Research and Reviews: Journal of Pharmacognosy and Phytochemistry

e-ISSN:2321-6182
p-ISSN:2347-2332

In vivo anticandidal Activities of Sub-fraction S (237-253) From Uvariondendron Calophyllum Ethanolic Leaf Extract

Zeuko'o Menkem Elisabeth[1,2*], Toghueo Kouipou Rufin Marie[2], Siwe Tchokomeni Gael[3], Ngouana Kammalac Thierry[2], Fekam Boyom Fabrice[2]

[1]Catholic University of Cameroon, Bamenda, School of Health and Medical Sciences. P.o box 782, Bamenda

[2]Antimicrobial and Biocontrol Agents Unit, Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, University of Yaoundé 1, P. O Box 8476, Yaoundé, Cameroon

[3]Laboratory of animal Physiology, Department of Animal Biology and physiology, Faculty of Science, University of Yaoundé 1, P. O Box 812, Yaoundé, Cameroon.

Research Article

Received date: 16/05/2017
Accepted date: 12/06/2017
Published date: 15/06/2017

*For Correspondence

[1]Catholic University of Cameroon, Bamenda, School of Health and Medical Sciences, P.O. Box 782, Bamenda.
[2]Antimicrobial and Biocontrol Agents Unit, Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, University of Yaoundé 1, P.O. Box 8476, Yaoundé, Cameroon

E-mail: mllemenkem@gmail.com

Keywords: Uvariodendron calophyllum, acute toxicity, vaginal candidiasis, systemic candidiasis, BALB/C mice, histopathological analysis

ABSTRACT

Background and objectives: Mucosal candidiasis can disseminate through the bloodstream and cause disseminated candidiasis. The acute toxicity and the effect of sub-fraction S (237-253) on the vaginal and systemic candidiasis in BALB/C mice model was evaluated.

Methodology: The leaf crude extract of Uvariodendron calophyllum was prepared using Ethanol 95% then partitioned using hexane, methylene chloride and methanol and the methylene chloride fraction was fractionated using column chromatography to obtain sub-fraction S (237-253). Vaginal and systemic candidiasis were induced using Candida albicans strains NR-29445 and NR-29450 on immunocompromised BALB/C mice model and treated with subfraction S at 100, 200 and 400 mg/kg bw. The number of colony forming units in the vaginal candidiasis on the vaginal smears was measured by culturing the vaginal smears on SDA during ten days of treatment. In the systemic candidiasis, the number of colony forming units in the blood, spleen, kidney, liver and lungs of the different groups of mice was cultured with their histopathological analysis evaluated.

Results: The results show that LD50 was > (2000 mg/kg). The healing effect was observed with the different doses (100 mg/kg, 200 mg/kg and 400 mg/kg) and was dose dependent. Inflammation of the stratified squamous epithelium (increased size) in the treatment with water 2 ml/100 g indicating the level of infection and progression was observed. The treatment with sub-fraction S at 400 mg/kg reduced the size of the stratified squamous epithelium. The healing effect was dose dependent for the different organs.

Conclusion: The sub-fraction S could be potential source of constituents with in-vivo anticandidal properties with little or no side effect.

INTRODUCTION

Candidiasis has emerged as an alarming opportunistic disease ranging from mucosal to life threatening invasive candidiasis[1]. *Vulvovaginal candidiasis* (VVC) has been estimated to be the second most common cause of vaginitis after bacterial vaginosis with about 70 to 75% of healthy adult women suffering from VVC during their lifetimes worldwide[2,3,4]. Moreover, a prevalence of 32.1%, was observed in 22 to 26 years age group in Nigeria[5]. Also, in Kenya, a prevalence of 42.7% of vaginal candidiasis

e-ISSN:2321-6182
p-ISSN:2347-2332

in pregnant women attending the antenatal clinic of thicka district hospital was also reported[6] with *Candida albicans* being the most prevalent. In addition to causing mucosal diseases, invasive infections have been reported with 40% mortality rate[7,9]. Moreover, the mortality associated with these invasive infections for adults ranges from 14.5% to 49%[9,10], and *C. albicans* is estimated to be responsible for 50-60% of the cases of invasive candidiasis[10,7]. As such, *C. albicans* is the most prevalent fungal pathogen in humans. The different classes of antifungals available for the treatment of candidiasis include polyens, azoles, pyrimidine analogues, echinocandins and allylamines. However, these drugs have some limitations such as high toxicity and low bioavailability (polyens) and reduced spectrum of action (echinocandins)[11]. Resistance has already been described for all these antifungal drugs and is more often associated with therapeutic failure[12,13].These limitations in treatments underline the urgent need for new antifungal agents with improved potency and innovative modes of action that could be further developed as antifungal treatments.

Natural products have proven efficacy in the treatment of a wide range of ailments. Plants from the Annonaceae family are used in traditional medicine to treat many infectious diseases including microbial infections and with its wide exploitation in a number of biological activities[14,15]. In an ongoing work on Uvariodendron calophyllum we reported the antifungal and antioxidant activity of its ethanolic extracts[8]. Moreover, the bioguided fractionation and study of the mode of action of the most active sub fraction[17] was also reported.

Therefore, this work describes the in-vivo study of the most active sub-faction, aiming to evaluate the acute toxicity and the effect of sub-fraction S (237-253) on the vaginal and systemic candidiasis in BALB/C mice model.

MATERIAL AND METHODS

Plant Material, Preparation and Extraction

The leaves, twigs, stem bark, and stem of U. calophyllum were harvested from mount Kalla in Yaoundé (Cameroon) on 11th September 2011 and identified at the National Herbarium of Cameroon, Yaoundé where voucher specimen was deposited under the identification number 28734/SFR/CAM. The leaves were dried at room temperature in the laboratory, and then ground to fine powder.

The ethanolic extracts were prepared by macerating 500 g of the fine powder in 95% ethanol (m/v 1:5) for 72 hours with regular stirring, and then filtered. The filtrate was evaporated using rotary evaporator to obtain a solid extract. The latter was stored at 4°C. The dried ethanolic leaf extracts were dissolved in water at 10 g/100 mL and subsequently partitioned[18]. Briefly, the extracts dissolved in water were successively washed using equal volumes (100 mL) of hexane, methylene chloride, and methanol to afford hexane fraction, methylene chloride fraction, and methanolic fraction. The solvents were separately evaporated under reduced pressure. The methylene chloride fraction of the leaf ethanolic extract (UCl EtOH CH2Cl2) which showed the overall best anti-yeast activity was selected and subjected to silica gel column chromatography. The column elution was done using solvent systems of increasing polarities, Hexane-Ethyl Acetate [100:0–0:100] and Ethyl Acetate - Methanol [95:5-0:100]. The sub-fraction S FS: (237–253) was selected as shown below[17].

Evaluation of the Acute oral toxicity profile

The oral acute toxicity of crude extracts sub-fraction S (237-253) *Uvariodendron calophyllum* was evaluated using the BALB/C mice according to the procedure outlined by the OECD 2001[18] protocol with some modifications. These animals were provided by the animal house of the Faculty of Medicine of the University of Yaoundé I aged 9-12 weeks old. A limit dose of 2000 mg/kg body weight of sub-fraction S, was administered to each mice of group 1 while the control group (group 0) received the vehicle (distilled water). Feed was provided to the mice 1 to 2 hours after treatment. The animals were observed after dosing during 14 days.

In-vivo Anti- candidiasis Activity (Anti- vulvovaginal and anti-systemic candidiasis)

The therapeutic effect of sub-fraction S was evaluated on the immunocompromised mice models of vaginal and systemic candidiasis. The animals were grouped into 6 groups of 6 mice each as follows: Group 0: Healthy controls, Group 1: Control infected treated with distilled water (2 mL/100 g), Group 2: Positive control infected received fluconazole, Group 3: Infected treated with 100 mg/ kg of plant sub-fraction S, Group 4: Infected treated with 200 mg/kg of plant sub-fraction S, Group 5: Infected treated with 400 mg/kg of plant sub-fraction S.

Immunosuppression of Mice for in Vivo Studies

Mice were immunosuppressed by intraperitoneal injection of Cyclophosphamide (Baxter Oncology GmbH- Kanststrasse2-33790Halle-Kunsebeck, Germany) at 200 mg/kg 4 days before and 1 day after inoculation with *Candida albicans* strains.

The oestrogen dependent vaginal candidiasis was used for the vaginal candidiasis experiment[19,20]. All the animals were injected subcutaneously with 0.2 mL (0.5 mg/mL) of estradiol solution (Oromone, Abbott Biologicals B.V. Veerweg 12 8121 AA Olst PAYS-BAS)[23] two days before inoculation, once a week after inoculation until the end of the experiment. This for the mice to be in a state of pseudooestrus.

e-ISSN:2321-6182
p-ISSN:2347-2332

Induction of Systemic and Vaginal Candidiasis

After the induction of pseudooestrus and immunosuppression, the animals were inoculated intravaginally with 10 µL of *C. albicans* NR-29445 suspension (1.3×10^7 CFU/mL) for vaginal candidiasis[23,21] and intraperitoneally with 0.2 mL of a cell suspension of 1×10^6 cells/mL of *C. albicans* NR-29450. These microorganisms were obtained from BEI resources. The evolution of the infection was monitored by plating vaginal and blood cultures from each animal on SDA.

Treatment and Determination of Healing Properties of the Potent Sub-Fraction S

The sub-fraction S at 100 mg/kg, 200 mg/kg and 400 mg/kg, Fluconazole at 150 mg/kg and distilled water at 2 mL/100 g were administered once daily starting 2 days after infection by oral gavage for 10 days in the vaginal candidiasis experiment. The vaginal fungal load from each mouse were collected using swaps and plated on SDA petri dishes and the number of CFUs were counted. The kinetic of evolution of the treatment was noted every 48 hours for 10 days. The results were expressed as Log CFU per 2 days per dose.

The sub-fraction S at 100 mg/kg, 200 mg/kg and 400 mg/kg, Fluconazole at 150 mg/kg and distilled water at 2 mL/100g were administered once daily starting 2 days after infection by oral gavage for 6 days in the systemic candidiasis experiment. The spleen, liver, lungs, kidneys and blood were submitted to microbiological evaluation in order to determine the fungal load. After collection, the samples were weighed and macerated in 1.0 mL of sterile PBS. A volume of 0.1 mL was spread over culture plates containing Sabouraud-dextrose agar using an inoculating loop. The procedures were performed in duplicate. The plates were then sealed and incubated at 37 °C for 2 days and the number of colony forming units (CFU) noted. Total colony-forming units (CFU) were expressed as the Log_{10} CFU per organ. Growth of more than 10 CFU per 0.1 mL tissue homogenate was considered indicative of systemic infection[24]. In addition, the relative organ weight of these organs were also calculated.

Histopathological Studies

In order to assess the level of damage of the organs and healing effect of the sub-fraction, kidneys, liver, lungs, spleen and vagina retrieved from sacrificed mice were fixed in 10% buffered formalin and processed by standard tissue processing techniques. Paraffin-embedded specimens were cut into thin sections and stained with haematoxylin and eosin for histological analysis. The investigations on each organ were to assess tissue structure and healing effect of sub-fraction S.

Statistical Analysis

The data were statistically analysed using the software SPSS 17.0 for Windows and analysis of variance (ANOVA) coupled with Tukey test with $p<0.05$ considered as statistically significant.

RESULTS AND DISCUSSION

Acute Toxicity

The animals in both vehicle treated and extract-treated groups were normal and did not display significant changes in their behaviour when compared to the control. The body weight of the mice increased during the observation period. No mortality was recorded in the female mice in all the groups.

The **Figure 1** shows the effect of extract on principal organ weights relative to body weight. There were no significant changes in body weight. All animals exhibited a normal increment in body weight without great difference between both control and treated groups.

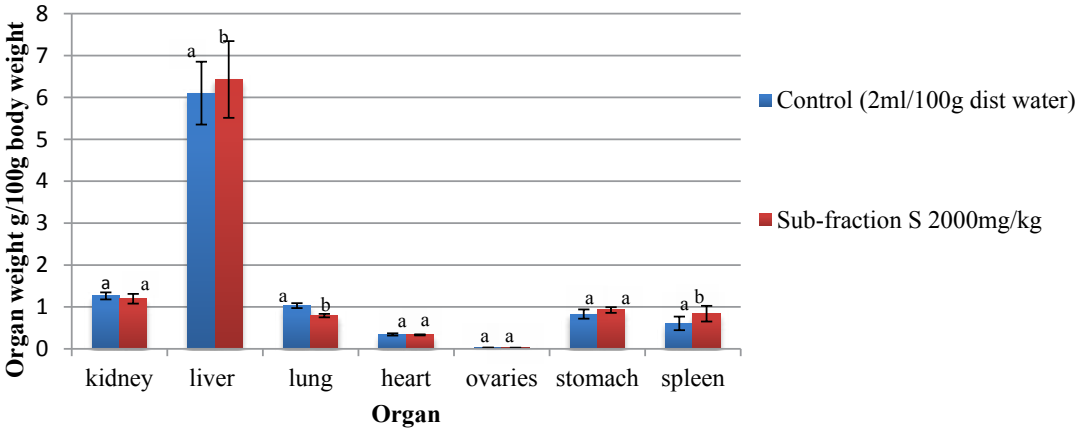


Figure 1. Relative Organ Weight of mice (ROW) per 100g body weight recorded at the end of the study from experimental mice after 14 days. A indicate significant difference at $p \leq 0.05$; b, $p \leq 0.05$. In the places where there are same letters means similar effects was observed in the organs.

The organs in the two groups with same letters showed similar weight variation and with different letters showed different variation.

e-ISSN:2321-6182
p-ISSN:2347-2332

IN-VIVO ANTI-CANDIDAL ACTIVITIES

Antivulvovaginal Candidiasis

Observation of mice during the vaginal candidiasis

During the experiment, the mice were observed for behaviour and symptoms. These included vaginal discharge, typically 'cottage-cheese-like' but varying from watery to thick mucus, itching. These symptoms confirmed the establishment of the infection.

The Effect of Plant Sub-fractions After 10 Days of Treatment

Treatment of vaginal candidiasis with sub-fraction S

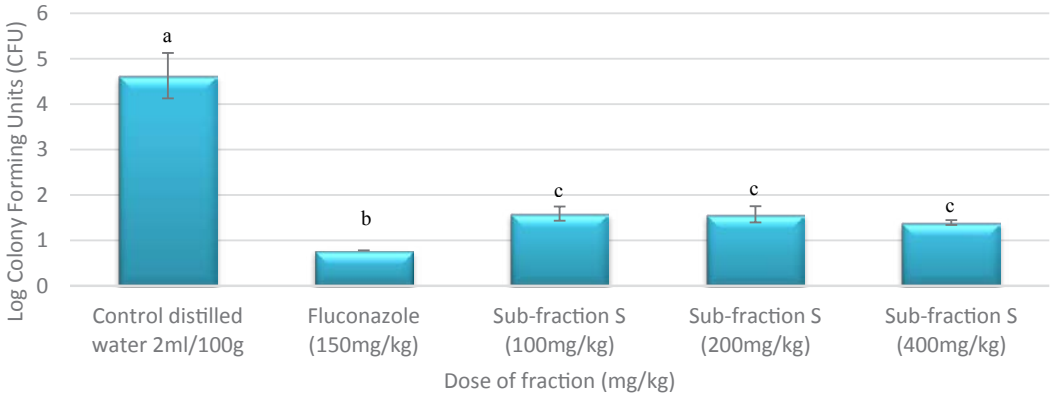


Figure 2: The effect of plant sub-fraction S after 10 days of treatment.
a, indicate significant difference at $p \leq 0.05$; b, $p \leq 0.05$; c, $p \leq 0.05$. a, b, c indicate there is significant difference among the treatment groups.

The results of the vaginal culture before infection showed no colony counts, indicating the mice were safe and free from infection. The number of the colonies formed was noted and expressed as shown in **Figure 2**. There was a significant difference between the different doses, 100 mg/kg, 200 mg/kg and 400 mg/kg compared to the control at $p \leq 0.05$. All the groups infected showed a positive infection with the highest number of colony counts of log CFU ± SD = 4.62 ± 0.50 in the control treated with 2 mL/100 g distilled water. In the control treated with fluconazole the log CFU was 0.77 ± 0.00. In the groups treated with 100 and 200 mg/kg, a reduction was observed with 66% healing compared to the control (with log CFU 1.58 ± 0.16 and 1.57 ± 0.17 respectively). The group treated with 400 mg/kg, the healing was observed at 70%.

The Kinetic of treatment of vaginal candidiasis over ten days

The therapeutic effect of the sub-fraction S on mice vaginal candidiasis model showed that the infection was established in all experimental animals two days after inoculation with *C. albicans* **(Figure3)**.

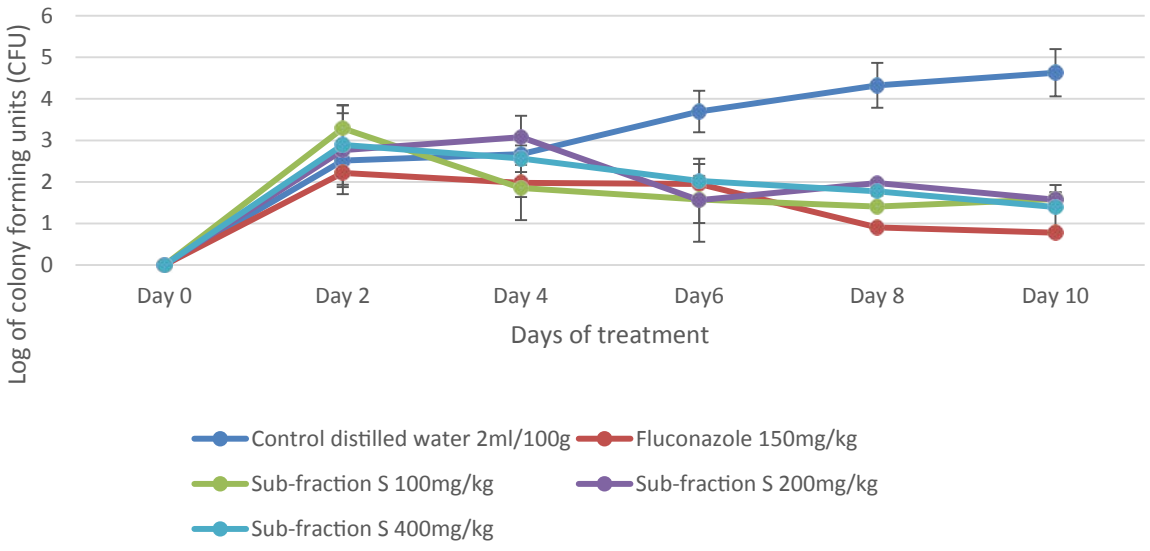


Figure 3. The kinetic of treatment of vaginal candidiasis over ten days.The different days with respect to the different treatment doses with variation in the number of colony units.

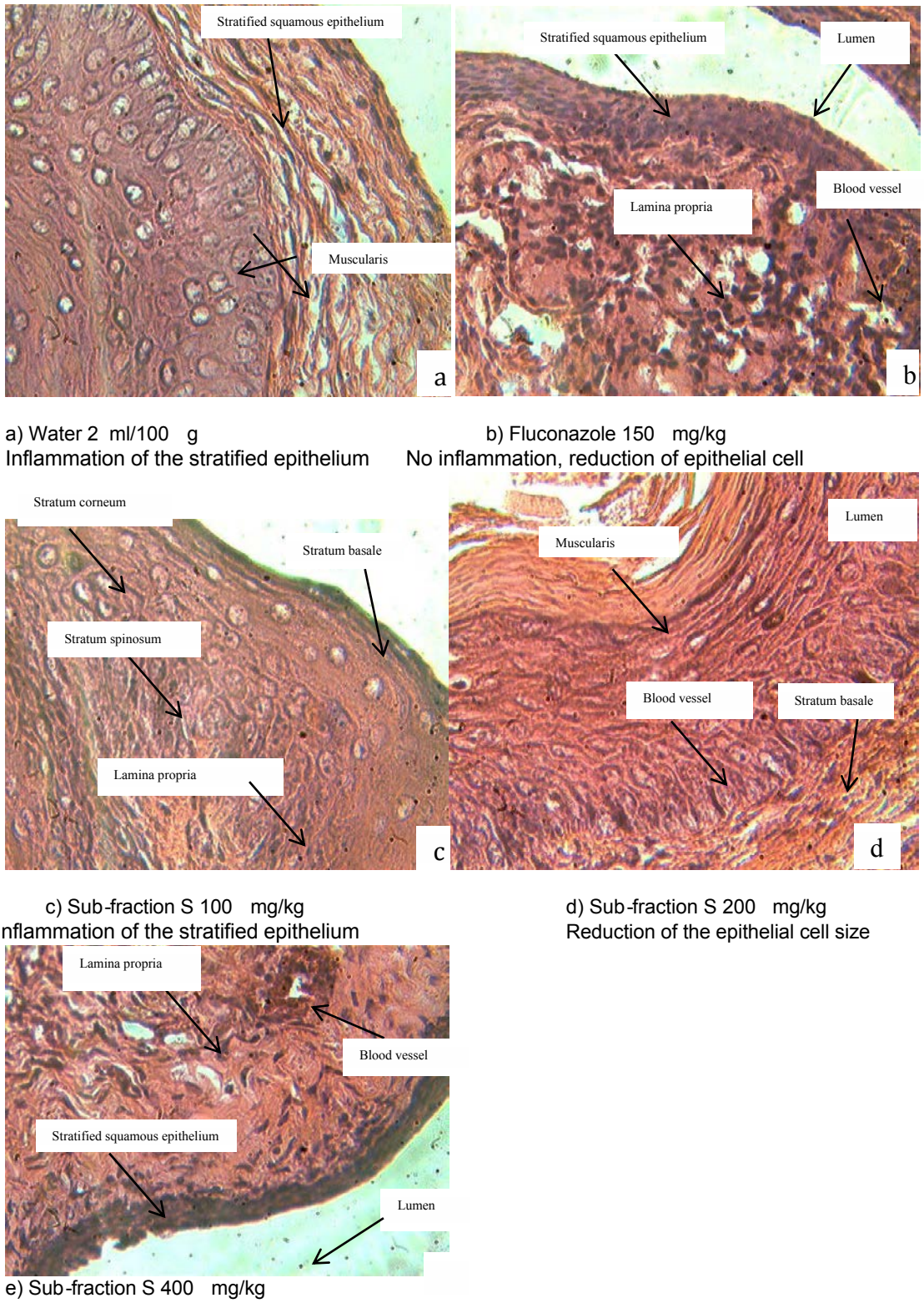


Figure 4. The effect of sub-fraction S at different concentrations on the walls of the vagina after 10 days of treatment.

The treatment of these animals with the different doses induced an important time-decrease in the number of yeast colonies in the vagina of infected animals. The treatment with sub-fraction S at 400 mg /kg was the most effective in reducing the *Candida* vaginal infection. Thus, the treatment with sub-fraction S is efficacious in resolving experimental *Candida* infection in mice. The results obtained could be linked to the bioactive components present in the sub-fraction S (flavonoids, terpenoids and glycosides)[16]. These bioactive compounds could also act in combination by producing the observed effects in the reduction of the number of colony forming units in the tissues[22].

Histopathological study of the vagina revealed inflammation of the stratified epithelium in the group treated with distilled

e-ISSN:2321-6182
p-ISSN:2347-2332

water and subfraction S at 100 mg/kg (picture a and c). No inflammation and reduction of epithelial cell reaching normal was observed in the groups treated with fluconazole at 150 mg/kg, sub-fraction S at 200 mg/kg and 400 mg/kg (picture b, d and e).The results in the different treatment groups showed healing effects after 10 days of treatment. However, an increase in the number of days of the treatment could completely eradicate this infection. Moreover, persistence of this infection may eventually cause systemic candidiasis and candidaemia.

SYSTEMIC CANDIDIASIS

Observation of the Mice During the Experiment

During the experiments, the mice eye balls were protruded, their fur was rougnened and they were generally less active as compared to the normal group with increased group huddle and sleep. They also appear very weak and thin with the curvatures of the bony structures beneath the mice visible to the naked eye. All the animals developed soft, pale stools the day after the first inoculation; and were used in subsequent investigations.

Metastatic deposits were found in the kidneys, lungs, liver and spleen. The pathogenesis of invasive candidiasis is thought to involve colonization/adhesion of the fungus to host cells, penetration and invasion of host cell barriers and finally dissemination via the blood stream[26].

Quantification of the Colony-forming Units in the Organs

The liver, lungs, kidneys, spleen and blood were cultured after 6 days of treatment at different doses and colony forming units counted. The liver had the highest number of colony forming units of *C. albicans*, followed by the kidneys, lungs and spleen.

Effect of the Different Doses of Plant Sub-fraction S on the Colony Forming Units of Organs

Effect of sub-fraction S at different doses on the colony forming units on liver

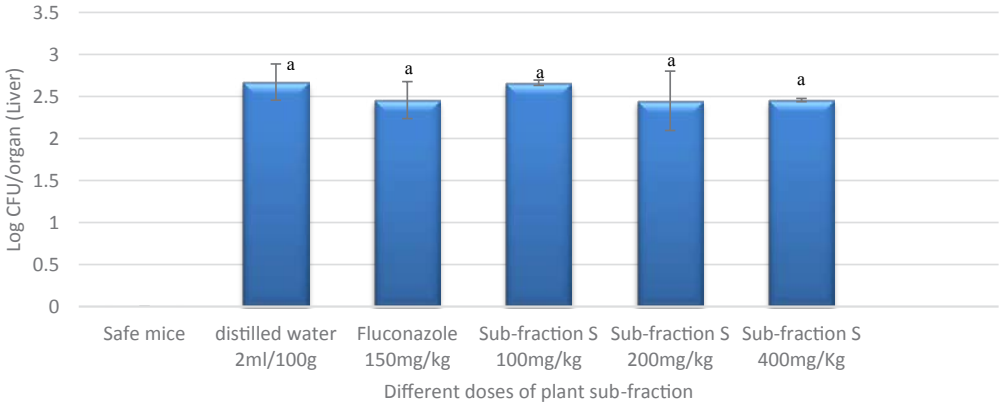


Figure 5. Effect of the different doses on the colony forming units of the liver.
a, p ≤ 0.05, same letter indicate similar effect is observed in all the groups.

There was a similar variation in the reduction of the colony forming units at the different doses tested. The liver being the organ for detoxification, the reduction of the colony forming units in the liver varied between 2.38± 0.16 to 2.67±0.21 at different doses. The highest number of colony forming units being the control group (treated with distilled water) as shown in **Figure 5**.

Effect of sub-fraction S at different doses on the colony forming units on spleen

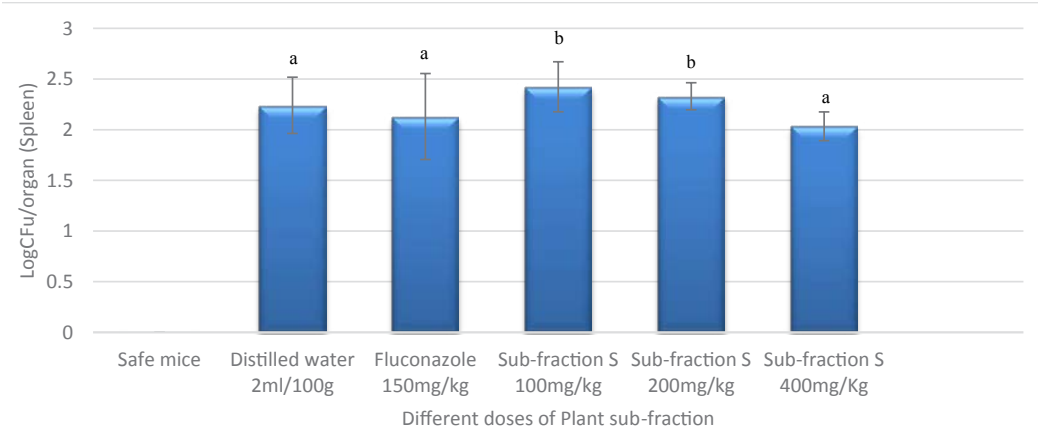


Figure 6. Effect of the different doses on the colony forming units of the spleen.
a, b p ≤ 0.05, same letter indicate similar effect is observed in all the groups.

e-ISSN:2321-6182
p-ISSN:2347-2332

Similar effects was observed with the groups treated with sub-fraction S at 100 mg/kg and 200 mg/kg. The group receiving distilled water had similar effects with fluconazole 150 mg/kg and 400 mg/kg. The higher the number of colony forming units the lower the effects of the sub-fraction S. The treatment with sub-fraction S at 400 mg/kg after 6 days was efficient and comparable to the group treated with fluconazole at 150 mg/kg.

The spleen plays an important role in clearing *Candida* cells from the bloodstream. The presence and growth of *Candida* in the spleen of the mice is indicative that infection was effective[27]. This indicated that an increase in the treatment period could have high possibility of observing very low colony count at 400 mg/kg **(Figure 6)**.

Effect of sub-fraction S at different doses on the colony forming units on the lungs

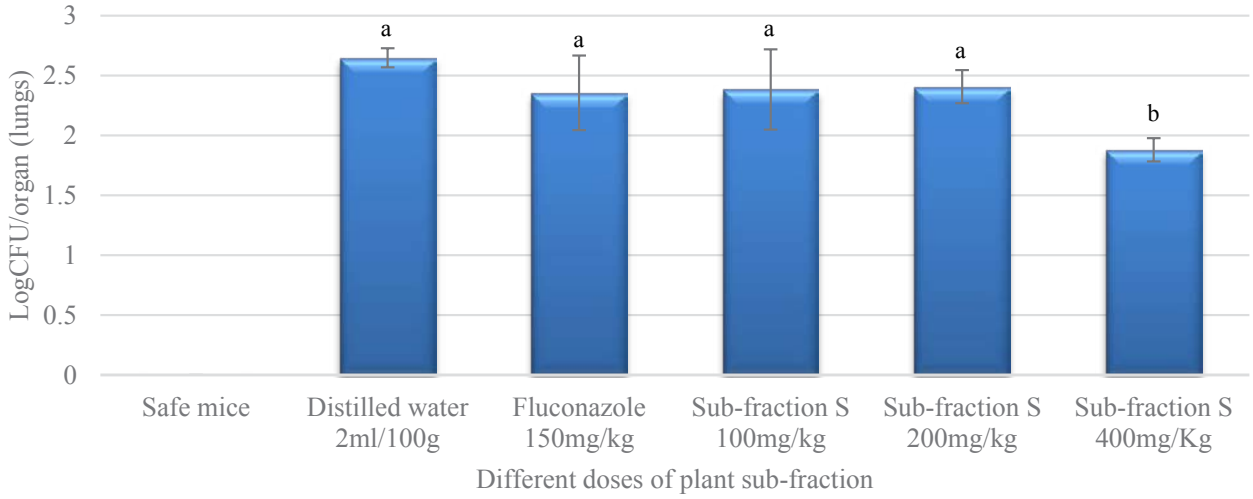


Figure 7. Effect of the different doses on the colony forming units of the lungs.
a, b, indicate significant difference at $p \leq 0.05$, same letter indicate similar effect is observed in the groups.

The same letters indicate similar effects was observed among the groups i.e sub-fraction S 100 mg/kg and 200 mg/ kg. The sub-fraction S at 400 mg/ kg reduced the number of colony forming units in the lungs.

The lungs are also effective against, and a target of, *Candida* infection. Indeed, the high colony forming units counts in the lungs observed compared to other organs confirm those previously reported, emphasizing the efficiency of the lungs in retaining and eliminating viable *Candida* (blastospores and pseudohyphae) in *in-vivo* experimental infection models[27,28]. The results showed **(Figure 7)** that groups treated with sub-fraction S at 100 mg/kg, 400 mg/kg and fluconazole 150 mg/kg performed similar clearing of *Candida albicans* at the level of the lungs. Those treated with sub-fraction S at 200 mg/kg showed the best reduction in the number of colonies **(Figures 8-10).**

Effect of sub-fraction S at different doses on the colony forming units on the kidneys

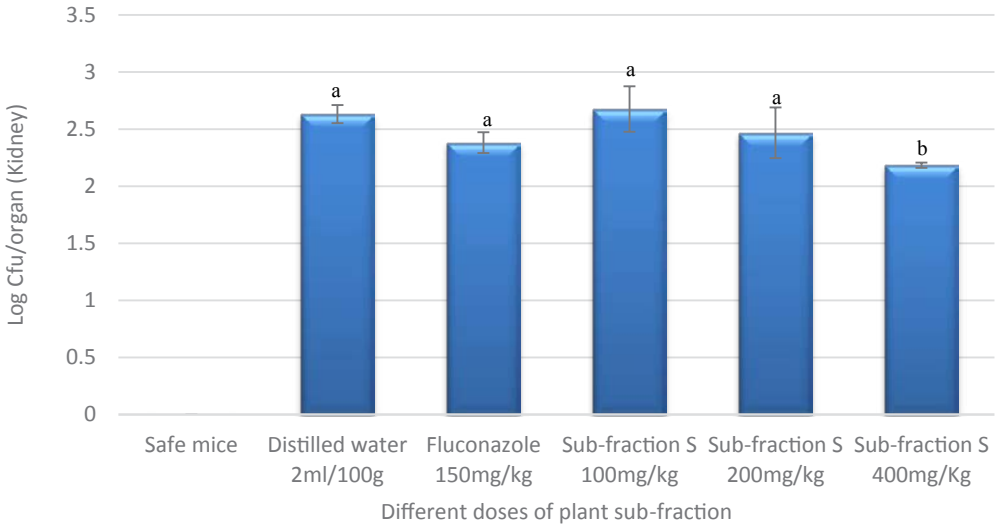


Figure 8. The effect of the different doses of plant sub-fraction on colony forming units in the kidneys.
a, b, indicate significant difference at $p \leq 0.05$, same letter indicate similar effect is observed in the groups.

The same letters indicate similar effects was observed among the groups i.e. sub-fraction S 100 mg/kg and 200 mg/kg. The sub-fraction S at 400 mg/kg reduced the number of colony forming units in the kidneys.

e-ISSN:2321-6182
p-ISSN:2347-2332

Effect of sub-fraction S at different doses on the colony forming units on blood

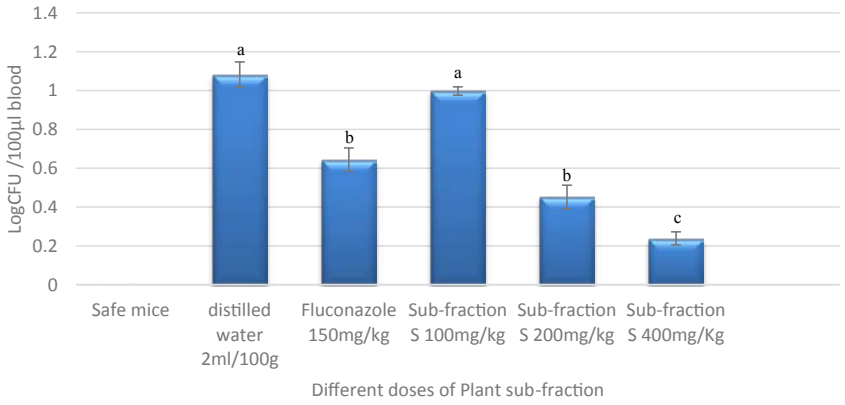


Figure 9. Effects of the different treatment groups on the colony forming units in blood at the end of the experiment. a, indicate significant difference at $p \leq 0.05$, b, indicate significant difference at $p \leq 0.05$; c, $p \leq 0.05$ indicate significant difference.

The same letters indicate similar effects was observed among the groups i.e distilled water 2 ml/100 g and sub-fraction S 100 mg/kg. The sub-fraction S at 200 mg/kg and 400 mg/kg reduced the number of colony forming units in the blood. This indicate that blood cleared the microorganisms very fast.

EVOLUTION OF THE WEIGHT OF MICE, RELATIVE ORGAN WEIGHT AND HISTOLOGY

Effects of the Relative Organ weight with Respect to the Groups

Effect of sub-fraction S at different doses on the relative organ weight of the lungs

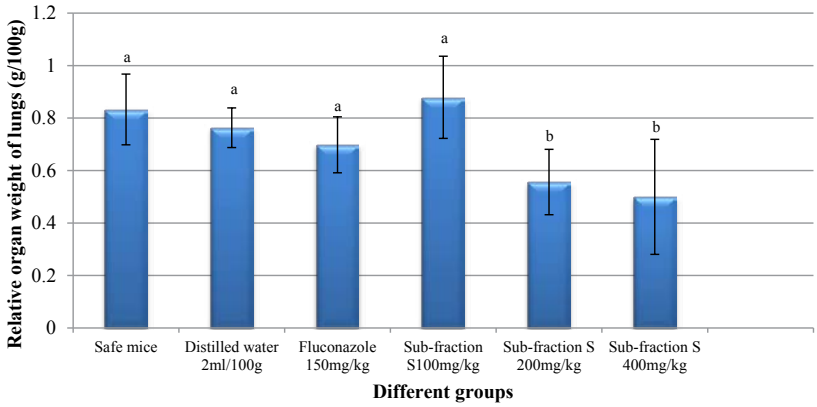
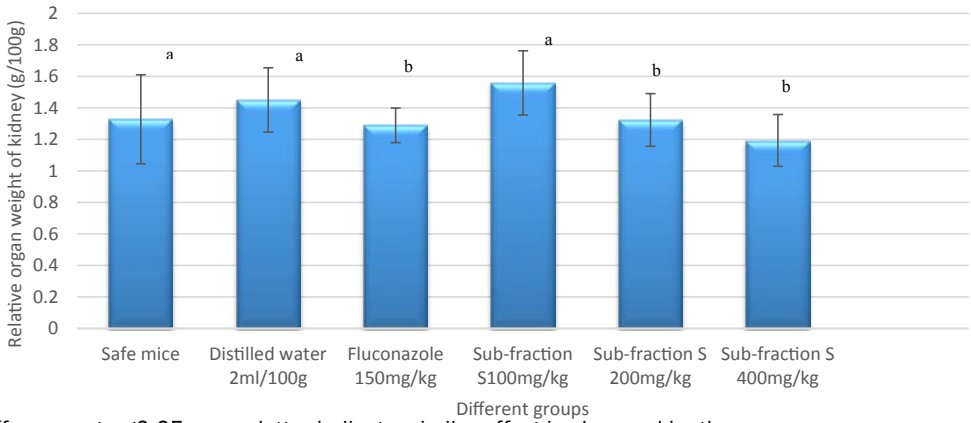


Figure 10. Relative weight of lungs in the different groups.
a, b, indicate significant difference at $p \leq 0.05$, same letter indicate similar effect is observed in the groups.

The results **(Figure 10)** showed there was reduction in the relative weight of the lungs in the entire group compared to the control. The group treated at the dose of 400 mg/kg also had a lower organ weight, this could be due to the lower effect of the sub-fraction S in this organs and high number of colony forming units observe.

Effect of sub-fraction S at different doses on the relative organ weight of the kidneys



a,b, indicate significant difference at $p \leq 0.05$, same letter indicate similar effect is observed in the groups

Figure 11. Relative weight of kidney in the different groups.

e-ISSN:2321-6182
p-ISSN:2347-2332

The kidneys also showed relative organ variation in most of the groups compared to the control. The group treated with 100 mg/kg of sub-fraction S showed the highest relative organ weight and that of 400 mg/kg showed the low weight compared to the control groups. The group treated with Fluconazole, 150 mg/kg, and that of 200 mg/kg of sub-fraction S showed similar weight compared to the control. A constant decrease in the relative organ weight **(Figure 11)** of the kidneys was observed at the dose of 200 mg/kg and 400 mg/kg. The observed results could be related to the high number of colony forming units in these groups.

Effect of sub-fraction S at different doses on the relative organ weight of the spleen

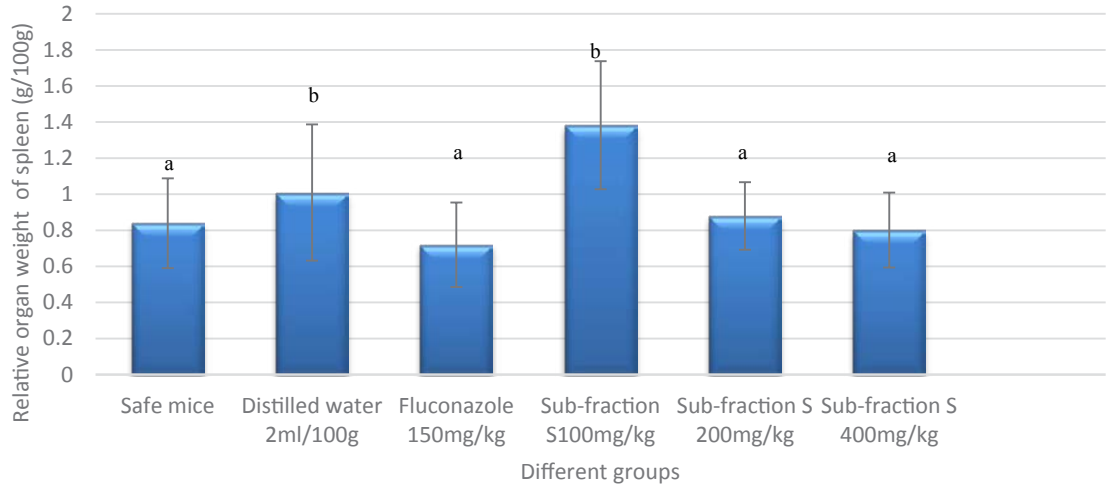


Figure 12. Relative weight of spleen in the different groups.

a, b, indicate significant difference at $p \leq 0.05$, same letter indicate similar effect is observed in the groups.

The results showed that sub-fraction S 100 mg/kg has the highest weight compared to the control. There is no great difference between the relative organ weight of sub-fraction S at 200 mg/kg and 400 mg/kg compared to the control **(Figure 12)**. This could be due to the lower number of colonies observed in the organs of the mice in the different groups.

Effect of sub-fraction S at different doses on the relative organ weight of the liver

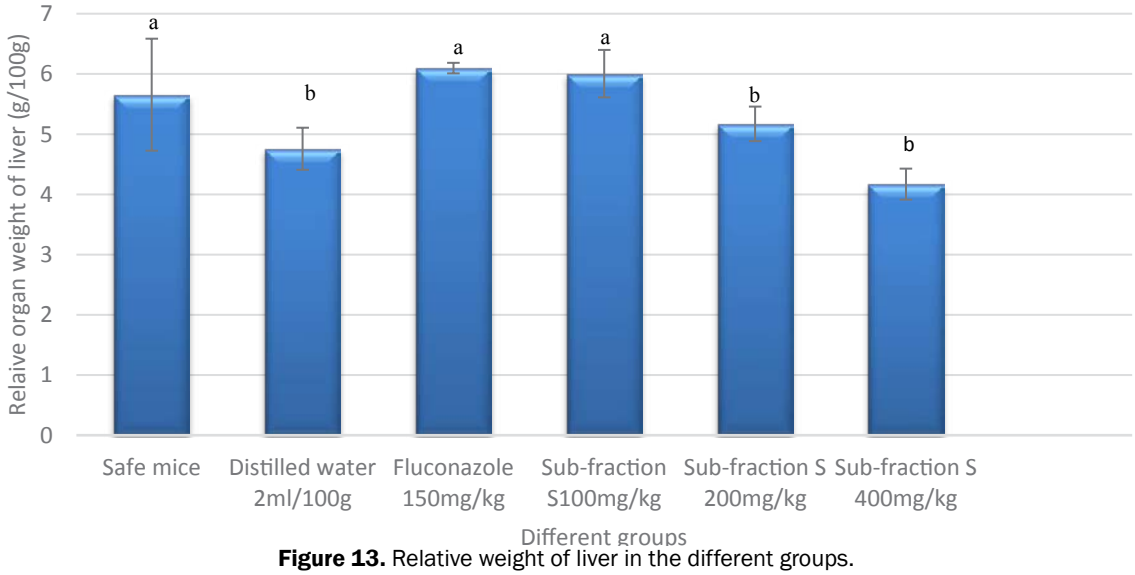


Figure 13. Relative weight of liver in the different groups.

The relative organ weight of the liver in the different groups with distilled water 2 mL/100 g and sub-fraction S, 400 mg/kg being the lowest **(Figure 13)**. Fluconazole, 150 mg/kg and sub-fraction S, 100 mg/kg showed similar weight. The variation in the relative organ weight of the liver is different from the number of colony obtained. The effect could be organ specific, i.e reducing the number of colony forming units on one organ, thereby reducing the intensity of the disease in the said organ.

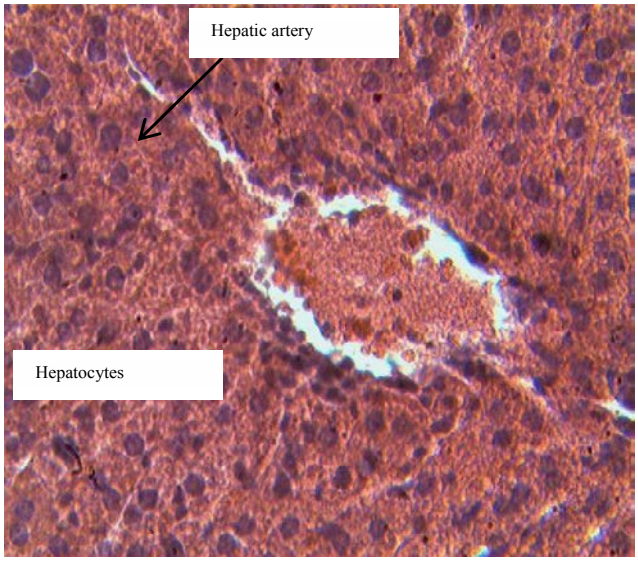
Results of Histopathological Parameters on the Different Organs

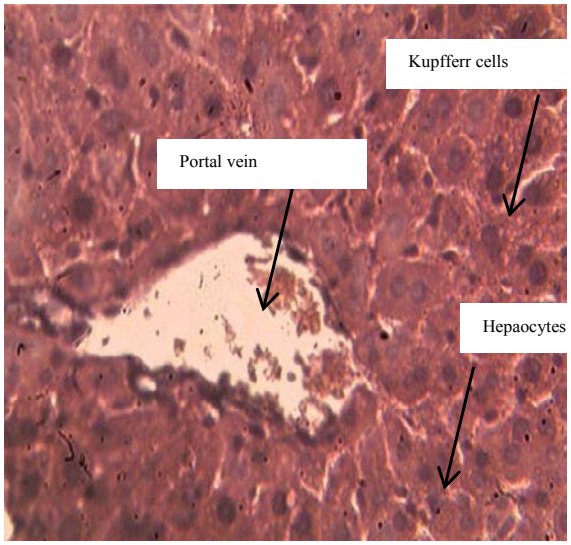
The different groups in **Figure 14** showed the effects of *Candida* cells as well as the therapeutic effect of sub-fraction S on the liver tissues of the mouse (BALB/c). The inflammation of the liver (picture d), disorganised sinusoids (picture c), vascular congestion (picture d), leucocytes infiltration (picture d), and sinusoids damaged and hemorage/bleeding (picture d) were

e-ISSN:2321-6182
p-ISSN:2347-2332

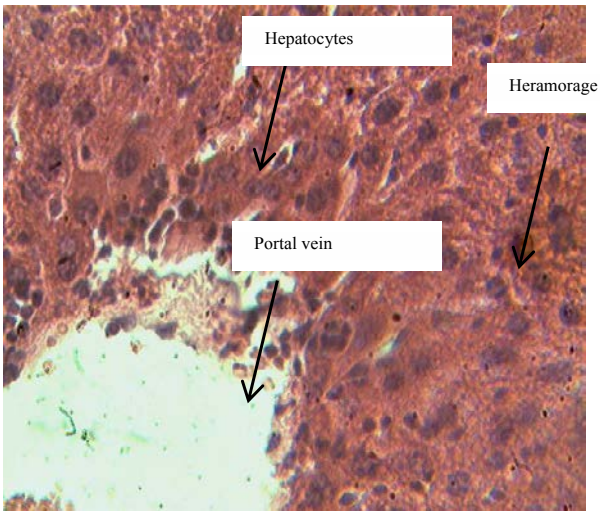
observed in the different groups of the treated mice.

Histopathological parameters of the liver

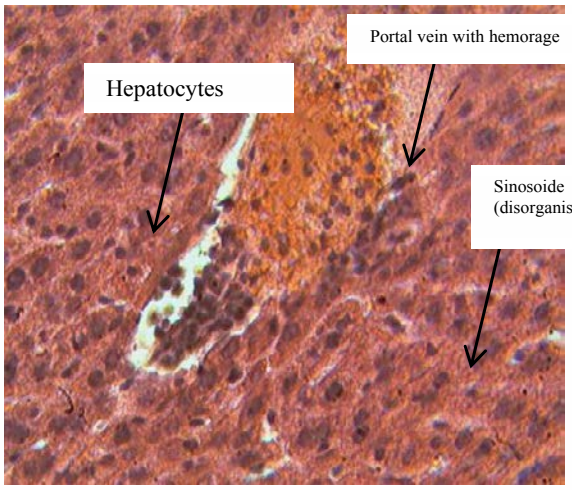




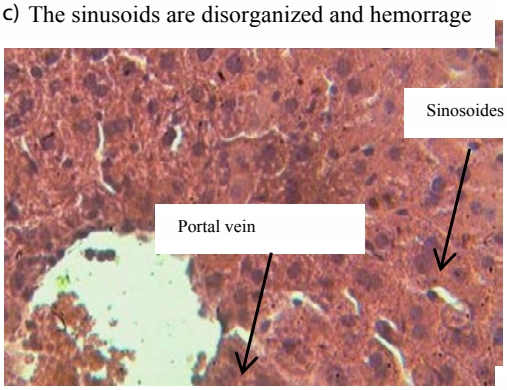
a) safe mice: normal leucocytes and hepatocytes



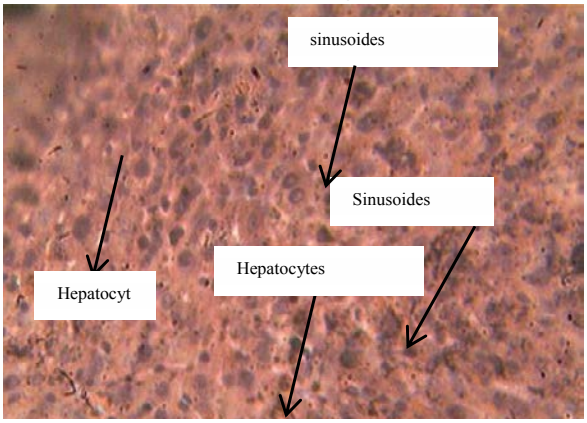
c) The sinusoids are disorganized and hemorrage



d) Inflammation of the liver, vascular congestion, leucocytes infiltration



e) Sub-fraction S 200 mg/kg: normal



f) Sub-fraction S 400mg/kg: leucocytes

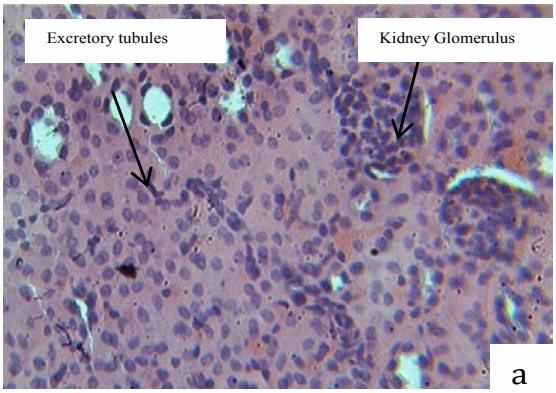
Figure 14. Histology of the liver of different group's 400x magnification.

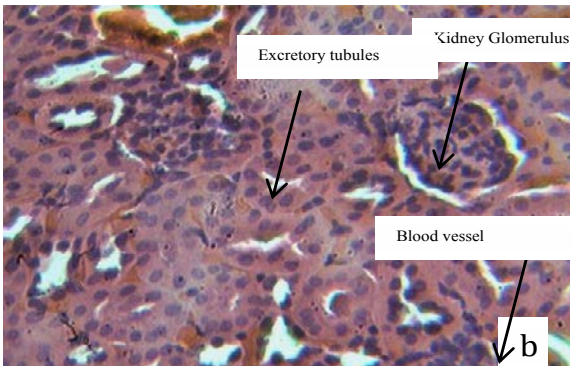
Histopathological parameters of the kidneys

The different groups showed **(Figure 15)** the tissues of the kidney during infection and after treatment with the subfraction S showing disorganized tubules (picture b: treated with distilled water) vasculation congestion, leucocytes filtration, Mesangiale expansion and hemorrage (Picture c: group treated with fluconazole), vasculation congestion, inflammation and hemorrage

e-ISSN:2321-6182
p-ISSN:2347-2332

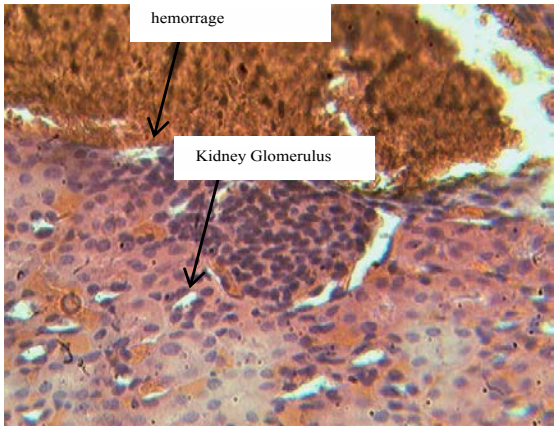
(picture d: treated with sub-fraction S 100 mg/kg). The group treated with sub-fraction S 200 mg/kg and 400 mg/kg showed no abnormalities.

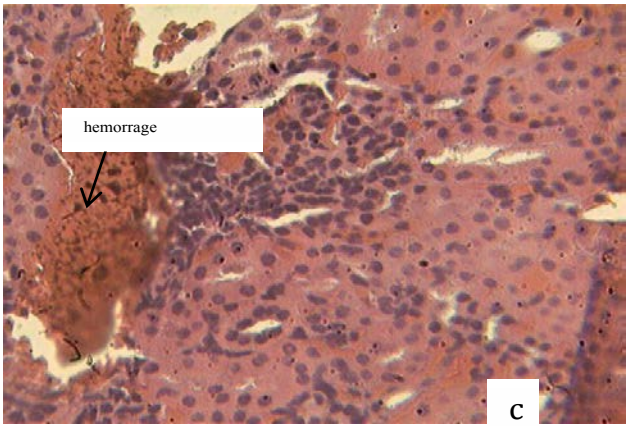




a) Safe mice: normal cells and

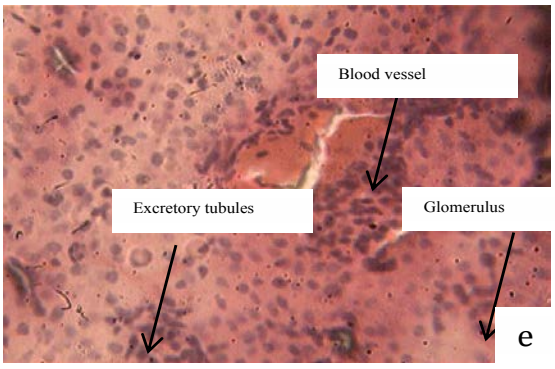
Excretory tubules

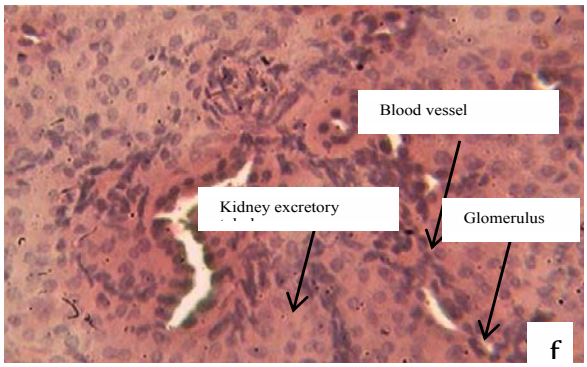




c) Fluconazole 150 mg/kg Vasculation congestion, leucocytes filtration,

d) Sub-fraction S 100 mg/kg Vasculation congestion, haemorrage





e) Sub-fraction S 200mg/kg: normal

f) Sub-fraction S 400mg/kg): normal

Figure 15. Histology of kidneys of different groups infected with *C. albicans* and treated with sub-fraction S.

DISCUSSION

In this study, the macroscopic examination of the organs of the animals treated with extract showed no changes in colour compared to control. Autopsy at the end of the experiment revealed no apparent changes in the liver, kidneys, lungs, heart and spleen from both control and treated mice. The clinical symptom is one of the major important observations to indicate the side effects on organs in the treated groups[22].The alterations of body and internal organ weights reflect the side effects after exposure to the toxic substances[23,24,25]. The heart, liver, kidneys, spleen, stomach, ovaries and lungs are the primary organs affected by metabolic reaction caused by toxicant[26]. This indicated that the LD50 value was greater than 2000 mg/kg. In principle, the limit test method is not intended for determining a precise LD50 value, but it served as a suggestion for classifying the sub-fraction or extract[27]. According to the chemical labelling and classification recommended by OECD, the sub-fraction was assigned class 5, which is the lowest toxicity class. According to the study by[28] substances with LD50 values greater than 2000 mg/kg by oral route are regarded as being safe or practically non-toxic. This is the first time the acute oral toxicity of this sub-fraction is highlighted.

e-ISSN:2321-6182
p-ISSN:2347-2332

C. albicans is known to be the most important microorganism responsible for about 20 to 25% of candidiasis[36]. However, the development of mucosal infection models generally requires the use of immunosuppressive agents, antibiotic, estrogen treatment, or the use of germ-free animals[29,30]. Local rather than systemic immunity is critical for anti-candida defense in the vaginal mucosa. Estrogen transforms the columnar epithelium into thicker stratified squamous epithelium and increases the glycogen content, pH, and growth substrates, all of which facilitate *C. albicans* infection. In addition, estrogen acts on both the fungus and the reproductive tract epithelium of the host to enhance fungal adhesion, hyphal growth, and colonization[39,31]. Estrogen may also inhibit innate or adaptive immune defenses, thus facilitating tissue evasion[31,33], but in the absence of pseudoestrus, vaginal infections are short-lived[34,35]. The use of immunosuppressive is a valid method of quickly inducing fungal colonization. In rodents, Cyclophosphamide (CPA) inhibits the production of antibodies, and is active in cells with high mitotic activity inhibiting cellular and humoral immune responses[33,35]. Studies have shown that immunosuppression are necessary to the success of vulvovaginal candidiasis model[36,37,38]. The different doses of sub-fraction S 100 mg/kg, 200 mg/kg and 400 mg/kg showed considerable healing effect of the vaginal candidiasis after 10 days of treatment. The reduction in the number of colony forming unit in the group of mice treated with sub-fraction S at the dose of 400 mg/kg showed similar effect to that obtained with the group treated with fluconazole 150 mg/kg. Moreover, after ten days of treatment some fungal colonies still remain but no symptoms were observed, there was complete dryness of the vagina of the mice in the different groups. This indicated that sub-fraction S could possibly treat the disease and eradicate the visible symptoms in the infected mice. However, fluconazole at 150 mg/kg confirms its use in the treatment of vaginal candidiasis by oral route. Fluconazole has now been in popular use for two decades, dominating the treatment choice for vagina candidiasis[48]. Moreover, this route of administration is convenient, the cheapest available route, easy to use, safe and acceptable[48,39]. *Candida* species caused infections which can emerge from mucocutaneous candidiasis to life-threatening candidaemia[50,40,7]. Thus, an increased colonization burden may predispose them to invasive candidiasis[41].Therefore, the oral treatment could be used for the treatment of vaginal candidiasis (Mucosal) and the prevention of invasive or disseminated candidiasis.

The results of histology of the vagina showed inflammation of the stratified columnar epithelium of the group of mice treated with distilled water while that of the group treated with sub-fraction S at the dose of 400 mg/kg showed reduced inflammation. The histopathological study of groups treated with sub-fraction S showed residual inflammatory infiltrates, epithelial desquamation and hyperkeratosis as signs of tissue reaction associated with the infection. Despite the therapeutic treatment with sub-fraction S on the eradication of the vaginal fungal burden from infected mice, the sub-fraction S was not able to decrease the signals of inflammation. This observations are similar to that observed by[36] who worked with methanolic extract of *Syngonanthus nitens.*

In this assay a greater weight loss and higher fungal burdens and increased virulence was observed[42]. Gross anatomical evaluation revealed inflammation in these organs. In the mice, the highest and lowest number of fungi were found in the liver and spleen, respectively. Although *C. albicans* cells adapt to the available nutrients in their local niche[43,44], our data show clearly that the initial fitness advantage conferred by growth due to immunosuppression is ultimately reflected in the infection outcome in both systemic and vaginal infections.

After systemic infection with the yeast *Candida albicans*, inbred mice show substantial differences in mortality, organ colonization, and severity of tissue damage[45]. Although there is a loose correlation between mortality and fungal burden as measures of infection[46,47], histological assessment of the lesions has shown a dichotomy in patterns of tissue destruction in the liver and other organs of different inbred mice[48]. The mean infection rate was higher in the liver, kidneys, lungs and spleen than blood. Papdimitriou and Ashman[60] reported that though significant yeast deposition of *C. albicans* occurred in the lungs, kidneys, spleen and liver, the only histological evidence of colonization by the fungus, with a mild transient infection, was observed in the liver. Brown *et al.* also reported that *Candida* organisms multiplied to a greater extent in the kidneys than in the liver, spleen and lungs of rats and mice. The transient infection in the spleen, lymph nodes and thymus with minimal inflammatory reaction towards the terminal stage of the experiment and the failure to produce chronic lesions is due to the larger population of tissue phagocytes.

These quantitative variations in the amounts of *Candida* cells in the tissues occur within the context of genetically determined differences in susceptibility to tissue damage[48]. During systemic candidiasis, fungal cells can disseminate to virtually every organ within the human host, each with potentially different availability of nutrients. In the liver for example, *C. albicans* has access to large quantities of glycogen, the main storage molecule of glucose. The brain has high concentrations of glucose and vitamins as potential nutrient sources[50] In other tissues, *C. albicans* faces relatively poor glucose concentrations and uses alternative metabolic pathways to utilize host proteins, amino acids, lipids and phospholipids. It was recently shown that adaptation to different nutrient sources by *C. albicans* not only promotes survival and growth, but also affects virulence[51]. Growth on alternative carbon sources, such as lactate or amino acids, rendered the fungus more resistant to environmental stress and increase its virulence potential in both a mouse model of systemic candidiasis and vaginal infection model[51].

Candida recovery from the blood was obtained in the different groups with the *Candida* strain. Indeed, it has been reported that yeast is generally recovered from the blood after only a few hours of inoculation[52]. This reduction in blood with sub-fraction S was dose dependent **(Figure 9)**. This result indicates that sub-fraction S could reach the blood faster. This result suggests that *C. albicans* cells were cleared from the blood. The observed disappearance of *C. albicans* from the blood could be attributed to the

e-ISSN:2321-6182
p-ISSN:2347-2332

combined killing effect of sub-fraction S and the immune cells. This observation could be explained further by the fact that some of the *C. albicans* cells moved into the deep organs like the kidneys, being the primary target organ in systemic candidiasis where the multiplication of *C. albicans* occurs. Further studies must be performed to follow the effects of *Candida* on the host as well as the defence mechanisms deployed by the sub-fraction S on host against *Candida* infection.

In this study, inducing the immunosuppression status promoted *Candida* infection, which concurs with previously reported studies and justify the fact that the immunocompromise patients are more susceptible to fungal infection than healthy people[53]. This results is similar to those obtained by[54] on the immunosuppression state of the mice. This study demonstrates the effect of sub-fraction S on *Candida* infection on various host tissues, further investigations is needed to study the interaction pathways between sub-fraction S on *Candida* and the host cells, which may help to identify key mechanisms to improve the host's innate immunity against *Candida* infection.

Nevertheless, it is noteworthy that the result obtained could be considered as significant bearing in mind that *C. albicans* survival in the kidney plays a primary role in mortality in patients with disseminated candidiasis[56]. The kidneys have been identified as the most affected organ following *Candida* infection in experimental models[57,58] and also in human[59] due to the ability of the yeast to produce pseudohyphae in the tubular renal lumen and to penetrate into the renal parenchyma[60]. This is confirmed in this study, which demonstrates the disorganization of the tissue and its invasion by the *Candida* strain, similar to that obtained by[54]. In addition, these results highlight the renal susceptibility to *Candida* infection and the effects of sub-fraction S at different doses on the elimination of *Candida* colonies in this organ. At the dose of 200 mg/kg a reduction in the number of CFU is observed which is lower than that observed at the dose of 100 mg/kg **(Figure 8)**. The histologic analyses showed the presence of severe inflammation, as ascertained by leukocyte infiltration, abscesses, necrosis, and varying degrees of hydronephrosis. These findings also support those previously reported showing the severe invasion of the kidneys by *C. albicans*[54].

Our results revealed that sub-fraction S displayed an inhibitory effect on fungal proliferation *in- vivo*. This could be explained by the presence of glycosides, terpenoids and flavonoids in these extracts. However, reports on the effects of these compounds on the immune system have been reported with flavonoids having the ability to stimulate the cell mediated immune system as well as to enhance antibody production.

The results obtained with the hepatic wall homogenates were consistent with the histological findings. The extent of mucosal invasion correlated well with the colonization of the hepatic surface. This is similar to that obtained by[54]. The histology of the liver during infection was examined to determine the level of inflammation and the invasiveness of the disease. The high number of positive cultures concurs with previously reported data showing that this organ is the least effective in clearing the microorganisms[54]. *Candida* infection led to the formation of multiple abscesses in the liver resulting in tissue necrosis.

To the best of our knowledge, the *in vivo* anticandidal properties of sub-fraction S are herein reported for the first time. Therefore, a possible prolongation of the period of treatment in future investigation will help stabilize the efficiency of sub-fraction S.

CONCLUSION

Overall, the results obtained showed the *in-vivo* anticandidal potentials and toxicity profile of the sub-fractions from *U. calophyllum.* The results showed that the sub-fraction S contain secondary metabolites with promising activity profile. However, more work is needed to characterise, quantify the active principle and formulate a phytodrug.

REFERENCES

1. Mohandas V and Ballal M. Distribution of Candida Species in Different Clinical Samples and Their Virulence:Biofilm Formation, Proteinase and Phospholipase Production: A Study on Hospitalized Patients in Southern India. J Global Infects Dis. 2011; 3: 4–8.
2. Sobel JD. Candida vulvovaginitis, Waltham, MA, 2012.
3. Das Neves J, et al. Local treatment of vulvovaginal candidosis: general and practical considerations. Drugs. 2008; 68: 1787-1802.
4. Špaček J, et al. Clinical aspects and luteal phase assessment in patients with recurrent vulvovaginal candidiasis. Euro J of Obst & amp; Gynecology and Reproductive Biology. 2007;131: 198-202.
5. Ugochukwu Dennis O, et al. Epidemiology of Candida vaginitis in women of reproductive age in selected hospitals in Onitsha metropolis, Anambra state, Nigeria and its environs 2007-2012. J Pub Health and Epidem. 2013; 5: 459-562.
6. Menza N, et al. Prevalence of Vaginal Candidiasis and Determination of the Occurrence of Candida Species in Pregnant Women Attending the Antenatal Clinic of Thika District Hospital, Kenya. Open J Med Micro. 2013; 3: 264-272.
7. Pfaller MA and Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbio Rev. 2007; 20: 133-163.
8. Zaoutis TE, et al. The epidemiology and attributable outcomes of candidemia in adults and children hospitalized in the

e-ISSN:2321-6182
p-ISSN:2347-2332

United States: a propensity analysis. Clinical Infect Dis, 2005; 41: 1232-1239.

9. Gudlaugsson O, et al. Attributable mortality of nosocomial candidemia, revisited. Clin Infect Dis. 2003; 37: 1172-1177.
10. Perlroth J, et al. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. Medical Mycology, 2007; 45: 321-346.
11. Lewis RE. Current Concepts in Antifungal Pharmacology. Mayo Clin. 2011: 86: 805–817.
12. Tscherner M, et al. Pathogenesis and Antifungal Drug Resistance of the Human Fungal Pathogen Candida glabrata. Pharmaceuticals. 2011; 4: 169–186.
13. Kanafani ZA and Perfect JR. Antimicrobial resistance: Resistance to antifungal agents: Mechanisms and clinical impact. Clin. Infect. Dis. 2008; 46: 120–128.
14. Thierry Kammalac Ngouana, et al .Potent and Synergistic Extract Combinations from Terminalia Catappa, Terminalia Mantaly and Monodora tenuifolia Against Pathogenic Yeasts. Medicines. 2015; 2: 220-235.
15. Zeuko'o Menkem Elisabeth. Antifungal and Antioxidant Activities of Piptostigma calophyllum, Uvariodendron calophyllum and Uvariodendron molundense growing in Cameroon. J of Biolog Active Products from Nature. 2012; 2: 110 - 118.
16. Zeuko'o Menkem Elisabeth, et al. Anti-yeast activity of extracts and fractions from Uvariodendron calophyllum (Annonaceae). Intern J biological and chemi sci. 2015; 9: 2500-2522.
17. Bolou GEK. Évaluation in vitro de l'activité antibactérienne des extraits de Terminalia glaucescens planch sur Salmonella typhi et Salmonella typhimurium. Bulletin de la Société Royale des Sciences de Liège 2011; 80: 772–790.
18. Organization for Economic Co-operation and Development (OECD). The OECD Guideline for Testing of Chemical. The Organization of Economic Co-Operation Development, Paris, 2001; 1-14.
19. Ryley JF and McGregor S. Quantification of vaginal Candida albicans infections in Rodents. J Med and Vetin Mycology. 1986; 24: 455 - 460.
20. Fidel PL, et al. Efficacy of D0870 treatment of experimental Candida vaginitis. Antimicrobial Agents Chemotherapy. 1997; 41: 1455-1459.
21. Williamson EM. Synergy and other interactions in phytomedicines. Phytomedicine. 2001; 8: 401–409.
22. Eaton DL and Klaassen CD. Principles of toxicology. In Casarett and Doull's Toxicology: The Basic Science of Poisons .5th ed. McGraw-Hill, New York, USA, 1996.
23. Carol SA. Acute, Subchronic and Chronic Toxicology. CRC Press Inc. Boca Raton, FL, USA, 1995.
24. Raza M, et al. Effect of prolonged vigabatrin treatment on hematological and biochemical parameters in plasma, liver and kidney of Swiss albino mice. *J Pharma* Sci. 2002; 70: 135-145.
25. Teo S, et al. A 90 days oral gavage toxicity study of d-methylphenidate and d,l-methylphenidate in Sprague-Dawley rats. Toxicology. 2002; 79: 183-196.
26. Dybing E, et al. Hazard characterization of chemicals in food and diet: dose response, mechanism and extrapolation issues. Food Chemistry and Toxicology. 2002; 42: 237-282.
27. Roopashree TS, et al. Acute oral toxicity studies of antipsoriatic herbal mixture comprising of aqueous extracts of Calendula officinalis, Momordica charantia, Cassia tora and Azadirachta indica seed oil. Thai J Pharm Sci. 2009; 33: 74-83.
28. Kennedy GL, et al. Estimation of acute toxicity in rats by determination of the appromate lethal dose rather than LD_{50}. J Appl Toxi. 1986; 6: 145-148.
29. Carrara MA, et al. A new model of vaginal infection by Candida albicans in rats. Mycopathologia. 2010; 170: 331–338.
30. Tavanti A, et al. Outcome of experimental rat vaginitis by Candida albicans isolates with different karyotypes. Microbial Pathogenesis. 2010; 49: 47–50.
31. Ferrer J . Vaginal candidosis: Epidemiological and etiological factors. Intern J Gyno and Obst. 2000; 71: 1-7.
32. De Bernardis F, et al. Protection against rat vaginal candidiasis by adoptive transfer of vaginal B lymphocytes. FEMS Yeast Research. 2010; 4: 432 – 440.
33. Naglik JR. Animal models of mucosal Candida infection. FEMS Microbiology Letters. 2008; 283: 129 -139.
34. Rahman D, et al. Murine model of concurrent oral and vaginal Candida albicans colonization to study epithelial hostpathogen interactions, Microbes and Infection. 2007; 9: 615–622.
35. Hamad M, et al.Utility of the oestrogen dependent vaginal candidosis murine model in evaluating the efficacy of various

e-ISSN:2321-6182
p-ISSN:2347-2332

therapies against vaginal Candida albicans infection. Mycoses. 2006; 49: 104–108.

36. Araujo Marcelo Gonzaga De Freitas, et al.Evaluation of Syngonanthus nitens (Bong.) Ruhl. Extract as antifungal and in treatment of vulvovaginal candidiasis. Medi myco Online. 2013; 1-10.
37. Dhawan S, et al. Efficacy of CIM 1 166, a combination of compounds derived from Mentha spp. in alleviating experimental vulvovaginal candidiasis in mice. World J of Microb and Biotec. 2009; 25: 161 – 163.
38. Chami F, et al. Evaluation of carvacrol and eugenol as prophylaxis and treatment of vaginal candidiasis in an immunosuppressed rat model. J of Antimim Chemo. 2004; 54: 909–914.
39. Watson MC, et al. Oral versus intravaginal imidazole and triazole antifungal treatment of uncomplicated vulvovaginal candidiasis (thrush). Coch Data of Syste Rev. 2001.
40. Kim J and Sudbery P. Candida albicans, a major human fungal pathogen. J of Micro. 2011; 49: 171–177.
41. Achkar JM and Fries BC. Candida infections of the genitourinary tract. Clin Microb Rev, 2010; 23: 253–273.
42. Mac Callum Donna M. Hosting Infection: Experimental Models to Assay Candida Virulence. Intern J of Micro. 2010.
43. Rodaki A. Glucose promotes stress resistance in the fungal pathogen Candida albicans. Molecular Biology of the Cell. 2009; 20: 4845 - 4855.
44. Lorenz MC et al. Transcriptional response of Candida albicans upon internalization by macrophages. Eukaryotic Cell. 2004; 3: 1076–1087.
45. Ashman Robert B. Strain-Dependent Differences in Host Response to Candida albicans Infection in Mice Are Related to Organ Susceptibility and Infectious Load. Infection and immunity. 1996; 64: 1866 -1869.
46. Marquis G. Genetics of resistance to infection with Candida albicans in mice. British *J* Experimental Pathology.1988; 69: 651 - 660.
47. Salvin SB and Neta R. Resistance and susceptibility to infection in inbred murine strains. Variations in the response to thymic hormones in mice infected with Candida albicans. Cellular Immunology. 1983; 75: 160-172.
48. Ashman RB and Papadimitriou JM. Murine candidiasis. Pathogenesis and host responses in genetically distinct inbred mice. Immunology and Cell Biology. 1987; 65: 163–171.
49. Brown Mr et al. Systemic candidiasis in an apparently immunocompetent dog. J Veter Diag Invest. 2005; 17: 272–276.
50. Fleck CB, et al. Nutrient acquisition by pathogenic fungi: nutrient availability, pathway regulation, and differences in substrate utilization. Intern J Medi Micro. 2011.
51. Ene IV, et al. Host carbon sources modulate cell wall architecture, drug resistance and virulence in a fungal pathogen. Cellular Microbiology. 2012; 14: 1319 - 1335.
52. Trnovec T, et al. The distribution in mice of intravenously administered labelled Candida albicans. Sabouraudia. 1978; 16: 299-306.
53. Lass-Flörl C. Invasive fungal infections in pediatric patients: a review focusing on antifungal therapy. Expert Rev Anti-Infective Ther.2010; 8: 127-35.
54. Noumi Emira, et al. Effect of glucose supplementation on Candida albicans virulence in immunosuppressed Swiss albino mice (Mus musculus). Afric J Micro Rese. 2011; 5: 2479-2487.
55. Baine WB. Clearance of Candida albicans from blood-stream of rabbits. *Infection* and *Immunity*. 1974; 10: 1420 - 1425.
56. Bendel CM. Systemic infection following intravenous inoculation of mice with Candida albicans INT1 mutant strains. Molecular Genetics and Metabolism. 1999; 67: 343–351.
57. MacCallum DM. Massive induction of innate immune response to Candida albicans in the kidney in a murine intravenous challenge model. FEMS Yeast Rese. 2009; 9: 1111-1122.
58. Mariné M. Combined antifungal therapy in a murine infection by Candida glabrata. Journal of Antimicrobial Chemotherapy. 2006; 58: 1295-1298.
59. Karlowicz MG. Candidal renal and urinary tract infection in neonates. Seminar Perinatology. 2003; 27: 393-400.
60. Winblad B. Experimental renal candidiasis in mice and guinea pigs. Acta Pathologica et Microbiologica Scandinavica A. 1975; 83: 406-414.