WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.453
Volume 13, Issue 8, 930-953. Research Article ISSN 2277–7105

THE IMPACT OF AN *IN SILICO* STUDY AND A POLYHERBAL FORMULATION "CLEAN V" ON VAGINAL FUNGAL INFECTION CAUSED BY CANDIDA ALBICANS, BOTH IN TERMS OF PREVENTION AND TREATMENT

Kamrudeen Samani[1]*, Kabita Kandel[2] and Dhirendra K. Sah[3]

[1,2,3]Dept. of Research and Development, Fleur Himalayan Ltd, Om Ashram, Birgunj Nepal.

Article Received on
01 March 2024,

Revised on 22 March 2024,
Accepted on 11 April 2024

DOI: 10.20959/wjpr20248-31990

***Corresponding Author**
Kamrudeen Samani
Dept. of Research and Development, Fleur Himalayan Ltd, Om Ashram, Birgunj Nepal.

ABSTRACT

Vaginal fungal infection is second after bacterial infection and Vulvovaginal Candidiasis (VVC), mainly caused by *Candida albicans*(with 40-45% recurrence), influencing women's reproductive function and morbidity in newborns is of global concern. Despite the prescription of oral antifungal agents, it is imperative to have natural products derived from local treatment formulation that can also restore the vaginal micro-ecosystem. Clean V, formulated with different natural oils as antifungal agents and lactic acid for pH modulation was developed. The antifungal activity of Clean V against *C. albicans*ATCC 10231 showed 20 mg/ml as minimum inhibitory concentration (MIC) in disk and well diffusion method with 2.0 cm of a zone of inhibition and 30 mg/ml. as minimum fungicidal concentration (MFC) in pour plate method with 2-minute exposure time against log phase growing pathogen. Comparative analysis of the commercial similar product that had the same MIC but in MFC assay at the same concentration showed $2.44X10^3$ colony forming units per mL (CFU/mL) indicating Clean V to be superior. Using lactic acid as a pH-modulating agent can aid in the restoration of vaginal microflora. Additionally, Clean V, which contains a surfactant free of sulphate and paraben, is suitable for individuals with sensitive skin. Therefore, it is proposed that Clean V may offer improved effectiveness as a substitute for treating VVC. After conducting *in-silico* research on ligand-protein interaction, it was found that 1,8-cineole, lactic acid, linalyl acetate, linalol, and terpinene-4-ol show good binding energies with the target C. albicans.

KEYWORDS: *Antifungal, Lactic acid, Natural oils, Vulvovaginal Candidiasis.*

1.0. INTRODUCTION

Vaginal complaints are most common nowadays, become the global concern.[1] In reproductive-aged women, vulvovaginitis, orinflammation of the vulva and vagina, is most ordinarily secondary to infectious agents where vulvovaginal candidiasis is responsible for about one third of the cases. Also, it affects up to 75% of the women at some points in their lifetime. About 8% of women suffer from recurrent VVC. 90% of VVC is caused by the *Candida albicans* and the remaining cases caused by *Candida glabrata.*[2] *C. albicans* normally a harmless commensal organism found in the gastrointestinal tract, oral and vaginal mucosa and is often asymptomatic. For some immunologically weak and immunocompromised people, however, it is opportunistic pathogen. The risk factors for VVC include the use of reproductive hormones, antibiotic treatment, use of oral contraceptives and diabetes.[3]

A polyherbal formulation, Clean V enriched with natural oils; *Melaleucaalternifolia* (Tea Tree) oil and *Lavandulaangustifolia* (Lavender) essential oil and lactic acid as a pH balancing formula could be a valid alternative to synthetic drugs for curing VVC. Essential oils are well known in traditional medicine characterized by the broad spectrum activity against bacteria and fungi.[4] *Melaleucaalternifolia*, known as Tea tree is an Australian native plant. The medicinal uses of its leaves were already known to heal wounds and cure cutaneous infection by squeezing them over the infected part. Oil obtained from steam-distillation from the leaves was used in a wide range of pathological conditions, such as tinea, ringworm, empyema, paronychia, tonsillitis, stomatitis and vaginal infections.[5][6] Tea tree oil alters the permeability of cells membrane, allowing K^+ leakage leads to loss of chemiosmotic control therefore cause rupture and disruption of cell wall.[7]

The essential oil of *Lavandulaangustifolia*is mostly used as a carminative, sedative agent, and as a relaxant in aromatherapy.[8] Traditionally, it was used in veterinary practice to kill lice and other animal parasites as well as in wounds and burns as an antiseptic agent.[9] The main chemical components; linalool and linalyl acetate are responsible for the inhibition of fungal growth, the formation of germ tubes and the killing of fungal cells. In the last few years, the antimicrobial activity of lavender oil has been carried out against antibiotic-resistant strains[10] and anti fungal activity including the inhibition of germ tube growth for *C.albicans.*[11]

Lactic acid maintains an acidic environment that promote a healthy vaginal milieu inhospitable to many pathogenic bacteria and is negatively correlated with vaginal infections.[12] Besides maintaining the acidic environment, lactic acid has potent antimicrobial property against both bacteria and fungi.[13] MIC and MBC for bacteria; ≥1.25 mg/mL & ≥2.50 mg/mL MIC and MFC of lactic acid for *C.albicans;* ≥18.75 mg/mL & 37.50 mg/mL respectively (Stanojević-Nikolić *et.al.*, 2016).

The objective of the present study was to evaluate the antifungal activity of Clean V against *C. albicans*ATCC 10231.To this end, we investigated the ability of the polyherbal formulation to inhibit fungal growth and kill time. In addition, a comparative study with the commercially available product was brought out to evaluate the efficacy of the product.

2.0. MATERIALS AND METHOD

2.1. Docking studies

The docking program requires three computation steps to carry out a docking study these are as follows:

(1) Preparation of the receptor

(2) Preparation of the ligand

(3) Set-up of the parameters of the docking program

The following subsections describe these three steps in detail.[30]

2.2. Receptor preparation

The three-dimensional structure of Candida albicans SC5314, with PDB code 4yde, was obtained from the Protein Data Bank (PDB) at http://www.rcsb.org/pdb/home/home.do.

The RCSB is a global archive that contains information about the 3D structure of macromolecules, such as proteins, DNA, and their complexes, as determined by X-ray crystallography, NMR spectroscopy, and cryoelectron microscopy.[30]

2.3. Ligand preparation

Clean V is a polyherbal formulation that contains three different compositions, each with its own active chemical constituent responsible for therapeutic benefits. These active chemical constituents, also known as ligands, were downloaded from the ChEMBL Database and loaded into Pyrex for molecular docking. We used Swiss adme to predict the ligand molecular properties, such as ADME parameters, pharmacokinetic properties, drug likeness nature, and medicinal chemistry.[30]

2.4. Target protein and active site prediction

The various literature surveys were taken into consideration for the evaluation of protein and the active sites.[30]

2.5. Molecular docking

Pyrex software is used for molecular docking and Ligand was downloaded from the ChEMBL Database. Protein was downloaded from Protein Data Bank which can be accessed at http://www.rcsb.org/pdb/home/home.do with Candida albicans SC5314 (PDB code 4YDE).

To prepare a protein, you can use "Discovery Studio Visualizer." First, you need to delete all water molecules and hetero atoms. To do this, select them and hit delete. Next, add polar hydrogen atoms. You can find this option by clicking on "Chemistry" and then "Polar Hydrogen." Once you've completed these steps, save the file in PDB mode. Adding hydrogen atoms will help the protein interact with the amino acids present in the 2D structure.

Now open Pyrex software, click on the "File" menu, choose "Load Molecule," select the protein, “left-click on it” and choose "Auto-Dock," followed by "Make Macromolecule."

To prepare the ligand, click on "Open Babel," choose "Insert New Item" (represented by an Excel format with a plus sign on the left-hand side), select the ligand, “left-click on it”, and choose "Minimize Selected," followed by "Convert Selected to Auto Dock Ligand (PDBQT)."

To interact with the protein and ligand, click on "Vina Wizard," click on "Start" (located on the left-hand side). To add the ligand and macromolecule, press "CTRL" and select the ligand and macromolecule files in PDBQT format. Click on "Forward" (a new dialog box appears), then click on "Forward" again. Docking will start after that. Once docking is complete, save the file by clicking on "Save As Comma-Separated Value" (represented by an excel format with a save icon on the left hand side).

To analyse the results in Auto Dock, follow these steps. First, click on the "Macromolecule" tab. Next, select the highest docking score “left-click on it”. Then, choose the "Display Option" feature. This will generate all of the models or conformations of the ligand. If you want to change the display of the protein, select the protein and click on "Macromolecule Surface". Here, you can see all of the poses of the ligand that have been displayed. You can remove unwanted poses one by one by self-selecting them. Keep the ligand of your choice for

better generalization and save it in PDB format, for example, "LigandName_Docked". To analyze the report (for example, a 2D image), open Discovery Studio Visualizer, load the docked ligand, press "CTRL + H," followed by opening the protein and pressing "CTRL + H." Copy all the protein files and paste them into the ligand file. Select the protein, click on "Define Receptor," select the ligand, click on "Define Ligand," followed by ligand interaction and click on "Show 2D Diagram.".[30]

3.0 Revival of Test Organism

For the revival of the test organism, a single colony of *Candida albicans*ATCC 10231 (Microbiologics) was taken and inoculated intoa test tube containing 10 mL of Sabouraud Dextrose Broth (Himedia) and incubated at 35°c for 48 hours. After that, one loop-full organism was taken and streaked on the Sabouraud Dextrose Agar with Chloramphenicol, Medium 4. (In accordance with IP 2014) (Himedia) plates. Then the plates were incubated at 35°c for 48 hours.

3.1. Antimicrobial Susceptibility Testing

3.1.1. Disk-Diffusion Assay

Disk-diffusion test or Kirby-Bauer method was performed to establish the zone of inhibition to gauge the efficacy of clean V. In this method, small disk having diameter of 5 mm were made from filter paper. Sabouraud Dextrose Agar with Chloramphenicol, Medium 4. (In accordance with IP 2014) (Himedia) plate was prepared and inoculated with the organism suspension equivalent to 0.5 McFarland and a small disk containing Clean V were placed on the agar surface aseptically. Then agar plates were incubated at 35 °C for 48 hours.

3.1.2. Well Diffusion Assay

Similarly, with the procedure used in the disk-diffusion method, a hole with a diameter of 6 to 8 mm is punched aseptically in the SDA plate with a sterile cork bore, and a volume (100 µL) of the Clean V was introduced into the well. This method is also used to establish the zone of inhibition.[12]

3.1.3. MIC and MFC Determination

Using broth dilution method, Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) were determined. For this, 100 µl of organism suspension equivalent to 0.5 McFarland was added to each tubes containing different concentrations; 0 mg/mL,1 mg/mL, 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL, 25 mg/mL, 30 mg/mL, 35

mg/mL, 40 mg/mL, 50 mg/mL of Clean V and incubated at 35°C for 48 hours. MIC was defined as the minimum concentration of the starter at which the tested micro-organism does not demonstrate visible growth was observed.[11] To determine MFC, 100 µl from each tubes showing no visible growth was spread on SDA plates and incubated at 35 °C for 48 hours and observed the plates compared to the control plate.

3.1.4. Time and Concentration Dependent Anti-fungal Activity Determination

Pour Plate Method giving 2 minutes' treatment time was used further to explore the time and concentration dependent anti fungal activity. For this, single colony of *C. albicans* was taken and inoculated in a test tube containing 10 ml SDB and incubated at 35°C for 48 hours. After that, 100 µl of culture is inoculated in 10 mL SDB and incubated at 35°C for 3 hours. Optical density was measured and maintain 0.5 McFarland turbidity standard (0.08 -0.1 at OD_{600}) which provides an optical density comparable to the density of an organism suspension $1.5*10^8$ colony forming units. Different concentrations of Clean V (0mg/mL, 15mg/mL, 20mg/mL, 25 mg/mL, 30 mg/mL, 35 mg/mL and 40 mg/mL and 50 mg/mL) was prepared in SDB media. On the other hand, plates containing SDA with 1.5% agar and tubes containing 9 mL SDB with 0.8% agar as a top agar were prepared for all concentrations. Then 100µl of test organisms was subjected in to the different concentrations of Clean V and a treatment time of 2 minutes was given then 1000µl was taken from each tubes and subjected in to the test tubes containing top agar then poured over the SDA plates. Plates were incubated at 35°C for 48 hours.

3.1.5. Comparative Analysis with Commercially Available Market Sample

A comparative study for the anti fungal activity of Clean V with the commercially available sample was done via well diffusion[12] and pour plate method with adopting the same procedure mention in the testing method of Clean V.

4.0. RESULT AND DISCUSSION

Table 1: Docking results with 4YDE.

Compound name	Binding Affinity (-)	Interacting amino acids	Interaction Ligand-residue
1,8-cineole	6	ARG A:292 PHE A:259	Attached to Oxygen
Lactic Acid	4.4	ARG B:413 ASN B:426 LYS B:427 HIS B:415	Attached to Oxygen Attached to Hydrogen

		ASP B: 422	
Linalool	5.4	TYR A:103	Attached to Hydrogen
Linalyl Acetate	5.8	GLN A:110 TYR A:103	Attached to Oxygen Attached to Hydrogen
Terpinene-4-ol	6.1	LEU B:381 ASN B:321	Attached to Oxygen Attached to Hydrogen

Here is a 2D image of the ligand docked with an active phytochemical component, illustrating their binding relationship.

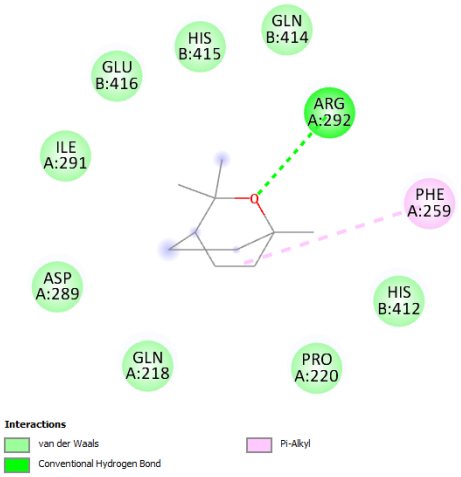


Fig. 1.0. Binding interactions between 1,8-cineole with 4YDE.

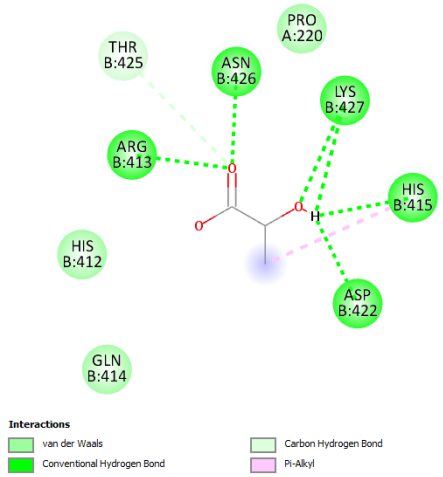


Fig. 2.0. Binding interactions between Lactic Acid with 4YDE.

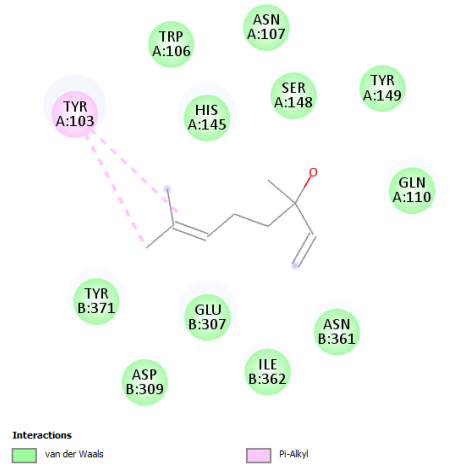


Fig. 3.0 Binding interactions between Linalool with 4YDE.

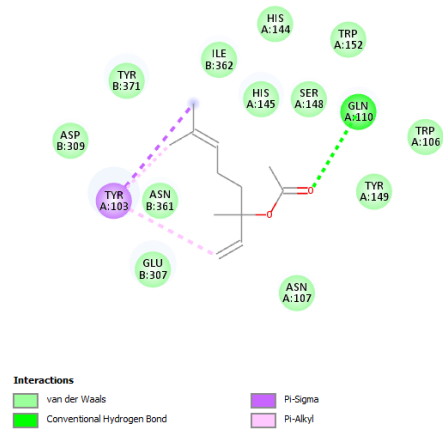


Fig. 4.0. Binding interactions between Linalyl Acetate with 4YDE.

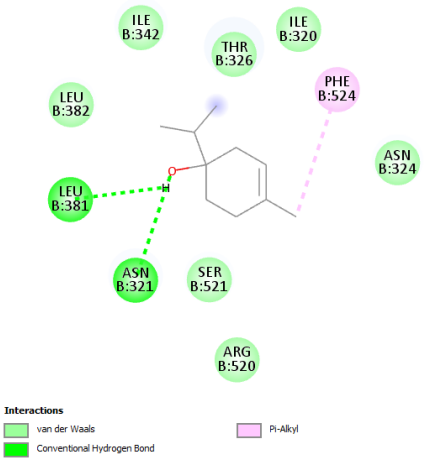


Fig. 5.0: Binding interactions between Terpinene-4-o l with 4YDE.

Table 2: Physicochemical Properties, Lipophilicity, Water Solubility, Pharmacokinetics, Druglikeness and Medicinal Chemistry of different active phytochemical constituent are shown below.

1. Compound name: 1,8-cineole

Physicochemical Properties	
Formula	C10H18O
Molecular weight	154.25 g/mol
Num. heavy atoms	11
Num. arom. heavy atoms	0
Fraction Csp3	1.00
Num. rotatable bonds	0
Num. H-bond acceptors	1
Num. H-bond donors	0
Molar Refractivity	47.12
TPSA	9.23 Å²
Lipophilicity	
Log $P_{o/w}$ (iLOGP)	2.58
Log $P_{o/w}$ (XLOGP3)	2.74
Log $P_{o/w}$ (WLOGP)	2.74
Log $P_{o/w}$ (MLOGP)	2.45
Log $P_{o/w}$ (SILICOS-IT)	2.86
Consensus Log $P_{o/w}$	2.67
Water Solubility	
Log *S* (ESOL)	-2.52
Solubility	4.63e-01 mg/ml ; 3.00e-03 mol/l
Class	Soluble
Log *S* (Ali)	-2.59
Solubility	3.98e-01 mg/ml ; 2.58e-03 mol/l
Class	Soluble
Log *S* (SILICOS-IT)	-2.45
Solubility	5.45e-01 mg/ml ; 3.53e-03 mol/l

Class	Soluble
Pharmacokinetics	
GI absorption	High
BBB permeant	Yes
P-gp substrate	No
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Log K_p (skin permeation)	-5.30 cm/s
Druglikeness	
Lipinski	Yes; 0 violation
Ghose	No; 1 violation: MW<160
Veber	Yes
Egan	Yes
Muegge	No; 2 violations: MW<200, Heteroatoms<2
Bioavailability Score	0.55
Medicinal Chemistry	
PAINS	0 alert
Brenk	0 alert
Leadlikeness	No; 1 violation: MW<250
Synthetic accessibility	3.65

2. Compound name:lactic acid

Physicochemical Properties	
Formula	C3H6O3
Molecular weight	90.08 g/mol
Num. heavy atoms	6
Num. arom. heavy atoms	0
Fraction Csp3	0.67
Num. rotatable bonds	1
Num. H-bond acceptors	3
Num. H-bond donors	2
Molar Refractivity	19.47
TPSA	57.53 Å²
Lipophilicity	
Log $P_{o/w}$ (iLOGP)	-0.02
Log $P_{o/w}$ (XLOGP3)	-0.72
Log $P_{o/w}$ (WLOGP)	-0.55
Log $P_{o/w}$ (MLOGP)	-0.85
Log $P_{o/w}$ (SILICOS-IT)	-0.80
Consensus Log $P_{o/w}$	-0.59
Water Solubility	
Log *S* (ESOL)	0.12
Solubility	1.19e+02 mg/ml ; 1.32e+00 mol/l
Class	Highly soluble

Log *S* (Ali)	-0.01
Solubility	8.76e+01 mg/ml ; 9.72e-01 mol/l
Class	Very soluble
Log *S* (SILICOS-IT)	0.98
Solubility	8.66e+02 mg/ml ; 9.62e+00 mol/l
Class	Soluble
Pharmacokinetics	
GI absorption	High
BBB permeant	No
P-gp substrate	No
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Log K_p (skin permeation)	-7.36 cm/s
Druglikeness	
Lipinski	Yes; 0 violation
Ghose	No; 4 violations: MW<160, WLOGP<-0.4, MR<40, #atoms<20
Veber	Yes
Egan	Yes
Muegge	No; 2 violations: MW<200, #C<5
Bioavailability Score	0.85
Medicinal Chemistry	
PAINS	0 alert
Brenk	0 alert
Leadlikeness	No; 1 violation: MW<250
Synthetic accessibility	1.29

Swiss Institute of Bioinformatics - © 2024 | SIB privacy policy

3. Compound name : Linalool

Physicochemical Properties	
Formula	C10H18O
Molecular weight	154.25 g/mol
Num. heavy atoms	11
Num. arom. heavy atoms	0
Fraction Csp3	0.60
Num. rotatable bonds	4
Num. H-bond acceptors	1
Num. H-bond donors	1
Molar Refractivity	50.44
TPSA	20.23 Å²
Lipophilicity	
Log $P_{o/w}$ (iLOGP)	2.71
Log $P_{o/w}$ (XLOGP3)	2.97
Log $P_{o/w}$ (WLOGP)	2.67

Log $P_{o/w}$ (MLOGP)	2.59
Log $P_{o/w}$ (SILICOS-IT)	2.35
Consensus Log $P_{o/w}$	2.66
Water Solubility	
Log *S* (ESOL)	-2.40
Solubility	6.09e-01 mg/ml ; 3.95e-03 mol/l
Class	Soluble
Log *S* (Ali)	-3.06
Solubility	1.35e-01 mg/ml ; 8.75e-04 mol/l
Class	Soluble
Log *S* (SILICOS-IT)	-1.84
Solubility	2.20e+00 mg/ml ; 1.43e-02 mol/l
Class	Soluble
Pharmacokinetics	
GI absorption	High
BBB permeant	Yes
P-gp substrate	No
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Log K_p (skin permeation)	-5.13 cm/s
Druglikeness	
Lipinski	Yes; 0 violation
Ghose	No; 1 violation: MW<160
Veber	Yes
Egan	Yes
Muegge	No; 2 violations: MW<200, Heteroatoms<2
Bioavailability Score	0.55
Medicinal Chemistry	
PAINS	0 alert
Brenk	1 alert: isolated_alkene
Leadlikeness	No; 1 violation: MW<250
Synthetic accessibility	2.74
Swiss Institute of Bioinformatics - © 2024 | SIB privacy policy	

4. Compound name: Linalyl acetate

Physicochemical Properties	
Formula	C12H20O2
Molecular weight	196.29 g/mol
Num. heavy atoms	14
Num. arom. heavy atoms	0
Fraction Csp3	0.58
Num. rotatable bonds	6
Num. H-bond acceptors	2
Num. H-bond donors	0

Molar Refractivity	60.17
TPSA	26.30 Å²
Lipophilicity	
Log $P_{o/w}$ (iLOGP)	3.08
Log $P_{o/w}$ (XLOGP3)	3.93
Log $P_{o/w}$ (WLOGP)	3.24
Log $P_{o/w}$ (MLOGP)	2.95
Log $P_{o/w}$ (SILICOS-IT)	2.98
Consensus Log $P_{o/w}$	3.24
Water Solubility	
Log *S* (ESOL)	-3.14
Solubility	1.43e-01 mg/ml ; 7.30e-04 mol/l
Class	Soluble
Log *S* (Ali)	-4.18
Solubility	1.29e-02 mg/ml ; 6.58e-05 mol/l
Class	Moderately soluble
Log *S* (SILICOS-IT)	-2.52
Solubility	5.97e-01 mg/ml ; 3.04e-03 mol/l
Class	Soluble
Pharmacokinetics	
GI absorption	High
BBB permeant	Yes
P-gp substrate	No
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Log K_p (skin permeation)	-4.71 cm/s
Druglikeness	
Lipinski	Yes; 0 violation
Ghose	Yes
Veber	Yes
Egan	Yes
Muegge	No; 1 violation: MW<200
Bioavailability Score	0.55
Medicinal Chemistry	
PAINS	0 alert
Brenk	1 alert: isolated_alkene
Leadlikeness	No; 2 violations: MW<250, XLOGP3>3.5
Synthetic accessibility	2.75
Swiss Institute of Bioinformatics - © 2024 | SIB privacy policy	

5. Compound name: Terpinene -4-ol

Physicochemical Properties	
Formula	C10H18O
Molecular weight	154.25 g/mol
Num. heavy atoms	11
Num. arom. heavy atoms	0
Fraction Csp3	0.80
Num. rotatable bonds	1
Num. H-bond acceptors	1
Num. H-bond donors	1
Molar Refractivity	48.80
TPSA	20.23 Å²
Lipophilicity	
Log $P_{o/w}$ (iLOGP)	2.56
Log $P_{o/w}$ (XLOGP3)	3.26
Log $P_{o/w}$ (WLOGP)	2.50
Log $P_{o/w}$ (MLOGP)	2.30
Log $P_{o/w}$ (SILICOS-IT)	2.44
Consensus Log $P_{o/w}$	2.61
Water Solubility	
Log *S* (ESOL)	-2.78
Solubility	2.54e-01 mg/ml ; 1.64e-03 mol/l
Class	Soluble
Log *S* (Ali)	-3.36
Solubility	6.75e-02 mg/ml ; 4.38e-04 mol/l
Class	Soluble
Log *S* (SILICOS-IT)	-1.91
Solubility	1.92e+00 mg/ml ; 1.24e-02 mol/l
Class	Soluble
Pharmacokinetics	
GI absorption	High
BBB permeant	Yes
P-gp substrate	No
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Log K_p (skin permeation)	-4.93 cm/s
Druglikeness	
Lipinski	Yes; 0 violation
Ghose	No; 1 violation: MW<160
Veber	Yes
Egan	Yes
Muegge	No; 2 violations: MW<200, Heteroatoms<2
Bioavailability Score	0.55
Medicinal Chemistry	

PAINS	0 alert
Brenk	1 alert: isolated_alkene
Leadlikeness	No; 1 violation: MW<250
Synthetic accessibility	3.28
Swiss Institute of Bioinformatics - © 2024 | SIB privacy policy	

4.1. Disk and well Diffusion Assay

Cream-colored colonies of the test organism, *C.albicans*ATCC 10231 were observed on the SDA plates. Antifungal susceptibility testing via disk-diffusion and well diffusion methods showed 2.0 cm of a zone of inhibition. As the Clean V diffuses into the agar plate, it inhibits germination and growth of organisms producing a significant zone of inhibition indicating the anti fungal property of Clean V.

(a) **(b)**

Figure 5: Antifungal susceptibility testing; (a) disk-diffusion method of Clean V using *C. albicans*as a test organism, (b) well diffusion method of Clean V against *C.albicans.*

4.2. MIC and MFC Determination

To determine the concentration-dependent anti fungal activity, the result from the broth dilution method was observed. While analysing the tubes of different concentrations, more turbidity was appeared in 0 mg/mL and 1 mg/mL, a moderate type of turbidity was observed in the tubes containing 5 mg/mL and 10 mg/mL of Clean V. In 15 mg/mL, mild turbidity was observed and concentrations at and above 20 mg/mL shows no turbidity indicating 20 mg/mL as MIC. The result observed from the culture of three tubes having the concentrations of 20 mg/mL 25 mg/mL and 30 mg/mL, indicates 20 mg/mL was the MFC as well. No visible colonies were obtained in these three plates after the incubation at optimal growth conditions.[13] As Clean V is a liquid wash preparation, MFC was further investigated as a time-dependent killing concentration via the pour plate method.

Table 3: Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of Clean V against *C. albicans.*

Concentration (mg/mL)	Turbidity	Appearance of Turbidity
0	++++++	More
1	+++++	More
5	++++	Moderate
10	+++	Moderate
15	++	Mild
20	+	Mild
25	-	No turbidity
30	-	No turbidity
35	-	No turbidity
40	-	No turbidity
50	-	No turbidity

4.3. Time and Concentration Dependent Anti fungal Activity Determination

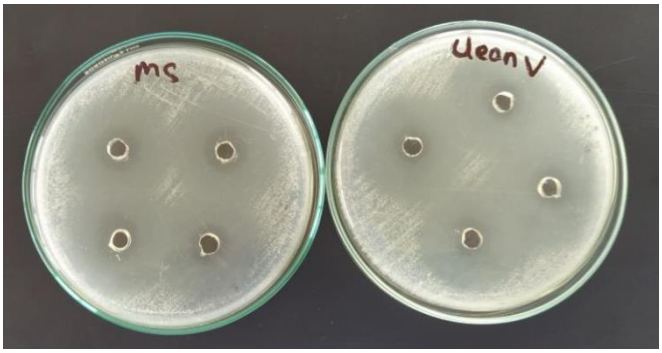
While investigating the time and concentration dependent anti fungal activity of Clean V via pour plate method, we came to know that concentration of 30 mg/mL was able to kill the inoculum used within 2 minutes. At 0 mg/mL where clean V was not used, too many to count colonies (TMTC) were observed. Similarly, the plate of concentration of 15 mg/mL also showed TMTC colonies indicating ineffective concentration at a particular time to kill the test organism used. However, at the concentrations 20 mg/mL and 25 mg/mL, many countable colonies of $1.6* 10^3$cfu/mL and $9.5* 10^2$cfu/mL respectively, were found to show some effectiveness of the product as the concentration was increased. And the concentration at 30 mg/mL, 35 mg/mL, 40 mg/mL and 50 mg/mL, no visible colonies were obtained on the respective plates. This result confirmed that the concentration of 30 mg/mL is enough to kill the inoculum of the test organism used.

Table 4: Time and concentration dependent antifungal activity of Clean V against *C. albicans*via pour plate method.

Concentration (mg/mL)	Cfu/mL	Treatment time (min)
0	TMTC	2
15	TMTC	2
20	$1.6* 10^3$	2
25	$9.5* 10^2$	2
30	0	2
35	0	2
40	0	2
50	0	2

4.4. Comparative Analysis with Commercially Available Market Sample

Further exploration for comparative study of antifungal activity of Clean V with commercially available Market Sample (MS) demonstrated that equal zone of inhibition of 2.0 cm was observed in SDA plates of both samples via well diffusion method. The obtained zone of inhibition in both sample plates indicated both have antifungal activity against the tested organism.



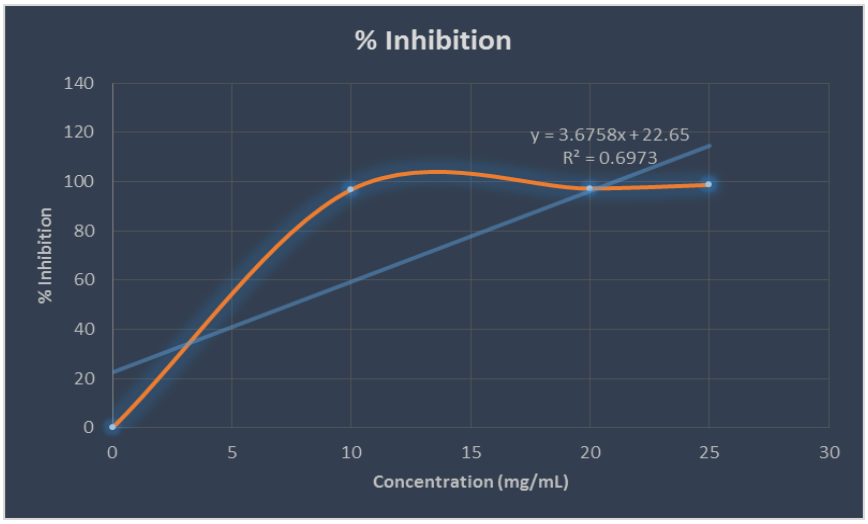
(a) **(b)**

Figure 7: Anti fungal susceptibility testing; (a) well-diffusion method of Clean V using *C. albicans*as a test organism, (b) well diffusion method of Clean V against *C. albicans.*

However, result obtained from pour plate method helps to scrutinize the time and concentration dependent efficacy of both products. Concentration of 30 mg/mL of MS showed $2.64*10^3$ cfu/mL whereas, Clean V kills 100% of the organism at the same concentration and same treatment time. Moreover, 50 mg/mL of MS showed the same effects shown by the 30 mg/mL of Clean V signify the effectiveness of Clean V over MS.

Table 5: Comparative analysis of anti fungal activity of Clean V with commercially available market sample (MS) against *C. albicans*via pour plate method.

Concentration (mg/mL)	Cfu/mL (Clean V)	Cfu/mL (MS)	Treatment time (min)
0	TMTC	TMTC	2
10	TMTC	TMTC	2
20	$1.6*10^3$	$2.64*10^3$	2
30	0	$2.44*10^3$	2
40	0	$1.1*10^2$	2
50	0	0	2
60	0	0	2
70	0	0	2
80	0	0	2



Graph 1: The concentration Vs inhibition graph of Clean V are shown below.

The from graph IC_{50} value is 7.44 mg/mL and IC_{90} value is 18.322 mg/mL

16.0. SUPPLIMENT DATA

(a) **(b)**

Figure 8: Revival of test organism; (a) after 48 hours of incubation at 35 °C, (b) after 1 week of incubation showing transition of yeast into hyphal form.

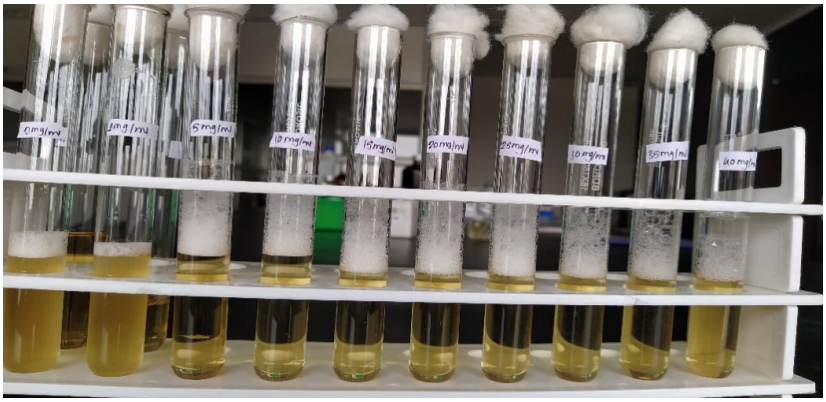


Figure 9: Test for Minimum inhibitory concentration (MIC) of Clean V via broth dilution method.

Figure 10: Test for minimum fungicidal concentration (MFC); culture the inoculum from the tubes having minimum visible growth to no visible growth.

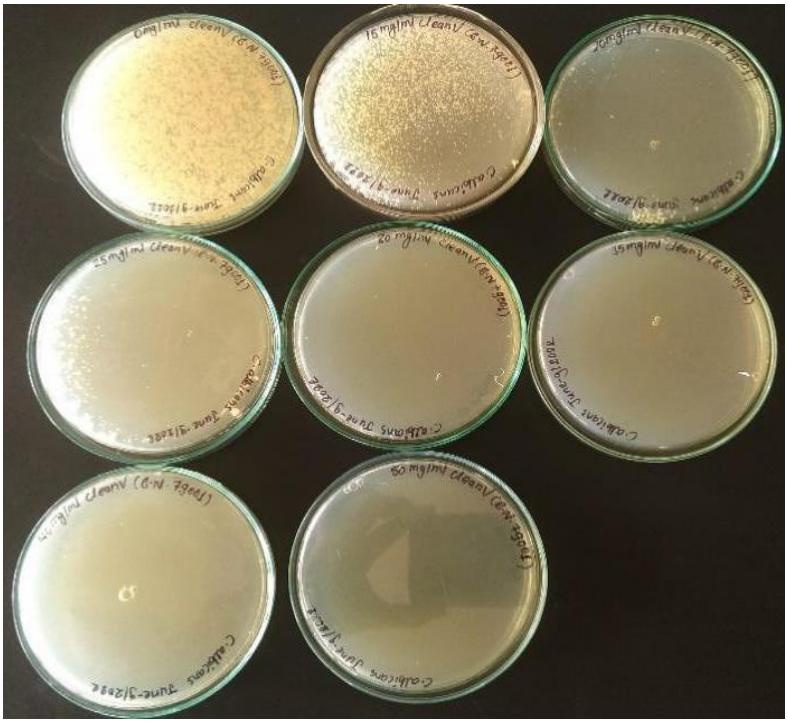


Figure 11: Antifungal susceptibility testing of Clean V giving 2 minutes of treatment time via pour plate method against *C.albicans.*

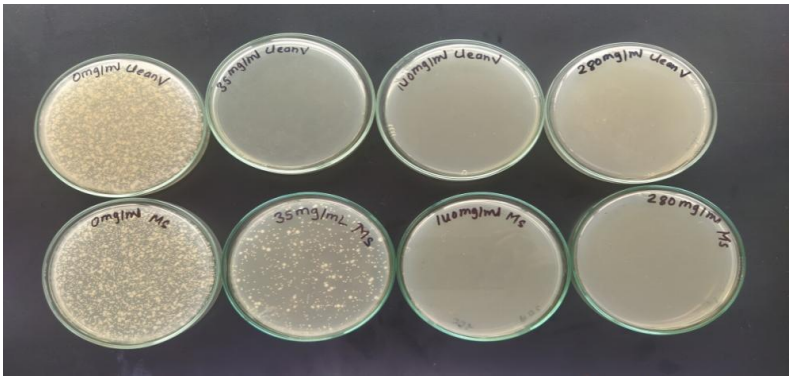
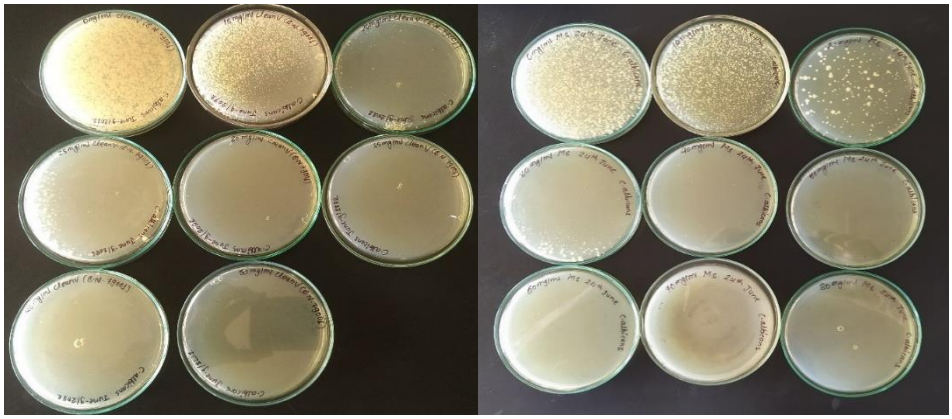


Figure 12: Comparative study about time and concentration dependent anti fungal activity of Clean V with commercially available market sample.



(a) **(b)**

Figure 13: Comparative study about time and concentration dependent anti fungal activity of (a) Clean V; (b) commercially available market sample.

5.0. DISCUSSION

C. albicans, a diamorphic (capable of reversible transition between the yeast and hyphal form) fungiis an opportunistic pathogen responsible for 60 % of both superficial and systemic mycoses.[14] Adhesion, morphological condition, physiological state of the host and the ability to penetrate the epithelial mucosa and other tissues are the factors predominantly involved in pathogenesis,[15],[16] Formation of germ tube followed by the transition to the hyphal form by the extension of its tip, can generate a strong pressure for tissue penetration. Penetration is facilitated by the secretions of protease, lipases and other histolytic enzymes from the hyphal tip and the hyphal form is often resistance to phagocytosis by macrophages and other leukocytes.[17],[18]

In the present study, Clean V showed fungicidal activity against *C. albicans* strains. The active phytochemical constituent; tea tree oil, lavender oil and lactic acid all are responsible for the antifungal property.[19],[20],[21] Tea tree oil has the major chemical components as terpinen-4-ol, 1-8-cineole, α-terpineol and terpinolene. Though each component individually shows some antimicrobial property, terpinen-4-ol is the principle active component responsible for tea tree oil's antimicrobial efficacy,[22] And the synergistic effect of the constituent components provides more promising antifungal effect of essential oil as a whole than single compounds.[23],[24],[25] The antifungal activity of tea tree oil is by the pronounced alteration in mycelial morphology, cellular organelles and membrane permeability.[20] By the same token, previous studies on lavender oil demonstrated that the major chemical components linalool and linalyl acetate were responsible for inhibition of germ tube

formation and hyphal elongation, indicating they are effective against *C.albicans* diamorphism and may thus reduce fungal progression and the spread of infection in the host tissue. Also, essential oil is responsible for fungicidal activities against *C. albicans.*[11]

The vaginal tract of women in their reproductive years normally contains a healthy microbiota, primarily composed of lactobacillus species. These lactobacilli play a crucial role in protecting against harmfulbacteria by producing lactic acid in sufficient quantities.[26] When the levels of lactobacilli are depleted, it disrupts the balance of the vaginal microbiota, creating an opportunity for anaerobic organisms to contribute to vaginal infections.[27],[28] Lactic acid helps maintain an acidic environment that promotes a healthy vaginal milieu, which is unfavourable for many pathogenic bacteria and is inversely associated with vaginal infections.[29] In addition to its role in maintaining acidity, lactic acid exhibits strong antimicrobial properties against both bacteria and fungi.[21]

According to *in-silico* research on ligand-protein interaction, 1,8-cineole, lactic acid, linalyl acetate, linalol, and terpinene-4-ol exhibit good binding energies with C. albicans target.[30]

6.0. CONCLUSION

In sum to, Clean V exhibits significant antifungal activity against Candida albicans, the causative agent of vaginal yeast infections (VVC). The combination of active chemical constituent present in polyherbal formulation used in Clean V enhances its ability to kill fungi and maintain a favourable environment for the dominance of beneficial microbiota, while inhibiting the growth of unwanted pathogens. This contributes to the restoration of lactobacilli in the vaginal tract. Therefore, Clean V can be utilized both as a preventive and therapeutic solution for women of childbearing age. Additionally, its sulphate and paraben-free formulation makes it suitable for daily use, particularly for individuals with sensitive skin.

The "clean V" is a herbal formulation that contains various active phytochemical constituents, each of which is directly linked to therapeutic benefits in case of illness. The formulation consists of five active chemical constituents, namely 1,8-cineole, lactic acid, linalyl acetate, linalool, and terpinene-4-ol. These constituents are regarded as ligands and were subjected to a docking investigation with Candida albicans strain (PDB ID-3E4E). The outcome of the investigation suggested that the formulation was interacting well with the target protein.[30]

7.0. ACKNOWLEDGMENTS

We express our sincere gratitude to management of Fleur Himalayan limited. Special appreciation to Managing Director Mr. Ajay Bahadur Pradhanang for his encouragement and support.

8.0. Funding: This work was supported by management of Fleur Himalayan limited.

9.0. Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

10.0. Ethics approval and consent to participate

Not applicable.

11.0. Consent for publication

Not applicable.

12.0. Conflict of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

13.0. Future Research

More experimental work such as Western blot analysis, Real-time PCR, Flow cytometer, ELISA analysis, histologic analysis and Immunofluorescence, Chromatin Immuno precipitation and Immuno cytochemistry is needed to carry out to find the more pharmacological feature required for the drug to possess good therapeutics activity.

14.0 Abbreviations

PDB ID: Protein data bank

CYP: Cytochrome

PCR: Polymerase chain reaction

ELISA: Enzyme-linked immunosorbent assay

VVC: Vaginal yeast infections

MIC: Minimum inhibitory concentration

MFC: Minimum Fungicidal Concentration

CFU/mL: Colony forming units per mL

TMTC: Too many to count colonies.

15.0 REFERENCES

1. Swidsinski, A., Guschin, A., Tang, Q., Dörffel, Y., Verstraelen, H., Tertychnyy, A., Khayrullina, G., Luo, X., Sobel, J. D., & Jiang, X. Vulvovaginal candidiasis: histologic lesions are primarily polymicrobial and invasive and do not contain biofilms. *American journal of obstetrics and gynecology*, 2019; *220*(1): 91.e1–91.e8. https://doi.org/10.1016/j.ajog.2018.10.023
2. Sobel J. D. Vaginitis. *The New England journal of medicine*, 1997; *337*(26): 1896–1903. https://doi.org/10.1056/NEJM199712253372607
3. Kim, J., & Sudbery, P. Candida albicans, a major human fungal pathogen. *Journal of microbiology (Seoul, Korea)*, 2011; *49*(2): 171–177. https://doi.org/10.1007/s12275-011-1064-7
4. Shimoni, M., Putievsky, E., Ravid, U., & Reuveni, R. Antifungal activity of volatile fractions of essential oils from four aromatic wild plants in israel. *Journal of chemical ecology*, 1993; *19*(6): 1129–1133. https://doi.org/10.1007/BF00987374
5. Penfold, A. R., & Morrison, F. R. *Some notes on the essential oil of Melaleucaalternifolia*, 1937.
6. Wilkinson, J. M., & Cavanagh, H. M. Antibacterial activity of essential oils from Australian native plants. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, 2005; *19*(7): 643-646.
7. Carson, C. F., Hammer, K. A., & Riley, T. V. Melaleucaalternifolia (Tea Tree) oil: a review of antimicrobial and other medicinal properties. *Clinical microbiology reviews*, 2006; *19*(1): 50–62. https://doi.org/10.1128/CMR.19.1.50-62.2006
8. Cannard, G. On the scent of a good night's sleep, 1995.
9. Grieve, M. M., and Leyel, Mrs. CF, A Modern Herbal, 1931; Vols. I and II.
10. Cavanagh, H. M. A., & Wilkinson, J. M. Biological activities of lavender essential oil. *Phytotherapy research*, 2002; *16*(4): 301-308.
11. D'auria, F. D., Tecca, M., Strippoli, V., Salvatore, G., Battinelli, L., & Mazzanti, G. Antifungal activity of Lavandulaangustifolia essential oil against Candida albicans yeast and mycelial form. *Medical mycology*, 2005; *43*(5): 391-396.
12. Magaldi, S., Mata-Essayag, S., Hartung de Capriles, C., Perez, C., Colella, M. T., Olaizola, C., & Ontiveros, Y. Well diffusion for antifungal susceptibility

testing. *International journal of infectious diseases: IJID: official publication of the International Society for Infectious Diseases*, 2004; *8*(1): 39–45. https://doi.org/10.1016/j.ijid.2003.03.002

13. Balouiri, M., Sadiki, M., & Ibnsouda, S. K. Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of pharmaceutical analysis*, 2016; *6*(2): 71–79. https://doi.org/10.1016/j.jpha.2015.11.005
14. White, T. C., Marr, K. A., & Bowden, R. A. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clinical microbiology reviews*, 1998; *11*(2): 382-402.
15. Drago, L., Mombelli, B., De Vecchi, E., Bonaccorso, C., Fassina, M. C., & Gismondo, M. R. Candida albicans cellular internalization: a new pathogenic factor? *International journal of antimicrobial agents*, 2000; *16*(4): 545-547.
16. Phan, Q. T., Belanger, P. H., & Filler, S.G. Role of hyphal formation in interactions of Candida albicans with endothelial cells. *Infection and immunity*, 2000; *68*(6): 3485-3490.
17. Cutler, J. E. Putative virulence factors of Candida albicans. *Annual review of microbiology*, 1991; *45*(1): 187-218.
18. Hube, B., & Naglik, J. Candida albicans proteinases: resolving the mystery of a gene family. *Microbiology*, 2001; *147*(8): 1997-2005.
19. Behmanesh, F., Pasha, H., Sefidgar, A. A., Taghizadeh, M., Moghadamnia, A. A., Adib Rad, H., & Shirkhani, L. Antifungal Effect of Lavender Essential Oil (Lavandulaangustifolia) and Clotrimazole on Candida albicans: An In Vitro Study. *Scientifica*, 2015; *2015:* 261397. https://doi.org/10.1155/2015/261397
20. Yu, D., Wang, J., Shao, X., Xu, F., & Wang, H. Antifungal modes of action of tea tree oil and its two characteristic components against Botrytis cinerea. *Journal of applied microbiology*, 2015; *119*(5): 1253-1262.
21. Stanojević-Nikolić, S., Dimić, G., Mojović, L., Pejin, J., Djukić-Vuković, A., & Kocić-Tanackov, S. Antimicrobial activity of lactic acid against pathogen and spoilage microorganisms. *Journal of Food Processing and Preservation*, 2016; *40*(5): 990-998.
22. Cox, S. D., Mann, C. M., & Markham, J. L. Interactions between components of the essential oil of Melaleucaalternifolia. *Journal of Applied Microbiology*, 2001; *91*(3): 492-497.
23. Hummelbrunner, L. A., & Isman, M.B. Acute, sublethal, antifeedant, and synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm,

Spodopteralitura (Lep., Noctuidae). *Journal of Agricultural and food chemistry*, 2001; *49*(2): 715-720.

24. Bouchra, C., Achouri, M., IdrissiHassani, L. and Hmamouchi, M. Chemical composition and antifungal activity of essential oils of seven Moroccan Labiatae against Botrytis cinereaPers: Fr. J Ethnopharmacol, 2003; 89: 165–169.
25. Deweer, C., Yaguiyan, A., Muchembled, J., Sahmer, K., Dermont, C. and Halama, P. In vitro evaluation of dill seed essential oil antifungal activities to control Zymoseptoriatritici. Commun AgricAppl Biol Sci., 2012; 78: 489–495.
26. Kumar, N., Behera, B., Sagiri, S. S., Pal, K., Ray, S. S., & Roy, S. Bacterial vaginosis: Etiology and modalities of treatment-A brief note. *Journal of pharmacy &bioallied sciences*, 2011; *3*(4): 496–503. https://doi.org/10.4103/0975-7406.90102
27. Borges, S., Silva, J., & Teixeira, P. The role of lactobacilli and probiotics in maintaining vaginal health. *Archives of gynecology and obstetrics*, 2014; *289*(3): 479-489.
28. Ventolini, G. Progresses in vaginal microflora physiology and implications for bacterial vaginosis and candidiasis. *Women's Health*, 2016; *12*(3): 283-291.
29. Skarin, A., &Sylwan, J. Vaginal lactobacilli inhibiting growth of Gardnerellavaginalis, Mobiluncus and other bacterial species cultured from vaginal content of women with bacterial vaginosis. *Actapathologica, microbiologica, etimmunologicaScandinavica. Section B, Microbiology*, 1986; *94*(6): 399–403. https://doi.org/10.1111/j.1699-0463.1986.tb03074.x.
30. Kamrudeen Samani et al. Ijppr. Human, 2023; 28(4): 9-27.