Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba

GAYAN KANCHANA WIJESINGHE

EFFECT OF TRUE CINNAMON *(Cinnamomum verum)* LEAF OIL AGAINST *IN VITRO Candida* BIOFILMS AND ITS CYTOTOXIC EFFECT

EFEITO DO ÓLEO ESSENCIAL DE FOLHA DE CANELA VERDADEIRA (*Cinnamomum verum*) CONTRA BIOFILMES *IN VITRO* DE *Candida* E SEU EFEITO CITOTÓXICO

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“ විජ්ජා උප්පත්තං සෙට්ඨා”

“Vijja Uppattan Setta”

(Among All That Arise, Knowledge Is the Greatest)

- Lord Buddha –

DEDICATION

This study is wholeheartedly dedicated to my beloved parents, who have been my source of inspiration and gave me strength when I thought of giving up, who continually provide their moral, spiritual, emotional, and financial support.

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ABSTRACT

The essential oil extracted from *Cinnamomum verum* leaves has been used as an antibacterial and antifungal agents for centuries. The objective of this research was to evaluate the antifungal activity of the *C. verum* leaf oil against standard strains of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646). Effect of *C. verum* leaf oil in vapor phase was evaluated using microatmosphere technique. CLSI M27-A3 broth microdilution was employed in determining MIC and MFC. Time requires to kill planktonic *Candida* cells, effect on initial adhesion and on germ tube formation were evaluated using standard protocols. Effect of *C. verum* leaf oil on forming biofilm was quantified using XTT viability assay, visualized using Scanning Electron Microscope (SEM) and the biofilm progression in the presence of essential oil was visualized using Time lapses microscope. Effect of Cinnamon leaf oil on established biofilms of test strains were quantified and visualized using XTT viability and SEM respectively. Post exposure intracellular changes were visualized using Transmission Electron Microscopy (TEM). Toxicological assessments were carried out with Human Keratinocyte cell line (HaCaT) and *Galleria mellonella* larvae animal model. Commercial antifungal agent Chlorhexidine digluconate was used as positive control. All experiments were done in triplicates. Multiple means of more than three data sets were compared using one way ANOVA and two way ANOVA. The level of significance was taken at 5% ($p < 0.05$). Eugenol was the most abundant chemical compound in Cinnamon leaf oil. All test strains were susceptible for *C. verum* leaf oil vapor. *C. verum* leaf oil exhibited MIC value 1.0 mg/ml and MFC value 2.0 mg/ml against all test strains while Chlorhexidine exhibited MIC value of 1.0 mg/ml and MFC, 2.0 mg/ml with *C. albicans* and *C. dubliniensis*. MIC and MFC for *C. tropicalis* with Chlorhexidine was 0.5 mg/ml. Killing time of Cinnamon leaf oil for *C. albicans* was 6h. 50% reduction in initial adhesion was achieved with 1.0 mg/ml, > 2.0 mg/ml and 0.34 mg/ml by *C. albicans*, *C. tropicalis* and *C. dubliniensis* respectively. 0.5 And 1.0 mg/ml significantly inhibit the germ tube formation of *C. albicans* and *C. dubliniensis.* Minimum Biofilm Inhibitory Concentration ($MBIC_{50}$) for forming and established biofilms were <0.2 mg/ml for all test strains. *C. verum* leaf oil caused retardation in biofilm development. SEM images exhibited cell wall damages, cellular shrinkages and decreased hyphal formation of *Candida*. TEM indicated intracellular vacuolation, cell wall damages and cytoplasmic granulation. Cinnamon leaf oil caused no inhibition of HaCaT cell at maximum concentration tested (1000 mg/ml). Essential oil of Cinnamon leaf didn't exhibit a lethal effect at any concentration tested on *G. mellonella in vivo* assay. *C. verum* leaf oil is a potential alternative anti-*Candida* agent with minimal toxicity.

Key words: *Cinnamomum verum*, essential oil, *Candida* spp., Biofilms, Antifungal agent.

RESUMO

O óleo essencial extraído das folhas de *Cinnamomum verum* tem sido usado a tempos como um promissor agente antibacteriano e antifúngico. O objetivo desta pesquisa foi avaliar a atividade antifúngica do óleo essencial das folhas de *C. verum* contra cepas padrão de *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) e *C. dubliniensis* (ATCC MYA-646). O efeito do óleo essencial *C. verum* na fase de vapor foi avaliado pela técnica da microatmosfera. A microdiluição em caldo foi empregada na determinação da CIM e MFC segundo protocolo CLSI M27-A3. O tempo necessário para agir sobre a *Candida* spp. planctônica, o efeito na adesão inicial e a formação do tubo germinativo foram avaliados usando protocolos padrão. O efeito do óleo essencial de *C. verum* na formação do biofilme foi quantificado usando o ensaio de viabilidade XTT, visualizado por Microscopia Eletrônica de Varredura (MEV) e a progressão do biofilme na presença do óleo essencial foi visualizada no microscópio Time Lapse. O efeito do óleo essencial de *C. verum* nos biofilmes formados foi quantificado por teste de viabilidade XTT e MEV, respectivamente. Alterações intracelulares pós-exposição foram visualizadas usando Microscopia Eletrônica de Transmissão (MET). As avaliações toxicológicas foram levadas a efeito com a linha de células de queratinócitos humanos (HaCaT) e larvas de *Galleria mellonella*. Antifúngico comercial e digluconato de clorexidina foi utilizado como controle positivo. Todos os testes foram realizados em triplicatas. Múltiplas médias de mais de três conjuntos de dados foram comparadas usando ANOVA unidirecional e ANOVA bidirecional. O nível de significância adotado foi de 5% ($p < 0,05$). Com as análises de CG o Eugenol foi o composto químico mais abundante presente no óleo comercial das folhas de *C. verum*. Todas as linhagens de teste foram susceptíveis ao vapor do óleo das folhas de *C. verum*. O óleo essencial de *C. verum* exibiu valor de MIC 1,0 mg/ml e valor de MFC 2,0 mg/ml contra todas as cepas de teste, enquanto a clorexidina exibiu valor de MIC de 1,0 mg ml e MFC, 2,0 mg/ml com *C. albicans* e *C. dubliniensis*. A CIM e a MFC para *C. tropicalis* com Clorexidina foram de 0,5 mg/ml. Foi observada uma redução de 50% na adesão inicial com 1,0 mg/ml,> 2,0 mg/ml e 0,34 mg/ml para *C. albicans*, *C. tropicalis* e *C. dubliniensis*, respectivamente. 0,5 e 1,0 mg/ml inibe significativamente a formação de tubos germinativos de *C. albicans* e *C. dubliniensis*. A concentração mínima inibidora de biofilme em formação ($MBIC_{50}$) e biofilmes estabelecidos foi <0,2 mg/ml para todas as cepas de teste. O óleo da folha de *C. verum* demonstrou inibicão no desenvolvimento do biofilme. As imagens SEM exibiram alterações morfologicas na parede celular, encolhimento celular e diminuição da formação de hifas de *Candida*. Nas imagens de MET foram observados vacuolação intracelular, dano na parede celular e granulação citoplasmática. O óleo essencial da folha de canela causou inibição de 5% da célula HaCaT na concentração máxima testada (1000 mg/ml). O óleo essencial de *C. verum* não apresentou efeito tóxico nas concentrações de 500 mg/ml no ensaio *in vivo* de larvas de *G. mellonella*. O óleo da folha de *C. verum* se mostra um potencial agente antifúngico alternativo com toxicidade baixa.

Palavras-chave: *Cinnamomum verum*, óleo essencial, *Candida* spp., Biofilmes, Antifúngico.

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standard Institute
CLSM	Confocal Laser Scanning Microscope
DM	Diabetes Mellitus
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EO	Essential Oil
FBS	Fetal Bovine Serum
HIV	Human Immunodeficiency Virus
Hrs/h	Hours
kV	Kilovolt
MBEC	Minimum Biofilm Eradication Concentration
MBIC	Minimum Biofilm Inhibitory Concentration
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MOPS	3-morpholinopropane-1-sulfonic acid
MRSA	Methicillin-Resistant *Staphylococcus aureus*
NCAC	Non- *C. albicans Candida*
PBS	Phosphate Buffered Saline
RPMI	Roswell Park Memorial Institute
SDA	Sabouroud's Dextrose Agar
SEM	Scanning Electron Microscope
SPSS	Statistical Package for Social Sciences
TEM	Transmission Electron Microscope
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-*2H*-Tetrazolium-5-Carboxanilide
YPD	Yeast Peptone Dextrose

TABLE OF CONTENT

1. INTRODUCTION

Biofilms are communities of microorganisms attached to a surface living in a matrix of extracellular material derived both from the cells themselves and from the environment. Those microorganisms are usually organized into a three-dimensional structure (Acker et al., 2014; Marsh, 1999) in order to form this complex biofilm eco system. There are two types of biofilms; Monospecies biofilm that involves one microbial species and Multispecies biofilms that involves more than one species (Yang, 2003). According to the species involved in formation of biofilms; the structure, properties, their sensitivity to anti microbial agents and metabolic activities can vary from one biofilm to another.

Many human microbial infections (more than 65%) (Pierce et al., 2008) involve in biofilm formation on implanted biomaterials and/or host surfaces (Ramage et al., 2005, Weerasekera et al., 2016). Among the medically important biofilms, dental plaque (Marsh, 2006) is a widely studied biofilm which is known to associate with many oral and non-oral infections such as periodontitis (Zijnge, 2010), dental caries (Marsh, 1999) and endocarditis (Nomura et al., 2006). Dental plaque is a structurally and functionally organized biofilm found on a oral surfaces, embedded in a matrix of polymers of host and microorganisms origin (Marsh, 2006). Dental plaque consists of more than 1000 different microbial species, where 50% are reported to be uncultivable (Runarsson, 2009). Opportunistic pathogens such as *Candida* species, pathogens such as *Streptococcus* spp. (*S. mutans*, *S. mitis* etc.), *Lactobacillus* spp. and *Actinomyces* spp. are some of these microbial species.

Candida spp. Is present in various sites of the human body and can causes many other non-oral infections and infections associated with medical devices. Systaemic Candidosis, Superficial *Candida* skin infections, ear infections, genitourinary tract infections, Central venous catheters, prosthetic heart valves and urinary catheters associated infections are some examples (Kojic et al., 2014; Achkar, 2010; Douglas, 2002 and Odds, 1987). Some of these infections are serious life threatning infections while some are non-invasive, non-life threatning infections.

Candida albicans has a greater capacity to form biofilms among other fungal species (Ramage et al., 2005 and Kuhn, 2002). In oral cavity *Candida* spp. play a major role in dental plaque formation. It is well known that diabetes mellitus predisposed to opportunistic Candidiasis

(Marsh 1999). Further as diabetes mellitus (DM) is now considered as global epidemic and become the number one non communicable disease in all over the worls, we are at risk of acquiring the disease (Weerasekera et al., 2017). *Candida* sessile cells offer greater resistance to antifungal treatments compared to its' planktonic free living counterpart (Pierce et al., 2008).

Same as oral infections, *C. albicans* is the most commenly isolated and most virulent species of *Candida* (Weerasekera et al., 2016) responsible for non-oral *Candida* infections followed by *C. tropicalis* and *C. dubliniensis*.

Biofilm formation is initiated with adhesion of microbial cells to host surfaces. *Candida* spp. being more virulent opportunistic yeast, possesses several virulent factors such as high ability to adhere to host surfaces, proteinases (enzyme) secretion, hyphal formation, phenotypic switching, germ tube formation (*C. albicans* and *C. dubliniensis*) and aldehyde production (Ramage et al., 2005; Yang, 2003) etc.

Other than microbial factors, Environmental and host factors can influence the biofilm activity of an organism. Dietary nutrients (sugars) (Marsh, 2006 and Jin, 2004), saliva flow (Jin, 2004), pH of the environment (Marsh, 2006), oxygen supply and temperature (Marsh, 1999) are some of environmental factors and host immune status (HIV) and diseases (diabetes) (Ramage, 2001) condition are some of host factors which can affect the biofilm formation of a given organism.

Therefore it is very important to conduct studies with regard to *Candida* biofilm formation. This will help to understand its properties, role in infections and to investigate possible mechanisms which minimize the oral colonization of *Candida* spp.

Since, biofilm structure acts as a protective shiled, routine antimicrobial agents or physical stress have less effect on biofilm eradication. So it is important to introduce alternative biofilm controling and eradication strategies in order to treat biofilm infections (Marsh, 1999).

The use of medicinal plants as a tratment option is an ancient practice as well as modern world (Petrovska, 2012). Specially ancient asians including Sri Lankans, Indiand and Japanese people used these phytochemicals as their theraapeutic agents. The knowledge acquired over time about the use of medicinal plants by these peoples, has been transmitted along the

generations and is used until today. The use of medicinal plants as therapeutic alternatives has helped many populations that do not have access to modern treatments and drugs of high cost and low availability in modern world, so, the use of plants for the treatment of diseases/infections has been empirically employed worldwide. In past few years, with the scientific advance, more research were conducted to isolate and introduce new compounds with medicinal properties, allowing scientists to find new, effective medicinal compounds with low side effects (Gupta et al., 2014).

Scientific studies involving the use of medicinal plants have already contributed to obtaining several therapeutic agents used in traditional/Ayurvedic medicine. But still, the scientific basis of certain medicinal phytochemicals use specially in Asian traditional/Ayurvedic medicine should be further evaluated. In the Dentistry area, Phytotherapy is already an integrative and complementary practice to oral health, and its recognition is a great step to regulate the use and performance of the dental professionals in this area (Sinha, 2014). Studies show that oils and extracts obtained from medicinal plants were efficient as an anti microbial agent, specially against oral colonizers. Another important use of medicinal plants is, the synergistic combination with other existing commercial drugs in an attempt to reduce or avoid the side effects of conventional medicines (Silva et al., 2010). Some reports in the literature indicate that plant derivatives may enhance the efficacy of the traditional drug as well as reduce the side effects. Previous studies point out that some essential oils and phytochemical compounds have synergistically improved activities with commercial antifungals (Shin et al., 2003; Malongane et al., 2017).

In this context, *Cinnamomum verum*/ True Cinnamon, is part of the genus most cited in therapeutic microbiological studies, including data from clinical studies of medicinal activity. In this sense, this plant has been shown to be of great economic and pharmaceutical-medicinal interest. Although, several *in vitro* microbiological studies with *C. verum* show antimicrobial activities related to many microbial species in the health care area, including those that exhibit multiple antimicrobial resistance, it's antifungal effect on *Candida* spp. is still less comprehensive.

2. LITERATURE REVIEW

2.1 Oral Cavity

The oral cavity is considered a complex ecosystem with different ecological conditions of distribution of nutrients and different atmospheres, which confers different habitats to the oral microorganisms that colonize it. The resident microbiota of the oral cavity is diverse and can harbor many species of bacteria, mycoplasmas, fungi and viruses (Marsh & Martin, 2005). However, both resident and transient colonizers may undergo variations depending on the individual's diet, immune conditions, saliva flow, treatments with antibiotic therapy, as well as dental treatments (Marsh & Martin, 2005). Due to the polymicrobial character of the oral ecosystem, local infections are usually caused by different types of microorganisms and even the interaction of more than one species, such as dental caries, periodontal disease and other fungal infections (Jenkinson & Lamont, 2010; Ghannoum et al., 2010). Among these oral colonizers, *Streptococcus* spp, are found in both mucous membranes and dental biofilms. These include: *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus mutans* and *Streptococcus sanguinis* (Kreth et al., 2005). The dental biofilm is a complex, polymicrobial structure that favors the survival of microorganisms that contribute, due to its relation of mutualism, allowing the expression of genes that favor communication between them, the evasion of the immune system, as well as the production of a specialized habitat for each microorganism present (Kreth et al., 2005).

2.2 Oral biofilm formation

Oral biofilm formation is a complex and dynamic process. First, pellicles are formed on oral surfaces by adsorbing salivary proteins and glycoproteins. This surface conditioning film is termed the acquired enamel pellicle. The major components of pellicle are salivary glycoproteins, phosphor proteins, lipids and other host molecules. After the pellicle formation, reversibly attached acidogenic microorganisms including *Streptococcus mutans* (Kolenbrander et al., 2010) undergo adhesin-receptor mediated stronger irreversible attachment within a short period of time. Oral bacteria generally possess more than one type of adhesin molecules on their surface and can participate in multiple interactions both with host surface molecules and similar receptors

on other microorganisms. The dental biofilm pioneer species modify local ecological conditions and further promotes colonization by other species that may lead to diseases, such as caries and periodontal diseases (Kolenbrander et al., 2002). Among these Secondary colonizers, *Candida* species considered as a major contributor.

2.3 *Candida* spp.

Candida spp. are the most common causative agent of fungal infections, leading to a range of serious life-threatening invasive to non-life-threatening mucocutaneous diseases including skin and ear infections, genitourinary candidiasis, nasocomial pneumonias, medical device associated infections and Candidaemias (Kojic et al., 2014, Achkar 2010, Douglas, 2002 and Odds, 1987).

Opportunistic fungal infections caused by *Candida* yeasts, also called candidiases and / or candidoses, are more frequent infections associated with the compromised immune system. Predisposing factors for the infection are: extremes of age, hormonal changes, nutritional deficiency, HIV incidence, frequent antimicrobial exposure, chemotherapeutic treatments, carbohydrate-rich (glucose) diets, use of prostheses and low immunity (Marsh & Martin, 2005; Asmundsdóttir et al., 2009; Samaranayake et al., 2002; Sant'Ana et al., 2002) as host factors.

According to available statistics, approximately 5–7% of infants develop oral candidiasis. Prevalence of oral candidiasis in AIDS patients is around 9–31% and 20% in the cancer patients. The oral carriage of candida is reported to be 30–45% in the healthy adults (Patil et al., 2015). Though, *C. albicans* is the frequently isolated organism from oral *Candida* biofilms, *C. tropicalis, C. glabrata, C. krusei, C. parapsilosis, C. pseudotropicalis,* and *C. stellatoidea* also found as causative agents. Recent studies found that the prevalence of above mentioned non- *C. albicans* Candida (NCAC) species is increasing in past few years. (Williams & Lewis, 2011).

2.4 Virulence of *Candida*

Among all *Candida* species, *Candida albicans* is the most frequent etiological agent in cases of fungal infections, being associated in up to 50% of cases of this disease (Pemán et al., 2012). This genus has the capacity to adapt and proliferate easily in the environment of the human body, in several places. This ability to survive in different tissues of the human body is related to

its capacity for morphological transition, between hyphal form and yeast form (Tsang *et al*., 2012). According to Mayer et al. (2013), hyphae and yeast, are involved in the infection process, and the yeast, being smaller, is able to disseminate, while the hyphae invades the host tissue by penetrating the epithelium with the help of proteinases and phospholipases enzymes, escaping from phagocytic cells (Mayer et al., 2013; Raju & Rajappa, 2011; Luo et al., 2013; Coutinho, 2009).

Another factor associated with virulence of *Candida* spp. is the ability to form biofilms, Promoting cell adhesion and formation of the extracellular matrix of the biofilm, as well as the synthesis of proteins that favor its further adhesion. The biofilm structure increases the resistance to antimicrobial agents, due to the difficulty of penetration into the extracellular matrix, and the action of the immune system, besides allowing the increase of the gene expression of mechanisms of resistance to antifungals as the efflux pumps (Santana et al., 2013).

2.4.1 Biofilms and their resistance

The resistance of biofilms to antimicrobial agents is often attributed to the failure of these agents to penetrate the biofilm matrix. Drug access into the biofilms is also assisted by the presence of water channels in the biofilm structure. Nevertheless, biofilm extracellular matrix components could reduce access to such an extent that cells lying deep within a biofilm escape exposure. This would occur through adsorption or neutralization of antimicrobial agent, and would depend on the thickness of the biofilm and on the chemical nature of both the antimicrobial agent and the extracellular matrix material. For example, fluoroquinolones penetrate *P. aeruginosa* biofilms readily, whereas penetration by positively charged aminoglycosides is decreased. Similarly, fluconazole penetrates single-species *Candida* biofilms more rapidly than flucytosine. Further, rates of drug diffusion through biofilms of *Candida glabrata* or *Candida krusei* are faster than those through biofilms of *Candida parapsilosis* or *Candida tropicalis*, while diffusion of antimicrobial drugs through mixed-species biofilms of *C. albicans* and *S. epidermidis* is significantly slow (Drenkard, 2003; Al-Fattani et al., 2006).

Since biofilms exhibit greater resistance to antimicrobials compared to suspension grown planktonic cells (Pierce et al., 2008), different types of strategies are used in oral biofilm control and treatment of oral biofilm infections. Traditional methods of biofilm removal include mechanical removal and the use of synthetic and natural antibacterial mouthwashes and dentifrices

(Soukos et al., 2011). Due to lack of availability, harmful side effects and polymicrobial resistance, it's very important to find new effective alternative antimicrobial strategies in order to control these oral biofilm infections. As a result of those studies, many plants and phytochemicals were identified as potential anti-biofilm remedies.

2.5 Medicinal plants

Anti-microbial resistance is an emerging problem in health care facilities in modern world. (CDC, Atlanta, 2013). Resistance of microorganisms against available antimicrobials has become a major problem, especially when it comes to infections in immunocompromised patients. Such resistance has resulted in a dramatic increase in the incidence of systemic and opportunistic microbial infections and mortality, which leads to an increasing need for the development of effective new antimicrobial therapeutic drugs. According to published data, pathogens with multidrug resistance are methicillin-resistant *Staphylococcus aureus* (MRSA), a common causative agent of nosocomial infections, and which is exhibiting a resistance to vancomycin (Van et al., 2013), *Pseudomonas aeruginosa* (Nathwani et al., 2014), Streptococcus pneumoniae, a common respiratory pathogen with multidrug resistance (Kim et al., 2016), multidrug-resistant *Mycobacterium tuberculosis* (Kempker et al., 2015), which are causing an increase in the prevalence of tuberculosis; and virulent strains of *Escherichia coli* (Krüger et al., 2015)

Azoles are group antifungal drugs used widely as a treatment of Candidiasis since they cause few side effects in human body. Resistance to azoles arises during long-term exposure to the drug as well as low-level prophylactic treatment regimes (Kontoyiannis et al., 2002)

Due to this increasing multiresistance of etiological agents, many scientists pay their attention to develop new antimicrobial remedies to combat these microorganisms based on medicinal plants derivatives with low side effects, lower toxicity and decreased development of resistance of microorganisms (Al-Mariri, 2013).

Though plants have provided western medicine with an abundance of medicines and treatments for a variety of infections, species used in traditional medicines continue to be the most reliable sources for the discovery of useful antimicrobial compounds. As a result, hundreds of

plants worldwide are used in traditional medicine as treatments for bacterial and fungal infections. But, among these vast range of plants, very few of these have subjected to *in vitro* screening.

Further, natural herbal antimicrobial products are not necessarily safer than synthetic antibiotics, some patients prefer to use herbal medicines due to various reasons. Thus healthcare professionals should be aware of the available evidence for herbal antibiotics in order to ensure safe and effective use of those herbal medicines (Karen et al., 2003).

2.6 True Cinnamon/ Ceylon Cinnamon/ *Cinnamomum verum*

There are different types of Cinnamon species all over the world. Among them, true cinnamon/ ceylon cinnamon/ *Cinnamomum verum* is native to Sri Lanka and belongs to the family Lauraceae.

Cinnamomum cassia (Chinese Cinnamon), *C. burmannii* (Indonesian cinnamon), *C. loureiroi* (Vietnamese cinnamon) and *C. citriodorum* (Malabar cinnamon) are other major cinnamon species all over the world (Chen et al., 2014). Though all of them belongs to same genus, *Cinnamomum,* they are exhibiting different physical and chemical properties.

Cinnamon oil can be extracted from leaves or bark of Cinnamon tree. Based on the part of the tree used for extraction, chemical composition of the extracted oil is vastly varying. Both cinnamon leaf and bark essential oils contain cinnamaldehyde and Eugenol as their key constituents but the leaf oil has higher levels of eugenol, and the bark oil has higher levels of cinnamaldehyde. Both contain trace amounts of 43 other chemical compounds including bicyclogermacrene, α-phellanderene, ß-carryophyllene, aromadendrene, p-cymene α-copaene, α-amorphene and 1,8-cineole (Singh et al., 2007).

2.6.1 Cinnamon oil as an antimicrobial agent

Essential oils are mixtures of various chemical compounds originating from the secondary metabolism of plant species, constituting a rich source of biologically active compounds. Thus, the novel antifungal drug discovery based on natural herbal products is exhibiting a great success. (Khan et al., 2012).

Some publish evidences revealed the potential antimicrobial activity of essential oils extracted from Cinnamon plant. For example, Nir et al. (2000) investigated the effect of extract of cinnamon (Chinese cinnamon/*Cinnamonum cassia*) against *Helicobacter pylori*, Azzouz et al. (1982) investigated the effect of Chinese cinnamon against seven mycotoxigenic molds *Aspergillus flavus*, *A. parasiticus*, *A. ochraceus*, *Penicillium* spp. M46, *P. roqueforti*, *P. patulum*, and *P. citrinum* and observed growth inhibition of those molds (Nir et al., 2000; Azzouz et al., 1982).

Similarly Suksrikarm (1987) reported theantimicrobial activity of Cinnamon oil against many microbial species including *Lactobacillus* spp., *Bacillus thermoacidurans*, *Salmonella* spp., *Corynebacterium michiganense*, *Pseudomonas striafaciens*, *Clostridium botulinum*, *Alternaria* spp., *Aspergillus* spp., *Canninghamella* spp., *Fusarium* spp., *Mucor* spp., and *Penicillium* spp. (Suksrikarm, 1987).

Further, Singh et al. (2007) demonstrated the antifungal effect of Cinnamon oil against pathogenic fungi, *Aspergillus niger, Aspergillus flavus, Aspergillus ochraceus, Aspergillus terreus, Fusarium moniliforme, Fusarium graminearum, Penicillium citrinum* and *Penicillium viridicatum* (Singh et al., 2007).

While the *in vitro* antimicrobial activity of Cinnamon oil against some pathogens have well documented, there are lack of published data for anti-*Candida* activity of Cinnamon oil. In this research we are trying to fill this gap using *in vitro* laboratory studies.

2.7 Conceptual Framework

Conceptual framework for Biofilm formation of *Candida* species: Effect of True cinnamon (*C. verum*) leaf oil against *in vitro Candida* biofilms and its cytotoxic effect.

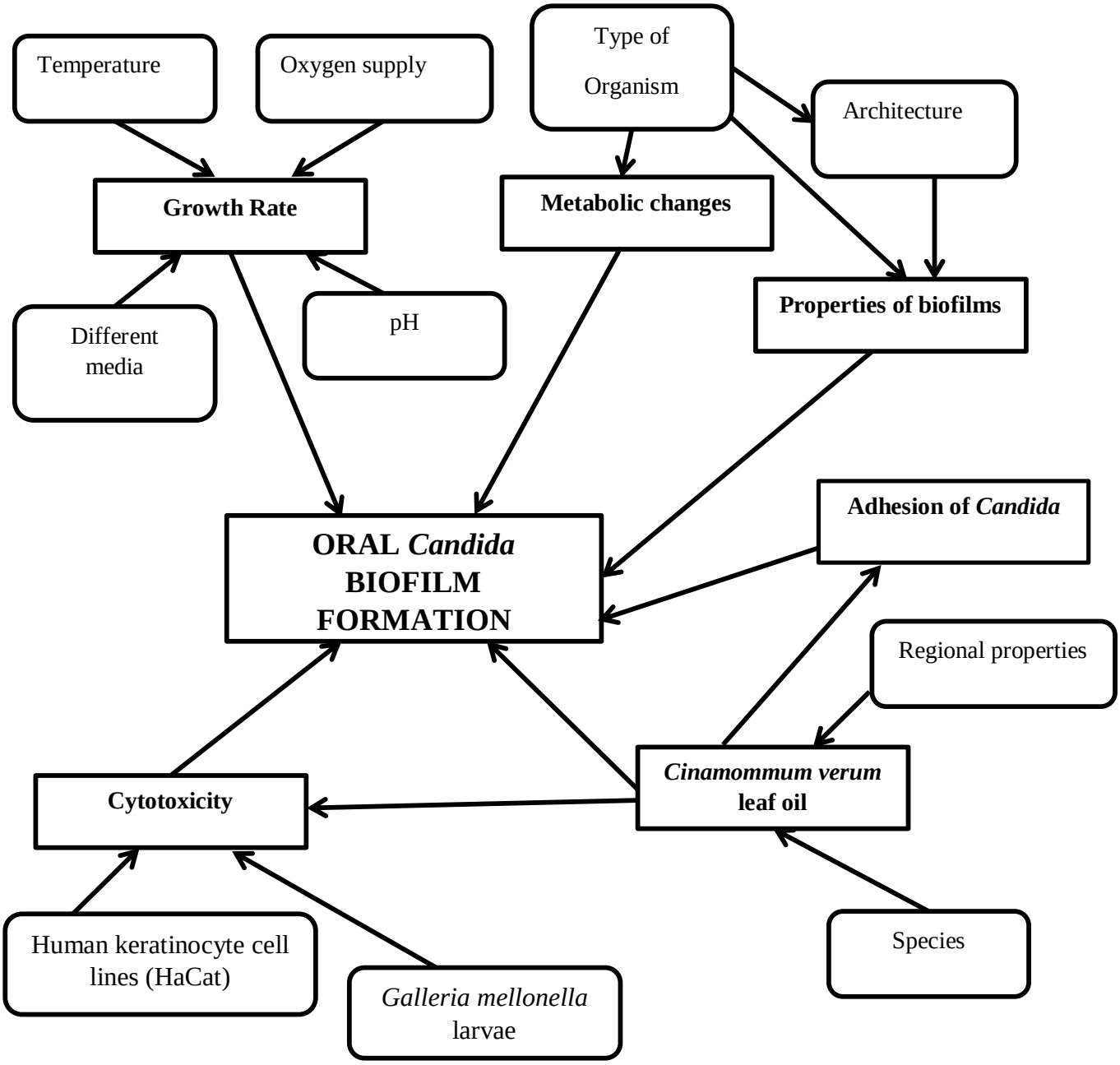

Figure 2.1: Conceptual Frame Work

Source: Author Developed

3. PROPOSITION

3.1. General Objective

To evaluate the anti-biofilm effect against *in vitro Candida* biofilms and the cytotoxic effect of true cinnamon (*Cinnamomum verum*) leaf oil.

3.2. Specific objective

1. To determine the effect of true cinnamon (*Cinnamomum verum*) leaf oil on Planktonic *Candida.*
2. To determine the effect of *Cinnamomum verum* leaf oil on initial adhesion and germ tube formation of *Candida*.
3. To determine the effect of true cinnamon (*Cinnamomum verum*) leaf oil on forming and established *in vitro Candida* biofilms.
4. To determine the post-exposure structural changes of *Candida in vitro* biofilms.
5. To determine the cytotoxic effect of true cinnamon (*Cinnamomum verum*) leaf oil on human keratinocyte (HaCaT) cell line.
6. To determine the *in vivo* lethal effect of *C. verum* leaf oil on larvae of *Galleria mellonella.*

4. MATERIAL AND METHODS

4.1. Study design

The study was an experimental laboratory based study.

4.2. Study setting

The study was conducted in Microbiology and Immunology laboratory of the Area of Microbiology and Immunology, Department of Oral Diagnosic, Piracicaba Dental School, University of Campinas, Brazil.

Address for correspondance: Piracicaba Dental School – UNICAMP, Area of Microbiology and Immunology, Limeira Avenue, 901. CEP:13414- 018, Piracicaba, SP, Brazil.

4.3. Essential oil

Commercially available essential oil of *C. verum* leaf was purchased for this study.

4.4. Dilution of essential oils and antifungals

The *C. verum essential oil* was diluted in Tween 80 (0.05%) solution and RPMI culture medium (Roswell Park Memorial Institute medium) buffered with MOPS (3-(N-morpholino) propane sulfonic acid) followed by sonication, 1 cycle of 20 seconds in to the final concentration of 32 mg/ml.

The antifungal Chlorhexidine digluconate (Sigma Aldrich, USA) was diluted in RPMI culture medium (Sigma Aldrich, USA) buffered with MOPS according to the manufacturers' guidelines in order to prepare 120mg/ml (0.12%) concentrations.

4.5. Gas Chromatography- Mass spectrometry (GC-MS) of *C. verum* leaf oil

Analyzes were performed on an HP-6890 gas chromatograph coupled with HP-5975 selective mass detector, under the following conditions:

HP-5MS Capillary Column (30 m x 0.25 mm x 0.25 µm)

Temperatures: Injector: 220 ° C

Column: 60 ° C, 3 ° C / min, 240 ° C

Detector: 250 ° C

Injected Volume: 1.0 µl

Carrier Gas Flow (He): 1.0 ml/min

4.6. Test strains of *Candida* used in the study

Three *Candida* type strains, *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) were used in this study. These strains were obtained from Microbiology and Immunology Area, Piracicaba Dental School, UNICAMP, Brazil.

4.7. Stocking and reactivation of test microorganisms

The standard strains of *Candida spp*. stocks were maintained in 80% grycerol in ultrafreezer at -80 °C. To reactivate stock organisms, they were subcultured in freshly prepared Saboraud Dextrose Agar (SDA, OXOID) culture medium, incubated aerobically at 37ºC for 24h.

All the experiments in this study were conducted in triplicate. Further all these experiments were conducted with maximum precautions to avoid any environmental contamination. The experiments were conducted inside a Class II Biosafety cabinet in the microbial culture laboratory and/or cell culture laboratory, department of Microbiology.

4.8. Effect on Planktonic *Candida*

4.8.1. Effect of *C. verum* leaf essential oil in vapour phase (Microatmosphere method, Kalemba et al., 2003 and Şerban et al., 2011)

Effect of *C. verum* leaf essential oil in vapour phase on planktonic *Candida* cells were qualitatively determined using Microatmosphere method explained by, Kalemba et al. (2003) and Şerban et al. (2011) previously with few modifications.

Standard suspensions of all test strains mentioned in 4.6 were prepared by dissolving portion of isolated colony in sterile normal saline by measuring and adjusting the absorbance to 0.08-0.1 at 600nm (0.5 McFarland standard).

The prepared suspensions were inoculated separately with a sterile cotton swab on to the prepared quality controlled SDA plates separately in order to get a confluent growth.

A 10 mm diameter sterile filter paper disc moistened with 100 µl of the working solution (1.046 g/ml) of essential oil was attached to the lid of a Petri dish. Plates were sealed with a piece of parafilm in order to prevent leakages of oil vapour. Agar plates were incubated overnight at 37^{o}C aerobically. Control plates were prepared without filter paper disks.

Presence or absence of growth inhibition of inoculated *Candida* on agar surface was observed after 24h incubation.

4.8.2. Determination of Minimum Inhibitory Concentration (MIC)

4.8.2.1. Preparation of *Candida* cell suspensions

Minimum inhibitory concentration (MIC) of *Candida* was determined by following the CLSI M27-A3 broth micro dilution method with modifications (CLSI, 2008). 1×10^{6} cells/ml suspensions of all test strains were prepared in sterile RPMI 1640 buffered with MOPS using 24 hours old fresh *Candida* cultures as described in 4.8.1.

4.8.2.2. Preparation of doubling dilutions of antifungal agents and assay

32 mg/ml mother solution of essential oil was prepared as mentioned in 4.4, 200µl of this mother solution was added to first well column and serially diluted with sterile RPMI 1640 buffered with MOPS, 100µl/well in order to prepare series of doubling dilutions of essential oil along the rows of the 96 well sterile flat bottomed microtiter plate.

Then the plate was inoculated with 100µl of prepared standard cell suspensions separately. (Each well received 100µl of the dilutions of the oil and 100µL of the suspension of each test strain of *Candida spp.* at a final concentration of 0.5 x 10^6 CFU/ml in each well)

Negative control group (Growth control): 100µl of RPMI 1640 instead of essential oil + standard cell suspension

Positive control group: 120 mg/ml Chlorhexidine gluconate (Sigma-Aldrich, USA) was tested.

The plates were incubated for 24 h at 37 °C aerobically and were visually examined for the presence and absence of growth (turbidity of the suspensions). Three independent experiments were performed.

4.8.3. Determination of Minimum Fungicidal Concentration (MFC)

MIC assay was performed as explained in 4.8.2 and 5 µl of solutions from each well (after 24h incubation) were plated in freshly prepared SDA plates and incubated at 37 °C for 24 h aerobically. MFC was defined as the lowest concentration of essential oil capable of inhibiting the fungal growth completely.

4.8.4. Killing Time Assay

Time-kill kinetics of leaf oil of *C. verum* was carried out following the procedure described by Tsuji et al. (2008). Concentrations equal to MIC (1mg/ml), half of MIC (0.5mg/ml) and twice the MIC (2mg/ml or MFC) of the oil was prepared.

An inocula of *C. albicans* (ATCC MYA-2876) were prepared with the concentration of 1.0×10^6 CFU/ml as explained in 4.8.1. and added to prepared dilutions separately. Microtiter plates were incubated at 37ºC for 24h aerobically.

Aliquots of 50µl of the above mixture were taken at time intervals of 0, 1, 2, 3, 4, 5, 6, 12, and 24h, make 10 fold dilutions in 50µl sterile normal saline and 100 µl was spread on SDA plates aseptically. Inoculated plates were incubated at 37 ◦C for 24h aerobically to determine CFU/ml. Negative control test was performed for the test organisms without oil or reference antifungal agent, 120mg/ml Chlorhexidine gluconate. Log CFU/ml was plotted against time (Tsuji et al., 2008). 120 mg/ml chlorhexidine gluconate was used as positive control.

4.9. Effect on Initial Adhesion

Adhesion to host surfaces is one of the main virulent factor of *Candida* spp. *Candida* biofilm formation begins with the adhesion of planktonic yeast cells to a surface (biotic or abiotic surfaces). The next step in biofilm formation is cell proliferation and early-stage hyphal formation of the adhered cells. This step is followed by biofilm maturation, resulting in a complex eco-system, including elongated hyphae, pseudohyphae, and budding yeast cells, encased in an extracellular matrix, giving the biofilm a thick and structured three dimensional appearance as well as providing protection from chemical and physical stresses.

Effect of *C. verum* leaf oil on *C. albicans* (ATCC MYA-2876), *C. dubliniensis* (ATCC MYA-646) and *C. tropicalis* (ATCC 750) adhesion to a sterile polystyrene surface was studied by using previously published methodology by Raut et al. (2013).

4.9.1. Preparation of standard cell suspension

Few colonies of 24 hours old fresh *Candida* cultures on SDA were inoculated into 50 ml of YPD broth in labeled sterile culture media bottle and incubated at 35-37 ºC for 18-24 hours in a shaking incubator at 30rpm (Nova Instruments, Brazil).

After incubation, cells were harvested and washed twice with sterile Phosphate Buffered Saline (PBS) and the inoculum was adjusted to 1.0×10^7 cells/ml in RPMI 1640 medium after counting cells using Neubauer improved counting chamber (Pierce et al.,2008).

4.9.2. Assay procedure

96 well sterile flat bottomed microtiter plates were seeded with these suspensions (100 µl/well) and allowed to adhere to polystyrene surface of plates for 2h at 37 ºC in a shaker incubator (75 rpm, Nova Instruments, Brazil), in the presence of different concentrations of *C. verum* leaf oil ranging from 8mg/ml to 0.0039mg/ml (100 µl/well). Wells without oil were kept as negative control. 120mg/ml chlorhexidine digluconate (Sigma-Aldrich, USA) was used as positive control.

After 2h incubation, wells were washed with 200 µl sterile normal saline in order to remove non-adherent cells. Attached cells in each well were quantified using XTT metabolic assay as explained below, and percentage reduction of adhered cells was calculated as compared to that of control (Raut et al., 2013).

4.9.3. XTT assay

For the quantification of biofilm cell viability, the plates were washed with sterile PBS and then reacted with 80µL of the XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-*2H*-Tetrazolium-5-Carboxanilide, Sigma-Aldrich) and menadione (Sigma-Aldrich) solution (4µl of menadione in 10ml XTT) for 2h. Then, the absorbance of resulting solution was measured at 490nm using microtiter plate reader /Versa MAX, molecular Devices, USA (Pierce et al., 2008).

4.10. Effect on Hyphae Formation (Germ tube)

Dimorphic fungi including *C. albicans* and *C. dubliniensis* have several morphological forms; yeast cells (blastoconidia) which divide by budding, germ tubes, true hyphae and pseudohyphae. The formation of germ tubes from blastoconidia is the first step in the development of true hyphae and the ability to switch between morphological forms has been suggested as a potential virulence factor. Compared to the unicellular blastoconidial form, hyphae have an increased ability to adhere to and penetrate host tissues.

An experiment described by Hammer et al. (2000) was carried out to examine the effects of true cinnamon leaf oil on the morphological transition (germ tube/hyphal formation) from blastoconida to hypha of *C. albicans* (ATCC MYA-2876) and *C. dubliniensis* (ATCC MYA-646). (Hammer et al., 2000)

Three concentrations, 0.25 mg/ml, 0.5 mg/ml and 1.0 mg/ml (1/4 MIC, 1/2 MIC and MIC) of *C. verum* leaf oil and Chlorhexidine digluconate were prepared in Fetal Bovine Serum (FBS, Sigma-Aldrich) in 1 ml volumes in sterile glass Bijou bottles at twice the desired final concentration as follows: 0.5, 1.0, 2.0 and 0 mg/ml.

Equal volumes (1 ml) of the prepared cell suspensions in YPD medium as explained in 4.9.1. were added to each *C. verum* leaf oil treatment and mixed thoroughly. All treatments were then incubated aerobically at 37 °C, without shaking, and were sampled at 0h, 2h, 4h and 6h after mixing thoroughly.

10 µl of prepared suspensions were loaded to Neubauer improved counting chamber at each time point and percentages of germinated cells (Cells with germ tubes) were calculated by counting the cells.

4.11. Effect on Forming *Candida* Biofilms

Biofilm experiments were performed as following, using *C. albicans* (ATCC MYA-2876), *C. dubliniensis* (ATCC MYA-646) and *C. tropicalis* (ATCC 750) as test strains.

120 mg/ml chlorhexidine digluconate was used as positive control of the experiment.

4.11.1. Effect on biofilm formation

100µl of standard inoculum of each organism was inoculated to a 96-well sterile flat bottomed microplate plate, followed by aerobic incubation for 120 minutes under agitation (75 rpm at 37 °C)(Nova Instruments, Brazil). The plate was then washed once with sterile Phosphate Buffered Saline (PBS) and 100µl of the dilutions of the essential oil (dilutions of essential oil were prepared according to 4.8.2.2.) was added to the treatment wells. 100µl of RPMI 1640 was added to the control wells instead of oil. The plate was then aerobically incubated for 24h at 37 °C (Silva et al. 2010).

Biofilm biomass was quantified after 24h incubation using XTT metabolic assay explained in 4.9.3.

4.11.2. Minimum toxic concentration for forming biofilms (MTC)

Minimum toxic concentration for forming biofilms (MTC) determines the minimum concentration of *C. verum* leaf oil requires to kill the forming biofilms of *Candida* spp. completely. The CFU assay was used to detect the MTC of the oil on forming biofilms. After the 24h treatment with different concentrations of *C. verum* leaf oil, the mass of the adhered biofilm was scraped using a sterile cell scraper and thoroughly homogenized in 100µl of sterile normal saline and then the suspension was serially diluted (10^{-1} to 10^{-6}). Subsequently, an aliquot of 100µL was plated on SDA medium. Agar plates were incubated at 37ºC for 24h. After incubation, the colony forming units were counted and the results were expressed in CFU/ml (Freitas-Fernandes et al. 2014).

4.11.3. Scanning Electron Microscopy (SEM) of forming biofilms in the presence of *C. verum* leaf oil

Ultrastructural properties of biofilms formed with the presence of different concentrations of true Cinnamon oil was evaluated using SEM.

4.11.3.1. Adhesion of *Candida*

10mm diameter glass cover slips were autoclaved and sterilized prior to biofilm growth. Sterile cover slips were placed in 12-well cell culture cluster separately. Coverslips were immersed in 1 ml standardized cell suspension ($1x10^7$ cells/ml) to ensure uniform *Candida* adhesion and incubated for 120 min at 37 ˚C in a shaker incubator (Nova Instruments, Brazil).

4.11.3.2. Biofilm formation and antifungal treatment

Then the cover slips were moved carefully using a sterile forceps and gently immersed in new 12-well plate containing 0.5mg/ml, 1.0mg/ml and 2mg/ml *C. verum* oil in RPMI 1640 (1ml/well). Coverslips were incubated aerobically for 24 h at 37˚C.

4.11.3.3. SEM procedure

The cover slips with treated biofilms were subsequently washed three times with sterile PBS. Then they were transferred to a new 24-well cell culture cluster containing 2.5% glutaraldehyde at 4 ˚C.

After fixing with glutraldehyde for 60 minutes, samples were dehydrated in a series of ethanol solutions (70% for 10 min, 80% for 10 minutes, 95% for 10 min and 10 minutes in absolute ethanol), and air-dried overnight in an incubator prior to sputter coating with gold.

Then the specimens were mounted on an aluminum stub, with carbon tape, and coated with gold under low-pressure with a gold sputter coater (JFC1 100; JEOL). The surface of the biofilms were visualized with the SEM (JEOL, JSM 5600LV) in high-vacuum mode at 10 kV, and the images processed.

4.11.4. Biofilm Progression Analysis (Time Lapse Microscopy)

Progression of *Candida in vitro* biofilms in the presence of *C. verum* leaf oil was evaluated using using Time Lapse Microscopy.

For analyzes of biofilm progression in the presence of *C. verum* leaf oil, *C. albicans* (ATCC MYA-2876); *C. dubliniensis* (ATCC MYA-646) and *C. tropicalis* (ATCC 750) were adhered on to the polystyrene surface of 24-well cell culture cluster and treated with three concentrations (0.5mg/ml, 1.0mg/ml and 2.0 mg/ml) of *C. verum* leaf oil as explained in 4.9.2. During the 24 hour incubation at 37 ^{o}C after adding the treatment, images of forming biofilms were captured at time 0, 4 h, 8 h, 12 h, 16 h, 20 h and 24 h using time-lapse microscope. (ZEISS ApoTome.2) (Oliveira, 2019).

4.12. Effect on Established *Candida* Biofilms

4.12.1. Minimum Biofilm Inhibitory Concentration ($MBIC_{50}$ and $MBIC_{75}$)

100µl of standard inoculum of each organism was inoculated to a 96-well sterile flat bottomed microplate plate, followed by aerobic incubation for 24h at 37˚C. The plate was then washed once with sterile Phosphate Buffered Saline (PBS) and 100µl of the dilutions of the essential oil (dilutions of essential oil were prepared according to 4.8.2.2.) was added to the treatment wells. 100µl of RPMI 1640 was added to the control wells instead of oil. The plate was then aerobically incubated for 24h at 37 °C (Pierce et al., 2008).

Biofilm viability was quantified after 24h incubation using XTT metabolic assay explained in 4.9.3.

4.12.2. Minimum biofilm eradication concentration (MBEC)

The CFU assay was performed to detect the MBEC of the oil on established 24h mature biofilms after the 24h treatment with different concentrations of *C. verum* leaf oil, as explained in 4.11.2. After incubation, the colony forming units were counted and the results were expressed in CFU/ml.

4.12.3. Ultrastructure of *Candida* biofilms after 24h exposure to *C. verum* leaf oil

Post-exposure architectural properties of *Candida* 24h mature biofilms after 24h treatment with essential oil was evaluated using SEM.

10mm diameter glass cover slips were autoclaved and sterilized prior to biofilm growth. Sterile cover slips were placed in 12-well cell culture cluster separately. Coverslips were immersed in 1 ml standardized cell suspension ($1x10^7$ cells/ml) and incubated for 24h at 37 ˚C.

After biofilm formation process, the cover slips were moved carefully using a sterile forceps and gently immersed in new 12-well plate containing 1.0mg/ml, 2mg/ml and 4mg/ml *C. verum* oil in RPMI 1640 (1ml/well). Coverslips were incubated aerobically for 24 h at 37˚C.

SEM procedure was carried out as explained in 4.11.3.3 in order to visualize ultrastructure of biofilms.

4.13. Transmission Electron Microscopy (TEM) for Determination of Post-Exposure cellular Changes

Antimicrobial agents act on microbial cells differently based on their mode of action. Some antimicrobial agents damage the cell wall of unicellular organism by inhibiting cell wall synthesis, some antimicrobial agents inhibit protein biosynthesis and some inhibit DNA replication. The three major groups of antifungal agents in clinical use, azoles, polyenes, and allylamine/thiocarbamates, exhibit their antifungal activity by inhibiting synthesis of major cell wall component ergosterol. (Ghannoum et al., 1999 and Kapoor et al., 2017)

To determine the effect of *C. verum* leaf oil on *Candida* cell wall, post exposure cellular changes were visualized by Transmission Electron Microscope as follows.

Briefly, standard cell suspensions with 1×10^6 cell concentration were prepared in RPMI 1640 as explained in 4.8.1. 1 ml of prepared suspensions were mixed with 9 ml of antifungal oil dilution in RPMI 1640 with the concentration of 10×MIC (10 mg/ml) separately and incubated at 37 ºC for 24h aerobically. After incubation, the resulting cell suspension was centrifuged and the cell pellet was resuspended with the Karnovsky's fixative for 18-24 hours at 4 ºC. After fixation, Wash the samples in 3 exchanges of 0.1M Sorensen phosphate buffer (pH 7.2-7.4) for 5 minutes. Then the samples were fixed with 1% osmium tetroxide. Samples were then air dried at room temperature for 2h followed by washing 3 times with 0.1M Sorensen phosphate buffer for 5 minutes each. Specimens were then dehydrated with increased concentrations of acetone (30%, 50%, 70%, 90% and 100%) for 5 minutes each. The prepared specimens were then embedded in Dr. SPURR resin followed by observation using TEM (JEM-1400, JEOL).

4.14. Effect of *C. verum* Leaf Oil on Host Tissues

4.14.1. Cytotoxicity of *C. verum* leaf oil

The experiments of anti-proliferative activity of true cinnamon leaf oil was carried out according to the protocol described by Monks et al. (1991) and Orellana et al. (2016).

Cellular growth was quantified spectrophotometry by using 3-(4,5-dimethyl2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide metabolic assay (MTT, Sigma-Aldrich) and analysis was based on the *in vitro* screening method for anticancer drugs conducted by National Cancer Institute (NCI, USA) using the panel of 60 human tumor cell lines (NCI_{60}) (Monks et al., 1991; Shoemaker, 2006).

4.14.1.1. Cell Line and Culture Medium

HaCaT (Human Keratinocyte Cell line) cell line obtained from Department of Oral Pathology, Faculty of Dentistry in Piracicaba, was used in this study.

Cells were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics, 100 µl/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). Cultured cells were maintained at 37ºC, under an atmosphere of 5% CO_2 and 95% air.

4.14.1.2. *In Vitro* Assay for Cytotoxicity

For testing, cells were washed with sterile phosphate buffer saline (PBS) and harvested by tripsinization using 2.5 g/l trypsin in EDTA (Cultilab, Brazil) and counted using Neubauer's improved counting chamber. Cell density was adjusted to $6.5x10^4$ cells/ml. Prepared standard cell suspension was plated (100µl/well) in sterile flat bottomed cell culture treated 96 well microtiter plates (Kasvi, Brazil) and incubated under 5% CO_2 and 95% air at 37ºC for 24 hours. After incubation, cell lines were treated with 100µl of different concentrations (8 mg/ml, 4 mg/ml, 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml) of *C. verum* leaf oil diluted in FBS and antibiotic supplemented RPMI 1640 medium for another 24h in same incubation conditions. Dilutions of 120 mg/ml (0.12%) chlorhexidine digluconate was used to compare the cytotoxicity with true cinnamon leaf oil. Control cells (negative) were incubated with culture medium only. All concentrations of leaf oil were in triplicates on the same cell batch.

4.14.1.3. Quantification of cellular proliferation

Cell viability/proliferation of HaCaT cell line after exposure to *C. verum* and chlorhexidine digluconate was quantified spectrophotometry by using 3-(4,5-dimethyl2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide (MTT) metabolic assay.

After the incubation period, the cell suspensions in each well were aspirated. All wells including control wells were filled with 100 µl of 5 mg/ml MTT solution per well. Plates were then incubated at 37ºC, under an atmosphere of 5% CO_2 and 95% air for 4h. After incubation, remaining MTT solution was replaced with 100 µl of Dimethyl Sulfoxide (DMSO) per well.

The spectrophotometric reading of the absorbance was performed at 492 nm using a microplate plate reader. With the mean absorbance values for each concentration, the percentage growth was calculated.

4.14.2. *In vivo* effect of *C. verum* essential oil on *Galleria mellonella* larvae (Minimum Lethal Concentration (MLC)

The *in vivo* toxicity test was performed on *Galleria mellonella* larvae as described by Scorzoni et al. (2013) with the collaboration of Pharmocology department at Piracicaba dental school, UNICAMP.

Study groups and control group (10 larvae per group/ n=10) of larvae (RJ Mous Livebait, The Netherlands) were selected by measuring their body weight (0.2 - 0.3 g larvae were included as study population) and selected larvae were placed in Petri dishes (10/petri dish). Larvae with color changes in their bodies including dark pigmantations or apparent melanization, were excluded.

Essential oil dilutions of *C. verum* leaf were prepared with sterile PBS as explained in 4.4. (1000.0, 500.0, 250.0, 128.0, 64.0, 32.0, 16.0, 2.0, 1.0, 0.5 mg/ml)

1 µl of essential oil dilutions were injected in the haemocele through the last left pro-leg of *Galleria mellonella* larvae using a 1 ml syringe. The pro-leg was decontaminated with 70% ethanol prior to administration of oil. Larvae containing petri dishes were then covered with lis and placed in an aerobic incubater at 37 °C.

Mortality of Larvae was monitored by visual inspection of the body colour (brown-dark brown) and by lack of body movement after touching them with forceps. The experiment was repeated twice.

4.15. Statistical analysis

The statistical analysis was carried out by using the software, Statistical Package for Social Sciences (SPSS) version 16. Multiple means of more than three data sets were compared using one way ANOVA and two way ANOVA. The level of significance was taken at 5% ($p < 0.05$).

5. RESULTS

5.1 Chemical composition of true cinnamon leaf oil

Chromatographic analysis of *C. verum* leaf essential oil was done using HP-6890 gas chromatograph coupled with HP-5975 selective mass detector. Detected chemical constituents and their relative abundance were represented in **Table 5.1**. Chromatogram of the analysis was attached in **Annex**. The most abundant compounds were Eugenol (77.22%), Benzyl benzoate (4.53%), Trans caryophyllene (3.39%), Acetyle eugenol (2.75%) and Linalool (2.11%) (**Table 5.1**).

Table 5.1: Compounds and relative abondunce of *C. verum* leaf essential oil detected by GC/MS.

Retention time (min)	Compound	% Abundance
21.41	Eugenol	77.22
36.65	Benzyl Benzoate	4.53
23.67	Trans caryophyllene	3.39
28.03	Acetyl eugenol	2.75
10.56	Linalool	2.11
17.45	Trans-Cinnamaldehyde	1.69
24.71	Acetic acid cinnamyl ester	1.49

5.2 Effect on Planktonic *Candida*

5.2.1 Effect of *C. verum* leaf essential oil in vapour phase

Microatmosphere plate method was used to screen the anti-*Candida* activity of *C. verum* leaf oil qualitatively. The protocol used here was the recommended antimicrobial assay for highly volatile plant derivatives including volatile essential oils. The results for antifungal properties of working solutions of true cinnamon leaf oil against selected seventeen *Candida* test strains are presented in **Table 5.2**. According to published studies, any zone of inhibition on agar surface was considered as sensitive for essential oil (Serban et al., 2011). Based on that, any visual

growth inhibition of *Candida* indicates the susceptibility/sensitivity of test strain to *C. verum* leaf oil **(Figure 5.1, Table 5.2)**.

According to obtained results from this qualitative assay, *C. verum* leaf oil exhibited an antifungal effect against all test strains in their planktonic state **(Table 5.2)**.

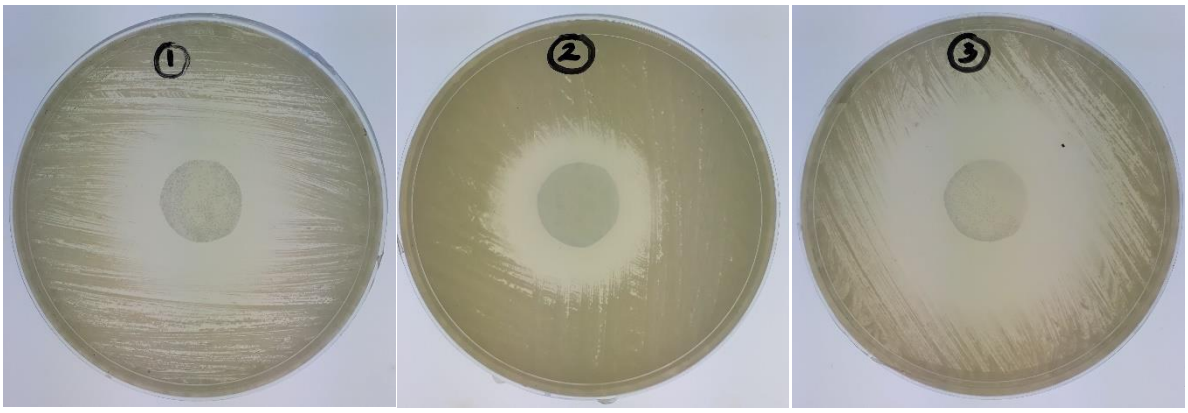

Figure 5.1: Inhibition zones of (01) *C. albicans* (ATCC MYA-2876), (02) *C. tropicalis* (ATCC 750), (03) *C. dubliniensis* (ATCC MYA-646) for true cinnamon (*C. verum*) leaf oil.

Table 5.2: Results of zone of inhibitions exhibited by planktonic test strains in the presence of working concentration (1.046g/ml) *C. verum* leaf oil. All experiments were done in triplicates.

Organism		**Presence/Absence of growth inhibition**	**Sensitive/Resistant**
01	*C. albicans* (ATCC MYA-2876)	+	Sensitive
02	*C. tropicalis* (ATCC 750)	+	Sensitive
03	*C. dubliniensis* (ATCC MYA-646)	+	Sensitive

5.2.2 Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The essential oil of *C. verum* leaves was tested on three test strains of *Candida*, in order to determine their inhibitory effect using the CLSI M27-A3 broth micro dilution method. Inhibitory activity of *C. verum* oil was tested with the concentration series starting from 32mg/ml. the minimum concentration tested was 0.0156mg/ml.

MIC results were obtained by visual observation and obtained results as follows including results of control experiments with Chlorhexidine digluconate. (**Table 5.3**)

Table 5.3: Results of MIC and MFC of *Candida spp*. Experiment was done in triplicates with three individual experiments. Chlorhexidine digluconate was used as positive control.

Microorganism		***Cinnamomum verum* oil**			**Control (Chlorhexidine digluconate)**		
		MIC (mg/ml)	**MFC** (mg/ml)	**MFC/ MIC**	**MIC** (mg/ml)	**MFC** (mg/ml)	**MFC/ MIC**
1	*C. albicans* (ATCC MYA-2876)	1.0	2.0	2.0	1.0	2.0	2.0
2	*C. tropicalis* (ATCC 750)	1.0	2.0	2.0	0.5	0.5	1.0
3	*C. dubliniensis* (ATCC MYA-646)	1.0	2.0	2.0	1.0	2.0	2.0

True cinnamon leaf oil exhibited both fungistatic and fungicidal activity on the planktonic cells of *Candida spp*. All test strains exhibited MIC of 1mg/ml, MFC of 2 mg/ml with MFC/MIC ratio of 2. **(Table 5.3)**.

C. albicans showed same MIC and MFC values for both *C. verum* oil and Chlorhexidine digluconate (1.0 and 2.0 mg/ml) and MFC value was two times higher than that value of Chlorhexidine digluconate.

C. tropicalis was more sensitive for chlorhexidine gluconate compared to *C. verum* leaf oil hence Both MIC and MFC values were lower with Chlorhexidine digluconate compared to true cinnamon oil.

C. dubliniensis exhibited same MIC and MFC pattern for both *C. verum* leaf oil and chlorhexidine digluconate.

Briefly, *C. verum* exhibited the similar efficacy as Chlorhexidine digluconate on planktonic *C. albicans* (ATCC MYA-2876) and *C. dubliniensis* (ATCC MYA-646). *C tropicalis* (ATCC 750) was more susceptible for chlorhexidine digluconate compaired to *C. verum* leaf oil (MFC/MIC was two times higher for *C. verum* leaf oil).

5.2.3 Killing Time Assay

This experiment determined the minimum time requires for true cinnamon leaf oil (or positive control Chlorhexidine digluconate) to eradicate the viable planktonic *Candida* cells from their *in vitro* culture.

Killing curves for *C. albicans* (ATCC MYA-2876) in the presence of *C. verum* leaf oil (0.5, 1.0 and 2.0 mg/ml) and 2.0 mg/ml Chlorhexidine digluconate were presented in **Figure 5.2.**

According to obtained results, 0.5 mg/ml and 1.0 mg/ml *C. verum* leaf oil do not kill *Candida* cells completely within 24h test period whereas 2.0 mg/ml kill *Candida* cells completely within 6h.

2.0 mg/ml Chlorhexidine digluconate has a rapid killing action on *Candida* cells compared to true cinnamon leaf oil hence killing time was 1h.

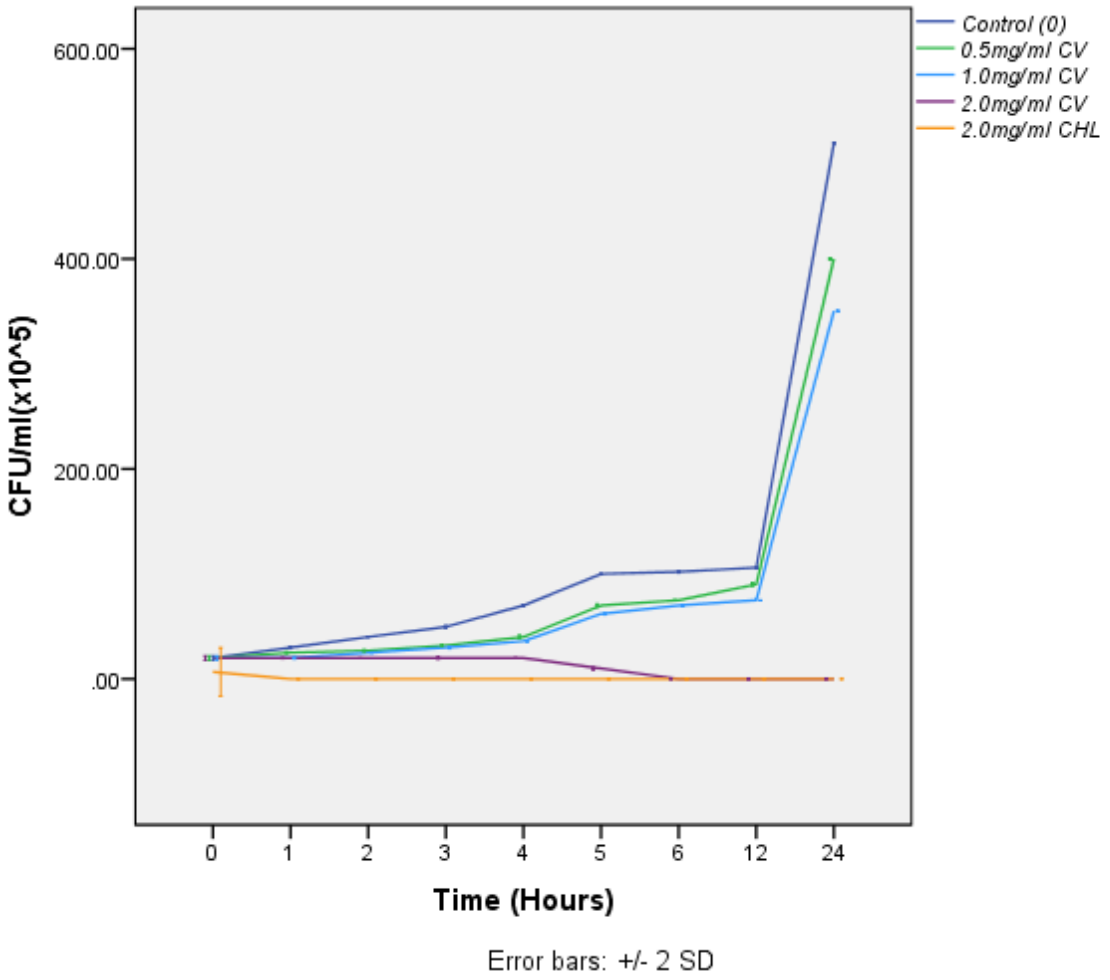

Figure 5.2: Killing curves of *C. albicans* (ATCC MYA-2876) for 0.5mg/ml, 1.0 mg/ml and 2mg/ml *C. verum* leaf oil and 2.0 mg/ml Chlorhexidine digluconate. (CV: *C. verum* leaf oil, CHL: Chlorhexidine digluconate). All error bars represent the ± 2 standard deviations (SD).

5.3 Initial Adhesion

Attachment to the host surfaces (followed by biofilm formation) is a virulent factor of *Candida* as well as the first step of microbial biofilm formation. This step consists of two major phases, namely initial attachment and irreversible attachment.

The results of the microtiter plate XTT method in order to determine the effect of *C. verum* leaf oil on *Candida* adhesion on to a polystyrene surfaces was presented in **Figure 5.3.**

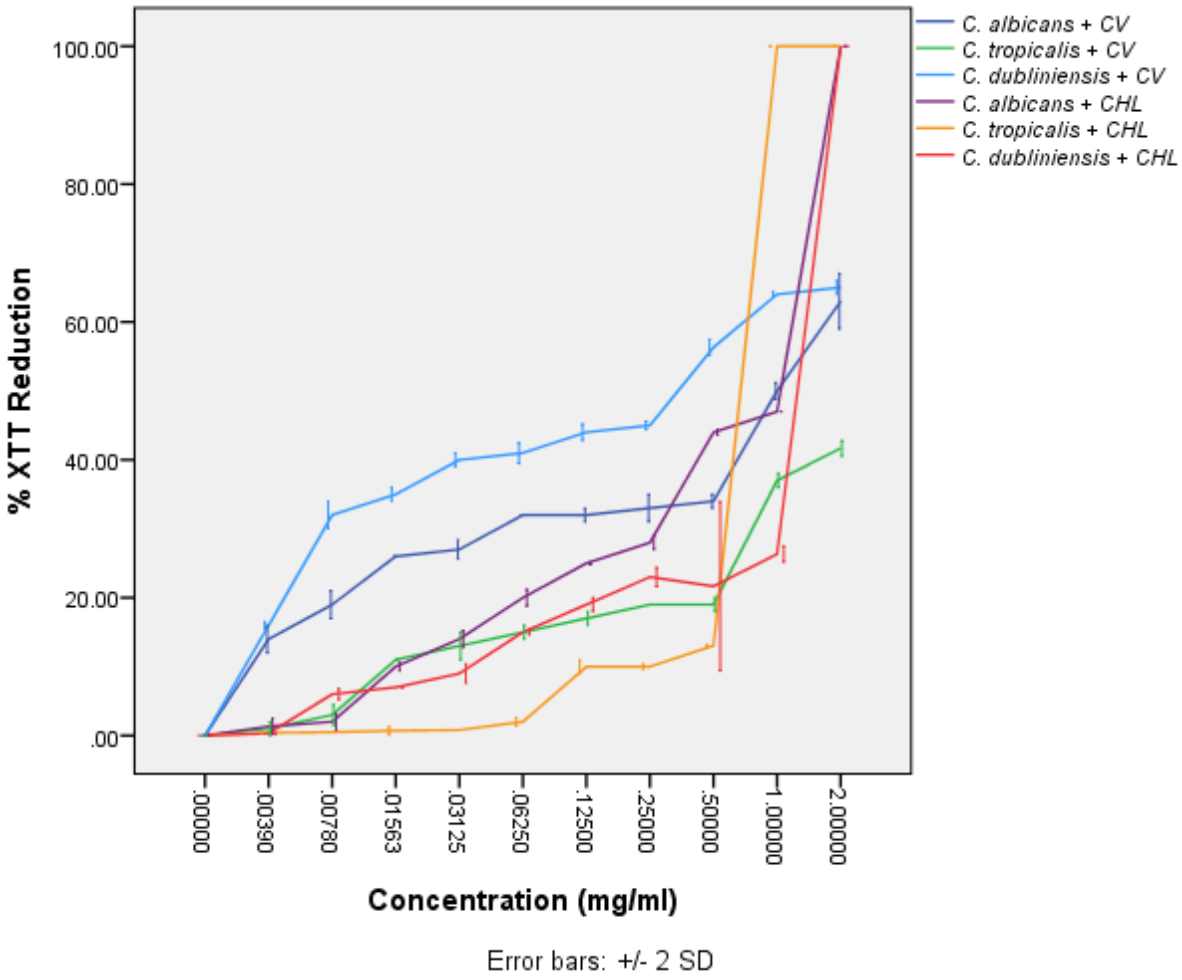

Figure 5.3: Percentage reduction in XTT metabolic activity of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) in the presence of different concentrations of CV: *C. verum* leaf oil and CHL: Chlorhexidine digluconate. All error bars represent the ± 2 standard deviations (SD).

According to obtained data, *C. verum* leaf oil effectively reduce the adhesion of all test strains on to polystyrene surface compared to negative control. *C. albicans* achieved 50% reduction in adhesion with 1.0 mg/ml concentration, whereas, *C. tropicalis* and *C. dubliniensis* showed 50% reduction in adhesion with >2.0 mg/ml and 0.34 mg/ml concentrations of oil respectively. Chlorhexidine digluconate exhibited 50% reduction in adhesion of *C. albicans*, *C.*

tropicalis and *C. dubliniensis* with the concentrations of 1.06 mg/ml, 0.72 mg/ml and 1.32 mg/ml respectively.

5.4 Effect of *C. verum* Leaf Oil on Germ Tube Formation

Germ tube formation is one of the key virulent factors of *C. albicans* and *Candida* non-albicans, *C. dubliniensis* which enables the absorption of nutrients from host tissues by invading them. This experiment determines the effect of Cinnamon leaf oil and chlorhexidine digluconate on germ tube formation of *C. albicans* (ATCC MYA-2876) and *C. dubliniensis* (ATCC MYA-646) *in vitro*.

Figure 5.4 shows the percentage of germ tube forming cells in the presence of 0.25, 0.5 and 1.0 mg/ml of true cinnamon leaf oil and chlorhexidine digluconate throughout 6h experiment period.

According to obtained data, both *C. verum* leaf oil and chlorhexidine digluconate significantly reduced the germ tube formation of *C. albicans* and *C. dubliniensis* ($p<0.05$) with all tested concentrations. Both true cinnamon leaf oil and chlorhexidine digluconate at 1.0 mg/ml (MIC) concentration completely inhibited the germ tube formation of both *Candida* species throughout the experiment period (6h).

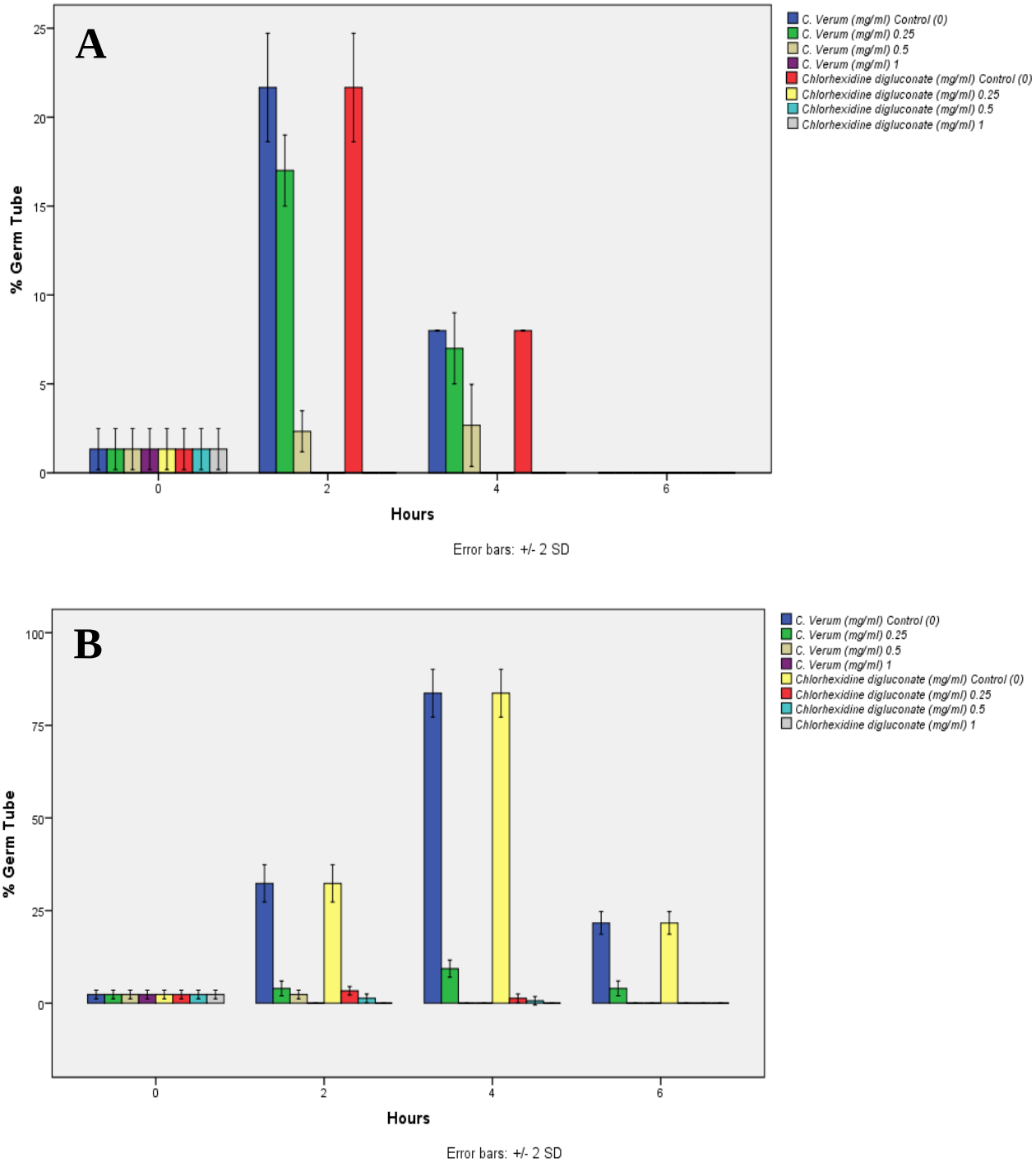

Figure 5.4: Percentage germ tube formation of **(A)** *C. albicans* (ATCC MYA-2876) and **(B)** *C. dubliniensis* (ATCC MYA-646) with 0.25, 0.5 and 1.0 mg/ml *C. verum* leaf oil and Chlorhexidine digluconate within 6h test period with 2h intervals. 0 mg/ml concentration indicates the negative control. All error bars represent the ± 2 standard deviations (SD).

5.5 Effect on *Candida* Biofilm Formation

Biofilm is a surface attached cells embedded in extracellular polymeric substances derived from attached cells and environmental origin. Biofilm formation is a virulent factor which causes higher resistant to antimicrobial agents of microbial pathogens. This experiments determines the effect of different concentrations of *C. verum* leaf oil on biofilm formation of surface attached *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646).

5.5.1 Minimum Biofilm Inhibitory Concentration of Biofilm Formation

In this experiment, minimum concentration of *C. verum* leaf oil required to inhibit the biofilm formation by 50% and 75% (compared to negative control) was determined. Biomass/viability of formed biofilms in the presence of essential oil was determined using XTT viability assay. **Figure 5.5** showed the percentage reduction of XTT metabolic activity of forming biofilms compared to negative control.

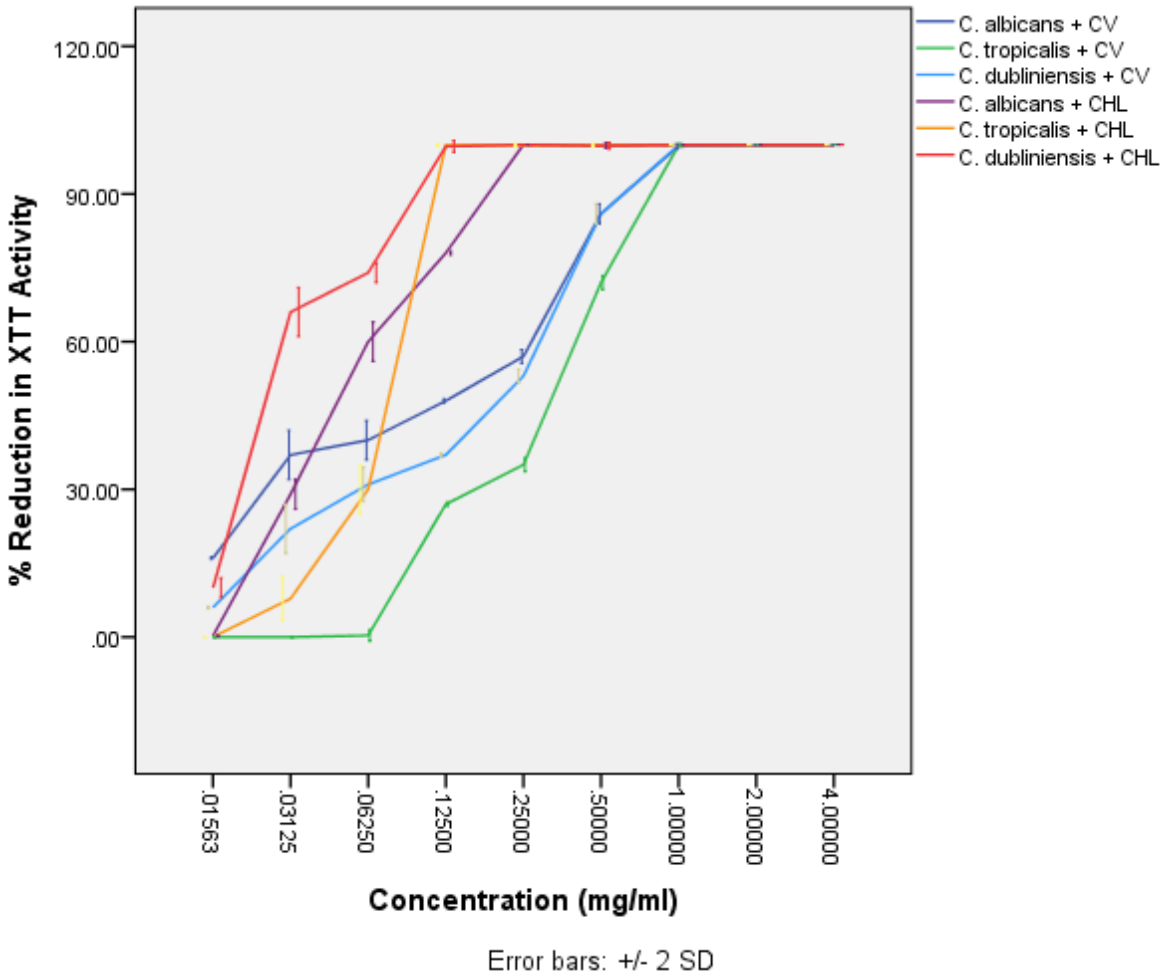

Figure 5.5: Percentage reduction of XTT metabolic activity of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) forming biofilms with the presence of different concentrations of *C. verum* leaf oil and Chlorhexidine digluconate. All error bars represent the ± 2 standard deviations (SD). CV: *C. verum* leaf oil, CHL: Chlorhexidine digluconate. All error bars represent the ± 2 standard deviations (SD).

According to obtained data, concentrations required to reduce the biofilm formation by 50% and 75% as follows **(Table 5.4).**

Table 5.4: Minimum biofilm inhibitory concentrations ($MBIC_{50}$ and $MBIC_{75}$) for forming biofilms of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646). CHL: Chlorhexidine digluconate.

	C. albicans		***C. tropicalis***		***C. dubliniensis***	
	C. verum	**CHL**	***C. verum***	**CHL**	***C. verum***	**CHL**
50% Reduction (mg/ml)	0.15	0.05	0.35	0.08	0.2	0.025
75% Reduction (mg/ml)	0.4	0.1	0.6	0.2	0.4	0.065

5.5.2 Minimum toxic concentration for forming biofilms (MTC)

Minimum concentration of *C. verum* leaf oil required to prevent the biofilm formation completely by killing *Candida* cells was determined using CFU assay. **Figure 5.6** represents the viability of forming biofilms with the presence of different concentrations of *C. verum* leaf oil and Chlorhexidine digluconate determined by CFU assay for forming biofilms. **Table 5.5** represents the minimum concentration of treatments required to prevent the biofilm formation of test strains completely.

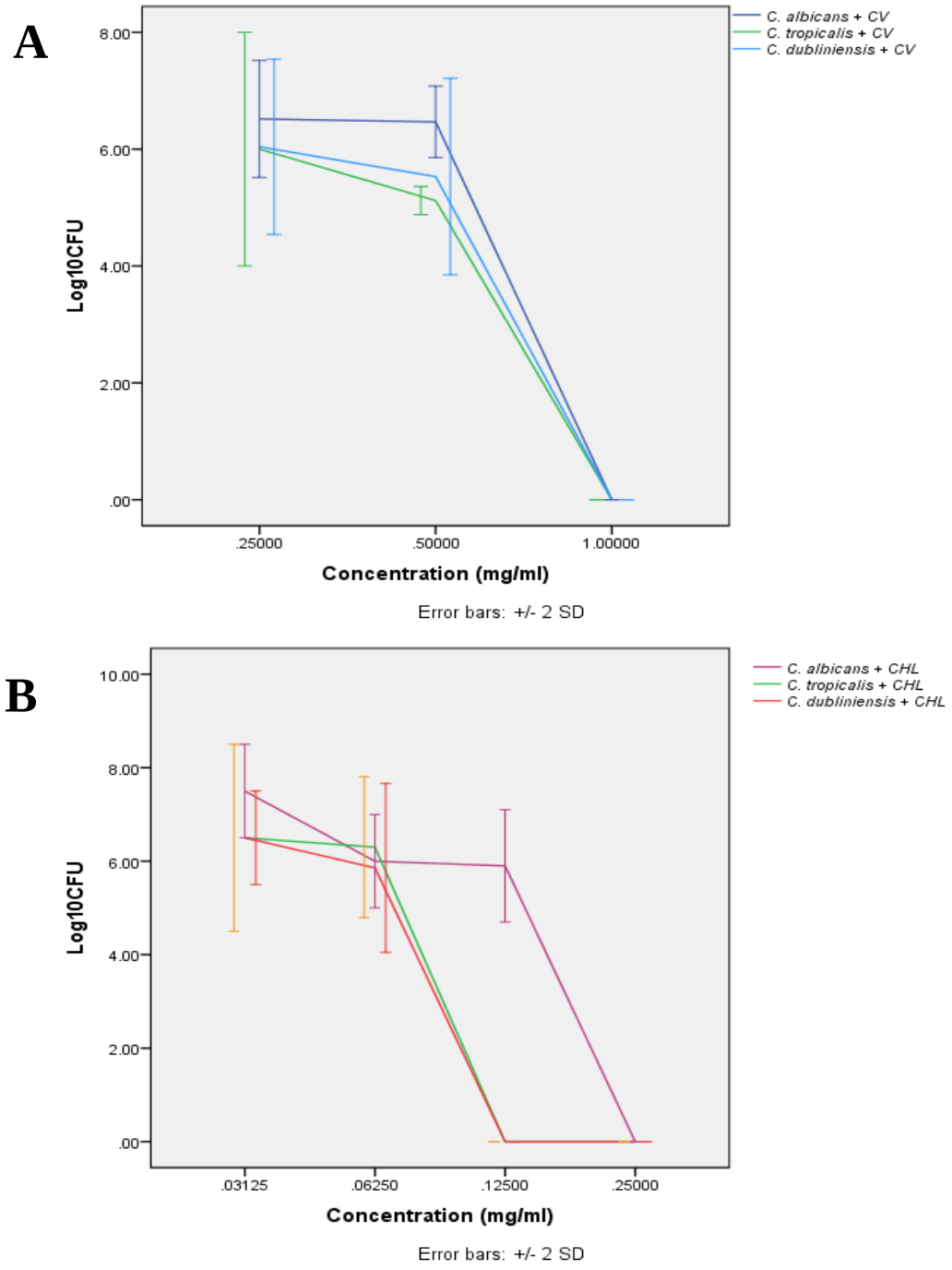

Figure 5.6: Log CFU values of forming CA: *C. albicans* (ATCC MYA-2876), CT: *C. tropicalis* (ATCC 750) and CD: *C. dubliniensis* (ATCC MYA-646) biofilms in the presence of different concentrations of (A) CV: *C. verum* leaf oil and (B) CHL: Chlorhexidine digluconate. All error bars represent the ± 2 standard deviations (SD).

Table 5.5: Concentrations of *C. verum* leaf oil and Chlorhexidine digluconate that kill the forming biofilms of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) completely.

Test strain	**Concentration requires to kill the forming biofilms (mg/ml)**	
	C. verum leaf oil	Chlorhexidine digluconate
C. albicans (ATCC MYA-2876)	1.0	0.25
C. tropicalis (ATCC 750)	1.0	0.125
C. dubliniensis (ATCC MYA-646)	1.0	0.125

1.0mg/ml *C. verum* kill the forming biofilms of all test strains, *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646). Chlorhexidine digluconate kills forming biofilms of *Candida* species with low concentrations compared to *C. verum* leaf oil. For *C. albicans*, the killing concentration with Chlorhexidine digluconate was 0.25 mg/ml (Four times low concentration compared to that value of *C. verum* leaf oil) and for *C. tropicalis* and *C. dubliniensis*, killing concentration was 0.125 mg/ml (Eight times less concentration compared to that value of *C. verum*).

5.5.3 Scanning Electron Microscopy (SEM) of forming biofilms

Ultrastructure of forming biofilms of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) in the presence of 0.5, 1.0 and 2.0 mg/ml *C. verum* leaf oil, Chlorhexidine digluconate and Fluconazole was qualitatively evaluated by SEM **(Figure 5.7, 5.8 and 5.9)**.

C. verum leaf oil caused the *Candida* cell shrinkage by damaging the cell wall and cause leakage of intracellular materials. These effects are concentration dependent. Maximum cell

damage was observed with 2 mg/ml *C. verum* leaf oil. And *C. verum* leaf oil reduced the biofilm development and biofilm cell density.

When considering Chlorhexidine digluconate, it exhibited the similar effect as *C. verum* leaf oil (damaging cell walls of *Candida* cells and cause cytoplasmic leakages). Further, according to SEM observations, Chlorhexidine digluconate decreased pseudo hyphae formation of *C. tropicalis* compared to negative control.

Similar observations were obtained for maturing biofilms of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) with Fluconazole. Biofilms formed in the presence of 0.008 mg/ml Fluconazole exhibited rough cellular surfaces indicating cell wall deformities. And there was no noticeable density reduction of sessile cells in biofilms formed in the presence of Fluconazole, compared to negative control.

Extracellular polymeric matrix that is characteristic of biofilms was not observed in any of the biofilms analysed using SEM since pre-treatments and electron beam of the equipment can cause the matrix destruction.

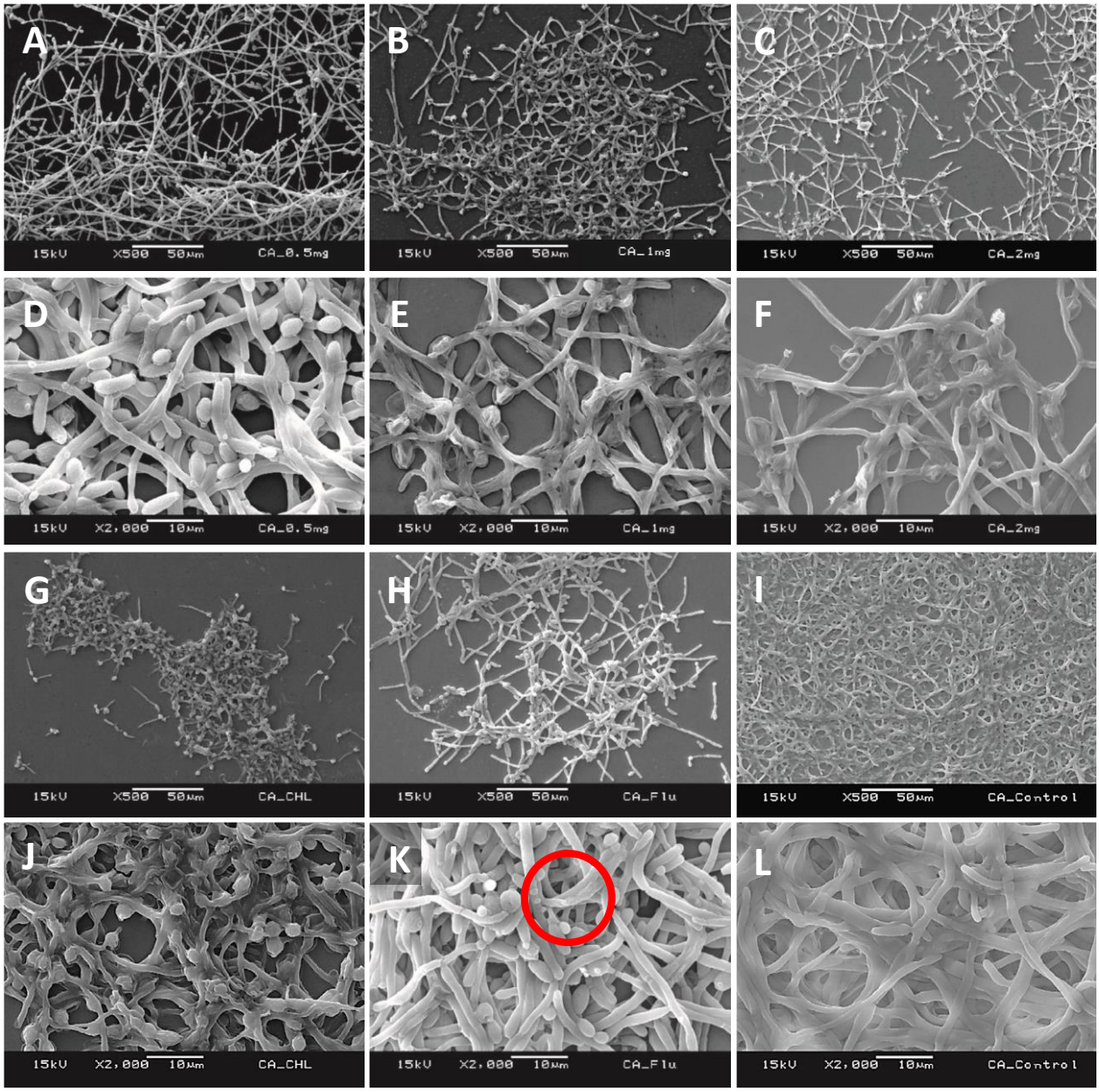

Figure 5.7: SEM images of *C. albicans* (ATCC MYA-2876) forming biofilms in the presence of 0.5 mg/ml *C. verum* leaf oil (A and D), 1.0 mg/ml *C. verum* leaf oil (B and E), 2mg/ml *C. verum* leaf oil (C and F), 0.25 mg/ml Chlorhexidine digluconate (G and J) and 0.008 mg/ml Fluconazole (H and K). I and L: Negative control. ○ – Cell wall deformities with treatments.

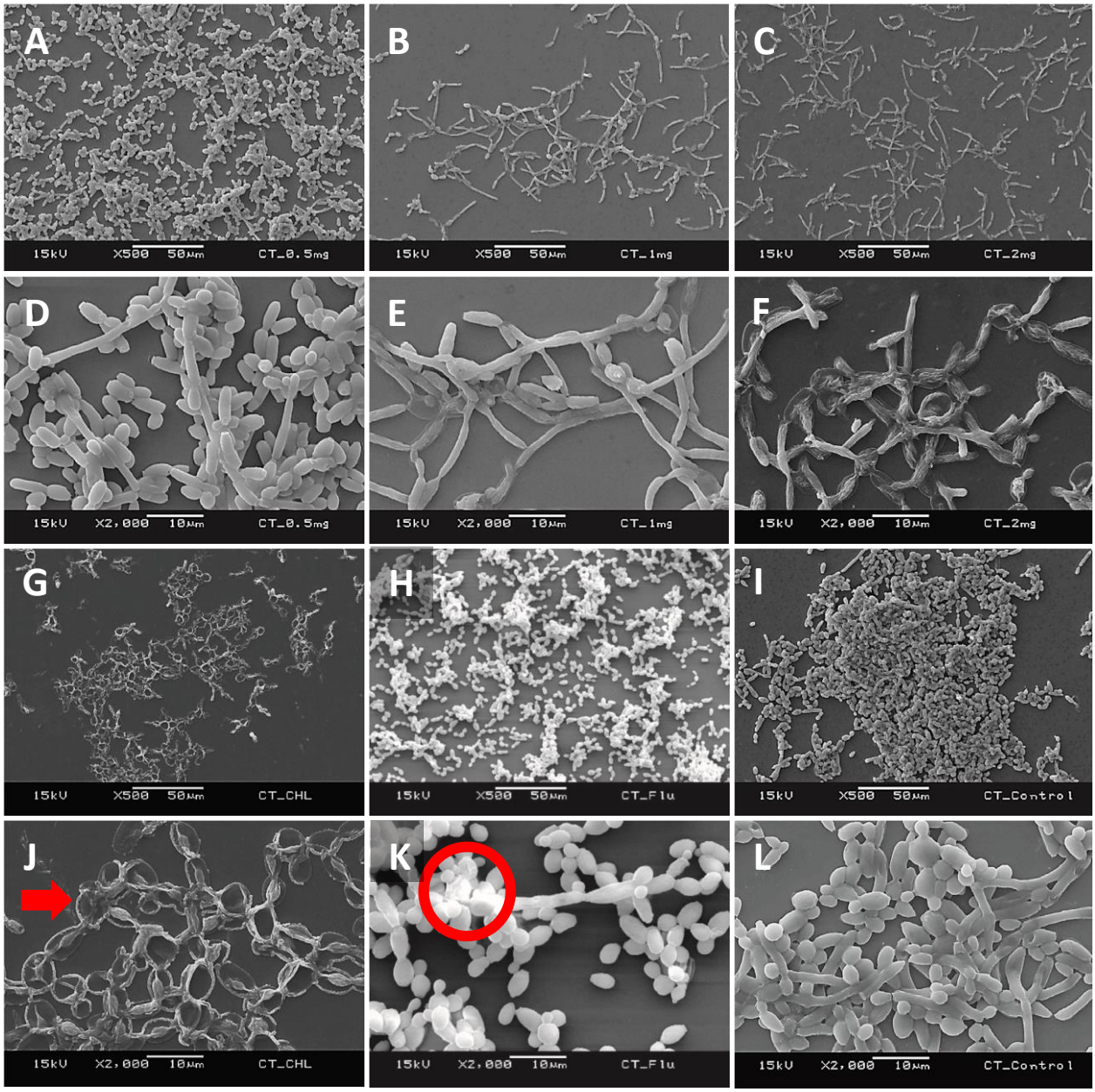

Figure 5.8: SEM images of *C. tropicalis* (ATCC 750) forming biofilms in the presence of 0.5 mg/ml *C. verum* leaf oil (A and D), 1.0 mg/ml *C. verum* leaf oil (B and E), 2.0 mg/ml *C. verum* leaf oil (C and F), 0.25 mg/ml Chlorhexidine digluconate (G and J) and 0.008 mg/ml Fluconazole (H and K) I and L: Negative control. ◯ – Cell wall deformities with treatments. ➡ – Leakages of intracellular components.

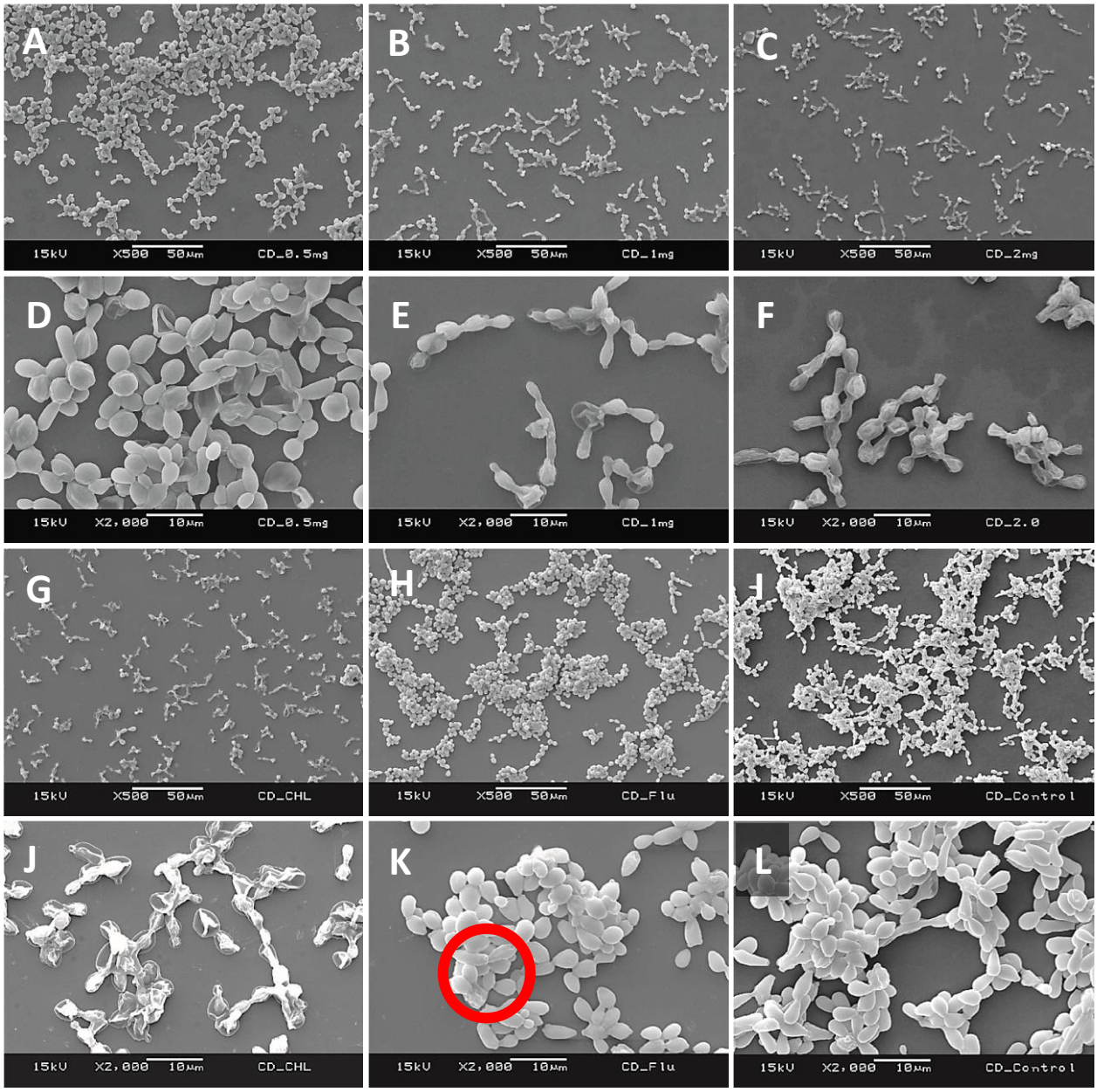

Figure 5.9: SEM images of *C. dubliniensis* (ATCC MYA-646) forming biofilms in the presence of 0.5 mg/ml *C. verum* leaf oil (A and D), 1.0 mg/ml *C. verum* leaf oil (B and E), 2mg/ml *C. verum* leaf oil (C and F), 0.25 mg/ml Chlorhexidine digluconate (G and J) and 0.008 mg/ml Fluconazole (H and K) I and L: Negative control. ○ – Cell wall deformities with treatments.

5.5.4 Biofilm Progression Analysis Using Time Lapses Microscope

Progression of *Candida in vitro* biofilms with initial microcolony formation, cell proliferation and biofilm establishment in the presence of *C. verum* leaf oil was qualitatively visualized using Time Lapse Microscopy imaging technique.

Figure 5.10 A, B and C indicate the Time lapses microscopic images of forming biofilms of *C. albicans* (ATCC MYA-2876); *C. dubliniensis* (ATCC MYA-646) and *C. tropicalis* (ATCC 750) without essential oil treatment, 0.5 mg/ml, 1.0 mg/ml and 2.0 mg/ml *C. verum* leaf oil treatment for 24h time period.

The progression of the untreated (negative) control biofilm demonstrates excessive biofilm developement throughout the observation period. 0.5 mg/ml concentration of *C. verum* leaf caused retardation of biofilm development of *Candida* spp. 1.0 mg/ml and 2.0 mg/ml *C. verum* leaf oil completely inhibited *C. tropicalis* and *C. dubliniensis* cell proliferation and biofilm development from 0h.

With 1.0 mg/ml *C. verum* leaf oil, *C. albicans* exhibited slight cell proliferation upto 8h, while 2.0 mg/ml completely inhibited biofilm development.

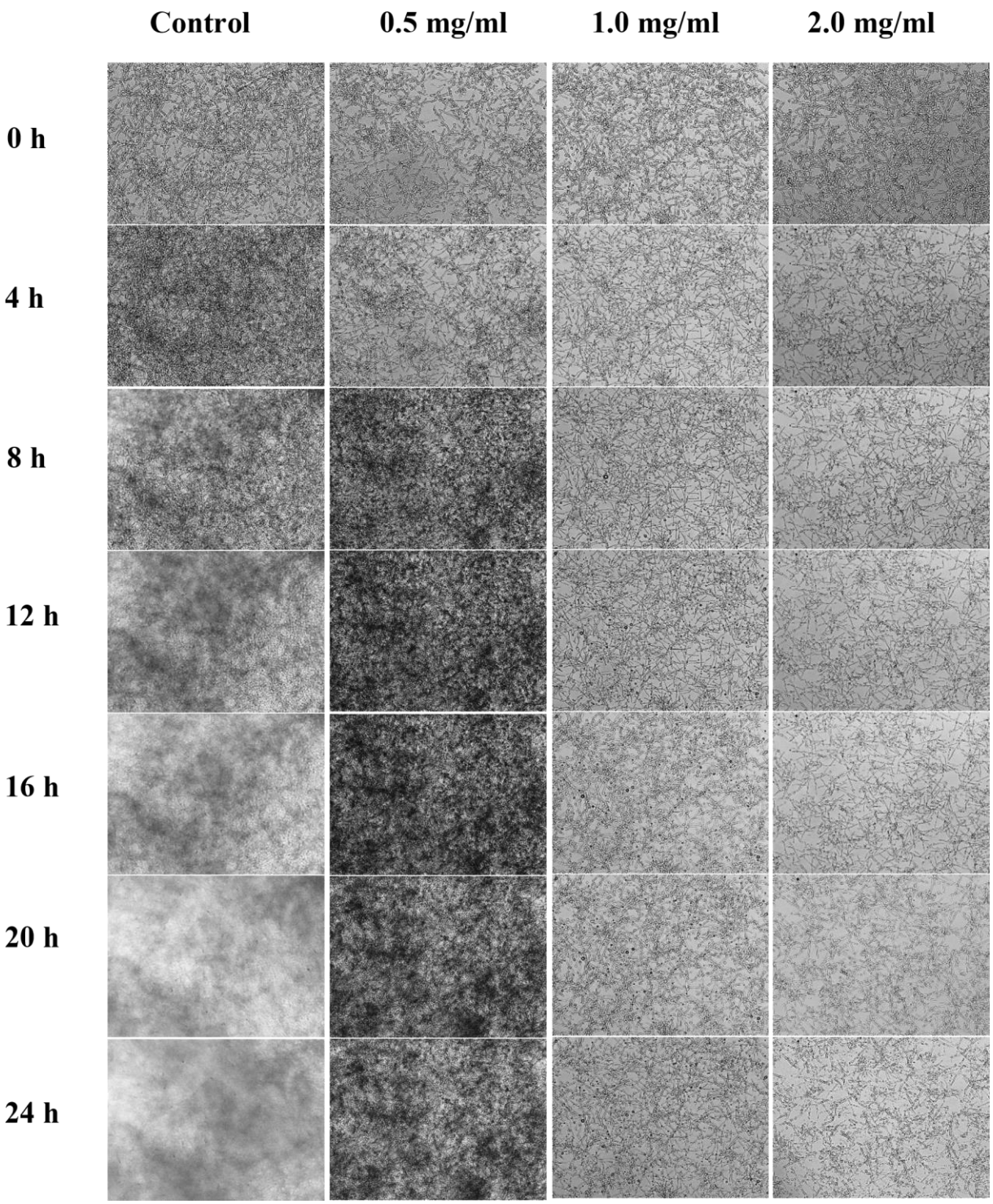

Figure 5.10 A: Time Lapses images of developing biofilms of *C. albicans* (ATCC MYA-2876). Each column represents the concentration of *C. verum* leaf oil treatment.

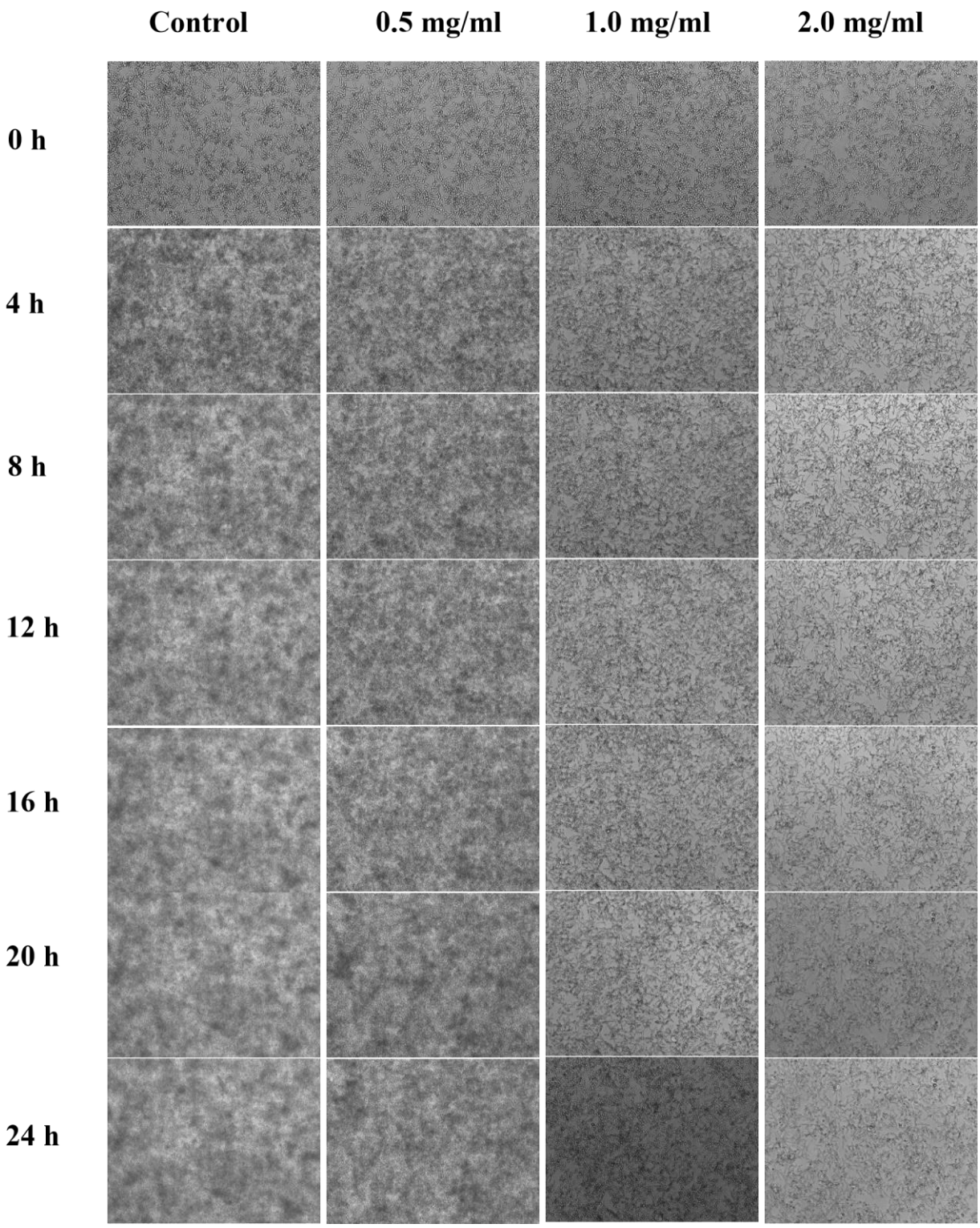

Figure 5.10 B: Time Lapses images of developing biofilms of *C. tropicalis* (ATCC 750). Each column represents the concentration of *C. verum* leaf oil treatment.

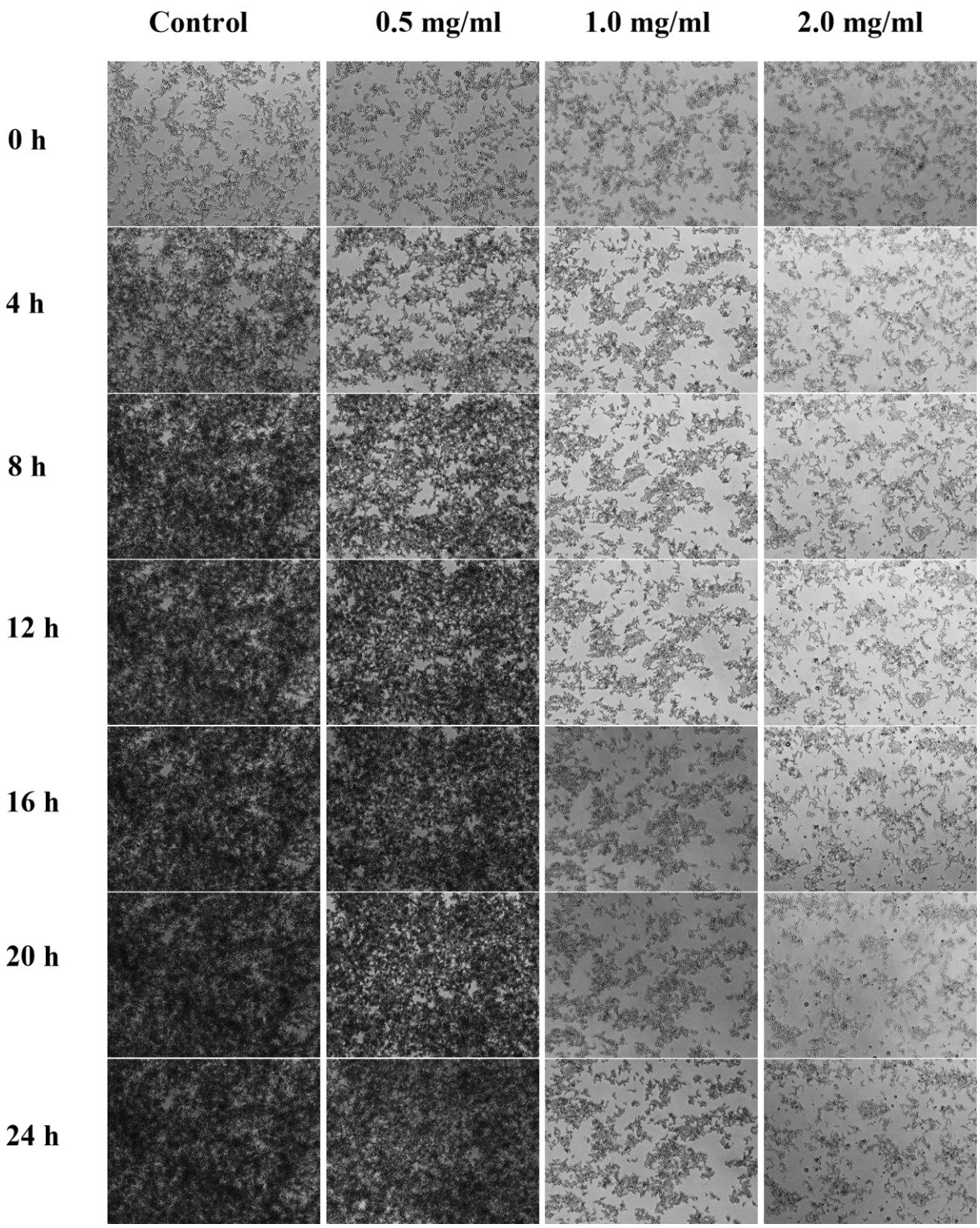

Figure 5.10 C: Time Lapses images of developing biofilms of *C. dubliniensis* (ATCC MYA-646). Each column represents the concentration of *C. verum* leaf oil treatment.

5.6 Effect on Established *Candida* Biofilms

5.6.1 Minimum Biofilm Inhibitory Concentration ($MBIC_{50}$ and $MBIC_{75}$)

Minimum concentrations of the essential oil required to reduce the biofilm cell viability by 50% and 75% of viability of negative control biofilms (biofilms without treatments) were defined as $MBIC_{50}$ and $MBIC_{75}$. $MBIC_{50}$ and $MBIC_{75}$were determined by treating 24h mature biofilms with different concentrations of *C. verum* leaf oil followed by XTT viability assay.

Figure 5.11 shows the percentage reduction of biofilm cell viability of 24h mature *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) biofilms determined by XTT viability assay after 24h treatment with *C. verum* leaf oil compared to negative control (biofilms without treatment).

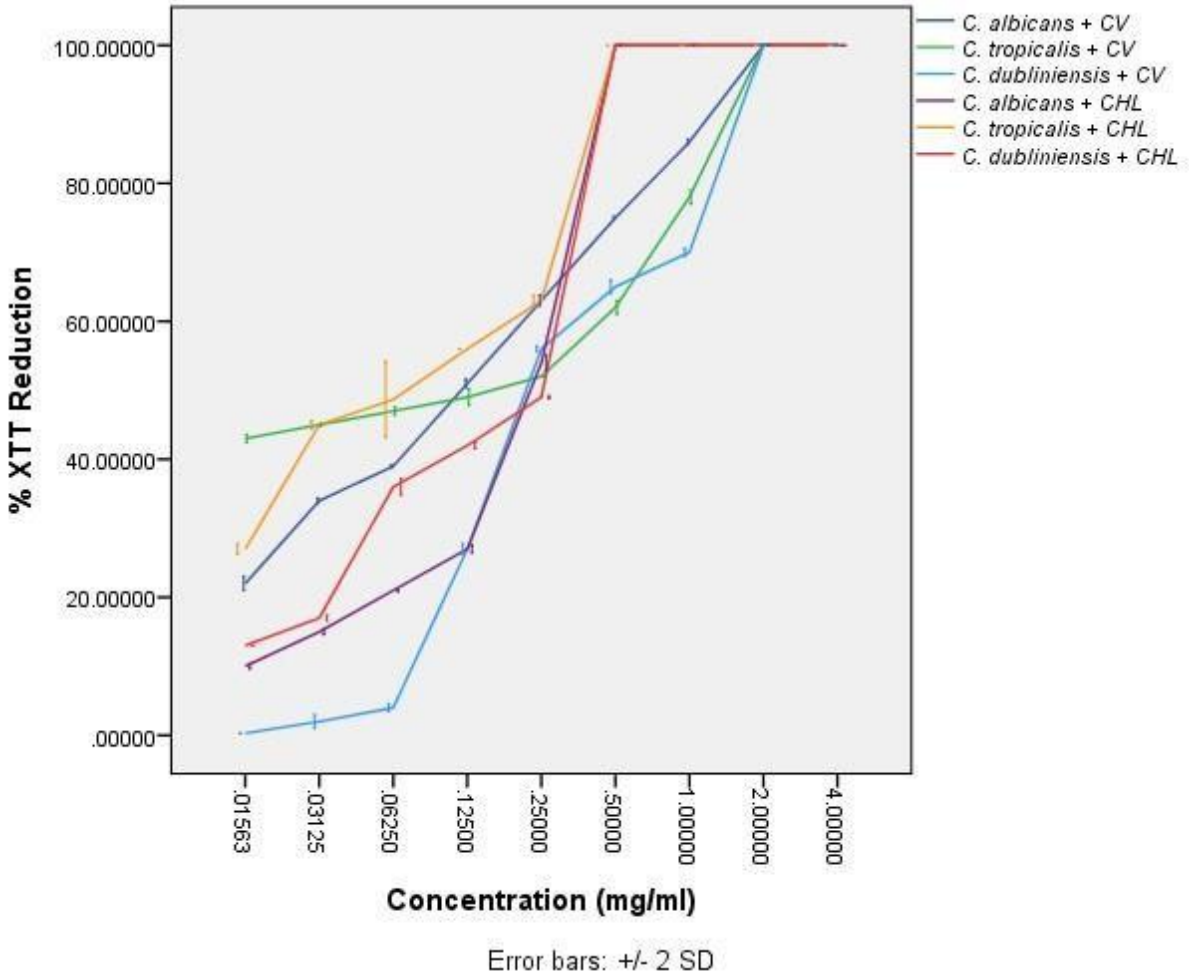

Figure 5.11: Percentage reduction of XTT metabolic activity of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) 24h established biofilms after treatment with different concentrations of *C. verum* leaf oil and Chlorhexidine digluconate. All error bars represent the ± 2 standard deviations (SD). CV: *C. verum* leaf oil, CHL: Chlorhexidine digluconate.

According to obtained data from XTT assay, concentrations required to reduce the biofilm viability by 50% and 75% as follows **(Table 5.6)**.

Table 5.6: Minimum biofilm inhibitory concentrations ($MBIC_{50}$ and $MBIC_{75}$) for established biofilms of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646). CHL: Chlorhexidine digluconate.

	C. albicans		***C. tropicalis***		***C. dubliniensis***	
	C. verum	CHL	*C. verum*	CHL	*C. verum*	CHL
$MBIC_{50}$ (mg/ml)	0.1	0.2	0.2	0.0625	0.2	0.3
$MBIC_{75}$ (mg/ml)	0.5	0.4	0.9	0.3	1.2	0.4

5.6.2 Minimum Biofilm Eradication Concentration (MBEC)

Minimum concentration of *C. verum* leaf oil required to kill the 24h mature biofilm completely (MBEC) was determined using CFU assay. **Figure 5.12** represents the viability of 24h mature biofilms after treating different concentrations of *C. verum* leaf oil and Chlorhexidine digluconate for 24h determined by CFU assay. **Table 5.7** shows MBEC values for mature *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) biofilms.

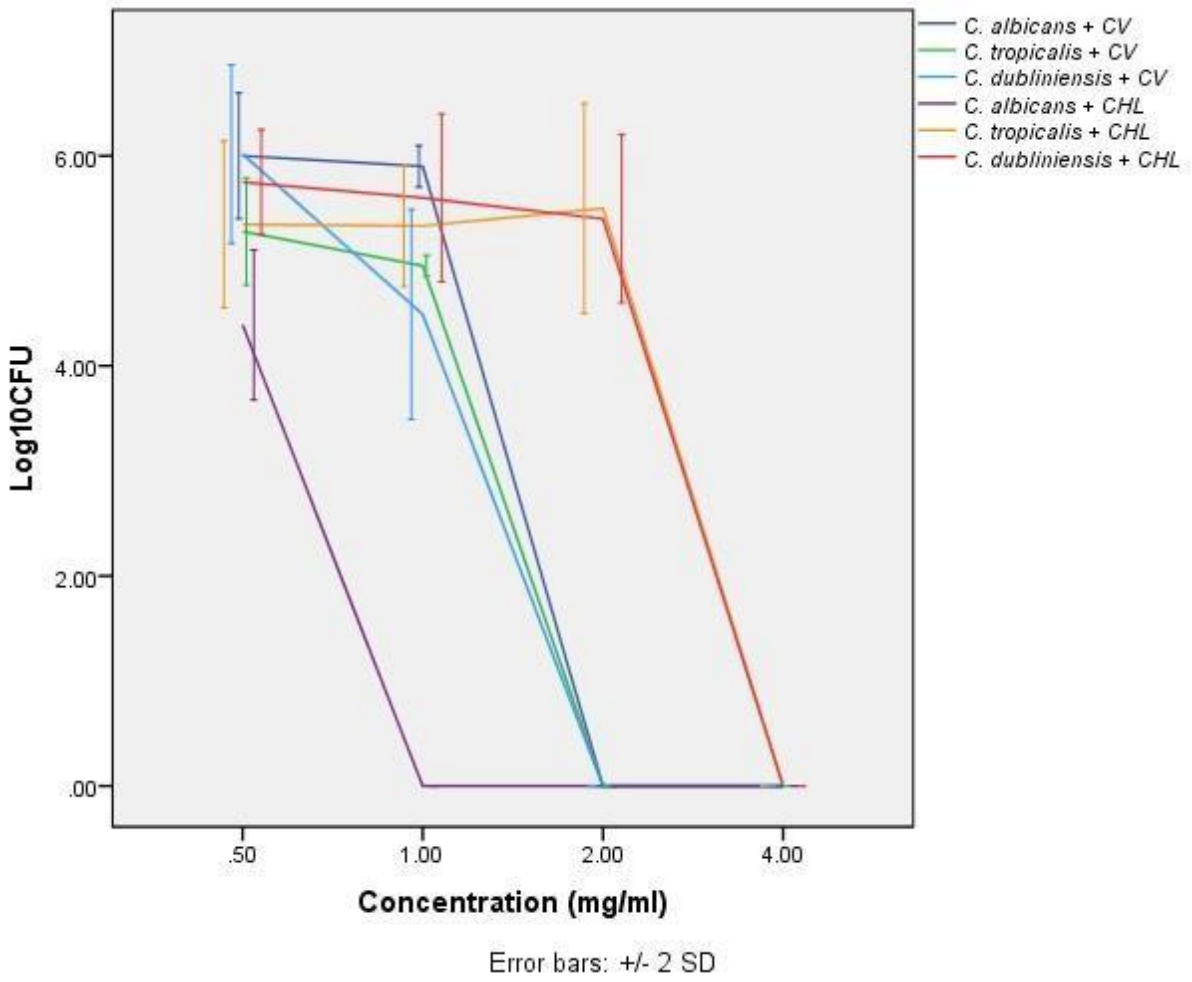

Figure 5.12: Log CFU values of forming *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) biofilms in the presence of different concentrations of CV: *C. verum* leaf oil and CHL: Chlorhexidine digluconate. All error bars represent the ± 2 standard deviations (SD).

Table 5.7: Concentrations of *C. verum* leaf oil and Chlorhexidine digluconate that kill the established biofilms of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA646) completely (MBEC).

Test strain	Concentration requires to kill the forming biofilms (mg/ml)	
	C. verum leaf oil	Chlorhexidine digluconate
C. albicans (ATCC MYA-2876)	2.0	1.0
C. tropicalis (ATCC 750)	2.0	4.0
C. dubliniensis (ATCC MYA-646)	2.0	4.0

5.6.3 Scanning Electron Microscopy (SEM) of established biofilms

Ultrastructure of established biofilms of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) after treating with 1.0, 2.0 and 4.0 mg/ml of *C. verum* leaf oil, 1 mg/ml (for *C. albicans* biofilms), 4.0 mg/ml (for *C. tropicalis* and *C. dubliniensis* biofilms) Chlorhexidine digluconate and 0.008 mg/ml Fluconazole was qualitatively evaluated by SEM (**Figure 5.13, 5.14 and 5.15**).

C. verum leaf oil showed the *Candida* cell shrinkage by damaging walls of mature sessile cells, pseudohyphae and hyphae and cause leakage of intracellular materials. These effects were concentration dependent. Maximum cell damage was observed with 4 mg/ml of *C. verum* leaf oil. Both 2 mg/ml and 4 mg/ml of *C. verum* leaf oil showed complete destruction of 24h mature biofilms of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) with 24h treatment.

When considering Chlorhexidine digluconate, it exhibited the similar effect as *C. verum* leaf oil (damaging cell walls of *Candida* cells and cause cytoplasmic leakages) of established *Candida* biofilms.

Similar observations were obtained for 24h established biofilms of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) with 0.008 mg/ml Fluconazole. Biofilms treated with 0.008 mg/ml Fluconazole exhibited rough cellular surfaces indicating that cell wall deformities and leakages of intracellular components were observed in *C. albicans* and *C. dubliniensis* biofilms.

Extracellular polymeric matrix was not observed in any of the biofilms analysed using SEM since pre-treatments and electron beam of the equipment may cause destruction of the matrix.

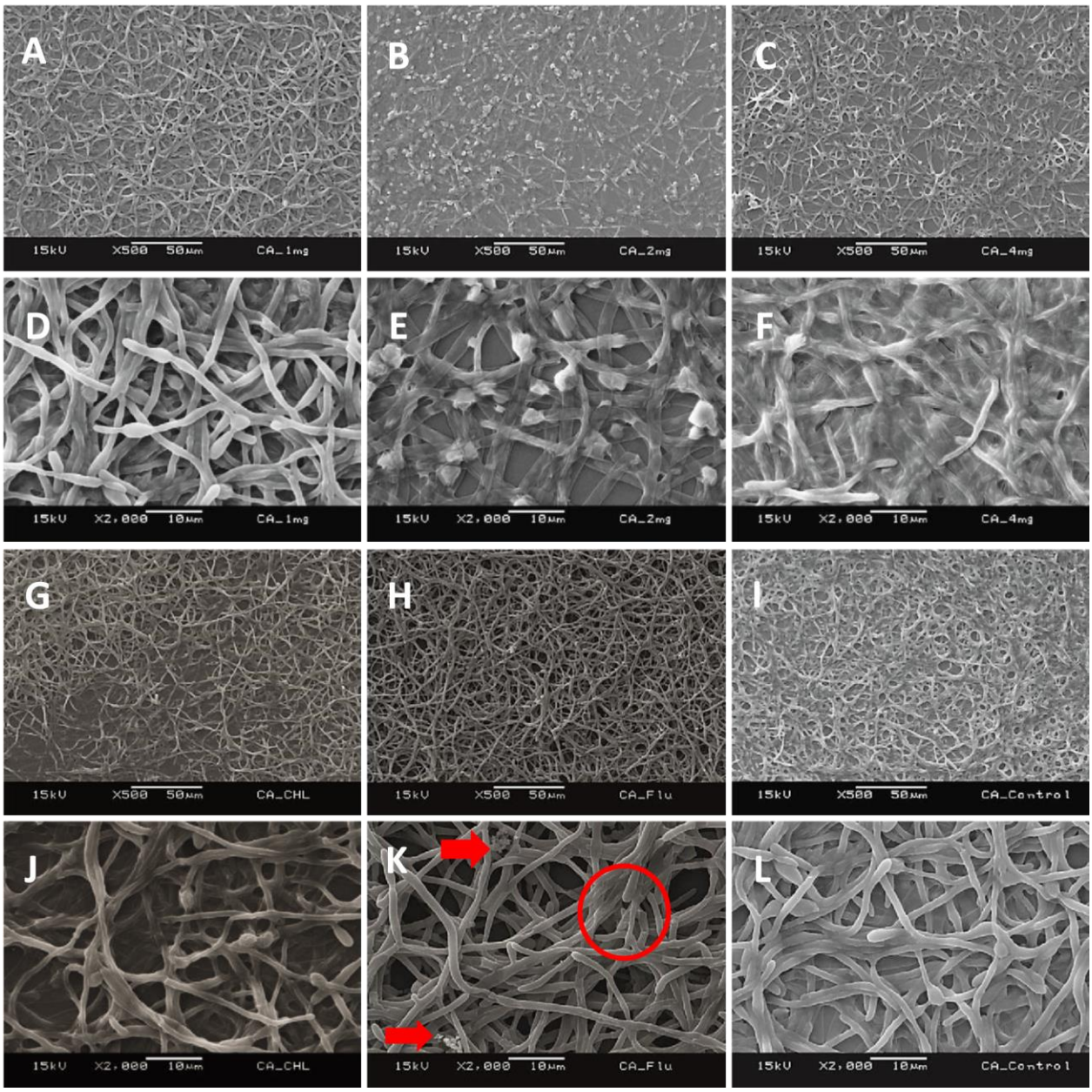

Figure 5.13: SEM images of *C. albicans* (ATCC MYA-2876) established biofilms after 24h treating with 1.0 mg/ml *C. verum* leaf oil (A and D), 2.0 mg/ml *C. verum* leaf oil (B and E), 4mg/ml *C. verum* leaf oil (C and F), 1.0 mg/ml Chlorhexidine digluconate (G and J) and 0.008 mg/ml Fluconazole (H and K). I and L: Negative control. ○ – Cell wall deformities with treatments. ➡ – Leakages of intracellular components.

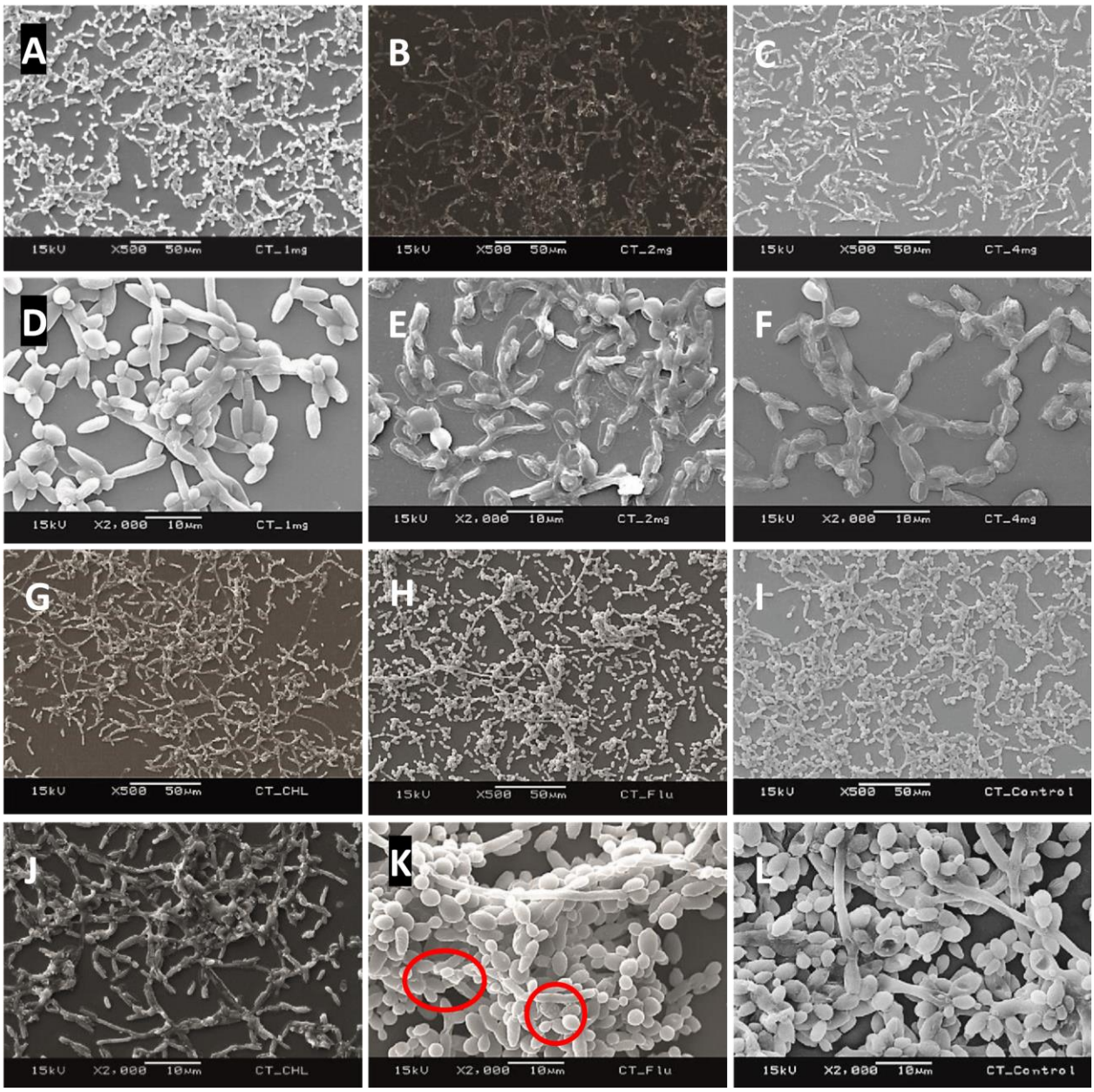

Figure 5.14: SEM images of *C. tropicalis* (ATCC 750) established biofilms after 24h treatment with 1.0 mg/ml *C. verum* leaf oil (A and D), 2.0 mg/ml *C. verum* leaf oil (B and E), 4.0 mg/ml *C. verum* leaf oil (C and F), 4.0 mg/ml Chlorhexidine digluconate (G and J) and 0.008 mg/ml Fluconazole (H and K) I and L: Negative control. O– Cell wall deformities with treatments.

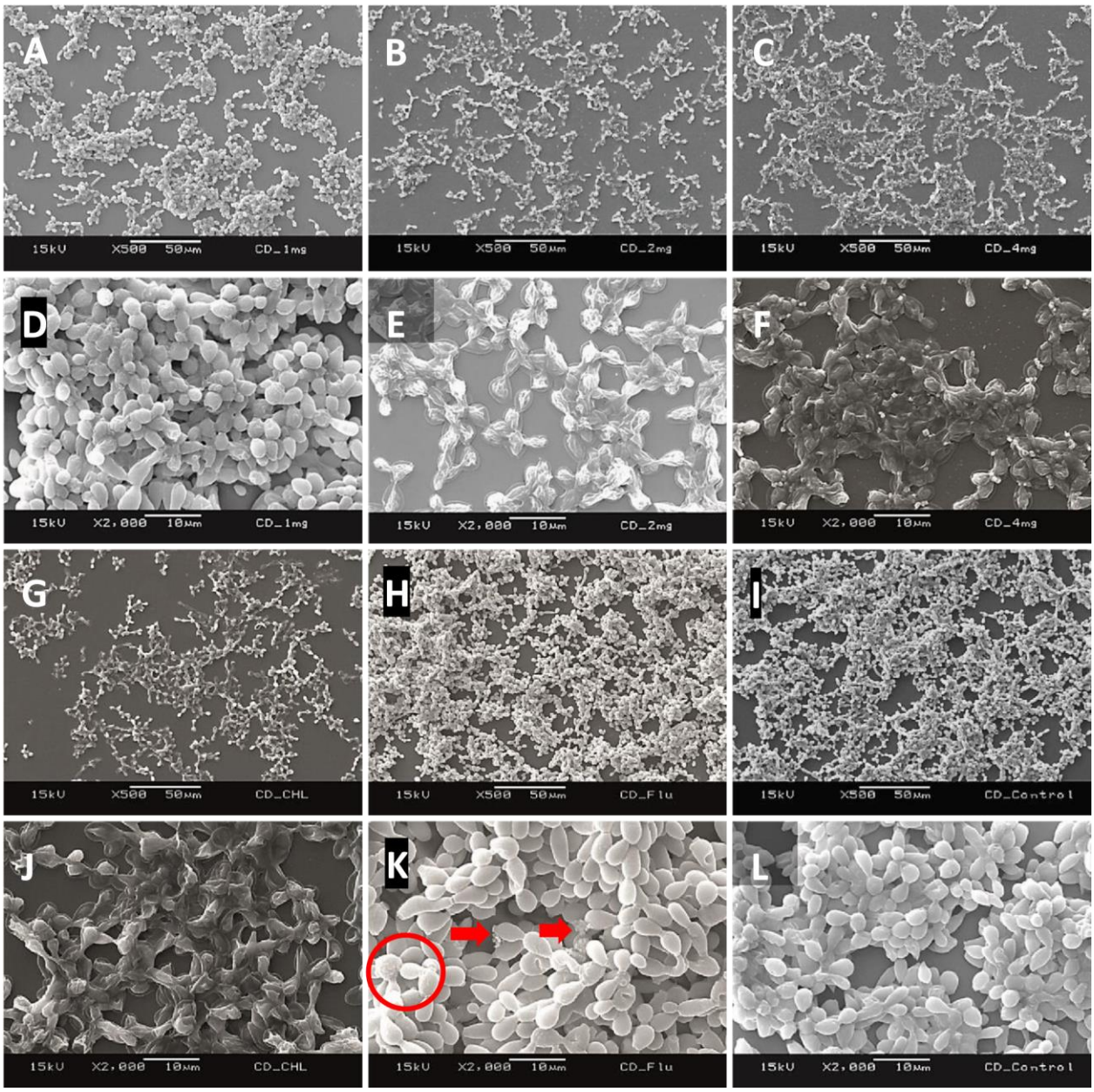

Figure 5.15: SEM images of *C. dubliniensis* (ATCC MYA-646) established biofilms after 24h treatment of 1.0 mg/ml *C. verum* leaf oil (A and D), 2.0 mg/ml *C. verum* leaf oil (B and E), 4.0 mg/ml *C. verum* leaf oil (C and F), 4.0 mg/ml Chlorhexidine digluconate (G and J) and 0.008 mg/ml Fluconazole (H and K) I and L: Negative control. ○ – Cell wall deformities with treatments. ➡ Leakages of intracellular components.

5.7 Transmission Electron Microscopy (TEM)

Post-exposure cellular morphology of planktonic *C. albicans* (ATCC MYA-2876); *C. dubliniensis* (ATCC MYA-646) and *C. tropicalis* (ATCC 750) was determined using TEM.

TEM images of test strains after 24h exposure to MIC of *C. verum* leaf oil and chlorhexidine digluconate were obtained as follows. (**Figure 5.16**)

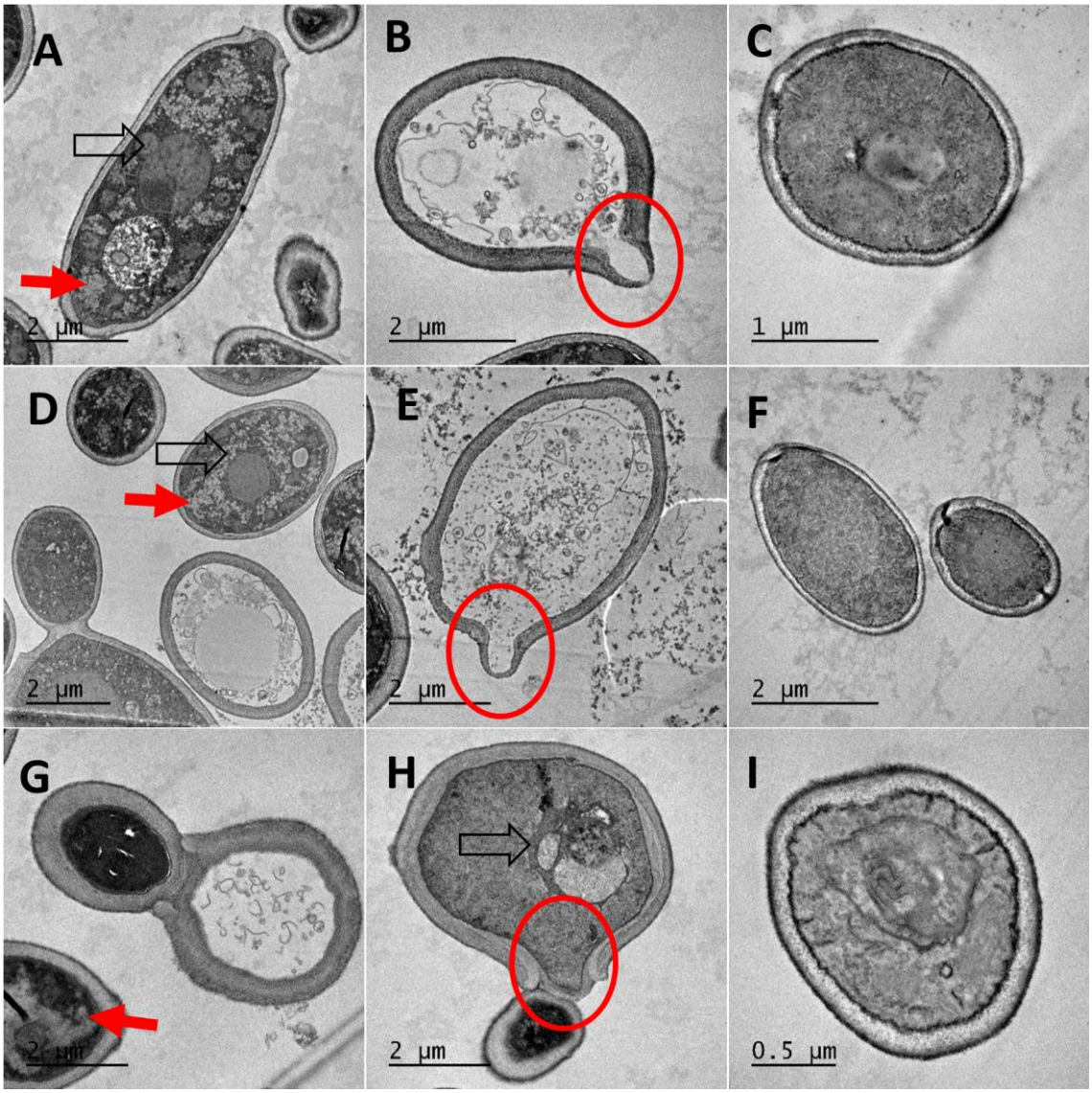

Figure 5.16: Transmission Electron Microscopic (TEM) images of *C. albicans* (A, B and C); *C. tropicalis* (D, E and F) and *C. dubliniensis* (G, H and I). White arrows indicate intra-cellular vacuoles, red circles indicate cell wall damages and red solid arrows indicate cytoplasmic coarse granular inclusion bodies. C, F and I are negative controls.

5.8 Effect of *C. verum* Leaf Essential Oil on Host Tissues

5.8.1 *In vitro* cytotoxicity of *C. verum* leaf oil (HaCaT human keratinocyte cell line)

In vitro antiproliferative/cytotoxic effect of *C. verum* leaf oil and chlorhexidine digluconate on human cells was determined using immortalized human keratinocytes (HaCaT).

Post-treatment cellular viability of cell line after treating with different concentrations of essential oil was quantified using MTT metabolic assay.

Figure 5.17 shows the percentage growth inhibition after treatment with different concentrations.

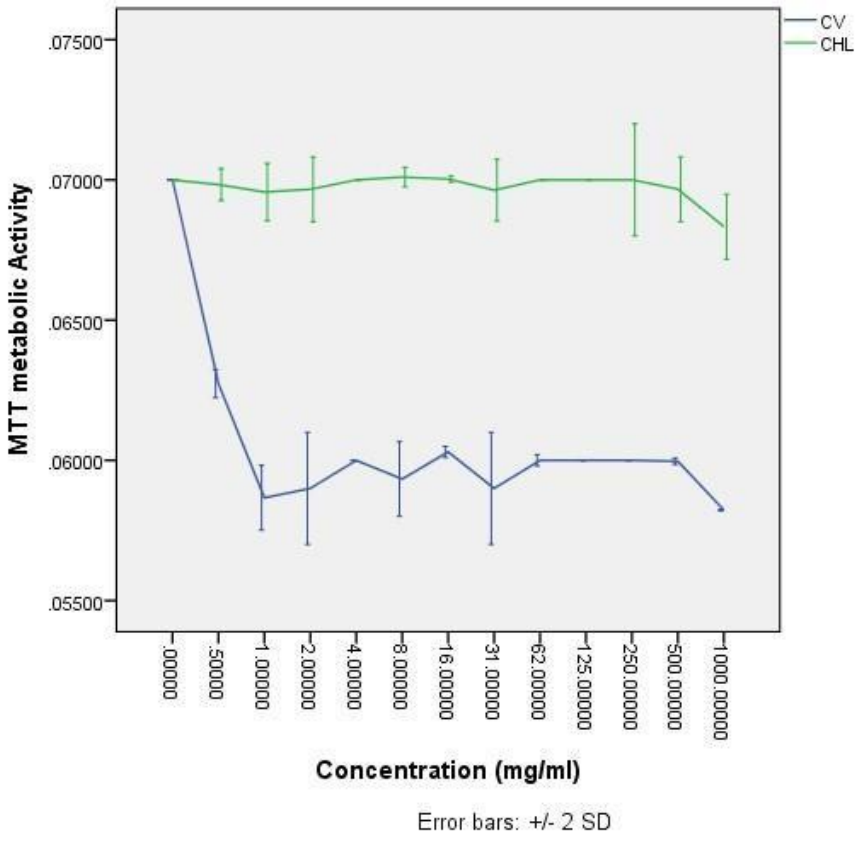

Figure 5.17: Percentage of average cellular inhibition of HaCaT cell line after 24h treatment with different concentrations of *C. verum* leaf oil and Chlorhexidine digluconate. All error bars represent the ± 2 standard deviations (SD).

5.8.2 *In vivo* toxicity of *C. verum* leaf oil (*Galleria mellonella* larvae)

In vivo toxicity of *C. verum* leaf essential oil was evaluated by using *in vivo* model of larvae of *Galleria mellonella* after treatment with different concentrations of essential oil. The test groups were treated with 0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml and 16 mg/ml of *C. verum* leaf essential oil and later its mortality was recorded for 5 days.

Figure 5.18 represents the survival rate of the larvae in each concentration to which they were exposed. All concentrations tested showed 100% survival of the treated larvae throughout whole experiment period.

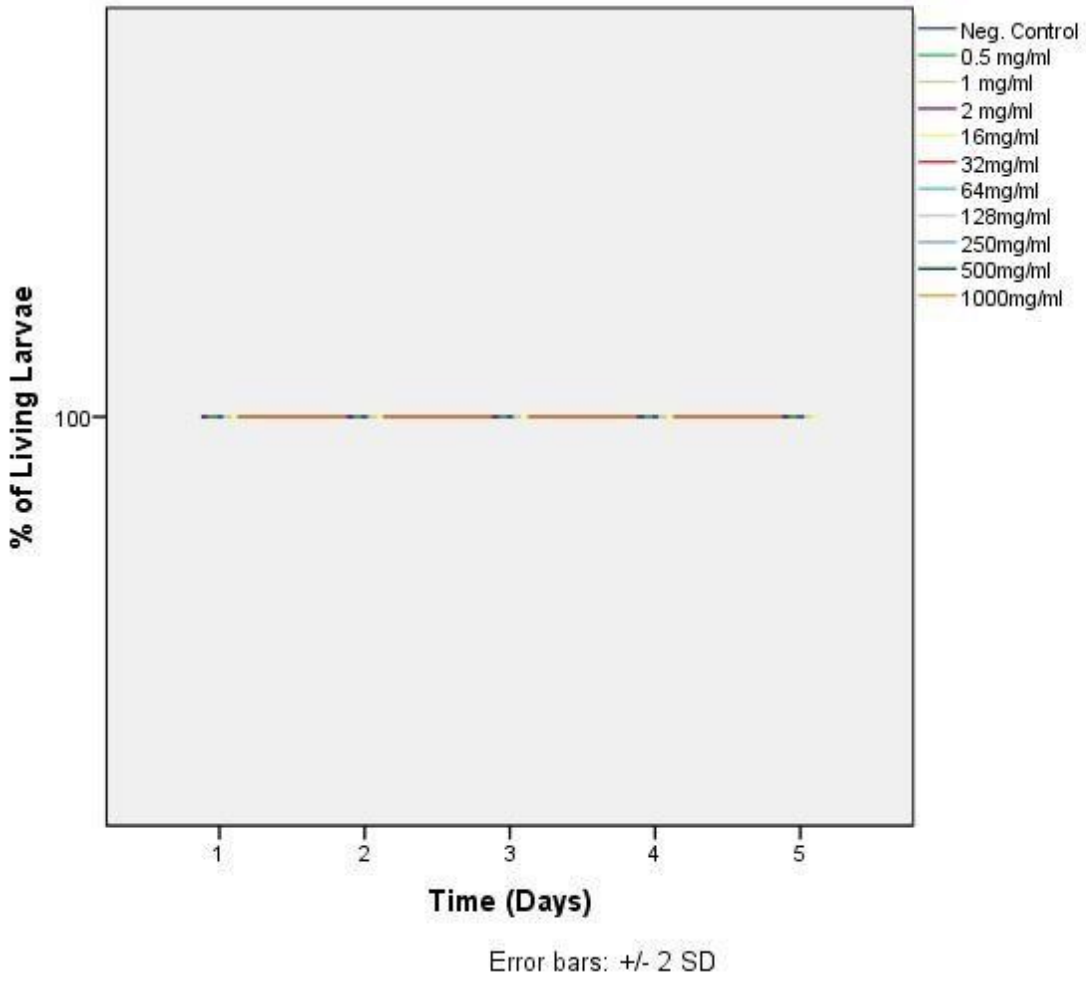

Figure 5.18: Survival rate of *G. mellonella* larvae after administration of *C. verum* leaf oil over 5 days experiment period. Control curve was obtained by administrating sterile PBS in to larvae.

6. DISCUSSION

Cinnamon has been used as a common culinary spice in several communities for centuries. Additionally, cinnamon has been employed as a folk medicine and phytomedicinal alternative in different cultures. Basically two species of genus Cinnamomum along with its some wild species are commonly using as approved herbal medicine. They are *C. verum* (True Cinnamon/Ceylon Cinnamon) and *C. cassia* (Chinese Cinnamon). The bark, flowers, fruits and leaves of above species comprise of useful medicinal properties (Gruenwald et al., 2010; Ranasinghe et al., 2013).The present study was conducted to find out the efficacy of *C. verum*/ true cinnamon leaf oil as a potential phytomedicinal therapeutic alternative against three commonest *Candida* strains, namely *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646).

Though anti-bacterial activity of *C. verum* bark essential oil is well documented, this research fills the deficiency in scientific evidences on antimicrobial activity and anti-candida activity of *C. verum* leaf essential oil as well as its effect on host tissues.

The current study assessed the chemical composition of true cinnamon leaf oil using HP-6890 gas chromatograph coupled with HP-5975 selective mass detector. The most abundant chemical component was Eugenol (77.22%) followed by Benzyl benzoate (4.53%), Trans caryophyllene (3.39%), Acetyle eugenol (2.75%) and Linalool (2.11%). Trans-Cinnamaldehyde (1.69%), Acetic acid cinnamyl ester (1.49%) were identified as minor compounds in *C. verum* leaf oil. Previous study carried out by Schmidt et al. (2006) assessed the essential oil extracted from leaves of *C. verum* from Sri Lanka and identified the chemical components as follows: 74.9% Eugenol, 4.1% β-caryophyllene, 3% benzyl benzoate 2.5% linalool as main compounds. Eugenyl acetate (2.1%), cinnamyl acetate (1.8%), safrole (1.3%) and cinnamaldehyde (1.1%) were contained as minor compounds (Schmidt et al., 2006). Another research carried out by Jantan et al. (2008) observed relatively high amounts of eugenol (90.2%) and low amounts of β−Caryophyllene (2%) and β-Phellandrene (0.5%) as key components (Jantan et al.,2008). Results for GC-MS analysis of *C. verum* leaf oil obtained in currents study was parallel to the observations of Schmidt et al. (2006). Though all above studies used same species (*C. verum*) and same part of the tree (leaves) to extract essential oil, the different chemical composition of the extracts were probabily due to effect of geographic and environmental conditions and technical reasons (Schmidt

et al., 2006). On the other hand, comparative analysis chemical composition of *C. verum* and Cassia cinnamon (*C. cassia*) leaf oil was carried out by wang et al. (2009) and they observed trans-Cinnamaldehyde (30.36%), o-Methoxy-cinnamaldehyde (25.39%), 3-Methoxy-1,2-propanediol (29.3%) and Coumarin (6.36%) as basic phytochemical components (Wang et al., 2009). The relatively low amount of cinnamaldehyde and coumarin in *C. verum* is responsible for its characteristic soft aroma and low toxicity on human body (Abraham et al., 2010). However, all studies identified Eugenol as key chemical component of cinnamon leaf oil and it is considered as the antimicrobial active compound which acts on microbial cell wall.

In the present study, observations obtained from microatmospheric plate method, demonstrated anti-*Candida* activity of *C. verum* leaf oil vapor. All three test strains exhibited a zone of growth inhibition with the administration of *C. verum* oil vapor **(Table 5.1)**. Since, in normal body temperature, *C. verum* leaf oil is a vapor, it is important to evaluate the efficacy of antimicrobial agent when it is not directly contact with the affected areas. Further, in traditional Ayurveda therapy, "Nasya Karma" is a basic treatment method where medications are introduced as a vapor inhalation specially for treatment of respiratory tract infections (Gupta et al., 2009). So this step can be consider as a screening test for anti-*Candida* activity of *C. verum* leaf oil in its vapor phase. In addition to that, routine antimicrobial screening tests were carried out using agar well diffusion or disk diffusion techniques. Though, these methods show reliable results with non-volatile antimicrobial substances, they provide erroneous results with highly volatile substances. Micro atmospheric plate method is recommended to prevent this error during the assay (Şerban et al. 2011).

Published literatures demonstrate differences on antimicrobial activity in direct contact liquid phase and its vapor phase. Lopez et al. (2007) observed a poor antimicrobial inhibitory effect of Eugenol in vapor phase compared to its direct contact liquid phase against common food borne pathogens. At the same time they observed an equal antimicrobial effect of Eugenol in vapor phase and liquid phase on *C. albicans* (López et al., 2007). Current study also demonstrated a good anti-*Candida* effect of vapor phase active compounds which is in agreement with previous data.

MIC and MFC of *C. verum* leaf oil on planktonic *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) were determined using CLSI M27-A3 broth micro dilution method with modifications (CLSI, 2008). Chlorhexidine digluconate was

used as positive control in this experiment. All test strains demonstrated a similar MIC and MFC patters with *C. verum* leaf oil. (MIC 1 mg/ml and MFC 2 mg/ml) and *C. albicans* (ATCC MYA-2876), and *C. dubliniensis* (ATCC MYA-646) showed similar susceptibility pattern for chlorhexidine digluconate with MIC of 1mg/ml and MFC of 2mg/ml. On other hand *C. tropicalis* (ATCC 750) exhibited lower and equal MIC and MFC value (0.5mg/ml) with chlorhexidine digluconate **(Table 5.3)**.

Though these data, in principle, demonstrate the antimicrobial action of *C. verum* leaf essential oil with an agreement with already publish data, pointing out that true cinnamon leaf oil have an antimicrobial activity due to the presence of bioactive compounds in their essential oil (López et al., 2007; Gruenwald et al., 2010; Ranasinghe et al., 2013), MIC and MFC values of current study are slightly different from recently published findings. Castro et al. (2013) determined MIC and MFC of *C. verum* blume essential oil using the microdilution technique. According to their findings, MIC and MFC of essential oil for *C. albicans* and *C. tropicalis* were 0.3 mg/ml and 2.5 mg/ml (Castro et al. 2013). Another study conducted by Rungel et al. (2018) observed 0.25 mg/ml MIC and MFC values of *C.verum* blume EO on *Candida* spp. These differences in MIC and MFC may be due to the concentration variations of active compounds in EO used. According to the classification of activity level of plant materials based on MIC value introduced by Duarte et al. (2005), MIC up to 0.5 mg/ml is considered as strong active, concentrations from 0.55 to 1.5 mg/ml is considered as moderately active and above 1.5 mg/ml is considered as weakly active plant materials against tested microorganisms (Duarte et al., 2005). According to this classification, the results obtained with the essential oil of *C. verum* leaves showed moderate anti-fungal activity on tested *Candida* strains. On the other hand commercial antifungal chlorhexidine digluconate exhibited a similar anti-*Candida* action as *C. verum* leaf oil for *C.albicans* and *C. dubliniensis* by demonstrating MIC value of 1 mg/ml. This is an important finding when the nontoxic or low toxic, cost effective antimicrobial phytomedicinal agents discovery is concerned.

As well as minimum effective concentrations, time requires to completely eradicate/kill the microbial population is one of the key determinant of antimicrobial potency of a given antimicrobial agent and plays a major role in designing the dosage regime of a given antimicrobial drug (Patel et al., 2009; Craig, 1993). Killing time of *C. verum* EO and reference

antifungal Chlorhexidine digluconate was determined using the standard procedure introduced by Tsuji et al. (2008). Standard cell suspension of reference strain of *C albicans* was mixed with MIC (1mg/ml), half of MIC (0.5mg/ml) and twice of MIC (2mg/ml or MFC) of the oil dilutions. At each time point sample of above mixtures were subjected to CFU assay to determine the viable cell count. Results obtained from killing time assay of *C. verum* leaf essential oil on test strain exhibited concentration dependent killing time. MFC (2.0 mg/ml) of *C. verum* kills *C. albicans* within 6h whereas 2.0 mg/ml Chlorhexidine digluconate kills *C. albicans* within 1h. Sub-MFC concentrations of *C. verum* EO does not exhibit any fungicidal effect within 24h experiment period. (**Figure 5.2**) Since killing time is concentration dependent, shorter killing times can be acquired by increasing the concentration of EO, but the toxicological assessments should be contemplated. Though killing time is considered as an essential component of antimicrobial profile, there is no evidence based data on time-kill kinetics of *C. verum* leaf EO on *Candida* spp. Present study fills this deficiency of available data with *in vitro* experimental design performed on ATCC type *Candida* strains.

Candida spp. displays many virulent factors that contribute to successfully colonize in host tissues. Adhesion to host surfaces (which is the initial step of biofilm formation), germ tube formation, biofilm formation and secretion of proteinases and other hydrolytic enzymes and aldehyde production are some of them (Calderone et al., 2001). In the current study, the effect of *C. verum* EO on *Candida* adhesion to polystyrene surfaces was evaluated by inoculating 96 well sterile flat bottomed microtiter plates with the 1:1 mixture of standard cell suspensions of reference *Candida* strains and different concentrations of EO. After allowing Candida cells to adhere on to polystyrene surface for 2h, adhered cell mass was quantified using XTT metabolic assay (Raut et al., 2013). *C. albicans* achieved 50% reduction in adhesion with 1.0 mg/ml concentration, whereas, *C. tropicalis* and *C. dubliniensis* showed 50% reduction in adhesion with >2.0 mg/ml and 0.34 mg/ml concentrations of oil respectively. Reference antifungal Chlorhexidine digluconate exhibited 50% of reduction in adhesion to *C. albicans*, *C. tropicalis* and *C. dubliniensis* with the concentrations of 1.06 mg/ml, 0.72 mg/ml and 1.32 mg/ml respectively (**Figure 5.3**). Importantly, 50% reduction of adhesion on *C. albicans* and *C. dubliniensis* was achieved with lower concentrations of *C. verum* leaf oil compared to chlorhexidine digluconate control which indicates the higher efficacy of *C. verum* EO in adhesion reduction of those two strains. According to data obtained, 2.0 mg/ml chlorhexidine digluconate completely inhibit the adhesion of all test strains,

but the observation may be due to killing of organisms completely during test period (2h). Since adhesion on to the host surfaces is one of the major virulent factor and initial step of biofilm formation, the anti-adhesion properties of *C. verum* EO on *Candida* spp. contributes to its' aptness as an antimicrobial agent.

Germ tube formation is another major virulent factor of *C. albicans* and *C. dubliniensis* which enables the absorption of nutrients from host tissues by invading them. Effect of *C. verum* EO on germ tube formation was determined by mixing dilutions of oil with *C. albicans* and *C. dubliniensis* standard cell suspensions prepared in FBS followed by counting the number of germinated cells at 2h, 4h and 6h experiment period. At sub-MIC (0.25 and 0.5 mg/ml), both *C. verum* leaf oil and chlorhexidine digluconate significantly reduces the germ tube formation of both *C. albicans* and *C. dubliniensis* whereas MIC (1.0 mg/ml) completely inhibit the germ tube formation of test strains (**Figure 5.4**). With time (for *C. albicans*, after 2h and for *C. dubliniensis*, after 4h) the germ tube formation in all groups including control group gradually decreased since produced germ tubes transformed to pseudo hyphae and hyphae. There are no published scientific evidences available on effect of true cinnamon leaf EO on adhesion and germ tube formation of *Candida* spp. Current study was designed to find the efficacy of Cinnamon leaf oil on controlling virulent factors of *Candida* spp. in order to fill this gap of available data. Adhesion and germ tube formation are key attributes of infection causing ability of *Candida* and therefore antimicrobial substances which interfere with these factors are considered as potential anti-*Candida* agents. Hence *C. verum* leaf EO can be developed as a therapeutic alternative against *Candida* infections.

Biofilm is a surface attached microbial communities embedded in an extracellular matrix derived from cells and environment (Acker et al., 2014). Biofilms are more resistant to physical and chemical stresses compared to their planktonic counterpart as biofilms exhibit various mechanisms to neutralize those external stresses. Extracellular biofilm matrix acts as a physical or chemical barrier for diffusion of antimicrobial agents into the biofilm. Further, with the limited availability of nutrients, the biofilm community shifts towards slow or no growth status from exponential growth. All above factors and induction of a biofilm phenotype and quorum sensing contribute to high resistance to antimicrobial agents of a biofilm (Simões et al., 2010; Mah et al., 2001). Since the resistance of biofilms to available antimicrobial strategies is becoming more prominent, more studies should be carried out in order to invent novel, non-toxic, inexpensive and

effective treatment options. In the current study, efficacy of *C. verum* leaf EO as a potential phytomedicinal agent was evaluated on forming biofilms and 24h established *Candida* biofilms. 96 well sterile flat bottomed microtiter plates were seeded with the standard cell suspensions of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) and adhered cells were then allowed to form biofilms in the presence of different concentrations of *C. verum* leaf EO. After 24h, viable biomass of formed biofilms were quantified using XTT viability assay. Similarly, biofilms of test strains were prepared in sterile 96 well flat bottomed microtiter plates and treated with different oil concentrations. Post-treatment biofilm viability was quantified using XTT assay. Viability of treated biofilms was further evaluated by performing the CFU assay on exposed biofilms. *C. verum* EO exhibited a potential antibiofilm effect on forming biofilms of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) test strains. Concentrations required to reduce the biofilm development by 75% (Compared to negative control) were~ 0.4, 0.6 and 0.4 mg/ml for three test strains respectively. And the concentrations of EO required to completely kill the forming biofilms (MTC) were 1.0 mg/ml for all test strains. Chlorhexidine digluconate kills Candida forming biofilms with lower concentrations than *C. verum* leaf oil (four times lower concentration for *C. albicans* and eight times lower concentration for *C. tropicalis* and *C. dubliniensis*) (**Table 5.5**). Importantly, these MTC values are lower than MIC and MFC values of test strains which indicates the high potency of killing of forming biofilms of *C. verum* EO. These data suggest the potential action of true cinnamon oil on interrupting the normal process of formation of *Candida* biofilms. Biofilm progression under chemical stress with *C. verum* leaf oil was visualized with time lapses microscope and results obtained from this experiment confirm above observation. 2×MTC (2 mg/ml) of *C. verum* EO completely inhibited biofilm development of all test strains from time 0h whereas slight cellular proliferation and biofilm development was observed in progressing *C. albicans* with MTC (1 mg/ml) from 0h to 8h. After 8h, no visible biofilm development was noted in *C. albicans* developing biofilms (**Figure 5.10 A, B and C)**. *C. tropicalis* and *C. dubliniensis* biofilm development was completely inhibited at 0h by MTC. Sub-MTC (0.5 mg/ml) causes retardation of biofilm development of all tested *Candida* strains. These results indicate the possible effective use of *C. verum* leaf EO as a biofilm preventive strategy. Kaneko et al. (2013) analyzed real-time Time-Lapse microscopy images to reveal the development process and to calculate the growth rates of *Candida* biofilms under the EO chemical stress (Kaneko et al., 2013). We observed

the progression of *Candida* biofilm under treatment with the essential oil, demonstrating that the effect of *C. verum* leaf EO on *Candida* biofilm development is dose dependent.

Effect of true cinnamon leaf essential oil do not limit only to planktonic and developing biofilms. It kills established *Candida* biofilms and present study quantified this effect using XTT metabolic activity. 75% reduction in cell viability (compared to negative control) of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) was observed with 0.5, 0.9 and 1.2 mg/ml EO respectively (**Table 5.6**). MBEC of *C. verum* leaf oil against *Candida* established biofilms was 2.0 mg/ml for all test strains (**Table 5.7**). These values are higher than that values of forming biofilms, which indicates higher resistance to chemical stress of mature biofilms.

SEM images were taken to understand the structure of biofilms developed under EO chemical stress and the structure of established *Candida* biofilms after EO treatment. All biofilms of test strains exhibited cell wall damages, cell wall deformities and leakages of intracellular materials with treatment of *C. verum* leaf oil **(Figure 5.7, 5.8, 5.9, 5.13, 5.14 and 5.15)**. Importantly, SEM images confirm the dose dependent nature of effects of *C. verum* leaf essential oil. Fluconazole is the known antifungal agent which belongs to azole group and inhibits synthesis of fungal sterol, ergosterol (Richardson et al., 1990). On the other hand Chlorhexidine digluconate is a biguanide which is used as an antibacterial mouth rinse. It alters the morphology cells and damages the cell wall of microorganisms and releases intracellular components. It has been suggested as a well-known therapeutic antifungal agent for oral candidiasis (Da Silva et al., 2011 and Bobichon et al., 1987). Since both antimicrobial agents have an effect on *Candida* cell wall, ultrastructure of forming *Candida* biofilms with 0.25 mg/ml Chlorhexidine digluconate (for *C. albicans*), 0.125 mg/ml Chlorhexidine digluconate (for *C. tropicalis* and *C. dubliniensis*) and 0.008 mg/ml Fluconazole (maximum recommended *in vitro* assay concentration, CLSI) were also visualized. Similar observations were obtained with the exposure to MTC of Chlorhexidine digluconate and 0.008 mg/ml Fluconazole. The intensity of the post-exposure response of Fluconazole was minimal due to low concentration. Similar observations to present study were obtained by Bennis et al. (2004) and Braga et al. (2007) after treatment of *Candida* established biofilms with Eugenol. According to their observations with SEM, Eugenol treatment induce morphological alterations in the envelope of *C. albicans*. Exposure to 500 µg/ml of Eugenol

significantly reduced the number of normal yeast and increased that of damaged cells with rough and wrinkled surfaces. These effects were also concentration dependent. Current study is in an agreement with already published data and suggestive of Eugenol as the antimicrobial active compound of true cinnamon leaf EO.

To confirm the SEM observations with cell wall damages, and to evaluate the intracellular/morphological changes of unicellular yeast forms, TEM images of *C. albicans* (ATCC MYA-2876), *C. tropicalis* (ATCC 750) and *C. dubliniensis* (ATCC MYA-646) test strains were taken after treatment with MIC of *C. verum* leaf oil. TEM images demonstrated cell wall damages and leakages of intracellular compartment (**Figure 5.16**). Importantly, TEM images revealed post-exposure cytoplasmic changes including intracellular vacuoles formation, scattered cytoplasm and formation of cytoplasmic granular inclusion bodies. These changes are suggestive of chemical stress on *Candida* cells by *C. verum* leaf oil.

All above experiments determined the pharmacodynamics (antimicrobial/anti-*Candida* properties) of *C. verum* leaf oil on *Candida*. Pharmacodynamics on host tissues was evaluated using two experimental models, *in vitro* cell culture model of human non-cancer keratinocytes (HaCaT) cell line and *in vivo Galleria mellonella* larvae. Those toxicology assessments provide proper understanding on effective, nontoxic dose and help in dose regimen designing by integrating both pharmacodynamics and pharmacokinetics of *C. verum* leaf oil. Further there are lack of published data on toxicology studies of *C. verum* leaf oil and current study fill that deficiency.

The half maximal inhibitory concentration (IC_{50}) quantitatively measure how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process) by half (Serra et al., 2018). In current study, *in vitro* HaCaT cell line was employed to determine the *in vitro* cytotoxicity assessment and determine the IC_{50} of *C. verum* leaf EO against human cells. Cells were grown in 96 well sterile flat bottomed microtiter plates and treated with different concentrations of *C. verum* leaf oil. Viability of cells after treatment was quantified using MTT metabolic assay. The maximum concentration tested was 1000 mg/ml (neat concentration of true cinnamon leaf EO oil/ oil without diluting) and this concentration did not show any inhibitory effect on HaCaT cells in current experiment (**Figure 5.17**). This finding

indicates that the safe use of *C. verum* leaf EO up to 1000 mg/ml without any toxic effect on human cells.

It is very important that compounds intended for use as therapeutic agent on human are adequately tested in suitable animal model systems. Usually Rodents (rabbits, rats and mice) are commonly used in this regard, but cost and ethical aspects have to be carefully considered. It is therefore useful to contemplate alternative simple experimental models. The *Galleria mellonella* (greater wax moth) larval stage is an immature life stage of an insect and it have been used for virulence and antimicrobial efficacy studies previously. There are number of benefits of use of *G. mellonella* larvae including easy to obtain pharmacokinetic and pharmacodynamic data, similar microbial pathogenicity and virulence determinants to humans and mice, short life span, smaller in size, low cost and without the need of specialized equipment and training and importantly functional and structural similarities of to the insect immune system to mammalian innate immune system make *Galleria mellonella* larvae (Ignasiak et al., 2017; Benaducci et al., 2016). Current study used *G. mellonella* larvae *in vivo* model to determine the lethal concentration (LC) of *C. verum* EO.

Different concentrations of EO were introduced to study population of *G. mellonella* larvae and kept the study model at 37 ^{o}C for five days under observation. In this experiment, 100% survival was observed with *G. mellonella* larvae after administration of all test concentrations ranging from 0.5 to 1000 mg/ml *C. verum* EO. This indicates the zero toxicity of true cinnamon leaf oil on test group. Since 1000 mg/ml is the highest possible concentration of *C. verum* leaf EO (neat solution) all dilutions including neat solution can be considered as non-toxic to experiment model (**Figure 5.18**).

Based on *in vitro* and *in vivo* toxicology study results and pharmacodynamics of *C. verum* leaf oil on *Candida* sessile and planktonic cells, *C. verum* leaf EO can be considered as a potential therapeutic alternative for *Candida* infections. Also authors suggest future research on effect of *C. verum* leaf EO on wide range of pathogenic fungi and bacteria.

7. CONCLUSIONS

- *C. verum* leaf oil contains Eugenol, Acetyle eugenol and Cinnamaldehyde as active compounds.
- *C. verum* leaf oil vapor has a potential anti-*Candida* activity on planktonic *Candida*.
- True Cinnamon leaf oil demonstrates both Fungicidal and Fungi static activity.
- True Cinnamon leaf oil negatively affect germ tube formation and adhesion of *Candida*.
- *C. verum* leaf oil exhibits anti biofilm activity on both forming and established *C. albicans*, *C. tropicalis* and *C. dubliniensis*.
- True Cinnamon leaf EO acts on *Candida* cell wall and causes cell wall damages and cell wall deformities.
- All these effects are concentration dependent.
- *C. verum* leaf oil does not exhibit any significant cytotoxicity in HaCaT *in vitro* cell culture model and *in vivo Galleria mellonella* larvae model at tested concentrations.

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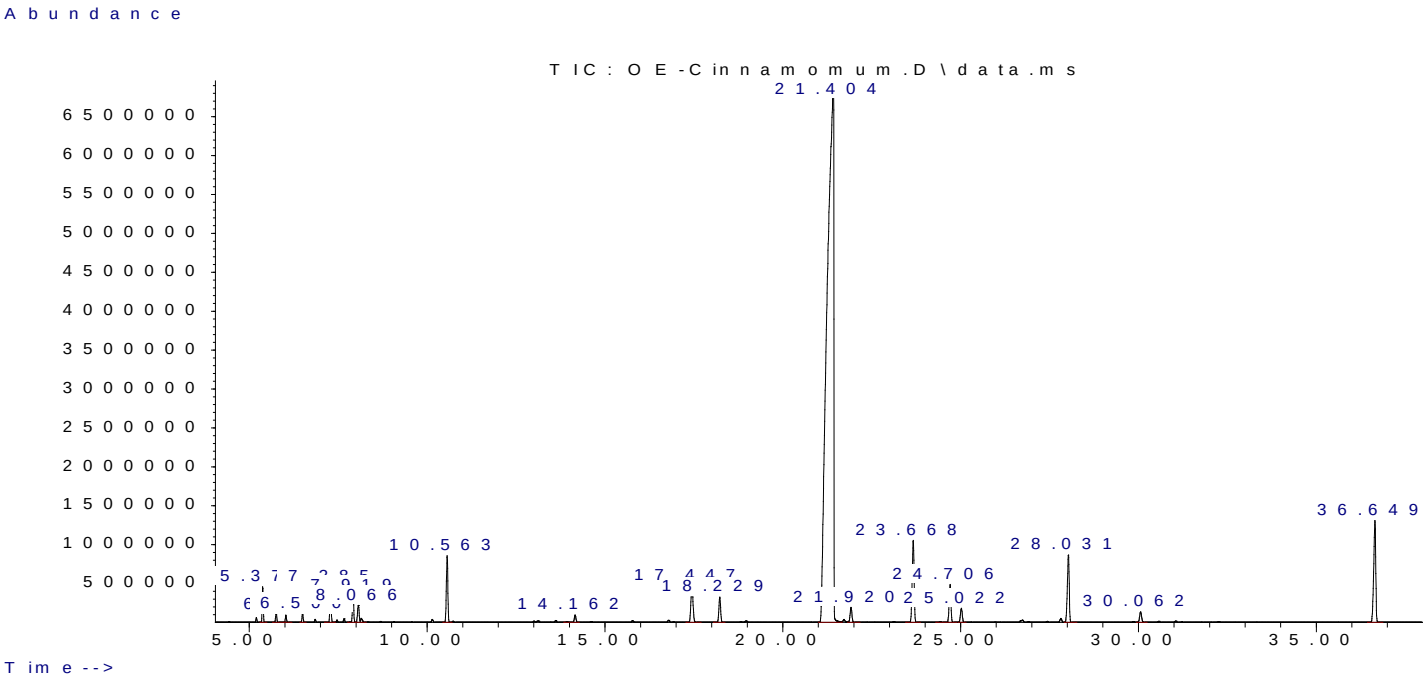
ANNEXES

1. Chromatogram of Cinnamon leaf oil
2. Similarity analysis report

Annex 1 - Chromatogram of Cinnamon leaf oil



Identified chemical compounds of *C. verum* leaf EO.

t_R (min) [a]	IR [b]	Identification	% rel. [c]
5.38	932	Alfa-Pineno	0.81
5.76	946	Canfeno	0.25
6.03	957	Benzaldehyde	0.18
6.50	975	Beta-Pineno	0.23
7.29	1004	Alfa-Felandreno	0.96
7.92	1022	Para-Cimeno	0.76
8.07	1027	Limoneno	0.75
10.56	1099	Linalol	2.11
14.16	1189	Alfa-Terpineol	0.28
17.45	1267	Trans-Cinamaldehyde	1.69
18.23	1285	Safrol	0.94
21.41	1361	Eugenol	77.22
21.92	1374	Alfa-Copaeno	0.60
23.67	1416	Trans-Caryophylene	3.39
24.71	1442	Acetic acid cinnamyl ester	1.49
25.02	1450	α-humulene	0.58
28.03	1526	Eugenol acetate	2.75
30.06	1579	Caryophylene oxide	0.46
36.65	1760	Benzyl benzoate	4.53

Notes: a) Retention Time

b) Retention Index

c) Percentage fraction of total integrated area for chromatogram

Annex 2 - Similarity analysis report

EFFECT OF TRUE CINNAMON (Cinnamomum verum) LEAF OIL AGAINST IN VITRO Candida BIOFILMS AND ITS CYTOTOXIC EFFECT

RELATÓRIO DE ORIGINALIDADE

7%	5%	4%	%
ÍNDICE DE SEMELHANÇA	FONTES DA INTERNET	PUBLICAÇÕES	DOCUMENTOS DOS ALUNOS