Evaluation Of Some Medicinal Plants For Their Antiseptic And Wound Healing Properties

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By

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TAMILNADU, INDIA.

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DECLARATION

I hereby declare that the thesis entitled **“Evaluation of some medicinal plants for their antiseptic and wound healing properties”** submitted by me for the award of the degree of Doctor of Philosophy of The Tamilnadu Dr. MGR Medical University, Chennai-600 032, is the result of my original and independent work at JSS College of Pharmacy, Ooty-643 001, during the years 2003-2007, under the supervision of **Dr.P.Vijayan** and has not formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title previously.

Ooty (**M.N.SATISH KUMAR)**

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Supervisor

CERTIFICATE

This is to certify that the thesis entitled **"Evaluation of some medicinal plants for their antiseptic and wound healing properties"** is the record of research work done by **Mr. M.N.Satish Kumar** at J.S.S. College of Pharmacy, Ootacamund-643 001, under my supervision, during the years 2003-2007 and this thesis has not previously formed the basis for the award of any Degree, Diploma, Associateship, Fellowship or other similar title. I also certify that the thesis represents independent work done by the candidate and that this has not formed in part or fully the basis for the award of any other previous research degree.

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This is to certify that the thesis entitled **"Evaluation of some medicinal plants for their antiseptic and wound healing properties"** is the record of research work done by **Mr. M.N.Satish Kumar** at J.S.S. College of Pharmacy, Ootacamund-643 001, during the year 2003-2007 under the supervision of **Prof. P.Vijayan**.

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(M.J.NANJAN)

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Abbreviations

%	Percentage
+ve	Positive
µg	Microgram
µl	Microlitre
µm	Micro molar
^0C	Degree Celsius
2M H_2SO_4	2 molar sulphuric acid
A. flavus	*Aspergillus flavus*
A.niger	*Aspergillus niger*
AIDS	Acquired immune deficiency syndrome
ALAT	Alanine amino transferase
ALP	Alkaline phosphatase
AMB	Amphotericin-B
ANOVA	Analysis of variance
ASAT	Aspartate amino transferase
b. wt	Body weight
B.coagulans	*Bacillus coagulans*
B.mageterium	*Bacillus mageterium*
BSA	Bovine serum albumin
C.albicans	*Candida albicans*
c.f.u	Colony forming unit
C.neoformans	*Candida neoformans*
C_{amp}	Cyclic adenine monophosphate
CC	Chemokine ligand
Cm	Centimeter
CMI	Cell mediated immunity
CS	Control and collagen
Cu	Copper
Cu-En	Copper- enfenamic
DC	Dendritic cells
DF	Degree of freedom
DMBA	Dimethyl benzanthracene
DMSO	Dimethyl sulphoxide
DNA	Deoxyrebo nucleic acid
E	Eosinophils
E. coli	*Escherichia coli*
ELISA	Enzyme linked immunosorbant assay
g	Gram

(*contd.,*)

Abbreviations (*contd.,*)

G-CSF	Granulocyte colony stimulating factor
GGT	Gamma glutaraldehyde
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hour
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HEOB	Hydroalcoholic extract of *Ocimum basilicum*
HEOS	Hydroalcoholic extract of *Ocimum sanctum*
HEPES	N-(2-Hydroxyethyl) Piperazine – N'-2- ethanesulphonic acid
$HGEC_S$	Human gingival epithelial cells
HIV	Human immune deficiency virus
HPLC	High performance liquid chromatography
HRP	Horse Radish Peroxidase
HRT	Hormone Replacement Therapy
i.e	That is
I.N.T	2-(4-iodophenyi)-3-(niyrophenyl)-5-phenyltetrozolium chloride
i.p	Intraperitoneal
i.v.	Intravenously
IC	Immunocompromised
IF	interferon
IFN- γ	Interferon Gamma
$I_g A$	Immunoglobulin -g
IL	Interleukin
Kg	Kilogram
L	Lymphocytes
LAK	Lymphokine- activated killer cells
LC	Liquid chromatography
LDL	Low density lipoproteins
M	Monocytes
MCP	Monocyte chemotactic protein
M-CSF	Macrophage colony-stimulating factor
mg	Milligram
mg/kg	Milligram/kilogram
MIC	Minimum inhibitory concentration
ml	Milliliter
mm	Millimeter
MRSA	Methicillin resistant staphylococcus aureus
MST	Mean survival time
N	Neutrophils
NCIM	National collection of industrial microorganism

(*contd.,*)

Abbreviations (*contd.,*)

NK	Natural killers
nm	Nanometer
NMDA	N-Methyl D-Aspartate
NO	Nitric oxide
O.basilicum	*Ocimum basilicum*
O.sanctum	*Ocimum sanctum*
O_2	Oxygen
ODN_S	Oligodeoxynucleotides
OECD	Organization for Economic Cooperation and Development
OPC	Oro pharyngeal candidiasis
P	para
P. aeruginosa	*Pseudomonas aeruginosa*
PAS	Periodic acid Schiff stain
PBS	Phosphate buffer saline
Pg	Picco gram
PGF	Platelet growth factor
PMN	Polymorpho nuclear
PMNL	Polymorphonuclear leukocytes
QIC	Quercetin incorporated collagen
R.arrhizus	*Rhizopus arrhizus*
R.B.C.	Red blood corpuscles
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Revolution per minute
RVVC	Recurrent vulvovaginal candidiasis
S.aureus	*Staphylococcus aureus*
S.cerevisiae	*Saccaromyces cerevisiae*
S.E.M	Standard Error Mean
S.No	Serial number
S.typhi	*Salmonella typhi*
SDA	Sabourard Dextrose Agar
SDB	Sabourard dextrose broth
SOD	Super oxide dismutase
t	Tertiary
T.rubrum	*Trichophyton rubrum*
T/C	Treated by control
TBARS	Thiobarbituric acid reactive substance
TEP	1,1,3,3-tetra ethoxy propane
Th	T helper
TLC	Total leucocytes count

(*contd.,*)

Abbreviations (*contd.,*)

TMB	Tetra Methyl Benzidine
TNF	Tumour Necrosis Factor
TP	Total Protein
U/l	Units/Litre
uv	Ultra violet
V/V	Volume by volume
-ve	Negative
vs.	Versus
VVC	Vulvovaginal candidiasis
W.B.C	White blood corpuscles
w/v	Weight by volume
WHO	World health organization
α	Alpha
β	Beta
γ	Gamma
M	Micron

1. INTRODUCTION

1.1. Natural products

Plant products have been used throughout human history for various purposes including medicine. Tens of thousands of secondary metabolites are produced by the higher plants as natural defense against disease and infection. Medicines derived from plants have played a pivotal role in the healthcare of many cultures, both ancient and modern. The Indian system of holistic medicine known as Ayurveda uses mainly plant-based drugs or formulations to treat various ailments including cancer. Of the approximately 877 small molecule drugs introduced world wide between 1981 and 2002, 61% can be traced back to their origins in natural products.

Ayurveda provides details for the therapeutic use of as many as 290 herbal drugs. Medicinal plants are an important resource to traditional society's healthcare systems. It is estimated that 70–80% of the rural population in developing countries depend on traditional medicine for primary healthcare today, eventhough allopathic medicine is available in these countries. Over the last two decades, however, successful attempts for the better understanding of molecular mechanisms of action of some natural products have kindled interest in their therapeutic use in modern medical settings. The role of natural products in human healthcare thus cannot be underestimated. Recent surveys suggest that one in three Americans use medicinal natural products daily and possibly one in two cancer patients use them as well (Goel et al., 2008).

For a long time, mankind throughout the world has been developing traditional medicine based on the knowledge of medicinal plants. This knowledge got enriched over numerous generations due to experimentation and through observations of animal behavior. Most of the time, this information is only orally inherited and is, therefore, in danger of being lost. However, it represents for the local population a possibility of simple and cheap treatment. In addition, it is a source of potentially important new pharmaceutical substances. The interest and urgency of ethnobotanical research is thus obvious. In particular, many plant extracts and essential oils isolated from plants have been shown to exert biological activity both, in *vitro* and *in vivo*, justifying further

research on the characterization of antimicrobial activity of these plants (Magassouba et al., 2007).

1.2. Need for new antiseptic, wound healing and antimicrobial agents

In the past century, microorganisms played an increasing role in the production of antibiotics and other drugs for the treatment of some serious infectious diseases. Advances in the description of the human genome, as well as the genomes of pathogenic microbes and parasites, has permitted the determination of the structures of many proteins associated with disease processes. With the development of new molecular targets based on these proteins, there is an increasing demand for novel molecular diversity for screening. Bacterial infection is one of the most serious global health issues of the present century. Antimicrobial resistance settings have failed to address this essential aspect of drug usage (Galvan et al., 2008).

Wounds are major concern for the patient and clinician alike; chronic wounds affect a large number of patients and seriously reduce their quality of life. Current estimates indicate that nearly 6 million people suffer from chronic wounds worldwide. The prevalence of chronic wounds in the community has been reported as 4.5 per 1000 population, whereas in the case of acute wounds, it is 10.5 per 1000 population. Nearly 1–3% of the drugs listed in Western pharmacopoeia are intended for use in the skin and for wounds out of which at least one third are herbal remedies. It has been estimated that in America 3–5% of all hospitalized patients with spinal cord injuries suffer from ulcers. The cost of institutional care on the same is said to be US$ 1000 per day while no such estimates are available for Indian institutions. The same demographic study has projected market expenditure of over US$ 7 billion world wide for provisions of wound healing properties. Both Traditional and Western systems of medicine for wound healing suffer from lack of resources and awareness. A need, therefore, arises for the advocacy and wider publication for relevant research to be pursued in this area. In broad terms, the relevant headings for consideration are utilization, safety and efficacy. Research on wound healing agents is, therefore, one of the developing areas in modern biomedical sciences (Kumar et al., 2007).

The current scenario of emergence of multiple drug resistance to human pathogenic organisms has necessitated a search for new antimicrobial substances from other sources including plants. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. The use of medicinal plants still plays a vital role to cover the basic health needs in developing countries. Active principles of many drugs are found in plants as secondary metabolites. The remarkable contribution of plants to the drug industry was possible because of the large number of phytochemical and biological studies carried out all over the world. Herbal remedies used in folk medicine provide an interesting and still largely unexplored source for the creation and development of potentially new drugs, which might help to overcome the growing problem of resistance and also the toxicity of the currently available commercial antibiotics. It is of great interest, therefore, to carry out screening of medicinal plants in order to validate their use in folk medicine (Oskay and Sari, 2007).

1.3. Physiology of wound healing

Wound healing is a highly complex physiological process. It involves the interplay of various cellular and biochemical components that drive the repair cascade. Apart from cellular and biochemical components, several enzymatic pathways also become active during repair and help the tissue to heal (Kapoor and Appleton, 2005). Tissue injury stimulates an ordered cascade of events, commencing with coagulation, through inflammation and culminating in repair. Whereas the initial responses are similar, subsequent events depend on the type of tissue as well as the degree of damage. Wound healing largely involves repair by replacement with connective tissue. Repair can be achieved by resolution, regeneration or replacement (with scar tissue, sometimes called organization). Many cell types and inflammatory mediators such as cytokines are implicated in the repair process. In addition, there are both intrinsic and extrinsic factors that influence the way in which wounds heal (Halloran and Slavin, 2002).

Acute wounds heal in four phases namely haemostasis, inflammation, new tissue formation (proliferation) and remodeling. In chronic wounds, this normal progression is disrupted and slow healing or non-healing can also occur (Williamson and Harding,

2004). Deodhar and Rana (1997) suggested the cutaneous healing is the interaction of a complex series of phenomena that eventuates in the resurfacing, reconstitution and proportionate restoration of tensile strength of wounded skin.

Wound healing process can be roughly divided into 3 overlapping phases of inflammatory reaction, proliferation, and remodeling (Figure 1).

1.3.1. Inflammatory phase

Inflammation is a highly effective component of the innate initial reaction of the body to injury. It is an important consequence of injury and one that normally leads to tissue repair and restoration of function. The inflammatory response can be subdivided into vascular and cellular responses. In the early wound healing process, local vasodilatation, blood and fluid extravasation into the extravascular space, and blocking of lymphatic drainage can produce cardinal signs of inflammation, including redness, swelling, and heat. This acute inflammatory response usually lasts between 24 and 48 hours and may persist up to 2 weeks in some cases. Tissue injury causes blood vessel disruption and bleeding. Platelets adhere, aggregate, and release many mediators to facilitate coagulation. Although haemostasis is the major function of blood coagulation, a secondary but equally important function of platelets is to initiate the healing cascade via release of chemoattractants and growth factors. At the same time, the clot provides a matrix scaffold for the recruitment of cells to an injured area. In responding to these important mediators, leukocytes, including neutrophils and macrophages, infiltrate the wounded area and assist in cleaning and removing damaged tissue debris and foreign particles. Once in the wound site, activated macrophages release several important growth factors and cytokines, initiating granulation tissue formation (Halloran and Slavin, 2002).

1.3.2. Proliferative phase

The initial inflammatory responses to injury provide the necessary framework to the subsequent production of a new functional barrier. In this phase of healing, cellular activity predominates. The major events during this phase are the creation of a

permeability barrier (i.e reepithelialization), the establishment of appropriate blood supply (i.e angiogenesis), and reinforcement of the injured dermal tissue (i.e fibroplasia) (Williamson and Harding 2004).

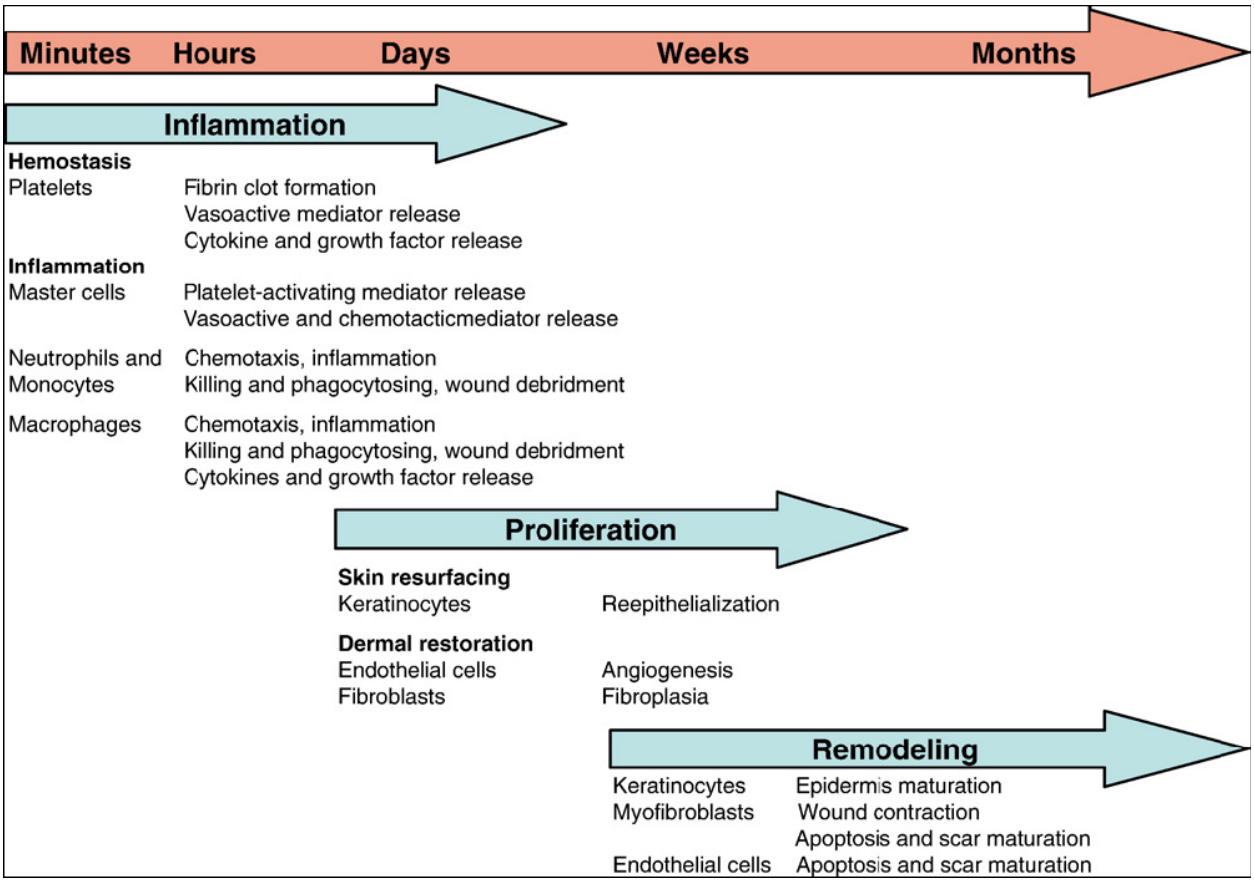


Figure 1: Major cells and their effects on normal wound healing (reprinted from Li et al., 2007)

1.3.3. Wound contraction

Contraction of the wound begins soon after wounding and peaks at 2 weeks. The degree of wound contraction varies with the depth of the wound. For full-thickness wounds, contraction is an important part of healing and accounts for up to a 40% decrease in the size of the wound. In partial-thickness wounds, contraction is less and is in direct proportion to their depth. Myofibroblasts are the predominant mediator of this contractile process because of their ability to extend and retract. During granulation tissue formation, fibroblasts are gradually modulated into myofibroblasts, which are characterized with actin microfilament bundles (not seen in networks of normal skin

fibroblasts), similar to those seen in smooth muscle cells, along their plasma membrane. There is an increased expression of smooth muscle differentiation markers of actin, myosin, and desmin starting on day 6 and reaching a maximum on day 15, after which these regress progressively. Myofibroblasts contain one of the highest concentrations of actinomyosin of any cell. The cells within the wound align along the lines of contraction, and contraction of the wound occurs in directions of skin tension lines. This muscle like contraction of myofibroblasts is mediated by prostaglandin F_1 (PGF_1), 5-hydroxytryptamine, angiotensin, vasopressin, bradykinins, adrenaline, and noradrenaline. This contraction is unified and requires cell-cell and cell-matrix communication. Fibronectin not only provides the multiple functions described previously but also assists in wound contraction. Myofibroblast pseudopodia extend, and cytoplasmic actin binds to extracellular fibronectin, attaches to collagen fibers, and retracts, drawing the collagen fibers to the cell, thereby producing wound contraction (Li et al., 2007).

1.4. Abnormal wound healing

There are many local and systemic factors that contribute to impaired wound healing. Local factors pertain mainly to persistence of debris within the wound; for example, devitalized tissue, clot, foreign material, including sutures and bacterial contamination. These can act as a physical barrier to the ordered development of granulation tissues and collagen deposition, or may exaggerate the evoked inflammatory response. Ultimately, this can change the way tissues heal, changing primary to secondary union or resolution to organization. Local tissue hypoxia, either chronic (e.g. caused by radiation enteritis, atherosclerosis or diabetic micro vascular disease) or more acute (e.g. caused by vascular damage or tight sutures) will compromise healing. Mobile wounds do heal but more slowly than those held together by sutures. Systemic factors that impair healing include nutritional status, diabetes, glucocorticoid treatment, irradiation, hypoxia, jaundice and renal failure. Old age is a contentious issue: certainly wounds in older patients take longer to heal, but there are no studies to prove age correlates with a poorer quality of healing (Halloran and Slavin, 2002).

One of the most common examples of delayed wound healing is seen with diabetic foot ulcers. Diabetic foot ulcers are estimated to affect 15% of all diabetics (Mancini and Ruotolo 1997). Factors such as cost, time and debility associated with this chronic inflammatory condition make it a major clinical concern. Furthermore, with an increasingly obese population, the incidence of diabetes and thus diabetic foot ulcers is likely to increase. Deregulation in the inflammatory and proliferative phase of wound healing along with neuropathy, stress and deformity have all been indicated to be associated with diabetic foot ulcer pathology. Several animal studies suggest that the dysregulation caused in diabetic wound healing may be attributed to an altered metabolism of collagen at the wound site, and abnormal granulation tissue formation. Consequently, the orderly process of wound repair is impaired and the chronic inflammatory phase persists resulting in an increased neutrophil infiltration along with an increase in reactive oxygen species (ROS) production (Diegelmann and Evans, 2004).

1.5. Wound healing property of traditional medicinal plants

The chloroform extract of *Ocimum sanctum* has been shown to have antibacterial and wound healing properties in infectious condition in rats (Thaker and Anjaria, 1985). Septilin, a proprietary preparation claimed to be useful in inflammatory conditions, has been tested for antiinflammatory and wound healing effects in rats. It significantly enhanced the tensile strength in incision wounds, wound contraction and epithelization in excision wounds (Udupa et al., 1989). The aqueous, alcoholic, petroleum ether, chloroform, propylene glycol and glycosylated and aqueous extracts with zinc oxide of the herb, *Centella asiatica,* have been applied daily on the excised areas. The aqueous extract suspension in 5% propylene glycol favored wound healing and the rest did not have any significant effect (Rao et al., 1996). The ointment and injection form of the methanol extract of *Leucas lavandulaefolia* has been examined for its wound healing activity and both produced a significant response against wounds (Saha et al., 1997).

Shoba and Rao, (1999) have reported a phytopharmaceutical product containing crude powders from eleven plants that exhibits wound healing activity. The ethanol extract of *Aristolochia bracteolata* has shown significant wound healing activity along with

increased levels of antioxidant enzymes (Shirwaikar et al., 2003). Pretreatment with curcumin (a naturally occurring phenolic compound of turmeric) significantly enhanced the rate of wound contraction, increased synthesis of collagen, hexosamine, and nitric oxide and improved fibroblast and vascular densities (Jagetia and Rajanikant, 2004).

Methanolic extracts of *Ageratum conyzoides*, *Anthocleista djalonensis, Napoleona imperialis*, *Ocimum gratissimum*, and *Psidium gaujava* have been evaluated for their antibacterial and wound healing properties. *Napoleona imperialis* showed 90% wound healing activity in rats along with antibacterial activity (*in vitro*) (Chah et al, 2006). *In vitro* antibacterial properties of nine different compounds including vinyl hexylether, shellsol, 2,4-dimethyl hexane, 2-methylnonane and 2,6-dimethyl heptane isolated from the leaf of *Tragia involucrate* have been studied against *Escherichia coli*, *Proteus vulgaris* and *Staphylococcus aureus.* The compound vinyl hexyl ether showed a broad spectrum of activity. The highest activity was found in shellsol against *Proteus vulgaris* and *Staphylococcus aureus*. Shellsol treated rats showed complete wound healing after 24 days. Histological examination revealed an increase in the fibroblast, revascularization, granulation and thickness of scar tissue after treatment with shellsol as compared to the control (Samy et al, 2006). *Arrabidaea chica* Verlot extract has been shown to stimulate fibroblast growth in a concentration dependent manner. It increased *in vitro* collagen production, reduced wound size and moderate antioxidant activity (Jorge et al., 2008).

1.5.1. Role of antioxidants in wound healing

Hypoxemia caused by disrupted vasculature, is a key factor that limits wound healing. Correcting hypoxemia through the administration of supplemental oxygen can have significant beneficial impact on wound healing. Oxygen may support vital processes such as angiogenesis, cell motility, and extracellular matrix formation (Gayle et al., 2003). Clinical experience with adjunctive hyperbaric oxygen therapy in the treatment of chronic wounds has shown that wound hyperoxia increases wound granulation tissue formation and accelerates wound contraction and secondary closure. The reports of clinical studies suggest topical oxygen has no detrimental effects on wounds but show

beneficial indications in promoting wound healing. Oxygen delivery is, therefore, considered as a critical element for wound healing (Kalliainen et al., 2003).

Prolonged exposure to high concentrations of oxygen, however, induces production of reactive oxygen species, causing damage to the cells. Antioxidant supplementation has, therefore, been proposed as an adjuvant to attenuate such deleterious secondary effects. α-Lipoic acid supplementation has been shown to efficiently reduce, both the lipid and DNA, oxidation induced by oxygen exposure (Alleva et al., 2005). Gomathi et al., (2003) have reported that quercetin incorporated collagen (QIC) treated animals when compared with control and collagen (CS) treated animals, show a better healing. In addition, antioxidant studies have indicated that QIC quench the free radicals more efficiently. Wound healing processes are known to involve a sharp increase of nitric oxide generation in wound tissue thus suggesting a possible therapeutic use of various nitric oxide donors for the acceleration of wound healing (Rawlingson, 2003; Grierson and Ormerod, 2004; Shekhter et al., 2005).

Wound healing and antioxidant studies of alcoholic and aqueous extracts of *Plagiochasma appendiculatum* have shown that these plant extracts have potent wound healing activity along with potent antioxidant activity as revealed by lipid peroxidation inhibition and increase in superoxide dismutase and catalase activity (Singh et al., 2006). Reddy et al., (2008) have studied the wound healing and antioxidant activity of the methanolic extract of *Holoptelea integrifolia.* More than 90% wound healing in excision wound model and higher breaking strength with increased hydroxyproline content in incision wound model were recorded in treated groups. The extracts also exhibited antioxidant activity.

1.6. Antimicrobial activity of traditional medicinal plants

Clinical microbiologists are interested in the topic of antimicrobial plant extracts for two reasons. First, it is very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians; several are already being tested in humans. It is reported that on an average, two or three antibiotics derived from microorganisms are launched each year. After a downturn in that pace in recent

decades, the pace is again quickening as scientists realize that the effective life span of any antibiotic is limited. Worldwide spending on finding new anti-infective agents (including vaccines) is expected to increase by 60% from the spending levels in 1993. New sources, especially plant sources, are also being investigated. Second, the public is becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics. In addition, many people are interested in having more autonomy over their medical care. A multitude of plant compounds (often of unreliable purity) is readily available over-the-counter from herbal suppliers and natural-food stores, and self-medication with these substances is commonplace. The uses of plant extracts, as well as other alternative forms of medical treatments, were enjoying great popularity in late 1990s. Earlier in this decade, approximately one third of people surveyed in the United States used at least one “unconventional” therapy during the previous year. It was reported that in 1996, sales of botanical medicines increased 37% over 1995 (Cowan, 1999).

Caceres et al., (1987) selected 89 plants based on ethanobotanical survey used in Guatemala for the treatment of dermatomucosal diseases. Ethanolic macerations were tested against *Candida albicans, Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The results showed that among 89 plants, 28 exhibit *in vitro* inhibition on the tested microorganisms.

Santolina chamaecyparissus volatile oil possesses significant effect in controlling experimental systemic and vaginal candidiasis (Suresh et al., 1995). In addition, it also showed antimycotic activity and effective in controlling experimental vaginal candidiasis (Suresh et al, (1997).

Crude methanol, hexane, chloroform, petroleum ether, ethyl acetate and *n*- butanol extracts of tar obtained from the roots and stems of *Pinus brutia* Ten., used in the folk medicine of Turkey, have shown antimicrobial activity against *Staphylococcus aureus, Streptococcus pyogenes*, *Escherichia coli* and *Candida albicans* (Kizil et al., 2002). Kokoska et al., (2002) have studied the antimicrobial activity of crude ethanolic extracts of 16 Siberian medicinal plants against *Bacillus cereus, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans*. Among the

16 plants studied, 12 showed antimicrobial activity against one or more species of microorganisms. The most active antimicrobial plants were *Bergenia crassifolia, Chelidonium majus, Rhaponticum carthamoides, Sanguisorba officinalis* and *Tussilago farfara.*

The aqueous extract of *Nigella sativa* seeds have been shown to exhibit inhibitory effect against systemic candidiasis in mice, thus validating the traditional use of this plant in fungal infections (Khan et al., 2003). Sahin et al., (2003) have evaluated the antimicrobial activity of *Satureja hortensis* L., which is an annual herb used as traditional medicine in Eastern Anatolia region of Turkey for the treatment of different infectious diseases and disorders. The results showed that the hexane extract has no antifungal activity. Instead it has antibacterial activity against four strains of *Bacillus* species. The methanolic extract has both antibacterial and anticandidal activity.

The fruit and leaf extracts of *Solanum aculeastrum* Dunal (Solanaceae) have been investigated for *in vitro* antimicrobial activity against 10 selected bacterial and 5 fungal strains (Koduru et al, 2006). The methanolic extracts of both the fruits and the leaves showed appreciable activity against Gram-positive and Gram-negative bacteria, whereas the water extracts showed the least activity against the bacteria. The methanol extracts were particularly inhibitory to the growth of *Aspergillus flavus* and *Pencillium notatum.* The most resistant organisms were *Aspergillus niger*, *Candida albicans*, and *Fusarium oxysporum*

Oskay and Sari, (2007) investigated the antimicrobial activity of the ethanolic extracts of 19 Turkish medicinal plants against bacterial species and a yeast, *Candida albicans*. Extracts of *Eucalyptus camuldulensis* (leaves)*, Rosmarinus officinalis* (leaves), *Ecballium elaterium* (leaves and fruits), *Liquidambar orientalis* (leaves), *Cornus sanguine*a (leaves, flowers, and stems), *Vitis vinifera* (leaves, raw fruits,and young branches), *Inula viscosa* (leaves), *Hypericum perforatum* (leaves, flowers,and stems), and *Punica granatum* (leaves and flowers) showed broad-spectrum antimicrobial activity. Chuang et al., (2007) evaluated the therapeutic properties of the seeds and leaves of *Moringa oleifera* Lam as herbal medicines. The ethanolic extract showed *in*

vitro antifungal activities against dermatophytes such as *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum* and *Microsporum canis*.

1.6.1. Antimicrobial activity of bioactive phytoprinciples

Phenols do not inhibit any one particular biochemical process but have a non-specific denaturing action on the cell wall (Zsolnai, 1960). Major groups of antimicrobial compounds from plants are phenolics, polyphenolics, quinines, flavones, flavonoids, flavonols, tannins, terpenoids, alkaloids, lectins and polypeptides (Cowan, 1999).

The ethanolic extract of *Adesmia aegiceras* has shown antimicrobial activity. A chemical study of the bioactive alcoholic extract revealed quercetin, isorhamnetin-3- rutinoside, isovitexin, pinitol and chlorogenic acid as its main components (Agnese et al., 2001). Antimicrobial activity of *Sebastiania brasiliensis* has been studied by Penna et al., (2001). The bioactive antimicrobial compounds identified are methylgallate and protocatechuic acid in addition to quercetin, kaempferol, quercetrin and gallic acid.

Rosmarinic acid a caffeic acid ester commonly found in species of Lamiaceae, possesses antibacterial and antifungal activities (Petersen and Simmonds, 2003). Grayer et al., (2003) have identified two characteristic phenolic compounds in the well-known genera of culinary herbs like mint, rosemary, sage, thyme and basil, namely, caffeic acid esters (Z,E)-[2-(3,5-dihydroxyphenyl) ethenyl] 3-(3,4-dihydroxyphenyl)-2-propenoate and (Z,E)-[2-(3,4-dihydroxyphenyl)- ethenyl] 3-(3,4-dihydroxyphenyl)-2-propenoate. Both the phenolic compounds have been show to possess antifungal activity. Sohn et al., (2004) have evaluated the antimicrobial activity of 18 flavonoids, purified from five different medicinal plants and tested against *Candida albicans, Saccaromyces cerevisiae, Escherichia coli, Salmonella typhimurium, Staphylococcus epidermis and S.aureus.* Papyriflavonol A, kuraridin, sophoraflavanone D and saphoraisoflavanone A exhibited good antifungal with strong antibacterial activity. Kuwanon C, mulberrofuran G, albanol B, kenusanone A,and sophoraflavanone G showed strong antibacterial activity. Morusin, sanggenon B and D, kazinol B,

kurarinone, kenusanone C, isosophoranone were effective to only Gram-positive bacteria, and broussochalcone A was effective to *C.albicans.*

Phenolic compounds and flavonoids possess antioxidant effect (Banerjee et al., 2005 and Pourmorad et al., 2006). Flavonoids possess antifungal, antiviral and antibacterial activity. For example, the activity of quercetin is partially attributed to inhibition of DNA gyrase. It has also been reported that Sophoraflavone G and (−)-epigallocatechin gallate inhibit cytoplasmic membrane function, and that licochalcones A and C inhibit energy metabolism (Cushnie and Lamb, 2005).

Gayoso et al., (2005) have evaluated the antifungal activity of *Eugenia cariophyllata* essential oil and eugenol, its major constituent, on fungal strains isolated from onychomycosis. *Anatolian propolis* samples have exhibited antimicrobial activity due to the presence of flavonoids such as pinocembrin, pinostropin, isalpinin, pinobanksin, quercetin, naringenin, galangine and chrysin (Uzel et al., 2005). Pycnogenol, a standardized extract of *Pinus pinaster* bark, has shown antimicrobial activity against different Gram-positive and Gram-negative bacteria and fungi. It also has significant activity against candidiasis (Torras et al., 2005).

The several low molecular weight compounds from higher plants possess strong inhibitory activity *in vitro* against *Candida* species. The main specific targets of natural anticandidals are the ergosterol pathway, respiratory chain and chitin biosynthesis (Pauli, 2006). Eugenol, the major phenolic component of clove essential oil, has been shown to possess potent antifungal activity. Eugenol displayed potent activity against *C. albicans* biofilms *in vitro* with low cytotoxicity and, therefore, has potential therapeutic implication for biofilm-associated candidal infections (He et al., 2007). Antioxidant with fungicidal effect may be a better candidate for candidiasis than pure antioxidant example, cinnamaldehyde with eugenol has revealed the strongest synergy against *Laetiporus sulphureus*. The synergism may possible due to the interference of fungal cell wall synthesis and cell wall destruction plus radical scavenging effect (Yen and Chang, 2008).

1.7. Candidiasis

Human infections, particularly those involving the skin and mucosal surface constitute a serious problem, especially in tropical and subtropical developing countries; methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* being the most frequent pathogens. The drug-resistant bacteria and the fungal pathogen have further complicated the treatment of infectious diseases in immunocompromised, acquired immunodeficiency syndrome and cancer patients (Oskay and Sari, 2007). In recent years, there has been a growing incidence of opportunistic fungal infections due to the increasing immunocompromised population, including organ transplant recipients, neonates, human immunodeficiency virus and cancer patients (Rojas et al., 2007).

1.7.1. Immunity and role of cytokines and major cells against candidiasis

Natural and synthetic immunomodulators that increase nonspecific resistance to infection are also known to induce interleukin 1 (IL-1) production. The effect of IL-1 on the survival of neutropenic mice with a lethal *Candida albicans* infection has been studied (Van Wout et al., 1988). The numbers of candida cultured from the blood, liver, spleen, and kidney were not significantly different in IL-1 treated and control animals. Passive transfer of serum obtained from mice pretreated with IL-1 to recipient mice did not provide protection against a subsequent lethal candidal infection. IL-1 prolonged the survival of neutropenic mice with lethal *C. albicans* infection.

The effect of human recombinant interleukin-1α (IL-1α) on a systemic candidal infection in mice under immunosuppression has been investigated by Kullberg et al., (1990). In normal mice and in mice pretreated with cyclophosphamide, hydrocortisone acetate, the outgrowth of *C. albicans* in the kidney was significantly reduced on the administration of IL-1α. The protective effect of IL-1α after injection of *C. albicans* in cyclophosphamide-treated mice, indicate that the enhancement of host resistance by IL-1α is not due to increased granulocytopoiesis or chemotaxis of granulocytes but strongly suggest that other mechanisms play a role in the protective effect of IL-1α against systemic infections.

T-cell mediated immunity is essential for recovery for both cutaneous and mucosal infections caused by Candida, Malassezia and dermatophytes and systemicinfections by Cryptococcus, Blastomyces and Coccidioides. An often chronic progressive disease caused by these fungi is associated with a depression or absence of T-cell-mediated immunity to antigens of the infecting fungus (Lehmann, 1985).

Studies from women with recurrent vulvovaginal candidiasis and from an animal model of experimental vaginitis suggest that deficiencies in immune function should be examined at the local rather than systemic level. Results showed that although constitutive Th1- and Th2-type cytokine expression was detectable in cervicovaginal lavage fluid from normal women, differences in cytokines were observed in recurrent vulvovaginal candidiasis patients (Fidel Jr et al., 1997a).

C. albicans can trigger secretion of potent chemo attractant and proinflammatory cytokines by oral mucosal fibroblasts. The secretion of pro-inflammatory cytokines by oral mucosal fibroblasts in response to *C. albicans* suggests that these cells have the potential to enhance the host defense against this organism *in vivo* (Bagtzoglou and Lamster, 1999).

An IL-10 deficient and wild-type mouse with disseminated or gastrointestinal candidiasis or invasive pulmonary aspergillosis has been evaluated. Unlike parasitic protozoan infection, *C. albicans* or *A. fumigatus* infection did not induce significant acute toxicity in IL-10 deficient mice. Instead it showed reduced fungal burden and fungal-associated inflammatory responses. The increased resistance to infections as compared to wild-type mice was associated with upregulation of innate and acquired antifungal Th1 responses, such as a dramatically higher production of IL-12, nitric oxide (NO) and tumor necrosis factor -α as well as interferon- γ by CD4+ T cells (Del Sero et al.,1999).

T cells are important and they are functioning without showing significant increases in numbers within the vaginal mucosa during infection. The resistance to *C. albicans* infection in mice results from the development of T helper (Th) type-1 cell responses. Cytokines produced by Th-1 cells activate macrophages and neutrophils to a candidacidal state. The development of Th-2 responses underlines susceptibility to

infection, because cytokines produced by Th-2 cells inhibit Th1 development and deactivate phagocytic effector cells. With the recognition of the reciprocal influences between innate and adaptive Th immunity, it appears that the coordinated action of these two lines of immune defense is required to efficiently oppose the infectivity of the fungus and to determine its lifelong commensalism at the mucosal level (Romani, 1999). The T-cell response to *C. albicans* in the regional lymph nodes, which correlates best with rapid oral clearance of *C. albicans*, is a balanced Th0 cytokine response involving early secretion of both IFN- γ and IL-4 (Elahi et al., 2000). There is a correlation between NK cell activation and the resistance to experimental systemic candidiasis. Thus, it seems that the immune surveillance of metastatic spread and the infection by *C. albicans* share some immune effector mechanisms, in particular activation of NK cells (Ortega et al., 2000).

Cell mediated immunity plays an important role in the resistance to mucosal candidiasis, but little is known about the mechanisms that protect mucosal surfaces against disease. While macrophages kill micro-organisms using oxygen-dependent and independent mechanisms, studies in mice have shown that nitric oxide (NO) produced by macrophages contribute to resistance to infection with *C. albicans*. Inhibition of NO synthesis *in vivo* leads to increased susceptibility of mice to systemic and mucosal candidiasis and reduced candidacidal activity of macrophages. *In vitro* studies have shown that NO inhibits the growth of *C. albicans* (Elahi et al., 2001). Khan et al., (2003) have suggested that candidacidal pathway in murine neutrophils is nitric oxide (NO) dependent. NO is responsible for defense against pathogen that survives and proliferates in the intracellular environment of many different types of somatic cells.

Both innate resistance and acquired immunity play some role in maintaining *C. albicans* in the commensal state and protecting the systemic circulation. Polymorphonuclear leukocytes (PMNL) are critical for protection against systemic infections, whereas cell-mediated immunity by Th1-type $CD4^+$ T-cells is important for protection against mucosal infections (Fidel Jr, 2002).

The dendritic cells (DCs) pulsed with *Aspergillus* antigens induced the activation of CD4+ Th1 cells capable of conferring resistance to the infection. Local delivery of

unmethylated CpG oligodeoxynucleotides (ODNs) and the Asp f 16 *Aspergillus* allergen have resulted in the functional maturation and activation of airway DCs capable of inducing Th1 priming and resistance to the fungus (Bozza et al., 2002a). The invasive pulmonary aspergillosis is the most common manifestation of *Aspergillus fumigatus* infection in immunocompromised patients and is characterized by hyphal invasion and destruction of pulmonary tissue. A Th1/Th2 dysregulation and a switch to a Th2 immune response may contribute to the development and unfavorable outcome of invasive pulmonary aspergillosis. DCs have a primary role in surveillance for pathogens at the mucosal surfaces and are recognized as the initiators of immune responses to them. Bozza et al., (2002b) have assessed the functional activity of pulmonary DC in response to *A. fumigates* conidia and hyphae, both *in vitro* and *in vivo*. They found that pulmonary DCs were able to internalize conidia and hyphae of *A. fumigatus* through distinct phagocytic mechanisms and recognition receptors and discriminate between the different forms of cytokine production.

Bozza et al., 2003 have reported that the unique nature of the fungal infections demands a better understanding of immunological mechanisms required to efficiently oppose fungal infections. To improve the immune response against these infections cell mediated immunity to the fungus may represent a useful strategy to develop vaccinations.

Wiseman and Mody (2007) have reported that the contribution of non-neutrophilic granulocytes and innate cytotoxic lymphocytes to fungal immunology are considerable. Collectively, these cells participate in allergy, inflammation and fungal infection in various tissues, but most notably in the respiratory tract. Mast cells, basophils and eosinophils are responsible for the initiation of allergic responses to fungi. Innate cytotoxic lymphocytes have demonstrated importance in fungal infections such as pulmonary cryptococcosis. These lymphocytes participate through the secretion of cytokines helping to balance the Th1/Th2 response and by promoting clearance of fungi, or through direct cytotoxic effector function against fungal pathogens (Figure. 2).

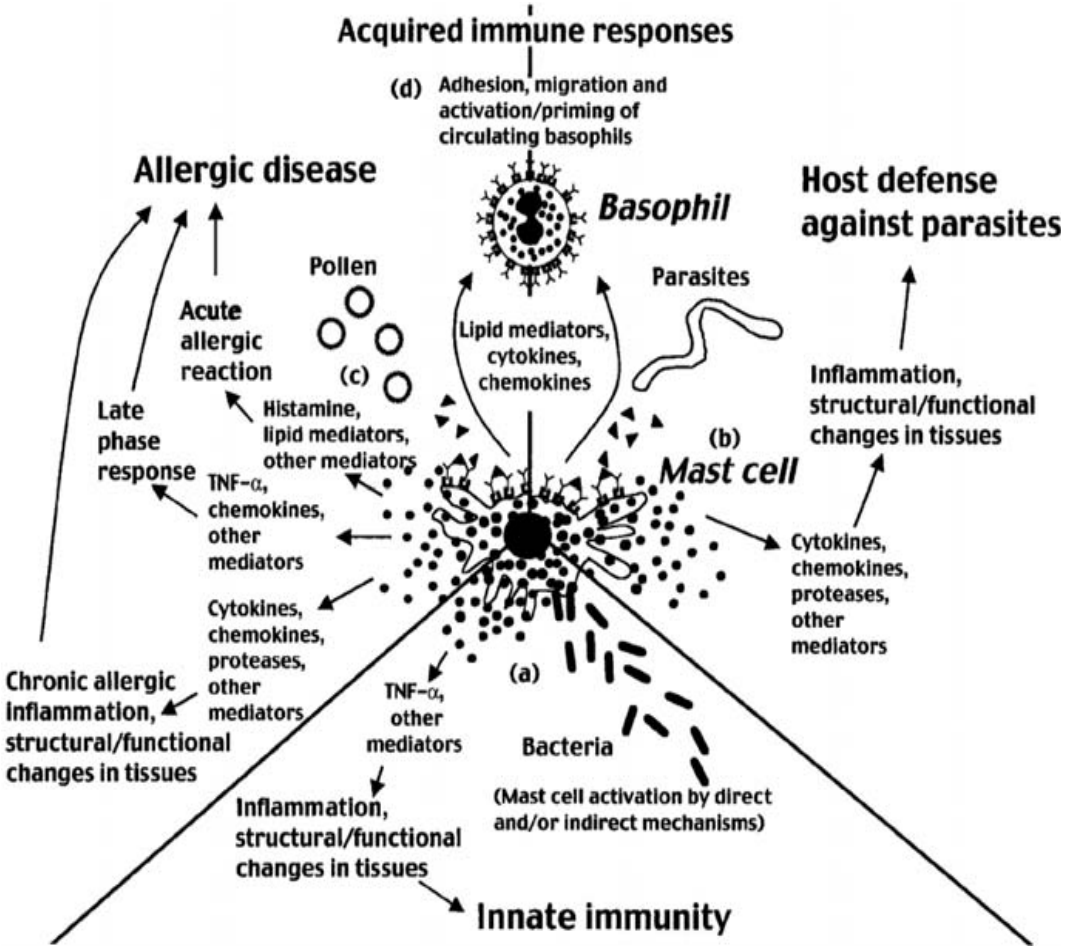


Figure 2. The diverse role of cytokines, non-neutrophilic granulocytes and innate cytotoxic lymphocytes and the regulation of host defense, allergy and Innate responses (reprinted from Wiseman and Mody, 2007).

1.7.2. Systemic candidiasis

In the United States of America, *Candida* species remains the fourth most common blood-stream pathogen, accounting for 8% of all hospital-acquired blood-stream infections. Notably, more than one third of candidal blood-stream infections are caused by *C. albicans* (Pfaller, 1995).

Systemic candida infections have been shown to increase mortality and morbidity in intensive care unit patients (Leleu et al., 2002). *C. albicans* is an opportunistic fungus which causes superficial infections and life-threatening systemic candidiasis in immunocompromised host such as AIDS patients, cancer patients, or other

immunocompromised individuals. Treatment of candidiasis patients is hampered by limited choice of antifungal agents and the appearance of clinical isolates resistant to azole drugs (Niimi et al., 1999). GM193663 and GM237354 are new sordarin derivatives that may play a major role in the treatment of systemic candidiasis (Martinez et al., 2000).

Gastrointestinal and systemic candidiasis in immunocompromised mice has been studied by Cole et al., (1989). The results have shown a high density of invasive fungal hyphae in stomach of immunocompromised animals challenged with *C.albicans.* In addition, homogenates of the oesophagus and selected body organs of mice examined were positive for *C.albicans*. It has been suggested that the animal model described in this study may be particularly useful both for exploring methods, and for testing the efficacy of anticandidal drugs. Mellado et al., (2000) has developed a simple and inexpensive mice model of sustained colonization of gastrointestinal tract. This model could be useful for analyzing prophylaxis, treatment, and diagnosis of systemic candida infections and for evaluating virulence of strains.

1.7.3. Oral candidiasis

Oral candidiasis is a common opportunistic infection of the oral cavity caused by an overgrowth of *C. albicans*. The incidence varies depending on age and certain predisposing factors which include impaired salivary gland function, drugs, dentures, high carbohydrate diet, and extremes of life, smoking, diabetes mellitus, Cushing's syndrome, malignancies, and immunosuppressive conditions (Akpan and Morgan, 2002).

Oral candidiasis is a common fungal infection in patients with an impaired immune system, such as those undergoing chemotherapy for cancer and or with AIDS. A major predisposing factor in HIV-infected patients is a decreased CD4 T-cell count. The majority of infections are due to *C. albicans*. Azoles namely ketoconozole, fluconazole and itraconazole have been an important in treatment, however, resistance to these drugs is a major problem (Hoepelman and Dupont, 1996).

Treatment of severe oropharyngeal candidiasis, particularly in patients with a compromised immune system, is often more difficult, and relapses are common. Reports of resistance to systemic agents, particularly in patients needing recurrent therapy, are increasing. Oral candidiasis is significantly associated with patients using higher doses of inhaled corticosteroids (Kennedy et al., 2000). The decrease in salivary flow rates, secretion of antimicrobial proteins in saliva, and salivary polymorphonuclear leukocytes (PMNs) activity are risk factors for oral candidiasis associated with aging and systemic diseases (Ueta et al., 2000). Pabuccuoglu et al., (2002) have reported on morphological observations in five cases of laryngeal candidiasis. These cases had pronounced epithelial hyperplasia, prominent ortho-parakeratosis, and predominantly neutrophilic infiltration in the upper layers of the mucosal epithelium. Takakura et al., (2003) have established a convention and easy method for oral candidiasis in murine model. Mice were immunocompromised and orally infected with *C.albicans* by means of cotton swab in a sedative state. On day 7 of post inoculation colony forming units of *C.albicans* were recovered from oral cavity. This model would be a useful experimental oral candidiasis for investigating the efficacy of various antifungal agents.

1.7.4. Vaginal candidiasis

The availability of several potent antifungal agents, systemic or topical, over the counter or prescription would suggest that therapeutic needs for *Candida* vaginitis are minimal or absent. A critical factor is duration of therapy and the need for maintenance of therapy in recurrent candidiasis. Azole therapy for candidiasis frequently fails, depleting the therapeutic armamentarium of successful options. Additional therapeutic challenges remain for women who can be easily controlled but not cured with intensive azole therapy in spite of the absence of *in vitro* antifungal resistance (Sobel, 1999).

Protective host defense mechanisms against vaginal *Candida albicans* infections are poorly understood. Although cell-mediated immunity (CMI) is the predominant host defense mechanism against most mucosal candida infections, the role of CMI against vulvovaginal candidiasis is uncertain, both in humans and in experimental mouse model. The role of humoral immunity is equally unclear. While clinical observations suggest a minimal role for antibodies against vaginal candidiasis, an experimental rat

model has provided evidence for a protective role for *Candida*-specific immunoglobulin A (IgA) antibodies (Wozniak et al., 2002). Recurrent vulvovaginal candidiasis (RVVC) is by no means uncommon and is a source of considerable physical discomfort in addition to serving as a major therapeutic challenge. The syndrome is multifactorial in etiology and hence management strategies must recognize the complex etiological pathways. Many women receiving the misplaced diagnosis of RVVC have a variety of other infectious and non-infectious entities presenting with identical symptoms. Hence the first step in the management of RVVC is confirming the diagnosis of RVVC including microbial confirmation and species identification. Efforts are required to identify and correct a causal mechanism. Maintenance suppressive azole antifungal regimens are highly effective in controlling symptoms, although cure is less common (Sobel, 2003).

Vulvovaginal candidiasis (VVC) is an opportunistic mucosal infection caused by *C.albicans* that affects a large number of healthy women of childbearing age. Acute episodes of VVC often occur during pregnancy and during the luteal phase of the menstrual cycle, when levels of progesterone and estrogen are elevated. Estrogen is an important factor in hormone-associated susceptibility to *C.albicans* vaginitis (Fidel Jr et al., 2000; Fidel Jr, 2004). Clinical features of vulvovaginal candidiasis are nonspecific, and the standard laboratory test to identify *Candida* organisms from a vaginal swab may take between 5 to 7 days to confirm the diagnosis. Due to this half of the women are misdiagnosed with VVC, resulting in overtreatment with antifungal agents (Tan, 2003).

Prevalence of vaginal candidiasis is significantly higher in non-secretor group. The absence of blood group antigens in the body fluids and the lack of enzyme glycosyl transferase enhance the attachment of yeasts to the epithelial cell and render the non-secretor more prone to infection (Kulkarni and Venkatesh, 2004). Rates of point prevalence asymptomatic yeast colonization (22%) are similar to adults and similarly dominated by *C.albicans*, but with uncharacteristically high vaginal yeast burden. In contrast with the high rate of sexually transmitted infections (18%), incidence of symptomatic vulvovaginal candidiasis is low (less than 2%) (Barousse et al., 2004).

1.8. Limitations of current antifungal agents

For several years, amphotericin-B was the most effective agent for the treatment and prevention of systemic fungal infections. The introduction of the triazoles like fluconazole and itraconazole, however, has challenged amphotericin-B as the gold standard. In particular, the triazoles have become the agents of choice in chemoprophylaxis; fluconazole has been widely used but the introduction of an itraconazole oral solution offers an agent with high bioavailability and a broader spectrum of activity than that of fluconazole. In the empirical treatment of systemic fungal infections, the lipid-associated formulations of amphotericin-B and the itraconazole intravenous formulation are at least as effective as conventional amphotericin-B and are less toxic. The high cost of lipid-associated formulations of amphotericin- B may make their use prohibitively expensive (Meis and Verweij, 2001). These compounds, however, show some limitations due to either their toxicity (amphotericin B) or the emergence of resistance to the antifungal treatments (azoles). In addition, most azoles are fungistatic giving rise to the possibility fungi not getting cleared from the patient, particularly in immunocompromised individuals. There is an urgent need, therefore, to develop fungicidal, nontoxic, new antifungal agents for therapeutic intervention (Bruneau et al., 2003).

1.9. Review of literature on *Ocimum santcum* and *Ocimum basilicum*

The *Ocimum* genus that belong to the family *Lamiaceae* comprises annul and perennial herbs and shrubs native to the tropical and sub tropical regions of Asia, Africa and Central South America. Pushpangadan et al., (1995) have identified more than 150 species in this genus. More recently, Paton et al., (1999) has proposed that only 65 species of *Ocimum* should be retained, and that other attributions should be considered synonyms or false attribution. *Ocimum sanctum* and *Ocimum basilicum* are considered as sacred plants in the Hindu culture and are known as "*Tulsi*" or "Tulasi" in Hindi. *Ocimum sanctum* (Appendix I) is known as Holy Basil and *Ocimum basilicum* (Appendix II) as Sweet basil in English. They are tropical annual herbs, up to 18 inches tall and grow into a low bush and are members of the family *Lamiaceae (Labiatae)*. The plants grow wild in India and are also widely cultivated in home and temple gardens.

Figure 3: Picture of *Ocimum sanctum* Linn.

Figure 4: Picture of *Ocimum basilicum* Linn.

1.9.1. Phytochemical reports

Grayer et al., (1996) have identified two major flavone aglycones from *Ocimum basilicum*, namely, salvigenin and nevadensin, and 10 minor ones, namely, cirsileol,

cirsilineol, eupatorin, apigenin, acacetin, genkwanin, apigenin 7, and 4'-dimethyl ether, cirsimaritin, ladanein and gardenin B. The essential oils produced from nine different cultivators of *Ocimum basilicum* L have been analyzed and the major components of these oils are estragol, linalool, methyl cinnamate, eugenol and methyl eugenol (Hasegawa et al., 1997). The amount and enantiomeric composition of linalool were determined in the essential oils of seven chemotypes of *Ocimum basilicum* L., *Ocimum sanctum* L., *Ocimum gratissimum* L., and *Ocimum canum* Sims of Thai origin, and in commercial basil oils. The linalol isolated from cultivars of *Ocimum basilicum* L. of various origin, and from commercial basil oils consisted of (R) (-) - linalool and has been proved to be optically pure in most cases. In essential oils of *Ocimum sanctum* L., and *Ocimum canum* Sims. (S) (+)-linalol is the main enantiomer (Ravid et al., 1997). *Ocimum basilicum* mainly contains either estragol or a linalool-estragole mixture.

Linalool, eugenol, *t-α-* bergamotene and thymol are the major constituents of essential oil of *Ocimum basilicum* L. (Keita et al., 2000). Grayer et al., (2000) have identified xanthomicrol, cirsimaritin, rutin, kaempferol 3-*O*- glucoside and vicenin-2 as major flavonoids in the ethanolic extract of *Ocimum gratissimum*. Rosmarinic acid is a caffeic acid ester present in *Ocimum basilicum* (Bais et al., 2002). The oil obtained from *Ocimum basilicum* L. var. *purpurascens* has been classified as monoterpene alcohol-type according to monoterpene alcohol content. The oil obtained from *Ocimum sanctum* L. green has been classified as eugenol/sesquiterpene, while oil from *Ocimum sanctum* purple as eugenol type, according to the content of eugenol (Mondello et al., 2002). Grayer et al., (2002) have identified vicenin-2, luteolin 5-*O*-glucoside, luteolin 7-*O*-glucoronide, apigenin 7-*O*-glucoronide in hydroalcoholic extract of *Ocimum sanctum*, and vicenin-2, quercetin 3-rutinoside and quercetin 3-*O*-glucoside in *Ocimum basilicum.*

Opalchenova and Obreshkova, **(**2003) have identified linalol, methylchavikol, methylcinnamat and linolen from essential oil of *Ocimum basilicum* and tested them against *Staphylococcus*, *Enterococcus*, and *Pseudomonas*. The results have shown strong inhibitory effect on test bacteria. Grayer et al., (2003) have identified two caffeic acid esters in mint, rosemary, sage, thyme and basil namely (Z,E)-[2-(3,5-

dihydroxyphenyl) ethenyl] 3-(3,4-dihydroxyphenyl)-2-propenoate and (Z,E)-[2-(3,4-dihydroxyphenyl)- ethenyl] 3-(3,4-dihydroxyphenyl)-2-propenoate.

The major constituents of basil oil are 3,7-dimethyl-1, 6-octadien-3-ol (linalool), 1-methoxy-4-(2-propenyl) benzene (estragole), methyl cinnamate, 4-allyl-2-methoxyphenol (eugenol), and 1, 8-cineole (Lee et al., 2005). Rosmarinic acid content of the *in vitro* produced plants as well as parent plants have been determined by HPLC analysis and subjected to randomly amplified polymorphic DNA (RAPD). Maximum rosmarinic acid production in plants produced from cultures grown on MS medium was noted (Rady and Nazif, 2005).

The composition of 18 Turkish basil (*Ocimum basilicum*) essential oils has been investigated by Telci et al., (2006). Seven different chemotypes namely linalool, methyl cinnamate, methyl cinnamate/linalool, methyl eugenol, citral, methyl chavicol, and methyl chavicol/citral were identified.

1.9.2. Pharmacological activities

Vaijayanthimala et al., (2000) have reported that the aqueous and alcohol extracts of *Ocimum sanctum* show anticandidal activity (*in vitro*) of against 30 *C.albicans* isolated from vaginal candidiasis patients. Vonshak et al., (2003) have reported that the aqueous extract of *Ocimum basilicum* show antifungal activity against *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton soudanense*, *Candida albicans*, *Torulopsis glabrata* and *Candida krusei.* Suppakul et al., (2003) have reported that the essential oil of *Ocimum basilicum* show antimicrobial activity against wide range of Gram-positive, Gram-negative bacteria, and fungi. The methanol extract of *Ocimum basilicum* has shown anticandidal activity (*in vitro*) against *Candida albicans* and *Candida glabrata* (Hamza et al, 2006).

The fixed oil of *Ocimum sanctum* has been reported to show antiinflammatory activity by Singh et al., (1996). The radioprotective effect of the leaf extract of *Ocimum sanctum* has been reported by Ganasoundari et al., (1997). The radioprotective effect of the leaf

extract of *Ocimum sanctum* in combination with WR- 2721 has been reported by Ganasoundari et al., (1998).

The ethanolic extract of *Ocimum sanctum* has been shown to bring back the noise stress induced changes leucopenia, increase corticosterone level and enhanced the neutrophil functions to normal levels (Archana and Namasivayam 2000). The methanol extract from the roots of *Ocimum sanctum* show antistress activity. The effect produced by the extract is comparable to desipramine (Maity et al., 2000). The essential oil of *Ocimum sanctum* and eugenol has show potent anthelmintic activity in *in vitro* (Asha et al., 2001).

The effect of *Ocimum sanctum* seed oil on immunological parameters in both non-stressed and stressed animals has been studied. The effects of restraint stress on humoral as well as cell-mediated immune responses were effectively attenuated by pretreating the animals with *Ocimum sanctum* seed oil (Mediratta et al., 2002). Antioxidant activity of *Ocimum basilicum* has been reported by Javanmardi et al., 2003 and Politeo et al., 2007.

Aqueous and alcohol extracts of *Ocimum sanctum* has been shown to inhibit lipid peroxidation activity in a dose dependent manner (Geetha et al., 2004). *Ocimum sanctum* leaf extract is very effective in elevating antioxidant enzyme response by increasing the hepatic glutathione reductase, superoxide dismutase, and catalase activities significantly. The extract induces antioxidant level that correlates with significant reduction of lipid peroxidation and lactate dehydrogenase formation. Moreover, the extract was highly effective in inhibiting carcinogen-induced tumor incidence in both the tumor models at primary initial level (Dasgupta et al., 2004).

The ethanolic extract of *Ocimum sanctum* has acted as radical scavenger and also as lipoxygenase inhibitor (Juntachote and Berghofer, 2005). The hydroalcoholic extract of Ocimum *sanctum* and MK-801, an NMDA receptor antagonist, prevented the chronic restraint stress induced rise in plasma cAMP level, myocardial SOD and catalase activity as well as light microscopic changes in the myocardium (Sood et al., 2006).

2. SCOPE AND OBJECTIVES

Bacterial and fungal infections are some of the most serious global health issues of the present century. Antimicrobial resistance settings have failed to address this essential aspect of drug usage. A number of clinically efficacious antibiotics are becoming less effective due to the development of resistance. The increased prevalence of fungal infections in immunocompromised individuals, including transplantation, cancer and acquired immune deficiency syndrome patients and the relative toxicity of amphotericin B and resistance to azole drugs are known (Fidel Jr, 1997b). Further, wounds are a major concern for the patient with bacterial infection and they affect a large number of patients and seriously reduce their quality of life. Current estimates indicate that nearly 6 million people suffer from wounds worldwide. The prevalence of chronic wounds in the community has been reported as 4.5 per 1000 population whereas that of acute wounds is nearly double at 10.5 per 1000 population. 1-3% of the drugs listed in western pharmacopeia are intended for use in the skin and for wounds. At least one third of herbal remedies are known for such use (Kumar et al., 2007).

Research on infectious diseases and wounds are one of the developing areas in biomedical sciences. Many traditional practitioners across the world, particularly in countries like India and China with age old traditional practices, have valuable information of plants for treating infectious diseases and wounds. Numerous biologically active plants have been discovered by evaluation of ethnopharmacological data, and these plants may offer the local population immediately accessible therapeutic products.

Ocimum sanctum and *Ocimum basilicum* popularly known as holy basil in English belong to the *Ocimum* genus and to the *Lamiaceae* (*Labiatae*) family. Different parts of these plants have been claimed to be effective in a wide spectrum of diseases (Dharmani et al., 2004). Traditionally, several ancient medical systems, including Ayurveda, Greek, Roman, Siddha and Unani medical systems have mentioned various therapeutic properties for *Ocimum sanctum* and *Ocimum basilicum.* Ahmad and Beg, (2001) and Vaijayanthimala et al., (2000) have reported anticandidal activity (*in vitro)* for *Ocimum sanctum*, Vonshak et al., (2003), Suppakul et al., (2003) and Hamza et al.,

(2006) have reported the same for *Ocimum basilicum*. Mediratta et al., (2002) have reported immunomodulatory activity for *Ocimum sanctum* seed oil.

The present investigations were, therefore, proposed to evaluate the efficacy of the hydroalcoholic extract of *Ocimum sanctum* and *Ocimum basilicum* against septic wounds using excision and incision models in rats, in addition to antibacterial, antifungal (*in vitro*), and *in vivo* anticandidal activity in both non immunocompromised and immunocompromised mice. Further, both the plant extracts were also proposed to be tested for possible toxic effects by repeated dose 28 day sub acute oral toxicity in rats.

Plan of work

Step I

Plant collection and authentication of plant species

Step II

Preparation of hydroalcoholic extract of *Ocimum sanctum* and *Ocimum basilicum*

Step III

Preliminary phytochemical studies on the hydroalcoholic extract of *Ocimum sanctum* and *Ocimum basilicum*

- Qualitative phytochemical studies
- Estimation of total phenolic compounds
- Estimation of total flavonoids
- Quantitative analysis of eugenol, quercetin, rutin and rosmarinic acid by HPLC

Step IV

In vitro studies

Evaluation of hydroalcoholic extract of *Ocimum sanctum* and *Ocimum basilicum* for the following studies;

- Nitric oxide scavenging property
- Antibacterial and antifungal studies (Cup plate method and Two fold serial dilution method)

Step V

In vivo studies

Evaluation of hydroalcoholic extract of *Ocimum sanctum* and *Ocimum basilicum* for the following studies;

- Experimental systemic candidiasis in non immunocompromised and immunocompromised mice
- Experimental systemic candidiasis under gastrointestinal colonization in non immunocompromised and immunocompromised mice
- Oral candidiasis in immunocompromised mice
- Repeated dose 28-day sub acute oral toxicity in rats

Step VI

Formulation development

- Formulation and evaluation of cream and gel for hydroalcoholic extract of *Ocimum sanctum* and *Ocimum basilicum*

Step VII

Evaluation of hydroalcoholic extract of *Ocimum sanctum* and *Ocimum basilicum* cream for the following studies;

- Experimental vaginal candidiasis in non immunocompromised and immunocompromised mice
- Antimycotic activity in normal guinea pigs
- Excision wound model with infection in rats
- Incision wound model with infection in rats

Step VIII

- Statistical analysis of the data

3. EXPERIMENTAL

3.1. Materials

The following chemicals, kits, reagents, drugs and instruments were used in the experimental studies;

Table 1

Name of the chemical/kits/reagents/drugs	Company	Catalogue number /Product number
1,1,3,3-tetra ethoxy propane	Sigma Aldrich, USA	T9889
Aluminum chloride	Sigma Aldrich, USA	06220
Eugenol	Sigma Aldrich, USA	E-51791
Flavine adenine dinucleotide	Sigma Aldrich, USA	46360
Gallic acid monohydrate	Sigma Aldrich, USA	267645
Griess reagent	Sigma Aldrich, USA	03553
n-butanol	Sigma Aldrich, USA	34193389
Phosphate buffer saline powder	Sigma Aldrich, USA	79378
Phosphomolybdic acid	Sigma Aldrich, USA	79560
Phosphotungstic acid	Sigma Aldrich, USA	79690
Quercetin	Sigma Aldrich, USA	RM-6191
Rosmarinic acid	Sigma Aldrich, USA	536954
Rutin hydrate	Sigma Aldrich, USA	R-2303
Xanthine oxidase	Sigma Aldrich, USA	X-1875
Crystal violet	Hi media laboratory, Mumbai	40576-0025
Lacto phenol cotton blue	Hi media laboratory, Mumbai	S 016
NADPH	Hi media laboratory, Mumbai	RM576
Nutrient agar Broth	Hi media laboratory, Mumbai	M002

Nutrient agar medium	Hi media laboratory, Mumbai	M001
Peptone	Hi media laboratory, Mumbai	M028
Saboraud's dextrose agar medium	Hi media laboratory, Mumbai	M063
Saboraud's dextrose agar broth	Hi media laboratory, Mumbai	M003
Bees wax	SD Fine Chem Ltd, Mumbai	43017 K 05
Borax	SD Fine Chem Ltd, Mumbai	26037
Carbopol	SD Fine Chem Ltd, Mumbai	56092 K 05
Chloramine-T	SD Fine Chem Ltd, Mumbai	37670 KO1
Folin-ciocalteu's reagent	SD Fine Chem Ltd, Mumbai	29058 LO2
Hydroxy proline	SD Fine Chem Ltd, Mumbai	47120 GO1
Iodine	SD Fine Chem Ltd, Mumbai	28081
Methyl paraben	SD Fine Chem Ltd, Mumbai	31020 L 03
Orthophosphoric acid	SD Fine Chem Ltd, Mumbai	39416 LO5
Paraffin	SD Fine Chem Ltd, Mumbai	39439 k05
Periodic acid- Schiff	SD Fine Chem Ltd, Mumbai	89842
Polyethylene glycol	SD Fine Chem Ltd, Mumbai	76311 L 25
Propyl paraben	SD Fine Chem Ltd, Mumbai	76311 L 25
Pyridine	SD Fine Chem Ltd, Mumbai	20225 L02
Saffranin	SD Fine Chem Ltd, Mumbai	44312
Sodium carbonate	SD Fine Chem Ltd, Mumbai	20916
Sodium dodecyl sulphate	SD Fine Chem Ltd, Mumbai	54801 G25
Thiobarbituric acid	SD Fine Chem Ltd, Mumbai	20904
Tween 20	SD Fine Chem Ltd, Mumbai	76368
Acetic acid	Loba Chemicals, Mumbai	008
Ehrlich reagent	Loba Chemicals, Mumbai	3657
Ethanol GR 99-100%	Loba Chemicals, Mumbai	3714

Ethyl acetate 99-100%	Loba Chemicals, Mumbai	138
Ethyl acetate GR	Loba Chemicals, Mumbai	G 0921
HEPES buffer	Loba Chemicals, Mumbai	4040
Methanol	Loba Chemicals, Mumbai	LB173906
Potassium acetate	Loba Chemicals, Mumbai	20350
Potassium ferricyanide	Loba Chemicals, Mumbai	5359
Sodium hydroxide	Loba Chemicals, Mumbai	5898
Dimethyl sulphoxide	Ranbaxy chemicals Ltd, Mumbai	D0175
Acetonitrile	Rankem, Mumbai	A 0755
Potassium dihydrogen phosphate	Qualigens fine chemicals, Mumbai	19465
Formic acid	Merck Ltd. Mumbai	AG 3A530207
Oestradiol	Merck Ltd. Mumbai	-
Clindamycin	Pfizer, Mumbai	-
Chloramphenicol	Pfizer, Mumbai	-
Gentamycin	German remedies, Mumbai	-
Ketamine hydrochloride	Aneket , Thane	-
Cyclophosphamide	Dabur India, New Delhi	-
Tetracycline hydrochloride	Dabur India, New Delhi	-
Nitrofurazone	EMCURE, Mumbai	-
Ketoconozole	Torrent Pharma Ltd, Mumbai	-
Thiopental sodium	Piramal HC, Mumbai	-
Tumor necrosis factor- alpha kit	Koma Biotech, South Korea	K 0331186
Interleukin 1- alpha kit	Koma Biotech, South Korea	K 0331141

Name of the instrument	Company	Model
UV Spectrophotometer	Shimadzu, Japan	UV-1700
HPLC	Shimadzu, Japan	LC-2010
Digital P^H meter	Systronics, India	335
Cell analyser	Medonic, Merck, India	CA-536
Autoanalyser	Merck microlab 200,Germany	3086-542
Tissue homogenizer	Remi, Mumbai	RQ 127A
Centrifuge	Remi, Mumbai	R8C
ELISA reader	Bio-Rad Laboratories Inc.,	Model 550

3.2. Collection of plants

Ocimum sanctum and *Ocimum basilicum* were collected from Pollachi, Coimbatore district during the month of August, 2004 and authentified by Botanical Survey of India (BSI), Coimbatore (Certificate No. BSI/SC/5/23/06-07/Tech).

3.3. Preparation of extracts

The dried plant parts (leaves and flowers) were shade dried, ground into coarse powder with the help of a grinder. The powdered materials of the plant parts were subjected to cold maceration using ethanol and distilled water. The coarse powder material was kept in a mixture of ethanol and distilled water (1:1) for 10 days with intermittent shaking, in a round bottom flask. After 10 days, it was filtered and the filtrate was subjected to distillation at temperature 60 ^{0}C for removing the ethanol and water. After the distillation, the semi solid obtained was kept in a vacuum dessicator for drying (Kokate et al., 1988). The percentage yield of the extract was 10.3% and 9.2% w/w for *Ocimum sanctum* and *Ocimum basilicum*, respectively. The hydroalcoholic extracts of *Ocimum sanctum* and *Ocimum basilicum*, will henceforth be called HEOS and HEOB, respectively.

3.4. Qualitative phytochemical screening of the extracts

The qualitative phytochemical screening was carried out as per standard methods (Raaman, 2006).

3.5. Estimation of total phenolic compounds in the extracts

Total phenol is usually determined in extracts by using the Folin-Ciocalteus method (Banerjee et al., 2005). This test is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. After oxidation a green blue complex, measurable at 765 nm is formed. The total phenol content of a tested material is related to its antioxidant activity.

A dilute extract of each plant extract (0.5 ml of 1:10 g/ml) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixture was allowed to stand for 15 min and the total phenols was determined by colorimetry at 765 nm. A standard curve was prepared using 0, 50, 100, 150, 200, 250 mg/L solutions of gallic acid in methanol: water (50:50, v/v). Linearity obtained in the range of 2.5 to 25 μg/ml and the absorbance of test was interpolated with the standard curve. The total phenol content was expressed as gallic acid equivalent in mg/g of the extracts.

3.6. Estimation of flavonoids in the extracts (Pourmorad et al., 2006)

Aluminum chloride colorimetric method was used for flavonoids estimation. Each plant extract (0.5 ml of 1:10 g/ml) in methanol was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It was kept at room temperature for 30 min and the absorbance of the reaction mixture was measured at 415 nm using UV visible spectrophotometer. The standard curve was prepared by using rutin at concentrations 12.5 to 100 g/ml in methanol. The absorbance of test was interpolated with standard curve. The flavonoids content was expressed as mg/g of the extracts.

3.7. Quantitative analysis of the phytoprinciples in the extracts

3.7.1. Preparation of standard and test sample solutions

10 mg of the standard (rutin, quercetin, rosmarinic acid and eugenol) was dissolved in 5 ml of methanol in a 10 ml volumetric flask and the volume was made up to 10 ml with methanol and kept as stock solution. 10 mg of extract was dissolved in 5 ml of methanol in a 10 ml volumetric flask and the volume was made up to 10 ml with the stock solution. Various concentrations were prepared from the stock solution and used for HPLC analysis and stored between 2 – 8^{o}C until use.

3.7.2. Chromatographic conditions

Chromatographic separation was performed on a Shimadzu® liquid chromatographic system equipped with a LC-10 AT-vp solvent delivery system (pump), SPD M-10AVP photo diode array detector and Rheodyne 7725i injector with 20 μl loop volume. Class-VP 6.01 data station was applied for data collecting and processing (Shimadzu, Japan). A Princeton SPHER C_{18} (150 x 4.6 mm i.d. 5μ) column was used for the separation. The mobile phase (a mixture of acetonitrile and potassium dihydrogen orthophosphate buffer pH 3.5 adjusted with 1:3 v/v orthophosphoric acid) was delivered at a flow rate of 0.7 ml/min with detection at 254 nm. The mobile phase was filtered through a 0.2 μ membrane filter and degassed. Analysis was performed at ambient temperature and the retention time of, rutin, quercetin, rosmarinic acid and eugenol were found to be, 4.52, 3.58, 6.65 and 12.80 min, respectively.

3.8. *In vitro* studies

3.8.1. Nitric oxide (NO) scavenging property of the extracts (Marcocci et al., 1994)

The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (1 ml) and the extracts (1000-31.25 μg/ml), the compound and standard solutions (1 ml) were incubated at 25^{o} C for 150 min. After incubation, 0.5ml of the reaction mixture was removed and 1mL of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was mixed and allowed to stand for 5 min for completion of diazotization reaction and then 1ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for another 30 min in diffused light. The absorbance

was measured at 540 nm against the corresponding blank solutions in a 96-well microtitre plate (Tarsons Product (P) Ltd., Kolkata, India) using ELISA reader.

3.8.2. Antibacterial and antifungal studies

The following bacterial and fungal organisms were procured from National collection of industrial microorganisms (NCIM), National Chemical Laboratory, Pune, Maharashtra India;

Name of the bacteria	**Culture type**	**Number**
Staphylococcus aureus (+)	NCIM	2079
Bacillus coagulans (+)	NCIM	2313
Bacillus subtilis (+)	NCIM	2063
Bacillus mageterium(+)	NCIM	2187
Escherechia coli (-)	NCIM	2065
Pseudomonas aeuruginosa(-)	NCIM	2200
Salmonella typhi (-)	NCIM	2501
Shigella (-)	NCIM	2068
Name of the fungi	**Culture type**	**Number**
Candida albicans	NCIM	3471
Cryptococcus neoformans	NCIM	3541
Trochophyton rubrum	NCIM	3382
Aspergillus niger	NCIM	1196
Aspergillus flavus	NCIM	0535
Rhizopus arrhizus	NCIM	1009
Saccharomyces cerevisiae	NCIM	3193

3.8.2.1. Preparation of test inoculum (Suppakul et al., 2003)

The bacterial and fungal strains were sub cultured from nutrient agar slants in an Erlenmeyer's flask containing 100.0 ml of nutrient broth and incubated at 37 ^{o}C for 24 h

for preparing stock cultures. Similarly fungal cultures were prepared in sabouraud's dextrose broth by incubating at 24 ^{o}C for 48 h.

3.8.2.2. Preparation of test drug

The extracts were dissolved in dimethyl sulphoxide (DMSO), to prepare 1000 µg/ml to 3.625 µg/ml concentrations and were used for the further *in vitro* studies

3.8.2.3. Cup plate method (Caceres et al., 1987)

A nutrient agar plates (made four wells) was prepared and 0.1ml of organism was spread on the quadrant plate by the help of a borer. The sample solution of the extract was added to two wells (10 µg/disc) and dimethyl sulphoxide to the other two wells as control. Gentamycin and ketoconozole were used as the positive control throughout the experiment.

3.8.2.4. Two fold serial dilution method (Suresh et al., 1995)

The extract at doses ranging from 1000 to 3.625 µg/ml in nutrient broth was tested for their antibacterial and antifungal activity against several bacterial and fungal strains using two fold serial dilution technique. Gentamycin and ketoconozole were used as the positive controls and the whole study was carried out in duplicate. The lowest concentration of the test drug that caused apparently a complete inhibition of growth of the microorganism was taken as the minimum inhibitory concentration of the drug.

3.9. Pharmacological studies (*In vivo*)

The extracts were subjected to *in vivo* pharmacological studies. Numerous pharmacological actions have been well documented for *Ocimum sanctum* and *Ocimum basilicum.* As per the methods of Maity et al., (2000), 200 and 400 mg/kg/b.wt dose levels of the extract was selected and dissolved in sterile saline for systemic candidiasis. The cream of the extract (2% and 4%) was selected for antimycotic and vaginal candidiasis and wound healing studies.

3.9.1. Animals

Adult male/female Swiss albino mice (25-30 gm), adult Wistar rats (180-200 gm) and adult albino guinea pigs (300- 350 gm) were obtained from the central animal house, JSS College of Pharmacy, Ootacamund. The animals were housed under laboratory conditions (relative humidity 85±2%, temperature 22 ±1^{o}C and 12 h light and 12 h dark cycle). They were fed with standard rodent pellet diet (Gold Mohar, Lipton –India, Ltd.,) and distilled water *ad libitum*. The study was approved by the institutional animal ethics committee for animal care and use (JSSCP/IAEC/Ph.D/ PH.COLOGY /01/2005-06 and 06/07)

3.9.2. Immunocompromised animals (Cole et al., 1989)

Cyclophosphamide was administered (i.p) on the second day (300 mg/kg) and fifth day (150 mg/kg) of post inoculation with *C. albicans*.

3.9.3. Effect of the extracts against experimental systemic candidiasis in non immunocompromised and immunocompromised mice (Suresh et al., 1995)

Grouping of animals

Healthy Swiss albino mice, weighing of 25-30 gm were selected. Groups of 10 animals of both sex in each group, were taken for the study and treated as shown below.

Groups	Treatment
Group 1	Vehicle control (saline 1ml/100gm) inoculated with *C.albicans*
Group 2	Immunocompromised control (inoculated with *C.albicans* and immunocompromised)
Group 3	HEOS 200mg/kg (inoculated with *C.albicans*)
Group 4	HEOS 400mg/kg (inoculated with *C.albicans*)
Group 5	HEOB 200mg/kg (inoculated with *C.albicans*)
Group 6	HEOB 400mg/kg (inoculated with *C.albicans*))
Group 7	HEOS 200mg/kg (immunocompromised and inoculated with *C.albicans*)
Group 8	HEOS 400mg/kg (immunocompromised and inoculated with *C.albicans*)
Group 9	HEOB 200mg/kg (immunocompromised and inoculated with *C.albicans*)

Group 10	HEOB 400mg/kg (immunocompromised and inoculated with *C.albicans*)
Group 11	Ketoconozole 60mg/kg (immunocompromised and inoculated with *C.albicans*)

Experimental systemic candidiasis was induced in mice by intravenous administration of about 0.1 ml of $1x10^{5-6}$ cells / ml of the culture suspension of *C.albicans* through tail vein. The extract, ketoconozole and vehicle were administered twice daily orally. The positive control and vehicle control (sterile saline) were maintained throughout the experiment. The animals were observed for mortality for 16 consecutive days and the percentage mean survival time (MST) was calculated using the following formula;

$$\text{Percentage MST} = \frac{X_1Y_1 + X_2Y_2 + \ldots\ldots X_nY_n - \text{number of surviving animals}}{\text{Total number of animals}}$$

Where X = the number of day (day on which animal death occurs)

Y = the number of animals dead on the particular day

The treated/control values (T/C %) were calculated by the ratio of mean survival time of treated and control group. The survived animals were euthanized on day 16 with excess dose of thiopental sodium (45 mg/kg) and the brain, heart, liver, lungs, kidney and intestine were removed and homogenized in 5 ml sterile saline. The number of viable units (cfu) in each 0.1 ml of homogenate was determined by plate counts on sabourouds dextrose agar (SDA) (containing 0.05 mg/ml of chloramphenicol) and the results were recorded. The whole experiment was done under aseptic conditions. The body weights of mice were recorded on day 0, 3, 6, 9, 12 and 16. The blood was collected on day 0 and day 16 for estimating total leucocytes using a cell analyzer and differential leukocytes were estimated according to the Ghai, (1987). A portion of brain, heart, liver, lungs, kidney and intestine were collected and fixed with 10% formalin solution. Sections were made from the paraffin block and stained with periodic acid-schiff (PAS) for histopathology examination (Mukherjee, 1989).

3.9.4. Effect of the extracts against experimental systemic candidiasis under gastrointestinal colonization in non immunocompromised and immunocompromised mice (Mellado et al., 2000 and Suresh et al., 1995)

Grouping of animals

Healthy Swiss albino mice, weighing at the range of 25-30 gm were selected. Groups of 10 animals of both sex in each group were taken for the study and treated as described below.

Group 1 (Vehicle control): Treated with antibiotics (Clindamycin 60mg/kg + Gentamycin 25mg/kg) and sterile saline (1ml/100gm) orally for the whole duration of the experiment of 24 days and inoculated with *C. albicans* (intragastric) on day 13 of the antibiotic treatment.

Group 2 (immunocompromised group): Treated with antibiotics (Clindamycin 60mg/kg + Gentamycin 25mg/kg) and sterile saline (1ml/100gm) orally for the whole duration of the experiment of 24 days and inoculated with *C. albicans* (intragastric) on day 13 of the antibiotics treatment and received cyclophosphamide (300mg/kg and 150mg/kg) on day 15 and 17, respectively.

Group 3 (non immunocompromised): Treated with antibiotics (Clindamycin 60mg/kg + Gentamycin 25mg/kg) orally for the whole duration of the experiment of 24 days and inoculated with *C. albicans* (intragastric) on day 13 and received HEOS (200mg/kg) orally on day 14 to 24 twice daily, 24 h after post-inoculam of *C. albicans.*

Group 4 (non immunocompromised): Treated with antibiotics (Clindamycin 60mg/kg + Gentamycin 25mg/kg) orally for the whole duration of the experiment for 24 days and inoculated with *C. albicans* (intragastric) on day 13 and received HEOS (400mg/kg) orally on day 14 to 24 twice daily, 24 h after post-inoculam of *C. albicans.*

Group 5 (non immunocompromised): Treated with antibiotics (Clindamycin 60mg/kg + Gentamycin 25mg/kg) orally for the whole duration of the experiment for 24 days and inoculated with *C. albicans* (intragastric) on day 13 and received HEOB (200mg/kg) orally on day 14 to 24 twice daily, 24 h after post-inoculam of *C. albicans.*

Group 6 (non immunocompromised): Treated with antibiotics (Clindamycin 60mg/kg + Gentamycin 25mg/kg) orally for the whole duration of the experiment for 24 days and inoculated with *C. albicans* (intragastric) on day 13 and received HEOB (400mg/kg) orally on day 14-24 twice daily, 24 h after post-inoculam of *C. albicans.*

Group 7 (immunocompromised): Treated with antibiotics (Clindamycin 60mg/kg + Gentamycin 25mg/kg) orally for the whole duration of the experiment for 24 days and inoculated with *C. albicans* (intragastric) on day 13 and received HEOS (200mg/kg) orally on day 14 to 24 twice daily, 24 h after post-inoculam of *C. albicans* and received cyclophosphamide (300mg/kg and 150mg/kg) on day 15 and 17, respectively.

Group 8 (immunocompromised): Treated with antibiotics (Clindamycin 60mg/kg + Gentamycin 25mg/kg) orally for the whole duration of the experiment for 24 days and inoculated with *C. albicans* (intragastric) on day 13 and received HEOS (400mg/kg) orally on day 14 to 24 twice daily, 24 h after post-inoculam of *C. albicans* and received cyclophosphamide (300mg/kg and 150mg/kg) on day 15 and 17, respectively.

Group 9 (immunocompromised): Treated with antibiotics (Clindamycin 60mg/kg + Gentamycin 25mg/kg/b.wt) orally for the whole duration of the experiment for 24 days and inoculated with *C. albicans* (intragastric) on day 13 and received HEOB (200mg/kg) orally on day 14 to 24 twice daily, 24 h after post-inoculam of *C. albicans* and received cyclophosphamide (300mg/kg and 150mg/kg) on day 15 and 17, respectively.

Group 10 (immunocompromised): Treated with antibiotics (Clindamycin 60mg/kg + Gentamycin 25mg/kg) orally for the whole duration of the experiment for 24 days and inoculated with *C. albicans* (intragastric) on day 13 and received HEOB (400mg/kg) orally on day 14 to 24 twice daily, 24 h after post-inoculam of *C. albicans* and received cyclophosphamide (300mg/kg and 150mg/kg) on day 15 and 17, respectively.

Group 11 (immunocompromised): Treated with antibiotics (Clindamycin 60mg/kg + Gentamycin 25mg/kg) orally for the whole duration of the experiment for 24 days and inoculated with *C. albicans* (intragastric) on day 13 and received ketoconozole (60mg/kg) orally on day 14 to 24 twice daily, 24 h after post-inoculam of *C. albicans* and received cyclophosphamide (300mg/kg and 150mg/kg) on day 15 and 17, respectively.

Experimental systemic candidiasis under gastrointestinal colonization was induced in mice by intragastric administration of about 0.1 ml of $1x10^{5-6}$ cells / ml of the culture suspension of *C.albicans*. Antibiotics and test drugs were dissolved in sterile saline and administered orally. The positive control and vehicle control (sterile saline) were maintained throughout the experiment. The animals were observed for mortality for 24 consecutive days and the percentage mean survival time (MST) was calculated using the following formula;

$$\text{Percentage MST} = \frac{X_1Y_1 + X_2Y_2 + \ldots\ldots X_nY_n - \text{number of surviving animals}}{\text{Total number of animals}}$$

Where X = the number of days (day on which animal death occurs)

Y = the number of animals dead on the particular day

The treated/control values (T/C %) were calculated by the ratio of mean survival time of treated and the control group. Fresh fecal samples were collected on day 14 and 24 of the experimental period. The sample (500 mg) was homogenized in 5 ml of sterile saline and 0.1 ml of homogenate was plated on SDA containing 0.05 mg/ml of chloramphenicol and the cfu was noted. The survived animals were euthanized on day 24 with excess dose of thiopental sodium (45 mg/kg body weight) and the liver, lungs, kidney and intestine were removed and homogenized in 5 ml sterile saline. The number of viable units (cfu) in each 0.1 ml of homogenate was determined by plate counts SDA containing 0.05 mg/ml of chloramphenicol and the results were recorded. The whole procedure was done under aseptic conditions. The body weights of the mice were recorded on day 0 and 24. The blood was collected on day 0 and 24 and total leucocytes using cell analyzer and differential leukocytes according to Ghai, (1987) were estimated. The collected blood was also centrifuged at 4000 rpm, the serum was separated and estimated for TNF-α and IL-1α on day 24 by using standard kits. A portion of liver, lungs, kidney and intestine were collected and fixed with 10% formalin solution. Sections were made from the paraffin block and stained with periodic acid-schiff (PAS) for histopathology examination (Mukherjee, 1989).

3.9.4.1. Estimation of TNF- α in mice serum

Standard curve range: 32-2000pg/ml

Kit contents

Component	Description	Amount
Pre-coated 96 well ELISA microplate	Antigen-affinity purified rabbit anti-mouse TNF-alpha pre-coated 96well plate	1 plate
Detection antibody(lyophilized)	Biotinylated antigen-affinity purified rabbit Anti-mouse TNF-alpha	1 vial
Standard protein(lyophilized)	Recombinant Mouse TNF-alpha	1 vial
Color Development Enzyme(lyophilized)	Avidin-HRP conjugate	1 vial
Assay diluent	0.1% BSA in PBS(50ml)	1 vial
Color development Reagent A	TMB Solution(10ml)	1 vial
Stop solution	2M H_2SO_4(10ml)	1 vial
Color development Reagent B	Substrate(H_2O_2)solution(20ml)	1 vial
PBS powder	Pouch for 1L	1 EA
Tween-20 (50%)	1ml	1 vial
Plate sealer		3 EA

Reconstitution and storage:

Mouse TNF-α standard: 100ng (1vial) of recombinant mouse TNF-Alpha reconstituted in 0.1ml sterile water to a concentration of 1.0µg/ml.

Detection antibody: 2.5µg (1vial) of biotinylated antigen-affinity purified anti-mouse TNF-alpha reconstituted in 0.25ml sterile water to a concentration of 10µg/ml.

Color development enzyme: Avidin-HRP reconstituted in 60µl sterile water.

Reagent preparations:

All preparations were mixed thoroughly and warmed up to room temperature prior to use.

Washing solution (PBST): The PBS powder (1 pouch) was resolved to sterile water and the volume made up to 1liter, added 1ml of Tween-20 (50%) and mixed well.

The standards and sample dilutions in assay diluents at 1:2 serial dilutions were as follows;

Step	Dilution method	Standard concentration
Step A	4µL of standard + 2ml of assay diluent	2000pg/ml
Step B	1ml of step A + 1ml of assay diluent	1000pg/ml
Step C	1ml of step B + 1ml of assay diluent	500pg/ml
Step D	1ml of step C + 1ml of assay diluents	250pg/ml
Step E	1ml of step D + 1ml of assay diluents	125pg/ml
Step F	1ml of step E + 1ml of assay diluents	62.5pg/ml
Step G	1ml of step F + 1ml of assay diluents	31.25pg/ml

Detection antibody: diluted the reconstituted detection antibody in the assay diluent to a concentration of 0.5µg/ml (1/2 dilution). Each well needs 100µl.

Color development enzyme: diluted the Avidin-HRP conjugate 1:200 in assay diluent. Each well needs 100µl.

Color development solution: 1 volume of color development reagent A and 2 volume of reagent B (1:2) were mixed prior to use and each well needs 100µl.

ELISA Protocol:

The washing solution (200µl) was added to each well. The cells were aspirated to remove liquids and the plate were washed three times using 300µl of washing solution per well. After the last wash, the plates were inverted to remove residual solution and blotted on paper towel. The standard/sample (100µl) was added to each well in duplicate and covered with the space sealer and incubated at room temperature for at least 2 h. The wells were aspirated to remove liquid and the plates were washed four times as in step 1. The diluted detection antibody (100µl) 0.25µg/ml per well was added and covered with the space sealer, incubated at room temperature for 2 h. The plates were aspirated and washed four times as in step 1. The diluted color development enzyme (100µl) was added per well and the plate was covered with sealer provided and

incubated for 30 min at room temperature. The plate was aspirated and washed four times like as in step 1. The color development solution (100µl) was added to each well and incubated at room temperature for a proper color development (3-30 min). To stop the color reaction, 50µl of the stop solution was added to each well. The plates were read at 450 nm wavelength using a microtiter plate reader. The results were expressed as pg/ml.

3.9.4.2. Estimation of IL-1α in mice serum

Standard curve range: 16-2500pg/ml

Kit contents:

Component	Description	Amount
Pre-coated 96 well ELISA microplate	Antigen-affinity purified rabbit anti-mouse IL-1Alpha pre-coated 96well plate	1 plate
Detection antibody(lyophilized)	Biotinylated antigen-affinity purified rabbit Anti-mouse IL-1Alpha	1 vial
Standard protein(lyophilized)	Recombinant Mouse IL-1Alpha	1 vial
Color Development Enzyme(lyophilized)	Avidin-HRP conjugate	1 vial
Assay diluent	0.1% BSA in PBS(50ml)	1 vial
Color development Reagent A	TMB Solution(10ml)	1 vial
Stop solution	2M H_2SO_4(10ml)	1 vial
Color development Reagent	Substrate(H_2O_2)solution(20ml)	1 vial
PBS powder	Pouch for 1L	1 EA
Tween-20 (50%)	1ml	1 vial
Plate sealer		3 EA

Reconstitution and storage:

Mouse IL-1α standard: 12.8ng (1vial) of recombinant mouse IL-1Alpha reconstituted in 0.1ml sterile water for a concentration of 128ng/ml.

Detection antibody: 5µg (1vial) of biotinylated antigen-affinity purified anti-mouse IL-1Alpha reconstituted in 0.25ml sterile water for concentration of 20µg/ml.

Color development enzyme: Avidin-HRP reconstituted in 60µl sterile water.

Reagent preparations:

All preparations were mixed thoroughly and warmed up at room temperature prior to use.

Washing solution (PBST): Dissolved the PBS powder (1 pouch) in sterile water and the volume made up to 1liter, then added 1ml of Tween-20 (50%) to this solution and mixed well.

Sample dilution: Diluted samples to a proper concentration in assay diluents, each well needs 100µl.

The standards and samples were diluted in assay diluents at 1:2 serial dilutions as follows;

Step	Dilution method	Standard concentration
Step A	19.5µL of standard + 1ml of assay diluents	2500pg/ml
Step B	0.5ml of step A + 0.5ml of assay diluents	1250pg/ml
Step C	O.5ml of step B + 0.5ml of assay diluents	625pg/ml
Step D	0.5ml of step C + 0.5ml of assay diluents	312.5pg/ml
Step E	O.5ml of step D + 0.5ml of assay diluents	156.25pg/ml
Step F	0.5ml of step E + 0.5ml of assay diluents	78.125pg/ml
Step G	O.5ml of step F + 0.5ml of assay diluents	34pg/ml

Detection antibody: the reconstituted detection antibody was diluted in the assay diluent to a concentration of 0.5µg/ml (1/40 dilution) and each well needs 100µl.

Color development enzyme: diluted the Avidin-HRP conjugate 1:200 in assay diluent. Each well needs 100µl.

Color development solution: 1 volume of color development reagent A and 2 volume of reagent B were mixed (1:2) prior to use. Each well needs 100µl.

ELISA Protocol:

The washing solution (200µl) was added to each well and aspirated the well to remove liquids and washed three times using 300µl of washing solution per well. After the last wash, the plates were inverted to remove residual solution and blotted on paper towel. The standard/sample (100µl) was added to each well in duplicate and covered with the space sealer provided and incubated at room temperature for 2 h. The wells were aspirated to remove liquid and the plates were washed four times as in step 1. The diluted detection antibody (100µl) 0.5 µg/ml was added per well and covered with the space sealer provided and incubated at room temperature for 2 h and aspirated and washed four times as in step 1. The diluted color development enzyme (100µl) was added per well and covered with the plate sealer provided and incubated for 30 min at room temperature, aspirated and washed four times as in step 1. The color development solution (100µl) was added to each well and incubated at room temperature for a proper color development (3-30 min). To stop the color reaction, 50µl of the stop solution was added to each well. The plates were read at 450nm wavelength using a microtiter plate reader. The results were expressed as pg/ml

3.9.5. Effect of the extracts against oral candidiasis in immunocompromised mice

(Takakura et al., 2003)

Grouping of animals

Groups	Treatment
Group 1	Vehicle control (sterile saline 1ml/100gm) inoculated with *C.albicans* and immunocompromised
Group 2	HEOS 200mg/kg (immunocompromised and inoculated with *C.albicans*)
Group 3	HEOS 400mg/kg (immunocompromised and inoculated with *C.albicans*)
Group 4	HEOB 200mg/kg (immunocompromised and inoculated with *C.albicans*)
Group 5	HEOB 400mg/kg (immunocompromised and inoculated with *C.albicans*)
Group 6	Ketoconozole 60mg/kg (immunocompromised and inoculated with *C.albicans*)

Groups of six male mice were taken for the study. Since the opportunistic fungus *C.albicans* is a major cause of oral and esophageal infections in immunocompromised condition, investigations were carried out in immunocompromised mice rather than normal animals. Tetracycline hydrochloride in drinking water at a concentration of 0.83 mg/ml was given to mice one day before the induction of infection. Animals were anesthetized by 0.2 ml of 2 mg/ml ketamine hydrochloride (50 mg/kg i.p). Small sterilized cotton pads (baby cotton buds; Johnson and Johnson, Mumbai) were soaked in *C.albicans* cell suspension ($1x10^{5-6}$ viable cells/ml) and used to swab the entire oral cavity of the anesthetized mice to produce oral infection. The animals were immunosuppressed with cyclophosphamide (300 mg/kg and 150mg/kg i.p) on day 2 and 5, respectively, of post inoculation with *C.albicans.* The drugs were administered orally twice daily the next day after the infection for six consecutive days.

On day 7 the animals were euthanized under excess dose of thiopental sodium (45 mg/kg body weight) and the whole oral cavity including the oral mucosa, tongue, soft palate and other mucosal surfaces were swabbed using sterilized cotton pad. The end of the cotton pad was then cut off and placed in a tube containing 5 ml of sterile saline, when C *.albicans* was released from the swab into the saline with the help of a vortex mixer. It was then made up to the required dilution of the cell suspension. The cell suspension (0.1 ml) was plated on SDA containing 0.05 mg/ml of chloramphenicol and incubated at 24 ^{0}C for 48 h. The colony forming units of *C.albicans* was counted. The whole experimental procedure was done in aseptic conditions. Tongue and buccal mucosal layers were collected from euthanized animals and fixed in 10% neutral formalin solution and embedded in paraffin. Five µm sections were cut from the paraffin block and stained with periodic acid-schiff (PAS) for histopathological examination (Mukherjee, 1989).

3.9.6. Formulation of cream and gel for topical application

3.9.6.1. Formulation of cream (Flynn 1990)

Oleaginous phase

Bees wax-10 g

Liquid paraffin-30 g

Glycerin – 5 g

Aqueous phase

Borax-1g

Methyl paraben- 0.01%

Propyl paraben- 0.01%

Purified water- q.s

Oil soluble ingredients from the formula were first dissolved in the oil phase and the ingredients which were water soluble were dissolved in aqueous phase. Both the phases were prepared separately and they were heated to a temperature 60 to 70^{o}C. Oil phase was slowly incorporated into the aqueous phase by vigorous stirring until the formation of the cream was smooth and uniform. The extract was incorporated into the aqueous phase at 60^{0}C with constant stirring. Creams (2% and 4%) were prepared for further pharmacological studies. The formulated creams were investigated for their physical parameters like consistency, homogeneity, spreadability, washability and tube extrudability.

3.9.6.2. Formulation of gel (Flynn 1990)

Carbopol (200 mg) was mixed with 5 ml water thoroughly, until it was completely soluble. Three to four drops of sodium hydroxide was added until a clear gel was obtained. The extract (200 mg) was dissolved in 1 ml of water and this was added to the gel by continuously stirring the same. The consistency was adjusted with sodium hydroxide and water. Polyethylene glycol (2ml) was added after addition of the extract. The formulated gel was evaluated for the visual appearance, feel upon application, texture, colour and odour, and extrudability. Further, the formulated gels were then evaluated for the following parameters;

- Particle size distribution of the dispersed phase,

  The particle size distribution of the gel was assessed using optical microscopy technique.

- Viscosity,

  The viscosity of the gel was determined using Brookfield Viscometer RVD VIII, with spindle S7 at 100 rpm.

- Loss of water and other volatile components

  Freeze thaw cycles were used to assess the physical stability of the prepared gel and to identify their syneresis and loss of volatile components. Freez thaw cycles were determined by alternating the temperature every 24 h from 25^{o}c to 5^{o}c. The formulation was alternatively stored in a refrigerator (5^{o}c) and at (25^{o}c) in an incubator and the cycle was repeated 5 times and the gel was observed for loss of water and volatile components using the following formula;

$$\frac{\text{Final weight}}{\text{Initial weight}} \times 100$$

- Microbial limit test

  A microbial test was carried out for bacteria (E. coli) and fungi (*C.albicans*) after three months of storage.

Among the formulated cream and gel, the cream showed good physical properties. The formulated cream 2% and 4% was studied for further pharmacological studies.

3.9.7. Effect of cream of the extracts against experimental vaginal candidiasis in non immunocompromised and immunocompromised mice (Suresh et al., 1997)

Grouping of animals

A total number of 66 female mice were divided into eleven groups of six animals and treated as described below.

Groups	Treatment
Group 1	Control (cream base without drug and inoculated with *C.albicans*)
Group 2	Immunocompromised control (cream base without drug and inoculated with *C.albicans* and immunocompromised)
Group 3	HEOS 2% cream (inoculated with *C.albicans*)
Group 4	HEOS 4% cream (inoculated with *C.albicans*)
Group 5	HEOB 2% cream (inoculated with *C.albicans*)
Group 6	HEOB 4% cream (inoculated with *C.albicans*))
Group 7	HEOS 2% cream (immunocompromised and inoculated with *C.albicans*)
Group 8	HEOS 4% cream (immunocompromised and inoculated with *C.albicans*)
Group 9	HEOB 2% cream (immunocompromised and inoculated with *C.albicans*)
Group 10	HEOB 4% cream (immunocompromised and inoculated with *C.albicans*)
Group 11	Ketoconozole 2% cream (immunocompromised and inoculated with *C.albicans*)

The mice were brought to pseudoestrous stage by injecting 0.2 ml of 2.5mg/ml of oestradiol (i.p) for four days. On day 5 (day 1 of experimental period), the animals were inoculated intravaginally with $1x10^{7-8}$ cells of *C.albicans* in 0.1 ml of sterile saline via segment butterfly tubing fixed on a tuberculin syringe until it ran out. The animals were immunosuppressed with 300 mg/kg and 150 mg/kg of cyclophosphamide on day 2 and 5, respectively, of post inoculation with *C.albicans.* Animals were treated with the extract, with applicator twice daily for 18 days as described above. The treatment was started 24 h after post-inoculum with *C.albicans*. The sample of vaginal scrapings were taken post inoculum with a wire loop on days 3, 6 ,9, 12, 15 and 18 and the sample of vaginal scrapings was suspended aseptically in 10 ml of sterile saline. Ten fold serial dilutions were prepared and each dilution was then plated on SDA containing 0.05 mg/ml chloramphenicol and incubated for 48 h at 24^{o}C.The number of colony forming units per ml (cfu/ml) of retrieved vaginal scrapings suspension was then determined and scored to assess the intensity of infection. Ketoconazole 2% cream as positive control and cream base without drug as control was maintained throughout the experiment. Blood was collected and total leucocytes count using cell analyzer and differential leucocytes were observed on day 0 and day 18, according to Ghai, (1987).

3.9.8. Antimycotic activity (hair root invasion) of cream of the extracts (Suresh et al., 1997)

Male albino guinea pigs (300-350 gm) with three animals in each group were used. The hairs at the back of guinea pigs were shaved off with electric clippers. An open glass cylinder (2cm high and 3.5cm diameter) was laid on the widest part of the back (lumbar region), and 0.5 ml of inoculum containing $1x10^{6-7}$ cells/ml suspension was abraded into the portion of the skin encircled by the cylinder with a sterilize glass rod. HEOS and HEOB (2% and 4% cream) were applied externally with the help of an applicator, twice daily for 7 days, 48 h after post inoculums with *C.albicans.* During the experimental period animals were observed for erythma, redness scales and incrustation patches. On day 8 hairs were removed from infected area with sterilized forceps and plated on SDA containing 0.05 mg/ml chloramphenicol and incubated for 48 h at 24^{o}C. The vehicle control (cream base without drug) and positive control (ketoconozole 2% cream) were maintained throughout the experiment.

3.9.9. Effect of cream of the extracts against infectious wound in excision model in rats (Udupa et al., 1989 and Thaker and Anjaria, 1985)

Rats (8 per group of both sex) were anaesthetized with ketamine hydrochloride (50mg/kg i.p) and wounds were generated by marking a 1 cm² area on the midline of the dorsum with a marker pen and then excised full thickness skin with a fine scissor. A loop full of mixed organisms containing 1 ml of each ($1x10^{6}$cells per ml) *Staphylococcus aureus*, *Bacillus subtillis*, *Escherchia coli*, *Salmonella typhi*, *Cryptococcus neoformans* and *Candida albicans* cultures were applied topically on wounded area. Forty eight hours were left for infection to set. The extract (2% and 4% cream) was applied externally with the help of an applicator, twice daily for 20 days, 48 h after wound infection. The wound area was traced at 0, 4, 8, 12, 16, and 20 days. Two animals on day 8 and day 16 from each group were euthanized and remaining animals on day 21 with excess dose of thiopental sodium and the regenerated skin along with wounded area was carefully excised and a piece was homogenized. The homogenate was estimated for hydroxyproline, superoxide dismutase (SOD) and thiobarbituric acid reactive substance (TBARS). Another piece of skin was stored in 10% buffered formalin

for histopathology studies. Control, vehicle control (cream base without drug) and positive control (0.2% nitrofurazone cream) was maintained throughout the experiment.

3.9.10. Effect of cream of the extracts against infectious wound in incision model in rats (Udupa et al., 1989 and Thaker and Anjaria, 1985)

Rats (8 per group of either sex) were anesthetized with ketamine hydrochloride (50 mg/kg i.p) during surgical procedure. Dorsal and ventral skin was shaved and cleaned with alcohol. Full thickness incisions, 15mm in length, were created on the dorsal and ventral surfaces lateral to the midline with a scalpel. A loop full of mixed organisms containing 1 ml of each ($1x10^6$cells per ml) *Staphylococcus aureus*, *Bacillus subtillis*, *Escherchia coli*, *Salmonella typhi*, *Cryptococcus neoformans* and *Candida albicans* cultures were applied topically on the wounded area. After the infection, the parted skin was kept together and stitched with black silk at 0.5 cm intervals with surgical thread and curved needle (No.11). The wound was left undressed and forty eight hours were left for infection to set. The extract (2% and 4% cream) was applied externally with the help of an applicator, twice daily for nine days. Control and positive control (0.2% nitrofurazone cream) was maintained throughout the experiment. Sutures were removed on day 9 and the tensile strength using tensiometer was recorded on day 10.

The tensiometer consists of a 6x12 wooden board with one arm of 4 inch long, fixed on each side of the possible longest distance of the board. The board was placed at the edge of table. A pulley with bearing was mounted on the top of one arm. An alligator clamp with one centimeter width was tied on the tip of the other arm by a fishing line (20lb test monofilament) in such a way that the clamp could reach the middle of the board. Another alligator clamp was tied on the longer fishing line with a graduated polyethylene bottle on the other end. The tensile strength of a wound represents the degree of wound healing. One day before measuring the tensile strength the sutures were removed from the stitched wound.

On day 10 the rats were again anesthetized as described earlier and each rat was placed on a stack of paper towels on the middle of the board. The number of towels could be adjusted in such a way that the wound was on the same level as the tips of the

arms. The clamps were then carefully attached to the skin on the opposite sides of the wound at distance of 0.5 cm away from the wound. The longer pieces of the fishing line were placed on the pully and finally on to the polyethylene bottle and the position of the board were adjusted so that the bottle receives a rapid and constant rate of water from a large reservoir until the wound began to open. The amount of water in the polyethylene bottle was weighed and this was considered as an indirect measure of the tensile strength of the wound. The mean determination of tensile strength on the two paravertebral incisions on both sides of the animals was taken as the measure of the tensile strength of the wound for an individual animal. The tensile strength increment indicates better wound healing.

The skin samples were collected on last day of the study and homogenized. The homogenate was estimated for hydroxyproline, superoxide dismutase (SOD) and thiobarbituric acid reactive substance (TBARS). Another piece of skin was stored in 10% buffered formalin for histopathology studies.

3.9.10.1. Estimation of hydroxyproline

The estimation was carried out using the spectrophotometric method (Reddy and Enwemeka, 1996). The skin samples were hydrolyzed by autoclaving at 120^0 C for 20 min. Chloramine- T (450 µl) was added to 50 µl of hydroxylate and mixed gently and oxidation was allowed to proceed for 25 min at room temperature. To this 500 µl of Ehrlich's reagent (p-dimethyl aminobenzaldehyde in n-propanol/perchloric acid) was added and mixed gently. The chromophore was developed by incubating the mixture at 65^0C for 20 min. The absorbance of the reddish purple complex was measured spectrophotometrically at 550 nm. A standard curve was prepared with hydroxyproline (1mg/ml). Hydroxyproline was expressed as µg/g of the tissue.

3.9.10.2. Estimation of superoxide dismutase (SOD)

Estimation of SOD was carried out with the standard kit available (RANSOD of Randox laboratories Ltd., UK). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(-nitrophenol)-5-

phenyltetrazolium chloride (INT) to from a red formazen dye. The SOD activity was then measured by the degree of inhibition of this reaction.

Skin homogenate (1%) was prepared using 0.9% sodium chloride. The homogenate (25 µl) was taken and 850 µl of mixed substrate and 125 µl of xanthine oxidase were added to the homogenate. The absorbance was observed at 505 nm as per standard procedure mentioned in the kit. The SOD level was expressed as units/mg protein.

3.9.10.3. Estimation of thiobarbituric acid reactive substance (TBARS)

The method of Ohkawa et al., (1979) was used to estimate the total amount of TBARS in tissue homogenate. Sodium dodecyl sulphate (200 µl of 8%), 1.5 ml of 205 acetic acid solution, 1.5 ml of 0.9% aqueous solution of thiobarbituric acid and 1.3 ml of distilled water were added to 500µl of the supernatant of tissue homogenate and heated in a boiling water bath for 30 min. After cooling, the red chromophore was extracted on to the 5 ml mixture of n-butanol and pyridine (15:1 v/v) and centrifuged at 4000 rpm for 10 min. The organic layer was taken and its absorbance was measured at 532 nm. 1, 1, 3, 3, Tetra ethoxypropane (TEP) was used as an external standard and the level of lipid peroxidase was expressed as µ moles /mg protein. The calibration curve was prepared by the above procedure taking TEP as standard.

3.9.10.4. Estimation of proteins

Total protein was estimated by using E-coline kit. The color intensity was measured with E-Merck, Auto analyzer Microlab 200.

3.9.11. Repeated dose 28 day sub acute oral toxicity of the extracts (OECD guideline, 407)

The extract at 200mg/kg, 400mg/kg and 800mg/kg dose levels were administered orally twice daily to three groups of Wistar albino rats of either sex (10 per group), one dose level per group for a period of 28 days. During the period of administration the animals were observed closely, each day for signs of toxicity and for mortality. All animals were weighed once a week during the study. Necropsy was done for the animals that died during the study and at the conclusion of the study surviving animals were sacrificed

with excess dose of anesthesia (thiopental sodium 45mg/kg; i.p). Blood was collected through abdominal aorta and haematology parameters were estimated (Hb, RBC, platelet count, total leukocytes) using a cell analyzer and differential count according to the method described by Ghai, (1987). The plasma/serum was collected by centrifugation of blood and glucose, ASAT, ALAT, total bilirubin, uric acid, creatinine, GGT, alkaline phosphatase, cholesterol and total protein were estimated by using standard diagnostic kits (E-coline, Merck, Mumbai). Gross pathological changes and absolute wet weight of organs were observed on day 29. Histopathology of lung, kidney, brain, heart, spleen, testis and ovary were done at end of the test period according to the method described by Mukherjee (1989).

3.9.12. Statistical analysis

Data were expressed as Mean ± SEM. One way Analysis of variance (ANOVA) followed by Dunnett post test and Two way repeated measures ANOVA followed by Bonferroni post test were applied for anticandidal and wound healing activities using GraphPad Prism 4.01 (GraphPad Software Inc., San Diego, CA, USA). Unpaired Student's *t* test was applied for biochemical estimations in wound healing studies.

4. RESULTS AND DISCUSSION

4.1. Qualitative phytochemical screening of the extracts

HEOS and HEOB were tested for qualitative phytochemical screening and the results obtained are recorded in Table 2. The results reveal the presence of carbohydrates, phenolic compounds and flavonoids, proteins and amino acids in the extracts.

4.2. Estimation of total phenolic and flavonoids compounds in the extracts

The amount of phenolic and flavonoids compounds present in the extracts were estimated and given in Tables 3 and 4. The data indicate 7.5 mg/g and 9.0mg/g of phenolic compounds and 2.5mg/g and 3.0mg/g of flavonoids are found in HEOS and HEOB, respectively.

4.3. Quantitative analysis of phytoprinciples in the extracts

The results of the quantitative analysis of phytoprinciples are given in Table 5 and Figures 5-7. The results reveal eugenol (1.32mg/g), rosmarinic acid (0.73mg/g), rutin (0.4mg/g) and quercetin (0.15mg/g) in HEOS, querectin (6.22mg/g), rosmarinic acid (1.24mg/g), rutin (0.9mg/g) and eugenol (0.4mg/g) are present in HEOB.

4.4. Nitric oxide scavenging property of the extracts

The nitric oxide scavenging property of the extracts was studied and the results are given in Figure 8. The results reveal that HEOB has a higher activity (IC_{50} 340.1) activity when compared with HEOS (IC_{50} 283.8). The standard sample rutin has an activity of IC_{50} 159.5.

Table 2: Qualitative phytochemical screening of the extracts

1. Detection of alkaloids

S.No	Name of the extract	Wagner's test	Mayers'test	Hager's test	Dragendorff's test	Observations
1	HEOS	-ve	-ve	-ve	-ve	Alkaloids are absent
2	HEOB	-ve	-ve	-ve	-ve	Alkaloids are absent

2. Detection of carbohydrates and glycosides

S.No	Name of extract	Molish's test	Fehling's test	Barfoerd's test	Benedict's test	Observations
1	HEOS	+ve	+ve	+ve	+ve	Carbohydrate and sugars are present
2	HEOB	+ve	+ve	+ve	+ve	Carbohydrate and sugars are present

3. Detection of glycosides

S.No	Name of extract	Legal's test	Borntrager's test	Observations
1	HEOS	-ve	-ve	Glycosides are absent
2	HEOB	-ve	-ve	Glycosides are absent

4. Detection of proteins and amino acids

S.No	Name of extract	Millon's test	Biuret test	Ninhydrin test	Observations
1	HEOS	+ve	+ve	+ve	Protein and amino acid are present
2	HEOB	+ve	+ve	+ve	Protein and amino acid are present

5. Detections of phenolic compounds and flavonoids

S.No	Name of extract	Ferric chloride test	Lead acetate test	Alkaline test	Gelatin test	Magnesium and hydrochloric acid reduction test (for flavonoids)	Observations
1	HEOS	+ve	+ve	-ve	+ve	+ve	Phenolic compounds and flavonoids are present
2	HEOB	+ve	+ve	-ve	+ve	+ve	Phenolic compounds and flavonoids are present

6. Detection of saponins

S.No	Name of the extract	Foam test	Observations
1	HEOS	-ve	Saponins are absent
2	HEOB	-ve	Saponins are absent

+ ve: positive

- ve: negative

Table 3: Estimation of total phenolic compounds in the extracts

S.No	Concentration (µg/ml)	HEOS	HEOB
		Absorbance	Absorbance
1	1000	2.575	2.575
2	500	2.282	2.282
3	250	1.922	1.922
4	125	1.063	1.063
5	62.5	0.482	0.482
6	31.25	0.184	0.184
7	15.675	0.089	0.089
8	7.84	0.044	0.044
9	Test sample (9.0)	0.056	0.056

Table 4: Estimation of total flavonoids in the extracts

S. No	Concentration (µg/ml)	HEOS	HEOB
		Absorbance	Absorbance
1.	100	0.109	0.109
2.	90	0.099	0.099
3.	80	0.089	0.089
4.	70	0.074	0.074
5.	60	0.064	0.064
6.	50	0.052	0.052
7.	40	0.040	0.040
8.	30	0.028	0.028
9.	20	0.016	0.016
10.	10	0.005	0.005
11.	Test sample (30)	0.022	0.032

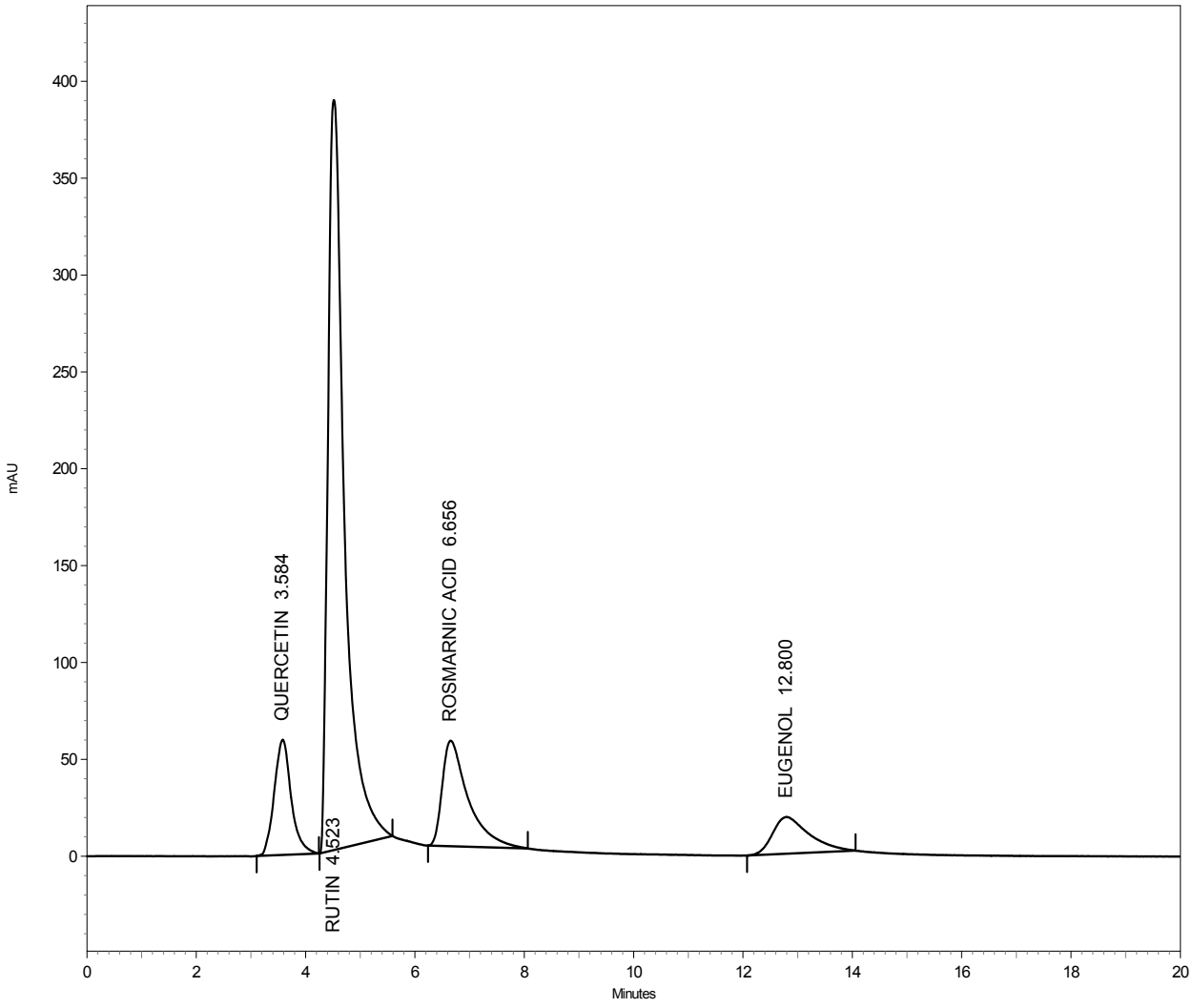
Table 5: Quantitative analysis of eugenol, rosmarinic acid, rutin and quercetin by HPLC in the extracts

Name of the plant	Eugenol (mg/g)	Rosmarinic acid (mg/g)	Rutin (mg/g)	Quercetin (mg/g)
HEOS	1.32	0.73	0.40	0.15
HEOB	0.40	1.24	0.90	6.22

Mobile phase: mixture of acetonitrile and potassium dihydrogen orthophosphate buffer (pH3.5) (1:3)

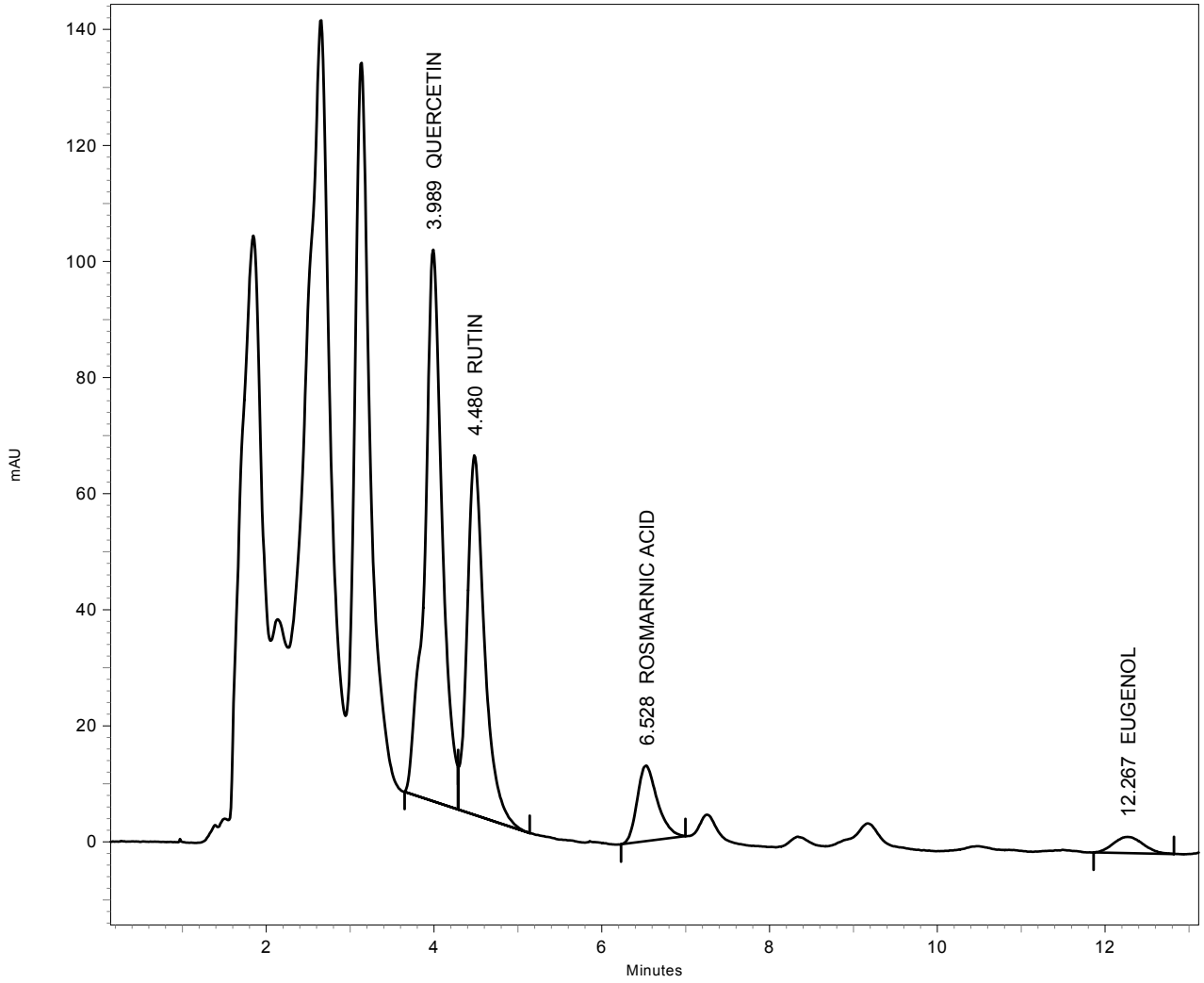
Retention time: eugenol (12.80), rosmarinic acid (6.65),rutin (4.52), quercetin (3.58)

Figure 5: Chromatogram of eugenol, rosmarinic acid, rutin and quercetin standards



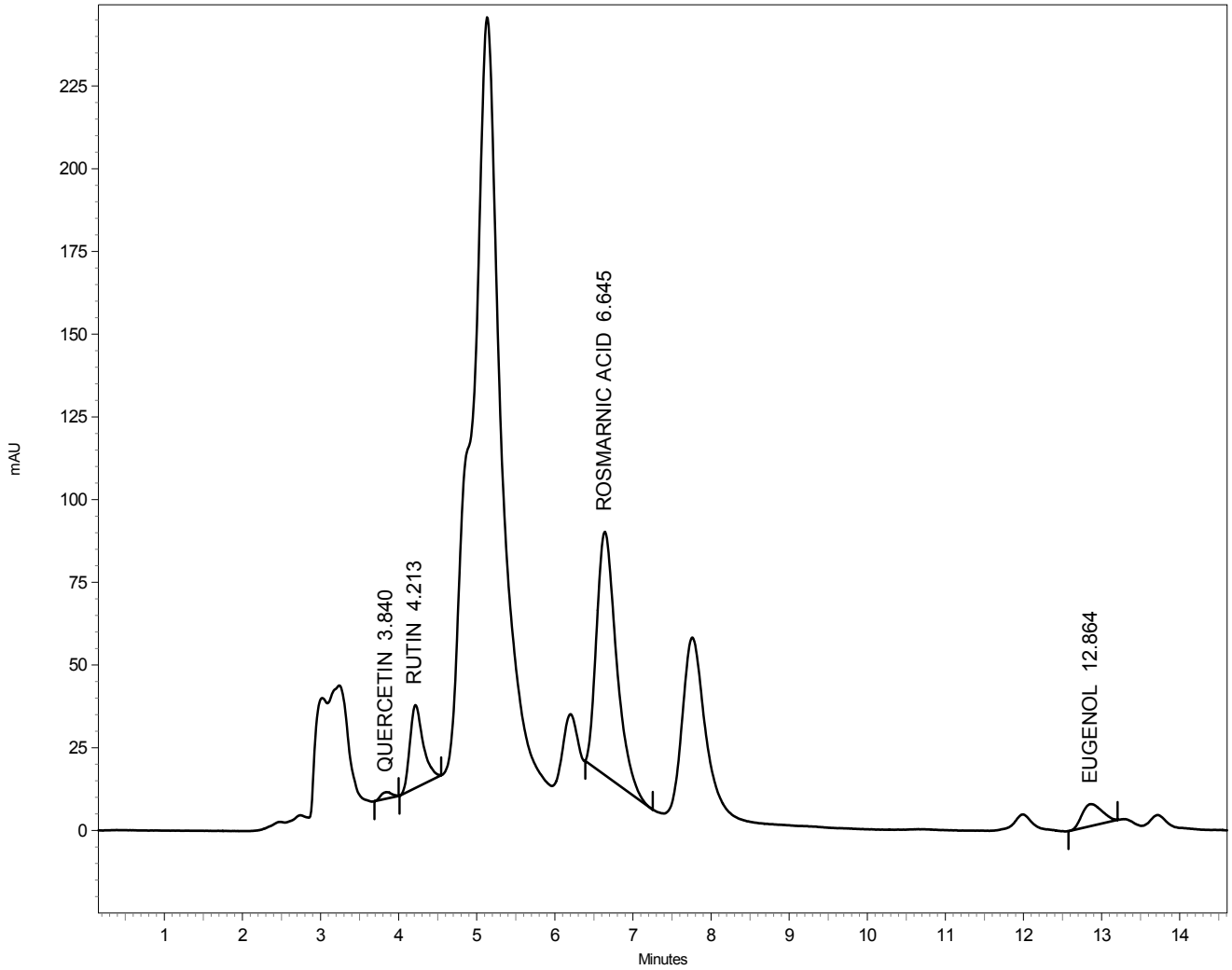
Retention time: eugenol (12.80), rosmarinic acid (6.65),rutin (4.52), quercetin (3.58)

Figure 6: Chromatogram of HEOS



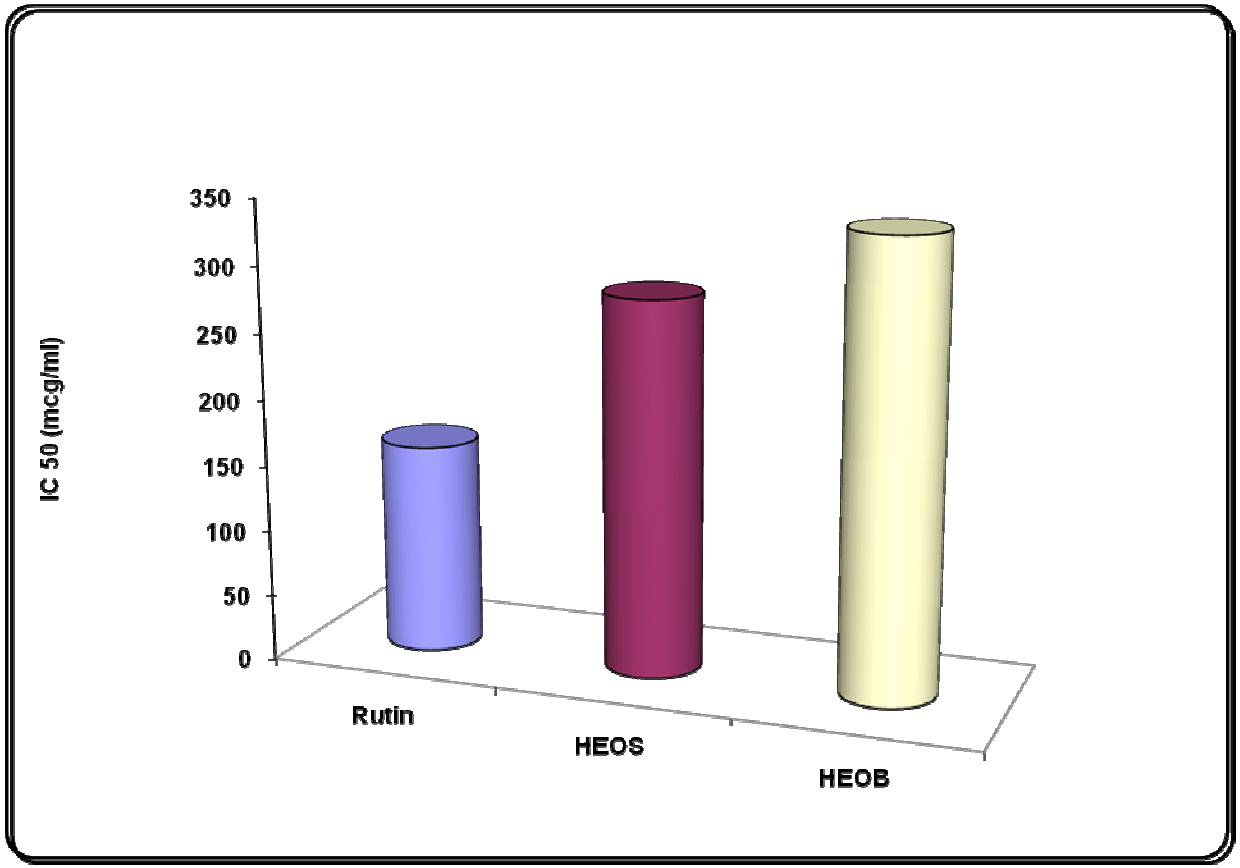
Retention time: eugenol (12.80), rosmarinic acid (6.65),rutin (4.52), quercetin (3.58)

Figure 7: Chromatogram of HEOB



Retention time: eugenol (12.80), rosmarinic acid (6.65),rutin (4.52), quercetin (3.58)

Figure 8: Nitric oxide scavenging activity of the extracts



4.5. *In vitro* antifungal and antibacterial studies

Doses of HEOS and HEOB ranging from 1000µg/ml to 3.625µg/ml of the broth were tested for antibacterial activity against eight strains of both Gram positive and Gram negative bacteria, 24 h cultures of containing $1x10^6$ cells /ml, using two fold serial dilution and the cup plate method. The results are recorded in Tables 6 and 7. The results reveal that HEOS has a zone of inhibition at 25 mm, 21 mm, and 20 mm for *S.aureus*, *B.subtilis* and *E.coli*, respectively in cup plate method. Zone of inhibition of 24 mm, 20 mm and 24 mm was observed for HEOB for *S.aureus*, *B.subtilis* and *E.coli*, respectively. An MIC at 500µg/ml in two fold serial dilution method was observed for these bacterial strains. There was no prominent action on the other tested bacterial strains.

The plant extracts were tested for antifungal activity against seven different fungal organisms. The results are recorded in Tables 8 and 9. The results reveal that both HEOS and HEOB possess zone of inhibition 24 mm against *C.albicans*, and 23 mm and 24 mm against *C.neoformans,* respectively. An MIC at 12.5µg/ml against *C.albicans* and *C.neoformans,* and 500µg/ml against other tested fungal organisms were observed. Both HEOS and HEOB possess maximum efficacy against *C. albicans*, thus giving a lead for further *in vivo* anticandidal studies. The results are comparable with ketoconozole, which show an MIC at 6.25µg/ml against all tested fungal organisms.

Table 6: Effect of the extracts on selected bacterial strains (cup plate method)

S. No	Organisms	Concentration of the extract (µg/ml)	Zone of inhibition (mm)		
			HEOS	HEOB	Standard drug
1	*S. aureus*(+)	1000	25	24	32
2	*B. coagulans*(+)	1000	16	18	31
3	*B. subtilis*(+)	1000	21	20	32
4	*B. mageterium*(+)	1000	19	19	28
5	*E. coli*(-)	1000	20	24	34
6	*P. aeruginosa*(-)	1000	15	18	20
7	*S. typhi*(-)	1000	16	16	32
8	*Shigella*(-)	1000	16	Nil	25

Strength of inoculums: $1x10^7$cells /ml (24h culture)

Drugs: Gentamycin, HEOS and HEOB (1 mg/ml stock solution; 10 µg/disc)

(+): Gram-positive bacteria and (-): Gram-negative bacteria.

Table 7: Effect of the extracts on selected bacterial strains (two fold serial method)

S.no	Organisms	Minimum Inhibitory Concentration (MIC) (µg/ml)		
		HEOS	HEOB	Standard drug
1	*S. aureus*(+)	500	500	6.25
2	*B. coagulans*(+)	1000	1000	6.25
3	*B. subtilis*(+)	500	500	6.25
4	*B. mageterium*(+)	1000	1000	3.625
5	*E. coli*(-)	500	500	6.25
6	*P. aeruginosa*(-)	1000	1000	6.25
7	*S. typhi*(-)	1000	1000	3.625
8	*Shigella*(-)	1000	1000	6.25

Strength of inoculums: $1x10^7$cells /ml (24h culture)

Drugs: Gentamycin, HEOS and HEOB (1mg/ml)

(+): Gram-positive bacteria and (-): Gram-negative bacteria.

Table 8: Effect of the extracts on selected fungal strains (cup plate method)

S.No	Organisms	Concentration of the extract (µg/ml)	Zone of inhibition (mm)		
			HEOS	HEOB	Standard drug
1.	*C. albicans*	1000	24	24	28
2.	*C. neoformans*	1000	23	24	29
3.	*T. rubrum*	1000	12	14	22
4.	*S. cerevisiae*	1000	14	16	26
5.	*A. niger*	1000	12	15	26
6.	*A. flavus*	1000	19	20	32
7.	*R. arrhizus*	1000	20	21	34

Strength of inoculums: $1x10^7$ cells /ml (48h culture)

Drugs: Ketoconozole, HEOS and HEOB (1 mg/ml stock solution; 10 µg/disc)

Table 9: Effect of the extracts on selected fungal stains (two fold serial dilution)

S.no	Organisms	Minimum Inhibitory Concentration (MIC) (µg/ml)		
		HEOS	HEOB	Standard drug
1.	*C. albicans*	12.5	12.5	6.25
2.	*C. neoformans*	12.5	12.5	6.25
3.	*T. rubrum*	500	500	6.25
4.	*S. cerevisiae*	500	500	6.25
5.	*A. niger*	1000	500	6.25
6.	*A. flavus*	500	500	6.25
7.	*R. arrhizus*	500	500	6.25

Strength of inoculums: $1x10^7$cells /ml (48h culture)

Drugs: Ketoconozole, HEOS and HEOB (1mg/ml)

4.6. Effect of the extracts against experimental systemic candidiasis in non immunocompromised and immunocompromised mice

4.6.1. Percentage mortality of non immunocompromised and immunocompromised mice treated with the extracts

The experimental systemic candidiasis, in both immunocompromised and non immunocompromised mice, was studied. The results given in Table 10 reveal 100% mortality in immunocompromised and 40% mortality in non immunocompromised mice treated with vehicle control, whereas only 20% mortality observed in non immunocompromised mice treated with HEOS 200mg/kg. However, HEOS at 400mg/kg, HEOB at 200mg/kg and 400mg/kg, treated groups show no mortality during the experimental period.

HEOS and HEOB (200mg/kg) treated immunocompromised group show 50% and 40% mortality, respectively. Similarly 10% mortality is observed in immunocompromised mice treated with HEOS and HEOB at 400mg/kg. No mortality is observed in ketoconozole treated immunocompromised mice. Since there was mortality in all groups, data of six mice from each group were taken for further observations.

4.6.2. Colony forming units of various organs of both non immunocompromised and immunocompromised mice treated with the extracts

The animals survived from different groups were euthanized at the termination of the study, various organs were collected and the cfu recorded. The data of six mice were subjected for repeated measures of Two way ANOVA and the results summarized as treatment versus cfu in organs (% variance 17.83, F-391015.34, DF- 50 and $p < 0.0001$), treatment versus result (% variance 56.54, F-6200869.0, DF-10 and $p<0.0001$), organs versus result (% variance 25.63, F-5622249.5, DF-5 and $p<0.0001$) are given in Table 11.

The data shown in Table 11a and Figure 9 reveal a significant ($p<0.001$) increase in the number of cfu in brain, heart, liver, lungs, kidney and intestine of both immunocompromised and non immunocompromised mice treated with vehicle control, whereas the mice treated with HEOS and HEOB reveal a significant ($p<0.001$) decrease

in the number of cfu in immunocompromised and non immunocompromised mice in all organs at tested dose levels, when compared to their respective control groups. Similar findings are observed in ketoconozole treated group.

The activity values calculated from log mean cfu of control and treated group exhibit that HEOS and HEOB at 400mg/kg are active on intestine, brain, heart, lungs and kidney of non immunocompromised mice, whereas at 200mg/kg they reveal a slight activity on all other organs tested for cfu. However, immunocompromised mice, treated with HEOS and HEOB at 400mg/kg, exhibit activity on all tested organs, whereas at 200mg/kg it show a slight activity on intestine and lungs. Not much activity was observed on brain, heart, liver and kidney. However, ketoconozole treated mice show activity on all tested organs.

4.6.3. Body weight of mice treated with the extracts in non immunocompromised and immunocompromised mice

The body weights of mice of various groups were recorded on day 0, 3, 6, 9, 12 and 16. As there was mortality in different days, the data of six mice in each group were subjected to statistical analysis of repeated measures of Two way ANOVA and the results summarized as treatment versus days (% variance 15.17, F-227.23.34, DF- 50 and p <0.0001), days versus result (% variance 1.01, F-151.3, DF-5 and p<0.0001), treatment versus result (% variance 63.53, F-17.54, DF-10 and p<0.0001) and effective matching (F-271.17, DF-55 and p<0.0001) are given in Table 12.

The data given in Table 12a reveal that there is a significant (p<0.05) decrease in body weights of non immunocompromised mice on day 16. No significant changes in other drug treated groups is observed except ketoconozole treated group, which shows a significant (p<0.001) increase in body weights on day 16.

4.6.4. Total leukocyte count and differential leukocyte count of non immunocompromised and immunocompromised mice treated with the extracts

The results obtained are given in Table 13 and Figures 10-13. The data reveal a significant ($p<0.001$) decrease in total leucocytes in non immunocompromised mice treated with vehicle control. A significant ($p<0.01$) increase in monocytes and neutrophils percentage and a significant ($p<0.05$) decrease in lymphocytes percentage are observed in non immunocompromised mice. No significant changes in total leucocytes count and percentage of differential leucocytes are observed in HEOS and HEOB and ketoconozole treated mice at tested dose levels in both immunocompromised and non immunocompromised condition. Percentage of eosinophils is not affected during the experimental period in all the tested groups.

4.6.5. Histopathology of liver, kidney and intestine of mice treated with the extracts

The animals survived from different groups were euthanized at the termination of the study (day 16) and the liver, kidney and intestine were collected and fixed in buffered formalin for histopathology. The reports reveal moderate inflammation of cells with fungal hyphae in liver, kidney and intestine of both immunocompromised and non immunocompromised mice. Mild inflammation with significance of fungal hyphae is seen in HEOS and HEOB treated group at 200mg/kg. No evidence of fungal hyphae is observed in both immunocompromised and non immunocompromised mice treated with HEOS and HEOB at 400mg/kg and ketoconozole (Plates 1- 33).

Table 10: Percentage mortality of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis

Groups	No. of dead animals on corresponding day																%Mortality	MST	%T/C
	Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16			
1		0	0	0	0	0	0	1	0	1	0	1	1	0	0	0	0	40	3.3	-
2		0	0	0	0	1	2	1	0	1	2	0	1	2	0	0	0	100	9.1	-
3		0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	20	0.3	9.1
4		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Nil	Nil	Nil
5		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Nil	Nil	Nil
6		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Nil	Nil	Nil
7		0	0	0	0	1	1	1	0	0	1	0	0	0	1	0	0	50	3.7	112
8		0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	10	0.2	6.1
9		0	0	0	0	0	1	2	0	0	1	0	0	0	0	0	0	40	2.4	72.7
10		0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	10	-	-
11		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Nil	Nil	Nil

Strength of inoculam (*C. albicans*): $1x10^6$ cells/ml (48 h culture)

Route of administration of inoculam: Intravenous

Route of administration of drug: oral

n: 10 (both sex)

Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated); Group: 3- HEOS 200mg/kg (non IC); Group: 4- HEOS 400mg/kg (non IC)

Group: 5- HEOB 200mg/kg (non IC); Group: 6 – HEOB 400mg/kg (non IC); Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC)

Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC); Group: 11- Ketoconozole 60mg/kg (IC)

Table 11: Statistical analysis of Two way ANOVA applied for colony forming units (cfu) of various organs of both non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis

S.No	Parameters	Percentage variance	F Value	DF	P value
1	Treatment vs. cfu in organs	17.83	391015.34	50	<0.0001
2	Treatment vs. result	56.54	6200869.0	10	<0.0001
3	Organs vs. result	25.63	5622249.5	5	<0.0001

Table 11a: Colony forming units of various organs of both non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis

Groups	Brain	Heart	Liver	Lung	Kidney	Intestine	Log Mean						Activity (Log Control-Log Treated)					
							B	H	Li	Lu	K	I	B	H	Li	Lu	K	I
1	4.80×10^2 ± 0.16	5.10×10^2 ± 0.23	9.05×10^2 ± 0.72	3.60×10^2 ± 0.22	8.00×10^2 ± 0.35	1.40×10^2 ± 0.09	2.7	2.7	3.0	2.6	2.9	2.2	-	-	-	-	-	-
2	5.2×10^2 ± 0.12***	6.3×10^2 ± 0.51***	12.12×10^2 ± 0.82***	4.8×10^2 ± 0.35***	9.6×10^2 ± 0.25***	1.9×10^2 ± 0.12***	2.7	2.8	3.1	2.7	3.0	2.3	-	-	-	-	-	-
3	1.18×10^2 ± 0.06***	1.24×10^2 ± 0.05***	3.08×10^2 ± 0.20***	0.89×10^2 ± 0.07***	2.52×10^2 ± 0.09***	0.37×10^2 ± 0.02***	2.1	2.1	2.5	2.0	2.4	1.6	0.6	0.6	0.5	0.6	0.5	0.5
4	0.51×10^2 ± 0.06***	0.50×10^2 ± 0.05***	0.92×10^2 ± 0.04***	0.09×10^2 ± 0.02***	0.89×10^2 ± 0.04***	Nil	1.7	1.7	2.0	1.0	2.0	Nil	1.0	1.0	1.0	1.5	0.9	2.1
5	1.20×10^2 ± 0.07***	1.25×10^2 ± 0.04***	3.10×10^2 ± 0.24***	0.90×10^2 ± 0.05***	2.40×10^2 ± 0.12***	0.40×10^2 ± 0.02***	2.1	2.1	2.5	2.0	2.4	1.6	0.6	0.6	0.5	0.5	0.5	0.5

6	0.60×10^2 ± 0.06***	0.62×10^2 ± 0.06***	1.20×10^2 ± 0.03***	0.10×10^2 ± 0.01***	0.94×10^2 ± 0.03***	Nil	1.8	1.8	2.1	1.0	2.0	Nil	0.9	0.9	0.9	1.5	0.9	2.1
7	2.49×10^2 ± 0.09^c	2.80×10^2 ± 0.12^c	6.54×10^2 ± 0.42^c	1.32×10^2 ± 0.04^c	5.14×10^2 ± 0.18^c	0.62×10^2 ± 0.04^c	2.4	2.4	2.8	2.1	2.7	1.8	0.3	0.4	0.3	0.6	0.3	0.5
8	0.82×10^2 ± 0.10^c	1.09×10^2 ± 0.03^c	1.51×10^2 ± 0.04^c	0.22×10^2 ± 0.03^c	1.34×10^2 ± 0.07^c	0.08×10^2 ± 0.01^c	1.9	2.0	2.2	1.3	2.1	0.9	0.8	0.8	0.9	1.4	0.9	1.4
9	2.60×10^2 ± 0.11^c	2.95×10^2 ± 0.16^c	6.80×10^2 ± 0.40^c	1.40×10^2 ± 0.05^c	5.40×10^2 ± 0.18^c	0.70×10^2 ± 0.04^c	2.4	2.5	2.8	2.1	2.7	1.8	0.3	0.3	0.3	0.6	0.3	0.5
10	1.00×10^2 ± 0.10^c	1.40×10^2 ± 0.02^c	1.95×10^2 ± 0.04^c	0.30×10^2 ± 0.02^c	1.65×10^2 ± 0.06^c	0.12×10^2 ± 0.01^c	2.0	2.1	2.3	1.5	2.2	1.1	0.7	0.7	0.8	1.2	0.8	1.2
11	0.10×10^2 ± 0.01^c	0.40×10^2 ± 0.01^c	0.78×10^2 ± 0.03^c	0.07×10^2 ± 0.00^c	0.72×10^2 ± 0.04^c	Nil	1.0	1.6	1.9	0.8	1.8	Nil	1.7	1.2	1.2	2.0	1.2	2.3

B- Brain; H- Heart; Li- Liver; Lu- Lungs; Kid- Kidneys; Ins- intestine *contd.,*

Data expressed in Mean ± SEM: ***p<0.001 vs. Group -1; ^{c}p<0.001vs. Group – 2; Two-way RM ANOVA followed by Bonferroni post test

Results are expressed as Log control– Log treated:

Activity values, > 3.0 maximum activity; 3.0 – 1.5 active; 1.5 – 0.5 slightly active; < 0.5 not active.

Strength of inoculam (*C.albicans*): $1x10^6$ cells/ml (48 h culture)

Route of administration of inoculam: Intravenous

Route of administration of drug: Oral

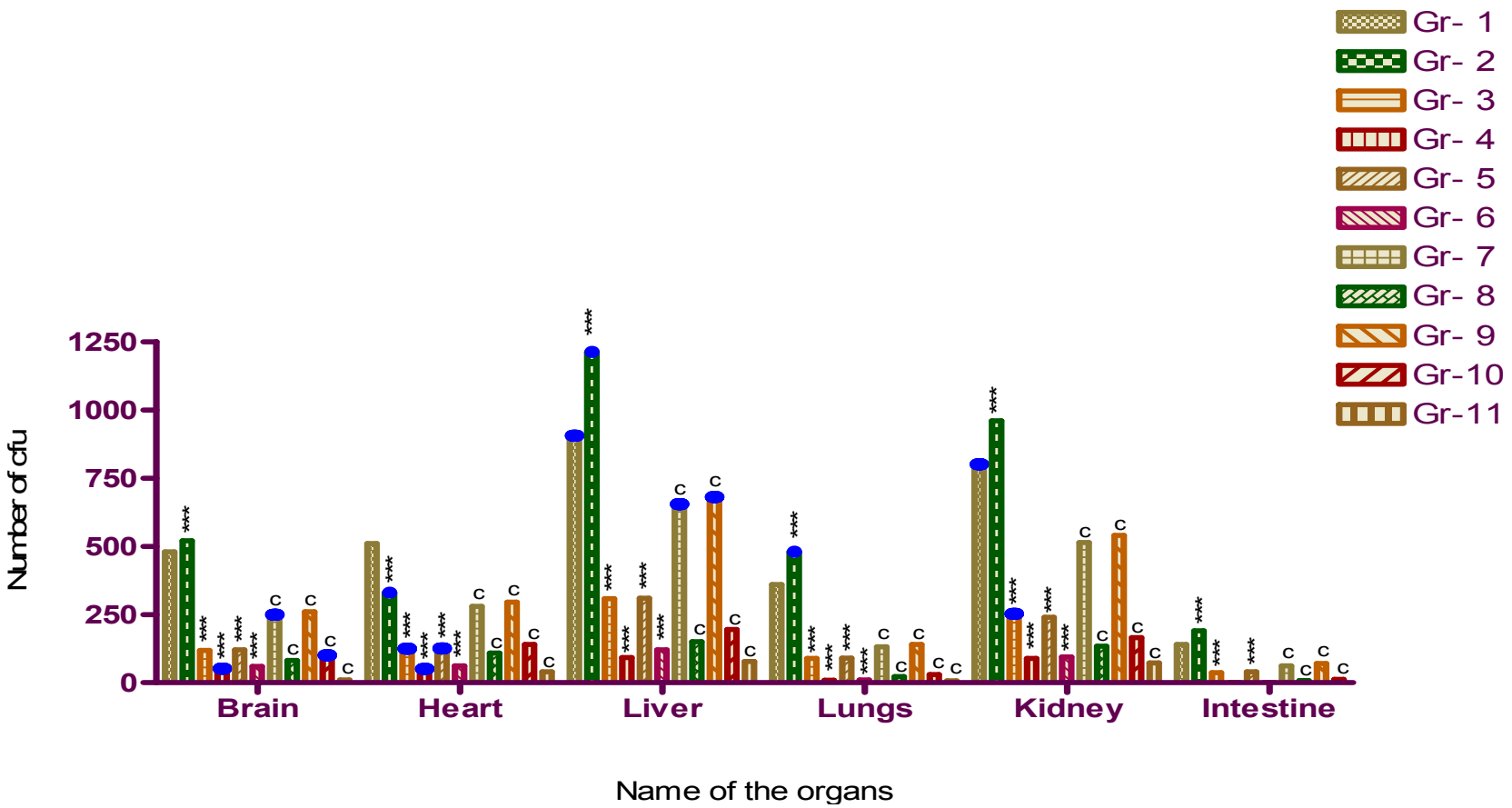
n: 6 (both sex)

Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated); Group: 3- HEOS 200mg/kg (non IC); Group: 4- HEOS 400mg/kg (non IC)

Group: 5- HEOB 200mg/kg (non IC); Group: 6 – HEOB 400mg/kg (non IC); Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC)

Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC); Group: 11- Ketoconozole 60mg/kg (IC)

Figure 9: Colony forming units of various organs of both non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis



Data expressed in Mean ± SEM: ***p<0.001 vs. Group -1; ^{c}p<0.001 vs. Group – 2; Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculam (*C.albicans*): 1x10^{6} cells/ml (48 h culture) Route of administration of inoculam: Intravenous

Route of administration of drug: Oral n: 6 (both sex)

Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated); Group: 3- HEOS 200mg/kg (non IC); Group: 4- HEOS 400mg/kg (non IC)

Group: 5- HEOB 200mg/kg (non IC); Group: 6 – HEOB 400mg/kg (non IC); Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC)

Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC); Group: 11- Ketoconozole 60mg/kg (IC)

Table 12: Statistical analysis of repeated measures of Two way ANOVA applied for body weights of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis

S.No.	Parameters	Percentage variance	F Value	DF	P value
1	Treatment vs. days	15.17	227.23	50	<0.0001
2	Days vs. result	1.01	151.30	5	<0.0001
3	Treatment vs. result	63.53	17.54	10	<0.0001
4	Effective matching	-	271.17	55	<0.0001

Table 12a: Body weights of mice treated with the extracts in experimental systemic candidiasis in non immunocompromised and immunocompromised condition

Groups	Day 0	Day 3	Day 6	Day 9	Day 12	Day 16
1	26.5± 0.7	25.9±0.7	25.8±0.6	26.0±0.7	25.1±0.6	24.6±0.6*
2	27.0±0.1	27.3±0.1	27.1±1.0	27.0±0.0	27.0±0.0	27.0±0.0
3	26.4±0.7	26.4±0.7	26.1±0.7	26.4±0.7	26.4±0.7	27.6±0.7
4	26.0±0.7	26.0±0.7	25.7±0.7	26.0±0.7	26.0±0.7	26.5±0.7
5	27.1±0.0	27.0±0.0	27.2±0.0	27.0±0.0	27.0±0.0	27.2±0.0
6	24.3±0.1	24.1±0.1	24.1±0.1	24.3±0.06	24.2±0.1	24.5±0.1
7	24.0±0.3	24.0±0.4	23.7±0.4	24.0±0. 3	24.0±0.4	24.3±0.3
8	26.0±0.2	26.0±0.2	25.7±0.2	26.0±0.2	27.0±0.7	25.7±0.2
9	30.2±0.3	30.0±0.3	30.0±0.3	30.2±0.3	30.1±0.3	30.0±0.3
10	26.7±0.3	26.9±0.3	27.5±0.3	27.0±0.3	27.3±0.5	31.0±0.2***

Data expressed as Mean ±SEM: *p<0.05, ***p<0.001 vs. 0 day of respective groups; n: 6 (both sex); Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculam (*C.albicans*): $1x10^6$ cells/ml (48 h culture); Route of administration of inoculam: Intravenous; Route of administration of drug: Oral

Group: 1-Vehicle control (non IC); Group: 2- HEOS 200mg/kg (non IC); Group: 3- HEOS 400mg/kg (non IC)

Group: 4- HEOB 200mg/kg (non IC); Group: 5 – HEOB 400mg/kg (non IC); Group: 6- HEOS 200mg/kg (IC)

Group: 7- HEOS 400mg/kg (IC); Group: 8- HEOB 200mg/kg (IC); Group: 9 – HEOB 400mg/kg (IC)

Group: 10- Ketoconozole 60mg/kg (IC)

Table 13: Total and differential leukocyte count of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis

Groups	TLC (mm^3)		Neutrophils (%)		Lymphocytes (%)		Eosinophils (%)		Monocytes (%)	
	Day 0	Day 16	Day 0	Day 16	Day 0	Day 16	Day 0	Day 16	Day 0	Day 16
1	6330±136.9	5100±197.7***	24.0±1.4	29.0±3.2**	72.0±1.9	65.0±1.1*	1.0±0.5	2.0±0.6	3.0±0.7	5.8±0.5**
2	6200±107.8	6266±79.4	24.0±1.2	24.0±0.8	72.0±3.2	71.0±2.9	1.7±0.5	2.0±0.4	2.7±0.7	3.0±0.4
3	6800±10.0	6909±10.9	23.5±1.6	22.8±1.8	70.5±3.6	70.3±2.5	2.0±0.4	2.0±0.4	4.0±0.4	4.0±0.5
4	6400±32.2	6475±14.5	25.0±1.3	25.8±1.9	71.0±1.2	69.0±1.5	1.0±0.5	2.0±0.5	3.0±0.5	3.0±0.4
5	6650±43.2	6800±60.8	23.5±1.7	22.8±1.7	70.0±2.8	71.0±3.5	2.3±0.4	2.7±0.7	4.0±0.5	3.3±0.5
6	6400±4.1	6455±9.6	27.0±1.8	26.0±2.3	68.0±1.5	69.0±2.9	1.3±0.5	1.8±0.4	3.5±1.0	3.3±1.1
7	6638±33.2	6772±54.7	2.1±1.9	23.3±0.8	73.0±3.2	71.0±2.9	1.8±0.5	2.2±0.5	3.2±0.6	3.7±0.4
8	6399±3.5	6460±15.3	28.0±2.5	26.5±2.0	67.0±3.5	68.5±2.8	2.0±0.6	2.0±0.4	3.0±0.8	3.0±0.4
9	6203±18.0	6295±19.8	29.0±1.7	29.0±1.0	66.5±2.1	65.0±1.5	1.5±0.2	2.0±0.4	3.0±0.7	3.5±0.9
10	6090±44.3	6166±49.0	24.0±1.6	23.0±1.5	70.0±2.2	69.0±2.5	2.0±0.4	2.0±0.4	4.0±0.9	4.0±0.5

Data expressed as Mean ±SEM: *p<0.05, **p<0.01, ***p<0.001 vs. 0 day of respective groups; n: 6 (both sex); Two way ANOVA followed by Bonferroni post test

Strength of inoculam (*C.albicans*): $1x10^6$ cells/ml (48 h culture); Route of administration of inoculam: Intravenous; Route of administration of drug: Oral

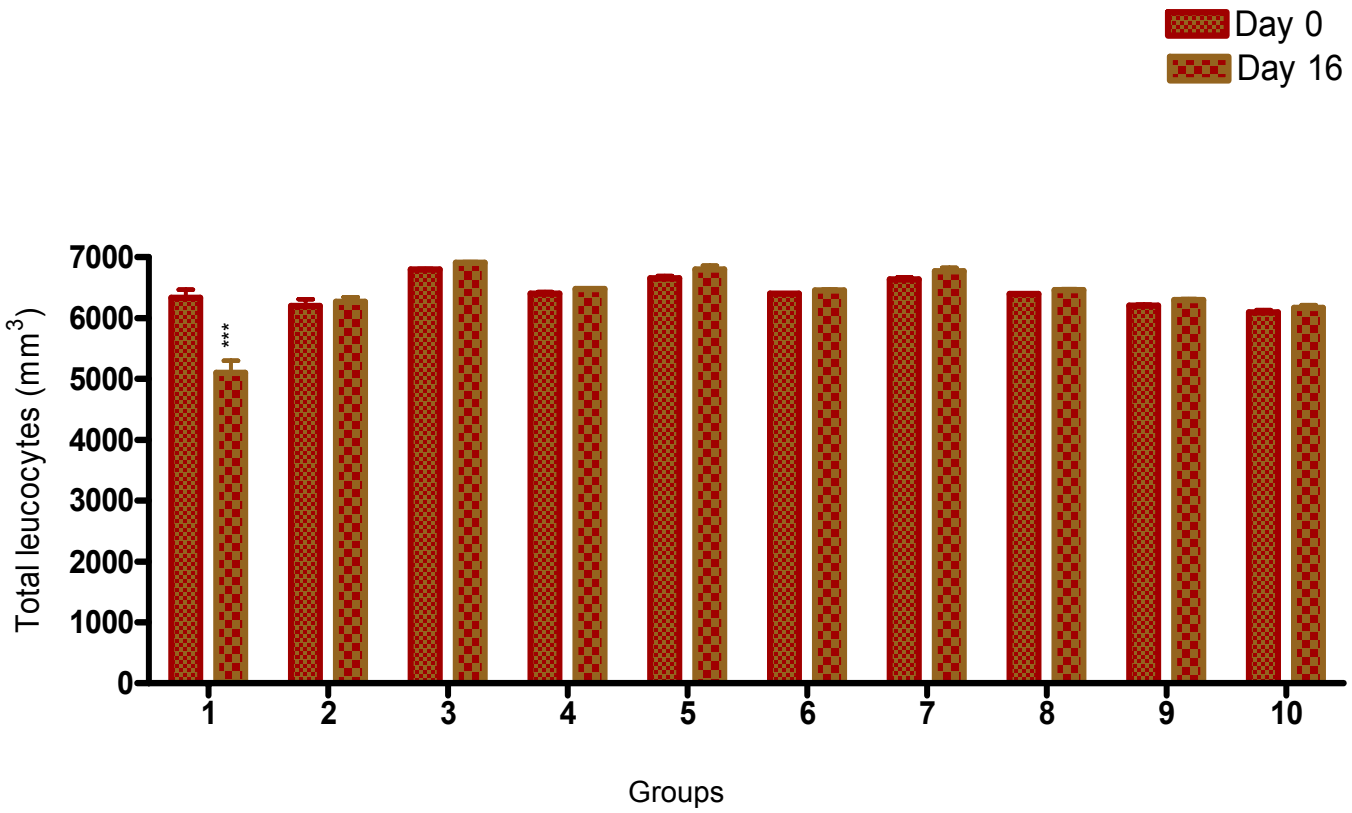
Group: 1-Vehicle control (non IC); Group: 2- HEOS 200mg/kg (non IC); Group: 3- HEOS 400mg/kg (non IC)

Group: 4- HEOB 200mg/kg (non IC); Group: 5 – HEOB 400mg/kg (non IC); Group: 6- HEOS 200mg/kg (IC)

Group: 7- HEOS 400mg/kg (IC); Group: 8- HEOB 200mg/kg (IC); Group: 9 – HEOB 400mg/kg (IC)

Group: 10- Ketoconozole 60mg/kg (IC)

Figure 10: Total leucocytes count of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis



Data expressed as Mean ±SEM: ***p<0.001 vs. 0 day of respective groups; n: 6; Strength of inoculam (*C.albicans*): $1x10^6$ cells/ml (48 h culture); Two-way RM ANOVA followed by Bonferroni post test

Route of administration of inoculam: Intravenous; Route of administration of drug: Oral

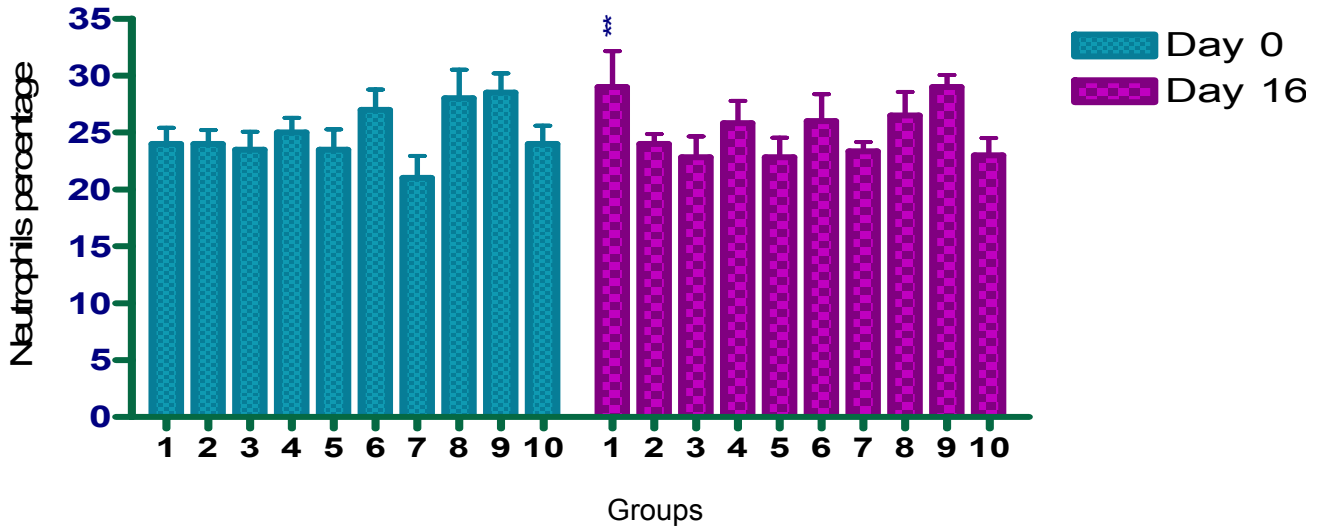
Group: 1-Vehicle control (non IC); Group: 2- HEOS 200mg/kg (non IC); Group: 3- HEOS 400mg/kg (non IC)

Group: 4- HEOB 200mg/kg (non IC); Group: 5 – HEOB 400mg/kg (non IC); Group: 6- HEOS 200mg/kg (IC)

Group: 7- HEOS 400mg/kg (IC); Group: 8- HEOB 200mg/kg (IC); Group: 9 – HEOB 400mg/kg (IC)

Group: 10- Ketoconozole 60mg/kg (IC)

Figure 11: Percentage neutrophils of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis



Data expressed as Mean ±SEM: **p<0.01 vs. 0 day of respective groups; n-6; Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculam (*C.albicans*): $1x10^6$ cells/ml (48 h culture); Route of administration of inoculam: Intravenous

Route of administration of drug: Oral

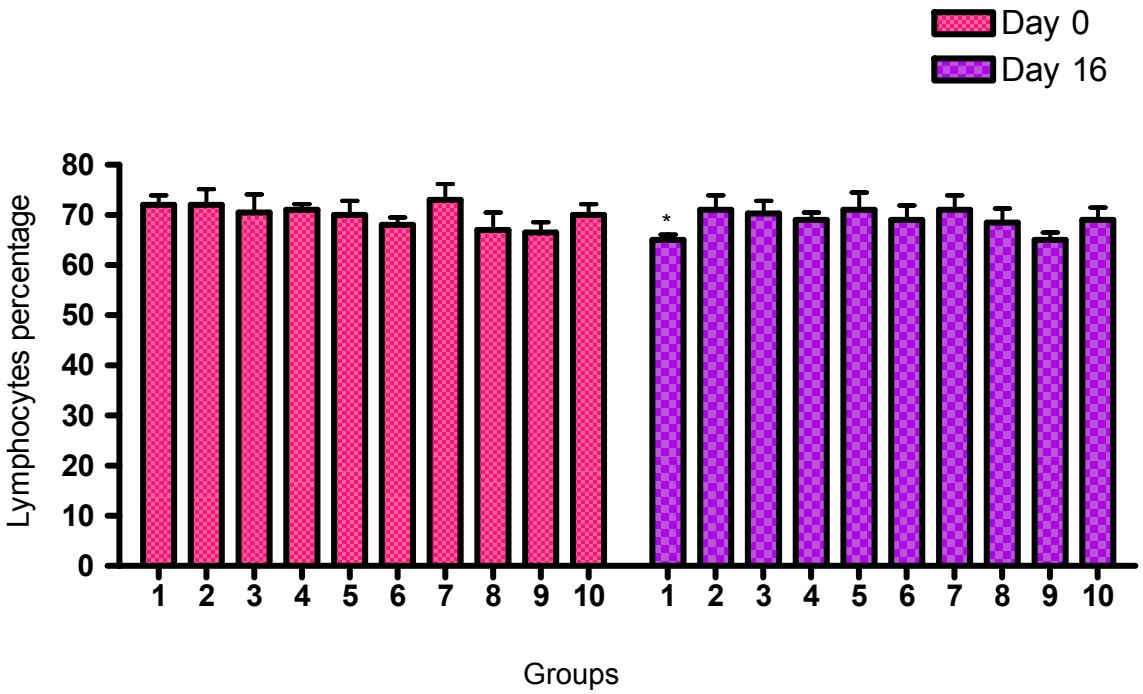
Group: 1-Vehicle control (non IC); Group: 2- HEOS 200mg/kg (non IC); Group: 3- HEOS 400mg/kg (non IC)

Group: 4- HEOB 200mg/kg (non IC); Group: 5 – HEOB 400mg/kg (non IC); Group: 6- HEOS 200mg/kg (IC)

Group: 7- HEOS 400mg/kg (IC); Group: 8- HEOB 200mg/kg (IC); Group: 9 – HEOB 400mg/kg (IC)

Group: 10- Ketoconozole 60mg/kg (IC)

Figure 12: Percentage lymphocytes of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis



Data expressed as Mean ±SEM: *p<0.05 vs. 0 day of respective groups; n-6; Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculam (*C.albicans*): $1x10^6$ cells/ml (48 h culture); Route of administration of inoculam: Intravenous

Route of administration of drug: Oral

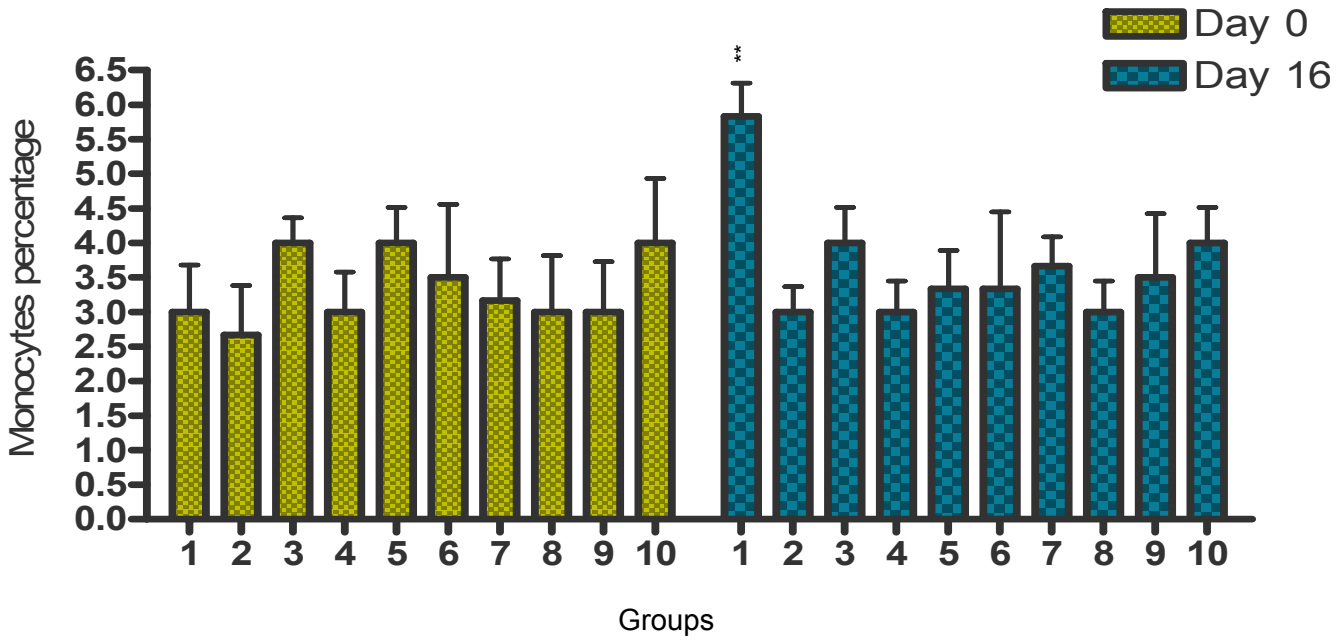
Group: 1-Vehicle control (non IC); Group: 2- HEOS 200mg/kg (non IC); Group: 3- HEOS 400mg/kg (non IC)

Group: 4- HEOB 200mg/kg (non IC); Group: 5 – HEOB 400mg/kg (non IC); Group: 6- HEOS 200mg/kg (IC)

Group: 7- HEOS 400mg/kg (IC); Group: 8- HEOB 200mg/kg (IC); Group: 9 – HEOB 400mg/kg (IC)

Group: 10- Ketoconozole 60mg/kg (IC)

Figure 13: Percentage monocytes of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis



Data expressed as Mean ±SEM: **p<0.01 vs. 0 day of respective groups; n-6; Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculam (*C.albicans*): $1x10^6$ cells/ml (48 h culture); Route of administration of inoculam: Intravenous

Route of administration of drug: Oral

Group: 1-Vehicle control (non IC); Group: 2- HEOS 200mg/kg (non IC); Group: 3- HEOS 400mg/kg (non IC)

Group: 4- HEOB 200mg/kg (non IC); Group: 5 – HEOB 400mg/kg (non IC); Group: 6- HEOS 200mg/kg (IC)

Group: 7- HEOS 400mg/kg (IC); Group: 8- HEOB 200mg/kg (IC); Group: 9 – HEOB 400mg/kg (IC)

Group: 10- Ketoconozole 60mg/kg (IC)

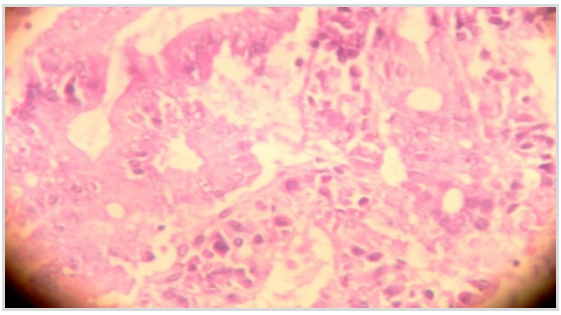


Plate 1.Histology of non IC mice intestine treated with solvent control in experimental systemic candidiasis shows fungal hyphae with moderate inflammation (PAS 40 x)

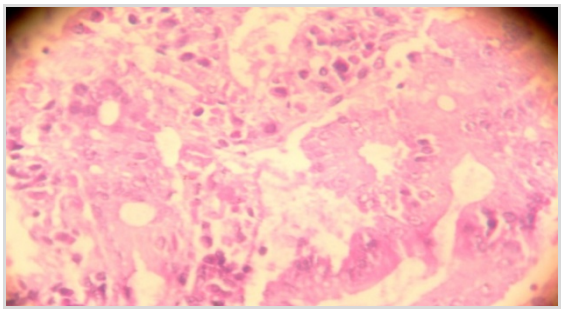


Plate 2.Histology of IC mice intestine treated with solvent control in experimental systemic candidiasis shows fungal hyphae with inflammation (PAS 40 x)

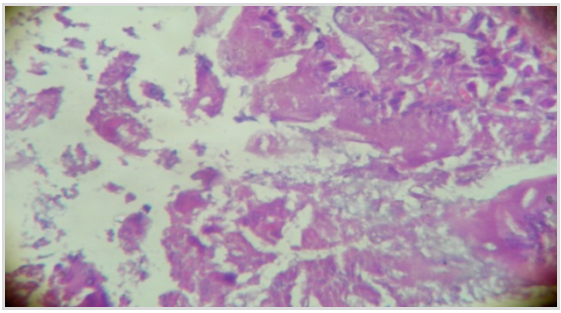


Plate 3. Histology of non IC mice intestine treated with HEOS (200mg/kg) in experimental systemic candidiasis shows no fungal hyphae with mild inflammation (PAS 40 x)

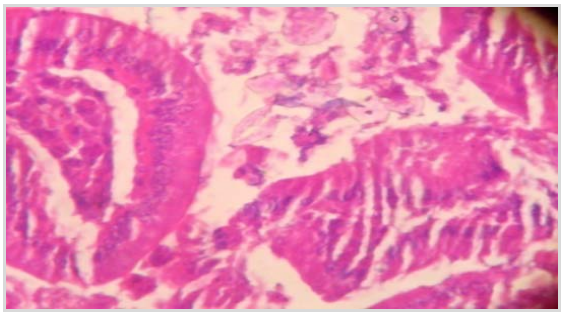


Plate 4. Histology of non IC mice intestine treated with HEOS (400mg/kg) in experimental systemic candidiasis shows normal (PAS 40x)

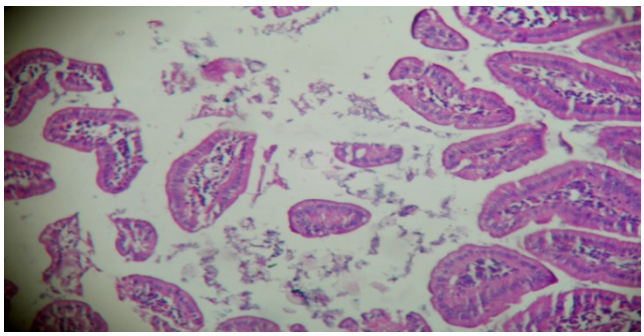


Plate 5. Histology of non IC mice intestine treated with HEOB (200mg/kg) in experimental systemic candidiasis shows no fungal hyphae with mild inflammation (PAS 40 x)

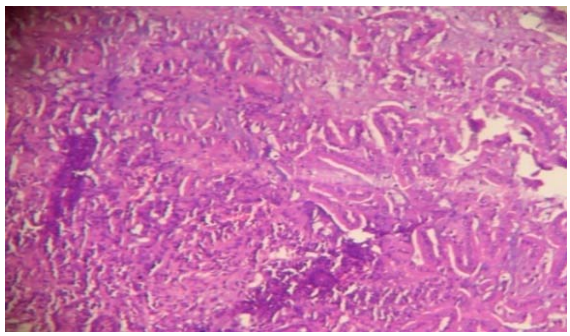


Plate 6. Histology of non IC mice intestine treated with HEOB (400mg/kg) in experimental systemic candidiasis shows normal (PAS 40x)

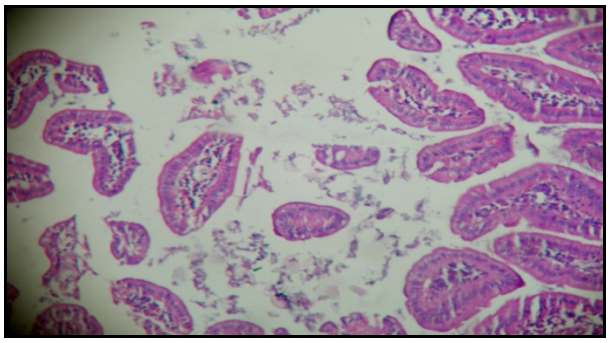


Plate 7. Histology of IC mice intestine treated with HEOS (200mg/kg) in experimental systemic candidiasis shows fungal hyphae with mild inflammation (PAS 40 x)

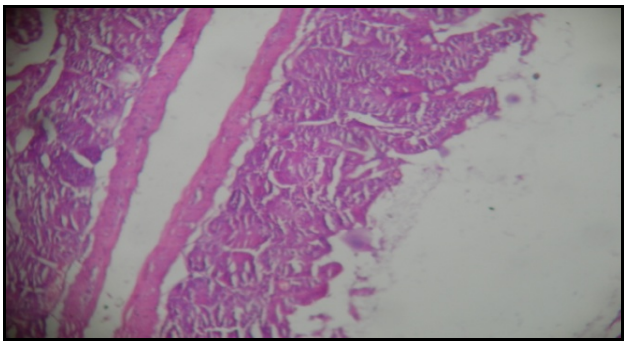


Plate 8. Histology of IC mice intestine treated with HEOS (400mg/kg) in experimental systemic candidiasis shows no fungal hyphae with mild inflammation (PAS 40 x)

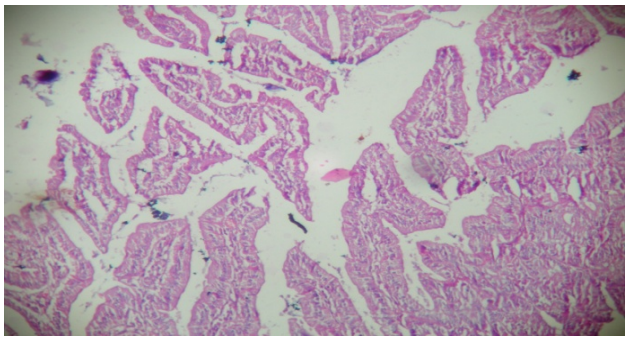


Plate 9. Histology of IC mice intestine treated with HEOB (200mg/kg) in experimental systemic candidiasis shows fungal hyphae with moderate inflammation (PAS 40 x)

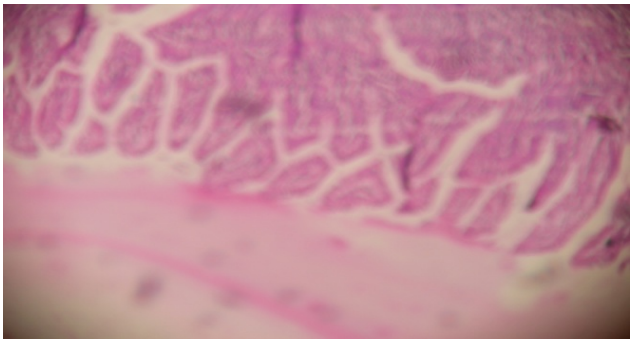


Plate 10. Histology of IC mice intestine treated with HEOB (400mg/kg) in experimental systemic candidiasis shows no fungal hyphae with mild inflammation (PAS 40 x)

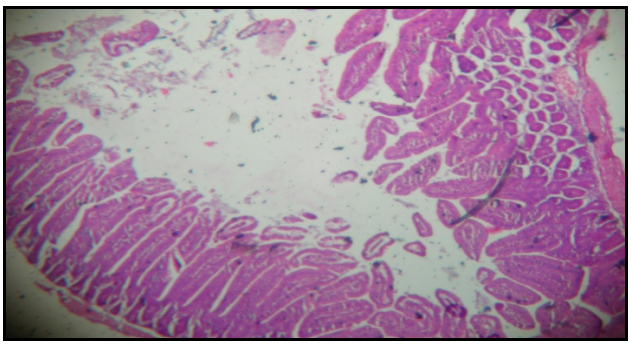


Plate 11. Histology of IC mice intestine treated with ketoconozole (60mg/kg) in experimental systemic candidiasis shows normal (PAS 40 x)

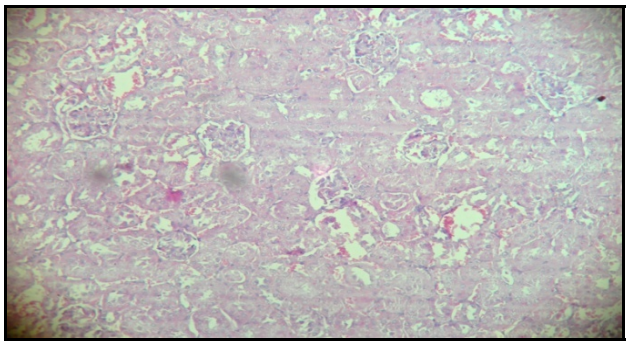


Plate12.Histology of non IC mice kidney treated with solvent control in experimental systemic candidiasis shows fungal hyphae with moderate inflammation (PAS 40 x)

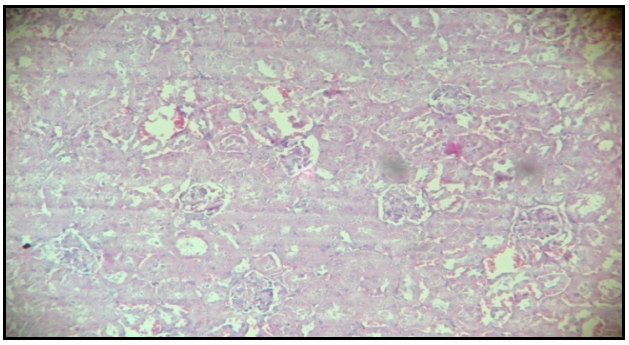


Plate13.Histology of IC mice kidney treated with solvent control in experimental systemic candidiasis shows fungal hyphae with inflammation (PAS 40 x

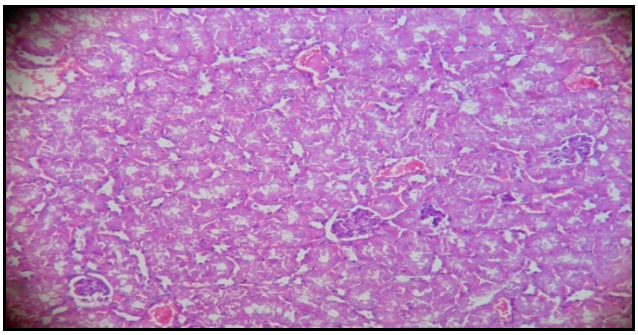


Plate 14. Histology of non IC mice kidney treated with HEOS (200mg/kg) in experimental systemic candidiasis shows no fungal hyphae with mild inflammation (PAS 40 x)

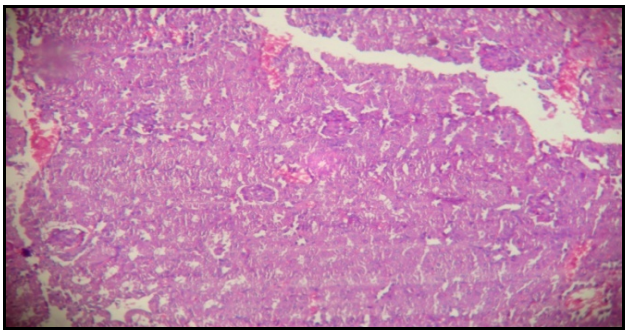


Plate 15. Histology of non IC mice kidney treated with HEOS (400mg/kg) in experimental systemic candidiasis shows normal (PAS 40 x)

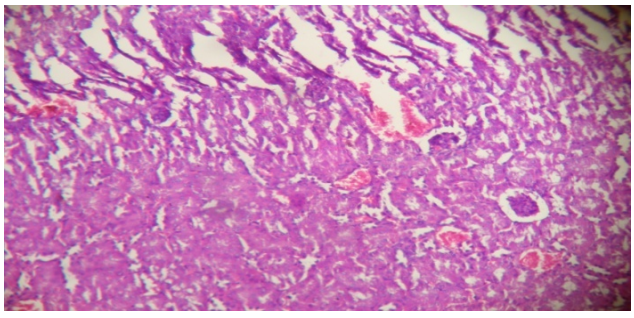


Plate 16. Histology of non IC mice kidney treated with HEOB (200mg/kg) in experimental systemic candidiasis shows no fungal hyphae with focal inflammation (PAS 40 x)

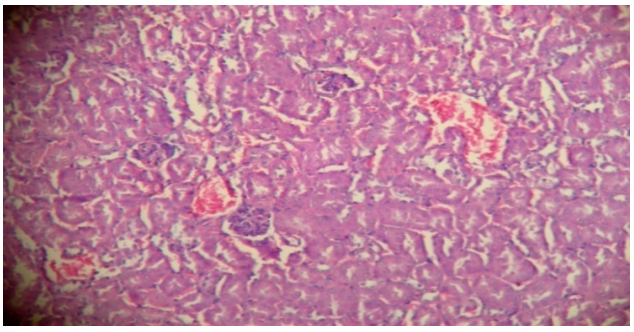


Plate 17. Histology of non IC mice kidney treated with HEOB (400mg/kg) in experimental systemic candidiasis shows normal (PAS 40 x)

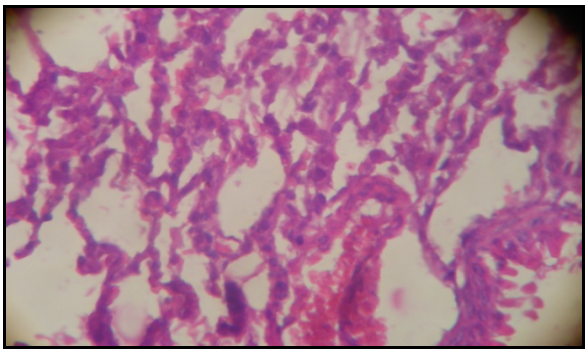


Plate 18. Histology of IC mice kidney treated with HEOS (200mg/kg) in experimental systemic candidiasis shows fungal hyphae with mild inflammation (PAS 40 x)

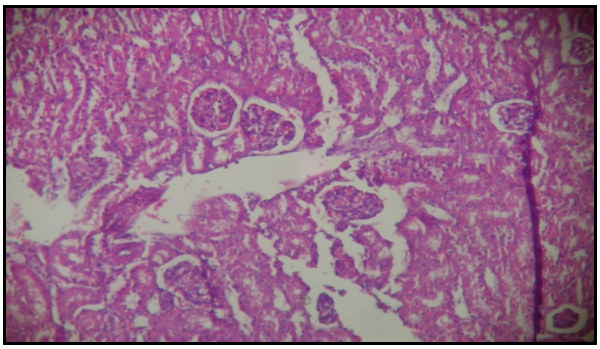


Plate 19. Histology of IC mice kidney treated with HEOS (400mg/kg) in experimental systemic candidiasis shows normal (PAS 40 x)

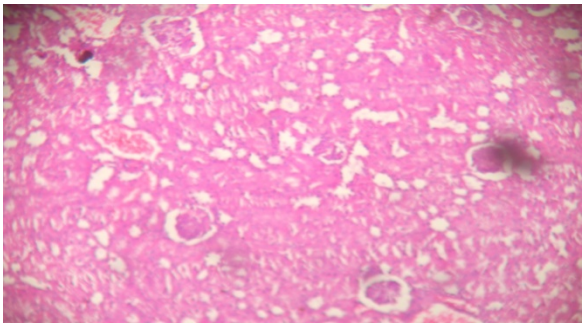


Plate 20. Histology of IC mice kidney treated with HEOB (200mg/kg) in experimental systemic candidiasis shows fungal hyphae with mild inflammation (PAS 40 x)

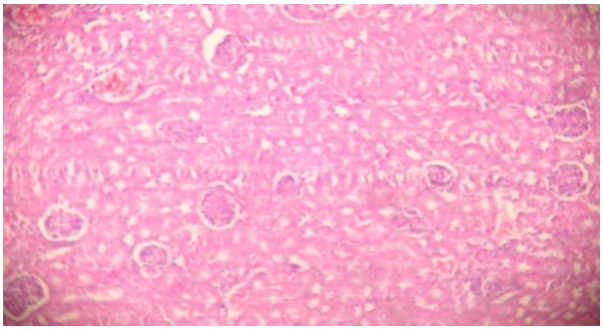


Plate 21. Histology of IC mice kidney treated with HEOB (400mg/kg) in experimental systemic candidiasis shows no fungal hyphae with mild inflammation (PAS 40 x)

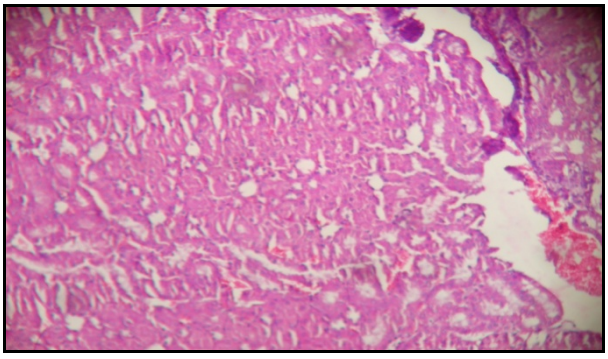


Plate 22. Histology of IC mice kidney treated with ketoconozole (60mg/kg) in experimental systemic candidiasis shows normal (PAS 40 x)

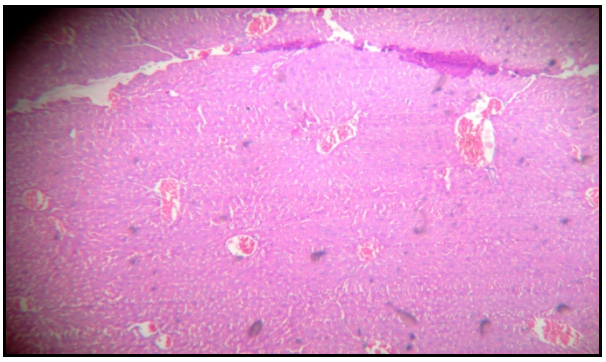


Plate 23.Histology of non IC mice liver treated with solvent control in experimental systemic candidiasis shows fungal hyphae with moderate inflammation (PAS 40 x)

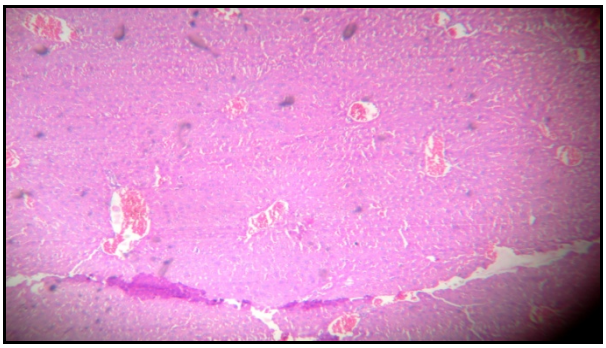


Plate 24.Histology of IC mice liver treated with solvent control in experimental systemic candidiasis shows fungal hyphae with moderate inflammation (PAS 40 x)

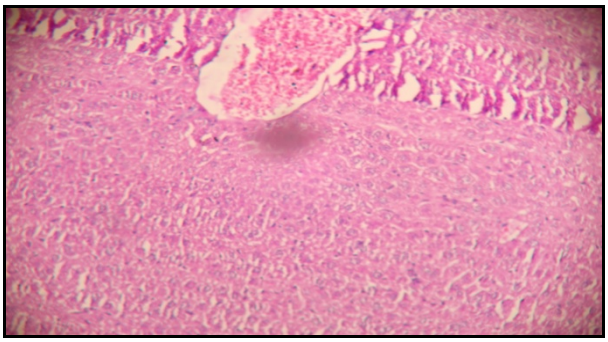


Plate 25. Histology of non IC mice liver treated with HEOS (200mg/kg) in experimental systemic candidiasis shows no fungal hyphae with mild inflammation (PAS 40 x)

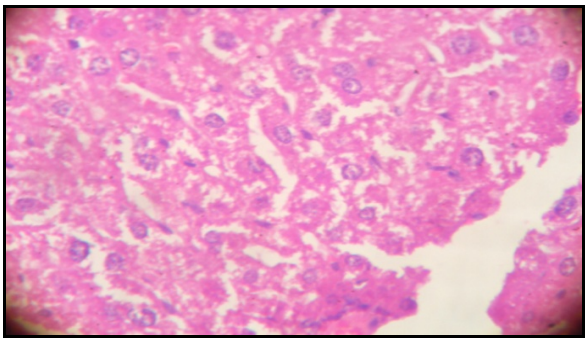


Plate 26. Histology of non IC mice liver treated with HEOS (400mg/kg) in experimental systemic candidiasis shows normal (PAS 40 x)

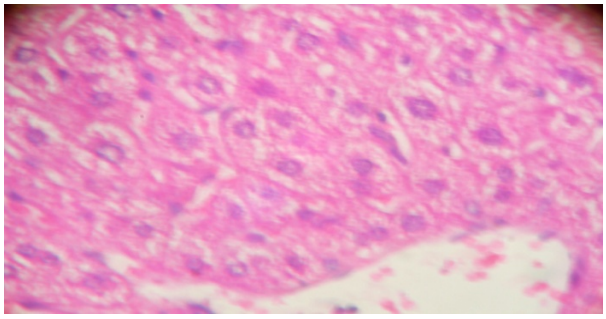


Plate 27. Histology of non IC mice liver treated with HEOB (200mg/kg) in experimental systemic candidiasis shows no fungal hyphae with moderatel inflammation (PAS 40 x)

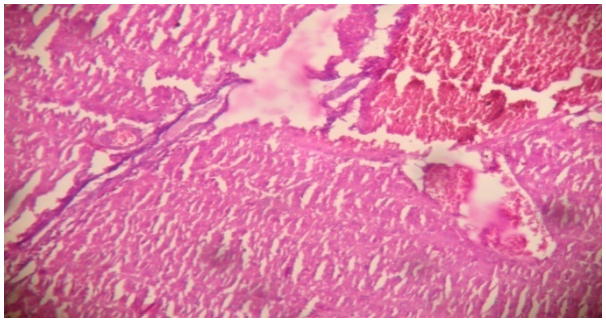


Plate 28. Histology of non IC mice liver treated with HEOB (400mg/kg) in experimental systemic candidiasis shows no fungal hyphae with mild inflammation (PAS 40 x)

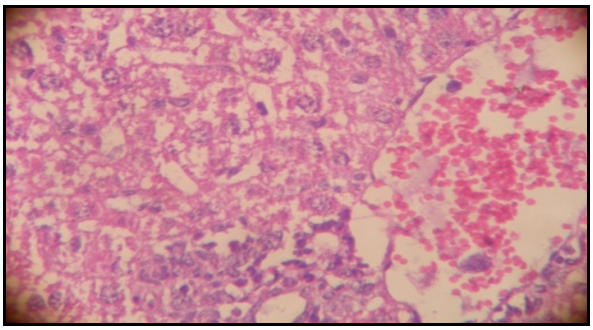


Plate 29. Histology of IC mice liver treated with HEOS (200mg/kg) in experimental systemic candidiasis shows fungal hyphae with mild inflammation (PAS 40 x)

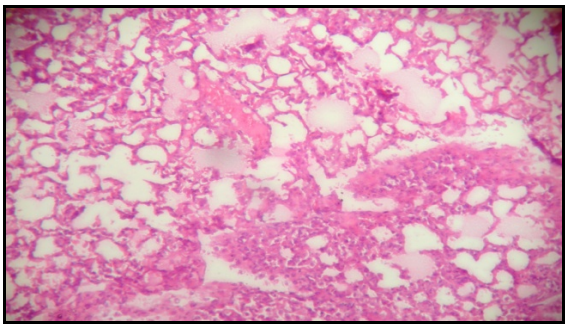


Plate 30. Histology of IC mice liver treated with HEOS (400mg/kg) in experimental systemic candidiasis shows normal (PAS 40 x)

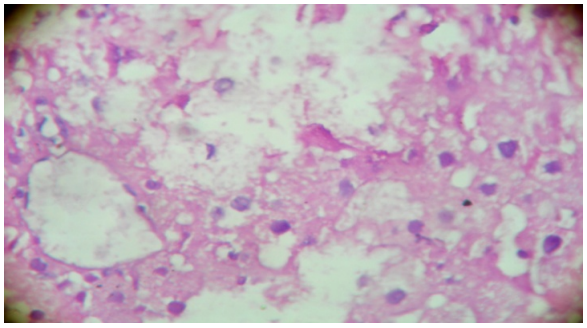


Plate 31. Histology of IC mice liver treated with HEOB (200mg/kg) in experimental systemic candidiasis shows fungal hyphae with mild inflammation (PAS 40 x)

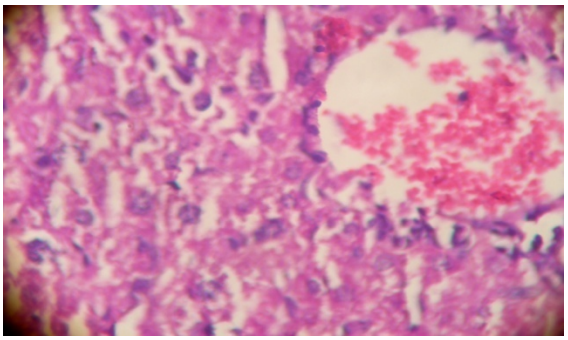


Plate 32. Histology of IC mice liver treated with HEOB (400mg/kg) in experimental systemic candidiasis shows normal (PAS 40 x)

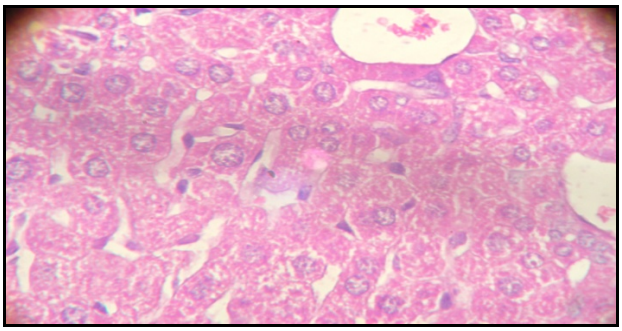


Plate 33. Histology of IC mice liver treated with ketoconozole (60mg/kg) in experimental systemic candidiasis shows normal (PAS 40 x)

4.7. Effect of the extracts against experimental systemic candidiasis in non immunocompromised and immunocompromised mice under gastrointestinal colonization

4.7.1. Percentage mortality of non immunocompromised and Immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization

Immunosuppression and gut colonization are well known predisposing factors for candidiasis. Mice were inoculated with *C.albicans* through intragastric route, immunosuppressive agents and antibiotics were administered orally as described in experimental design.

The results obtained on the effect of the extracts on percentage mortality of both immunocompromised and non immunocompromised mice systemically inoculated with *C.albicans* under gut colonization are given in Table 14. The results reveal that 40% mortality in vehicle control treated immunocompromised and non immunocompromised mice and 20% mortality in non immunocompromised mice treated with 200mg/kg of the extracts, whereas 20% morality was found in immunocompromised mice treated with 200 and 400 mg/kg of the extracts. There was no mortality in non immunocompromised group treated with 400mg/kg of the extracts and ketoconozole, since there was mortality in all groups, data of six mice from each group were taken for the further observations.

4.7.2. Colony forming units (cfu) in fecal samples of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization

The data of six mice from each group were taken for observations. The fecal samples were collected on day 14 and 24 and analyzed for cfu. The data obtained were subjected for repeated measures of Two way ANOVA and summarized as days versus treatment (% variance 5.83, F-37.42, DF- 10 and $p < 0.0001$), treatment versus result (% variance 90.38, F-575.15, DF-10 and $p<0.0001$), days versus result (% variance 2.41, F-154.48, DF-1 and $p<0.0001$) and effective matching (F-1.01, DF-44 and $p<0.4892$) are given in Table 15,15a and Figure 14. The results reveal a significant ($p<0.001$)

increase in the number of cfu in fecal samples of both immunocompromised and non immunocompromised mice treated with vehicle control, on day 14 and 24. On day 14, the non immunocompromised mice treated with the extracts shows a significant ($p<0.001$) decrease in the number of cfu in fecal samples at tested dose levels. Similarly a significant ($p<0.05$) decrease in the number of cfu is seen in immunocompromised mice treated with HEOB (200 and 400 mg/kg) and a significant ($p<0.001$) decrease in the number of cfu treated with (HEOS 400mg/kg) and ketoconozole, when compared to their respective control groups.

On day 24 there is a further increase in the number of cfu in fecal samples of vehicle control treated in both immunocompromised and non immunocompromised mice, whereas a significant ($p<0.001$) decrease in the number of cfu is found in the extracts and ketoconozole treated groups in both immunocompromised and non immunocompromised mice at the tested dose levels.

4.7.3. Colony forming units of various organs of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization

Since both the extracts showed significant anticandidal activity on intestine, lungs, kidney and liver in systemic candidiasis model, these four organs of six mice were collected for observations in gut colonization model. Homogenates of intestine, lungs, liver and the kidney showed extensive colonization by *C.albicans* in gastrointestinal tract. The data given in Table 16 and Figure 15 reveal that intestine, kidney, liver and lungs exhibit a significant ($p<0.001$) number of cfu in both immunocompromised and non immunocompromised mice, whereas, the extracts and ketoconozole treated mice show a significant ($p<0.001$) decrease in the number of cfu in intestine, kidney, liver and lungs at the doses tested.

4.7.4. Body weights of mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization in non immunocompromised and immunocompromised condition

Body weights of mice were recorded on day 0, 6, 9, 12, 18 and 24. The data of six mice subjected for statistical analysis of repeated measures of Two way ANOVA and the results summarized as treatment versus days (% variance 11.23, F-27.73, DF- 50 and p <0.0001), days versus result (% variance 4.01, F-99.05, DF-5 and p<0.0001), treatment versus result (% variance 51.60, F-7.24, DF-10 and p<0.0001) and effective matching (F-88.03, DF-44 and p<0.0001) are given in Table 17.

Table 17a shows the effect of the extracts on body weights of mice in experimental systemic candidiasis under gut colonization. A significant (p<0.001) decrease in body weights are seen in both immunocompromised and non immunocompromised mice on day 6 onwards. As the study progressed a further decrease (p<0.001) in body weights are seen in both immunocompromised and non immunocompromised mice treated with vehicle control. No significant change in body weights of mice are seen in the extracrs and ketoconozole treated mice at tested dose levels during the experimental period.

4.7.5. Total and differential leucocytes count of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization

The effect of the extracts was studied on the total and differential leucocytes count of normal and immunocompromised mice in experimental systemic candidiasis under gastrointestinal colonization. The results obtained are given in Table 18 and Figures 16-19. The results reveal a significant (p<0.001) decrease in total leucocytes and lymphocytes percentage and a significant (p<0.001) increase in neutrophils and monocytes percentage in immunocompromised and non immunocompromised mice treated with vehicle control. A significant (p<0.001 and p<0.01) increase in total leucocytes is observed in both immunocompromised and non immunocompromised mice treated with the extracts, and ketoconozole at tested doses, when compared with 0 day of their respective groups. HEOB (400mg/kg) treated immunocompromised mice

shows a significant ($p<0.001$) increase in neutrophils percentage and a significant ($p<0.001$) decrease in lymphocytes percentage when compared with 0 day.

4.7.6. Effect of the extracts on TNF-α and IL-1α in non immunocompromised and immunocompromised mice against experimental systemic candidiasis under gastrointestinal colonization

The effect of the extracts on TNF-α and IL-1α in non immunocompromised and immunocompromised mice against experimental systemic candidiasis under gastrointestinal colonization are given in Table 19 and Figures 20 and 21 reveal a significant ($p<0.001$) increase in the levels of both TNF-α and IL-1α in the extracts, and ketoconozole treated mice at doses tested, when compared to the normal.

4.7.7. Histopathology of intestine, liver and kidney of mice treated with the extracts in non immunocompromised and immunocompromised mice against experimental systemic candidiasis under gastrointestinal colonization

Histology of intestine reveal moderate to dense inflammation including lymphoid follicles of sub mucosa and congestion with fungal hyphae in both immunocompromised and non immunocompromised mice treated with vehicle control. Mild inflammation in lumen with fungal hyphae is seen in non immunocompromised mice treated with HEOB 200mg/kg and normal appearance in HEOS 200mg/kg treated mice. Similar observations are seen in immunocompromised mice treated with the extracts (200mg/kg). However, no histological changes are observed in both non immunocompromised and immunocompromised mice treated with 400mg/kg of the extracts and ketoconozole (60mg/kg) (Plates 34-44).

Histology of non immunocompromised mice kidney treated with vehicle control reveal focal congestion, mild inflammation with fungal hyphae, whereas, immunocompromised mice treated with vehicle control show tubules with inflammation and fungal hyphae. The extracts (200mg/kg) treated non immunocompromised mice reveal mild inflammation with fungal hyphae. However, 400mg/kg of the extracts and ketoconozole (60mg/kg) treated groups show normal appearance (Plates 45-55).

Histology of non immunocompromised and immunocompromised mice liver treated with vehicle control revealed dilated sinusoids and central vein, focal necrosis with fungal hyphae. The extracts (200mg/kg) treated non immunocompromised mice shows congestion of central vein with absence of fungal hyphae, whereas, the extracts (400mg/kg) and ketoconozole (60mg/kg) treated non immunocompromised and immunocompromised mice show normal appearance (Plates 56-66).

Table 14: Percentage mortality of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization

Groups	Days	Number of animals dead on corresponding days																							% Mortality	MST	% T/C
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24			
1		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	40	9.2	-
2		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	40	8.0	86.9
3		0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	1.8	20.4
4		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Nil	-	-
5		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	20	3.6	39.1
6		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Nil	-	-
7		0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	1.2	13.6
8		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	20	3.6	40.9
9		0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	20	2.4	26.0
10		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	20	3.6	39.1
11		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Nil	-	-

Strength of inoculum (*C. albicans*):1x10^7 cells/ml (48 h culture) Route of administration of inoculum: intra gastric

Route of administration of drug: oral n: 10 (both sex)

Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated); Group: 3- HEOS 200mg/kg (non IC); Group: 4- HEOS 400mg/kg (non IC)

Group: 5- HEOB 200mg/kg (non IC); Group: 6 – HEOB 400mg/kg (non IC); Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC)

Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC); Group: 11- Ketoconozole 60mg/kg (IC)

Table 15: Statistical analysis of repeated measures Two way ANOVA applied for colony forming units (cfu) in fecal samples of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization

S. No	Parameters	Percentage Variance	F Value	DF	P value
1	Days vs. treatment	5.83	37.42	10	<0.0001
2	Treatment vs. result	90.38	575.15	10	<0.0001
3	Days vs. result	2.41	154.48	1	<0.0001
4	Effective matching	_	1.01	44	0.4892

Table 15a: Colony forming units in fecal samples of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization

Groups	Day 14	Day 24
1	$1.84x10^3$±24.5	$2.22x10^3$±37.4
2	$3.04x10^3$±40.0***	$3.6x10^3$±31.6***
3	$1.58x10^3$±58.3***	$1.26x10^3$±67.8***
4	$1.38x10^3$±37.4***	$9.40x10^2$±40.0***
5	$1.62x10^3$±58.3***	$1.32x10^3$±58.3***
6	$1.44x10^3$±24.5***	$9.80x10^2$±37.4***
7	$2.88x10^3$±80.0[a]	$2.68x10^3$±58.3[c]
8	$2.78x10^3$±37.4[c]	$2.38x10^3$±37.4[c]
9	$2.98x10^3$±86.0[a]	$2.78x10^3$±20.0[c]
10	$2.88x10^3$±37.4[a]	$2.44x10^3$±74.5[c]
11	$1.68x10^3$±37.4[c]	$7.6x10^2$±24.5[c]

Data expressed as mean ± S.E.M: ***p< 0.001 vs. Group-1: [a] p<0.05, [c]p< 0.001 vs. Group-2; Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculum (*C.albicans*): $1x0^7$ cells/ml (48 h culture); Route of administration of inoculum: intragastric; Route of administration of drug: Oral; n: 6 (both sex)

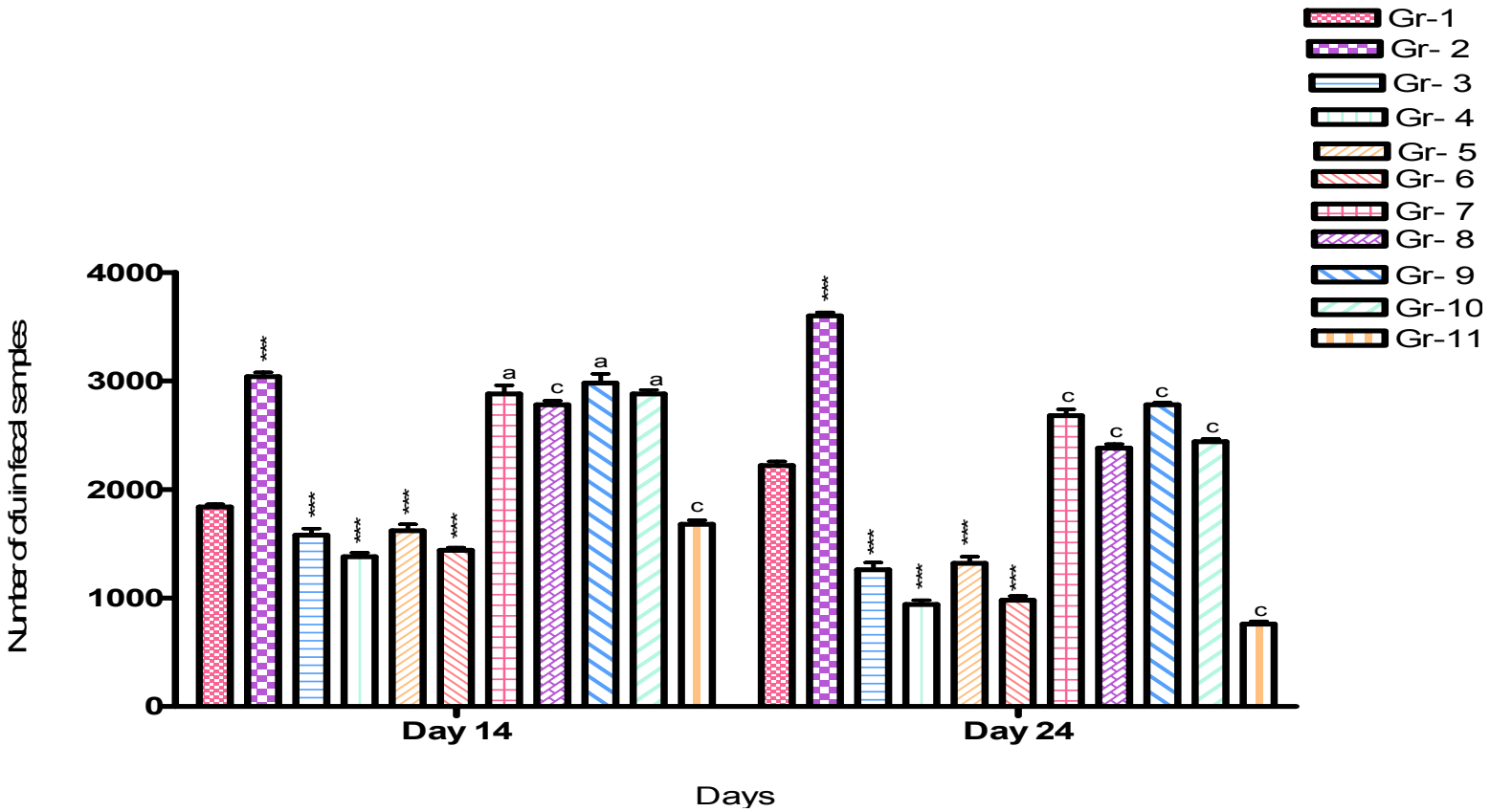
Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated); Group: 3- HEOS 200mg/kg (non IC)

Group: 4- HEOS 400mg/kg (non IC); Group: 5- HEOB 200mg/kg (non IC); Group: 6 – HEOB 400mg/kg (non IC)

Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC); Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC)

Group: 11- Ketoconozole 60mg/kg (IC)

Figure 14: Colony forming units in fecal samples of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization



Data expressed as mean ± S.E.M:*** p< 0.001 vs. Group-1; [a] p<0.05, [c] p< 0.001 vs. Group-2; Two-way RM ANOVA followed by Bonferroni post test; Strength of inoculum (*C.albicans*): $1x0^7$ cells/ml (48 h culture)

Route of administration of inoculum: intragastric; Route of administration of drug: Oral: n: 6 (both sex)

Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated); Group: 3- HEOS 200mg/kg (non IC)

Group: 4- HEOS 400mg/kg (non IC); Group: 5- HEOB 200mg/kg (non IC); Group: 6 – HEOB 400mg/kg (non IC)

Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC); Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC)

Group: 11- Ketoconozole 60mg/kg (IC)

Table 16: Colony forming units of various organs of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization

Groups	Intestine	Kidney	Liver	Lungs	Log Mean				Activity (Log control-Log treated)			
					Intestine	Kidney	Liver	Lungs	Intestine	Kidney	Liver	Lungs
1	$6.1x10^3$±44.7	$6.2x10^3$±70.7	$6.1x10^3$±54.8	$6.2x10^3$±86.0	3.8	3.8	3.8	3.8	-	-	-	-
2	$6.5x10^3$±104.9***	$6.7x10^3$±31.6***	$7.0x10^3$±171.5***	$7.2x10^3$±91.7***	3.8	3.8	3.8	3.9	-	-	-	-
3	$2.6x10^2$±24.5***	$3.6 x10^2$±24.5***	$3.8 x10^2$±37.4***	$7.2 x10^2$±37.4***	2.2	2.3	2.6	2.9	1.6	1.5	1.2	0.9
4	$2.8 x10^2$±20.0***	$3.2 x10^2$±20.0***	$3.0 x10^2$±31.6***	$4.8 x10^2$±20.0***	2.3	2.1	2.5	2.8	1.5	1.7	1.3	1.0
5	$1.3 x10^2$±4.5***	$1.6 x10^2$±3.2***	$4.2 x10^2$±37.4***	$7.6 x10^2$±51.0***	2.1	2.2	2.6	2.9	1.7	1.6	1.2	0.9
6	$1.1 x10^2$±4.5***	$1.3 x10^2$±5.5***	$3.4 x10^2$±24.5***	$6.0 x10^2$±31.6***	2.0	2.1	2.5	2.8	1.7	1.7	1.3	1.0
7	$5.2 x10^2$±20.0^c	$6.0 x10^2$±31.6^c	$7.6 x10^2$±24.5^c	$8.0 x10^2$±31.6^c	2.5	2.6	2.8	2.9	1.3	1.2	1.0	0.9
8	$4.4 x10^2$±24.5^c	$4.6 x10^2$±24.5^c	$4.8 x10^2$±20.0^c	$7.2 x10^2$±58.3^c	2.5	2.4	2.6	2.9	1.3	1.4	1.1	0.9
9	$3.2 x10^2$±7.0^c	$3.4 x10^2$±5.5^c	$6.0 x10^2$±54.8^c	$8.6 x10^2$±24.5^c	2.5	2.5	2.8	2.9	1.3	1.3	1.1	0.9
10	$2.1 x10^2$±3.2^c	$2.2 x10^2$±3.2^c	$5.0 x10^2$±31.6^c	$8.4 x10^2$±24.5^c	2.3	2.3	2.7	2.9	1.5	1.5	1.2	0.9
11	$1.0 x10^2$±3.2^c	$1.1 x10^2$±4.5^c	$2.0 x10^2$±31.6^c	$2.0 x10^2$±31.6^c	2.0	2.0	2.3	2.3	1.8	1.8	1.5	1.6

Data expressed as mean ±SEM: ***p< 0.001 vs. Group-1; ^{c}p< 0.001 vs.Group-2; Two-way ANOVA followed by Bonferroni post test

Results are expressed as: **Activity values: >3.0-maximum activity; 3.0-1.5-active; 1.5-0.5-slightly active ;< 0.5-not active**

Strength of inoculum (*C. albicans*): $1x10^7$cells/ml (48 h culture)

Route of administration of inoculum: intragastric

Route of administration of drug: Oral

n: 6 (both sex)

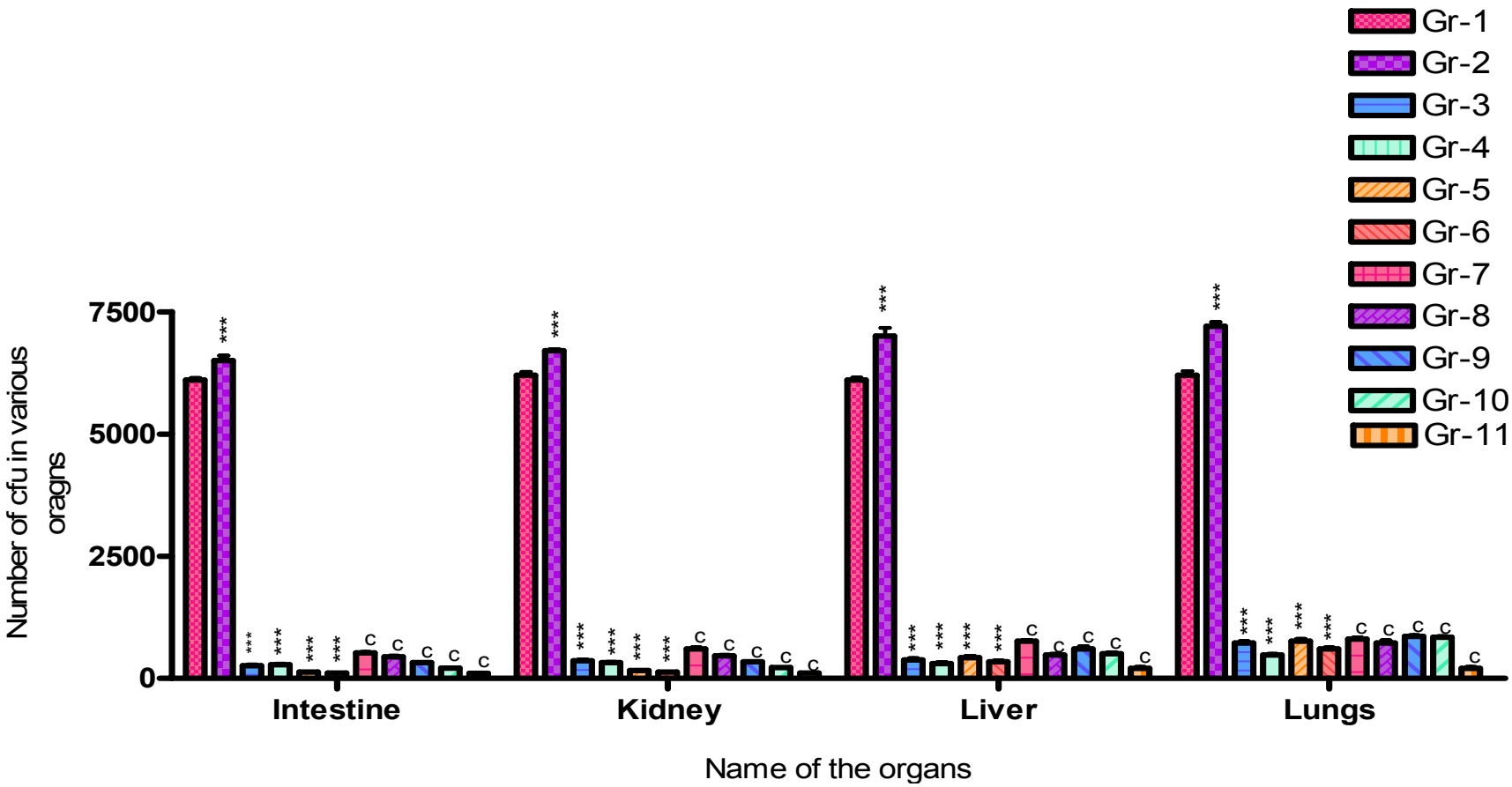
Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated); Group: 3- HEOS 200mg/kg (non IC)

Group: 4- HEOS 400mg/kg (non IC); Group: 5- HEOB 200mg/kg (non IC); Group: 6 – HEOB 400mg/kg (non IC)

Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC); Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC)

Group: 11- Ketoconozole 60mg/kg (IC)

Figure 15: Colony forming units of various organs of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization



Data expressed as mean ±SEM: ***p< 0.001 vs. Group-1; [c]p< 0.001 vs.Group-2; Two-way ANOVA followed by Bonferroni post test

Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated); Group: 3- HEOS 200mg/kg (non IC)

Group: 4- HEOS 400mg/kg (non IC); Group: 5- HEOB 200mg/kg (non IC); Group: 6 – HEOB 400mg/kg (non IC)

Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC); Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC)

Group: 11- Ketoconozole 60mg/kg (IC)

Table 17: Statistical analysis of repeated measures Two way ANOVA applied for body weights of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization

S. No	Parameters	Percentage Variance	F Value	DF	P value
1	Treatment vs. days	11.23	27.73	50	<0.0001
2	Days vs. result	4.01	99.05	5	<0.0001
3	Treatment vs. result	51.60	7.24	10	<0.0001
4	Effective matching	-	88.03	44	<0.0001

Table 17a: Body weights of mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization in non immunocompromised and immunocompromised condition

Groups	Day 0	Day 6	Day 9	Day 12	Day 18	Day 24
1	25.6±0.9	21.4±1.2***	20.0±1.1***	18.2±1.4***	18.0±1.3***	17.2±1.2***
2	26.0±0.5	20.2±1.3***	18.0±0.9***	16.4±0.7***	14.4±0.2***	14.2±0.2***
3	24.6±1.1	24.2±1.2	24.0±1.2	23.8±1.1	24.2±1.3	24.6±1.3
4	24.2±1.8	23.6±1.8	23.2±1.8	23.2±1.9	23.6±1.6	23.6±1.8
5	24.4±1.0	24.0±0.9	23.6±1.2	23.6±1.2	23.2±1.1	23.2±1.2
6	24.0±1.7	23.8±1.6	23.4±1.5	23.2±1.6	23.2±1.5	23.0±1.7
7	26.0±1.1	25.6±0.8	25.4±1.0	25.2±0.9	25.0±1.0	25.2±0.9
8	26.2±0.4	26±0.3	25.8±0.5	25.8±0.2	26.0±0.3	25.8±0.4
9	26.6±0.5	26.4±0.6	26.2±0.5	26.2±0.4	26.2±0.5	26.2±0.4
10	26.0±0.3	25.8±0.2	25.6±0.2	25.6±0.2	26.4±0.5	26.0±0.4
11	28.6±1.3	28.6±1.3	28.6±1.3	28.6±1.3	28.6±1.3	28.6±1.3

Data expressed as mean ± S.E.M: ***p< 0.001 vs. 0 day of respective groups; n: 6 (both sex); Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculum (*C.albicans*):1x10^7 cells/ml (48 h culture); Route of administration of inoculum: intragastric

Route of administration of drug: Oral Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated)

Group: 3- HEOS 200mg/kg (non IC); Group: 4- HEOS 400mg/kg (non IC); Group: 5- HEOB 200mg/kg (non IC)

Group: 6 – HEOB 400mg/kg (non IC); Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC); Group: 9- HEOB 200mg/kg (IC);

Group: 10 – HEOB 400mg/kg (IC); Group: 11- Ketoconozole 60mg/kg (IC)

Table 18: Total and differential leucocyte count of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization

Groups	TLC (mm^3)		Neutrophils (%)		Lymphocytes (%)		Eosinophils (%)		Monocytes (%)	
	Day 0	Day 24	Day 0	Day 24	Day 0	Day 24	Day 0	Day 24	Day 0	Day 24
1	5980±37.4	4860±128.8***	24.8±0.4	31.4±0.9***	69.6±0.6	59.8±1.0***	1.2±0.2	1.4±0.2	4.4±0.2	7.4±0.2***
2	5940±74.8	3980±73.4***	24.6±0.5	34.5±1.0***	69.6±0.4	56.5±1.5***	1.2±0.2	1.4±0.4	4.6±0.2	7.6±0.2***
3	5880±58.3	5960±97.9**	22.0±1.0	22.6±0.5	72.4±0.2	72.8.0±0.7	1.8±0.3	1.4±0.4	3.8±0.2	3.2±0.2
4	6060±50.9	6540±156.8***	21.4±0.7	23.4±0.4	72.6±0.8	70.0±2.5	1.6±0.3	1.4±0.3	4.4±0.2	5.2±0.3
5	5900±54.7	5980±101.9	21.9±0.4	22.4±0.4	72.2±0.3	72.4±0.8	1.1±0.2	0.8±0.4	4.8±0.2	4.4±0.2
6	6080±37.4	6380±162.4***	24.2±0.6	23.4±0.5	70.8±0.7	70.3±2.9	0.8±0.2	1.8±0.3	4.2±0.2	4.5±0.2
7	5720±58.3	5880±37.4**	21.6±1.2	22.8±0.7	72.6±1.0	71.4±3.2	1.6±0.2	0.7±0.3	4.2±0.3	5.1±0.2
8	6120±96.9	6380±124.0***	22.2±0.4	24.1±0.3	72.4±0.3	69.6±0.5	1.6±0.3	1.1±0.5	4.7±0.2	5.2±0.2
9	5740±50.9	5990±44.7**	22.2±1.0	23.2±0.7	71.5±0.5	70.5±3.2	1.6±0.2	0.9±0.2	4.7±0.3	5.4±0.2
10	6140±92.7	6400±130.3***	22.1±0.9	34.2±0.3***	71.5±0.8	58.9±0.9***	1.6±0.2	1.3±0.2	4.8±0.4	5.6±0.2
11	5900±70.7	6100±44.7***	23.8±0.4	23.6±0.2	70.4±0.2	70.1±0.8	1.3±0.3	1.0±0.3	4.5±0.2	5.2±0.3

Data expressed as mean ± S.E.M: **p< 0.01, ***p< 0.001 vs. 0 day of respective groups; n: 6 (both sex); Two-way RM ANOVA followed by Bonferroni post test;Strength of inoculum (*C.albicans*):1x10^7 cells/ml (48 h culture); Route of administration of inoculum: intragastric

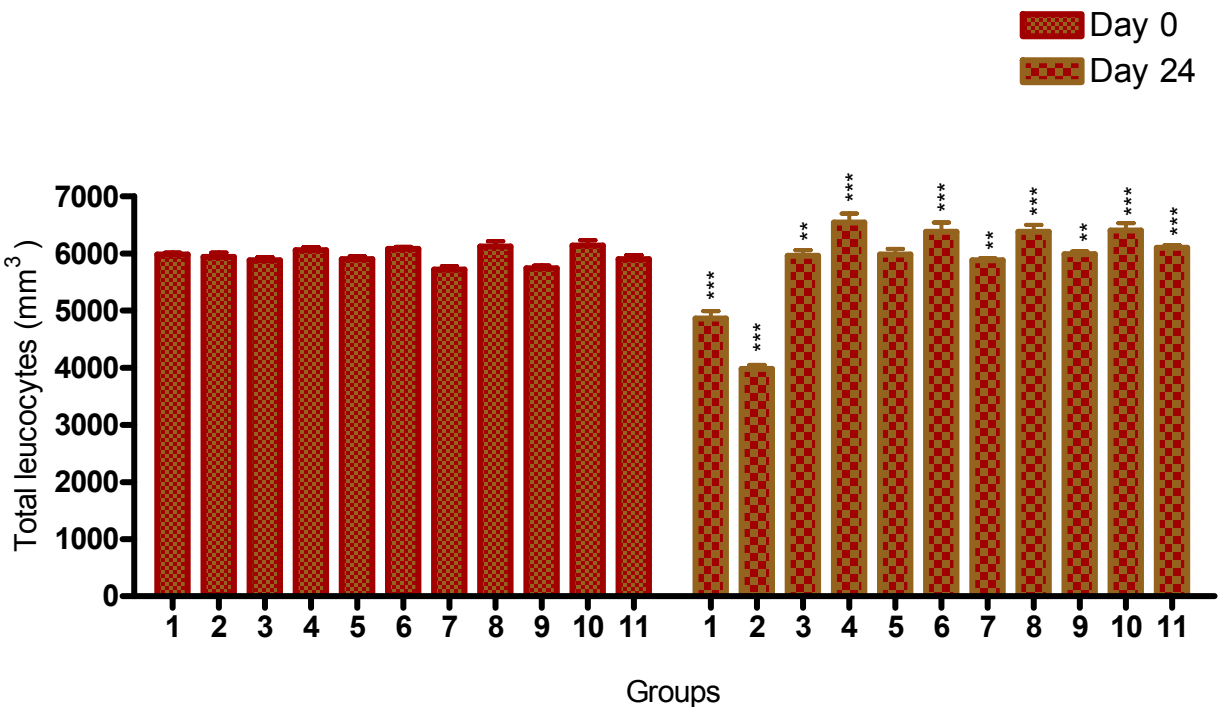
Route of administration of drug: Oral Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated)

Group: 3- HEOS 200mg/kg (non IC); Group: 4- HEOS 400mg/kg (non IC); Group: 5- HEOB 200mg/kg (non IC)

Group: 6 – HEOB 400mg/kg (non IC); Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC); Group: 9- HEOB 200mg/kg (IC);

Group: 10 – HEOB 400mg/kg (IC); Group: 11- Ketoconozole 60mg/kg (IC)

Figure 16: Total leukocyte count of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization



Data expressed as mean ± S.E.M: **p< 0.01, ***p< 0.001 vs. 0 day of respective groups; n: 6 (both sex); Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculum (*C.albicans*):1x10^7 cells/ml (48 h culture); Route of administration of inoculum: intragastric

Route of administration of drug: Oral

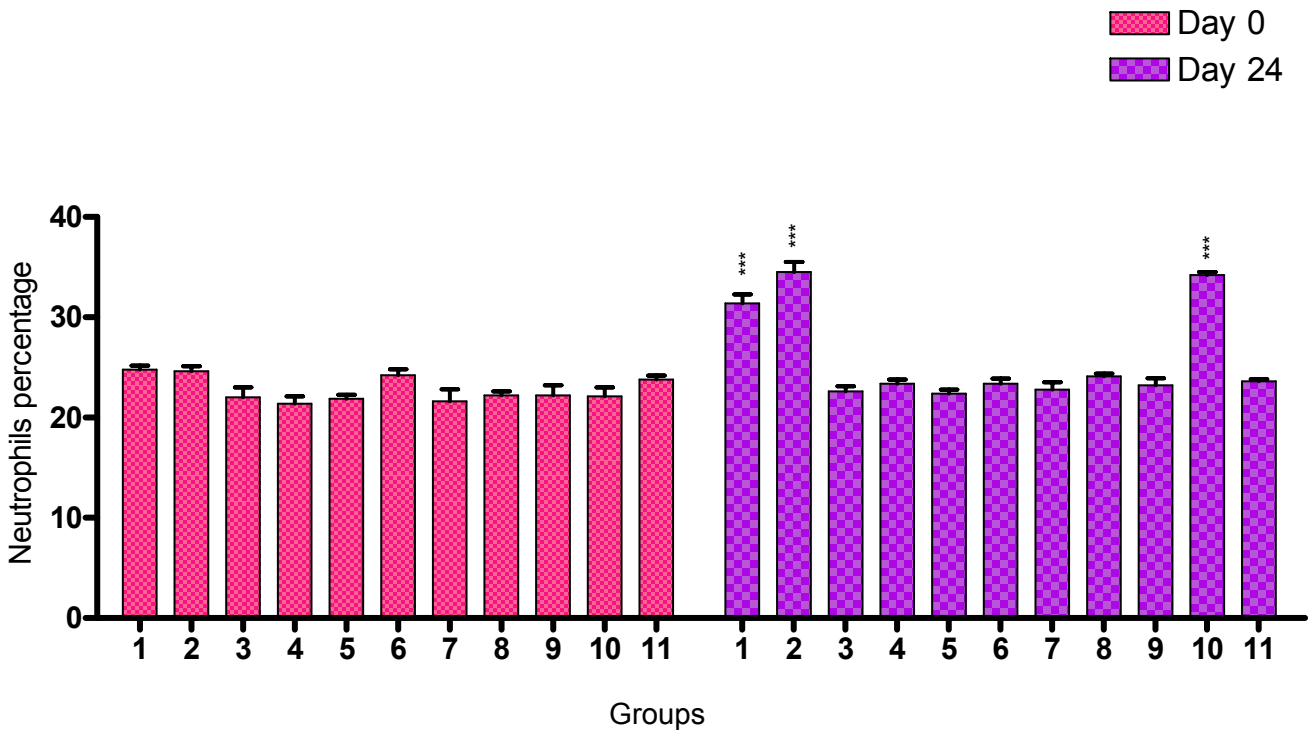
Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated)

Group: 3- HEOS 200mg/kg (non IC); Group: 4- HEOS 400mg/kg (non IC); Group: 5- HEOB 200mg/kg (non IC)

Group: 6 – HEOB 400mg/kg (non IC); Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC)

Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC); Group: 11- Ketoconozole 60mg/kg (IC)

Figure 17: Percentage neutrophils of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization



Data expressed as mean ± S.E.M: ***p< 0.001 vs. 0 day of respective groups; n: 6 (both sex); Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculum (*C.albicans*):1x10^7 cells/ml (48 h culture); Route of administration of inoculum: intragastric

Route of administration of drug: Oral

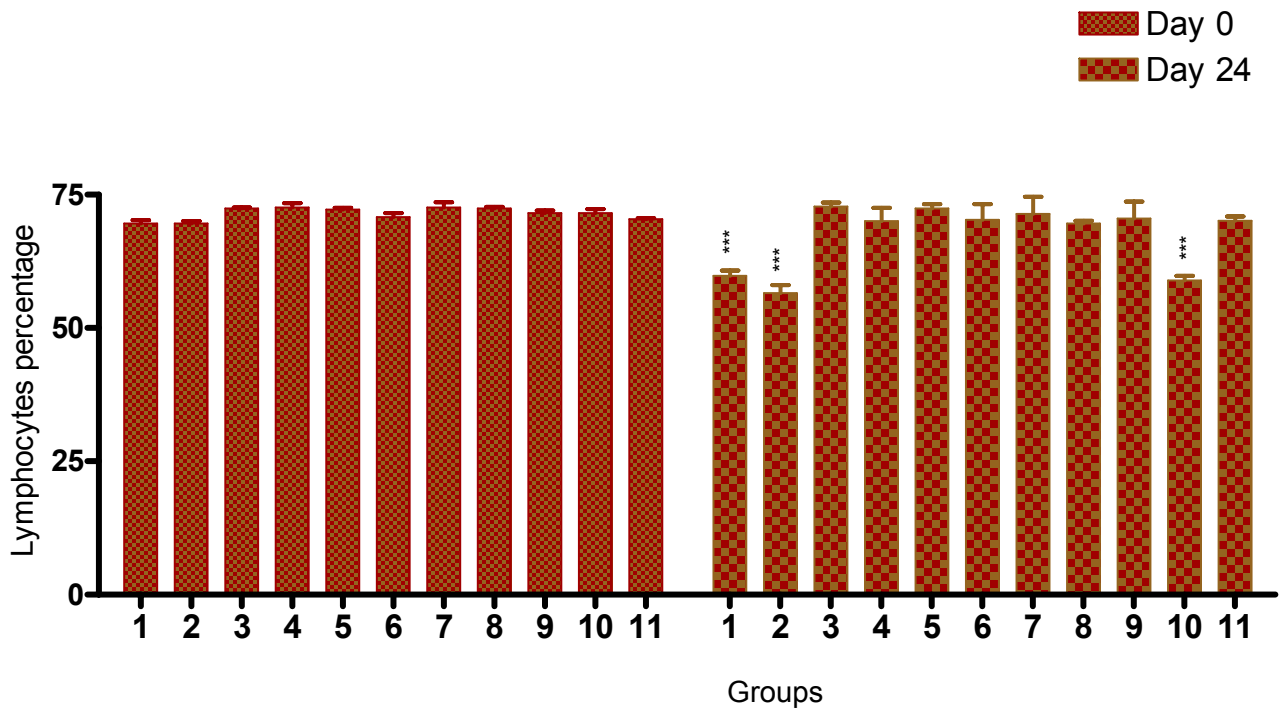
Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated)

Group: 3- HEOS 200mg/kg (non IC); Group: 4- HEOS 400mg/kg (non IC); Group: 5- HEOB 200mg/kg (non IC)

Group: 6 – HEOB 400mg/kg (non IC); Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC)

Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC); Group: 11- Ketoconozole 60mg/kg (IC)

Figure 18: Percentage lymphocytes of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization



Data expressed as mean ± S.E.M: ***p< 0.001 vs. 0 day of respective groups; n: 6 (both sex); Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculum (*C.albicans*):1x10^7 cells/ml (48 h culture); Route of administration of inoculum: Intragastric; Route of administration of drug: Oral

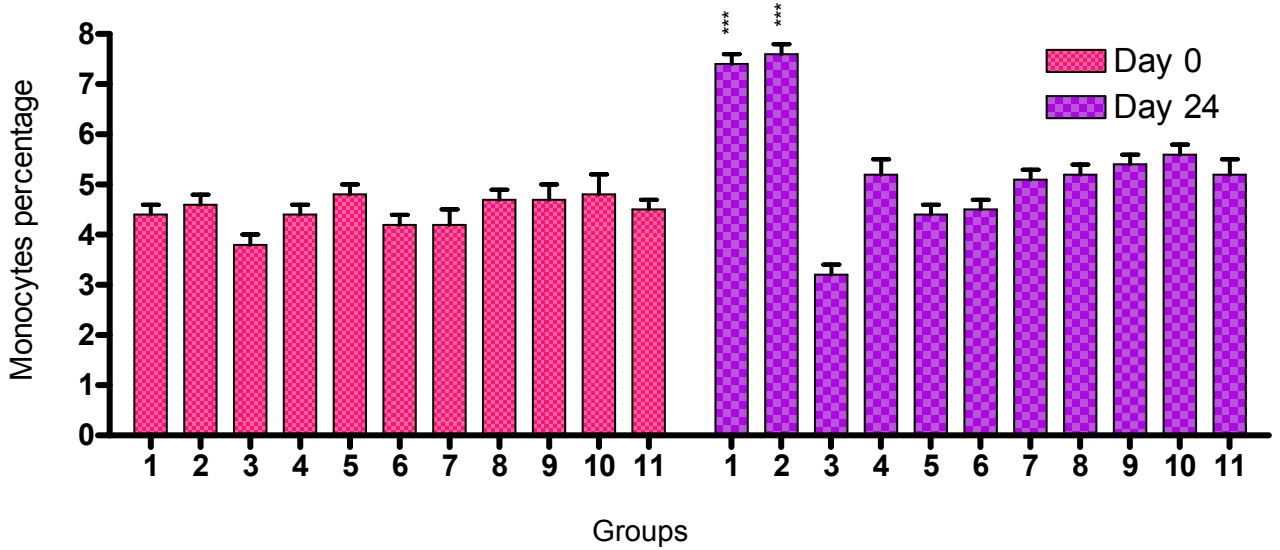
Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated)

Group: 3- HEOS 200mg/kg (non IC); Group: 4- HEOS 400mg/kg (non IC); Group: 5- HEOB 200mg/kg (non IC)

Group: 6 – HEOB 400mg/kg (non IC); Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC)

Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC); Group: 11- Ketoconozole 60mg/kg (IC)

Figure 19: Percentage monocytes of non immunocompromised and immunocompromised mice treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization



Data expressed as mean ± S.E.M: ***p< 0.001 vs. 0 day of respective groups; n: 6 (both sex); Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculum (*C.albicans*):1x10^{7} cells/ml (48 h culture); Route of administration of inoculum: Intragastric; Route of administration of drug: Oral

Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated)

Group: 3- HEOS 200mg/kg (non IC); Group: 4- HEOS 400mg/kg (non IC); Group: 5- HEOB 200mg/kg (non IC)

Group: 6 – HEOB 400mg/kg (non IC); Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC)

Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC); Group: 11- Ketoconozole 60mg/kg (IC)

Table 19: Tumor necrosis factor alpha (TNF- α) and Interleukin 1 alpha (IL-1 α) levels in non immunocompromised and immunocompromised mice serum treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization

Groups	TNF α (pg/ml)	IL- 1 α (pg/ml)
1	38.2±2.6	62.2±4.1
2	78.7±7.1	31.8±3.2
3	681.7±11.4***	474.2±13.8***
4	908.3±9.5***	926.7±12.3***
5	745.0±13.8***	563.3±12.0***
6	1208.0±52.3***	570.0±5.2***
7	798.3±13.3***	1250.0±17.5***
8	1517.0±69.1***	1550.0±36.5***
9	926.7±13.6***	1450.0±65.8***
10	1843.0±58.8***	1732.0±25.2***
11	1673.0±47.7***	1673.0±59.0***

Data expressed as mean ± S.E.M:***p<0.001 vs. Normal (**TNF α 35.5±2.0 pg/ml** and **IL- 1 α 59.3±3.2 pg/ml)**; One way ANOVA followed by Dunnett post test

Strength of inoculum (*C.albicans*):1x10^7 cells/ml (48 h culture) Route of administration of inoculum: Intragastric

Route of administration of drug: Oral n: 6 (both sex)

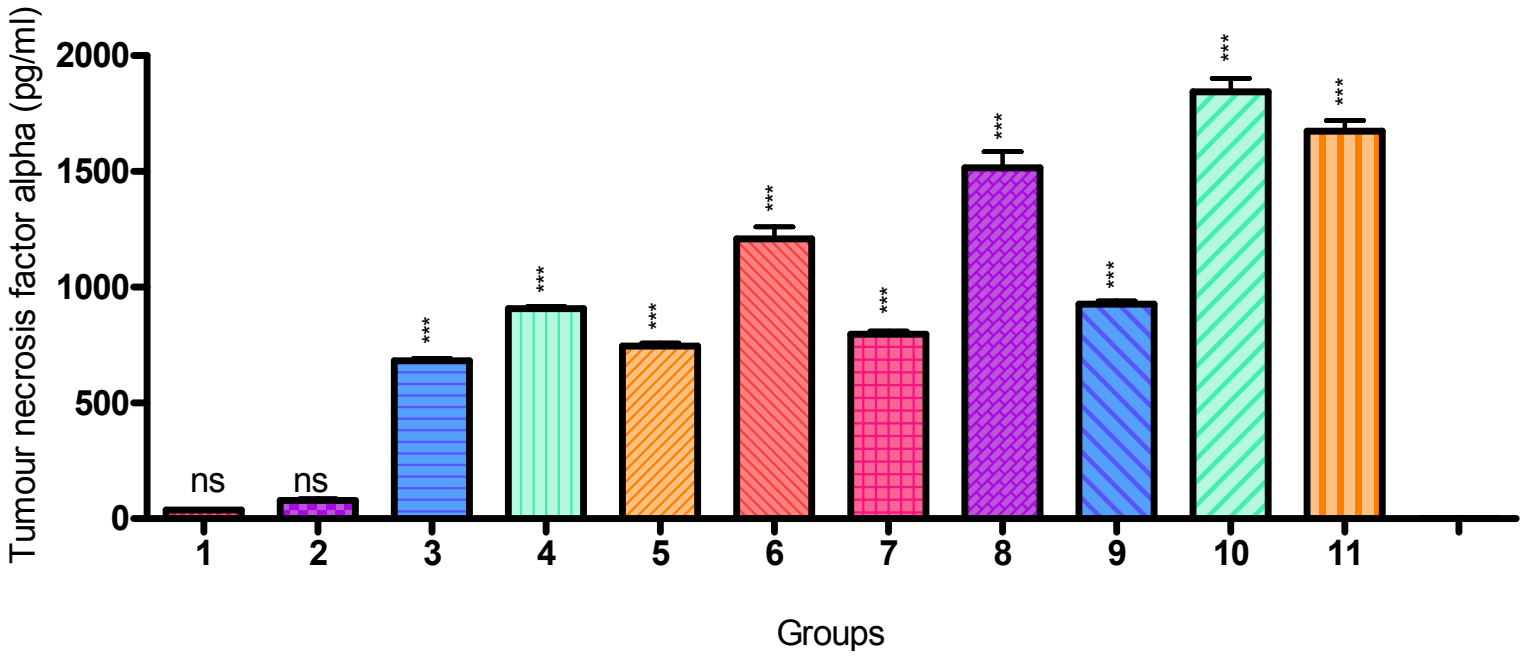
Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated)

Group: 3- HEOS 200mg/kg (non IC); Group: 4- HEOS 400mg/kg (non IC); Group: 5- HEOB 200mg/kg (non IC)

Group: 6 – HEOB 400mg/kg (non IC); Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC)

Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC); Group: 11- Ketoconozole 60mg/kg (IC)

Figure 20: Tumor necrosis factor alpha (TNF-α) levels in non immunocompromised and immunocompromised mice serum treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization



Data expressed as mean ± S.E.M:***p<0.001 vs. Normal (**TNF-α 35.5±2.0 pg/ml**); One way ANOVA followed by Dunnett post test

Strength of inoculum (*C.albicans*):1x10^7 cells/ml (48 h culture) Route of administration of inoculum: Intragastric

Route of administration of drug: Oral n: 6 (both sex)

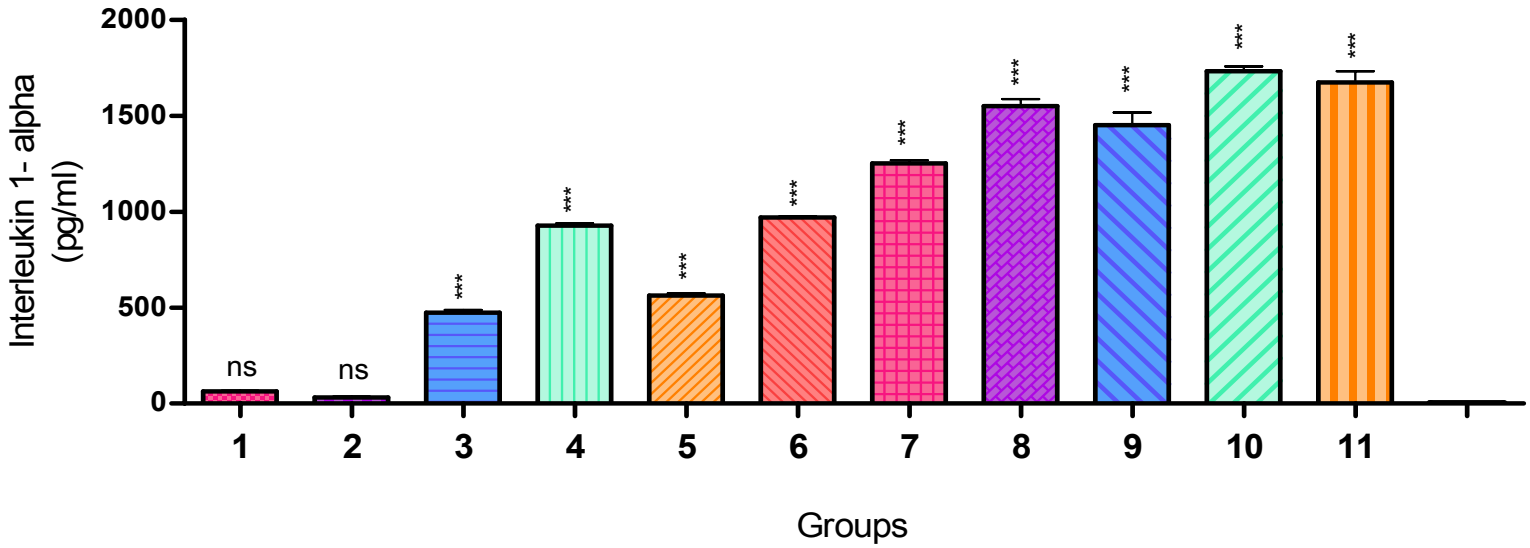
Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated)

Group: 3- HEOS 200mg/kg (non IC); Group: 4- HEOS 400mg/kg (non IC); Group: 5- HEOB 200mg/kg (non IC)

Group: 6 – HEOB 400mg/kg (non IC); Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC)

Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC); Group: 11- Ketoconozole 60mg/kg (IC)

Figure 21: Interleukin 1 alpha (IL-1 α) levels in non immunocompromised and immunocompromised mice serum treated with the extracts in experimental systemic candidiasis under gastrointestinal colonization



Data expressed as mean ± S.E.M:***p<0.001 vs. Normal (**IL-1 α 59.3**±3.2 **pg/ml**); One way ANOVA followed by Dunnett post test

Strength of inoculum (*C.albicans*):1x10^7 cells/ml (48 h culture) Route of administration of inoculum: Intragastric

Route of administration of drug: Oral n: 6 (both sex)

Group: 1-Vehicle control (non IC); Group: 2- IC control (vehicle treated)

Group: 3- HEOS 200mg/kg (non IC); Group: 4- HEOS 400mg/kg (non IC); Group: 5- HEOB 200mg/kg (non IC)

Group: 6 – HEOB 400mg/kg (non IC); Group: 7- HEOS 200mg/kg (IC); Group: 8- HEOS 400mg/kg (IC)

Group: 9- HEOB 200mg/kg (IC); Group: 10 – HEOB 400mg/kg (IC); Group: 11- Ketoconozole 60mg/kg (IC)

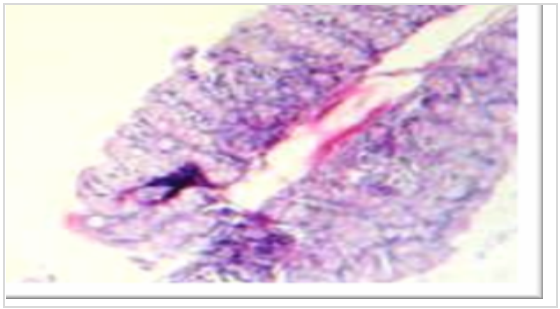


Plate 34. Histology of intestine of non IC mice treated with vehicle control in experimental candidiasis under GI colonization showing moderate to dense inflammation including lymphoid follicles of sub mucosa and congestion with fungal hyphae (PAS 40x)

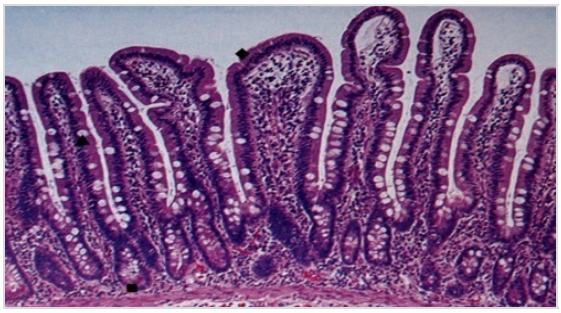


Plate 35. Histology of intestine of IC mice treated with vehicle control in experimental candidiasis under GI colonization showing moderate to dense inflammation including lymphoid follicles of sub mucosa and congestion with fungal hyphae (PAS 40x)

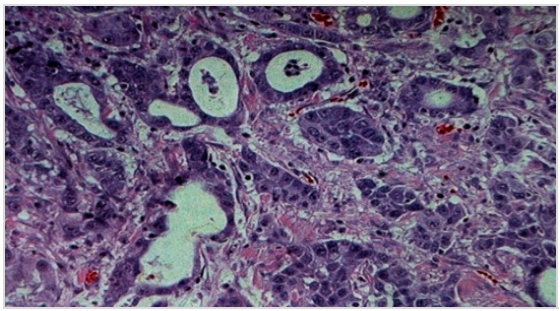


Plate 36. Histology of intestine of non IC mice treated with HEOS (200mg/kg) in experimental candidiasis under GI colonization showing normal appearance (PAS 40x)

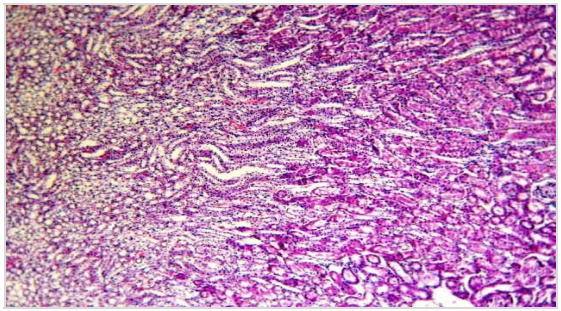


Plate 37. Histology of intestine of non IC mice treated with HEOS (400mg/kg) in experimental candidiasis under GI colonization showing normal appearance (PAS 40x)

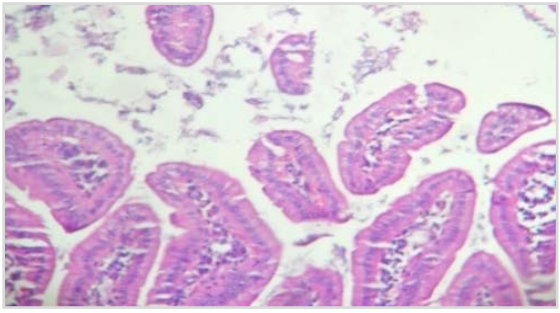


Plate 38. Histology of intestine of non IC mice treated with HEOB (200mg/kg) in experimental candidiasis under GI colonization showing mild inflammation in lumen with fungal hyphae (PAS 40x)

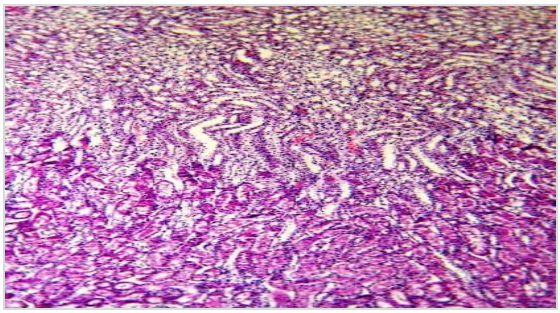


Plate 39. Histology of intestine of non IC mice treated with HEOB (400mg/kg) in experimental candidiasis under GI colonization showing normal appearance (PAS 40x)

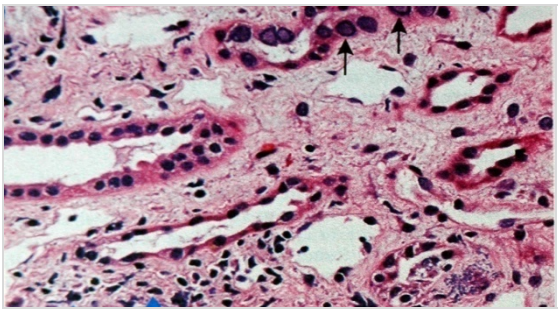


Plate 40. Histology of intestine of IC mice treated with HEOS (200mg/kg) in experimental candidiasis under GI colonization showing normal appearance (PAS 40x)

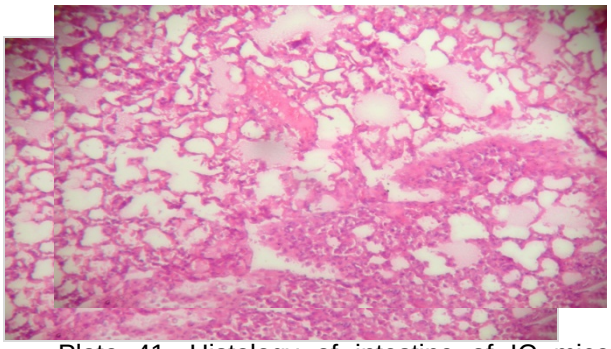


Plate 41. Histology of intestine of IC mice treated with HEOS (400mg/kg) in experimental candidiasis under GI colonization showing normal appearance (PAS 40x)

Plate 10 Histology of *C.albicans* infected immunocompromised mice intestine treated with *Ocimum sanctum* (400mg/kg/b.wt) showing mild inflammation in lumen with fungal hyphae PAS 40x

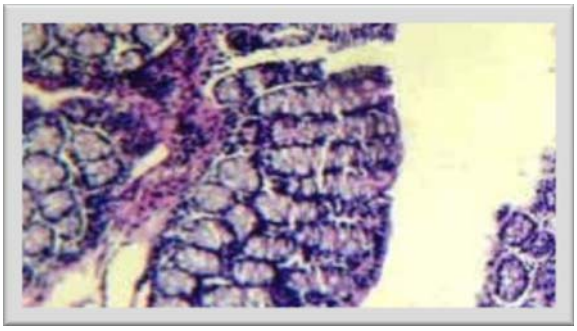


Plate 42. Histology of intestine of IC mice treated with HEOB (200mg/kg) in experimental candidiasis under GI colonization showing normal appearance (PAS 40x)

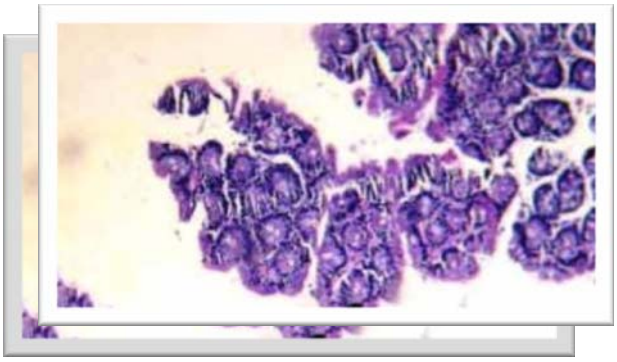


Plate 43. Histology of intestine of IC mice treated with HEOB (400mg/kg) in experimental candidiasis under GI colonization showing normal appearance (PAS 40x)

Plate 8 Histology of *C.albicans* infected immunocompromised mice intestine treated with *Ocimum basilicum* (400mg/kg/b.wt) showing mild inflammation PAS 40x

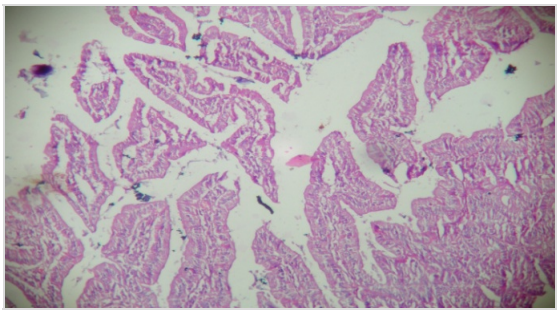


Plate 44. Histology of intestine of IC mice treated with ketoconozole (60 mg/kg) in experimental candidiasis under GI colonization showing parenchyma with no inflammation (PAS 40x)

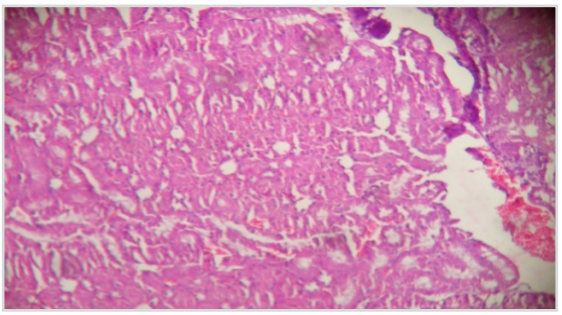


Plate 45. Histology of kidney of non IC mice treated with vehicle control in experimental candidiasis under gi colonization showing dense inflammation of interstitium with fungal hyphae (PAS 40x)

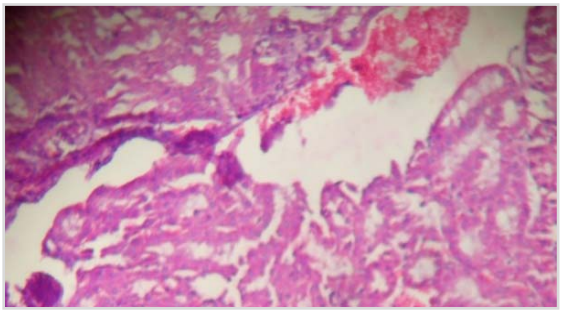


Plate 46. Histology of kidney of IC mice treated with vehicle control in experimental candidiasis under gi colonization showing dense inflammation of interstitium with fungal hyphae (PAS 40x)

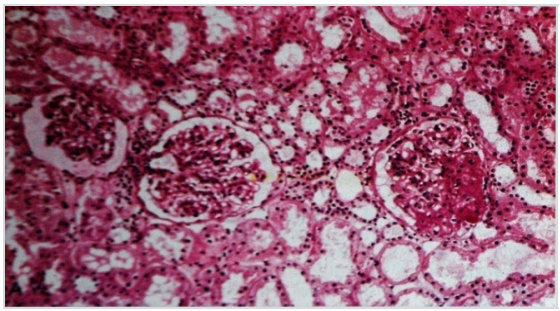


Plate 47. Histology of kidney of non IC mice treated with HEOS (200mg/kg) in experimental candidiasis under gi colonization showing renal tissue and congestion of the interstitium with focal inflammation (PAS 40x)

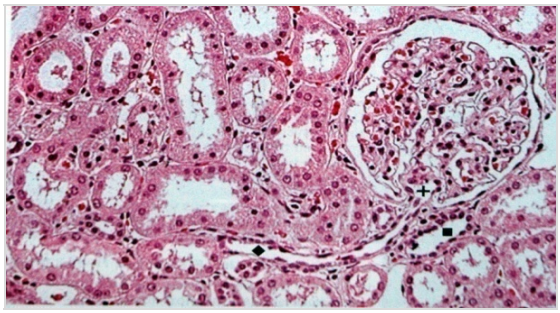


Plate 48. Histology of kidney of non IC mice treated with HEOS (400mg/kg) in experimental candidiasis under gi colonization showing renal tissue with predominantly tubules and interstitium with normal surface (PAS 40x)

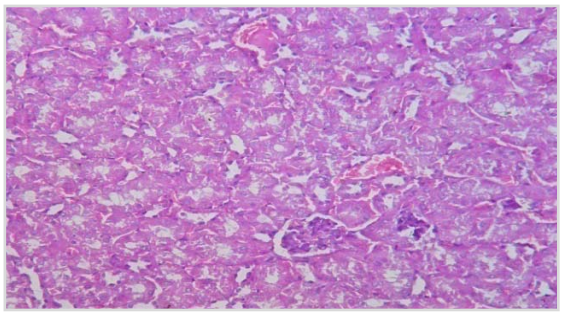


Plate 49. Histology of kidney of non IC mice treated with HEOB (200mg/kg) in experimental candidiasis under gi colonization showing mild inflammation of tubules with fungal hyphae (PAS 40x)

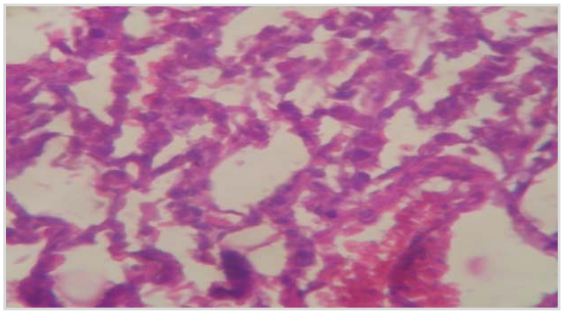


Plate 50. Histology of kidney of non IC mice treated with HEOB (400 mg/kg) in experimental candidiasis under gi colonization showing normal renal tissue and tubules (PAS 40x)

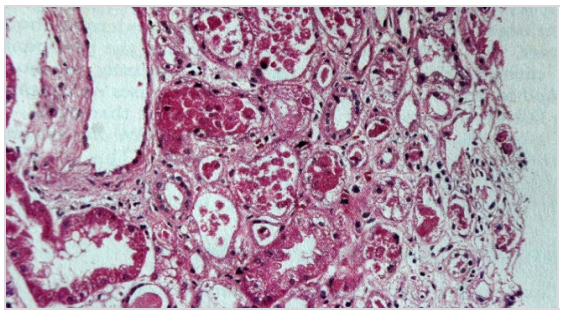


Plate 51. Histology of kidney of IC mice treated with HEOS (200mg/kg) in experimental candidiasis under gi colonization showing renal tissue with few rows of parenchyma and congestion of the interstitium with mild inflammation (PAS 40x)

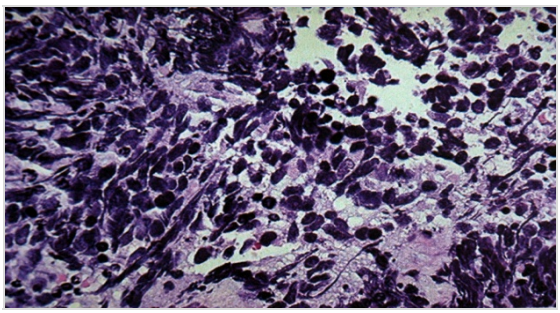


Plate 52. Histology of kidney of IC mice treated with HEOS (400mg/kg) in experimental candidiasis under gi colonization showing renal tissue with normal surface (PAS 40x)

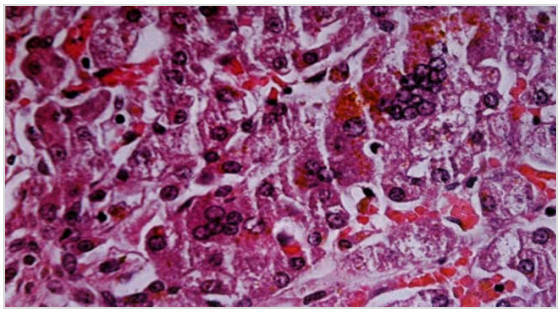


Plate 53. Histology of kidney of IC mice treated with HEOB (200mg/kg) in experimental candidiasis under gi colonization showing mild inflammation of tubules with fungal hyphae (PAS 40x)

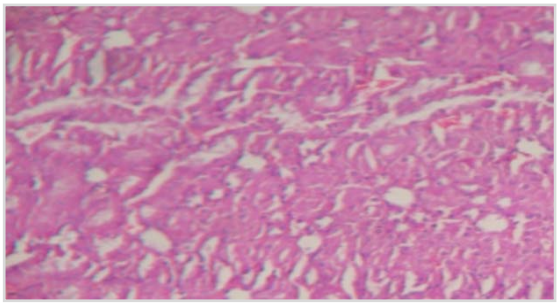


Plate 54. Histology of kidney of IC mice treated with HEOB (400 mg/kg) in experimental candidiasis under gi colonization showing normal appearance (PAS 40x)

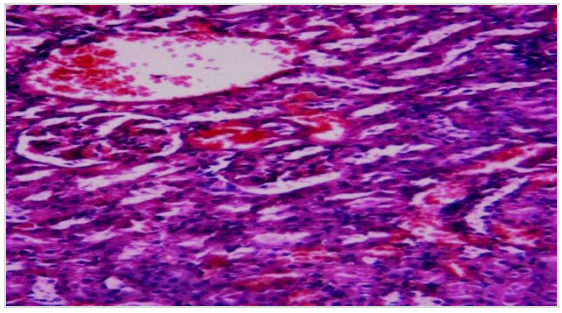


Plate 55. Histology of kidney of IC mice treated with ketoconozole (60 mg/kg) in experimental candidiasis under gi colonization showing no inflammation (PAS 40x)

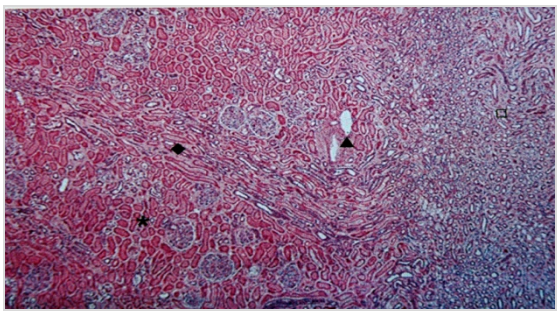


Plate 56. Histology of liver of non IC mice treated with vehicle control in experimental candidiasis under gi colonization showing congestion with focal necrosis (PAS 40x)

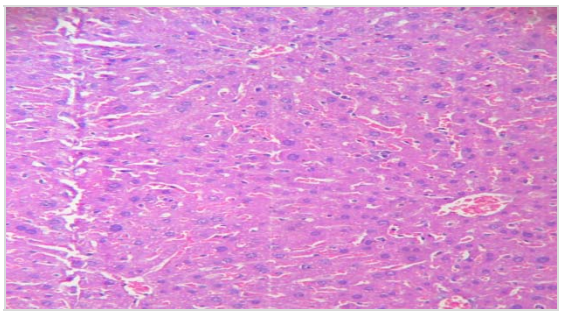


Plate 57. Histology of liver of IC mice treated with vehicle control in experimental candidiasis under gi colonization showing dense inflammation, congestion with focal necrosis (PAS 40x)

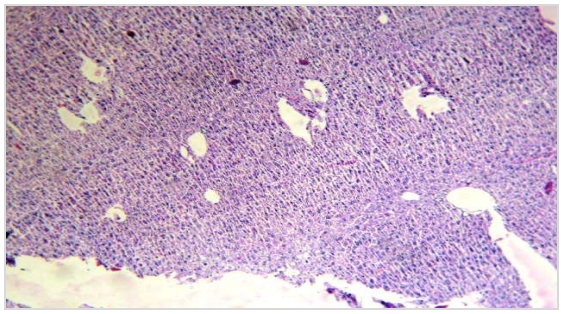


Plate 58. Histology of liver of non IC mice treated with HEOS (200mg/kg) in experimental candidiasis under gi colonization showing hepatic parenchyma (PAS 40x)

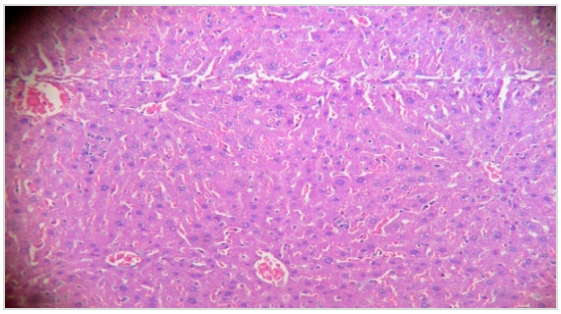


Plate 59. Histology of liver of non IC mice treated with HEOS (400mg/kg) in experimental candidiasis under gi colonization showing normal surface (PAS 40x)

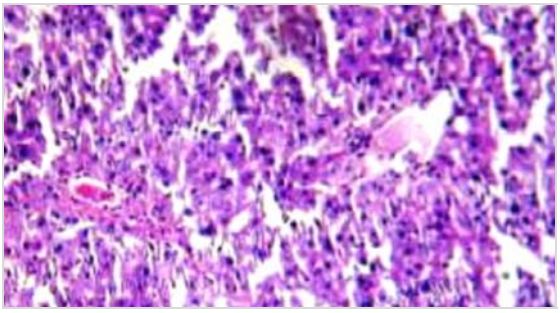


Plate 60. Histology of liver of non IC mice treated with HEOB (200 mg/kg) in experimental candidiasis under gi colonization showing congestion of central vein (PAS 40x)

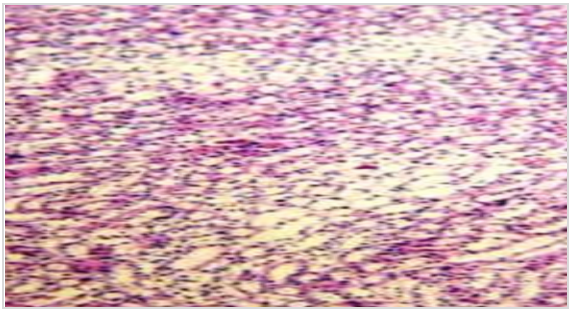


Plate 61. Histology of liver of non IC mice treated with HEOB (400 mg/kg) in experimental candidiasis under gi colonization showing normal appearance (PAS 40x)

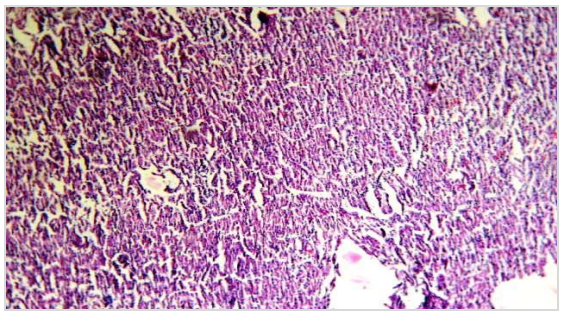


Plate 62. Histology of liver of IC mice treated with HEOS (200mg/kg) in experimental candidiasis under gi colonization showing hepatic parenchyma and hexagonal cells (PAS 40x)

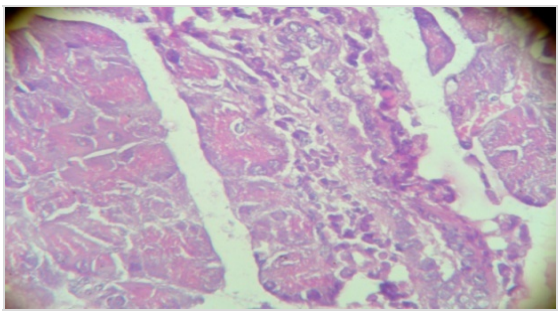


Plate 63. Histology of liver of IC mice treated with HEOS (400mg/kg) in experimental candidiasis under gi colonization showing hepatic parenchyma and hexagonal cells (PAS 40x)

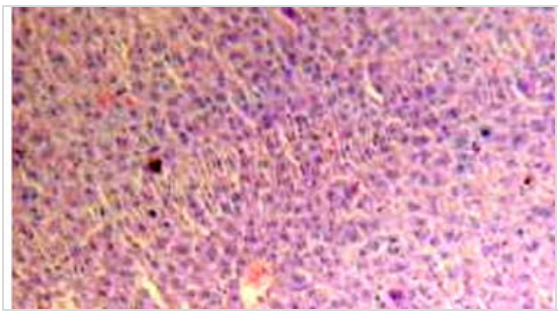


Plate 64. Histology of liver of IC mice treated with HEOB (200 mg/kg) in experimental candidiasis under gi colonization showing few fungal hyphae structure (PAS 40x)

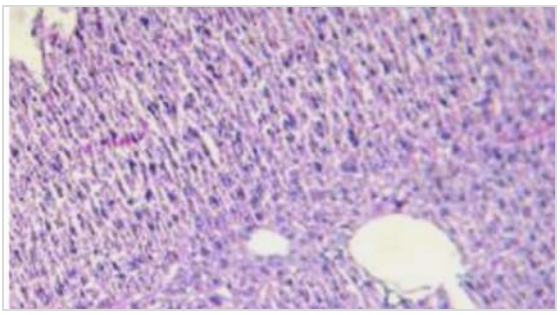


Plate 65. Histology of liver of IC mice treated with HEOB (400 mg/kg) in experimental candidiasis under gi colonization showing normal appearance (PAS 40x)

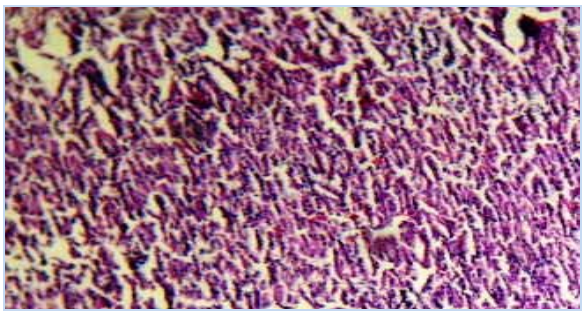


Plate 66. Histology of liver of IC mice treated with ketoconozole (60 mg/kg) in experimental candidiasis under gi colonization showing parenchyma with no inflammation (PAS 40x)

4.8. Effect of the extracts against experimental oral candidiasis in immunocompromised mice

Oral inoculation of *C.albicans* and immunosuppressive drugs were administered to the mice as described in the experimental design and the results obtained are given in Table 20 and Figure 22. The results reveal a maximum number of cfu in oral swabs collected from buccal mucosa of immunocompromised mice. Oral treatment with the extracts (200 and 400mg/kg) and ketoconozole (60mg/kg) show a significant ($p<0.01$) decrease in the number of cfu, when compared with solvent control.

All the animals survived were euthanized on day 7 of the study and oral mucosal layer and tongue were dissected for histopathological examination. The examination reveals moderate inflammation with appearance of fungal hyphae in oral mucosa and tongue of immunocompromised mice. Normal histological appearance is observed in drug treated (HEOS 200, 400mg/kg and HEOB 400mg/kg and ketoconozole) mice. However, a mild inflammation of oral mucosal cells with fungal hyphae is observed in HEOB 200mg treated mice (Plates 67-78).

Table 20: Effect of the extracts against experimental oral candidiasis in immunocompromised mice

Groups	Colony forming units (cfu) in buccal mucosa	Log mean	Activity (Log control-Log treated)	% activity (test activity/standard activity)x100
1	$244.0x10^2 \pm 5.8$	4.4	-	-
2	$192.6x10^2 \pm 5.4$**	4.3	0.1	11.2
3	$134.0x10^2 \pm 1.2$**	4.1	0.3	26.5
4	$235.2x10^2 \pm 5.9$**	4.4	0.02	2.04
5	$160.0x10^2 \pm 2.8$**	4.2	0.2	19.4
6	$26.0x10^2 \pm 0.3$**	3.4	1.0	100

Data expressed as mean ± SEM: **p<0.01 vs. Group-1 (IC control); One way ANOVA followed by Dunnett post test

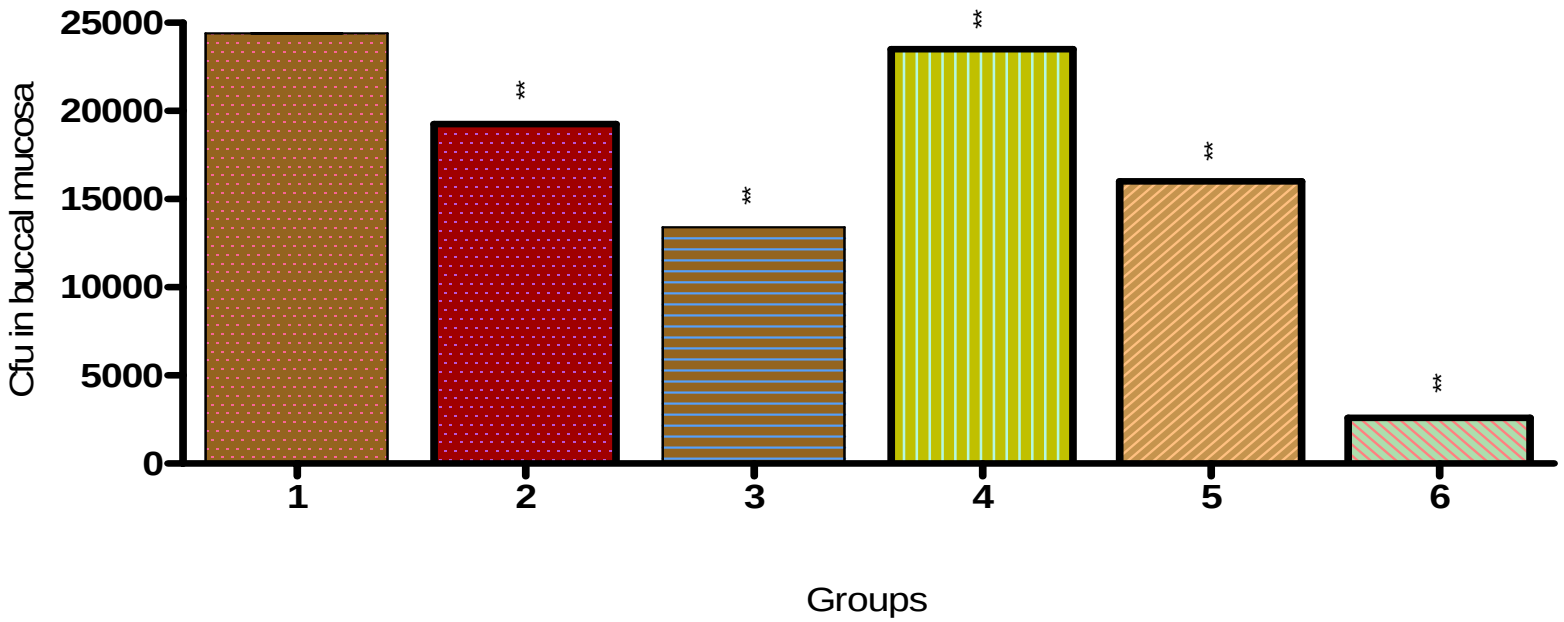
Strength of inoculum (*C.albicans*):$1x10^7$ cells/ml (48 h culture) Route of administration of inoculum: Oral swabbing

Route of administration of drug: Oral n: 6 (both sex)

Group: 1- IC control; Group: 2- HEOS 200mg/kg (IC); Group: 3- HEOS 400mg/kg (IC)

Group: 4- HEOB 200mg/kg (IC); Group: 5 – HEOB 400mg/kg (IC); Group: 6- Ketoconozole 60mg/kg (IC)

Figure 22: Effect of the extracts against experimental oral candidiasis in immunocompromised mice



Data expressed as mean ± SEM: **p<0.01 vs. Group-1 (IC control); One way ANOVA followed by Dunnett post test

Strength of inoculum (*C.albicans*):1x10^7 cells/ml (48 h culture) Route of administration of inoculum: Oral swabbing

Route of administration of drug: Oral n: 6 (both sex)

Group: 1- IC control; Group: 2- HEOS 200mg/kg (IC); Group: 3- HEOS 400mg/kg (IC)

Group: 4- HEOB 200mg/kg (IC); Group: 5 – HEOB *4*00mg/kg (IC); Group: 6 – Ketoconozole 60mg/kg (IC)

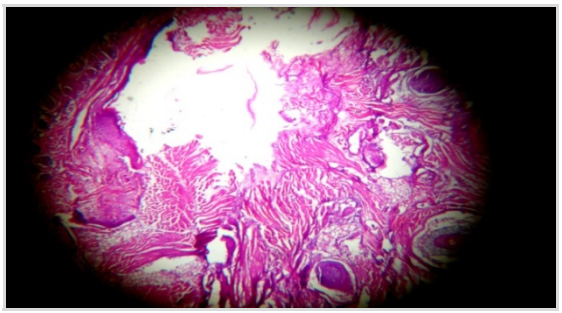


Plate 67. Histology of buccal mucosa of IC mice treated with vehicle control in oral candidiasis showing moderate inflammation with fungal hyphae (PAS 40x)

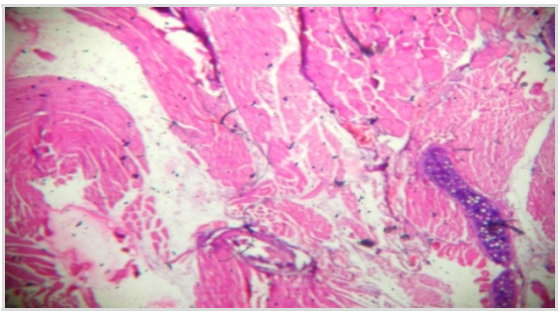


Plate 68. Histology of buccal mucosa of IC mice treated with HEOS (200 mg/kg) in oral candidiasis showing mild inflammation with fungal hypahe (PAS 40x)

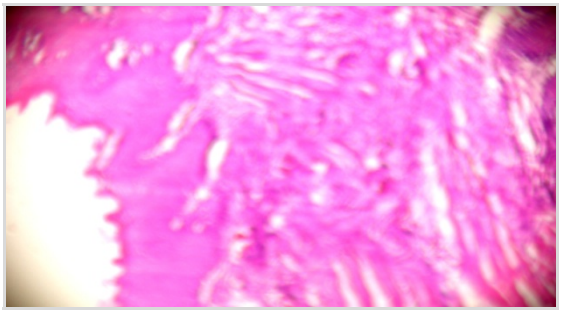


Plate 69. Histology of buccal mucosa of IC mice treated with HEOS (400 mg/kg) in oral candidiasis showing normal appearance (PAS 40x)

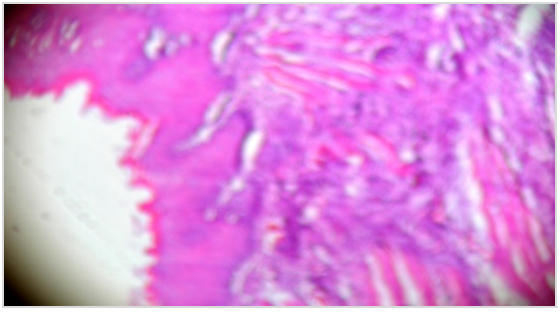


Plate 70. Histology of buccal mucosa of IC mice treated with HEOB (200 mg/kg) in oral candidiasis showing mild inflammation with fungal hyphae (PAS 40x)

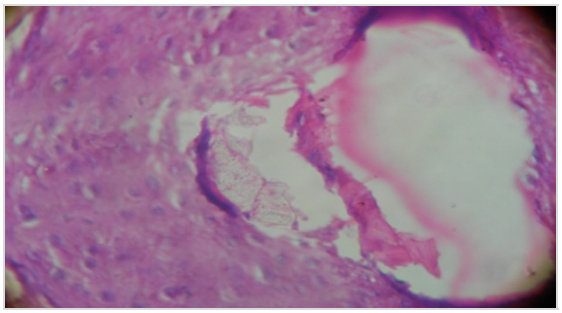


Plate 71. Histology of buccal mucosa of IC mice treated with HEOB (400 mg/kg) in oral candidiasis showing normal appearance (PAS 40x)

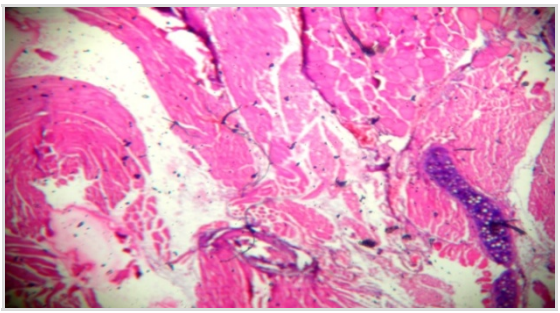


Plate 72. Histology of buccal mucosa of IC mice treated with ketoconozole (60 mg/kg) in oral candidiasis showing normal appearance (PAS 40x)

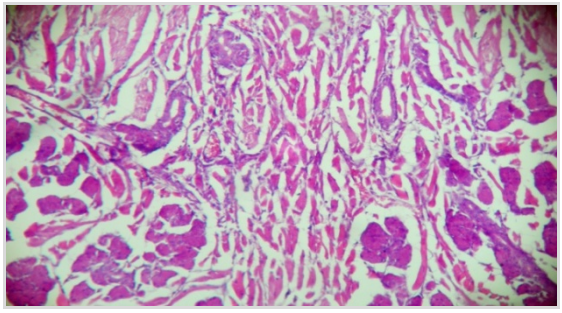


Plate 73. Histology of tongue of IC mice treated with vehicle control in oral candidiasis mild inflammation with fungal hyphae (PAS 40x)

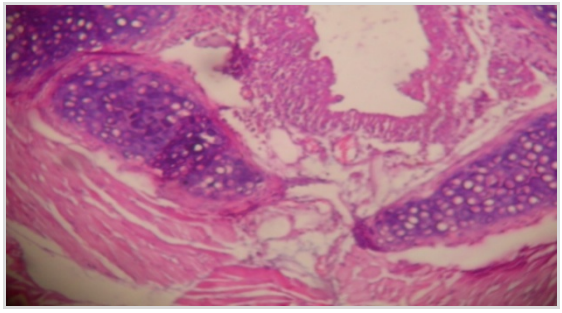


Plate 74. Histology of tongue of IC mice treated with HEOS (200 mg/kg) in oral candidiasis showing mild inflammation with fungal hyphae (PAS 40x)

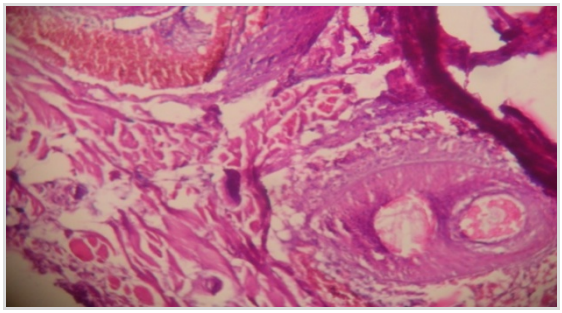


Plate 75. Histology of tongue of IC mice treated with HEOS (400 mg/kg) in oral candidiasis showing normal appearance (PAS 40x)

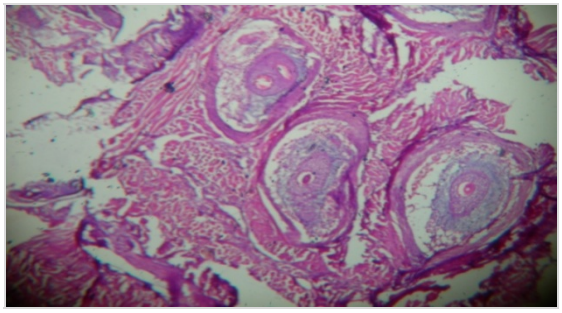


Plate 76. Histology of tongue IC mice treated with HEOB (200 mg/kg) in oral candidiasis showing normal appearance (PAS 40x)

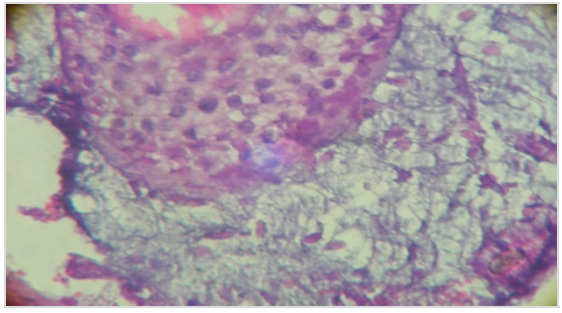


Plate 77. Histology of tongue IC mice treated with HEOB (400 mg/kg) in oral candidiasis showing normal appearance (PAS 40x)

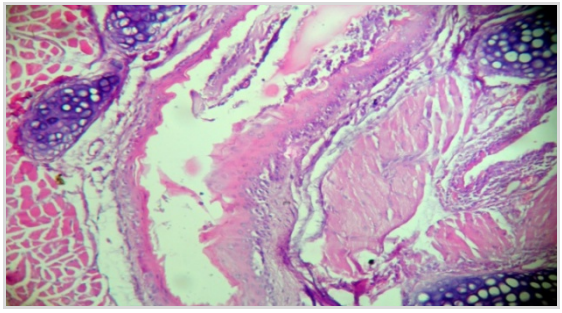


Plate 78. Histology of tongue of IC mice treated with ketoconozole (60mg/kg) in oral candidiasis showing mild inflammation (PAS 40x)

4.9. Formulation development and evaluation of the extracts cream and gel

The formulated creams were investigated for their physical parameters like consistency, homogeneity, spreadability, washability and tube extrudability and it was found that 2% cream was more effective in terms of consistency, homogenity, and tube extrudability in comparison with 4% cream. The results are given in Table 21.

The particle size for the gel was determined through optical microscopy. The average particle size of the 2% gel was found to be 369.48 -498.68 µM and for 4% gel it was 369.8-498.67 µM. The viscosity (determined using a Brookfield viscometer) for 2% gel at 5 rpm was found to be 664 cps and for 4% gel 682 cps.

The loss of water and volatile compounds were observed by Freez thaw cycling test. The prepared gel was weighed initially then a Freez thaw cycle was carried out. After performing five cycles the gel was reweighed and the loss of water was calculated for the loss of water. The results indicate that there are no changes in the loss of water and other volatile components. The microbial limit test for the formulation was done using bacterial and fungal cultures. The results suggest that there is no growth of microorganisms. The sample, therefore, passes the microbial limit test. This may be due to the antimicrobial activity of the extract. The results are given in Table 22.

Table 21: Evaluation of the extracts cream

S.no	Formulation	Consistency	Homogenity	Spreadability	Washability	Tube extrudability
01	HEOS 2%	+++	+++	+++	+++	+++
02	HEOS 4%	++	++	+++	+++	++
03	HEOB 2%	+++	++	+++	++	+++
04	HEOB 4%	++	++	++	++	++

Table 22: Evaluation of the extracts gel

S.no	Formulation	Particle size (µM)	Viscosity (cps)	Microbial limit test	Freez thaw cycling test
01	HEOS 2%	368.48-498.68	664	Nil	Nil
02	HEOS 4%	369.8-498.67	682	Nil	Nil
03	HEOB 2%	370.45-490.75	660	Nil	Nil
04	HEOB 4%	372.60-495.85	685	Nil	Nil

4.10. Effect of the extracts against experimental vaginal candidiasis in non immunocompromised and immunocompromised mice

4.10.1. Effect of extracts against experimental vaginal candidiasis in non mmunocompromised and immunocompromised mice

The vaginal scrapings were collected on day 3, 6, 9, 12 and 18 and the data subjected to repeated measures of Two way ANOVA and summarized as days versus treatment (% variance 43.60, F-16443.39, DF- 50 and p <0.0001), treatment versus result (% variance 52.42, F-120702.77, DF-10 and p<0.0001), days versus result (% variance 3.91, F-14955.39, DF-5 and p<0.0001) and effective matching (F-0.82, DF-55 and p<0.8124) and are given in Table 23.

The cfu obtained (Table 23a and Figure 23) from the vaginal scrapings of the control (cream base) treated both non immunocompromised and immunocompromised mice exhibit an increase in fungal burden. However, 2% and 4% creams of the extracts treated both non immunocompromised and immunocompromised groups show a significant (p<0.001) decrease in the number of cfu on day 3 onwards. Similar findings are observed on day 6, 9, 12 and 18 in both non immunocompromised and immunocompromised groups when compared with their respective control groups. The complete cure of vaginal infection was observed on day 30 (data not shown). The results are comparable with ketoconozole which shows a higher activity.

4.10.2. Effect of the extracts on body weight in experimental vaginal candidiasis in non immunocompromised and immunocompromised mice

Body weights of mice were recorded on day 0, 6, 9, 12 and 18 and the data subjected to statistical analysis of repeated measures of Two way ANOVA and results summarized as treatment versus days (% variance 16.76, F-2.94, DF- 50 and p <0.0001), days versus result (% variance 8.76, F-15.36, DF-5 and p<0.0001), treatment versus result (% variance 30.29, F-13.01, DF-10 and p<0.0001) and effective matching (F-2.04, DF-55 and p<0.0001) are given in Table 24.

The results in Table 24a reveal a significant (p<0.001) decrease in body weights of both non immunocompromised and immunocompromised mice treated with control (cream

base). Similarly, there is a significant ($p<0.01$) decrease in body weights observed in HEOS 200mg/kg treated group on day 15 and 18. However, there is no significant changes in HEOB 200 and 400 mg/kg HEOS and ketoconozole treated groups when compared to 0 day.

4.10.3. Effect of the extracts on total leukocyte count and differential leukocyte count of non immunocompromised and immunocompromised mice in experimental vaginal candidiasis

the results given in Table 25 and Figures 24-27. The data reveal a significant ($p<0.001$) decrease in total leucocytes, lymphocytes percentage and a significant ($p<0.001$) increase in neutrophils and monocytes percentage, in non immunocompromised and immunocompromised mice treated with control (cream base), when compared with 0 day of their respective groups. No significant changes are seen in the extracts, and ketoconozole treated mice at tested dose levels. Eosinophils count is not affected in all groups during the experimental period.

Table 23: Statistical analysis of repeated measures Two way ANOVA applied for colony forming units in vaginal scrapings of non immunocompromised and immunocompromised mice treated with the extracts in experimental vaginal candidiasis

S.No	Parameters	Percentage Variance	F Value	DF	P value
1	Days vs. treatment	43.60	16443.39	50	<0.0001
2	Treatment vs. result	52.42	120702.77	10	<0.0001
3	Days vs. result	3.91	14955.39	5	<0.0001
4	Effective matching	-	0.82	55	0.8124

Table 23a: Effect of the extracts against experimental vaginal candidiasis in non immunocompromised and immunocompromised mice

Groups	Colony forming units in vaginal scrapings						Log mean	Log control-Log treated	Remarks
	Day 3	Day 6	Day9	Day 12	Day15	Day 18			
1	$7.9 \times 10^5 \pm 0.04$	$9.7 \times 10^4 \pm 0.02$	$10.3 \times 10^4 \pm 0.08$	$13.3X10^4 \pm 0.15$	$23.4X10^4 \pm 0.11$	$19.5 \times 10^4 \pm 0.08$	5.4	-	-
2	$4.2X10^4 \pm 0.08^{***}$	$10.5X10^4 \pm 0.01^{***}$	$14.9 \times 10^3 \pm 0.004^{***}$	$16.8X10^4 \pm 0.16^{***}$	$25.6X10^4 \pm 0.04^{***}$	$28.4 \times 10^4 \pm 0.15^{***}$	5.2	-	-
3	$3.6X10^4 \pm 0.01^{***}$	$8.1X10^3 \pm 0.03^{***}$	$6.1 \times 10^3 \pm 0.01^{***}$	$4.8X10^3 \pm 0.01^{***}$	$3.6X10^3 \pm 0.06^{***}$	$2.5 \times 10^3 \pm 0.02^{***}$	3.9	1.4	Slightly active
4	$1.6X10^4 \pm 0.007^{***}$	$5.1X10^3 \pm 0.04^{***}$	$3.4 \times 10^3 \pm 0.04^{***}$	$1.5X10^3 \pm 0.03^{***}$	$5.3X10^2 \pm 0.04^{***}$	$4.5 \times 10^2 \pm 0.03^{***}$	3.6	1.8	Active
5	$2.8X10^4 \pm 0.01^{***}$	$2.8X10^3 \pm 0.014^{***}$	$5.7 \times 10^3 \pm 0.01^{***}$	$4.1X10^3 \pm 0.01^{***}$	$3.2X10^3 \pm 0.01^{***}$	$2.7 \times 10^3 \pm 0.05^{***}$	3.9	1.5	active
6	$1.4X10^4 \pm 0.04^{***}$	$4.4X10^3 \pm 0.02^{***}$	$2.9 \times 10^3 \pm 0.01^{***}$	$0.9X10^3 \pm 0.01^{***}$	$4.2X10^2 \pm 0.04^{***}$	$1.6 \times 10^2 \pm 0.04^{***}$	3.6	1.8	active
7	$1.8X10^4 \pm 0.003^{c}$	$4.2X10^4 \pm 0.02^{c}$	$4.6 \times 10^3 \pm 0.03^{c}$	$5.1X10^3 \pm 0.04^{c}$	$5.5X10^3 \pm 0.08^{c}$	$3.2 \times 10^3 \pm 0.04^{c}$	4.1	1.1	Slightly active
8	$2.8X10^4 \pm 0.003^{c}$	$6.3X10^3 \pm 0.03^{c}$	$4.7 \times 10^3 \pm 0.03^{c}$	$2.6X10^3 \pm 0.03^{c}$	$1.1X10^3 \pm 0.02^{c}$	$2.6 \times 10^2 \pm 0.02^{c}$	3.8	1.4	Slightly active
9	$1.6X10^4 \pm 0.009^{c}$	$3.9X10^4 \pm 0.08^{c}$	$4.1 \times 10^3 \pm 0.03^{c}$	$4.9X10^3 \pm 0.02^{c}$	$4.1X10^3 \pm 0.03^{c}$	$2.9 \times 10^3 \pm 0.02^{c}$	4.1	1.1	Slightly active
10	$1.4X10^4 \pm 0.004^{c}$	$0.5X10^2 \pm 0.001^{c}$	$4.3 \times 10^3 \pm 0.03^{c}$	$2.1X10^3 \pm 0.02^{c}$	$1.1X10^3 \pm 0.01^{c}$	$2.3 \times 10^2 \pm 0.03^{c}$	3.7	1.5	active
11	$6.1 \times 10 \pm 0.05^{c}$	$7.7X10 \pm 0.05^{c}$	$6.7 \times 10^2 \pm 0.05^{c}$	$4.2X10^2 \pm 0.06^{c}$	$2.1X10^2 \pm 0.03^{c}$	Nil	2.8	2.4	active

contd.,

Data expressed in Mean ± SEM: ***p<0.001 vs. Group -1; ^{c}p<0.001vs. Group -2; Two-way RM ANOVA followed by Bonferroni post test

Results are expressed as Log control– Log treated: **Activity values, > 3.0 maximum activity; 3.0 – 1.5 active; 1.5 – 0.5 slightly active; < 0.5 not active.**

Strength of inoculam (*C.albicans*): $1x10^{7}$ cells/ml (48 h culture) Route of administration of inoculam: Intravaginal

Route of administration of drug: Intravaginal n: 6 (female)

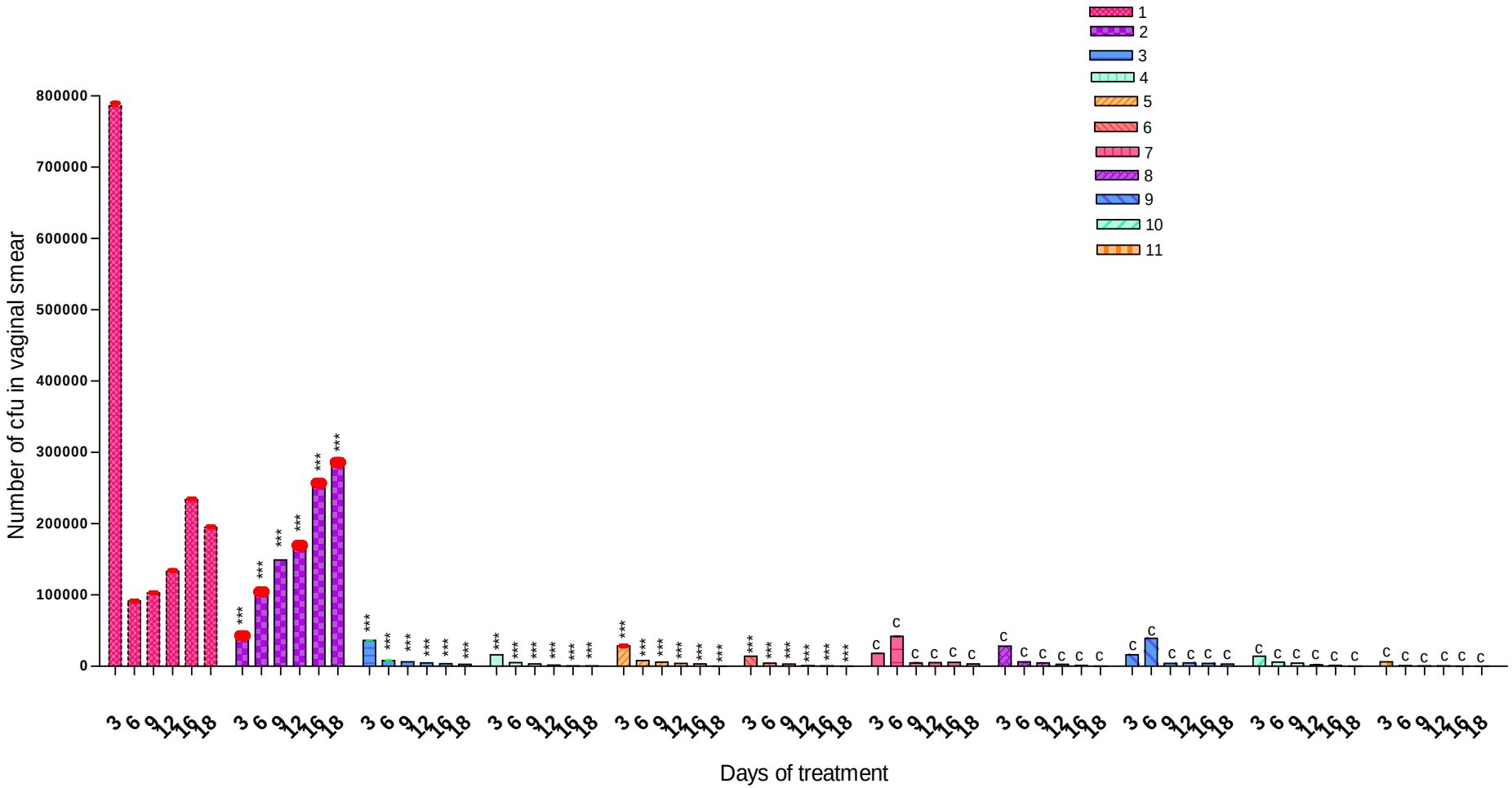
Group: 1- Control (cream base) (non IC); Group: 2- IC control (cream base); Group: 3- HEOS 2% cream (non IC)

Group: 4- HEOS 4% cream (non IC); Group: 5- HEOB 2% cream (non IC); Group: 6 – HEOB 4% cream (non IC)

Group: 7- HEOS 2% cream (IC); Group: 8- HEOS 4% cream (IC): Group: 9- HEOB 2% cream (IC)

Group: 10 – HEOB 4% cream (IC); Group: 11- Ketoconozole 2% cream (IC)

Figure 23: Effect of the extracts against experimental vaginal candidiasis in normal and immunocompromised mice



Data expressed in Mean ± SEM: ***p<0.001 vs. Group -1; [c]p<0.001vs. Group -2; Two-way RM ANOVA followed by Bonferroni post test

Results are expressed as Log control– Log treated: **Activity values, > 3.0 maximum activity; 3.0 – 1.5 active; 1.5 – 0.5 slightly active; < 0.5 not active.**

Strength of inoculam (*C.albicans*): $1x10^7$ cells/ml (48 h culture)

Route of administration of inoculam: Intravaginal

Route of administration of drug: Intravaginal

n: 6 (female)

contd.,

Group: 1- Control (cream base) (non IC); Group: 2- IC control (cream base); Group: 3- HEOS 2% cream (non IC)

Group: 4- HEOS 4% cream (non IC); Group: 5- HEOB 2% cream (non IC); Group: 6 – HEOB 4% cream (non IC)

Group: 7- HEOS 2% cream (IC); Group: 8- HEOS 4% cream (IC): Group: 9- HEOB 2% cream (IC)

Group: 10 – HEOB 4% cream (IC); Group: 11- Ketoconozole 2% cream (IC)

Table 24: Statistical analysis of repeated measures Two way ANOVA applied for body weights of non immunocompromised and immunocompromised mice treated with the extracts in experimental vaginal candidiasis

S.No	Parameters	Percentage Variance	F Value	DF	P value
1	Treatment vs. days	16.76	2.94	50	<0.0001
2	Days vs. result	8.76	15.36	5	<0.0001
3	Treatment vs. result	30.29	13.01	10	<0.0001
4	Effective matching	-	2.04	55	<0.0001

Table 24a: Body weights of mice treated with the extracts in experimental vaginal candidiasis in both normal and immunocompromised condition

Groups	Day 0	Day3	Day 6	Day 9	Day 15	Day 18
1	27.5±0.3	26.7±0.4	25.7±0.6	21.3±0.5***	19.3±0.5***	17.7±0.4***
2	28.0±1.3	26.1±1.3	25.0±1.2	22.8±1.2***	21.7±1.0***	19.0±0.4***
3	25.0±0.6	23.5±0.7	22.2±0.6	22.8±1.3	20.0±0.4**	20.1±0.4**
4	24.9±1.7	25.3±1.6	25.2±1.5	25.3±1.7	25.2±1.3	25.4±1.5
5	26.4±0.9	26.6±0.7	26.0±0.6	25.4±0.7	26.1±1.0	26.0±0.6
6	23.3±1.4	22.2±1.2	21.1±1.2	20.4±1.0	21.4±1.3	25.0±1.7
7	27.0±0.5	26.8±0.5	25.4±0.4	25.0±0.4	25.5±0.8	26.8±0.8
8	25.0±0.6	24.9±0.6	24.8±1.0	25.0±0.4	24.8±0.8	25.0±0.8
9	26.4±1.1	26.2±1.4	26.0±0.8	26.0±0.8	26.2±1.1	26.1±1.2
10	23.8±1.0	22.6±0.9	21.4±0.8	20.8±1.0	21.8±0.8	21.7±0.4
11	28.0±1.1	27.8±0.6	26.3±1.5	24.9±1.0	216.8±0.4	28.0±0.8

Data expressed as Mean ±SEM: **p<0.01, ***p<0.001 vs. 0 day of respective groups; Two-way RM ANOVA followed by Bonferroni post test; Strength of inoculam (*C.albicans*): $1x10^7$ cells/ml (48 h culture); Route of administration of inoculam: Intravaginal

Route of administration of drug: Intravaginal n: 6 (female)

Group: 1- Control (cream base) (non IC); Group: 2- IC control (cream base); Group: 3- HEOS 2% cream (non IC)

Group: 4- HEOS 4% cream (non IC); Group: 5- HEOB 2% cream (non IC); Group: 6 – HEOB 4% cream (non IC)

Group: 7- HEOS 2% cream (IC); Group: 8- HEOS 4% cream (IC): Group: 9- HEOB 2% cream (IC)

Group: 10 – HEOB 4% cream (IC); Group: 11- Ketoconozole 2% cream (IC)

Table 25: Total and differential leukocyte count of non immunocompromised and immunocompromised mice treated with the extracts in experimental vaginal candidiasis

Groups	TLC (mm^3)		Neutrophils (%)		Lymphocytes (%)		Eosinophils(%)		Monocytes (%)	
	Day 0	Day 18	Day 0	Day 18	Day 0	Day 18	Day 0	Day 18	Day 0	Day 18
1	9140±3.1	5269±3.3***	31.0±1.1	41.17±0.3***	61.0±4.3	49.7±0.3***	4.0±0.6	2.0±0.6	4.0±0.6	7.3±0.6***
2	9043±2.1	5433±176.9***	30.0±0.8	44.0±4.3***	65.5±0.7	47.54±0.4***	3.0±0.4	2.0±0.6	2.0±0.3	7.7±0.4***
3	9142±3.2	9196±170.0	34.0±2.0	29.7±2.4	61.0±1.3	64.2±0.5	2.0±0.4	3.0±0.4	2.8±0.5	3.0±0.7
4	9545±2.2	9066±76.20	37.0±1.7	34.0±3.4	60.0±1.0	53.2±0.5	1.0±0.3	1.0±0.3	2.0±0.3	2.0±0.6
5	9333±0.8	9355±5.60	38.0±1.3	32.0±3.0	56.0±0.7	61.2±0.7	3.0±0.7	3.0±0.3	3.0±0.4	4.0±0.3
6	8269±19.1	8100±22.74	37.0±2.5	30.0±3.4	60.0±3.4	66.7±0.6	1.0±0.4	1.0±0.4	2.0±0.8	2.0±0.4
7	9283±3.6	9250±334.3	38.0±2.8	32.0±2.2	56.0±1.6	61.0±2.0	3.0±0.7	3.0±0.3	3.0±0.7	4.0±0.8
8	9620±35.1	9257±152.0	38.0±2.4	35.7±1.8	58.7±0.3	59.0±0.6	2.0±0.7	3.0±0.7	2.0±0.6	2.0±0.4
9	9620±35.1	9293±119.5	39.0±2.9	34.0±1.5	53.5±0.4	58.7±0.8	4.2±0.87	3.0±0.7	3.3±0.5	4.0±0.9
10	8400±15.3	8503±132.1	33.0±1.1	32.3±1.6	61.7±4.4	61.5±0.6	2.0±0.4	2.0±0.4	3.0±0.7	3.0±0.9
11	9375±26.7	9340±241.4	30.2±1.6	33.0±1.1	52.2±1.1	59.0±1.1	4.0±1.0	4.0±0.4	3.0±1.0	4.0±0.5

Data expressed as Mean ±SEM: ***p<0.001 vs. 0 day of respective groups; Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculam (*C.albicans*): $1x10^7$ cells/ml (48 h culture) Route of administration of inoculam: Intravaginal

Route of administration of drug: Intravaginal n: 6 (female)

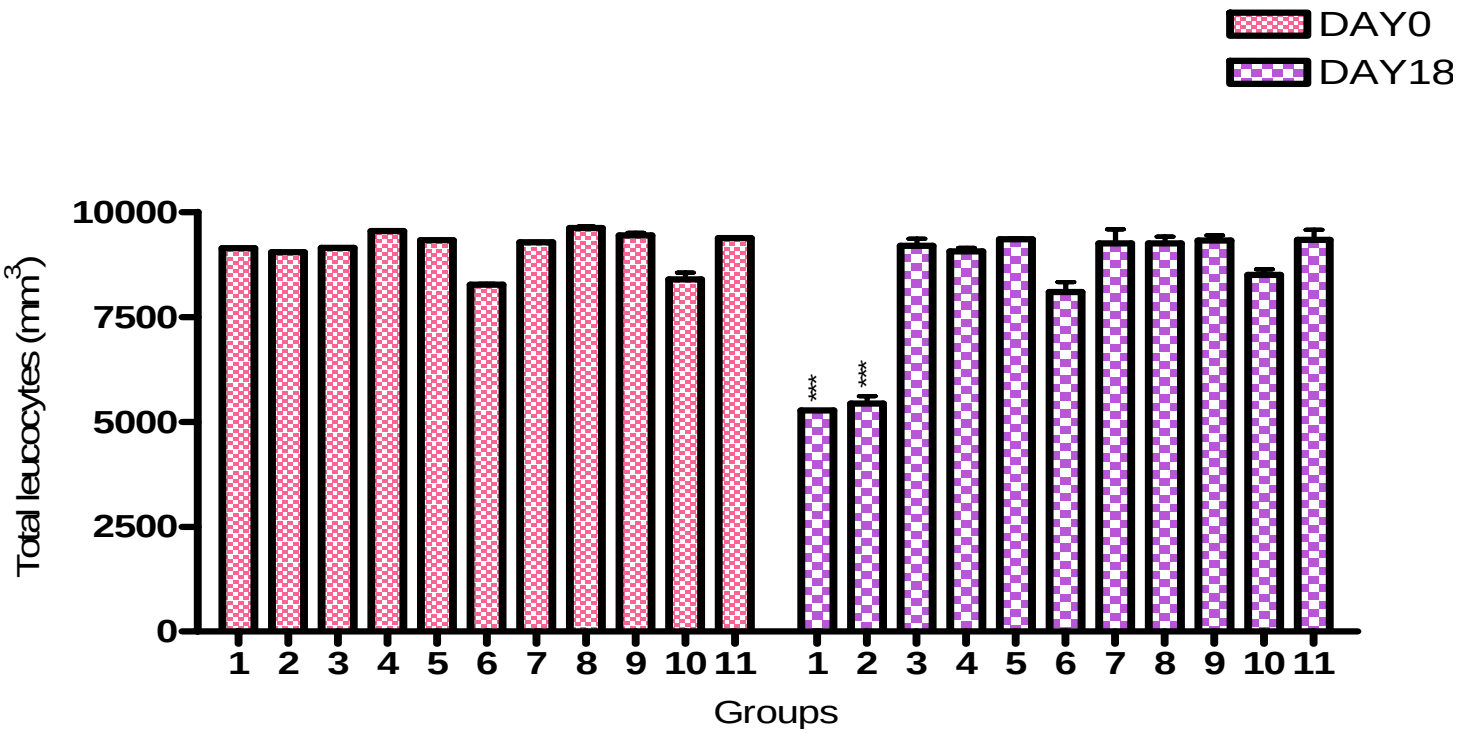
Group: 1- Control (cream base) (non IC); Group: 2- IC control (cream base); Group: 3- HEOS 2% cream (non IC)

Group: 4- HEOS 4% cream (non IC); Group: 5- HEOB 2% cream (non IC); Group: 6 – HEOB 4% cream (non IC)

Group: 7- HEOS 2% cream (IC); Group: 8- HEOS 4% cream (IC): Group: 9- HEOB 2% cream (IC)

Group: 10 – HEOB 4% cream (IC); Group: 11- Ketoconozole 2% cream (IC)

Figure 24: Total leukocyte count of in non immunocompromised and immunocompromised mice treated with the extracts in experimental vaginal candidiasis



Data expressed as Mean ±SEM: ***p<0.001 vs. 0 day of respective groups; Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculam (*C.albicans*): $1x10^7$ cells/ml (48 h culture) Route of administration of inoculam: Intravaginal

Route of administration of drug: Intravaginal n: 6 (female)

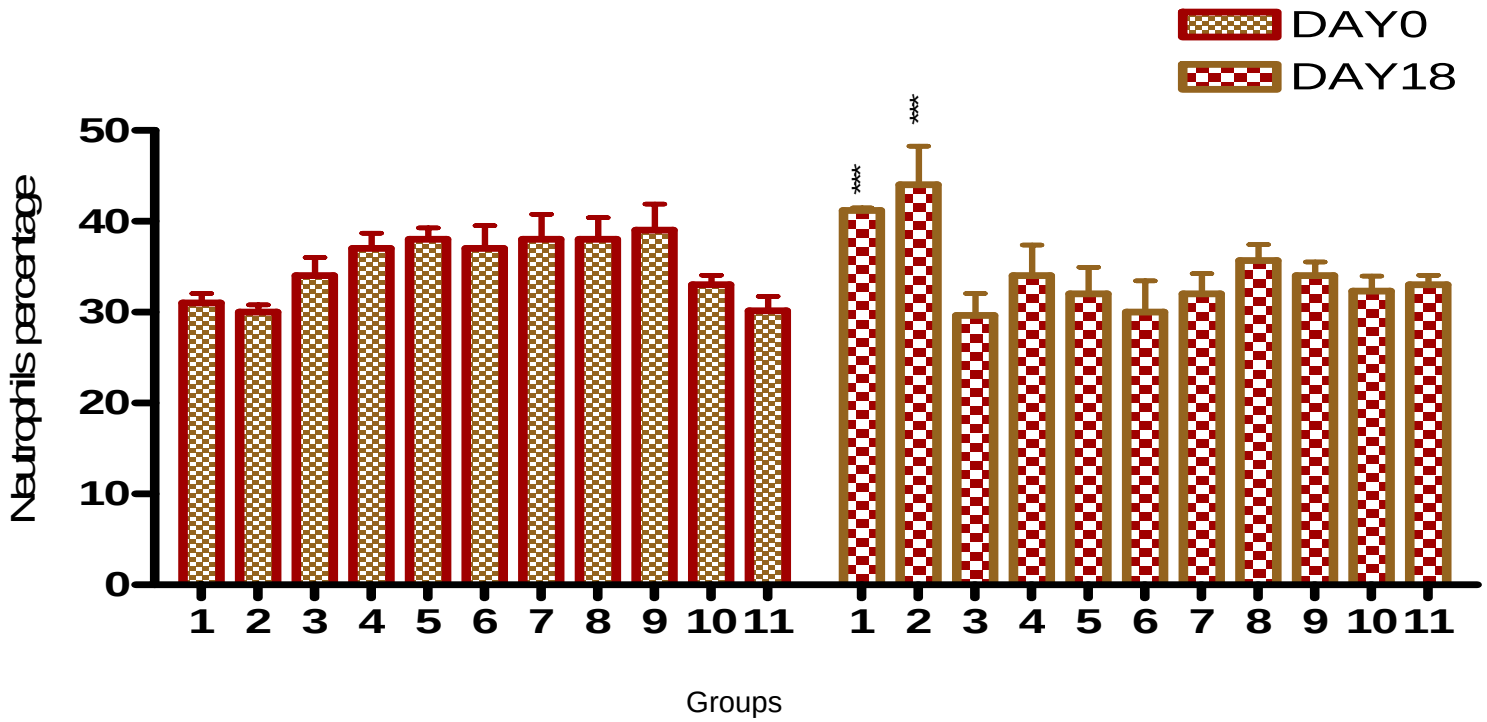
Group: 1- Control (cream base) (non IC); Group: 2- IC control (cream base); Group: 3- HEOS 2% cream (non IC)

Group: 4- HEOS 4% cream (non IC); Group: 5- HEOB 2% cream (non IC); Group: 6 – HEOB 4% cream (non IC)

Group: 7- HEOS 2% cream (IC); Group: 8- HEOS 4% cream (IC): Group: 9- HEOB 2% cream (IC)

Group: 10 – HEOB 4% cream (IC); Group: 11- Ketoconozole 2% cream (IC)

Figure 25: Percentage neutrophils of non immunocompromised and immunocompromised mice treated with the extracts in experimental vaginal candidiasis



Data expressed as Mean ±SEM: ***p<0.001 vs. 0 day of respective groups; Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculam (*C.albicans*): $1x10^7$ cells/ml (48 h culture) Route of administration of inoculam: Intravaginal

Route of administration of drug: Intravaginal n: 6 (female)

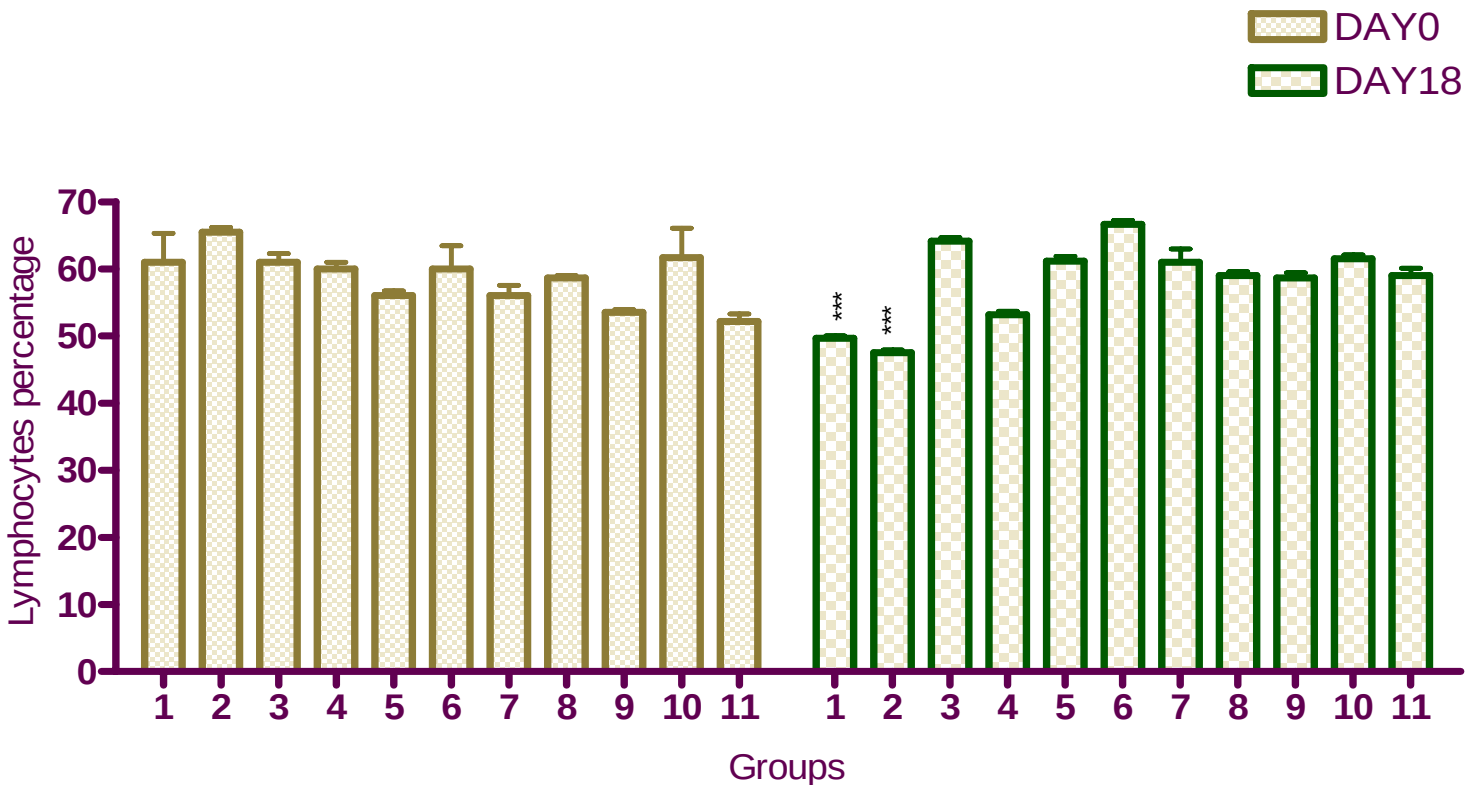
Group: 1- Control (cream base) (non IC); Group: 2- IC control (cream base); Group: 3- HEOS 2% cream (non IC)

Group: 4- HEOS 4% cream (non IC); Group: 5- HEOB 2% cream (non IC); Group: 6 – HEOB 4% cream (non IC)

Group: 7- HEOS 2% cream (IC); Group: 8- HEOS 4% cream (IC): Group: 9- HEOB 2% cream (IC)

Group: 10 – HEOB 4% cream (IC); Group: 11- Ketoconozole 2% cream (IC)

Figure 26: Percentage lymphocytes of non immunocompromised and immunocompromised mice treated with the extracts in experimental vaginal candidiasis



Data expressed as Mean ±SEM: ***p<0.001 vs. 0 day of respective groups; Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculam (*C.albicans*): $1x10^7$ cells/ml (48 h culture) Route of administration of inoculam: Intravaginal

Route of administration of drug: Intravaginal n: 6 (female)

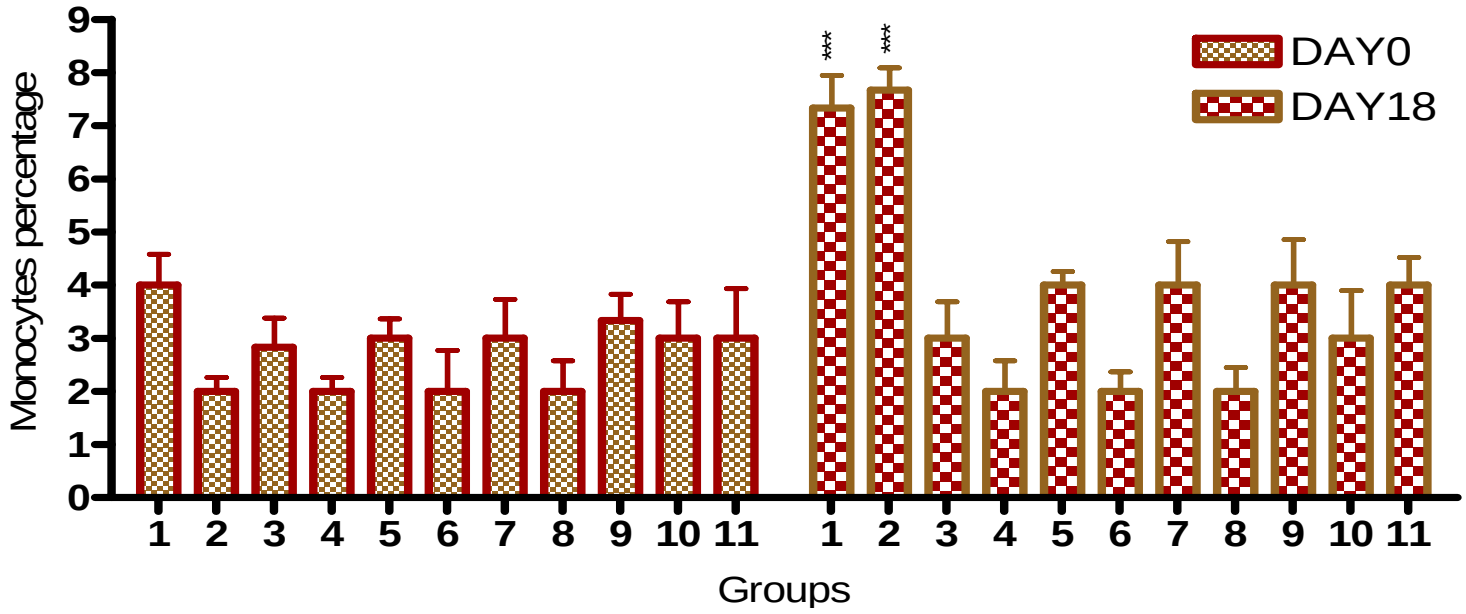
Group: 1- Control (cream base) (non IC); Group: 2- IC control (cream base); Group: 3- HEOS 2% cream (non IC)

Group: 4- HEOS 4% cream (non IC); Group: 5- HEOB 2% cream (non IC); Group: 6 – HEOB 4% cream (non IC)

Group: 7- HEOS 2% cream (IC); Group: 8- HEOS 4% cream (IC): Group: 9- HEOB 2% cream (IC)

Group: 10 – HEOB 4% cream (IC); Group: 11- Ketoconozole 2% cream (IC)

Figure 27: Percentage monocytes of non immunocompromised and immunocompromised mice treated with the extracts in experimental vaginal candidiasis



Data expressed as Mean ±SEM: ***p<0.001 vs. 0 day of respective groups; Two-way RM ANOVA followed by Bonferroni post test

Strength of inoculam (*C.albicans*): $1x10^7$ cells/ml (48 h culture) Route of administration of inoculam: Intravaginal

Route of administration of drug: Intravaginal n: 6 (female)

Group: 1- Control (cream base) (non IC); Group: 2- IC control (cream base); Group: 3- HEOS 2% cream (non IC)

Group: 4- HEOS 4% cream (non IC); Group: 5- HEOB 2% cream (non IC); Group: 6 – HEOB 4% cream (non IC)

Group: 7- HEOS 2% cream (IC); Group: 8- HEOS 4% cream (IC): Group: 9- HEOB 2% cream (IC)

Group: 10 – HEOB 4% cream (IC); Group: 11- Ketoconozole 2% cream (IC)

4.11. Antimycotic activity (hair root invasion) of the extracts in guinea pigs

In this method a primary infection is induced with *C.albicans,* which leads to an uniform invasion of hair follicles over the infected area. The efficacy of the test drugs is expressed both clinically and mycologically in terms of percentage, the results obtained in the prior study are given in Table 26. The results reveal that HEOS 4% cream possess 55.88 % of mycological activity and 44.41% of clinical efficacy, and the 2% cream 50% mycological activity and 55.55% clinical efficacy. Mycological activity and clinical efficacy of 64.7% and 66.66% respectively, are observed for 4% HEOB cream. HEOB cream (2%) shows 58.82% mycological activity and 55.55% clinical efficacy when compared to the control (cream base). Both plant extracts markedly decrease erythema and scale induced by *C.albicans* in guinea pigs at tested dose levels. However, ketoconozole possesses maximum percentage of mycological (70.88) and clinical (66.66) efficacy when compared to the plant extracts.

Table 26: Antimycotic activity (hair root invasion) of the extracts in guinea pigs

S. No	Groups	Mycological positive hair sample (mean)	Clinical scoring (mean)	% efficacy	
				Mycological	Clinical
1	Control (cream base)	34	03	-	-
2	HEOS *2* % cream	17	1.66	50.00	55.55
3	HEOS *4*% cream	15	1.33	55.88	44.44
4	HEOB 2% cream	14	1.33	58.82	55.55
5	HEOB 4% cream	12	01	64.70	66.66
6	Ketoconozole 2% cream	10	01	70.58	66.66

Strength of inoculum (*C.albicans*): $1x10^7$ cfu/ml (48 h culture); Route of administration of inoculam and drug: Topical; n: 3 (male)

Score	Activity
0	no findings
1	few erythmatous patches
2	well defined redness scaly area
3	large scale of marked redness, incrustation, scaling, bold and untreated patches

4.12. Effect of the extracts on infected wound in excision model in rats

Effect of HEOS and HEOB on infected wound in excision model in rats was studied and the results obtained are given in Table 27 and Figure 28. The results reveal a significant ($p<0.001$) wound contraction on day 4, 8, 12 and 16 in rats treated topically with the extracts 2% and 4% creams when compared to the control. Similar findings are observed in nitrofurazone treated rats. The rate of wound contraction is maximum on day 8 in rats treated with 4% HEOB cream, when compared with other groups. On day 16 maximum wound contraction is seen in all drug treated groups when compared to the control.

Hydroxyproline, SOD and lipid peroxides level were estimated in regenerated wound skin on day 8, 16 and 21. Also histopathology of regenerated wound skin was carried out on day 8, 16 and 21. The results in Table 28 indicate a significant ($p<0.001$and $p<0.01$) increase in hydroxyproline content on day 8 in rats treated with the extracts 2% and 4% creams. A significant ($p<0.01$) decrease in lipid peroxides in rats treated with HEOB 4% cream is also observed. However, a significant ($p<0.05$) decrease in the level of lipid peroxides is recorded in HEOS and HEOB 2% cream treated rats. Nitrofurazone treated rats shows a significant ($p<0.001$) increase in hydroxyproline content and a significant ($p<0.01$) decrease in lipid peroxides level when compared to the control.

The results obtained on the histopathology of regenerated skin on day 8 are given in Table 29 and Plates 79-85. The data reveal a faster epithelization and capillary proliferation with maximum degree of fibroblasts deposition, besides lesser degree of inflammation and lower extent of migration of polymorphs lymphocytes and macrophages in rats treated topically with the extracts 4% cream, whereas, the extracts 2% cream treated rats reveal lesser degree of capillary proliferation and migration of polymorphs, lymphocytes, macrophages and fibroblast when compared to 4% cream.

The observations on day 16 given in Table 30 indicate no significant change in hydroxyproline content suggesting that the levels were almost normal. However, a significant ($p<0.001$ and $p<0.05$) decrease in the levels of lipid peroxides level is observed in all drug treated groups when compared to the control. The histopathology

report in Table 31 and Plates 86-92 reveal almost completely healed lesion with minimal inflammation in HEOB 4% and nitrofurazone treated rats. Reepithelization, capillary proliferation, fibroblasts deposition and migration of leucocytes with inflammation appear in HEOS 2%, 4% and HEOB 2% treated rats.

Similar findings on hydroxyproline content and lipid peroxides level are observed on day 21. Histopathology of skin reveals moderate inflammation, migration of polymorphs, lymphocytes, macrophages, fibroblasts and capillary proliferation in control rats. HEOS 2% and 4% cream and HEOB 2% cream treated rats show mild inflammation, migration of fibroblasts, migration of polymorphs, lymphocytes and macrophages and minimal capillary proliferation. Completely healed wound is observed in HEOB 4% and nitrofurazone treated rats (Tables 32 and 33 and Plates 93-99). No significant change in SOD levels is observed in all drug treated rats at tested dose levels during the experimental period.

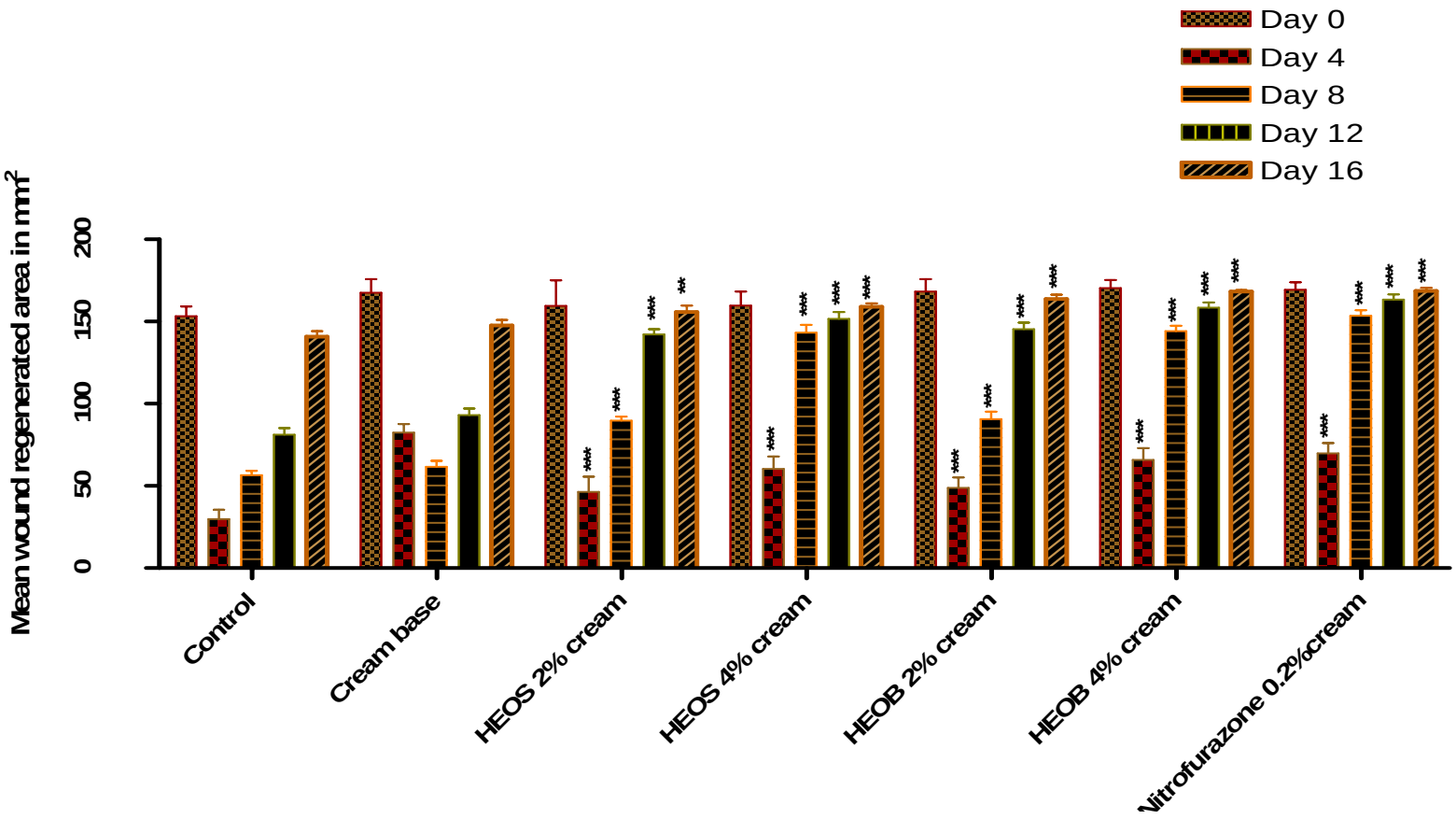
Table 27: Effect of the extracts on infected wound in excision model in rats

Groups	Wound area on day 0	Mean wound regenerated area in mm^2			
		Day 4	Day 8	Day 12	Day 16
Control	153.3±5.9	29.8±5.8	56.5±2.6	81.2±4.0	140.8±3.4
Cream base	167.5±8.3	82.5±5.1	61.7±3.6	93.0±4.0	147.7±3.4
HEOS 2% cream	159.5±15.6	46.3±9.2***	89.7±2.5***	142.2±3.2***	155.8±3.9**
HEOS 4% cream	159.7±8.6	60.3±7.6***	143.3±4.7***	151.7±4.0***	159.0±2.1***
HEOB 2% cream	168.3±7.5	48.7±6.5***	90.7±4.4***	145.3±4.0***	163.8±2.6***
HEOB 4% cream	170.3±4.9	65.8±7.2***	144.3±3.2***	158.5±3.2***	168.3±1.0***
Nitrofurazone cream (0.2%)	169.5±4.4	69.7±6.4***	153.5±3.5***	163.3±3.2***	168.7±1.8***

Data expressed as mean ±SEM: ** $p < 0.01$, *** $p < 0.001$ vs. Control; One way ANOVA followed by Dunnett post test)

Route of administration of drug: Topical

Figure 28: Effect of the extracts on infected wound in excision model in rats



Data expressed as mean ±SEM: ** p < 0.01, *** p < 0.001 vs. Control; One way ANOVA followed by Dunnett post test

Route of administration of drug: Topical

Table 28: Effect of the extracts on biochemical parameters of infected wound in excision model in rats on day 8

Groups	**Hydroxyproline µg/g tissue**	**SOD Units/mg protein**	**TBARS µ moles/mg protein**
Control	923±49.0	6.03±0.3	50.6±2.2
Cream base	959.15±11.8	5.4±0.8	34.05±5.3
HEOS 2% cream	2031.85±44.5***	5.39±.07	24.25±2.4*
HEOS 4% cream	5097.35±15.5***	6.1±0.6	21±2.6*
HEOB 2% cream	2012.68± 23.6**	5.6±1.0	22.16±1.9*
HEOB 4% cream	5372.4±125.0***	5.97±0.3	16.8±0.4**
Nitrofurazone (0.2%)	5332.4±118.7***	5.11±0.4	17.1±1.3**

Data expressed as mean ± SEM: * p < 0.05, ** p < 0.01 *** p< 0.001 vs. Control; Unpaired Student's *t* test

Table 29: Histological features of rat skin treated with the extracts in infected wound in excision model on day 8

Treatment	Degree of inflammation	Degree of Polymorphs	Degree of lymphocytes	Degree of macrophages	Degree of epithelization	Degree of capillary proliferation	Degree of Fibroblasts
Control	++++	++++	++	+	-	+	+
Cream base	+++	++++	+	-	-	+	-
HEOS 2% cream	++	+++	+	+	-	++	++
HEOS 4% cream	++	++	-	-	-	+++	++++
HEOB 2% cream	++	++	-	-	-	+++	++++
HEOB 4% cream	+	+	-	-	-	+++	++++
Nitrofurazone (0.2%)	Minimal inflammation				++++	+++	++++

Minimal - +

Moderate - ++

More than +++ - maximum

Table 30: Effect of the extracts on infected wound in excision model in rats on day 16

Groups	Hydroxyproline µg/g tissue	SOD Units/mg protein	TBARS µ moles/mg protein
Control	429.85±31.0	6.5±0.3	44.33±3.6
Cream base	387.48±12.1	5.5±0.3	36.7±2.5
HEOS 2% cream	361.01±7.5	5.73±0.4	26.57±1.5*
HEOS 4% cream	366.34±11.4	5.5±0.3	19.07±1.6***
HEOB 2% cream	359.21±6.5	5.25±0.7	24.6±1.9*
HEOB 4% cream	358.83±12.0	6.4±0.3	14.85±0.9***
Nitrofurazone (0.2%)	360.1±12.6	5.43±0.05	16.45±0.4***

Data expressed as mean ± SEM: * p < 0.05, *** p< 0.001 vs. Control;Unpaired Student's *t* test

Table 31: Histological features of rat skin treated with the extracts in infected wound in excision model on day 16

Treatment	Degree of inflammation	Degree of Polymorphs	Degree of lymphocytes	Degree of macrophages	Degree of epithelization	Degree of capillary proliferation	Degree of Fibroblasts
Control	++	+++	+	+	-	+	+
Cream base	++	++	+	+	-	++	+++
HEOS 2% cream	+	+	+	+	+	+	+++
HEOS 4% cream	+	-	+	-	+	++	+++
HEOB 2% cream	+	+	+	+	+	+	+++
HEOB 4% cream	Very minimal inflammation, almost completely healed						
Nitrofurazone (0.2%)	Very minimal inflammation, almost completely healed						

Minimal - +

Moderate - ++

More than +++ - maximum

Table 32: Effect of the extracts on infected wound in excision model in rats on day 21

Groups	Hydroxyproline µg/g tissue	SOD Units/mg protein	TBARS µ moles/mg protein
Control	92.53±5.9	3.28±0.5	45.1±0.0
Cream base	82.00±3.4	2.60±0.2	33.7±5.8
HEOS 2% cream	76.5±3.9	2.9±0.2	23.3±0.9**
HEOS 4% cream	70.5±1.9	3.55±0.05	22.4±1.0**
HEOB 2% cream	78.9±2.6	3.12±0.2	24.62±2.4**
HEOB 4% cream	73.45±3.0	3.5±0.2	15.8±1.6**
Nitrofurazone (0.2%)	74.65±2.2	2.95±0.2	12.2±0.0***

Data expressed as mean ± SEM: ** p < 0.01 *** p< 0.001 vs. Control; Unpaired Student's *t* test

Table 33: Histological features of rat skin treated with the extracts in infected wound in excision model on day 21

Treatment	Degree of inflammation	Degree of Polymorphs	Degree of lymphocytes	Degree of macrophages	Degree of epithelization	Degree of capillary proliferation	Degree of Fibroblasts
Control	++	+	+	+	-	++	++
Cream base	++	+	+	+	-	++	+++
HEOS 2% cream	+	-	+	-	+	+++	+++
HEOS 4% cream	+	-	-	-	+++	+++	+++
HEOB 2% cream	+	-	+	-	+	+++	+++
HEOB 4% cream	Healed lesion, epithelization completed						
Nitrofurazone (0.2%)	Completed healed lesion						

Minimal - +

Moderate - ++

More than +++ - maximum

4.13. Effect of the extracts on infected wound in incision model in rats

In this model, tensile strength is observed on day 10 that demonstrates the degree of collagen deposition in wound healing. The data obtained in the present are given in Table 34 and Figure 29, the data reveal that the tensile strength increases significantly ($p<0.01$) in rats treated with the extracts 4% cream, and positive control, when compared with control. HEOS and HEOB 2% creams also exhibit a significant ($p<0.05$) increase in tensile strength. However, it is less when compared with 4% cream.

A significant ($p<0.01$) increase in hydroxyproline content and decrease in the level of lipid peroxides are recorded in the extracts 4% cream treated rats, whereas significant ($p<0.05$) increase in hydroxyproline content and decrease in lipid peroxides level is noted in HEOS and HEOB 2% treated rats. However, nitrofurazone treated rats show a significant ($p<0.001$) increase in hydroxyproline and a significant ($p<0.01$) decrease in level of lipid peroxides. The results are shown in Table 35.

The histopathology of regenerated skin exhibit (Table 36 and Plates 100-106) faster epithelization, neovasculization and migration of fibroblasts and no migration of polymorphs, lymphocytes and macrophages, but minimal inflammation in rats treated with the extracts 4% cream when compared to the control. The extracts cream (2%) also exhibit neovasculization and migration of fibroblasts in regenerated tissue. However, there is inflammation and migration of polymorphs, lymphocytes and macrophages. Nitrofurazone treated rats show completely healed wound.

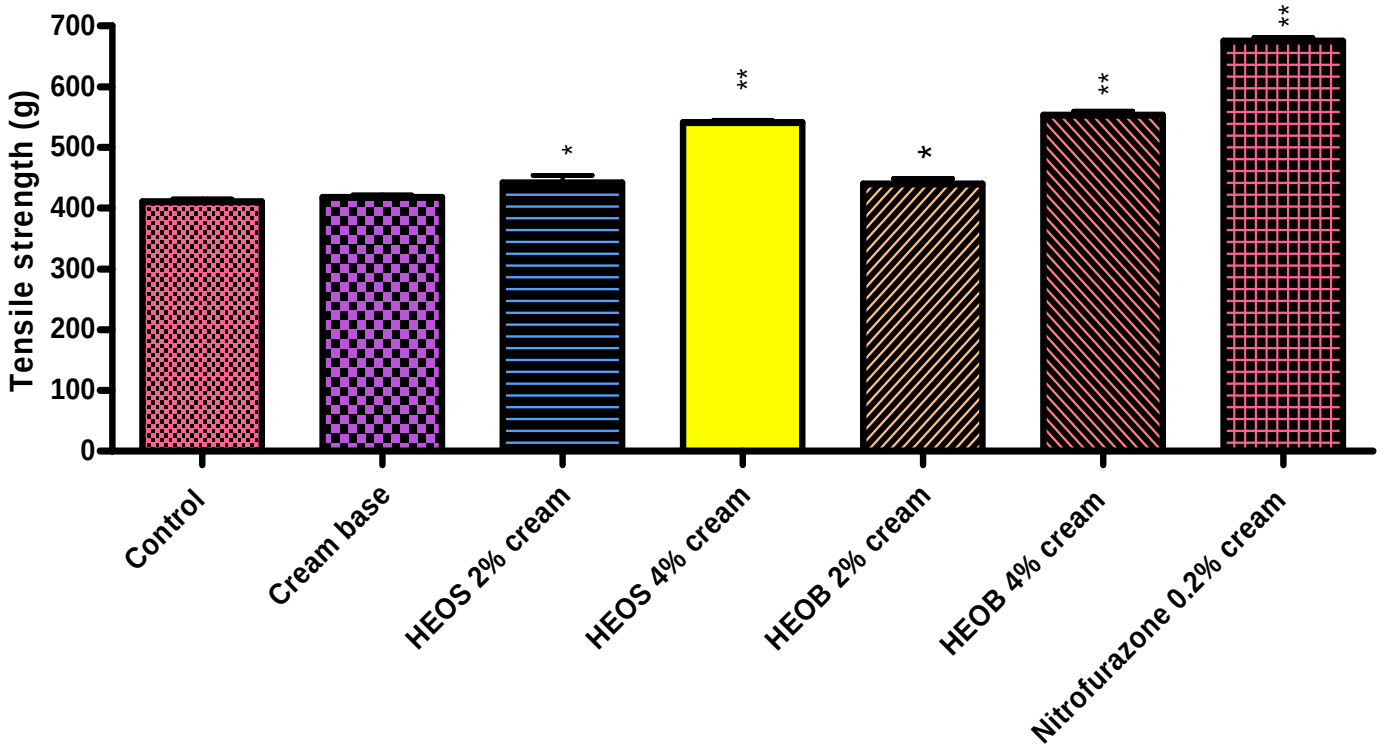
Table 34: Effect of the extracts on infected wound in incision model in rats on day 10

Treatment	Tensile strength (g)
Control	410.33 ± 4.69
Cream base	417.67 ± 4.02
HEOS 2% cream	441.67 ± 12.63*
HEOS 4% cream	540.67 ± 4.26**
HEOB 2% cream	439.92± 8.93*
HEOB 4% cream	553.5 ± 6.09**
Nitrofurazone (0.2%)	675.33 ± 5.38**

Data expressed mean ± SEM: * $p < 0.05$, ** $p < 0.01$ vs. Control; One way ANOVA followed by Dunnett post test

Route of administration of drug: Topical

Figure 29: Effect of the extracts on infected wound in incision model in rats on day 10



Data expressed mean ± SEM: * $p < 0.05$, ** $p < 0.01$ vs. Control; One way ANOVA followed by Dunnett post test

Route of administration of drug: Topical

Table 35: Effect of the extracts on biochemical parameters of rats in infected wound in incision model on day 10

Groups	Hydroxyproline µg/g tissue	SOD Units/mg protein	TBARS µ moles/mg protein
Control	430.75±8.9	4.8±0.20	37.42±1.39
Cream base	454.03±13.30	3.78±0.20	32.87±0.98
HEOS 2% cream	497.6±6.22*	4.9±0.17	27.9±1.25*
HEOS 4% cream	515.27±8.08**	4.72±0.25	18.61±0.96**
HEOB 2% cream	486.23±5.62*	4.82±0.16	29.67±2.1*
HEOB 4% cream	596.15±10.34**	5.46±0.09	7.38±0.53**
Nitrofurazone (0.2%)	779.00±12.811***	4.88±1.88	19.17±1.04**

Data expressed as mean ± SEM: * $p < 0.05$, ** $p < 0.01$ *** $p< 0.001$ vs. Control; Unpaired Student's *t* test

Table 36: Histological features of rat skin treated with the extracts in infected wound in incision model on day 10

Treatment	Degree of inflammation	Degree of Polymorphs	Degree of lymphocytes	Degree of macrophages	Degree of epithelization	Degree of capillary proliferation	Degree of Fibroblasts
Control	+++	+++	+	+	-	+	-
Cream base	++	++	++	+	-	++	++
HEOS 2% cream	++++	+++	+	+	-	++	++
HEOS 4% cream	++	-	-	-	-	++	+++
HEOB 2% cream	++++	+++	+	+	-	++	++
HEOB 4% cream	+	-	-	-	++	++	++++
Nitrofurazone (0.2%)	No lesions						

Minimal - +

Moderate - ++

More than +++ - maximum

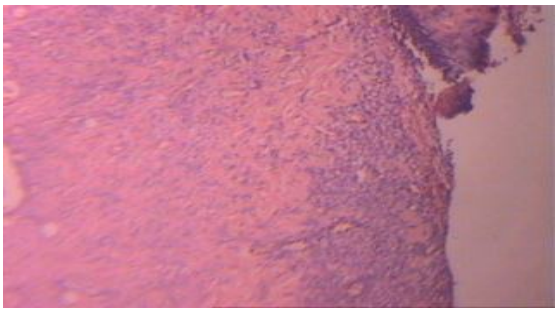


Plate 79. Histology of control rat skin in excision model with infected wound on day 8

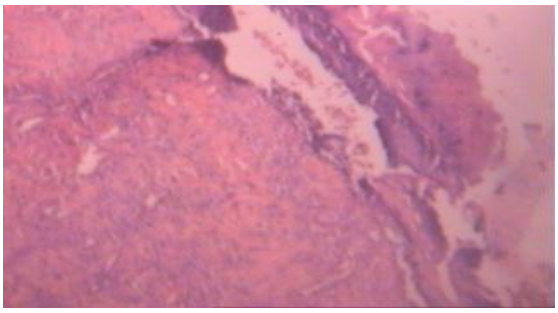


Plate 80. Histology of rat skin in excision model with infected wound treated with cream base on day 8

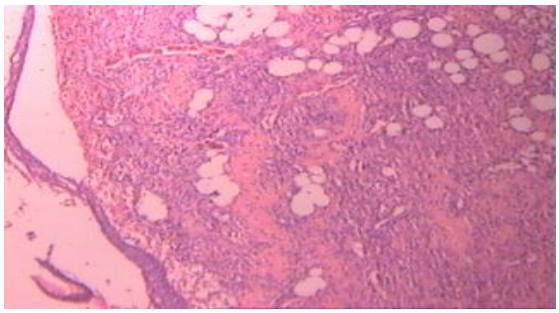


Plate 81. Histology of rat skin in excision model with infected wound treated with HEOS 2% cream on day 8

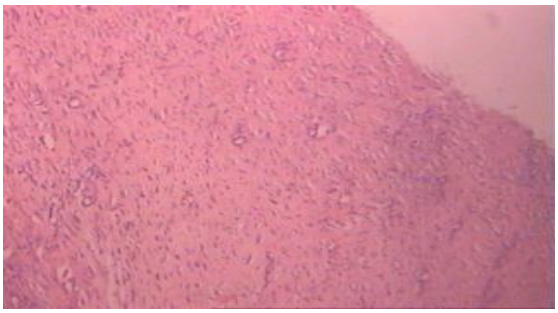


Plate 82. Histology of rat skin in excision model with infected wound treated with HEOS 4% cream on day 8

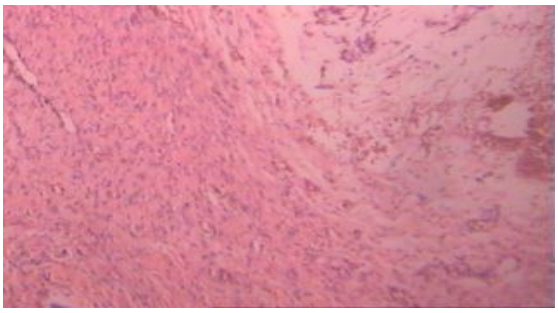


Plate 83. Histology of rat skin in excision model with infected wound treated with HEOB 2% cream on day 8

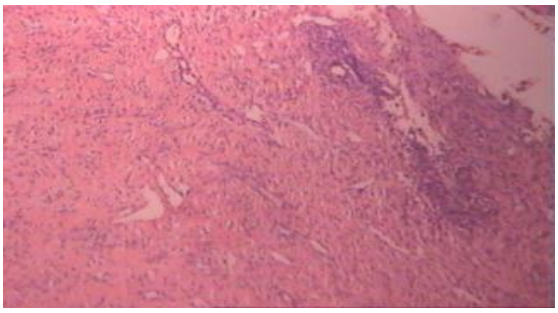


Plate 84. Histology of rat skin in excision model with infected wound treated with HEOB 4% cream on day 8

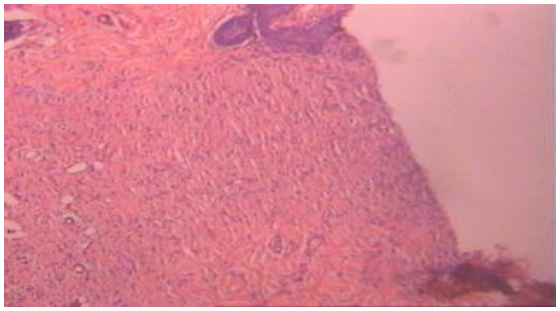


Plate 85. Histology of rat skin in excision model with infected wound treated with nitrofurazone (0.2% cream) on day 8

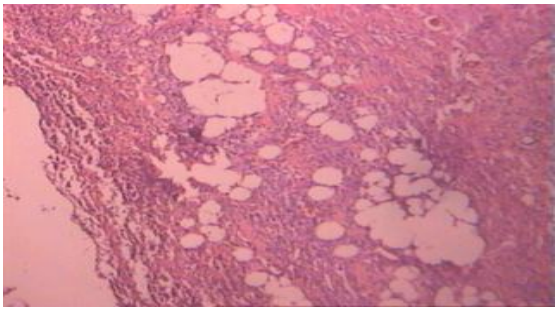


Plate 86. Histology of control rat skin in excision model with infected wound on day 16

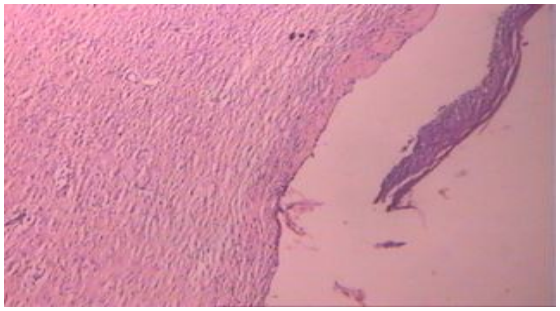


Plate 87. Histology of rat skin in excision model with infected wound treated with cream base on day 16

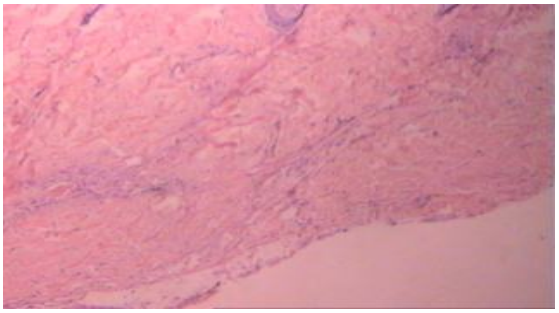


Plate 88. Histology of rat skin in excision model with infected wound treated with HEOS 2% cream on day 16

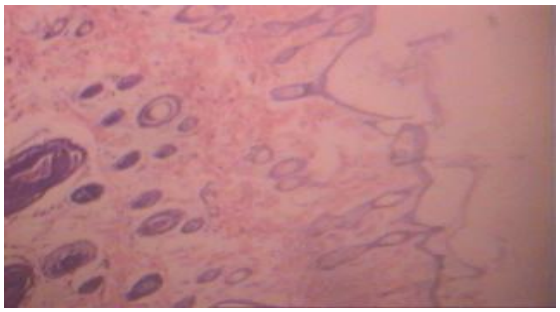


Plate 89. Histology of rat skin in excision model with infected wound treated with HEOS 4% cream on day 16

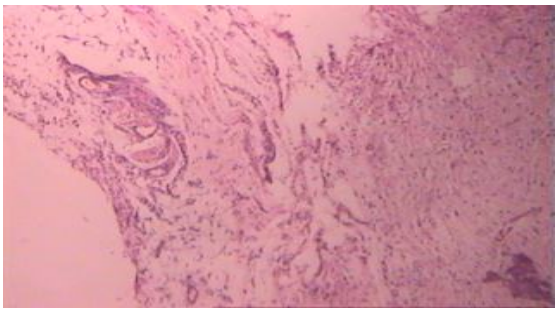


Plate 90. Histology of rat skin in excision model with infected wound treated with HEOB 2% cream on day 16

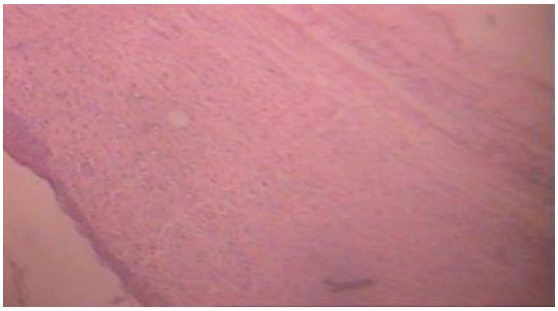


Plate 91. Histology of rat skin in excision model with infected wound treated with HEOB 4% cream on day 16

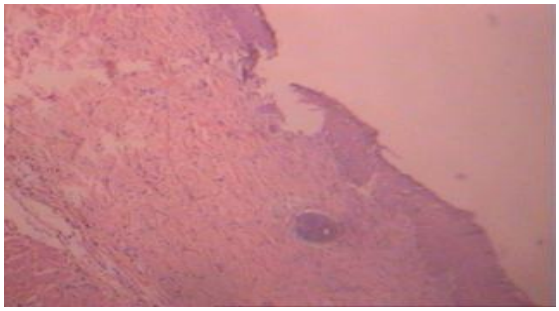


Plate 92. Histology of rat skin in excision model with infected wound treated with nitrofurazone (0.2% cream) on day 16

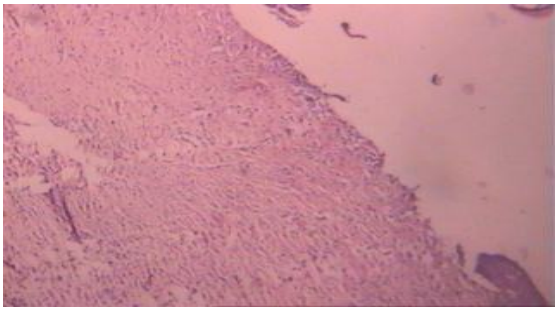


Plate 93. Histology of control rat skin in excision model with infected wound on day 21

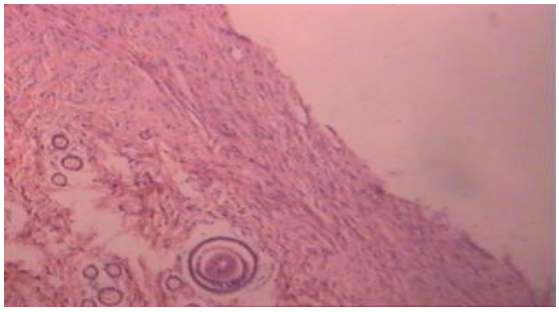


Plate 94. Histology of rat skin in excision model with infected wound treated with cream base on day 21

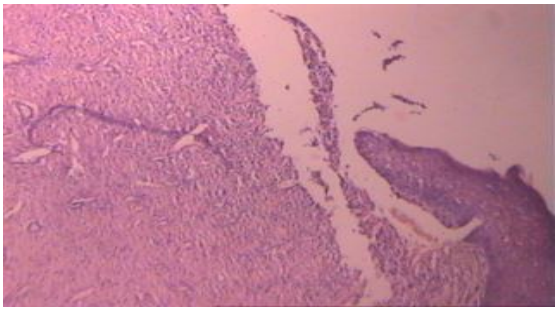


Plate 95. Histology of rat skin in excision model with infected wound treated with HEOS 2% cream on day 21

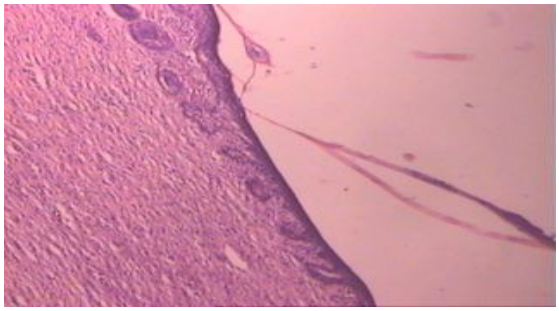


Plate 96. Histology of rat skin in excision model with infected wound treated with HEOS 4% cream on day 21

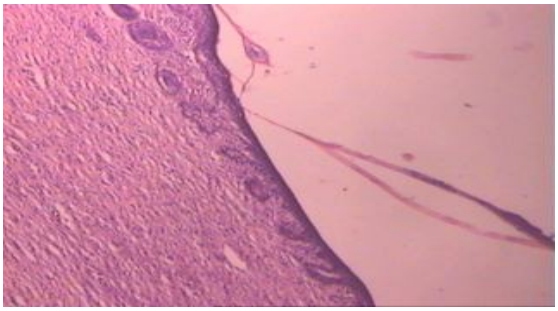


Plate 97. Histology of rat skin in excision model with infected wound treated with HEOB 2% cream on day 21

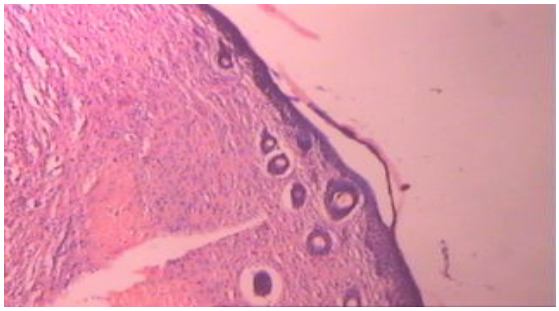


Plate 98. Histology of rat skin in excision model with infected wound treated with HEOB 4% cream on day 21

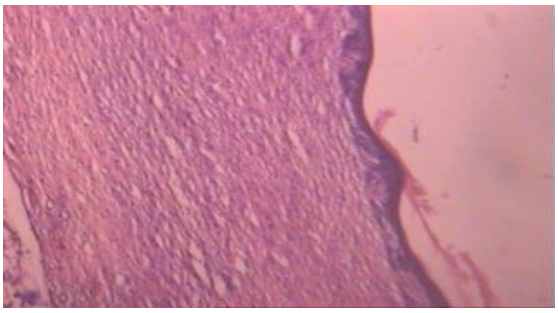


Plate 99. Histology of rat skin in excision model with infected wound treated with nitrofurazone (0.2% cream) on day 21

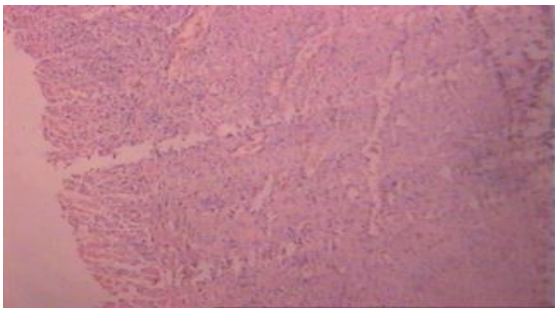


Plate 100. Histology of control rat skin in incision model with infected wound on day 10

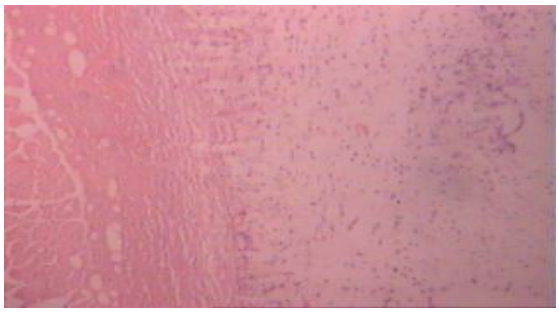


Plate 101. Histology of rat skin in incision model with infected wound treated with cream base on day 10

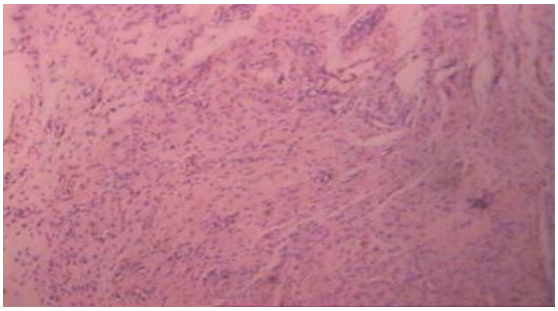


Plate 102. Histology of rat skin in incision model with infected wound treated with HEOS 2% cream on day 10

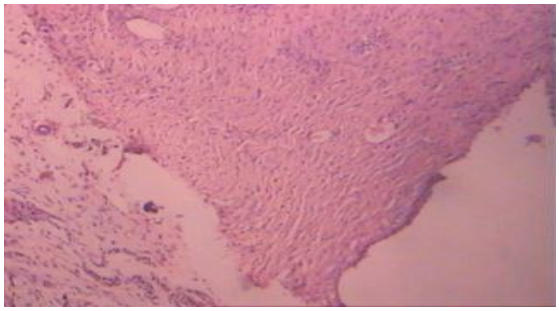


Plate 103. Histology of rat skin in incision model with infected wound treated with HEOS 4% cream on day 10

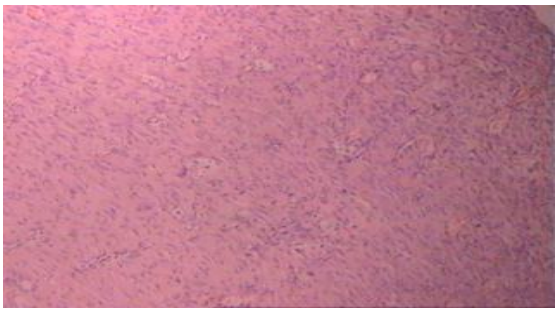


Plate 104. Histology of rat skin in incision model with infected wound treated with HEOB 2% cream on day 10

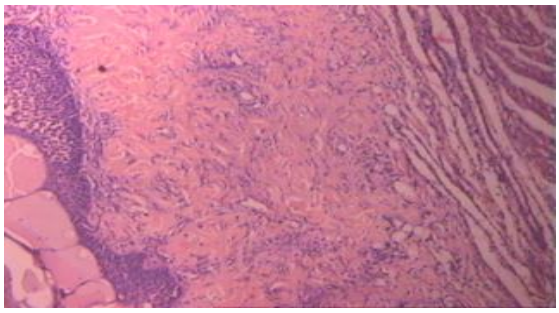


Plate 105. Histology of rat skin in incision model of with infected wound treated with HEOB 4% cream on day 10

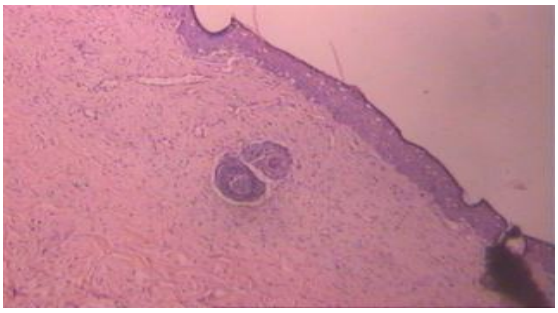


Plate 106. Histology of skin in incision model with infected wound treated with nitrofurazone (0.2% cream) on day 10

4.14. Effect of the extracts on repeated dose 28-day sub acute oral toxicity in rats

The data of body weight of rats in repeated dose 28 day sub acute toxicity test was subjected to repeated measures of Two way ANOVA and the results summarized as body weight versus treatment (% variance 0.46, f-0.39, df- 12 and $p < 0.9648$), treatment versus result (% variance 4.37, f-0.35, df- 3 and $p< 0.7881$), days versus result (% variance 4.40,f -11.01, df-4 and $p< 0.0001$), effective matching (f-41.45, df-20 and $p<0.0001$) are given in Table 37.

Table 37a indicates a significant ($p<0.01$) increase in body weight of rats treated with a high dose (800 mg/kg) and middle dose (400 mg/kg) of the extracts. There is no significant change in body weights of rats treated with low dose (200mg/kg). Table 38 shows the effect of the extracts on urine analysis. There is no prominent change in urine colour, appearance and pH. Tests for protein and glucose in urine are negative.

The hematological estimations and clinical biochemistry was carried out on termination of the study and the results obtained are given in Table 39 and 40, respectively. The results reveal a significant ($p<0.001$and $p<0.01$) increase in total leucocytes and platelet count in rats treated with HEOS and HEOB 800 and 400 mg/kg, respectively. Red blood corpuscles count and hemoglobin percentage increases significantly ($p<0.01$) in rats treated with the extracts 800 mg/kg. No significant change is observed in differential leucocyte count in all drug treated groups. The effect of the extracts on clinical biochemistry shows, a significant ($p<0.01$) increase in ALAT level in 800mg/kg of the extracts treated rats, when compared to control. No significant change is observed in all other parameters.

As shown in Table 41, there is no significant change in organ weights of rats treated with the extracts at tested dose levels. Histological sections of lung, kidney, brain, heart, spleen, testis and ovary show normal appearance in all drug treated rats. However, histology of liver show mild periportal and lobular inflammation in rats treated with high dose (800 mg/kg) of the extracts.

Table 37: Statistical analysis in repeated measure Two way ANOVA of body weight s of rats treated with the extracts on repeated dose 28 day sub acute oral toxicity

S. No	Parameters	Percentage variance	F Value	DF	P value
1	Body weight vs. treatment	0.46	0.39	12	0.9648
2	Treatment vs. results	4.37	0.35	3	0.7881
3	Days vs. results	4.40	11.01	4	0.0001
4	Effective matching	--	41.45	20	0.0001

Table 37a: Body weights of rats treated with the extracts on repeated dose 28 day sub acute oral toxicity

Groups	Day 0	Day 7	Day 14	Day 21	Day 28
Solvent control	144.7±2.0	145.3±1.9	145.7±1.7	146.2±1.8	146.3±1.8
HEOS 200mg/kg	147.5±2.8	148.5±3.7	149.8 ±3.2	149.3±3.2	150.8±3.9*
HEOS 400mg/kg	146.7±2.8	146.7±2.4	148.3±3.1	150.2±2.6	153.8±3.7**
HEOS 800mg/kg	146.3±2.0	147.0 ±2.2	149.2±2.6	152.5 ±2.6	155.8±3.0**
HEOB 200mg/kg	147.5±2.8	149.3±3.2	149.6±3.2	150.5±3.5	150.6±3.6
HEOB 400mg/kg	146.6±2.9	147.1±2.6	147.6±2.9	149.0±3.3	150.0±3.5*
HEOB 800mg/kg	146.3±2.0	149.0±1.4	149.5±2.6	151.1±3.0**	152.1±3.6**

Data expressed as mean ±SEM:*p<0.05; **p< 0.01 vs. 0 day of respective groups; Two way RM ANOVA followed by Bonferroni post test

Route of administration of drug: Oral n: 10 (both sex)

Table 38: Urine analysis of rats treated with the extracts in repeated dose 28 day sub acute oral toxicity

Groups	Colour	Appearance	pH	Protein	Glucose
Solvent control	Pale Yellow	Clear	7.4	Nil	Nil
HEOS 200mg/kg	Pale Yellow	Clear	7.3	Nil	Nil
HEOS 400mg/kg	Pale Yellow	Clear	7.2	Nil	Nil
HEOS 800mg/kg	Pale Yellow	Clear	7.6	Nil	Nil
HEOB 200mg/kg	Pale Yellow	Clear	7.8	Nil	Nil
HEOB 400mg/kg	Pale Yellow	Clear	7.4	Nil	Nil
HEOB 800mg/kg	Pale Yellow	Clear	7.3	Nil	Nil

Data expressed as mean ± SEM: One way- ANOVA followed by Dunnett post test

Route of administration of drug: Oral n: 10 (both sex)

Table 39: Haematological parameters of rats treated with the extracts on repeated dose 28 day sub acute oral toxicity

Groups	Hb (g %)	R.B.C(10^6cu. mm)	Platelet Count (10^6cu.mm)	T.L.C (10^3cu.mm)	Differential Count (%)			
					N	L	E	M
Solvent control	14.1 ±0.3	6417 ±174	219.2±0.6	5983±30.7	23.7±0.6	72.5±0.6	1.1±0.7	2.83±0.1
HEOS 200mg/kg	14.5 ±0.2	6633±156	223.0±1.8	6150±42.8	22.7±0.5	73.83±05	1.16±0.7	2.33±0.4
HEOS 400mg/kg	15.2 ±0.2*	7217±278	230.0±2.9**	6350±99.1**	22.0±0.3	73.83±0.3	1.17±0.1	2.83±0.1
HEOS 800mg/kg	15.6 ±0.2**	7550 ±251**	234.2±2.3***	6783±79.2***	24.0±0.0	73.2±0.37	0.83±0.1	2.0±0.3
HEOB 200mg/kg	14.5±0.2	6650±173	223.8±2.3	6183±40.14	22.83±0.3	73.00±0.3	1.167±0.1	2.83±0.2
HEOB 400mg/kg	15.2±0.2**	7250±308	231.2±2.6**	6400±124**	23.33±0.3	73.50±0.3	1.50±0.2	2.50±0.3
HEOB 800mg/kg	15.9±0.3**	7550±250**	236.2±2.3***	6867±33.3***	23.50±0.4	73.50±0.6	1.33±0.2	2.62±0.2

Data expressed as mean ± SEM: *p<0.05, **p< 0.01, ***p< 0.001 vs. Control; One way- ANOVA followed by Dunnett post test

Route of administration of drug: Oral n: 10 (both sex)

Table 40: Biochemical parameters of rats treated with the extracts on repeated dose 28 day sub acute oral toxicity

Parameters	Solvent control	HEOS 200mg/kg	HEOS 400mg/kg	HEOS 800mg/kg	HEOB 200mg/kg	HEOB 400mg/kg	HEOB 800mg/kg
Glucose (mg/dl)	184.8±8.2	185.2±8.1	186.2±8.3	187.7±8.5	185.5±8.2	186.9±8.5	187.8±8.3
ASAT(IU/L)	159.9±2.6	159.2±1.8	160.3±1.8	169.4±2.8	158.5±2.0	159.9±2.0	164.9±2.3
ALAT(IU/L)	38.21±0.4	38.30±0.4	39.02±0.7	45.96±0.3**	38.42±0.3	38.64±0.5	45.26±0.3**
Bilirubin (mg/dl)	0.24±0.1	0.26±0.0	0.27±0.0	0.31±0.0	0.24±0.0	0.31±0.0	0.38±0.06
Uric acid (mg/dl)	3.56±0.4	3.63±0.3	3.90±0.3	3.83±0.3	3.68±0.3	3.78±0.3	3.83±0.3
Creatinine (mg/dl)	0.91±0.0	0.92±0.0	0.95±0.0	0.96±0.0	0.92±0.0	0.94±0.0	0.96±0.0
GGT(U/L)	5.67±0.6	5.67±0.6	5.73±0.5	5.77±0.5	5.66±0.5	5.73±0.5	5.74±0.5
Alk. Phosphatase (IU/L)	36.76±0.9	37.20±0.6	37.52±0.3	38.73±0.3	37.86±0.5	37.69±0.4	37.89±0.4
Cholesterol (g/dl)	87.87±1.6	89.69±2.2	89.50±2.3	90.75±2.5	88.36±1.4	87.84±1.7	88.42±1.8
Total protein (g/dl)	6.35±0.1	6.35±0.2	6.40±0.2	6.87±0.2	6.26±0.2	6.57±0.1	6.91±0.1

Data expressed as mean ±SEM: **p< 0.01, vs. Control; One way- ANOVA followed by Dunnett post test

Route of administration of drug: Oral n: 10 (both sex)

Table 41: Organ weight of rats treated with the extracts in repeated dose 28 day sub acute oral toxicity

Groups	Brain	Heart	Liver	Lungs	Kidney	Stomach	Spleen	Testis	Ovary
Solvent control	2.02±0.05	1.55±0.08	13.57±0.48	3.15±0.03	1.37±0.09	2.16±0.09	1.26±0.04	0.96±0.43	0.28±0.12
HEOS 200mg/kg	2.20±0.04	1.38±0.05	14.83±0.34	3.28±0.04	1.50±0.07	2.26±0.09	1.32±0.02	0.94±0.42	0.26±0.11
HEOS 400mg/kg	2.62±0.09	1.42±0.05	15.23±0.39	3.50±0.06	1.54±0.08	2.21±0.18	1.39±0.03	0.96±0.43	0.33±0.15
HEOS 800mg/kg	2.82±0.03	1.40±0.03	15.29±0.57	3.84±0.06	1.36±0.06	2.11±0.16	1.32±0.04	0.95±0.43	0.31±0.14
HEOB 200mg/kg	2.13±0.04	1.28±0.06	14.51±0.56	3.34±0.05	1.45±0.07	2.30±0.05	1.29±0.04	1.02±0.05	0.30±0.13
HEOB 400mg/kg	2.56±0.13	1.23±0.05	15.32±0.54	3.59±0.05	1.52±0.07	2.34±0.05	1.34±0.04	1.03±0.46	0.31±0.14
HEOB 800mg/kg	2.68±0.10	1.27±0.04	15.29±0.57	3.86±0.04	1.15±0.06	2.38±0.05	1.37±0.04	1.05±0.47	0.25±0.15

Data expressed as mean ±SEM: Route of administration of drug: Oral n: 10 (both sex)

4.15. DISCUSSION

There is a need for searching for new potentially effective compounds against pathogenic microorganisms due to the development of microbial resistance towards antibiotics. One of the most promising targets in the search for new biologically active compounds is plants used in folk medicine, many of which have never been investigated for their chemical composition or pharmacological activity. In this search for finding potential leads from biologically active compounds from medicinal plants that may be effective against various strains of pathogenic fungi and for their antiseptic and wound healing properties, hydroalcoholic extracts of *Ocimum sanctum* (HEOS) and *Ocimum basilicum* (HEOB) were investigated in the present study for their *in vitro* antibacterial and antifungal activities and anticandidal (*in vivo*) activity in both non immunocompromised and immunocompromised mice, in addition to their safety (repeated dose 28 day sub acute oral toxicity) in rats. Efforts were also made to identify the phytoconstituents that are responsible for the antifungal and antibacterial activity.

The preliminary phytochemical investigations carried out on HEOS and HEOB reveal the presence of carbohydrates, proteins, amino acids and flavonoids. The total phenolic and flavonoids content present in HEOS and HEOB and *Ocimum basilicum* estimated. The study reveals 7.5 mg/g and 9.0mg/g of total phenolic compounds and 2.5mg/g and 3.0mg/g of flavonoids in HEOS and HEOB, respectively. Quantitative analysis by high performance liquid chromatography shows the presence of 1.3 mg/g eugenol, 0.73mg/g rosmarinic acid, 0.4 mg/g rutin and 0.15mg/g quercetin in HEOS. In the case of HEOB 6.2 mg/g querectin, 1.2 mg/g rosmarinic acid, 0.9 mg/g rutin and 0.4 mg/g eugenol are present. Cowan, (1999) and Sohn et al., (2004) have reported that various phenyl propanes and flavonoids are effective against candidiasis. They have also elucidated the various chemical structures of phytoconstituents responsible for the anticandidal activity namely phenolic compounds and flavonoids. Carson and Riley, (1995) and Mario et al., (1998) have reported that phenolic components of essential oils possess maximum antimicrobial activity. Zsolani, (1960), Cushnie and Lamb, (2005) and Pauli, (2006) have reported that plants having both, the flavonoids and phenolic compounds as major phytoconstituents possess anticandidal activity by causing either membrane

damage or membrane leakage. Flavonoids have thus been the focus of antimicrobial research and many investigators have isolated and identified flavonoids possessing antifungal, antiviral and antibacterial activities (Cushnie and Lamb, 2005). Eugenol is a major phytoconstituent present in the essential oils of *Ocimum sanctum* (Mondello et a., 2002) and *Ocimum basilicum* (Hasegawa et al., (1997) and Telci et al., 2006). Also antifungal activity of eugenol has been reported by He et al., (2007) and Gayoso et al., 2005. In addition, Yen and Chang, (2006) suggested that eugenol inhibit the growth of yeast either by blocking the cell wall synthesis or altering the cell wall structure, resulting in dysfunction of cell wall and increase of permeability to allow foreign particles entering fungal cell causing yeast death. Rosmarinic acid, a phenolic compound is widely distributed in *Ocimum* species (Rady and Nazif, 2005). Petersen and Simmonds, (2003) have reported antifungal activity of rosmarinic acid.

Rutin (Grayer et al., 2000) and quarcetin (Cushnie and Lamb, 2005) have been identified as major flavonoids in various *Ocimum* species. Earlier reports suggest antifungal activity of rutin (Agnese et al., 2001) and quarcetin (Uzel et al., 2005).

The anticandidal activity of both HEOS and HEOB observed in the present study is due to the presence of phenolic compounds and flavonoids viz., eugenol, rosmarinic acid, quercetin and rutin. Phenolic compounds and flavonoids are known to have antioxidant properties (Banerjee et al., 2005). Nitric oxide scavenging is one of the methods employed to find the antioxidant property of the plant extracts. The present investigations reveal an IC_{50} of 340.1 for HEOS and 283.8 for HEOB when compared to rutin (IC_{50} 159.5) thus indicates both HEOS and HEOB enhance the release of nitric oxide.

It has been proposed that the candidacidal pathway in murine neutrophils is nitric oxide (NO) dependent. NO is responsible for defense against pathogen that survives and proliferates in the intracellular environment of different types of somatic cells (Khan et al., 2003). The cell mediated immunity plays an important role in the resistance to candidiasis but little is known about the mechanisms that protect against the diseases. While monocytes/macrophages kill microorganisms using oxygen- dependent and independent mechanisms, NO is an antimicrobial factor generated by nitric oxide

synthase in activated macrophages and plays a role in the killing of bacteria, protozoa and fungi (Elahi et al., 2001). HEOS and HEOB, doses ranging from 1000µg/ml to 3.625µg/ml were tested *in vitro* for antibacterial activity against eight strains of both Gram positive and Gram negative bacteria, 24 h cultures of containing $1x10^6$ cells /ml, using two fold serial dilution and cup plate method. The results reveal that HEOS and HEOB are active with MIC at 500 and 1000 µg/ml, respectively on all tested bacterial strains. The plant extracts were tested for antifungal activity against seven different fungal strains. The study reveals that both HEOS and HEOB possess antifungal activity with MIC at 12.5 µg/ml of broth against *C.albicans* and 500 µg/ml against other tested fungi. Ahmed and Beg, (2000); Vaijayanthimala et al., (2000); Vonshak et al., (2003); Hamza et al., (2006) have also reported anticandidal activity (*in vitro*) of *Ocimum sanctum* and *Ocimum basilicum* species. Our findings concur with these reports.

The growing population of immunocompromised patients receiving immunosuppressive or anticancer therapy has resulted in an increased incidence of opportunistic mycoses. Deep-seated infections due to *C.albicans* are an important cause of infection in the immunocompromised population and treatment for these infections is still limited to a few agents (Martinez et al., 2000).

In our investigations both HEOS and HEOB showed maximal efficacy against *C. albicans*, and hence were taken up for further *in vivo* anticandidal study. The *in vivo* studies against experimental systemic candidiasis reveal that the mortality rate of mice treated with HEOS and HEOB (200 and 400 mg/kg) reduce when compared to the vehicle control. The mortality rate among non immunocompromised group treated with HEOS (200mg/kg) was 20%, whereas in the immunocompromised group it was 50% and 40% mortality for HEOS and HEOB (200mg/kg), respectively. Similarly 10% mortality was observed in immunocompromised group treated with HEOS and HEOB (400mg/kg). However, in immunocompromised group 100% mortality and 40% mortality in non immunocompromised mice treated with vehicle control was observed. No mortality was observed in non immunocompromised mice treated with HEOS (400mg/kg) and HEOB (200mg/kg and 400mg/kg).

These findings clearly suggest that both HEOS and HEOB have anticandidal activity and are also able to reverse the immunocompromised condition in mice.

The present investigations also reveal dissemination of *C.albicans* in brain, heart, liver, lungs, kidney and intestine in both non immunocompromised and immunocompromised mice. An increased number of colony forming units (cfu) in brain, heart, liver, lungs, kidney and intestine were observed. However, this is reversed in HEOS and HEOB treated non immunocompromised and immunocompromised groups at the dose levels tested. HEOS and HEOB treated mice show a two fold decrease of cfu in brain, heart, liver, lungs, kidney and intestine. The present findings are supported by the histopathology studies that show a moderate inflammation with fungal hyphae in liver, kidney and intestine of both non immunocompromised and immunocompromised mice treated with the vehicle control, whereas, HEOS and HEOB treated mice show no evidence of fungal hyphae and inflammation. HEOB (200 mg/kg) treated mice show the presence of fungal hyphae with mild inflammation of cells HEOB and HEOS(400mg/kg) groups show absence of inflammation and fungal hyphae completely.

Decreased body weights were found in both non immunocompromised and immunocompromised groups treated with vehicle control. However, this was reversed in HEOS and HEOB treated groups at the doses tested. The studies on hematological profile of experimental mice reveal leucopenia and increased monocytes and neutrophils percentage in both non immunocompromised and immunocompromised mice treated with vehicle control. This indicates induction of candidal infection and immunocompromised condition. Earlier reports also support these observations (Miller and Schaefer, 2007). However, these conditions were reversed in HEOS and HEOB treated mice, indicating immunomodulatory and cell mediated immunity properties of both the plant extracts. Mediratta et al., (2002) have reported immunomodulatory activity of *Ocimum sanctum*, which concurs with the present.

The findings once again suggest that both HEOS and HEOB possess significant anticandidal activity in experimentally induced candidiasis by decreasing the fungal burden at systemic level and also cell mediated immunity and immunomodulatory activity. Both HEOS and HEOB are equipotent against experimentally induced

candidiasis. Both the plants have shown better activity in non immunocompromised than immunocompromised mice. The anticandidal activity of HEOS and HEOB at 400 mg/kg is comparable to that of ketoconozole.

Candida species are not a component of indigenous microflora of the mouse gut. Gastrointestinal colonization by this yeast has been achieved usually in neonate mice or in immunocompromised animals. Neonate models of candida infection have been developed in order to avoid innate defenses and indigenous microflora suppression of yeast colonization (Cole et al., 1989). Nevertheless, infant models have shown some disadvantages such as high mortality rate attributable to inoculation procedure and overestimated virulence due to less effectiveness of the new born immune system. This condition makes experimental model unreliable for many laboratories. Adult mouse model of gastrointestinal colonization is more suitable for wide use (Mellado et al., 2000).

The effect of HEOS and HEOB on experimentally induced candidiasis under gastrointestinal colonization was also studied. In this study, broad spectrum antibiotics were administered to facilitate sustained gastrointestinal colonization in both non immunocompromised and immunocompromised mice. The present investigations reveal that mice that received antibiotics show a significant colonization by *C.albicans* in vehicle treated non immunocompromised and immunocompromised mice as seen by the number of colony forming units (cfu) in fecal samples analyzed on day 14 and 24, indicating severity of *C.albicans* dissemination at gastrointestinal tract. However, both in non immunocompromised and immunocompromised mice treated with HEOS and HEOB a significant decrease the number of cfu in fecal samples is observed on day 14 and 24 at tested dose levels.

The systemic dissemination of *C.albicans* in the gastrointestinal colonization was studied. The findings demonstrate that a significant colonization by *C.albicans* on intestine, kidney, liver and lungs of both immunocompromised and immunocompromised mice in the vehicle control group, whereas HEOS and HEOB at both the dose levels significantly decrease the number of cfu in intestine, kidney, liver and lungs of both immunocompromised and immunocompromised mice.

The above findings are also supported by the histopathological studies of intestine, kidney and liver. Histology of intestine, kidney and liver show fungal hyphae in both non immunocompromised and immunocompromised mice treated with vehicle control. However, both non immunocompromised and immunocompromised mice treated with HEOS and HEOB (400mg/kg) reveal absence of fungal hyphae completely.

The present findings suggest that *C.albicans* colonizes gastrointestinal tract under antibiotic therapy in both non immunocompromised and immunocompromised condition. Both HEOS and HEOB exhibit anticandidal activity in both non immunocompromised and immunocompromised conditions, as evident by a decrease in number of cfu in fecal samples and intestine, kidney, liver and lungs. The results are comparable to animals treated with ketoconozole that served as positive control.

Decreased body weights of mice were observed in both non immunocompromised and immunocompromised groups treated with vehicle control, when compared to their 0 day weights. However, this was reversed in HEOS and HEOB treated groups at doses tested.

The study of the hematological profile of systemic candidiasis under gastrointestinal colonization reveal an increased total leucocytes count, lymphocytes percentage, and increased neutrophils and monocytes percentage in both non immunocompromised and immunocompromised mice treated with the vehicle control. Neutrophils, monocytes and lymphocytes were brought to normal on day 24 in both non immunocompromised and immunocompromised mice treated with HEOS and HEOB at dose levels tested, when compared with 0 day of their respective groups.

Cyclophosphamide is toxic to dividing cells in the gastrointestinal tract and thereby alters the normal integrity of the mucosal epithelium of the gut. Cyclophosphamide has shown leucopenia and enhances infectivity of *C.albicans* (Cole et al., 1989). Weight loss, leucopenia, lymphocytopenia and increased neutrophils and monocytes in this study are characteristic of an infection and immunocompromised condition (Gupta et al., 1990; Miller and Schaefer, 2007). The present finding suggests that both HEOS and HEOB helps to develop cell mediated immunity in the system.

The pro-inflammatory cytokines like TNF-α and Il-1α play an important role in infectious diseases (Kullberg et al. 1990 and Del Sero et al., 1999). The present investigations on estimation of TNF-α and IL-1α in systemic candidiasis under gastrointestinal colonization reveal increased levels of both TNF- α and IL 1- in both non immunocompromised and immunocompromised groups treated with HEOS and HEOB. These findings suggest that both HEOS and HEOB possess anticandidal activity due to their capability to induce pro inflammatory cytokines viz., TNF- α and IL 1- α.

The regulatory role of natural killer (NK) cells in *C. albicans* infection is well established. These cells have the ability to regulate immune responses to this fungus through the production of cytokines and directly though interactions with other innate immune cells. Direct stimulation of NK cells with fungi results in the production of IFN-γ, TNF-α and GM-CSF. The production of these cytokines appears to be important in the regulation of neutrophils activity against *C. albicans*. Large granular lymphocytes (LGL) or NK cells were first reported to regulate the antifungal activity of polymorphonuclear (PMN) cells through a soluble PMN activating factor. The soluble PMN activator is likely to be TNF-α, although NK cell-derived GM-CSF might synergize with TNF-α to provide a potent recruitment and activation of PMN-mediated fungicidal activity. In addition to the regulation of neutrophils through cytokine production, lymphokine-activated killer cells (LAK) play an interesting role in the direct regulation of the monocyte/macrophage system. LAK suppresses the antifungal activity of GM-CSF and IL-3 cultured monocytes. The observed suppression, however, is alleviated by the presence of IFN-γ in monocyte culture. Monocyte antigen presenting ability, and IL-1 production have revealed a similar pattern of suppression following exposure to LAK cells. The evidence suggests that fungal activation of NK cells can lead to selective pressures on monocyte activation, and alter T-cell mediated responses accordingly. The contribution of NK cells to immune responses against *C. albicans* appears to be significant. The regulatory role of NK cells in response to *Candida* is highly active and involves both cytokine and direct regulation. There is substantial evidence for direct cytotoxic effector function (Wiseman and Mody 2007). Recent reports point out to a significant contribution also of natural killer (NK), Th1 lymphocytes and dendritic cell recruitment and/ or activation in the

defense against fungal growth (Bozza et al., 2002a; Bozza et al., 2002b; Bozza et al., 2003).

Cytokines or chemokines have been found to be key orchestrators of the cellular response. NK cells kill infected target cells by inducing apoptosis of the target cell and/or by releasing molecules such as perforin, which damage the cell membrane, leading to cell death. NK cells can produce substantial amounts of TNF-α, which are possibely involved in this process. *In vitro* TNF-α increases the capacity of neutrophils to damage *A. fumigatus* hyphae and enhances macrophage conidial phagocytosis. Both genetic deletion and antibody neutralization of IL-10 have been shown to reduce susceptibility of C57BL/6 mice to infection with *A. fumigatus* and *C. albicans*. The reduced fungal burden and increased survival of IL-10-deficient mice has been attributed to an increased production of type 1 cytokines including IFN-γ, TNF-α and IL-12, and associated with increased NO production (Del Sero et al., 1999). A single injection of either recombinant IL-1α or IL-1ß was shown to protect neutropenic mice against lethal disseminated candidiasis (Van‘t Wout et al., 1988 and Kullberg et al., 1990). Treatment also significantly decreased the numbers of *C. albicans* in the organs of infected normal mice and of mice rendered immunocompromised by cyclophosphamide, hydrocortisone acetate, or total body irradiation (Kullberg et al., 1990).

In the present investigations HEOS and HEOB against experimental systemic candidiasis in the gut colonization model suggest that HEOS has better anticandidal activity than HEOB at the dose levels tested, which is comparable to that of ketoconozole treated mice.

Immunosuppressive drugs such as antineoplastic agents like cyclophosphamide has shown to predispose to oral candidiasis by altering the oral flora and disrupting the mucosal surface (Akpan and Morgan, 2002). The present study involving oral candidiasis reveal the protective effect of HEOS and HEOB administered orally in immunocompromised mice. In this model, the severity of *Candida* infection was estimated by measuring the number of cfu in the oral swabs of buccal cavity. When compared to vehicle control, HEOS and HEOB facilitated recovery from oral candidiasis

7 days after infection. The study demonstrates that both HEOS and HEOB at 400mg/kg dose reduce the fungal counts in the oral cavity. A dose dependent activity was observed. The histological examination of tongue and oral mucosal layer exhibit similar results. These findings support the protective effect of HEOS and HEOB against oral candidiasis in immunocompromised condition.

Using HEOS and HEOB extracts separate cream and gel formulatios were developed in the present investigations. As the cream formulations showed better physical properties when compared to the gel formulations, the cream formulations were taken up for further studying their efficacy against vaginal candidiasis and superficial infections and in healing of infectious wounds.

The effect of HEOS and HEOB on experimental vaginal candidiasis was studied and the severity of infection was recorded on day 3, 6, 9 and 18. The results reveal an increase fungal burden in the vaginal smears obtained from mice treated with control (cream base) in both non immunocompromised and immunocompromised mice. Creams (2% and 4%) of HEOS and HEOB significantly decreased the fungal burden in both non immunocompromised and immunocompromised mice from day 3 onwards. A Similar effect was observed in successive days against vaginal fungal infection by both HEOS and HEOB at tested doses. However, both the plant extracts were unable to cure the infections completely even on day 18, when compared to ketoconozole treated mice. The present findings suggest that HEOB possess higher efficacy than HEOS against vaginal candidiasis.

Decreased body weights were recorded in vehicle control treated mice in both non immunocompromised and immunocompromised groups on day 18 when compared to day 0. No significant changes were observed in body weights of mice in both non immunocompromised and immunocompromised groups treated with HEOS and HEOB at the dose levels tested.

The hematological profile of mice in the present study reveal leucopenia, lymphocytopenia and an increased neutrophils and monocytes percentage in both non immunocompromised and immunocompromised mice treated with control (cream base).

However, these conditions were reversed in HEOS and HEOB (2% and 4% cream) treated mice. Polymorphonuclear leucocytes (PMN) are an important innate host defense mechanism against *C.albicans* in systemic circulation and have significant anticandidal activity *in vitro* (Sobel, 2003).

Antimycotic activity of HEOS and HEOB was also studied in the present investigations. In this model primary infection is induced by *C.albicans,* leading to an uniform invasion of hair follicles topically over the infected skin area. The antimycotic activity studies reveal that HEOS (4% cream) possess is 55.88 % antimycotic activity and 44.41% clinical efficacy and the 2% cream possess 50% antimycotic activity and 55.55% clinical efficacy. HEOB (4% cream) possess is 64.7% and 66.66% of antimycotic activity and clinical efficacy, respectively. The 2% cream possess is 58.82% antimycotic activity and 55.55% clinical efficacy when compared to control (cream base). Both plant extracts markedly decrease erythema induced by *C.albicans* in guinea pigs at tested dose levels. The present findings are also supported by earlier reports given by Vonshak et al., (2003) that *Ocimum basilicum* inhibits the dermatophytes in pathogenic moulds in *in vitro* system. The present findings on antimycotic activity suggest that HEOB has better antimycotic activity when compared to HEOS and it is comparable to that of ketoconozole.

The Present investigations on infected wounds in excision model by HEOS and HEOB revealed a significant wound contraction on day 4 in drug treated animals. Similarly, significant wound contraction is maintained on day 8, 12 and 16 in rats treated with 2% and 4% creams, when compared to the control. The rate of wound contraction is maximum on day 8 in HEOB (4%) treated rats, when compared to other groups. Significantly increased hydroxyproline and decreased lipid peroxides level are observed on day 8 in both HEOS and HEOB treated rats. The histopathology of regenerated skin shows a faster epithelization and capillary proliferation with maximum degree of fibroblast deposition on day 8 in HEOS and HEOB (4%) treated rats, whereas, HEOS and HEOB (2% cream) treated rats reveal a lesser degree of capillary proliferation and migration of polymorphs, lymphocytes, macrophages and fibroblast when compared to 4% cream.

On day 16, maximum wound contraction is observed in all drug treated groups when compared to the control. No significant change in hydroxyproline content is observed suggesting that the levels reached are almost normal. However a significant decrease in lipid peroxides level is observed in all the drug treated groups. The histopathology report reveals a complete healed lesion with minimal inflammation in HEOB (4%) and nitrofurazone (0.2%) cream treated rats. Reepithelization, capillary proliferation, fibroblasts deposition and migration of leucocytes with inflammation appear in HEOS (2%, 4%) and HEOB (2%) treated rats.

Similar findings on hydroxyproline and lipid peroxides are also observed on day 21. Histopathology of skin reveals moderate inflammation, migration of polymorphs, lymphocytes, macrophages, fibroblasts and capillary proliferation in control rats. HEOS (2% and 4%) cream and HEOB (2%) cream treated rats show mild inflammation, migration of fibroblasts, migration of polymorphs, lymphocytes and the macrophages with minimal capillary proliferation. Completely healed wound is observed in HEOB (4%) and nitrofurazone cream treated rats

Wound healing is a highly complex physiological process. It involves the interplay of various cellular and biochemical components, which drive the repair cascade. Apart from cellular and biochemical components, several enzymatic pathways also become active during repair and help the tissue to heal (Kapoor and Appleton, 2005). The process of wound repair differs from one kind of tissue to another and is generally independent of the form of injury. Although the different elements of the wound healing process occur in a continuous integrated manner, it is convenient to divide the overall process in to three overlapping phases namely inflammatory phase, proliferative phase and maturation phase (Li et al., 2007).

The process of epithelial resurfacing is critical in order for wound to be considered “healed”. The initial event in epithelization is the migration of undamaged epidermal cells from the wound margins and from the epithelium of hair follicles and other adnexal structures. After migration, an increase in epithelial proliferation begins at the wound margins to provide the need of additional cells for wound cover.

The cellular elements important in the inflammatory phase of wound healing are the polymorphonuclear leukocyte (PMN) and the macrophages. The PMN is short lived, and though initially predominant cell type, is largely replaced by the macrophages later after wounding. The prime function of these cells is one of the phagocytosis and killing of concomitant bacteria and tissue debridement (Deodhar and Rana, 1997).

The healing process begins with tissue reposition by cell proliferation presented in connective tissue. The main cells that trigger the wound healing process are the macrophage cells that remove foreign bodies and direct granular tissue development. Sequentially fibroblast and endothelial cells migrate towards the injured area increasing tissue permeability and collagen fiber production. The events of this phase are fibroblast proliferation with collagen synthesis that decreases vascularization by a contraction process that triggers the scar process. Neovascular tissue and macrophage cells carry chemical mediators, such as enzymes, oxygen and vitamins that are essential to establish high quality fibroblast and collagen formation. Fibroblast is the most important cell behind the synthesis of collagen. Rough endoplasmic reticulum in the fibroblast is the site of collagen synthesis. Hydroxyproline is an amino acid essential for collagen fibre synthesis. For this reason, hydroxyproline content has been used as an indicator to determine collagen content (Jorge et al., 2008). The collagen molecules cross linked with each other to form the fibrils and fibers that provide the tensile strength to the wound (Somayaji and Bairy, 1997).

Lipid peroxides are the one which inhibits the maintenance of the structural integrity of the connective tissues in the damaged tissues. A drug that inhibits lipid peroxidation is believed to increase the viability of cells by improving the circulation, preventing cell damage and promoting DNA synthesis. Many constituents are known to reduce lipid peroxidation thereby preventing or slowing the onset of cell necrosis (Shoba and Rao 1999 and Geetha et al., 2004).

The findings in the present investigations on HEOS and HEOB for the treatment of infectious wounds reveal a significant wound contraction, faster epithelization and capillary proliferation with maximum degree of fibroblasts, polymorphs and macrophages in regenerated skin. In addition increased hydroxyproline and decreased

lipid peroxides levels are observed in rats treated with HEOS and HEOB. This suggests that both HEOS and HEOB are effective against infectious wounds in the excision model, by causing reepithelization, migration of polymorphs and macrophages, neovasculization, increased hydroxyproline content and decreased lipid peroxides levels.

The data obtained for incision wound on day 10 shows increased breaking strength in the rat tissue treated with both HEOS and HEOB. The breaking strength/tensile strength of tissue demonstrate the degree of deposition of collagen. Increased hydroxyproline and decreased levels of lipid peroxides are recorded. However, HEOB (4% cream) treated rats reveal increased tensile strength (553.5) when compared to HEOS treated rats (540.67). The histopathology of skin shows a faster epithelization, neovasculization and migration of fibroblasts. Migration of polymorphs, lymphocytes and macrophages are not observed and inflammation is minimal in HEOS and HEOB treated rats. The present findings suggest that HEOB has better healing property against infectious wound in incision model.

It is known that reactive oxygen species (ROS) are deleterious to wound healing process due to their harmful effects on cells and tissues. ROS can induce severe tissue damage and even lead to neoplastic transformation decreasing the healing process by damages in cellular membrane, DNA, proteins and lips as well. Exposure to free radicals from a variety of sources has led organisms to develop a series of antioxidant defense mechanisms (Jorge et al., 2008). Free radical scavenging enzymes has an essential role in the reduction, deactivation and removal of ROS as well as regulating the wound healing process (Singh et al., 2006). Hence, if a compound is having antioxidant potentials and antimicrobial activity additionally, it can be a good therapeutic agent for accelerating the wound healing process.

The findings in the present investigations on *in vitro* nitric oxide scavenging property reveal that both HEOS and HEOB increase the release of nitric oxide that confirms with IC_{50} values. Rawlingson (2003); Grierson and Ormerod (2004) and Shekhter et al., (2005) have reported that enhanced release of nitric oxide is an important activity in wound healing process. The present findings suggest that enhanced release of nitric

oxide by HEOS and HEOB are one of the positive factors for their wound healing property.

In addition to our findings antioxidant activity of *Ocimum sanctum* and *Ocimum basilicum* has also been earlier reported by earlier workers (Javanmardi et al., 2003; Politeo et al., 2006). The antioxidant activity of these plants may, therefore, be responsible for wound healing property in both excision and incision models. Shirwaikar et al., (2003) and Jagetia and Rajanikant, (2004) have reported on the wound healing property of plants that possess antioxidant property.

Evaluation safety profile of a drug is equally important to that its efficacy. In the present study, the safety profile of both HEOS and HEOB was evaluated by repeated dose 28 day sub acute oral toxicity. The results show that there is no mortality, change in body weights and characters and content of urine in HEOS and HEOB treated rats at all three doses levels when compared to the control. The hematological results show an increase in total leucocyte count, platelet count, red blood corpuscles and hemoglobin percentage, which are dose dependent these hematological parameters are not altered at lower doses. ALAT level is increased in high dose treated rats, there is no change in other biochemical parameters. These studies support that the toxicity profile/safety profile of the extracts at the dose levels tested.

5. SUMMARY AND CONCLUSIONS

In the present investigations an attempt has been made to study the antiseptic and wound healing properties of hydroalcoholic extract of *Ocimum sanctum* (HEOS) and *Ocimum basilicum* (HEOB). Studies were also directed towards the evaluation of *in vitro* antibacterial and antifungal and *in vivo* antifungal activity of HEOS and HEOB in non immunocompromised and immunocompromised mice. Safety studies (repeated dose 28 day sub acute oral toxicity) of these extracts were also carried out in rats.

The phytochemical studies of HEOS and HEOB reveal the presence of carbohydrates, proteins, amino acids and flavonoids. Studies reveal 7.5 mg/g and 9.0mg/g of phenolic compounds, 2.5mg/g and 3.0mg/g of flavonoids in HEOS and HEOB, respectively. The quantitative analysis of phytoconstituents by high performance liquid chromatography revealed, 1.3 mg/g eugenol, 0.73mg/g rosmarinic acid, 0.4 mg/g rutin and 0.15mg/g quercetin in HEOS and 6.2 mg/g querectin, 1.2 mg/g rosmarinic acid, 0.9 mg/g rutin and 0.4 mg/g eugenol in HEOB. Nitric oxide scavenging test reveal an IC_{50} value of 340.1 for HEOB and 283.8 for HEOS. The *in vitro* antifungal studies reveal an MIC at 12.5µg/ml against *C.albicans* for both HEOS and HEOB.

The effect of HEOS and HEOB on experimental systemic candidiasis in non immunocompromised and immunocompromised mice was studied. The data reveal that the mortality rate is high in both vehicle treated, non immunocompromised and immunocompromised mice. However, the mortality rate is lower in HEOS and HEOB treated groups. Dissemination of *C.albicans* in various organs reveals significant number of cfu in brain, heart, liver, lungs, kidney and intestine. Number of cfu reduce significantly in both non immunocompromised and immunocompromised mice treated with HEOS and HEOB. Histopathology of intestine, liver and kidney also supports these findings.

The investigations on experimentally induced candidiasis under gastrointestinal colonization reveal that mice that received antibiotics show a significant colonization. However, mice treated with HEOS and HEOB show a sustained decreased colonization at tested dose levels upon analysis of the number of cfu in fecal samples. The present

findings demonstrate that a highly significant colonization by *C.albicans* on intestine, kidney, liver and lungs of both vehicle treated non immunocompromised and immunocompromised mice. These reduce significantly in HEOS and HEOB treated mice. The histological examination of these organs also confirms the same.

The results obtained in the investigations on oral candidiasis shows the protective effect of HEOS and HEOB in immunocompromised mice at tested dose levels. The histological examination of tongue and oral mucosal layer support these findings.

Our findings on HEOS and HEOB against experimental induced candidiasis models suggest that HEOS has better anticandidal activity in all three models when compared to HEOB. The total phenolic compounds, eugenol and rosmarinic acid content in these plants may play an important role against systemic candidiasis, which was high in HEOS than HEOB. The findings suggest that HEOS may be potential candidate for the treatment of systemic candidiasis.

Formulations of cream and gel were developed according to standard methods. Among the cream and gel, the cream shows better physical properties when compared to gel. The cream was therefore, tested against vaginal candidiasis, antimycotic and for would healing property.

The effect of 2% and 4% creams on experimental vaginal candidiasis reveal increased fungal burden in vaginal secretions of vehicle treated in both non immunocompromised and immunocompromised mice, whereas 2% and 4% cream significantly decreased the fungal burden in both conditions from day 3 onwards.

Investigations on antimycotic activity reveal that both HEOS and HEOB creams exhibit antimycotic activity and they also markedly decrease erythema induced by *C.albicans* in guinea pigs at tested dose levels.

The present studies on experimental vaginal candidiasis and antimycotic activity suggest that HEOB has a better efficacy against both these models when compared to HEOS. Further, a comparison of phytochemical reports among these plants suggests that flavonoids may play a major role against vaginal and superficial fungal infections.

HEOB may, therefore, be potential candidate for the treatment of vaginal and superficial fungal infections than HEOS.

The investigations on infectious wounds in excision and incision model reveal that both HEOS and HEOB (2% and 4% creams) have significant antiseptic and wound healing property in rats. The present findings also suggest that both HEOS and HEOB cause faster epithelization, and capillary proliferation with maximum degree of fibroblast deposition, and facilitate the migration of polymorphs, lymphocytes and macrophages over the infected wound area.

The investigations of repeated dose 28 day oral toxicity demonstrate increased total leucocyte, platelet, red blood corpuscles count and haemoglobin percentage and increased ALAT levels at 800 mg/kg dose of both HEOS and HEOB. It suggests that both HEOS and HEOB have mild dose related toxicity on liver and hematological parameters.

It may be concluded from the present investigations on HEOS and HEOB that both the plant extracts possess anticandidal activity against systemic, vaginal and superficial infections in non immunocompromised and immunocompromised conditions. The findings further suggest the anticandidal activity of both *Ocimum sanctum* and *Ocimum basilicum*, may be due to the following factors;

- **antioxidant activity of phenolic compounds and flavonoids of both plants may contribute to anticandidal activity,**
- **nitric oxide mediated candidacidal pathway,**
- **quercetin, rosmarinic acid, rutin, and eugenol are bioactive phytoprinciples responsible for anticandidal activity,**
- **increased total leucocytes and lymphocytes develop cell mediated immunity,**
- **induction of pro-inflammatory cytokines such as TNF- α and IL-1α**

In addition, both HEOS and HEOB possess antiseptic and wound healing property, due to multiple positive factors, such as,

- **faster epithelization,**
- **increased collagen deposition,**
- **neovasculization,**
- **inhibition of lipid peroxidation levels and**
- **antioxidant activity of total phenolic compounds and flavonoids, and bioactive phytoprinciples such as quercetin, rutin and rosmarinic acid.**

It is suggested, therefore, that both HEOS and HEOB are potential candidates for the treatment of septic wounds and for systemic, vaginal and superficial candidal infections. They may be used as complementary medicine in antifungal chemotherapy. Further clinical studies are required to establish this.

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Appendix I

***Ocimum sanctum* Linn** (Anonymous 1966)

Genus: *Ocimum*

Species:

Ocimum sanctum (Sacred Basil)

Ocimum basilicum (Sweet Basil)

Ocimum gratissimum

Ocimum menthaefolium

Ocimum canum (Heavy Basil)

Ocimum minimum (Bush Basil)

Ocimum kilimandscharicum (Camphor Basil)

Synonyms

Moschoma tenuiflorum(L.)Heynhold, Ocimum album Balco, O. anisodorum Muell., O.brachiatum Hasskarl, O. flexuosum Blanco, O. frutescens Burn., O. gratissimum Lour., O. inodorum Burm., O.monachorum L., O.nelsoni Zipp ex Span., O. teniflorum L., O. virgatum Blanco .

Selected vernacular names

Badroojj, basilic des moines, bazsalikom level, daun lampes, garden balsam,green tulsi,holy basil, jagu lu myah, kamimebouki, kaphrao, kaprao, kemangi, kemangi laki, kra phrao, lampas, monk'basil, peiteh, solasi, sulasi, sursa, tamole, thulasi, Tulashi, tulasi ,tulsi.

***Ocimum sanctum* Linn**

Geographical distribution

Indigenous to India parts of north and eastern Africa, Hainan Island and Taiwan china. It is cultivated in south-east Asia.

Description

An herb or shrub, up to 1 m high, often much branched. Stem square, lower parts sub-serrate, higher parts slightly furrowed and more densely pubescent or sub glabrous. Leaves simple, opposite, oblong, ovate or oval- oblong, 2.7-7.5 cm long,1-3 cm wide, with acute top, cuneate, obtuse to rounded base, margin entire, undulate or serrate, both surface thinly pubescent and dotted; petiole 0.2-3.0 cm long. Calyx 0.2-0.4 cm long with or without long or short hairs, ciliate, densely glandulose; upper lip 2.0-3.5 mm long, oval short acuminate; lower lip 1.0-2.5 mm long, dentate, teeth linear -acuminate from an equal- or unequal- sided triangular to ovate base, 2 anterior teeth equaling or slightly surpassing the upper lip; fruiting calyx not completely closed by teeth. Upper part of the corolla villous and glandulose, in the upper part; lobes of upper lip and

lobes of lower lip obtuse to rounded. Nutlets obovoid, dark brown or black, 1-2mm long; pericarp swells into a slimy mass when moistened.

General appearance

Leaves greenish brown, 2.5-7.5 cm long, 1-3 cm wide, oblong, ovate or oval- oblong, with acute top, cuneate, obtuse to rounded base, pinnate veins, serrate or entire or undulate margin; thin but flashy, both surface thinly pubescent; petiole cylindrical, 1-2 cm long, thinly pubescent.

Organoletic properties

Odour: characteristic, aromatic; taste; slightly pungent

Microscopic characteristics

Transverse section of the leaf through its midrib: upper epidermis consists of a layer of small, quadrangular transparent cells with thin walls and thin smooth cuticle. On tangential view, these cells are polygonal with straight or wavy walls. Lower epidermis consists of a layer of small quadrangular transparent cells with thin walls and thin smooth cuticle. Trichomes bent, consisting of 2-6 cells: glandular trichomes short, Lamiaceae type, consisting of 1 stalk cell and 2-4 cells with rounded heads. Palisade parenchyma consists of layer of long cylindrical cells containing chlorophyll; spongy parenchyma consists of polygonal cells with thin, straight or slightly wavy side walls. Vascular bundles collateral type with collenchyma cell. Stomata diacytic, on upper and lower epidermis.

Powdered plant material

Upper epidermis with diacytic stomata, glandular trichomes and palisade cells; lower epidermis with diacytic stomata, and underlying spongy cells;2- and 4-celled glandular trichomes, uniseriate, multicellular trichomes with collapsed cells; lignified fibers; spiral vessels; pollen grain rare; parenchyma and collenchyma from petioles.

Purity Tests

Total ash	Acid-Insoluble ash
Not more than 13%	Not more than 1%
Sulfated ash	Water-soluble extractive
Not more than 20%	Not less than 5%
Alcohol-soluble extracts	Loss on drying
Not less than 5%	Not more than 14%

Major chemical constituents

The main components are tannins (4.6%) and essential oil (up to2%). The amount of the primary constituent of the essential oil vary according to the geographical distribution and variety of the source plants material; eugenol (up to 62%), Methyleugenol (up to 86%), and α and β caryophyllene (up to 42%). Also present are methylchavicol, linalool and 1, 8-cineole.

Collection

Ocimum sanctum was collected and cultivated from the tropical and subtropical region of Asia, Africa and central and South America. However, the major place of diversity appears to be in Africa. This genus is characterization by a great variability in its morphology and chemo types.

Uses

In traditional systems of medicine, different parts (leaves, stem, flower, root, seeds and even whole plant) of *Ocimum sanctum* Linn have been recommended for the treatment of bronchitis, bronchial asthma, malaria, diarrhea, dysentery, skin diseases, arthritis, painful eye diseases, chronic fever, insect bite etc. The *Ocimum sanctum* has also been suggested to possess antifertility, anticancer, antidiabetic, antifungal, antimicrobial, hepatoprotective, cardioprotective, antiemetic, antispasmodic, analgesic, adaptogenic and diaphoretic actions. Eugenol (1-hydroxy-2-methoxy-4-allylbenzene), the active constituent present in *Ocimum sanctum*, has been found to be largely responsible for

the therapeutic potentials of Tulsi. Although because of its great therapeutic potentials and wide occurrence in India the practitioners of traditional systems of medicine have been using *Ocimum sanctum* for curing various ailments, a rational approach to this traditional medical practice with modern system of medicine is, however, not much available. In order to establish the therapeutic uses of *Ocimum sanctum* in modern medicine, in last few decades several Indian scientists and researchers have studied the pharmacological effects of steam distilled, petroleum ether and benzene extracts of various parts of Tulsi plant and eugenol on immune system, reproductive system, central nervous system, cardiovascular system, gastric system, urinary system and blood biochemistry and have described the therapeutic significance of Tulsi in management of various ailments. These pharmacological studies have established a scientific basis for therapeutic uses of this plant.

Appendix II

***Ocimum basilicum* Linn** (Anonymous 1966)

Synonyms

Ocimum anisatum (*Basilicum citratum*)

Selected vernacular names

English: Basil, Common Basil, Common Sweet Basil, Sweet Basil.

Sanskrit: Ajagandhika, Barbari, Manjariki, Surasa, Hindi: Babui tulsi, Babul, Bahari, Kalitulsi. Tamil: Tirnut-patchi, Tirunitru. Telugu: Bhu-tulasi, Rudrajada, Vebudi-patri

***Ocimum basilicum* Linn**

Geographical distribution

Indigenous on the lower hills of the Punjab and cultivated throughout the greater parts of India, Ceylon, and Burma.

Description

An erect branching herb, 0.6-0.9m.high, glabrous or more or less hispidly pubescent. Stem and branches green or sometimes purplish. Leaves 2.5-5cm or more long, ovate, acute, entire or more or less toothed or lobed; base cuneate, entire, petiole 1.3-

2.5cm.long. Whorls densely rasemose, the terminal raceme usually much longer that the lateral ones; bracts stalked, shorter than the calyx, ovate, acute. Calyx 5mm. long, enlarging in fruit, very shortly pedicelled; lower lip with the 2 central teeth longer than the rounded upper lip. Corolla 8-13mm. long white pink or purplish, glabrous or variously pubescent. Stamens slightly exerted, upper filaments toothed at the base. Nutlets about 2mm long, ellipsoid, black and pitted.

Cultivation

It is an herbaceous species, natives of warm regions of Asia, Africa and Iran, which emits a warm, sweet and peculiar aroma. This species is commercially cultivated for extraction of essential oil in southern France, Italy, Spain, Germany, North America, Bulgaria, Egypt, Sicily, Haity, Comomoros, Madagascar and the Seychelles islands. It is also cultivated in India. It is an erect glabrous herb up to 75 cm tall. Leaves are petiolate, ovate or oblong, narrowed at both ends, almost entire, they have numerous dot like oil glands which contains aromatic volatile oil. There are several varieties in it like “bullatum”, “minimum” and “violacerum”.

Chemical constituents

The chemical constituents in the oil are pinene, cineole, linalool, camphor, methyl chavicol, eugenol, sesquiterpenes, methyl cinnamate, geraniol, borneol and ocimene. The leaves contain protein, carbohydrate, volatile oil, fixed oil, thymol, xanthomicrol, butyl caffeate, cellulose, mineral matter and vitamin A, B and C.

Uses

The oil is used for various kinds of flavours in confectionary, baked goods, chili sauces, catsups, tomato pastes, pickles and vinegars. In dental and oral products this oil finds place. Oil is also used in perfumes and for scenting soaps. They are medicinally used for fever and also for bladder and kidney disorders. It is one of the most popular culinary herbs. This plant possesses stimulating properties. An infusion of leaves is used to treat gonorrhea, burning urine and nephritic infections. The plant is useful in the treatment of worms, stomach complaints, fever, cough and gout. Leaf juice is employed as a nasal douche and also to treat skin disorders. For relieving constipation and piles seeds are

eaten. The juice of leaves is poured into the ear to cure otitis. The seeds are said to have refreshing and sedative properties.