



Effect of Methyl Chavicol on the *Candida albicans* Intracellular Glutathione

¹Jabbi, A.M., ²Sani,Z.R., and ²Yusuf,B.R,

¹ Department of Biological Sciences, Faculty of Science, Nigeria Police Academy, Wudil Kano State.

² Department of Biological Sciences, Faculty of Science, Federal University Gusau, Zamfara State.

Corresponding author: ajgusau@gmail.com

Received: November 2015

Revised and Accepted: January 2016

Abstract

Herbs, have long been recognized for their medicinal values. *Ocimum sanctum* belongs to the family Labiatae and is known as Tulsi in Hindi and Holy Basil in English. Tulsi constituents are chiefly femchol, methyl chavicol, linalool, eugenol and β -caryophyllene, responsible for its anti-bacterial, anti-septic and anti-fungal action. Inhibitory effects of aqueous and alcoholic extracts of Tulsi have been observed on enteric pathogens by Agar diffusion method. Tulsi is also known to have numerous pharmacological activities, such as antidiabetic, antistress, immunomodulatory, analgesic, antipyretic, anti-inflammatory, antiulcerogenic, antihypertensive, CNS depressant, hepatoprotective, radioprotective, antitumor and antimicrobial activities. Methyl chavicol a major constituent of Holy Basil (*Ocimum sanctum*) essential oil has been evaluated against the effect of methyl chavicol on the intracellular Glutathione. Cytosolic reduced glutathione was determined by the method of Jollow with slight modifications. In this method 1.0 ml of the cell free extract was precipitate with 1.0 ml of 4 % sulphosalicylic acid. The samples were then kept for 1 hour at 4°C and centrifuged at 1200 g for 15 minutes at 40°C. The assay mixture consisted of 0.1 ml of above supernatant, 2.7 ml of Phosphate buffer (0.01 M, pH 7.4) and 0.2 ml of freshly prepared 5, 5'- dithiobis-2-nitrobenzene (DTNB) in a total volume of 3.0 ml. The colour developed due to the formation of a yellow colored complex, 5-thio-2-nitrobenzoate, was measured immediately at 412 nm at 25°C. It has been found in our study that the levels of glutathione were found to be 144 μ mole/gm at zero hours i.e. in yeast uninduced cells. Post 2 hours induction shows that levels of reduced glutathione in control untreated cells were found to be 124.4375 μ mole/gm cells. It went down as the induction period increases; at the end of 24hours induction the GSH levels were found to be 86.56775 μ mole/gm. These results in agreement with the previous studies. All treated cells showed decreased levels of GSH as the induction period increases. Decrease in GSH was found to be in dose dependent manner. A subsequent fall in the value of reduced glutathione was observed with the increasing concentrations of MC with the lowest being 28.452 μ mole/gm cells at 50 μ g/ml concentration of MC after 24 hrs inducton. This result is in coordination with the assumption that methyl chavicol may also affect the glutathione metabolic enzymes and may have affected the cell defense armory.

Keywords: *Candida albicans*; Glutathione; Methyl Chavicol; *Ocimum sanctum*: intracellular inhibition.

1.0 INTRODUCTION

Candida albicans is a diploid fungus that grows both as yeast and filamentous cells and a causal agent of opportunistic oral and genital infections in humans, and *Candida onychomycosis*, an infection of the nail plate. Systemic fungal infections (fungemias) including those by *C. albicans* have emerged as important causes of morbidity and mortality in immunocompromised patients (e.g., AIDS, cancer chemotherapy, organ or bone marrow transplantation). *C. albicans* biofilms may form on the surface of implantable medical devices. In addition, hospital-acquired infections by *C. albicans* have become a cause of major health concerns. About 85-95 % of vaginal infections cases are responsible for physician office visits every year. The fungus exists as a single celled organism rather than as hyphae. There are about 500 species of yeast or yeast-like organisms. *Candida* is a dimorphic fungus i.e. it can take two forms. Most of the time it exists as oval, single yeast cells, which reproduce by budding. Normal room temperature favours the yeast form (blastocoonidial) of the organism, but under physiological conditions (temperature, pH and the presence of serum) it may develop into hyphal form. Pseudohyphal, composed of chains of cells, are also common. Among these, six are most frequently isolated in human infections. While *Candida albicans* is the most abundant and significant species, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, and *Candida lusitanae* are also isolated as causative agents of *Candida* infections. Nevertheless, the diversity of *Candida* spp. that are encountered in infections is expanding and the emergence of other species that were rarely in play in the past is now likely.

Candida frequently encountered as a harmless commensal of digestive system and vaginal tract and less frequently as opportunistic pathogen. Mycelial form of *Candida albicans* is considered more invasive, difficult to phagocytosed and therefore more pathogenic. In addition to its dimorphism, *C. albicans* possesses well organized phenotypic switching system that probably helps the pathogen to invade the host immune defense (D'Souza and Heitman, 2001). It is responsible for Candidiasis, a disease that can vary from superficial mucosal to life threatening

systemic disorders. The frequency of both superficial and invasive candidiasis has increased markedly over the last few decades. Patients that become severely immune-compromised because of underlying disease such as leukemia, acquired immunodeficiency syndrome or patients who undergo cancer therapy, organ transplant are particularly susceptible to opportunistic fungal infections. A number of antifungal agents are available for the treatment of candidal infection (Gupta and Thomas, 2003; Carrillo-Munoz *et. al.*, 2006). The major agents belong either to Polyenes, such as Amphotericin B and Nystatin or to the azoles, such as Itraconazole and Fluconazole. Currently, use of standard antifungal therapies can be limited because of toxicity, low efficacy rates, and drug resistance. Recent studies have indicated *Candida* resistance to azoles or hepatotoxicity and nephrotoxicity linked to Polyenes use, particularly Amphotericin B. In order to avoid these drawbacks, a search for new and effective product is needed for the treatment of this fungal infection. Various new antifungals have demonstrated therapeutic potential. This new agents may provide additional options for the treatment of superficial fungal infections and they may help to overcome the limitations of current treatments.

Localized disease, such as oral *Candidiasis*, onychomycosis, and vaginitis, results. These infections are usually limited to surface of host, and can be quickly and successfully controlled by the use of one of the available anti fungal agents. *Candida* infection typically become systemic and life threatening when the host is immunocompromised. *Candida albicans* is principal pathogen responsible for such infections, although other pathogen such as *Cryptococcus*, *Neoformans*, *Aspergillus*, *fumigatus*. *Histoplasma capsulatum* and other *Candida* species are more important as well (Dixon *et. al.*, 1996). Mucosal *Candida* infection are frequently observed in AIDS and other immunocompromised patients as oral and aesophageal thrust are as vaginitis in women, which account more than 10 million case per year.

Candidiasis is mostly an endogenous infection, arising from overgrowth of the fungus

inhabiting in the normal flora. However, it may occasionally be acquired from exogenous sources (such as catheters or prosthetic devices) (Band *et. al.*, 1979) or by person-to-person transmission (such as oral candidiasis in neonates of mothers with vaginal candidiasis or endophthalmitis following corneal transplantation from an infected donor) (Behrens *et. al.*, 1991).

The holy basil (*Ocimum sanctum*) is native to Indian and is commonly known as Tulsi. It has been now widely grown all over the globe. In India, Tulsi (meaning 'Incomparable One') is cultivated almost everywhere. The herb is bitter and pungent there are many varieties of basils, found all over the world. White and black basils are the most commonly found varieties. The black Tulsi is the best as regards its properties. Basil acts as an appetizer and help digestion, it is beneficial to heart, suppresses excessive bile, cough and flatulence remove blood impurities. All the parts of the plants i.e seeds, leaves, shoots, flowers, stems and even the roots have medicinal value and are helpful in eradicating diseases. It belongs to the family Labiatae and is known as Holy Basil in English, which

1.1 *In-vitro* Cultivation

The in-vitro cultivation of the yeasts of the genus *Candida* require Sabouraud's medium, corn meal agar and rice infusion agar, Visible colonies grow over night when incubated at a precise temperature of 37°C. The nutritional requirements of the media are met with ammonium (but not nitrate) nitrogen and most (but not all) strains need to supply with growth factor biotin. Adding Tween-80 to corn-meal agar or rice infusion agar enhances colony development. The colonies are first seen to be tiny, white coloured which later fuse, becoming dry with the colour varying from white to cream colour. Depending on the species, their textures may be pasty, glistening

2.0 METHODOLOGY

2.1 Reduced Glutathione (GSH) levels

GSH levels were measured spectrophotometrically (LABOMED, INC. UV-VIS Dual Beam, USA).

2.1.1 Preparation of cell free extract:

constituents are chiefly fenchol, methyl chavicol, linalool, eugenol and β -caryophyllene, responsible for its anti-bacterial, anti-septic and anti-fungal action. Inhibitory effects of aqueous and alcoholic extracts of Tulsi have been observed on enteric pathogens by Agar diffusion method (Geeta *et. al.*, 2001). Tulsi is also known to have numerous pharmacological activities, such as antidiabetic, antistress, immunomodulatory, analgesic, antipyretic, anti inflammatory, antiulcerogenic, antihypertensive, CNS depressant, hepatoprotective, radioprotective, antitumor and antimicrobial activities (Archana *et. al.*, 2000).

Basil oil has a clear color, thin consistency and a sweet, herbaceous, licorice like, slightly camphorous. Linalool, Fenchol, Eugenol, Methyl chavicol and Beta-caryophyllene are the active components present in basil oil. It has been used over the century as medicated oils and ointments for respiratory infections especially for children. It is used in flavouring, cosmetics, soap, pharmaceuticals and perfumery (Ghannoum *et al.*, 1990; Jethwaney *et al.*, 1997 Archana *et al.*, 2000; Bhartiya *et al.*, 2006).

or dry, wrinkled and dull. The culture shows a diminished growth when the temperature is below 35°C. The suitable pH for growth is 7.0, with initial blast spore concentration not exceeding 10⁶/ml. The chemicals used for suitable growth include N, acetyl-D-glucosamine, and amino acid biotin, zinc and serum.

1.2 Objectives of the study:

1. To study the effect of different concentrations of Methyl Chavicol on intracellular Glutathione at different intervals of time
2. To grow and maintain the culture of *Candida albicans* ATCC 90028.

Cell free extract of *Candida* was prepared according to described method of Jethwaney *et. al.* (1997) with minor modifications. Cells were grown in the presence of 0, 10, 25 and 50 μ g/mL of methyl chavicol in Lee's media and pH is adjusted to 6.5 at each 90 mins. Cells

were harvested at an interval of 2 hours, 6 hours and 24 hours by centrifugation at 5000rpm for 5 minutes. Cell pellets (1 g wet wt) were suspended in 2 ml grinding medium (250 mM sucrose, 10 mM Tris HCl, pH 7.5, 1 mM PMSF) and 2 g glass beads (0.45 mm). The suspension was mechanically disrupted in cell homogenizer (MSK, Braun) by agitating it for a total of nine cycles of 10s each at 4000 vibrations per minute. Homogenate was collected and centrifuged at 1000 g for 5 min at 4 °C to remove unbroken cells and glass beads and further centrifuged for 40 min at 15 000 g at 4°C. The supernatant was used as cell free extract to assess levels of reduced glutathione using a LaboMed spectrophotometer (USA).

2.1.2 Reduced Glutathione

3.0 RESULTS AND DISCUSSION

Natural plant products have been used since antiquity and their use is increasing nowadays. either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads, because of the matchless availability of chemical diversity. (Geeta *et al.*, 2001). This study aims towards discovering novel antifungal drugs of natural origin.

3.1 Effect on Reduced Glutathione

Glutathione (GSH) is a tripeptide. It contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side chain. Glutathione, an antioxidant, helps protect cells from reactive oxygen species (ROS) such as free radicals and peroxides. Glutathione is nucleophilic at sulfur and attacks poisonous electrophilic conjugate acceptors.

Thiol groups are kept in a reduced state at a concentration of approximately ~5 mM in animal cells. In effect, glutathione reduces any disulfide bond formed within cytoplasmic proteins to cysteines by acting as an electron donor. In the process, glutathione is converted to its oxidized form glutathione disulfide (GSSG). Glutathione is found almost exclusively in its reduced form, since the enzyme that reverts it from its oxidized form, glutathione reductase, is constitutively active and inducible upon oxidative stress. In fact,

Cytosolic reduced glutathione was determined by the method of Jollow *et al.* (1974) with slight modifications. In this method 1.0 ml of the cell free extract was precipitated with 1.0 ml of 4 % sulphosalicylic acid. The samples were then kept for 1 hour at 4°C and centrifuged at 1200 g for 15 minutes at 40°C. The assay mixture consisted of 0.1 ml of above supernatant, 2.7 ml of Phosphate buffer (0.01 M, pH 7.4) and 0.2 ml of freshly prepared 5, 5'- dithiobis-2-nitrobenzene (DTNB) in a total volume of 3.0 ml. The colour developed due to the formation of a yellow colored complex, 5-thio-2-nitrobenzoate, was measured immediately at 412 nm at 25°C.

The GSH levels were calculated using the formula;

O.D. x Dilution factor x100/1.36.

Where the dilution factor =

Total volume/ volume of supernatant = 3/0.1 = 30.

the ratio of reduced glutathione to oxidized glutathione within cells is often used scientifically as a measure of cellular toxicity (Archana and Namasivayam, 2000).

The intracellular level of glutathione, which helps to maintain the redox potential of the cell, is decreased significantly during the thermal induction of yeast-to-mycelium conversion. In a study, the physiological role of the tripeptide glutathione (GSH) and its oxidized form (GSSG) was investigated during the initial steps of dimorphism (formation of germ-tubes), which is induced by human serum in exponential yeast-like cells (blastospores) of the *Candida albicans* strain CAI-4 (wild type) and its congenic *tps1/tps1* mutant, deficient in trehalose synthesis. The content of glutathione, measured both as GSH and the ratio GSH/GSSG, underwent a moderate drop in parallel with the induction of a significant degree of germ-tube emergence. Whereas the supply of exogenous glutathione did not affect the degree of dimorphic transition, depletion of intracellular glutathione by addition of 1-chloro-2,4 dinitrobenzene (CDNB) caused a clear reduction in the percentage of hyphae formation; although this effect must be due to the severe cell mortality produced by CDNB. Simultaneous measurements of GSH-metabolizing activities revealed a moderate

decrease of glutathione reductase concomitant with the activation of glutathione peroxidase.

3.2 Levels of Reduced glutathione

It has been found in our study that, the levels of glutathione were found to be 144 μ mole/gm at zero hrs i.e. in yeast uninduced cells. Post 2 hours induction shows that levels of reduced glutathione in control/untreated cells were found to be 124.4375 μ mole/gm cells. It went down as the induction period increases, at the end of 24hrs induction the GSH levels were found to be 86.56775 μ mole/gm. These results are concordant with the previous studies. All treated cells showed decreased levels of GSH as the induction period increases. Decrease in

GSH was found to be in dose dependent manner. Values are shown in the Table 1, below and represented in figure 1-3. A subsequent fall in the value of reduced glutathione was observed with the increasing concentrations of MC with the lowest being 28.452 μ mole/gm cells at 50 μ g/ml concentration of MC after 24 hrs induction. This result is in agreement with the assumption that methyl chavicol may also affect the glutathione metabolic enzymes and may have affected the cell defense armory, therefore the germ tube formation has not been furnished properly, as a result of Glutathione effect on intracellular.

Table 1: Data showing the effects of different concentrations of methyl chavicol on intracellular reduced glutathione levels after different periods of germ tube induction in *C. albicans*.

Sample	2 hrs	6 hrs	24 hrs
Control	124.4375	114.745	86.56775
10mg/mL Methyl Chavicol	110.5968	93.6875	82.925
25mg/mL Methyl Chavicol	100.9418	62.83375	52.213
50mg/mL Methyl Chavicol	63.1735	36.238	28.452

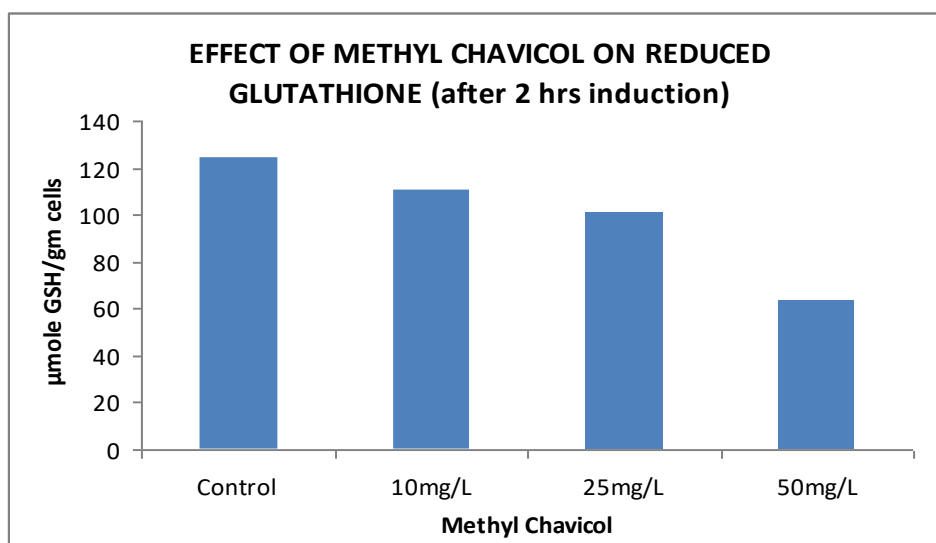


Figure 1: Effect of methyl chavicol on the levels of intracellular reduced Glutathione after 2 hrs of the germ tube induction in *C. albicans*. X-axis represents concentrations of Methyl chavicol.

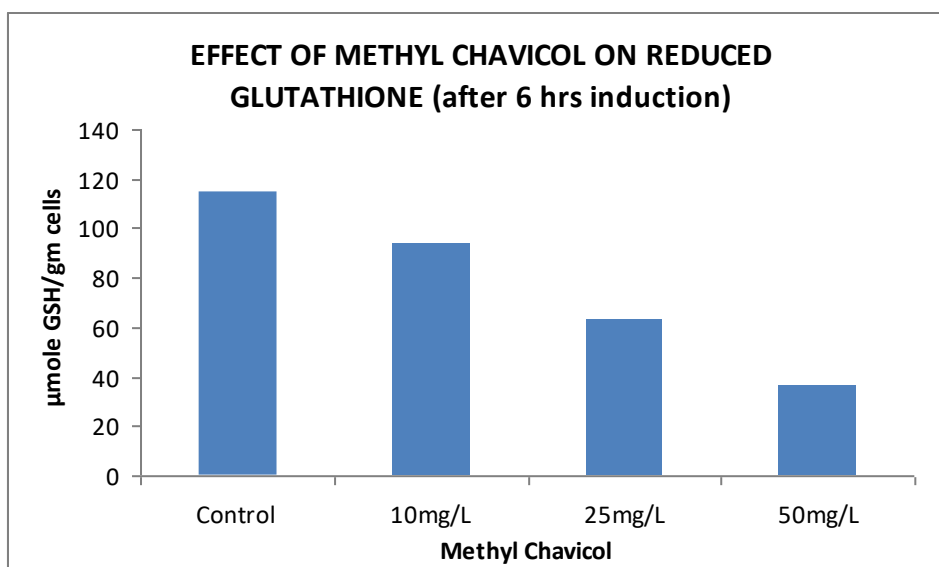


Figure 2: Effect of different concentrations of methyl chavicol on the levels of intracellular reduced Glutathione after 6 hrs of the germ tube induction in *C. albicans*. X-axis represents concentrations of Methyl chavicol

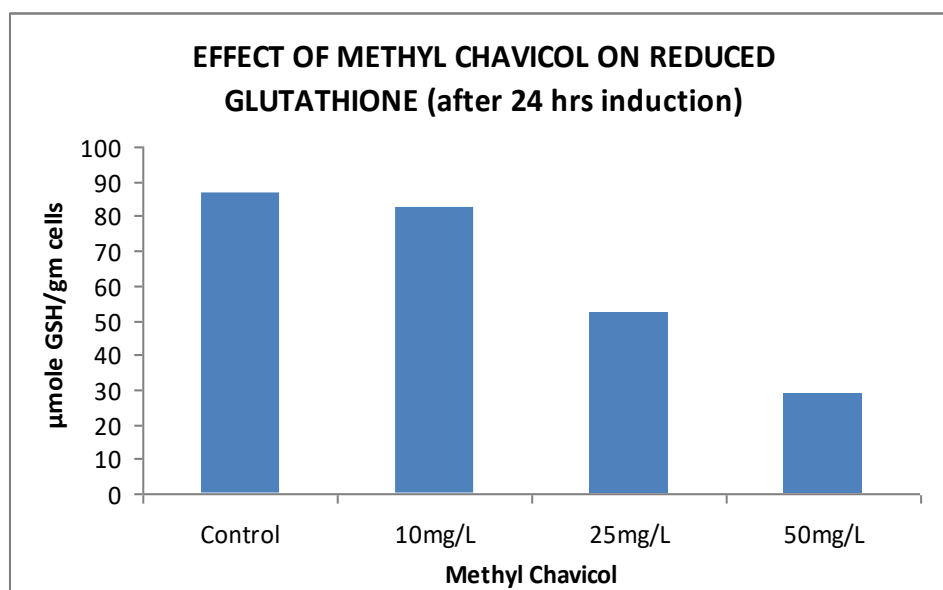


Figure 3: Effect of different concentrations of methyl chavicol on the levels of intracellular reduced Glutathione after 24 hrs of the germ tube induction in *C. albicans*. X-axis represents concentrations of Methyl chavicol.

4.0 CONCLUSION

The above study evaluated the effect of methyl chavicol on the *Candida albicans* Intracellular Glutathione. The Studies of host cell adherence and other pathogenicity markers including the production of phospholipases

and proteinase will also facilitate the possibility of methyl chavicol to be explored as an antifungal Drug. Post 2 hrs induction shows that levels of reduced glutathione in control/untreated cells were found to be

124.4375µmole/gm cells, as indicated in Fig.1. It went down as the induction period increases, at the end of 24 hrs induction the GSH levels, were found to be 86.56775µmole/gm.

Therefore, all treated cells showed decreased levels of GSH as the induction period increases, as shown in Figures 1,2 and 3.

5.0 REFERENCES

- Archana R, Namasivayam A. (2000). Effect of Ocimum sanctum on noise induced changes in neutrophil functions. *Journal of Ethnopharmacology* ; 73: 81-85
- Band, J. D., and D. G. Maki (1979). Infections caused by arterial catheters used for hemodynamic monitoring. *Journal of Ethnopharmacology*, 67:735-741.
- Bhartiya US, Raut YS, Joseph LJ. Protective effect of Ocimum sanctum L after high-dose 131iodine exposure in mice: an in vivo study. *Indian Journal of Experimental Biology* 2006;44(8):647-52.
- Behrens-Baumann, W., R. Ruechel, O. Zimmermann, and M. Vogel. (1991). *Candida tropicalis* endophthalmitis following penetrating keratoplasty. *British Journal of Ophthalmology*. 75:565.
- Dixon, D. M., McNeil, M. M., Cohen, M. L., Gellin, B. G. and La Montagne, J. R. (1996). Fungal Infections. *Public Health Reports* 111: 226-235.
- Geeta , V. D.M., Kedlaya R., Deepa S., and Ballal M. (2001) Activity of Ocimum sanctum (the traditional Indian medicinal plant) against the enteric pathogens; *Indian Journal of Medical Science*, 55(8):434-8, 472.
- Ghannoum, M.A., Abu-Elteen, K.H. 1990. Pathogenicity determinants of *Candida*. *Mycoses*. 33, 265-282
- Jethwaney D, Hofer M,Raj K, Prasad R. (1997) Functional reconstitution of a purified praline permease from *Candida albicans*: interaction with the antifungal cispentacin, *Microbiology*. 143: 397-404.
- Jollow D.J., Mitchell J.R., Zampagtrone N., Gillete J.R. (1974) Bromobenzene induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolite. *Pharmacology*. 11: 151-169.