ANTIMICROBIAL, ANTICANCER AND ANTIOXIDANT ACTIVITIES OF METHANOLIC EXTRACT OF *Elaeis guineensis* Jacq. LEAVES

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By

VIJAYARATHNA A/P SOUNDARARAJAN

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For any errors or inadequacies that may remain in this work, of course, the responsibility is entirely my own.

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LIST OF ABBREVIATIONS

a.m.u	atomic mass units
A. salina	*Artemia salina*
ATP	Adesino triphosphate
BHT	Butylated Hydroxytoluene
BSA	Bovine Serum Albumin
C. albicans	*Candida albicans*
CFU	Colony-Forming Units
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPPH•	Radical 2,2-diphenyl-1-picrylhydrazyl
E. guineensis	*Elaeis guineensis*
e.V	electron volt
FBS	Fetal Bovine Serum
FLZ	Flucanazole
FTIR	Fourier Transform Infrared
GAE	Gallic Acid Equivalent
GC-MS	Gas Chromatography-Mass Spectrometry
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HPSA	Hydrogen Peroxide Scavenging Activity
IC_{50}	Inhibitory Concentration 50%
KBr	Potassium bromide
LC_{50}	Lethality concentration 50%
LD_{50}	Lethal Dose 50%
mA	milli-Amperes
MCF-7	Michigan Cancer Foundation – 7
MHA	Mueller-Hinton Agar
MHB	Mueller-Hilton Broth
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NOS	Nitric Oxide Scavenging
OD	Optical density
OH	Hydroxide group
OPF	Oil Palm Fronds
PBS	Phosphate buffered saline
PDA	Potato Dextrose Agar
PUFA	Polyunsaturated Fatty Acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
SNP	Sodium nitroprusside
SOD	Superoxide dismutase

SPSS	Statistical Package for Social Sciences
TPC	Total Phenolic Content
U/mL	Units per millilitre
XOI	Xanthine Oxidase Inhibitory

AKTIVITI ANTIMIKROB, ANTIKANSER DAN ANTIOKSIDAN EKSTRAK METANOL DARI DAUN *Elaeis guineensis* Jacq

ABSTRAK

Malaysia merupakan sebuah negara penghasil minyak kelapa sawit berskala industri, juga pernah diberi pengiktirafan berperingat antarabangsa berasaskan kesungguhan dalam penghasilannya yang memuncak. Meskipun perladangan kelapa sawit menyumbang banyak ke negara, tetapi sisa buangan yang diperoleh daripada penanaman semula, penuaian dan pengeringan telah mancetus jalan ke pelbagai masalah. Hasil sampingan berkepelbagaian kelapa sawit dan daun kelapa sawit, dilaporkan menyumbang 70% kepada keseluruhan sisa industri kelapa sawit negara dengan mencapai 30 juta tan biomas setahun termasuk batang, daun dan tandan buah. Salah satu punca masalahnya yang ketara boleh dilihat dalam aspek pengurusan hasil sisa buangan sampingan semasa pemprosesan buah kelapa sawit. Maka dari itu, kajian ini telah dijalankan demi mengubah hasil buangan industri ke bahan bernilai industri farmaseutikal dengan mengambil kira penilaian aktiviti antimikrob, antikanser, antioksidan dan sitotoksik. Aktiviti antimikrob telah dikaji melalui kaedah peresapan disk dan kaedah pencairan kaldu. Ekstrak didapati berkesan terhadap mikroorganisma ujian dan nilai kepekatan perencatan minimum (MIC) didapati dalam julat 6.25- 50.00 mg/mL. Ekstrak daun *E. guineensis* menunjukkan aktiviti yang amat baik terhadap kulat patogen *C. albicans* dengan mencapai nilai MIC 6.25 mg/mL dan keputusan ini disahkan melalui kajian asai bunuh mengikut masa. Kajian mikroskopi elektron pengimbasan (SEM) dan transmisi (TEM) telah diamalkan untuk memerhati dan membezakan metamorfosis morfologi *C. albicans* yang diolah ekstrak dengan kawalannya tanpa olahan. Keputusan SEM dan TEM menunjukkan kesan yang signifikasi terhadap dinding sel dan kandungan sitoplasma

sel *C. albicans* yang kemudiannya diperhatikan musnah apabila didedah pada ekstrak daun *E. guineensis* berkepekatan 6.25 mg/mL selama 36 jam. Kajian aktiviti antikanser pula telah dijalankan dengan menggunakan sel kanser MCF-7 dan sel bukan kanser Vero melalui kaedah ujian MTT. Ekstrak didapati merencatkan sel kanser MCF-7 dengan nilai perencatan 50% (IC_{50}) iaitu 15.26 μg/ml dan sel Vero dengan nilai 22.53 μg/ml (IC_{50}). Secara kesimpulannya, keputusan kajian antikanser menunjukkan ekstrak bertindak sebagai agen antikanser terhadap sel MCF-7. Selain daripada kajian antimikrob dan antikanser, kajian antioksidan dengan menggunakan kaedah penjerapan radikal difenil-1-pikrilhidrazil (DPPH), kandungan jumlah fenol, asai perencatan xantin oksidase (XOI), asai perencatan nitrik oksida (NOI), asai perencatan hidrogen peroksida (HPSA) juga telah dijalankan. Ekstrak daun *E. guineensis* didapati mempamerkan aktiviti perencatan DPPH, XOI, NOI dan HPSA dengan nilai IC_{50} sebanyak 814.00 μg/mL, 37.48 μg/mL, 534.04 μg/mL dan 1052.02 μg/mL masing-masing manakala jumlah kandungan fenol dicatatkan sebanyak 333.3 μg GAE/g ekstrak kering. Kajian sitotoksik ekstrak daun *E. guineensis* telah dilakukan dengan menggunakan kaedah kematian anak udang brin (*Artemia salina*) dan kaedah ketoksikan oral akut yang dijalankan ke atas mencit albino Swiss, dimana kajian mencit diakhiri dengan kajian histopatologi. Nilai LC_{50} sebanyak 9.00 dan 3.87 mg/mL untuk masa olahan selama 6 dan 24 jam telah diperolehi bagi ekstrak daun *E. guineensis* dimana ia tidak menunjukkan sebarang kesan toksik terhadap *Artemia salina*, manakala mencit yang diberi ekstrak pada dos yang tinggi iaitu sebanyak 5,000 mg/kg berat badan melalui oral pula, tidak menunjukkan sebarang kematian atau bukti kesan sampingan. Kajian pemeriksaan histology organ-organ yang penting juga tidak menunjukkan sebarang perbezaan diantara mencit yang dirawat ekstrak dengan mencit kawalan. Ini nyata mengesahi *E. guineensi* sebagai

unsur bukan toksik. Pencirian kehadiran sebatian kimia aktif dalam ekstrak *E. guineensis* telah diperhatikan melalui kaedah analisis spektroskopi FTIR dan kromatografi gas–spektroskopi-jisim (GC-MS). Keputusan kajian FTIR ekstrak *E. guineensis* menunjukkan kehadiran kumpulan berfungsi yang mengesahkan aktiviti antimikrob dan antioksidaan manakala analisis GC-MS menunjukkan kehadiran 11 komponen aktif. Empat daripada komponen ini didapati menyumbang kepada aktiviti antimikrob sementara 6 komponen pula bertanggung jawab terhadap aktiviti antioksidaan. Sebagai kesimpulan boleh dikatakan bahawa ekstrak daun *E. guineensis* telah menunjukkan aktiviti antikandida yang sangat baik bersama-sama dengan aktiviti antikanser dan antioksidaan berkesan tanpa menunjukkan kesan toksik. Maka, penggunaan daun kelapa sawit secara komersial daripada bahan buangan industri kelapa sawit untuk diubah sebagai produk farmaseutikal yang berguna boleh membawa pulangan yang lumayan kepada bidang industri farmaseutikal negara.

ANTIMICROBIAL, ANTICANCER AND ANTIOXIDANT ACTIVITIES OF METHANOLIC EXTRACT OF *Elaeis guineensis* Jacq. LEAVES.

ABSTRACT

Malaysia, being one of the economically industrious producers of oil palm around the world correspondingly, had recognized for its assiduous and phenomenal expansion of oil palm (*Elaeis guineensis*) plantings. Though the plantation had considerably contributed to the nation, the by-products yielded during replanting, harvesting and pruning became controvertible. There are several by-products of oil palm, explicitly oil palm fronds (OPF) had been contributing 70% to the overall oil palm industry waste in the country which reportedly produced around 30 million tons annually of oil palm biomass, including trunks, fronds, and empty fruit bunches. One of the significant problems in palm fruit processing is managing the wastes generated during the process. Hence, this study was executed to transmute oil palm industrial waste into remedial pharmaceutical products to which activities such as antimicrobial, anticancer, antioxidant and cytotoxicity were evaluated. The antimicrobial activity of the extract was completed using disc diffusion method and minimum inhibitory concentration (MIC) assay per broth macrodilution method. The extract was found to be potent towards the tested microorganism with the MIC reading between 6.25- 50.00 mg/mL. It was found that the extract works effectively against pathogenic yeast, *C. albican*, under the MIC value of 6.25 mg/mL and this result was again reinstated through time killing assay. Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) were conducted to observe the morphological metamorphosis between extract treated *C. albicans* and untreated control cells. The visual image results of SEM and TEM further testify the significant effects of the extract against the cell wall and the cytoplasmic contents of

C. albicans cells eventuating in the destruction of cells that are exposed to the concentration of 6.25 mg/mL *E. guineensis* extract throughout 36 hours. The anticancer activity of the extract was performed using cancerous MCF-7 cell lines and non-cancerous Vero cell lines through MTT method. The extract inhibits the cancerous cell proliferation at the concentration of (15.26 μg/mL) while the normal Vero cells were inhibit at (22.53 μg/mL). It was concluded that the extract was toxic towards the cancerous MCF-7 cell lines. Alongside antibacterial and anticancer activities, the antioxidant activities of *E. guineensis* leaf extract were determined using Diphenyl-1-picrylhydrazyl (DPPH) Scavenging Assay, Total Phenolic Content (TPC), Xanthine Oxidase Inhibitory (XOI) Assay, Nitric Oxide Inhibitory (NOI) Assay and Hydrogen Peroxide Scavenging Assay (HPSA). The extract exhibited scavenging activities of DPPH, XOI, NOI and HPSA methods with the IC_{50} values of 814.00 μg/mL, 37.48 μg/mL, 534.04 μg/mL and 1052.02 respectively, while a total of 333.3 μg GAE/g of dry extract was achieved for total phenolics contents. The cytotoxicity activities of the extract were evaluated using brine shrimp assay (*Artemia salina*) and an oral acute toxicity using Swiss albino mice which were then followed by mice histopathalogical study. The values of LC_{50} obtained from brine shrimp assay were 9.00 and 3.87 mg/mL for 6 and 24 hours respectively which were interpreted as non toxic to *A. salina* whereas the mice administrated with the oral dose of 5000 mg/kg did not display any signs of adverse effects. The subsequent histophatological study revealed similar pathological patterns present in both *E. guineensis* treated and non-treated mice (control) vital organs which clearly state that *E. guineensis* is non-toxic. Identification of active ingredients present in *E.guineensis* was conducted using Fourier Transform Infrared (FTIR) Spectroscopy Analysis and Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis. The FTIR exhibited

chemical functional groups supporting the antimicrobial and antioxidant properties of *E. guineensis* extract while the GC-MS analysis indicated 11 active compounds out of which 4 compounds were believed to be part of the contribution factor of antimicrobial activities while 7 were responsible for antioxidant activities. In adumbration, the leaf extract of *E. guineensis* had explicit good anticandidal activity, standing as a potential source of antioxidant and anticancer agent without significantly causing toxicity effects. Therefore the economic utilization of the leaf material from the oil palm industry waste can be converted into a beneficial medicinal value which will in return ameliorate the pharmaceutical industry of the nation.

CHAPTER 1.0: INTRODUCTION

Malaysia is one of the largest producers of oil palm (*Elaeis guineensis* Jacq.) in the world, stating 17.7 million barrels of crude oil in February 2012 (DOS, 2012). Total planted area of oil palm increased from 73, 000, reaching 3.87 million hectares in 2004 and 5 million hectares in 2011 (Clark, 1996; MPOB, 2012). Oil palm fronds (OPF) contribute 70% to the overall oil palm industry waste in the country (Alper, 1998), which reportedly produces about 30 million tons annually of oil palm biomass, including trunks, fronds, and empty fruit bunches (Joseph, 2010). One of the significant problems in palm fruit processing is managing the wastes generated during the process. In Malaysia, for example, 9.9 million tons of solid wastes consisting of oil palm empty fruit bunches, fiber, and fruit shells, and 10 million tons of palm oil mill effluent (POME) were generated every year. The country uses millions of hectare for plantations of oil palm, and large quantities of cellulosic and non-cellulosic raw materials were produced during harvesting (Joseph, 2010). The expansion of plantations in the country has generated large amounts of agro waste, creating problems in replanting operations and tremendous environmental concerns. When left on the plantation floor, these waste materials produce serious environmental problems (Afolayan and Meyer, 1997; Hernández *et al.,* 2003).

Meanwhile, the discovery and the development of penicillin by Alexander Fleming had changed the entire direction to treating infectious diseases and saved the lives of millions of people (Berger, 1989). Antibiotics are probably the most successful family of drugs so far developed for improving human health. Besides this fundamental application, antibiotics (antimicrobials at large) have also been used for preventing and treating animals and plants infections as well as for promoting growth in animal farming (McManus *et al.,* 2002; Smith *et al.,* 2002; Singer *et al.,*

2003; Cabello, 2006). All these applications made antibiotics to be released in large amounts in natural ecosystems. Little is known on the overall effects of antibiotics on the population dynamics of the microbiosphere (Sarmah *et al.,* 2006). However, the effect of antibiotics used for treating infections or for farming purposes in the selection of antibiotics–resistant microorganisms, which may impact human health has been studied in more detail (Witte, 1998; Ferber, 2003; Singer *et al.,* 2003). As stated by the World Health Organization, the increasing emergence of antibiotic resistance in human pathogens is a special concern, not only for treating infectious disease, but also for other pathologies in which antibiotic prophylaxis is needed for avoiding associated infections. In this regard, the spread of antibiotic-resistant bacteria means that commonplace medical procedures once previously taken for granted could be conceivably consigned to medical limbo. The repercussions are almost unimaginable (WHO, 2000).

Bacterial resistance is a perfect example of natural selection. The widespread use or misuse of these drugs had killed off susceptible cells, leaving the rare resistant strains to survive and repopulate the ranks. Many infectious disease specialists are predicting that the problem of antibiotic resistant will soon be acute in the coming years and the fatalities from once curable diseases will increase sharply. Finally, pharmaceutical companies responding to this threat and are beginning to search for new types of antibiotics. Most of the antibiotics derived from natural products, produced by microorganisms or plants to kill other microorganisms. Antibiotics work by interfering with bacterial activities without affecting the eukaryotic cells (Karp, 2005).

The rise in microbial drug resistance and opportunistic infections especially the ones affecting individuals on immunosuppressive chemotherapy and AIDS

patients had all together created a different scenario. The discovery of many antifungal started but went in vain due to toxicity, where else other infectious were yet to be found cure. These problems have triggered the search for new alternative substances from other sources such as plants (Cowan, 1999). Natural products in the form of pure compounds or as standardized plant extracts provide unlimited opportunities for the discovery of new drugs since plant contains unmatched availability of chemical diversity (Cos *et al.*, 2006). According to WHO (2000), about three quarter of the world population depends on traditional remedies for their health care (Gilani and Atta-ur-Rahman, 2005).

Therapies developed along the principles of Western medicine are often limited in efficacy, carry the risk of adverse effects, and are often too costly, especially for the developing world. Therefore, treating diseases with plant-derived compounds which are accessible and do not require laborious pharmaceutical synthesis seems highly attractive. Furthermore, in spite of the advances in conventional medicine in the last decades, professionals and the lay public of developed countries pay increasing attention to phytomedicine. Several recent surveys from Europe and the United States have demonstrated a sharp rise in the use of botanical drugs within a few years, and up to 65% of patients with liver disease take herbal preparations (Flora *et al.*, 1996; Kessler *et al*., 2001; De Smet, 2002; Strader *et al.*, 2002). Similar figures exist for Europe where the expenses for silymarin, an herbal preparation used to treat chronic liver diseases, reaches $180 million in Germany alone (Pradhan and Girish, 2006). Many factors contribute to herbal medicine's appeal. Supporters of herbal medicine claim that herbs may both treat and prevent diseases upon where these treatments are believed to be safe because they are "natural" and fit into the image of a gentle and, therefore, harmless

alternative to conventional medicine. More so, patients are often dissatisfied with the latter because of disappointing treatment success or unfavorable side effects. In addition, herbal products are often exempt from rigorous regulations, such as in the U.S., and prescriptions are usually not required for these inexpensive products (Stickel and Schuppan, 2007).

E. guineensis is one of the plants that are central to the lives of traditional societies in West Africa. All parts of this plant are useful. The wood is used as frames for buildings, and the sap is fermented into palm wine. The oil from the fruit mesocarp and the seeds are used for cooking, and making soaps, creams, and other cosmetics. The fresh sap is used as a laxative, and partially fermented palm wine is administered to nursing mothers to improve lactation. The fruit-husk is used in the preparation of soaps used to treat skin infections. A root decoction is used to treat headaches in Nigeria. The pulverized roots are added to drinks as a cure for gonorrhea, menorrhagia, and bronchitis (Muthu *et al.,* 2006). The leaf extract and juice from young petioles are applied to fresh wounds. The fruit mesocarp oil and palm kernel oil are administered as a poison antidote and used externally with several other herbs as a lotion to treat skin diseases. Palm kernel oil is applied to convulsive children to regulate their body temperature. Oil palm is a folk remedy for cancer, headaches, and rheumatism, and is considered an aphrodisiac, a diuretic, and a liniment (Muthu *et al.,* 2006).

Since oil palm have been used in many ways to treat diseases caused many microbes, there is notably great potential for findings of a new pytochemical substances in the leaves of *Elaies guineensis* which could reduce the emergence of antibiotic resistance bacterial. In conjunction of these new findings, a large part of

the oil palm trees would also, not to go waste annually and instead, might benefit the country economically in the pharmaceutical sector.

1.1 Objectives

The current study was conducted with the following objectives:

1) To obtain the optimum methanol extracts from *E. guineensis* leaves by means of maceration methods for antimicrobial, anticancer, antioxidant and cytotoxicity testings.
2) To scientifically investigate the rationale behind traditional usage in searching for curative properties by conducting antimicrobial, anticancer and antioxidant activities testing on the methanol extracts of *E. guineensis* leaves.
3) To further extract and isolate fractions or bioactive compound(s) of therapeutic potential from *E. guineensis* leaves using FTIR and GC-MS techniques.

CHAPTER 2.0: LITERATURE REVIEW

2.1 Antimicrobial

2.1.1 Antimicrobial Agents

The rising of infections acting in conjunction with the global spread of drug-resistant bacterial pathogens had been a superlative denotation in executing new requisite approaches. The death toll cause by infectious diseases over the 20th century had designated a scrutinous pattern; from 1900 to 1980, the toll dropped from 797 per 100,000 people to 36 per 100,000 people, a reduction by a factor of more than 20 and a testament in part to the efficacy of antibiotics (NRC, 2006). Even so, from 1980 to 2000, the toll doubled, chiefly due to HIV and also with the spread of drug-resistant bacterial pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, multiple-drug-resistant gram-negative bacteria, and multiple-drug-resistant tuberculosis (Cohen, 2000). The manifestation of infections coinciding with patients of serious illness or immuno-compromised relatively institutes an elevation in mortality rate that constrains inevitable necessitation for new techniques in treating pathogens which are resistant towards coeval antibiotics. However, these present days sparked more imperative desolation due to untreatable pathogen infections, which had never been an issue since the pre-antibiotic era (Cohen, 2000).

Antibiotic resistance that develops in one or more patients insinuates a global promulgation threatening everyone's health. Formerly, antibiotic resistance was incontrovertibly afflicted with the most ailing patients in the intensive- care wards; notwithstanding, currently it became a pervasive throughout the world. The pathogen strains, markedly bacteria, carries commonly clustered resistance genes which are now recognized as multiple-drug-resistant strains with the ability to spread resistance

together. The severity is increased along with the contribution from international travel frequency blended with the lack of standard knowledge on antibiotic usage, leading to expedite spread of resistance to every environment around the globe. Cognizance of antibiotic- resistance crisis generally pertains to predictable and severely loss of efficacy of current antimicrobial repository, yet considerable economic status had permitted the development of new antimicrobial agents and therapies to persevere (Nathan, 2004).

An antimicrobial agent exerts to kill or inhibit the growth of infectious bacteria by further hindering deterioration while antifungal agent combats against fungi in the same manner (McDonnell, 2007). These powerful substances namely known as antibiotics constitute an array of agents recognized as antibacterial, antifungal, antiprotozoal, antihelminthic and antiviral (Baron *et al.,* 1994). In nature, bacteria and fungi obviate intruders (e.g, other microorganisms) by secreting compounds known as antibiotics (Greenwood and Whitley, 2003). The branch of knowledge colligating with antimicrobials was initiated by Pasteur and Joubert who ferreted out the aptitude of one type of bacteria in preventing the growth of another. It was later conceptualize that antibiotic produced by the antecedent was the cause of the other bacterium cessation. Like a shot, antibiotic became a prevailing term tributary to any drug that cures bacterial infection. Antimicrobial agents do not only pertain to naturally synthesized antibiotic compounds, but also to synthetically form compounds (Greenwood and Whitley, 2003).

Presently the disclosure of cardinal target (s) found on bacteria had embark a new strategy in understanding the metabolism and the sequencing genes of bacteria (Brazas and Hancock, 2005). The principle of a good antimicrobial agent and drug discovery is anticipated upon the discernment of bacterial genomics and their related

technologies. However, the progress on establishing new antimicrobial agents based on genomic approaches is still under trial at the stage of late clinical development (Coates *et al.*, 2002).

2.1.2 Characteristics of antimicrobial Agent's Activities *in vitro*

2.1.2.1 Antimicrobial Activity is Measurable

Adhering to accepted standards, the clinical microbiology laboratories had effectuated numerous susceptible tests encompassing disc diffusion, broth dilution and agar dilution methods that literally engage the measurement of minimal inhibitory concentration (MIC) of an antimicrobial agent. These tests are passable in thwarting bacterial replication at new sites efficaciously acting in conjunction with most clinical situations as well as boosting the competence of host's defense mechanism into extirpating the bacterial invasion. The combination of antimicrobial assays with MICs provides trenchant information on the pharmacokinetics of the agent (s) allowing the eradication of bacteria to be predicted (NCCLS, 1999).

The lowest concentration of an antimicrobial agent required to inhibit the visible growth of microorganism is defined as MICs. MICs are employed to ascertain the range of accepted antibiotic concentrations with the implication of doubling dilution steps up and down from 1 mg/L as required. The inhibition of bacterial growth occurs after overnight incubation upon where the length of period enjoins contingently with the type of microorganism, say anaerobes, which call for longer incubation for growth (Andrew, 2001). This method begets results that can be either interpreted as semi-quantitative or qualitative (Andrew, 2001).

Apart from MIC tests, typical clinic or veterinary laboratories transact *in vitro* susceptibility testing by employing disc diffusion method. The method discharges measurable activity relatively to the size of the inhibition zone generated,

encompassing the embedded agent disc. The elucidation of these *in vitro* results relies comparatively solid upon the standards of United States recommended earlier by the National Committee for Clinical Laboratory Standards (NCCLS, 2003). The NCCLS is now known as Institute of Clinical Laboratory Standards (ICLS), British Society for Antimicrobial Chemotherapy (BSAC) and the European Committee for Antimicrobial susceptibility testing (EUCAST), which exist as guidelines for antimicrobial susceptibility testing of conventional drugs (Ncube *et al.*, 2008).

There are two modes referred to the action of an antimicrobial agent skirmishing bacteria that congruent well with the term bacteriostatic or bactericidal. Bacteriostatic prevents bacterial growth while bactericidal means killing them. Certain antimicrobials become lethal to bacteria at the concentration equal or higher than MIC while others generally possess bacteriostatic activity. Nonetheless, in reality, these agents act exceptionally. The "bactericidal" effect would not kill every microorganism implied chiefly to be existing in large colonies, within 18 to 24 hours of test and in such wise the "bacteriostatic" agents kill some bacteria within the 18 to 24 hours after the test; often more than 90–99% of the inoculum, yet, not enough to be called "bactericidal." On those grounds, the absolute indication on whether an antibacterial agent is bactericidal or bacteriostatic confides within the conditions of bacterial growth and density, duration of test and the reduction tendency in bacterial rates. Along these lines, the clinical definition falls vagarious. Generally, antibacterials are better described as potentially being both bactericidal and bacteriostatic (Pankey and Sabath, 2004).

At all events, *in vivo* resolve into an alternate option to stipulate a typical drug's contingency of being bactericidal but in many instances were waived due to

technical and interpretive adverse circumstances (Lopes and Moreno, 1991; Amsterdam, 1996).

Killing curve stands as a collateral method in observing microbes responding to antimicrobials *in vitro.* The measurement of the killing effect betides reciprocally to the exposure of the microbes to a given concentration of drug and the population reduction is measured over time. The bactericidal effect can also be measured in the serum, commiserating the *in vivo* antimicrobial activity, often derivable from the subsisting serum components (MacGowan *et al.*, 1997).

2.1.2.2 Antimicrobial Activity is Specific

Microbes have one or more specific target components on their cells requisite in regulating physiological and replication activities. These targets are submissible in transit to the action of antimicrobial agents, explaining the classification of antimicrobials being based on their action mechanism. For example, β-lactam drugs achieve their effect by inhibiting a group of bacterial membrane proteins called penicillin-binding proteins resolute for cell wall synthesis (Spratt, 1980; Spratt and Cromie, 1988) while those with high molecular weights normatively proceed through multiple enzymatic functions. Hence, these proteins serve as docking targets for β-lactam drug activity.

Come what way, the antimicrobial drugs efficacious either through a direct event subsequent to the inhibition of the same cellular targets or an indirect one continuously appearing after a cascade of reaction exerting from a particular drug-bacteria interaction (Yan and Gilbert, 2004).

The antimicrobial agents have variegated mechanisms in extirpating or ceasing the growth of microbial cells as illustrated in Figure 2.1; (i) Inhibition of cell

wall synthesis (e.g. penicillins); (ii) Inhibition of protein synthesis (e.g. tetracyclines); (iii) Alteration of cell membrane (e.g. polymyxins); (iv) inhibition of nucleic acid synthesis (e.g. quinolones); and (v) antimetabolite activity (e.g. sulfonamides) (Greenwood and Whitely, 2003). Multitudinous mechanisms ascribing antibiotic reaction, relate to obliterating the synthesis mechanisms of bacterial cell wall. It is sensible that most of living bacteria cells are assembled from cell membranes playing imperious role in regulating the movement of substances across the inner and the outer surroundings.

In no manner, the future is still on dubitancy on the adequacy of antimicrobial therapy. Bacteria are felicitously resisting antimicrobial agents to a greater extension. Hospitals emerge to aggregate concrete mutably bacteria eliciting drawbacks in case of susceptible patients. As of now, the discovery of drugs is the only tool to content against bacterial resistance. Yet, microorganisms are becoming resistant faster than the rate at which new drugs are being made available; thus, future research in antimicrobial therapy may focus on finding how to overcome resistance to antimicrobials, or how to treat infections with alternative means.

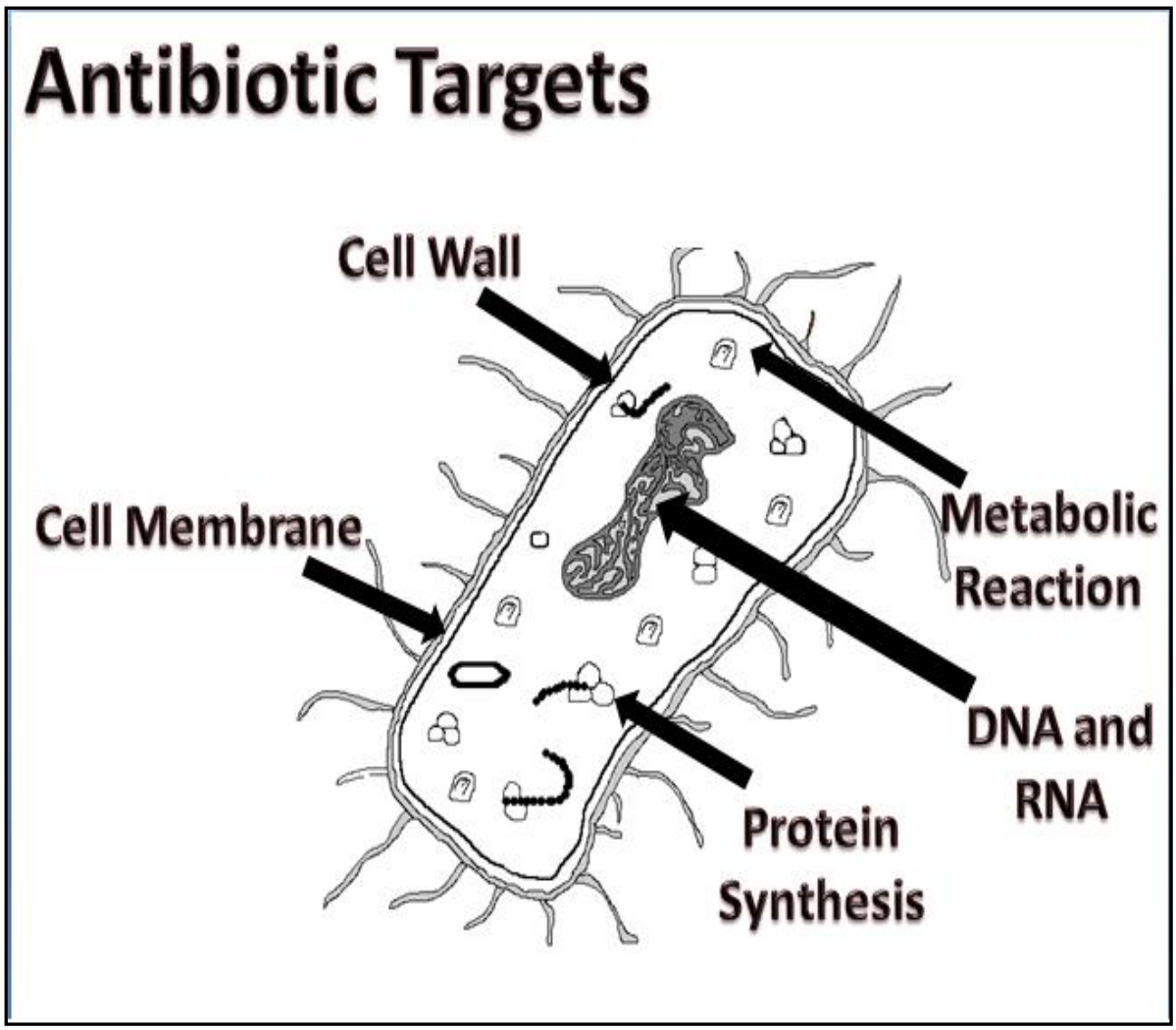

Figure 2.1: Ways on how antimicrobial agents target in ceasing the growth of microbial cells.

2.1.3 Evaluation of Antimicrobial Activity

A successful management of bacterial infections co-exists with a good comparative integration analysis of antimicrobial agents that gives rise to an accurate determination of bacterial susceptibility towards antibiotic. Various microorganisms are allowed to react towards natural extracts and pure compounds while observation of their growth response is measured as the rate of antimicrobial activity. A number of techniques were adapted that generally grouped into two major classes including the disc diffusion and the broth dilution method. Since, these methods do not possess the same level of sensitivity or standards, the results naturally subjected in term to their respective methods.

2.1.3.1 Agar-Diffusion Method

A simple way was introduced to determine the susceptibility of a microorganism to an antimicrobial agent involving the usage of micro-seeded agar plate which also infuses the diffusion of the agent into the agar medium. A reservoir imbued with the agent is exposed directly on the seeded agar surface. A reservoir can be of filter discs or stainless steel cylinders placed on the surface or holes punched in the medium. The hole -punch is applicable for aqueous extracts due to the interference by particulate matter is much lesser that with other reservoir types. Again, only a speck of sample is required and the possibility to test up to six extracts per plate against a single microorganism is its unique way of being advantageous (Hadacek and Greger, 2000). Non- polar samples or samples that find hard to diffuse into agar will not be appropriate to use hole-punch method.

The seeded system along with the disc is placed at lower temperature for several hours before incubation to provoke compound diffusion over the growth of

microbial. The substances start to diffuse from the disc into the agar taking after decrease in the concentration as a function of the square of the diffusion distance. The substances from the antimicrobial agent will travel to the line of demarcation upon where it exist more diluted and no longer have the aptitude to impound the microbial growth.

The interaction between a specific antimicrobial agent and bacterial isolate will be revealing clear zones formations simply known as growth-inhibition zones surrounding the disc. The zones that manifest as clear areas are easily measurable with a ruler and the outcome of the diameter can be used to build an antibiogram (Atlas *et al*. 1995). A swift assessment of the antimicrobial activity for any water soluble compound is procured from the agar diffusion test that accommodates basic antimicrobial data concerning manufacturing and quality control assurance of finished product (Block, 1991; Ascenzi, 1996; Paulson, 1999).

Samples of different types are incongruous to be compared in yielding the potentiality of a certain antimicrobial. This may be contributed by the differences in physical properties that comprise solubility, volatility and diffusion characteristics in agar (Cos *et al.*, 2006).

2.1.3.2 Dilution Method

Broth dilution is a vulgar method appointed in resolving the minimal inhibitory concentration (MIC) of antimicrobial agents that clap together antibiotics and other substances that kill (bactericidal activity) or inhibit the growth (bacteriostatic activity) of bacteria. Broth dilution utilizes a suspension of microorganism growing in liquid medium reacting against geometrically increasing concentrations (commonly, a twofold dilution series) of the antimicrobial agent (Wiegand *et al.*,

2008). The affiliated antimicrobial agent and its liquid medium are serial diluted, in consideration of the definite number of bacterial cells in an inoculum and time of incubation. The final volume of the investigation will allocate the method as macrodilution (employing a total volume of 2 mL) or microdilution (achieving using microtiter plates using ≤ 500 mL per well). Succeeding incubation, the presence of turbidity or sediment betokens the growth of microorganisms. The turbidity is consummated by visual estimation or through optical density at 405 nm. Test samples that dissolve partially at any rate will contravene with turbidity reading, calling attention for the stipulation of negative or sterility control (Cos *et al.,* 2006). The broth dilution herein, incorporates MIC that defines as the lowest concentration of the antimicrobial agent requirement to prevent the growth of microorganisms obligated to defined conditions (Wiegand *et al.,* 2008).

2.2 *Candida*

The ordinary human system avouches *Candida* being in favour with flora found in skin, mouth, vagina and stool. *Candida* is thin walled, small yeast stretching from 4 to 6 microns that reproduces by budding. Being the most common opportunistic mycoses pathogen and a colonizer, *Candida* is widely known to be existing in environment subsisting namely on leaves, flowers, water and soil. There are 154 species known to be abiding within the genus *Candida*, italicizing six as the most frequently isolated from human infections. The *Candida* colonies that come in pasty, smooth, dry, wrinkled and dull appear cream to yellowish in colour, growing swiftly routing maturity within 3 days. The *Candida* is unicellular yeast and transpires alternatively to multicellular mold giving the chance to reproduce in such sexual or asexual manner by forming spores or budding.

A certain number of *Candida* species turn up to be harmless commensally manifesting along the gastrointestinal and genitourinary tract. All the same time eliciting a range of painful medical conditions ranging from painful superficial infections, such as vaginitis and in nay rather healthy women causing severe surface infections of the mouth and esophagus in human immunodeficiency virus (HIV) patients, and life threatening blood stream infections among vulnerable intensive care patients (especially those undergoing cancer chemotherapy or immunosuppressive therapy following organ or bone marrow transplant procedures) (Kao *et al.*,1999). Candidemia is a very common hospital acquired (Beck-Sagué and Jarvis, 1993; Edmond *et al.,* 1999) infections with annual medical expenditure amounting to exceed $1 billion (Miller *et al.,* 2001) not to mention the mortality rates caused per year, apparently being the major public health concern.

Even though, infections caused by other *Candida* species are elevating worldwide, still candidiasis continues to be preponderant, attributing these facts to *Candida albicans.* A staggering feature of *C. albicans* morphology is its ability to colonize in variety forms scoping from unicellular budding yeast (Figure 2.2a) to true hyphae with parallel-sided walls (Figure 2.2 b–d) (Odds, 1985; 1988; Merson-Davies and Odds, 1989; Gow, 1994; 1997; Berman and Sudbery, 2002; Gow, 2002). The fungus also displays numbers of growth forms that can be cumulatively referred to as pseudo-hyphae upon the elongation of daughter bud take place (Figure 2.2e) and, after septum formation, the daughter cell remains attached to the mother cell. Along these lines, filaments yielded by elongated cells with constrictions at the septa are formed (Figure 2.2 f–h). The buds elongation from pseudohyphae springs up in extreme

resembling superficial hyphae (Figure 2.2 c, g). Thus the pseudo-hyphae and hyphae collectively referred to the term filamentous.

The membrane of *Candida* contains large quantities of sterols, usually ergosterol. Apart from a few exceptions, the macroscopic and microscopic cultural characteristics of the different *Candida* species are similar. They can metabolize glucose under both aerobic and anaerobic conditions. Several studies have demonstrated that infection with *Candida* is associated with certain pathogenic variables. Adhesion of *Candida* to epithelial cell walls, an important step in initiation of infection, is promoted by certain fungal cell wall components such as mannose, C3d receptors, mannoprotein, and saccharins (Ghannoum *et al.,* 1986; Kanbe *et al.,* 1991; Brassart *et al.,* 1991). The degree of hydrophobicity (Hazen *et al.,* 1991) and ability to bind to host fibronectin (Klotz and Smith, 1991) has also been reported to be important in the initial stages of infection. Other factors implicated are germ tube formation, presence of mycelia, persistence within epithelial cells, endotoxins, induction of tumor necrosis factor, and proteinases. (Cutler *et al.,* 1972; Saltarelli *et al.,* 1975; Sobel *et al.,* 1984; Kwon-Chung *et al.,* 1985; Smith, 1985; Riipi and Carlson, 1990). Phenotypic switching which is the ability of certain strains of *C. albicans* to change between different morphologic phenotypes has also been implicated (Slutsky *et al.,* 1985).

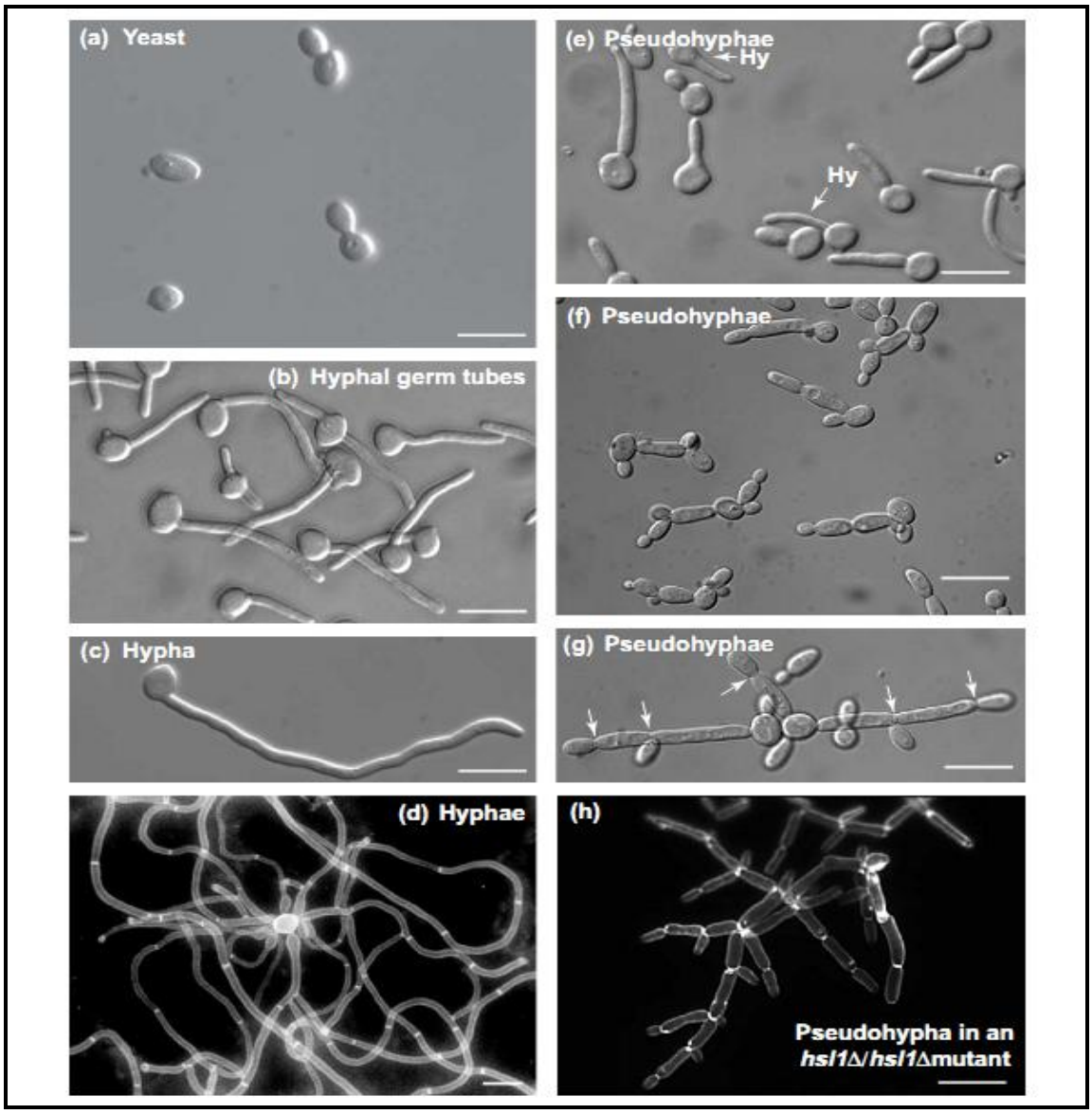

Figure 2.2: Yeast, hyphal and pseudohyphal morphologies. (a) Budding yeast cells (b) Hyphal germ tubes (e) Pseudohyphal buds in the conditions used, 25% of the cells are hyphal, examples of these are indicated by an arrow plus 'Hy'. (c) Hyphae continue to display parallel-sided walls with no constrictions or branches. (d) Mature hyphal mycelia. (e) Pseudohyphae exhibit morphologies ranging from short pseudohyphae that are appear to be polarized yeast cells to (g) two long pseudohyphae that superficially resemble hyphae. (h) Mature pseudohyphal mycelium. The images in (d) and (h) are of cells stained with Calcofluor white, which stains chitin in the cell walls and septa. All scale bars represent 10 mm (Adapted: Sudbery *et al.*, 2004).

2.2.1 *Candida albicans*

Candida albicans are diploids acquainted with 6 to 8 chromosomes and primitively heterozygous since neither sexual nor parasexual cycle is known (Odds, 1988; Scherer and Magee, 1990; Pla *et al.,* 1996). There is no known symptomatology associated to these fungi unless the compliance of propitious mode is assimilated with long-term antibiotic treatment and compromised local immune or barrier defences that initiate the conversion into becoming pathogenic. Those which are pathogenic have been classified into three broad categories: superficial, cutaneous, and systemic. Superficial mycoses (systemic fungal infections) normally are confined to the keratinized layer of the skin and its appendages (Assaff and Weil, 1996). The manifestation of cutaneous or subcutaneous mycoses of skin and cutaneous tissues prescriptively by way of traumatized area (wounded area); they conventionally remain localized but may spread throughout system with the aid of lymphatic. The systemic mycoses inveterately subjected to pulmonary inception, however may also affect most areas of the body (Chandler and Watts, 1996; Body, 1996). Being the most pathogenic among the *Candida* species is the *Candida albicans* reputable as causative agent of oral mucosal layers, vaginal infections, skin and diaper rash to lethal disseminated candidiasis in those with compromised immune systems and those who had medical implantation devices such as artificial joint, a pacemaker and invasive bloodstream infections (O'Dwyer *et al.,* 2007; Terrier *et al.,* 2007; ten Cate, *et al.,* 2009; Bhavan *et al.,* 2010) elevating severity in millions of individuals globally.

Aberrant environmental conditions elicit the switch of one morphological to different morphology formations in *C. albicans* such as blastospores or blastoconidia, pseudohyphae, true hyphae and clamydospores. The unicellular yeast

form that divides by budding is termed a blastospore. Budding is associated with the growth of new cellular material from a small, selected site on the blastospore surface which observes the development of a daughter bud at the pole distal to the birth scar that eventually enters into a growing phase. Subsequently, the process of nuclear division eventuates in septum being formed in between the parent and daughter cell units contemporaciously known to form two blastspores. A hypha on the other hand, is a long microscopic tube consisting of one or more cells surrounded by a tubular cell wall that is divided by septa. The yeast – hyphal transition is denoted with the initial formation of germ tube. A blastospore produces new cellular material in the shape of a cylinder, known as the germ tube, which grows continuously by extending through the distal pole. Mitotic cell division takes place within the extending hypha and septa are formed at intervals along the hyphae without interrupting the rate of extension. Hyphae may arise either as branches of already existing hyphae or by germination of spores. A mycelium is the vegetative part of fungus and consists of the entire hyphae including all their branches (Bartegs *et al.,* 1969; Molero *et al.,* 1998). This unique ability to switch between the yeast and the hyphal mode of growth has been implicated in its pathogenicity.

There were few influential factors that governs the hyphal phenotype *in vitro*; pH, temperature or compounds such as N-acetyl glucosamine, serum and proline (Molero *et al.,* 1998). The laboratory has a unique environmental condition that favours the *C. albicans* to develop true hypha from blastospores as a response to serum. Additionally, factors such as high temperature, high ratio of CO_2 and O_2, neutral pH and poorly nourished media also contribute to the formation of hyphal growth. Acidic pH, low temperature, air and enriched media nonetheless, trigger the growth of blastospore (Mattia *et al.*, 1982; Molero *et al.*, 1998; Li *et al.*, 2007).

The interaction between the host and the *C. albicans* encounter in such that the host apprehends the general survivance, fitness and persistence throughout the invasion of the virulent yeast that associate specific factors such as adhesion, invasion, cell damage and induction or evasion of host responses (Calderone and Braun, 1991; Naglik *et al.,* 2003; Zhu and Filler, 2010). In retaliation, the host counterfires by concatenating mechanical barriers that resist fungal penetration such as epithelial surfaces, soluble antimicrobial factors, and innate and adaptive cellular immune mechanisms. This promulgates that a frail alterations of the physiology of host system may transform the harmless commensal yeast into a treacherous pathogen apt in rendering feeble illness emphasizing the importance of host defence system in maintaining *C. albicans* in the commensal state.

2.2.2 Treatment for Candidiasis

Topical antifungal therapy is the recommended first line treatment for uncomplicated oral candidiasis and where systemic treatment is needed topical therapy should continue as this reduces the dose and duration of systemic treatment required (Epstein and Polsky, 1998). The systemic adverse effects and drug interactions that occur with the systemic agents do not occur with topical agents (Epstein *et al.,* 1984). Treatment in the early part of the 20th century was with gentian violet, an aniline dye, but because of resistance developing and side effects, such as staining of the oral mucosa, it was replaced by a polyene antibiotic, nystatin, discovered in 1951 and amphotericin B, discovered in 1956. They act by binding to sterols in the cell membrane of fungi, and, altering cell membrane permeability (Bennett, 1990; Gupta *et al.,* 1994). Nystatin and amphotericin are not absorbed from the gastrointestinal tract and are used by local application in the mouth.

Miconazole, an imidazole, can be used as a local application in the mouth but its use in this way is limited because of potential side effects such as vomiting and diarrhoea. Other drugs belonging to this class are clotrimazole and ketoconazole. Nystatin is the most widely used topical agent for the treatment of oral candidiasis (Guida, 1988; Epstein, 1990). It is available as an oral rinse, pastille, and suspension. It should be used as a rinse four times a day for two weeks. It can cause nausea, vomiting, and diarrhoea. The oral rinse contains sucrose and is useful in edentulous patients and those with xerostomia such as patients receiving radiotherapy and those with HIV infection (Epstein, 1993).

Clotrimazole troche can be an alternative for those patients who find nystatin suspensions unpalatable. Systemic antifungal therapy in oral candidiasis is appropriate in patients intolerant of or refractory to topical treatment and those at high risk of developing systemic infections (Epstein and Polsky, 1998). Both nystatin oral rinses and clotrimazole troches have high sucrose content and if tooth decay is a concern or the oral candidiasis is complicated by diabetes, steroid use or an immunocompromised state, triazoles which include fluconazole or itraconazole once per day has been found to be effective in these cases (Blatchford, 1990). Ketoconazole is also as effective as fluconazole and itraconazole but its use in elderly patients is not recommended due to drug interactions and side effects, which include hepatotoxicity.

Fluconazole is a potent and selective inhibitor of fungal enzymes involved in the synthesis of ergosterol, an important constituent of the plasma cell membrane. It therefore disrupts cell wall formation leading to leakage of cellular contents eventuating in and cell death. It is well absorbed by the gastrointestinal tract and the plasma levels are found to be over 90% of the levels achieved with intravenous

administration similarly achieved for the levels in saliva and sputum are also similar to that in the plasma. It is preferred, as it does not have the same hepatotoxicity as the imidazoles. It is now listed in the dental practitioners' formulary as well as the British National Formulary, the utilization spread between the dental and medical practice, yet there were no records with resistance problems. Itraconazole has a wider spectrum of activity than fluconazole and is therefore valuable in salvage treatment patients with immunocompromised patients with fluconazole resistant candidiasis. Increasing resistance to antifungals has become increasingly common since the introduction of fluconazole especially in patients with advanced HIV disease, and recurrent and long term treatment (Heinic *et al.,* 1993; Rex *et al.,* 1995).

Angular cheilitis is treated with antifungal steroid creams and ointments and any concurrent intraoral lesion is also treated at the same time and dietary deficiencies will be excluded and treated if found. Failure to respond to therapy especially in chronic atrophic candidiasis is usually due to non-compliance with treatment. Prophylaxis with antifungal agents reduces the incidence of oral candidiasis in patients with cancer undergoing treatment (Clarkson *et al.,* 2000) and fluconazole has been found to be more effective than topical polyenes (Lumbreras *et al.,* 1996). Prophylaxis on either a daily or weekly basis with antifungals reduces the incidence of oral candidiasis in patients with HIV with the reductions being most marked in those with low CD4 counts and recurrent oral candidiasis (Powderly *et al.,* 1995; MacPhail *et al.,* 1996; Schuman *et al.,* 1997; Smith *et al.,* 1999). The use of a chlorhexidine rinse only in bone marrow transplant patients as prophylaxis was found to be very effective (Ferretti *et al.,* 1987).

2.3 Free Radicals and Antioxidants

2.3.1 Free Radicals

Stable atoms are predicated for being in ground state to which the word ‘ground’ connotes every electron in the outermost shell of an atom being complimentary with electron that spins in the opposite direction. When any atom (e.g. oxygen, nitrogen) procures with the least presence of one unpaired electron in the outermost shell collaterally existing independently, it is then accredited with the term free radical (Karlsson, 1997). A free radical eventuates, in which time a covalent bond disintegrates between entities resulting in unpaired electron in each newly formed atom (Karlsson, 1997). The formation of unpaired electron (s) is highly reactive as free radicals. Radicals are capable of existing as reactive oxygen species (ROS) or reactive nitrogen species (RNS).

Free radicals are formed within the human system either as an intercessor during indispensable biological processes that concerns neurotransmission and inflammatory reactions or simply as a byproduct that had no corresponding value in an actual process. In many instances, the aerobic reactions execute free radicals by reducing a minor part of the oxygen (approximately 1-3 %) during the oxidative phosphorylation (Madger, 2006).

Come what way, there are also other external factors that coerced the accumulation of free radicals such as air pollution, ozone, nitrous oxide, cigarette smoke through active o passive, drugs, pesticides, contaminated or rancid foods, unsaturated fats and exposure to ionizing radiation example ultraviolet light, X-rays and cosmic (Greenly, 2004). The radicals will in turn oxidize other stable compounds transforming into free radicals themselves. These partly reduced forms of oxygen in unison are delineated as ROS and similarly RNS when nitrogen is involved. The