ANTIFUNGAL ACTIVITIES OF THREE ALKALOIDS FROM *RUTA ANGUSTIFOLIA* (L.) PERS. AGAINST ATCC SUSCEPTIBLE STRAINS OF *CANDIDA ALBICANS* AND *CANDIDA GLABRATA*

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by

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LIST OF ABBREVIATIONS

ACT1	Actin1
cDNA	Complementary deoxyribonucleic acid
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
HCL	Hidrochloric Acid
HPLC	High Performance Liquid Chromatography
ICL1	Isocitrate lyase gene and enzyme
INT	p-iodonitrotetrazolium violet
MIC	Minimum Inhibitory Concentration
MDR	Multidrug Resistance
MFC	Minimum Fungicidal Concentration
MLS1	Malate synthase 1
MTT	Methylthiazolyltetrazolium chloride
NaOH	Sodium Hydroxide
PCK1	Phosphoenolpyruvate carboxykinase 1
Rf	Retention factor
RNA	Ribonucleic acid
YPD	Yeast Peptone Dextrose
YPL	Yeast Peptone Lactate

LIST OF SYMBOLS

ºC	Degree Celcius
™	Trademark
®	Registered Trademark
nM	Nano Molar
M	Molar
µg	microgram
mg	miligram
ng	nanogram
g	gram
mmol	milimolar
µL	micromolar
mL	mililitre
cm	Centimetre
mm	Milimetre
CFU/mL	Colony Forming Unit/mililiter

AKTIVITI ANTIKULAT TIGA ALKALOID DARIPADA POKOK *RUTA ANGUSTIFOLIA* (L.) PERS TERHADAP STRAIN RENTAN ATCC *CANDIDA ALBICANS* DAN *CANDIDA GLABRATA*

ABSTRAK

Keupayaan tumbuh-tumbuhan ubatan melawan mikroorganisma patogenik telah menyebabkan ramai saintis mengasingkan sebatian aktif dan seterusnya mengkaji mekanisme tumbuhan ini. Ini adalah penting untuk melawan kejadian patogen yang rintang dadah. Dalam kajian ini, tumpuan adalah untuk mencari agen antikulat baru dari alkaloid *Rutaceae*. Pengasingan berpandukan biocerakian telah dilakukan untuk mengasingkan alkaloid aktif antikulat. Kesan alkaloid ini terhadap gen yang disasarkan telah dipilih dan diperhatikan dalam kajian ini melalui analisis kuantitatif rantai polimerase masa nyata (qRT-PCR). Daun dari tumbuhan *Ruta angustifolia* (L.) Pers. telah diekstrak dan difraksikan dengan menggunakan teknik kromatografi. Melalui pengasingan biocerakian, gabungan fraksi H3-3 dan H4-8, H10-9 dan DA-10 telah menghasilkan sejumlah tiga alkaloid aktif antikulat. Alkaloid yang telah diasingkan kemudiannya dikenalpasti melalui profil Kromatografi Lapisan Nipis, suhu takat lebur dan gelombang maksimum untuk penyerapan UV dalam metanol (UVλ max-MeOH) berbanding piawai alkaloid. Pengenalpastian selanjutnya disahkan oleh data spektroskopik HPLC (High Performance Liquid Chromatography) dan NMR (Nuclear Magnetic Resonance) telah dicirikan sebagai acridone, furoquinoline dan 4-quinolone, masing-masing dikenali sebagai arborinine, skimmianine dan graveoline. Aktiviti antikulat bagi ketiga-tiga alkaloid ini telah diuji terhadap *Candida albicans* dan *Candida glabrata* dari rangkaian ATCC. Ujian

microdilusi alkaloid memberikan nilai kepekatan perencat minimum (MIC) antara 250 μg/ml hingga 1000 μg/ml. Nilai Kepekatan Fungisidal Minimum (MFC) direkodkan pada 500 μg/ml dan 1000 μg/ml. Antibiotik fluconazole telah dipilih sebagai standard dalam kajian ini. Hasil daripada analisis qRT-PCR ke atas *C. albicans* menunjukkan bahawa arborinine mampu menurunkan kadar *CDR1* dan gen *ERG11*. Sel yang dirawat dengan skimmianine menunjukkan penemuan yang lebih baik apabila kadar ekspresi *ICL1, MLS1, MDR1* dan *CDR2* berjaya diturunkan. Bagi sel-sel yang dirawat dengan graveolin, pengurangan ekspresi telah dikesan dalam gen *CDR1, CDR2* dan *ERG11*. Analisis ke atas *C. glabrata*, sel yang dirawat dengan arborinine menunjukkan penurunan yang signifikan terhadap tiga gen yang disasarkan iaitu *ICL1, PCK1* dan *MLS1*. Kesan Skimmianine dan graveoline ke atas ekspresi gen sel adalah sama apabila kedua-dua sebatian tersebut dapat menurunkan kadar ekspresi *ICL1* dan *PCK1*. Kesan ke atas protin CaIcl1 di dalam *C. albicans* juga berjaya dikurangkan oleh tiga alkaloid aktif tersebut melalui teknik western blot. Secara keseluruhannya, kami menyimpulkan bahawa aktiviti antikulat alkaloid yang diasingkan ini adalah komponen yang mempunyai potensi sebagai perencat ekspresi gen yang virulen dan gen rintangan ke atas *C. albicans* and *C. glabrata.*

ANTIFUNGAL ACTIVITIES OF THREE ALKALOIDS FROM *RUTA ANGUSTIFOLIA* (L.) PERS AGAINST ATCC SUSCEPTIBLE STRAINS OF *CANDIDA ALBICANS* AND *CANDIDA GLABRATA*

ABSTRACT

The vital ability of medicinal plants to resist pathogenic microorganisms has led many scientists to isolate the active compounds and further investigate their mechanism of action. This is important as to fight the growing incidence of drug-resistant pathogens. Overall, this study is focusing towards searching for new antifungal agent from alkaloids belong to Rutaceae family. The bioassay guided isolation was performed to isolate the antifungal active alkaloids. The impacts of these alkaloids on selected targeted genes have been experimented through quantitative real-time polymerase chain reaction (qRT-PCR). Leaves of the plant *Ruta angustifolia* (L.) Pers. was extracted and fractionated by using standard chromatographic techniques. Through bioassay-guided isolation, combined fraction of H3-3 and H4-8, fraction H10-9 and DA-10 yielded a total of three antifungal active alkaloids. These isolated alkaloids were then characterized and compared to the standard alkaloids by means of Thin Layer Chromatography (TLC) profile, melting point and maximum absorption of UV spectra in methanol (UVλ max-MeOH). The identification was further confirmed by HPLC spectroscopic data and was characterized as acridone, furoquinoline and 4-quinolone so named arborinine, skimmianine and graveoline respectively. The antifungal activites of the three alkaloids were tested against *Candida albicans* and *Candida glabrata* of ATCC strains with that of the standard antibiotics fluconazole. Broth microdilution assay of

the alkaloids gave Minimum Inhibitory Concentration (MIC) values ranging from 250 µg/ml to 1000 µg/ml. Minimum Fungicidal Concentration (MFC) values were recorded at 500 µg/ml and 1000 µg/ml. The results from qRT-PCR analysis in *C. albicans* revealed that arborinine, skimmianine and graveoline down regulate all genes related to the central metabolism of *Candida* which are *PCK*, *ICL* and *MLS*. However, in the regulation of resistant genes, there is a decreased in *ERG11*, *CDR2* and *MDR* after treated with arborinine, skimmianine and graveoline respectively. As in *C. glabrata* gene expression analysis, arborinine treated cells showed a significant decrease against three targeted genes namely *ICL1*, *PCK1* and *MLS1*. Skimmianine and graveoline impact on the gene expression of the cell revealed the same impact when both of the compounds were able to down regulate *ICL1* and *PCK1* while MLS and *ERG11* increased. The three isolated compounds were also able to reduce protein expression of Caicl1 in *C. albicans* through Western Blot. Overall, this study has proven that arborinine, skimmianine and graveoline can be remarkable component as antifungal agents with potential capacity to inhibit the growth and suppress the expression of the targeted genes and protein of *C. albicans* and *C. glabrata*.

CHAPTER 1.0: INTRODUCTION

1.1. Problem Statement

Candida species are opportunistic fungal pathogens that have become the second most common responsible life-threatening infections worldwide (Liu et al. 2017). Over the past decade, the prevalence of candidiasis increased due to rising number of immunocompromising conditions including human immunodeficiency virus (HIV) infection, cancer, old age, endocrine disorders, radiotherapy, malignant diseases and other critical illnesses (Kaomongkolgit et al. 2009). Among the many *Candida* species, *C. albicans* has been reported as the most prevalent pathogen in both mucosal and systemic fungal infections (Pfaller et al. 2002) while *C. glabrata* is the second or third most isolated pathogen in patients with oral candidiasis (Fidel & Vazquez 1999). This is consistent with epidemiology report of fungal infections in an Infectious Disease Reference Centre (IDRC) in Malaysia, whereby increasing morbidity and mortality among hospitalized patients and immunocompromised individuals is a major factor in the emergence of opportunistic fungal infections with isolation frequency of 29.9% of *Candida albicans* and 3.9% of *Candida glabrata* (Tzar et al. 2013). A retrospective study were also evaluated on all cases of candidemia in Hospital Universiti Sains Malaysia (HUSM) among adult patient and collectively, *C. albicans* and *C. glabrata* were responsible for 20.1% of the distribution (Haydar 2018).

1.2 Significance of the Study

Candida carriages a multitude of virulence attributes to its adaptability for growth (Nordin et al. 2013). One of the attributes is metabolic flexibility in which it enables *Candida* to survive in nutrient-limited host during infection. Among the metabolic pathways, glycolysis, gluconeogenesis and the glyoxylate cycle are the most studied and all thought to contribute to *Candida* survival (Cheah et al. 2014). The key enzymes involve in those pathways are isocitrate lyase (*ICL*), malate synthase (*MLS*) and phosphoenolpyruvate Kinase (*PCK*). It has been suggested inhibitors of these enzymes should decrease survival of these pathogens inside macrophages (Lorenz & Fink 2001; Brown, Budge, et al. 2014). Therefore, the identification of this pathway as a target for antifungal compounds has potential applications in the development of new antifungal therapies.

Besides the fitness attributes of *Candida*, the resistance in current antifungal agents also restricts the effectiveness in treating *Candida*. Drug resistance emerges due to widespread application of antifungal drugs for both preventive and therapeutic purposes (Cowen et al. 2015). Cellular and molecular mechanisms underlying antifungal drug resistance may include reduction of drug absorption due to increased drug efflux and mutations in genes of target protein, involving overexpression of candida drug resistance (*CDR*) and multidrug resistance (*MDR*) genes. In addition, presence of point mutations in *ERG11* was also identified as the mechanism of resistance. *ERG11* gene encodes the enzyme of 14-α-sterol demethylase which involves in the biosynthesis of the fungal membrane (Cowen et al. 2015; Whaley et al. 2017).

Considering the above exceptional fitness attributes that *Candida* possesses to survive, development of new antifungal drugs are in crucial need as to fulfil the therapeutic resources which is limited in the spectrum of their activity (Shin et al. 2005) and reduce the incidence of drug-resistant fungi. As reported by Clinical and Laboratory Standard Institute, percent of resistant isolates in *C. albicans* and *C. glabrata* were 3.5% and 7.8% respectively (Whaley et al. 2017). One of the effort in developing new antifungal drugs is by utilizing secondary metabolites of natural plant products as a potential source for antifungal drugs with more effective and safe derivatives (Liu et al. 2017). Therefore, the present study was undertaken to isolate and identify the antifungal active alkaloids of *R. angustifolia* and evaluating their potential as gene inhibitors against the virulence genes namely *ICL*, *MLS*, *PCK* and the resistant genes which are *CDR*, *MDR* and *ERG11* genes in *C. albicans* and *C. glabrata*. The plant *R. angustifolia* was chose due to the antimicrobial bioautography-guided isolation of alkaloidal fractions of *R. angustifolia* leaves which led to the identification of a major antimicrobial active alkaloid identified as graveoline, together with arborinine, and the minor alkaloid, skimmianine (Zarisa, 2013; Zarisa et al. 2018). Belongs to the family of Rutaceae and genus of Ruta (rue), *Ruta angustifolia* is a main source of alkaloids derived from anthranillic acid precursors that promotes a pharmacologically active compound with antiasthmatic, anticancer, antibacterial and antifungal activities (Adamska-Szewczyk et al. 2016). The mechanism by which *R. angustifolia* mediate their biological activities has not been studied in great detail. Previous study on furoquinoline and pyranoquinolone alkaloids from Rutaceae have indicated that it targets a

specially designed 1.5kb DNA fragments, but the precise mechanism by which it inhibits which enzyme has not been established (Hanawa et al. 2004).

In the present study, phytochemical screening was first applied through bioassay guided isolation of antifungal active alkaloids of *Ruta angustifolia*. This bioassay is a simple and easy technique to localize and isolate which alkaloid contained in the crude extract that exert an antifungal activity. Once isolated and elucidated, the targeted alkaloids were further identified by certain phytochemical analysis. The identified antifungal active alkaloids were then subjected to broth microdilution assay to quantify the antifungal activity. Lastly, the selected potential target genes of *C. albicans* and *C. glabrata* were screened using the antifungal active alkaloid compound through quantitative real-time inhibitory polymerase chain reactions (qRT-PCR) and western blot to observe the up and down regulation of the genes and effects on its protein respectively.

1.3 Hypothesis

The anthranillic acid derived alkaloids of various structural types from *Ruta angustifolia* (L.) Pers. may possess antifungal activity and might downregulate the selected virulence and resistant genes and reduce CaIcl protein expression.

1.4 Objectives

General Objective:

This study is purposely done to identify the antifungal active alkaloids of *R. angustifolia* and to investigate its effect on virulence gene expression of *C. albicans* and *C. glabrata.*

Specific Objectives:

1) To extract and elucidate the antifungal active alkaloids isolated from the leaves of *Ruta angustifolia* (L.) Pers.

2) To determine Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of antifungal active alkaloids derived *Ruta angustifolia* against *C. albicans* and *C. glabrata*.

3) To investigate the effect of the isolated antifungal active alkaloids on the expression of virulence genes (*PCK1*, *ICL1*, *MLS1*,) and resistant genes (*ERG11*, *CDR1*, *CDR2* and *MDR1*) using qPCR analysis.

4) To examine the impact of antifungal active alkaloid derived *Ruta angustifolia* on *C. albicans* (Caicl1) protein using western blot.

CHAPTER 2.0: LITERATURE REVIEWS

2.1 Plants as Potential Source of Antifungal Agent.

Nature is the source of medicine, and a huge number of modern drugs have been isolated from the natural sources used in traditional medicine (Ghorbani et al. 2006). In Peninsular Malaysia itself, more than 1300 medicinal plant species have been document (Sultana et al. 2014) and most of them have involved in multidimensional study combining botanical, phytochemical, biological, and molecular (Balunas & Kinghorn 2005). It has been estimated that 14 to 28% of higher plant species which are traditionally used as medicine, 74% of those plant are pharmacologically active (Ncube et al. 2008). Plus, around 119 bioactive compounds from 90 plants were originally used as single entity medicinal agent, which means a specific secondary metabolite were extracted and are utilized with established application for instance caffeine is use as stimulant (Pan et al. 2013). Out of this 90 plants, 77% were initiate by ethno medical screening (Ghorbani et al. 2006).

Antifungal drugs originated from natural sources were found to be significantly increasing due to the treatment failure or recurrence fungal infections reported in oral (Darwazeh & Darwazeh 2014) and vaginal candidiasis (Belayneh et al. 2017). Besides, increasing incidence of drug-resistance also has led to the challenges in prevention and treatment of fungal infections (Hani et al. 2015). Based on ethnopharmacognosy research, a wealth of experience on the application of herbal in drugs discovery is due to its long-term history of utilization by human and are likely to be safe and less adverse effect (Sasidharan et al. 2011). In addition, as human beings eat both plants and

meats, plant-derived medicines may interact favourably with the human body and hence produce beneficial effects in terms of health promotion (Pan et al. 2013). The unlimited source of significant, complex and stable chemical structures that plants provided would never be the subject of synthetic establishment. Thus, the structural diversity that is still largely untapped can be guaranteed (Veeresham 2012).

Another reason that have increased the importance of medicinal plants is that the plants are relatively cheap source (Arif et al. 2009). Compared to average prescription drug price, herbal medicines are more affordable to the lower income group. However, some herbal products are quite expensive nowadays because consumers are also paying for the so-called research and development by the company involved in synthesizing the active compounds from its main ingredients (Krause & Tobin 2013). This also contribute to the availability of information about herbal medicines and have increased the trend of consuming them. Since 1997, herbal medicine sales in the United States reaching an estimated total of about US$3.24 billion (~RM12.3 billion). Malaysians spent about RM2.0 billion on herbals which amounts to about RM45.00 spent on herbals per person per year in the United States by comparison to about RM91.00 per person per year in Malaysia taking into account populations of 273 million and 22 million respectively. This figures the potential of the herbal market in our country (Hussin 2001).

2.2 Major groups of Antifungal Compounds from Plants

As biological material, plant is carrying a vast variety primary and secondary metabolites which can be selected as the molecule with desired biological activity (Arif et al. 2009). Primary metabolites in plants is fundamentally synthesized through pathways that generally modify and produce carbohydrates, proteins, fats, and nucleic acids that governs all basic physiological that allow plant growth, development and reproduction (Nwokeji et al. 2016). In contrast, secondary metabolite basically derived through several interacting metabolic pathways which also depends on primary processes which include tricarboxylic acid cycle (TCA), methylerithrotol phosphate (MEP) pathway, mevalonic and shikimic acid pathway (Wang et al. 2016). There are three groups of secondary metabolites namely Terpenes, Phenolic compounds and Nitrogen-containing compounds. Note that, the importance of these metabolites are due to their role in antimicrobial, pharmaceuticals, plant defence against herbivory, fragrance, stimulants, toxicity, attractant, plant breeding, physiological stress response, and allelopathtic effect (Nwokeji et al. 2016). They were also probably evolved from plants purposely for chemical defense against predation or infection (Satish et al. 2008). Of all the three classified group of the secondary metabolites, our focus is on the nitrogen-containing compound which is alkaloid. The plant of interest in the present study which belongs to *Rutaceae* family, has been a great source of anthranillic acid derived alkaloids with biologically active antibacterial and antifungal properties (Adamska-Szewczyk et al. 2016).

A few studies have been carried out on the antifungal activity of alkaloids of natural origin. These include plant-derived aporphinoid alkaloids

namely eupolauridine and liriodenine that exhibit potent inhibitory activity against the opportunistic fungal pathogens *Candida albicans* and *Cryptococcus neoformans* (Tripathi et al. 2017). Another isolated alkaloid was successfully identified as 2-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1-methylethylpentanoate from the plant *Datura metel*, showed a great activities both *in vitro* and *in vivo* against *Aspergillus* and *Candida* species (Dabur et al. 2005). An isoquinoline alkaloid known as berberine was also reported to be an antifungal alkaloid against *C. albicans* and non-albicans *Candida* species with variable susceptibilities. Notably, berberine also was reported to be non-toxic to human cells (Dhamgaye et al. 2014).

2.3 *Ruta angustifolia* (L.) Pers

The Rutaceae family has about 140 genera, grow in all parts of the world consisting of herbs, shrubs and small trees (Riyanto et al. 2001; Sandjo et al. 2014). Belongs to the family of Rutaceae and genus of *Ruta* (rue), *Ruta angustifolia* (L.) Pers, **Figure 2.1 (a)** is a medicinal plant which originates from Southern Europe and North Africa. According to CRC World Dictionary of Medicinal and Poisonous Plant, this plant was identified by Swedish biologist, Carolus Linnaeus in 1805 and later by Christiaan Hendrick Persoon (2010). *R. angustifolia* is said to be the diffusion of other species which is *R. chalepensis*. Significantly, this species has been abundantly consumed in the most ancient systematic record of medical practice of the Mediterranean world (Pollio et al. 2008).

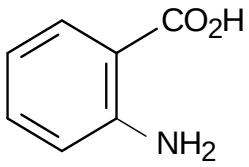
Figure 2.1 (a) *Ruta angustifolia* (whole plant) and **(b)** (The leaves)

The plant often grows up to 1.5 m height with slender woody stems and light green leaves with very strong foetid odour (Richardson et al. 2016) . The leaves of *Ruta angustifolia* **Figure 2.1 (b)** are in spiral arrangement crenate, narrow or oval leaves, 2–3 times divided into segments oblong and ciliate-fringed sepals. It is well grown on preferably well-drained calcareous clayey soil under fair dry conditions and partial shade (Kannan & Babu 2012).

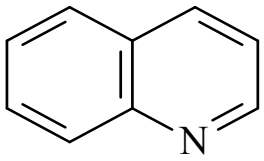
This plant is used traditionally for treating snake bites and parasites, stomatitis, rheumatism and bronchitis (Sandjo et al. 2014). In many parts of the world, the leaves will be first heated to boil before they consumed or used it as medicine. Based on this healing ability, several pharmacological activities were later proved by this plant includes antioxidant, analgesics, anti-cancer (Shuib et al. 2015; Richardson et al. 2016) and antifungal activities (Ncube et al., 2008). Therefore, it is necessary to focus and develop bioactive compounds of this plant as a potential drugs target against *Candida.*

2.3.1 Alkaloids of Ruta Species

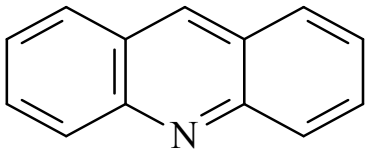
Alkaloids are a group of important secondary metabolite. This substance are presence in more than 15,000 nitrogen containing secondary metabolites which is approximately 20% of the species of vascular plant (Kennedy & Wightman 2011). Nitrogen atom in these substances is usually part of the heterocyclic ring, a ring that comprise both nitrogen and carbon atom (Nwokeji et al. 2016). Alkaloids in the *Ruta* species were completely to be derived from amino acids precursors, anthranillic acid particularly which possess quinoline and acridine skeletons **(Figure 2.2)** (Basco et al. 1994; Nugroho et al. 2010).



Anthranillic acid **(1)**



Quinoline **(2)**



Acridine **(3)**

Figure 2.2 Alkaloids derived from anthranillic acid (1) which gives rise to quinoline (2) and acridine (3) (Riyanto et al. 2001).

Acridine origin, is basically one of the biochemical pathways which involves condensation of anthranillic acid (1) and acetate or malonate **(Figure 2.3)**. The extension of N-methyl anthraniloyl-CoA chain's (4) three malonate units were integrated with lead to form polyketide (5). The Claisen condensation plays a role in nucleophilic addition, dehydration as well as the C-N linkage. This way, acridine skeleton was formed which bring a stable aromatic tautomer (8). Acridone alkaloids are found exclusively in plants belonging to the Rutaceae family and have been proven as anticandidiasis and antiplasmodium agent (Lukačin et al. 1999).

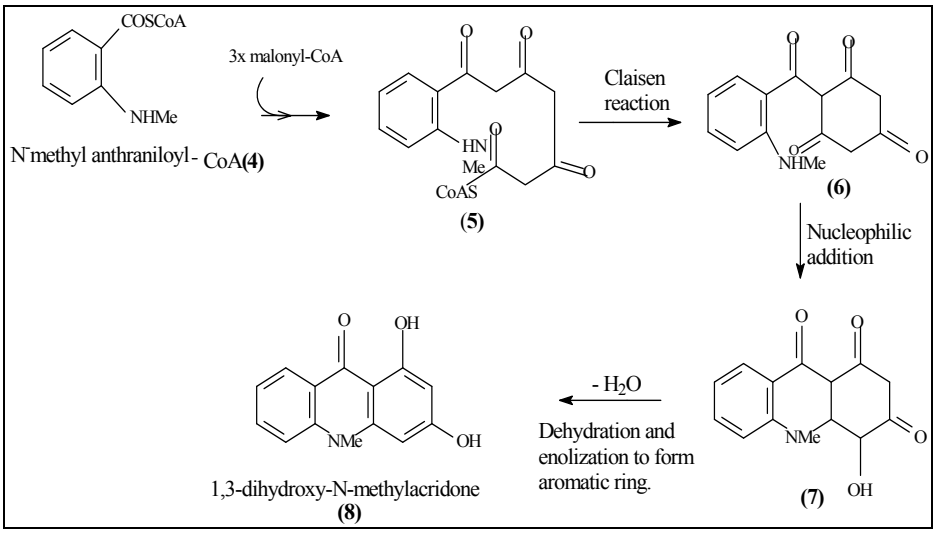


Figure 2.3 Biosynthetic pathway of acridone (Paul 2002).

Anthraniloyl-CoA (9) is the starter unit in chain extension via one molecule of malonyl-CoA. This will lead to amide formation and later produces the heterocyclic system comprising of di-enol tautomer (12). Finally, more stable 4-hydroxy-2-quinolone (13) were formed **(Figure 2.4)** (Paul 2002).

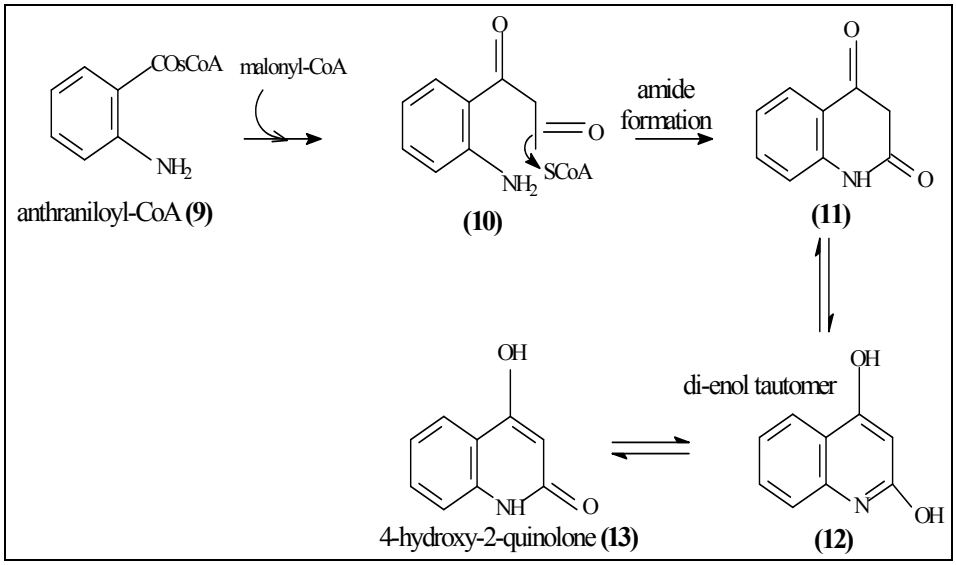


Figure 2.4 Biochemical pathway of 4-hydroxy-2-quinolone (13) (Paul 2002).

Two and 4-quinolones are among the derivatives of rutaceae alkaloids including furoquinoline and acridones. These quinolone alkaloids contribute to more pharmacological active substance isolation which is aryl-, alkyl- as well as alkylarylquinolone/ines. Hydroxyl group on carbons is adjacent to the nitrogen ring in the stable quinolone structure (13). In **figure 2.5**, 2-hydroxyquinolines and 4-hydroxyquinolines were formed through the camps cyclization which depends on exact starting materials and reaction conditions. Pairing of quinolines hydroxyl-substitutions was commonly known as 2-quinolones. 2-quinolones, the oxygen is a neighbour to N-ring while in 4-quinolone, the oxygen located crossly from the N-ring (Adamska-Szewczyk et al. 2016).

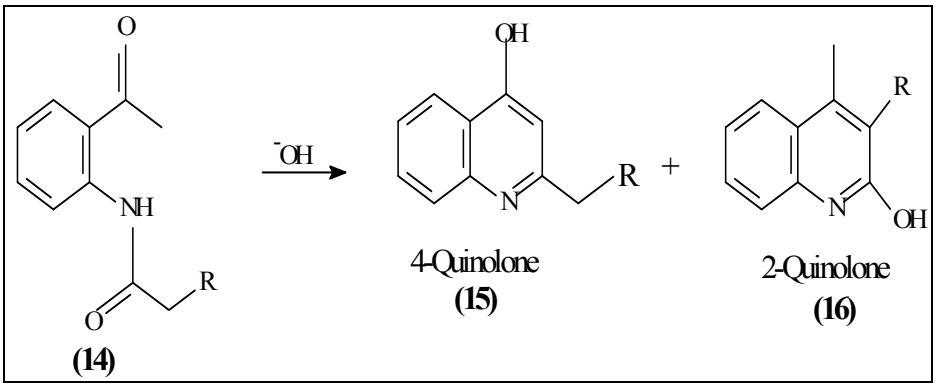


Figure 2.5 Formation of 4-quinolone (15) and 2-quinolone (16) (Abdou 2014).

It is highly nucleophilic by looking at position three of 4-hydroxy-2-quinolone structure. When dimethylallyl diphosphate took place, this is where alkylation is susceptible. As a results additional six- and five-membered oxygen heterocyclic rings will formed. To form furoquinoline alkaloids **(figure 2.6)** for example skimmianine (20), the dimethylallyl derivative will act as a precursor in and undergo an analogous series of reactions (Fournet et al. 1993; Paul 2002).

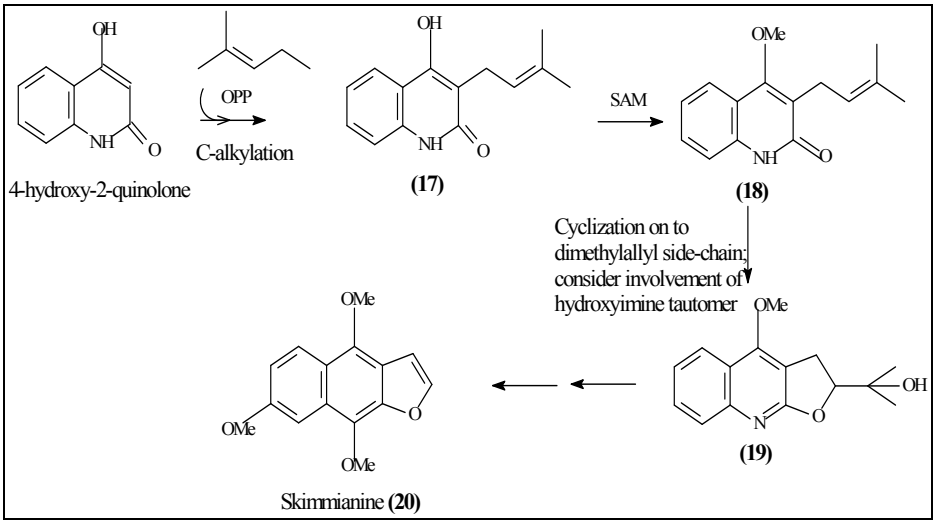


Figure 2.6 Biosynthetic pathway of furoquinoline/skimmianine (20) (Paul 2002).

Two new alkaloids were reported in the literature search, for instance 2-6-(2H-benzo[d]1,3-dioxolen-5-yl)hexyl-hydroquinolin-4-one and 2-{6'(2Hbenzo[d]1", 3"-dioxolen-5"yl)hexyl}-4-methoxy-quinoline. These were yielded from *R. chalepensis*, collected from the northern Saudi desert. Another alkaloids from this plant are dictamnine, pteleine, skimmianine, isogravacridonechlorine, maculosidine, graveoline, graveolinine, and 4-methoxy-1-methyl-2(1H)-quinolinone, and chalepensin (Emam et al. 2010).

2.2.2 Antifungal Active Alkaloids

A lot of studies have been conducted for so many years regarding phytochemical and pharmacological activities of materials extracted from members of the Rutaceae family (Adamska-Szewczyk et al. 2016). In such work, anthranillic acid derived alkaloids of the *Rutaceae* is taking one of the most attended due to their widened variety of medicinal application for instance antimicrobial, antiviral, antitumor and also analgesic effects (Sandjo et al. 2014). The literature search of this study has revealed a few Rutaceaea alkaloids which have completed phytochemical analysis, isolated and fully identified with antibacterial and antifungal properties. The focus is more towards the bioactivity of acridone, furoquinolines and 4-quinolone alkaloids.

Previously isolated alkaloids from *R. angustifolia* includes dictamnine, skimmianine, kokusaginine (Adamska-Szewczyk et al. 2016), berberine (Alam et al. 2011), rutaverine, arborinine, rutin, xanthotoxin, fagarine and graveolinine (Strigácová et al. 2000). The alkaloid berberine produced by *Ravenia spectabilis* from Rutaceae has an inhibitory activity against

C. albicans (Pepeljnjak & Petricic, 1992). Two fractions containing skimmianine and arborinine obtained from *R. angustifolia* of hexane and acetone extracts were screened for antifungal activity and reported to exhibit activity against *C. albicans* (Zarisa, 2013).

Besides *Ruta angustifolia*, *Ruta graveolens* is another popular species among *Rutaceae* family native to Mediterranean region but widely distributed all over the tropical regions. The searched for their antimicrobial activities were based on ethanolic, methanolic, chloroform and water extracts from the stem of the plant. By implementing agar-disc diffusion assay, each extract were then tested for anti-fungal activity against *Aspergillus niger*, *Aspergillus. flavus*, *Penicilium crysogenum*, *Rhizopus stolonifer* and *Fusarium oxosporium*. Out of the four extracts, ethanolic stem extract demonstrated the most pronounced inhibition of growth while the rest showed moderate antifungal activity except against *Flasparum oxosporium* (Pandey et al, 2011).

2.4 Virulence and Pathogenicity of Fungal Pathogen

Candidiasis is a common fungal infection that generally inhabit and localized the skin, fingers, oral cavity, esophagus, bronchi, lungs, gastrointestinal tract and vagina (Calderone et al. 2014). The most frequently identified species are *C. albicans* (70%) and *C. glabrata* (7%) (Pfaller et al. 2002). The condition is usually benign in healthy individual but can become infectious with widely range clinical syndrome from non-life threatening mucocutaneous illnesses to invasive progressions of bloodstream if immune

system are impaired or environmental niche of the host are disturbed (Pappas et al. 2004). Environmental niche is basically the local environment of the host which includes the interaction of normal microbiome that interrelate with hormones, diet and immune responses. There are presence of symbionts, which is the microbes that support health, commensals with no actual benefits to the host and pathobionts, a permanent resident with possibility to become pathogenic. In HIV-infected patients for instance, they often have low levels of *Bifidobacteria* and *Lactobacilli* species in the gut which allowed *C. albicans* to inhabit and become pathogenic (Salas & Chang 2014).

The ability of Candida to colonize the epithelial surfaces and to adapt the tissue environment of the host are prerequisite for pathogenesis development (Kobayashi et al. 2006). There are two types of infections which are superficial and invasive candidiasis. Superficial infection, include oral and vaginal thrush and also chronic mucocutaneous candidiasis. This type of infection is usually non-lethal, but still it significantly lowers the quality of life (Bondaryk et al. 2013).

Invasive candidiasis on the other hand normally occur once epithelial tissues are breached and enter the blood stream which termed as candidemia (Perlroth et al. 2007). This will further lead to systemic candidiasis, characterized by haematogenous spread to one or more vital organ such as kidney, liver, spleen and brain (Ahmad et al. 2010; Hashim et al. 2012). Severity of candidiasis depends on factors such as size of inoculum, ability of fungi to multiply in the tissue, magnitude of tissue damages and immune status of the host cells (Sajjad et al. 2010). A published literature has suggest that inoculum size of the cells in patients with candidemia is 10^4 CFU/ml and

10^3 CFU/ml of blood for *C. albicans* and *C. glabrata* respectively (George et al. 2005).

Dimorphic properties of *Candida* allow them to be able to multiply in the host tissue whereby transition between yeast and filamentous forms is often stimulated by growth within the host and can be observed in infected tissues during infection (Mayer et al. 2013; Chin et al. 2016). Magnitude of tissue damage is also due to this filaments formation in which they can escape from macrophages killing (Chin et al. 2016). *C. glabrata* on the other hand, is strictly haploid and normally grows only in the yeast form (Kaur et al. 2005). Immune status of the host cells also contributed to the severity of the infection where in normal condition, *Candida* will be phagocytosed by macrophages and neutrophils that secretes cytokines and induce hyphal development. Thus, in neutropenic patients, deficient in these immune cells will particularly lead to systemic candidiasis (Lorenz & Fink 2001). Overall, these factors contribute to the pathogenicity of fungal infection and often referred to as virulence factor. Virulence factors are of pronounced interest in fungal pathogenesis because they are often the target of the immune response (Casadevall & Pirofski 1999). For example, upon phagocytosis by macrophages, *C. albicans* undergoes a massive metabolic reorganization activating genes involved in alternative carbon metabolism including the glyoxylate cycle and gluconeogenesis (Brown, Brown, et al. 2014) which will be explain later in this chapter. Besides metabolic adaptation, *Candida* also possesses a range of virulence attributes including adherence to epithelial and endothelial cells, proteinase and phospholipase production, hypha and pseudohypha formation, phenotypic

switching, and antigenic modulation as a result of pseudohypha formation (Ahmad et al. 2010).

Like other *Candida* species, *C. glabrata* is a part of the normal microbiota of the mouth, gastrointestinal and vaginal tracts in humans and, in healthy individuals, it does not cause disease. However, *C. glabrata* becoming a cause for disease when there is disturbances in the normal environment especially in immunosuppressed hosts (Rodrigues 2013). However, the virulence factors associated with *C. glabrata*, relative to other pathogenic yeast species like *C. albicans* are poorly understood. Though, there is agreement that pathogenicity of *C. glabrata* could be attributed to its ability to form biofilms, inability to form true hyphae and to secrete certain proteases (Tam et al. 2015; Rodrigues 2018). Instead, *C. glabrata* continues to replicate as yeasts inside the phagosome until the host cell bursts by an unknown mechanism (Seider et al. 2011). Compared with *C. albicans*, *C. glabrata* can sometimes be considered to be less virulent (Fidel & Vazquez 1999).

Candidiasis provide a range of symptom which is depending on the site of infection and the degree of immunosuppression. Patients may be asymptomatic or experiencing dysphagia, odynophagia, retrosternal pain, epigastric pain, nausea and vomiting. If the case of Candidemia, they may have fever, chills, skin rash, fatigue, low blood pressure, muscle aches, vision changes, headaches and neurological deficit (Pappas et al. 2004; Ahmad et al. 2010).

2.5 Metabolism in Pathogenesis of Candida

Candida albicans and *Candida glabrata*, are among the successful pathogen due to their metabolic adaptation on the available nutrients. They are able to multiply under both nutrient-rich and nutrient-poor conditions (Ende et al. 2019). The fundamental essential for the Candida to grow and survive is nutrient and carbon assimilation (Calderone & Fonzi 2001). Once they inhabit a dynamically contrast niches, adaptation in availability of nutrient will take place to enhances the fitness of the fungus. This is very important for pathogenicity as virulence factors, thereby signifying an attractive target for potential therapeutic intervention (Ene et al. 2014). As mentioned previously, *Candida* responded to phagocytosis of macrophages by inducing pathways required for alternative carbon metabolism namely gluconeogenesis, glyoxylate cycle and fatty acid β-oxidation. Obviously, these pathways are important for their survival in the macrophage which try to kill them off by oxidative burst and low nutrient availability (Lorenz & Fink 2001; Rodaki et al. 2009).

The pathway relies on the efficient uptake and metabolism of available carbon sources which include fermentable sugars (such as glucose, fructose, and galactose) and non-fermentable carbon sources (such as amino acids and organic acids) (Fernando & Ludovico 2005). Analysis of fungal cells from different infection models showed that the carbon metabolism pathways are activated in mucosal and intraperitoneal infection models (Thewes et al. 2007) and were thought to reflect the heterogeneous nature of fungal populations in complex host microenvironments, whereby phagocytosed cells that are exposed to carbon starvation activate the glyoxylate cycle, whereas non-

phagocytosed cells retain access to glucose and activate glycolysis (Brown, Brown, et al. 2014).

2.5.1 Glycolysis and gluconeogenesis

Glycolysis is a sequence of reactions in Candida involves the conversion of one molecule of glucose into two-molecules of pyruvate. This is a conserved metabolic pathway, consists of the anaerobic conversion of glucose to pyruvate with the net production of two molecules of ATP **(Figure 2.7).** Pyruvate generated by glycolysis may be fermented to ethanol, or may enter the TCA cycle under aerobic conditions to generate additional energy and biosynthetic precursors (Eschrich et al. 2002).

This pathway, occurs in two phases, consist of phosphorylation of glucose to generate two molecules of glyceraldehyde-3-phosphate followed by conversion of glyceraldehyde-3-phosphate to pyruvate (Barelle et al. 2006). Prior to glycolysis, glucose enters cells and is phosphorylated by ATP to form glucose-6-phosphate. The next step in glycolysis is the isomerization of glucose 6-phosphate to fructose 6-phosphate. Fructose 6-phosphate is then phosphorylated by ATP to fructose-1, 6-bisphosphate (F-1, 6-BP). The second stage of glycolysis begins with the splitting of fructose 1,6-bisphosphate into glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) which are interconverted by triphosphate isomerase. The final phase in the glycolytic pathway involve the conversion of phosphoenolpyruvate (PEP) into pyruvate which is catalysed by pyruvate kinase. The products consist of three-carbon units rather than six-carbon units (Fernando & Paula 2005).

Gluconeogenesis on the other hand is the generation of glucose from non-sugar carbon substrates such as pyruvate, lactate, glycerol, and gluconeogenic amino acids. The process is essentially the reversal of the glycolysis pathway but not the exact reverse of glycolysis, some of the steps are the identical in reverse direction and three of them are new ones **(Figure 2.11)**. The starting point of gluconeogenesis is pyruvic acid, although oxaloacetic acid and dihydroxyacetone phosphate also provide entry points. Notice that oxaloacetic acid is also the first compound to react with acetyl CoA in the tricarboxylic acid cycle (TCA). Two essentially irreversible steps of the glycolytic pathway, which are catalysed by phosphofructokinase and pyruvate kinase, must be bypassed during gluconeogenesis. Fructose-1,6-biphosphate (encoded by FBP1 gene) catalyses the reverse reaction to phosphofructokinase. Meanwhile, phosphoenolpyruvate carboxykinase (encoded by *PCK1* gene) generates phospoenolpyruvate from oxaloacetate thereby passing pyruvate kinase (Eschrich et al. 2002). Therefore, any compounds that can down generate *PCK1* gene, may stop the Candida from growing and inhabit the host.

Both glycolysis and gluconeogenesis take place in the cytosol however, several enzymes of these pathways are also present in the cell wall (Urban et al. 2003). Similar to *C. albicans*, changes in gene expression was found in consistent with severe carbohydrate deprivation, including upregulation of genes in the glyoxylate cycle, beta-oxidation of long-chain fatty acids, and gluconeogenesis after incubation of *C. glabrata* with neutrophils (Fukuda et al. 2013).

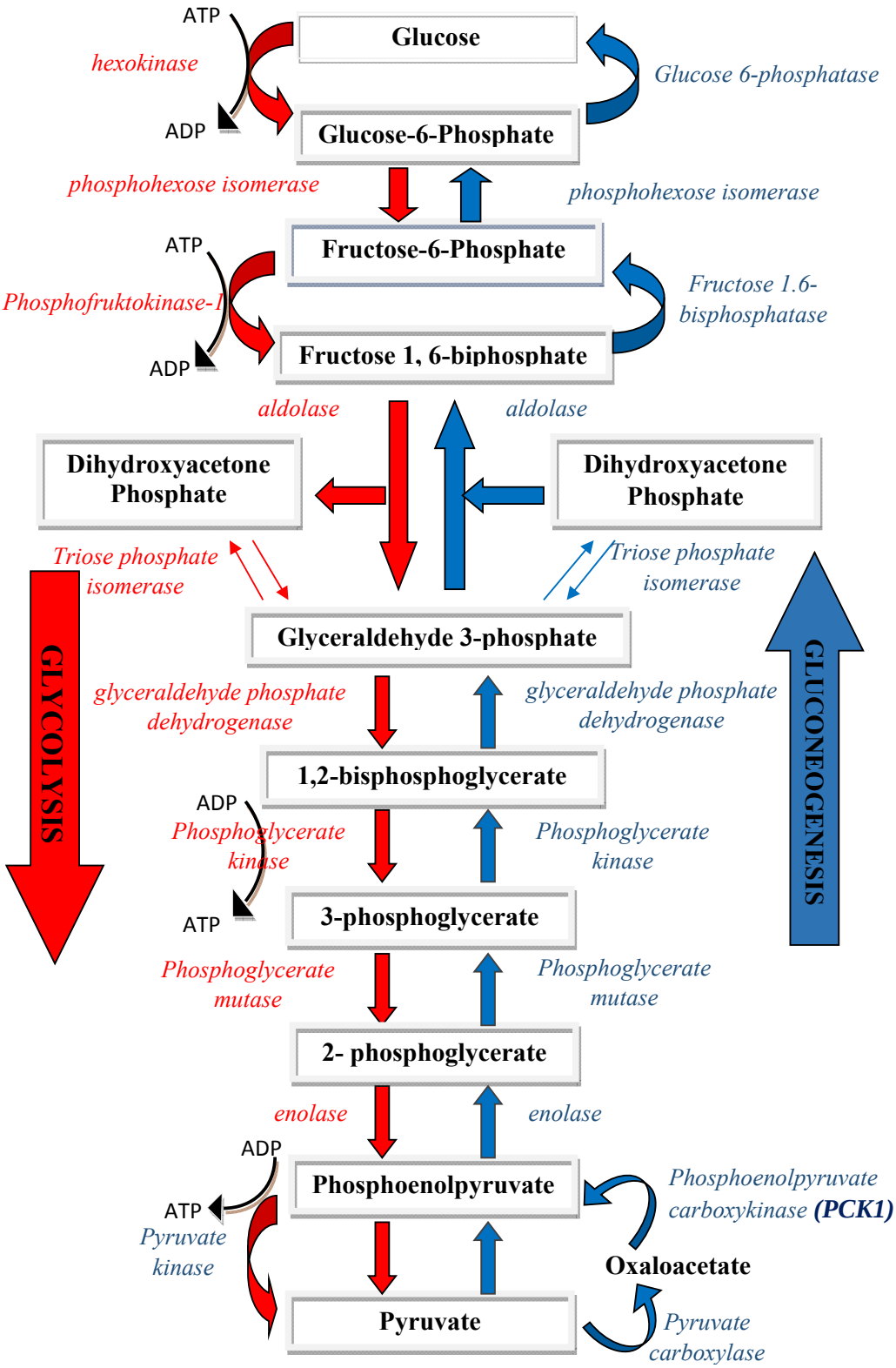


Figure 2.7 Substrates and enzymes involved in Glycolysis and gluconeogenesis pathway in Candida which take place in cytosol (Dwong, 2018).

2.5.2 The Tricarboxylate Acid (TCA) cycle and Glyoxylate cycle

The ubiquitous tricarboxylic acid (TCA) cycle, the second stage of respiration after glycolysis is a central pathway for the metabolism of carbon sources, lipids, and amino acids, and generate a major energy source for the cell under aerobic conditions (Tao et al. 2017). Basically, TCA cycle **(Figure 2.8)** consumes two-carbon units in the form of acetyl-CoA which is formed from pyruvate by the pyruvate dehydrogenase complex under oxidative and non-pressing conditions. This is the part that link between glycolysis and the TCA cycle. Acetyl-CoA and oxaloacetate are catalysed by citrate synthase in the formation of citrate. Citrate is then oxidized by a series of reactions to yield two molecules of CO_2 and one oxaloacetate. The energy generated by this oxidation is mainly occur in the mitochondrial matrix and conserved in the form of reducing equivalences NADH and $FADH_2$ (Kruckeberg & Dickinson 1999; Lee et al. 2011).

The function of TCA cycle is to provide reducing equivalents to the respiratory chain (Lorenz & Fink 2001; Fernando & Ludovico 2005) and also plays key roles in generating metabolic intermediates for various biosynthesis pathways and in catabolising of products from other pathways. These include fatty acid β-oxidation, the catabolism of the amino acids isoleucine, methionine or valine, and the transamination and deamination of other amino acids thereby generating oxaloacetate and α-ketoglutarate (Lorenz & Fink 2001). A recent proteomics study indicates that the TCA cycle is involved in the control of antifungal tolerance and biofilm formation (Li et al. 2015).

However, when *Candida* is grown on two-carbon compounds, such as acetate, the TCA cycle by itself cannot supply adequate amounts of biosynthetic precursors unless alternative reactions are available. Therefore, they employs a modification of the TCA cycle called the glyoxylate cycle **(Figure 2.8)**. In this cycle, two-carbon acetate units are convert into four-carbon dicarboxylic acids by bypassing oxidative decarboxylation during which they are incorporated into new molecules of glucose. The glyoxylate cycle regulates macrophage phagocytosis and virulence of *C. albicans*, whereby disruption of this prevents the growth of *C. albicans* inside macrophages by blocking nutrient availability (Lee et al. 2011).

Following phagocytosis by macrophages or neutrophils of the infected host, cytokines were secreted and hyphal-development occurred in the fungi. The glyoxylate cycle enables fungi to survive and grow in the nutrient-limited environment inside the phagocytic cells by utilizing alternative carbon sources such as lipid and amino acids (Lorenz & Fink 2001). Isocitrate lyase (*ICL1*) and malate synthase (*MLS*) are the key enzymes for this pathway which are highly conserved in bacteria, fungi and plants (Dunn et al. 2009).