mu$ L of ciclopirox olamine (10 mg/mL) (synthetic antifungal agent), SDB, and dimethyl sulfoxide (DMSO) was used as positive, negative, and vehicle control, respectively [18].

The minimal inhibitory concentration (MIC) of LE against *C. albicans* was studied by modified broth dilution method. Briefly, *C. albicans* was raised as  $1.5 \times 10^5$  cells/mL in SDB and mixed with serially diluted test extracts in a 96-well plate, followed by incubating at 37°C for 24 h. The MIC concentration was determined by spectrophotometric analysis of the growth of *C. albicans* at 600 nm. 100  $\mu$ L of ciclopirox olamine (10 mg/mL), SDB, and DMSO was used as positive, negative, and vehicle control, respectively [19].

#### Effect of LE on the growth of representative strains of normal microbial flora

MIC assay was performed to assess the antimicrobial activity of LE against *Lactobacillus casei* and *Lactobacillus acidophilus*. Strains were cultured in de Man, Rogosa, and Sharpe (MRS) broth to achieve the cell density of  $1.5 \times 10^8$  cell/mL. 100  $\mu$ L of ampicillin (2 mg/mL),

MRS, and DMSO was used as positive, negative, and vehicle control, respectively.

## Development and evaluation of SF

### Development of suppository base and SF with LE

The raw materials such as polyethylene glycol (PEG)-6000, PEG-400, propylene glycol, glycerin, and water were used. These ingredients were mixed in different combination and assessed for the hardness, distribution of ingredients, and disintegration time. The different concentration of LE (1, 2, and 5%) was added with a selected best combination of base formula (PEG-6000, PEG-400, propylene glycol, glycerin, and water). The prepared SFs were studied [20] for the physical appearance (color, surface, and silkiness), variations in weight (mean of four decimal weighing of 20 samples), hardness (by cutter shearing), distribution of ingredients (by spot and migration of ingredients), melting point, and disintegration time (time required for complete dissociation of formula at 37°C).

### Stability evaluation

The stability of SF was measured as per the guideline of Center for Drug Evaluation and Research, U.S.A (ICH Q1A (R2)) and Department of Health and Human Services Food and Drug Administration. The stability at different temperature (5 $\pm$ 3, 30 $\pm$ 2, and 40 $\pm$ 2°C) with 75% of relative humidity for 3 months was studied. The changes in phytochemicals content, *C. albicans* inhibition property, and antioxidant property of the formula were assessed.

### Determination of active compounds

Glabridin and 18- $\beta$ -glycyrrhetic acid content extract have been assessed by high-performance liquid chromatography technique. The extract was analyzed using isocratic condition (Shimadzu LC 10AV, Japan) with Shodex<sup>®</sup> RP-C18 (250 mm  $\times$  4.6 mm, 5  $\mu$ m, Japan) column. The isocratic condition of mobile phase was prepared with 70% of acetonitrile with 30% of 0.05% trifluoroacetic acid at a flow rate of 1.0 mL/min. The UV detector (Shimadzu LC 10AV, Japan) was set at 240 nm to analyze a linear range of glabridin (0.01–0.05 mg/mL) and 18-glycyrrhetic acid (0.02–0.10 mg/mL) and LE.

### Statistical analysis

The results were determined in triplicates to confirm the reproducibility. The data were given as mean  $\pm$  standard deviation. Analysis of variance was performed using statistical SPSS software version 17 (Chicago, SPSS Inc., U.S.A). The least significant difference *post hoc* test was performed to analyze the significant differences in antioxidant activities and  $p < 0.05$  was considered as statistically significant.

## RESULTS AND DISCUSSION

The licorice sample was extracted with 95% ethanol and 26.91 $\pm$ 1.35% of yield was achieved. Then, the LE was found to contain 167.70 $\pm$ 3.18 mg GAE/g extract and 162.53 $\pm$ 9.95 mg QE/g extract of phenolic and flavonoids content, respectively. The major active compounds of the licorice, such as glabridin and 18- $\beta$ -glycyrrhetic acid, were assessed in the LE and only glabridin (3.90 $\pm$ 0.05 mg/g extract) was detected (Table 1). The antioxidant nature of the LE has been evaluated by various assays such as ABTS, FRAP, LPO, NO, and SO. LE showed the scavenging

**Table 1: The yield and phytochemical content of ethanolic extract of *Glycyrrhiza glabra* L.**

S. No	Content	Values
1	Percentage of yield (%)	26.91 $\pm$ 1.35
2	Total phenolic content (mg GAE/g extract)	167.70 $\pm$ 3.18
3	Total flavonoid content (mg QE/g extract)	162.53 $\pm$ 9.95
4	Glabridin content (mg/g extract)	3.90 $\pm$ 0.05
5	18- $\beta$ -Glycyrrhetic acid content (mg/g extract)	ND

GAE: Gallic acid equivalent, QE: Quercetin equivalent, ND: Not detected

activity in terms of  $165.04 \pm 5.10 \mu\text{M}$  of  $\text{FeSO}_4$  equivalent/mg of extract and  $810.53 \pm 25.37$ ,  $3750.35 \pm 1.25$ ,  $68.25 \pm 0.07$ , and  $511 \pm 0.80 \mu\text{M}$  of Trolox equivalent/mg of extract in FRAP, ABTS, LPO, NO, and SO assays, respectively (Table 2). The data indicated that LE contains more free radical scavenging compounds, especially glabridin.

The studies suggested that nystatin vaginal suppositories were effective to treat VVC caused by *C. glabrata* and fluconazole-resistant *Candida*, whereas nystatin suppositories were less effective against *C. albicans*. Moreover, miconazole nitrate (1200 mg) vaginal suppository was active as oral fluconazole (150 mg) for the treatment of severe VVC [21,22]. The VVC in diabetes patients is commonly caused by *C. glabrata* and *C. tropicalis*. The boric acid vaginal suppositories based therapy cure the VVC in diabetes patients when compared to the oral fluconazole treatment, and boric acid suppositories are superior for the acute treatment of VVC [23,24]. The 14-day use of vaginal suppositories prepared with amphotericin B (50 mg) cures the non-albicans VVC effectively [5]. Whereas, the impact of the use of antibiotic as suppositories on normal microbial flora has not been reported in detail. In the present study, the MIC of LE against *C. albicans* and representative normal flora such as *L. casei* and *L. acidophilus* were studied. About  $\geq 62.50$ ,  $\geq 125$ , and  $\geq 250 \mu\text{g/mL}$  of LE was recorded as MIC for *C. albicans*, *L. casei*, and *L. acidophilus*, respectively. The results of the present study also proved the anti-*Candida* property of LE, and

the data indicated that the LE extract has not affected the representative normal microbial flora *in vitro*, which revealed that the use of LE in SF is safe and effective against infection in terms of antioxidant potential (Table 2) and antifungal nature (Table 3).

The desired quality of the base material, which is used in the SF preparation, is amorphous solid at room temperature, whereas, at body temperature (35–37°C), it should melt and facilitates the release of active compounds. The base materials are stable, non-irritating, and miscible with water and vaginal secretions. Oleaginous (triglycerides and cocoa butter [the obroma oil]) and water-soluble (PEG and glycerinated gelatin) bases are commonly used. The base of SF was made with different combinations of PEG6000, PEG400, water, propylene glycol, and glycerin at various concentrations (Table 4).

The dimension of the SF was about 25 mm×10 mm. The suitability of base material was assessed in three diverse categories such as hardness, distribution, and disintegration time (min). The combination of PEG6000, PEG400, water, propylene glycol, and glycerin at the ratio of 40:30:10:10:10 was found to be the best base material for SF. The optimum hardness, good distribution, and 20 min of disintegration time were observed in formula F6 (Table 4).

Four types of SFs were prepared with selected base (F6) and different concentration of (1, 2, and 5%) LE (Table 5). The SFs were assessed for the physical appearance, weight (g), hardness, distribution, melting point, and disintegration time. The formula "B" was found to be the optimum composition for the SF with LE extract, which contains 2% of LE. The weight, melting temperature, and disintegration time of formula "B" was  $2.45 \pm 0.03 \text{ g}$ ,  $34.33 \pm 0.58^\circ\text{C}$ , and  $27.40 \pm 0.70 \text{ min}$ , correspondingly (Table 6).

The stability of the SF with LE was assessed after the storage of samples at different temperature for 3 months as per the guideline of ICH Q1A (R2) Center for Drug Evaluation and Research, U.S., Department of Health and Human Services Food and Drug Administration. The MIC value ( $\geq 31.25 \mu\text{g/mL}$ ) and color of SFs did not differ on storage. Moreover, no spot formation was observed (Table 7).

**Table 2: Antioxidant activities of the licorice extract**

Antioxidant assays	Mean±SD
ABTS	$810.53 \pm 25.37 \mu\text{M}$ of Trolox equivalent/mg of extract
FRAP	$165.04 \pm 5.10 \mu\text{M}$ of $\text{FeSO}_4$ equivalent/mg of extract
LPO	$3,750.35 \pm 1.25 \mu\text{M}$ of Trolox equivalent/mg of extract
NO	$68.25 \pm 0.07 \mu\text{M}$ of Trolox equivalent/mg of extract
SO	$511 \pm 0.80 \mu\text{M}$ of Trolox equivalent/mg of extract

ABTS-2': 2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, FRAP: Ferric reducing antioxidant power, LPO: Inhibition of lipid peroxidation, NO: Nitric oxide, SO: Superoxide radical scavenging assay

**Table 3: Minimal inhibitory concentration of fresh LE and LE loaded suppository formula (stored at different conditions for 3 months) against tested microbes**

Samples	Minimal inhibitory concentrations		
	<i>C. albicans</i>	<i>L. casei</i>	<i>L. acidophilus</i>
DMSO	$\geq 125.00 \mu\text{L/mL}$	$\geq 500 \mu\text{L/mL}$	$\geq 500 \mu\text{L/mL}$
Ciclopirox olamine (positive control; anti-yeast)	$\geq 15.625 \mu\text{g/mL}$	-	-
Ampicillin (positive control; antibacteria)	-	$\geq 31.25 \mu\text{g/mL}$	$\geq 31.25 \mu\text{g/mL}$
LE	$\geq 62.50 \mu\text{g/mL}$	$\geq 125 \mu\text{g/mL}$	$\geq 250 \mu\text{g/mL}$
After 3 months of storage at different temperature			
SFLE (stored at -20°C)	$\geq 31.25 \mu\text{g/mL}$	$\geq 125 \mu\text{g/mL}$	$\geq 250 \mu\text{g/mL}$
SFLE (stored at 4°C)	$\geq 31.25 \mu\text{g/mL}$	$\geq 125 \mu\text{g/mL}$	$\geq 250 \mu\text{g/mL}$
SFLE (stored at 25°C)	$\geq 31.25 \mu\text{g/mL}$	$\geq 125 \mu\text{g/mL}$	$\geq 250 \mu\text{g/mL}$
SFLE (stored at 40°C)	$\geq 62.50 \mu\text{g/mL}$	$\geq 250 \mu\text{g/mL}$	$\geq 500 \mu\text{g/mL}$

SFLE: Suppository formula with licorice extract, LE: Licorice extract, DMSO: Dimethyl sulfoxide, *C. albicans*: *Candida albicans*, *L. casei*: *Lactobacillus casei*, *L. acidophilus*: *Lactobacillus acidophilus*

**Table 4: Different combination of base material and assessment of favorable conditions for the optimal preparation of suppository base formula**

Formula	Concentration (%)					Assessed parameters		
	PEG6000	PEG400	Water	Propylene glycol	Glycerin	Hardness	Distribution	Disintegration time (min)
F1	50	30	20	-	-	++++	Poor	~15
F2	50	30	-	20	-	+++	Poor	~25
F3	50	30	-	-	20	+++	Poor	~25
F4	40	20	10	20	10	+	Very poor	~25
F5	40	20	20	20	0	-	Poor	>30
F6	40	30	10	10	10	++	Good	~20
F7	40	30	20	10	0	-	Poor	~25

PEG: Polyethylene glycol

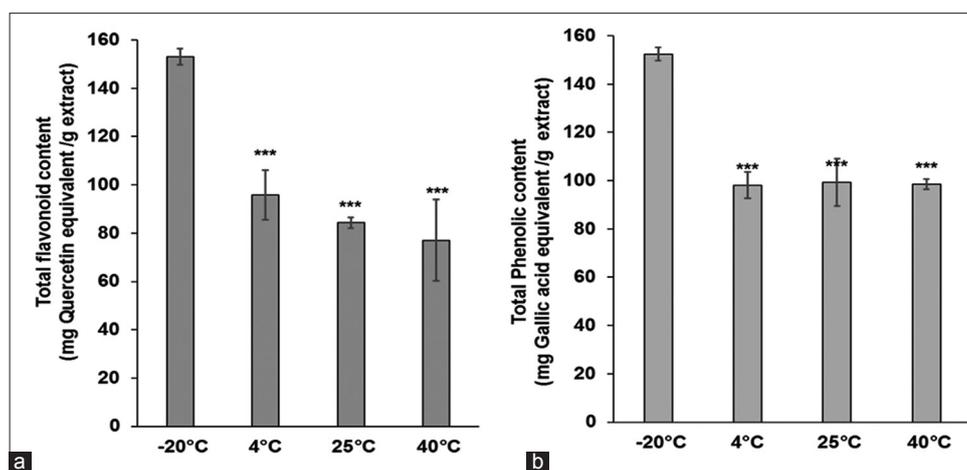


Fig. 1: The total flavonoid (a) and phenolic (b) content of suppository formula at different storage temperatures

Table 5: Formulation of suppository formula with different concentration of *Glycyrrhiza glabra* L. extract

Formula	Concentration (%)					
	PEG6000	PEG400	Propylene glycol	Glycerin	Water	Licorice extract
Base	40	30	10	10	10	0
A	40	30	10	10	9	1
B	40	30	10	10	8	2
C	40	30	10	10	5	5

PEG: Polyethylene glycol

Table 6: Evaluation of suppository formula with different concentration of *Glycyrrhiza glabra* L. extract

Formula	Evaluation					
	Physical appearance	Weight (g)	Hardness	Distribution	Melting point (°C)	Disintegration time (min)
Base	Good	2.40±0.05	++	Good	33.50±0.87	25.20±1.03
A	Poor	2.43±0.02	++	Poor	34.01±0.75	27.00±0.50
B	Good	2.45±0.03	++	Good	34.33±0.58	27.40±0.70
C	Poor	2.47±0.05	+++	Poor	34.55±0.62	30.75±0.81

+: Delicate, ++: Optimum, +++: Hard

Table 7: The stability assessment of selected suppository formula with *Glycyrrhiza glabra* L. extract after 3 months of storage

Condition	MIC (µg/mL)	Color	Uniformity
MS	≥125.00 µL	-	-
Ciclopirox olamine	>78.125	-	-
SFLE (stored at -20°C)	≥31.25	Orange	Yes
SFLE (stored at 4°C)	≥31.25	Orange	Yes
SFLE (stored at 25°C)	≥31.25	Orange	Yes
SFLE (stored at 40°C)	≥31.25	Orange	Yes

SFLE: Suppository formula with licorice extract

The total flavonoid and phenolic content of the SF were reduced concerning the storage temperature. The flavonoid concentration was reduced as 153.15±3.32, 95.84±10.35, 84.3±2.25, and 77.08±16.87 mg QE/g of extract, when stored at -20, 4, 25, 40°C, respectively. Likewise, the phenolic concentration was reduced as 152.15±2.71, 98.11±5.42, 99.28±9.90, and 98.53±2.0 mg GAE/g of extract, when stored at -20, 4, 25, 40°C, respectively (Fig. 1).

The antioxidant property of the SFs was estimated by five different assays such as FRAP, ABTS, LPO, NO, and SO, after storage and found that the storage at more than 25°C significantly affected the quality of the SFs regarding the free radical scavenging property. The scavenging properties were reduced from 146.84±0.46 mg of FeSO<sub>4</sub> equivalent/g

of extract and 775±112.29, 3550.64±17.46, 64.14±0.02, and 490.77±1.90 mg of Trolox equivalent/g of extract to 129.32±0.79 mg of FeSO<sub>4</sub> equivalent/g of extract and 569.33±14.24, 2261.77±456.89, 58.79±0.27, and 472.41±14.15 mg of Trolox equivalent/g of extract, after 3 months of storage at 40°C (Fig. 2).

The perfect SF should be non-toxic, non-irritating, and stable during storage. The SFs were active against *C. albicans* and relatively inert against tested *Lactobacillus*. The antioxidant nature was preserved during the storage of SF preparation at 4°C for 3 months. Room temperature affected the free radical scavenging nature but not the anti-*Candida* property of active compounds.

## CONCLUSION

The developed SF with LE was stable, non-toxic, and non-irritating in nature and active against *C. albicans*. Further, clinical studies are required to confirm the activity of developed SFs in real situation. In future, the SF could be in market, after the successful clinical trials.

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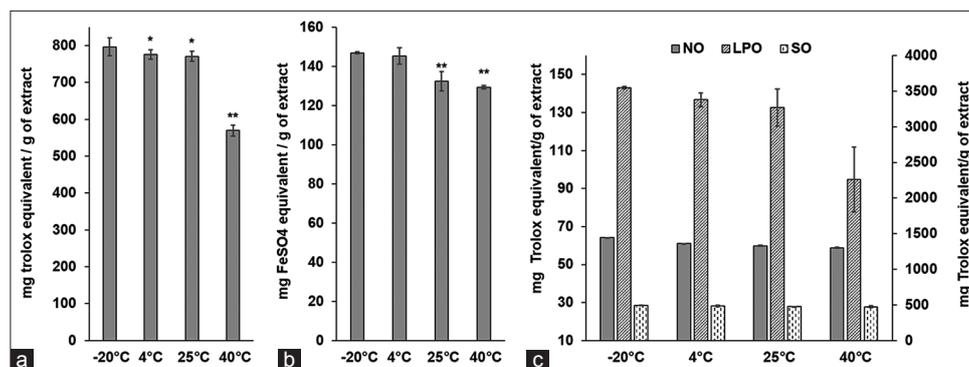


Fig. 2: The antioxidant property of suppository formula at different storage temperatures. (a) 2, 2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, (b) ferric reducing antioxidant power, and (c) nitric oxide, inhibition of lipid peroxidation and superoxide assays

#### AUTHOR'S CONTRIBUTIONS

CC involved in the study design, experiments, review, and finalization of the manuscript. BSS and PK contributed to data analysis, manuscript preparation, and critical revision of the manuscript. SS, NP, KC, NT, and SP are responsible for wet lab experiments, data collection, and analysis. All the authors agree with the content of the manuscript.

#### CONFLICTS OF INTEREST

There are no conflicts of interest.

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