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Review article

A review on *Wickerhamomyces anomalus* yeast: a promising eco-friendly approach to biological control of malaria

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ABSTRACT. Malaria is a deadly parasitic disease transmitted by female *Anopheles* mosquitoes. One of the most extensive malaria control measures proposed by the World Health Organization (WHO), which has received better attention in recent years, is the biological control of *Anopheles* mosquitoes. In this concept, *Wickerhamomyces anomalus* yeast has received more attention from researchers in this field. In the present review, we have investigated the anti-malaria effect of *Wickerhamomyces anomalous*. In the present review, we searched PubMed, ProQuest, Scopus, Embase, Google Scholar, Science Direct, and Wiley databases for relevant articles. Keywords used in the inquiries were biological control, yeast, *Wickerhamomyces anomalus*, malaria, *Anopheles* mosquito, and *Plasmodium*. *Wickerhamomyces anomalus* has a wide range of anti-microbial activity. By producing killer toxins (KT), this yeast can kill microorganisms, so it has called killer yeast. This was investigated and proven using monoclonal antibody, western blot analysis and immunofluorescence (IFA) technique. It has also been used in various studies regarding the biological control of malaria by killing *Anopheles* mosquito larvae. Considering the proven lethal effect of toxins produced by *Wickerhamomyces anomalus*, the results could be a big step forward towards ending the life cycle of malaria parasites in the body of vector mosquitos.

Keywords: biological control, fungi, malaria, *Wickerhamomyces anomalous*

Introduction

Malaria being the most important parasitic disease, especially in tropical countries, is caused by parasitic protozoa species of the genus *Plasmodium* and is transmitted by female *Anopheles* mosquitoes [1,2]. Malaria kills millions of people worldwide with approximately 350 million affected cases and 405,000 deaths each year. These epidemic diseases that have created many problems and concerns for public health around the world are all transmitted by mosquitoes [3–5]. One of the ways suggested by WHO to prevent malaria is controlling the disease around the world. Despite the use of anti-malarial drugs such as chloroquine, pyrimethamine, primaquine, sulfadoxine, and pyrimethamine, the emergence of drug resistance is an obstacle in controlling the disease [6,7]. On the other hand, the side effects of these drugs have also reduced their use for treatment. Another problem is the increased insecticide resistance in mosquito vectors [8]. Therefore, many research teams have been formed around the world to find appropriate solutions and strategies with greater effectiveness for malaria control and elimination [6,9]. Disrupting the transmission of pathogens from mosquito vectors to

humans is a compelling route to controlling and preventing the disease [10,11].

One of the most important strategies to achieve this goal is to fight *Anopheles* mosquitoes. There are three ways to do this; physical, chemical, and biological struggles. The biological combat against mosquitoes, sterilization of mosquitoes; use of larval-eating organisms; the destruction of larvae by bacteria, fungi, and protozoa that has attracted more attention than ever before [12,13]. The mosquito microbial symbionts have a crucial role in the immunological and physiological processes of insects. Additionally, they remarkably affect the transmission of the pathogen and are an effective means of combating malaria as well as other mosquito-borne diseases. Therefore, among biological struggles, symbiotic control (SC) has been suggested as a significant approach to defeating malaria [1,13,14]. The yeast *Wickerhamomyces anomalus* (formerly *Hansenula anomala* and *Pichia anomala*) isolated from *Anopheles* mosquitoes, resident in mosquito gut and gonads, produces natural anti-microbial secretions (KT) which can act as a disinfectant in the mosquitoes [4,9,15].

In a study by Mateo [16] after purifying the killer toxins of *Wickerhamomyces anomalus* strains isolated from *Anopheles stephensi*, an effective and potent anti-plasmodial effect against this parasite was observed in the culture medium. Although in this study it was noted that further research is needed, it has opened up new perspectives on the possible use of lethal strains in innovative strategies against the development of malaria parasites in vector mosquitoes using mosquito microbial symbionts [14,17]. Therefore, in the present review, we aimed to evaluate the anti-plasmodial effect of the yeast.

Methods

The scientific databases PubMed, ProQuest, Scopus, Embase, Google Scholar, Science Direct, and Wiley were searched for all the articles in the English language on the subject from database inception 1990 to 2021 by two researchers independently. After the screening of records obtained from these searches, full-text articles were also surveyed. We also applied the key concepts (alone or in combination) *Plasmodium*, Infection, Remittent fever, Marsh fever, Prophylaxis, Preventive therapy, Prevention and control, Prevention, Insect control, Vector-borne diseases, Protozoan infections, Anti-malaria, *Wickerhamomyces anomalus*, Symbiotic, Fungal, Fungi. Furthermore, to provide a more complete search strategy, we manually reviewed the reference lists of selected articles and related reviews. Finally, selected 2170 studies for this review. After screening, 9 papers were eligible as an anti-malaria effect of *Wickerhamomyces anomalous*.

Results

Symbiotic associations

The development of symbioses between microbial symbionts and many invertebrates, especially insects, has occurred to expand their environmental conditions in unfavorable environments. Symbiotic relationships in insects are very broad and often mutualistic. For example, fungi provide nutritional supplements for insects in return for a suitable habitat from the host. It is interesting to know that various types of yeasts form a wide range of symbiotic associations with insects, including bees, beetles, waps, and lacewings [18,19].

For instance, lacewings provide the amino acids needed in their diets through yeasts resident on crops, or termites use fungi to destroy dead plants. In some cases, beetles use fungal enzymes and yeasts to destroy the woody parts of plants and also remove tobacco toxins. As a matter of fact, the dependence of vertebrates on microbes is due to various metabolic functions, including detoxification of compounds and the synthesis of amino acids, lipids, vitamins, sterols, and pheromones [20,21].

Over the past decade, the microbe's resident in the mosquito gut has been extensively investigated because of their possible involvement of them in transmitting the disease. Therefore, researchers have proposed paratransgenesis (the genetic manipulation of insect symbiotic microorganisms) as an innovative way to control insect-borne diseases [22,23].

This method is more practical than the genetic manipulation of mosquitoes which had been previously suggested. There is little information about the yeast strains associated with mosquitoes because most of the information in this field is related to bacterial microorganisms such as *Wolbachia* [22].

Several studies conducted on the bacterial microbiota of mosquitoes have demonstrated that most bacterial symbionts inhabit the midgut of

mosquitoes. However, these microorganisms may also be present in other organs and tissues including salivary glands, reproductive organs, head, muscle, and Malpighian tubules [24]. In addition to Gram-negative bacteria including *Acinetobacter*, *Aeromonas*, *Asaia*, *Pantoea*, *Pseudomonas*, and *Serratia* are the most prevalent genera found in vector mosquitoes, *Comamonas*, *Elizabethkingia*, *Enterobacter*, and *Klebsiella* are also frequently found in *Anopheles* [25]. The intracellular *Wolbachia* is another genus, which lives in the mosquito non-gut tissues [26].

In the case of malaria, there have been few studies on the role of mosquito gut bacteria in the growth or non-growth of *Plasmodium* protozoa in insects. Many midgut symbiotic bacteria of some malaria vector mosquitoes have been studied, but their role in malaria transmission has not yet been properly investigated. Apart from bacteria, the other microorganisms inhabiting the mosquito gut, including yeasts, are largely unknown. In 1996, researchers isolated some candida species from different species of mosquitoes. Recently, several genera of *Candida* and *Pichia* have been detected in the middle intestine of the *Aedes aegypti* species, a mosquito species that transmits several viral infections including dengue fever and yellow fever [27,28].

Mosquitoes need to boost their nutrition to have a better diet. Male mosquitoes nourish exclusively on flower nectar and fruit juices, whereas in female mosquitoes, blood-feeding is a behavioral adaptation as well as essential for completing the gonotrophic cycle. On the other hand, due to the insufficient digestive enzymes in the mosquito body, the presence of microorganisms in the digestion process seems necessary to help the proper nutrition of female mosquitoes for fertility.

Wickerhamomyces anomalous

The yeast *Wickerhamomyces anomalous*, reported as an opportunistic pathogen in humans has been investigated for many years because of its extensive biotechnological potential, especially in food industry applications. Also, the production of deadly toxins (mycotoxins or killer toxins) in this yeast due to its anti-microbial activity, has attracted a lot of attention [29,30].

It has been revealed that *Wickerhamomyces anomalous* species, known as „killer" yeast, is highly competitive in the environment as well as tolerant to environmental changes so that it can withstand temperatures of 3° to 37°C and pH values of 2.0 to 12.0. Interestingly, the isolation of different strains of *Wickerhamomyces anomalous* even from insects demonstrates the diversity of its habitat [31,32].

The relationship between this yeast and four mosquito species – *Aedes aegypti*, *Aedes albopictus*, *Anopheles gambiae*, and *Anopheles stephensi* – from different geographical areas was confirmed by using culture-dependent and independent methods. However, further investigations were needed to clarify this subject. So, the stable presence of the *Wickerhamomyces anomalous* yeast in the mosquito organs and tissues including in the female midgut and the reproductive systems of male and female mosquitoes, indicating multiple transmission patterns, was revealed by transmission electron microscopy, fluorescent in situ hybridization, and PCR methods [27,33].

Yeast analysis in Anopheles stephensi

Previous studies have shown that mosquito tissues are a favorable environment for this yeast. Therefore, to detect yeast *anomalous*, an analysis was performed using molecular and culture-dependent methods. Ricci and Daminani [27] reported that 22 of the 34 cases of polymerase chain reaction (PCR) analyses in insects, according to the 18S rRNA gene sequence, were phylogenetically related to *Wickerhamomyces anomalous* species. This fungus species was widely identified among insects, especially in male and female mosquitoes, and it was found that the fungus can specifically form a stable symbiotic relationship with *Anopheles stephensi* [27].

In this study, to further investigate the presence of *Wickerhamomyces anomalous*, the bred insects were examined by the two specific tests of PCR and fluorescence in situ hybridization (FISH). The stages of prepuberty and puberty were tested in both sexes of mosquitoes and the results confirmed the presence of *Wickerhamomyces anomalus* throughout the growth stages of mosquitoes and in adult mosquitoes as well. The presence of *Wickerhamomyces anomalous* was confirmed using PCR methods in 69% of the tested samples. Detection of the yeast in 30 larvae, 29 pupae of 45 specimens, and 107 of 150 adult mosquitoes were very remarkable.

To find out whether the yeast could enter the larval mosquito habitats and be eaten by them, the water from a larval habitat was collected and

examined by PCR; however, negative screening results were reported. So, to identify the exact location of the yeast in the mosquito body, mosquito tissues were examined, and the results of this study indicated the presence of yeast in the intestines, glands, and sexual organs, particularly in both ovaries, the male reproductive system including the testes, secretory ducts, and salivary glands [27,28].

As a result, the presence of yeast in the larvae can be due to vertical transmission from mother to offspring. Analysis of newly emerged and non-blood-fed mosquitoes, by using fluorescence in situ hybridization assay, showed a weak fluorescence signal compared with blood-fed mosquitoes, which supports the hypothesis that the yeast uses nutrients in the mosquito diet (Fig.1).

Anti-fungal and anti-parasitic activities of yeast

One of the characteristics of *Wickerhamomyces anomalous*, which is very common in yeasts, is its broad-spectrum anti-microbial activity. Killer toxins (KT) are produced by the yeast *W. anomalous*, which kills some microorganisms. Moreover, several mechanisms of growth inhibition by this yeast, such as competition for nutrient uptake and ethyl acetate production, have also been reported [34].

KTs (killer toxins) are a group of variable molecular weight glycoproteins, whose activity is influenced by temperature and pH. The anti-microbial activities of *Wickerhamomyces anomalous* KTs, such as growth inhibition of *Penicillium* spp., *Aspergillus* spp., Entero-bacteriaceae spp. on cereal grains, and *Botrytis cinerea* on fruits, can make this yeast a valuable biological inhibitor (a bio-control agent). Although these inhibitory mechanisms are still unknown, KTs of *Wickerhamomyces anomalous* (WaKTs) probably play a major role [35,36].

Importantly, it has also been shown that these toxins have an inhibitory effect on some treatment-resistant fungal strains including *Candida albicans*, *Yarrowia lipolytica*, and *Saccharomyces cerevisiae* as well as protozoan parasites *Leishmania infantum*, *Leishmania major*, and *Acanthamoeba castellanii* [37,38]. One of the known mechanisms of action of the killer toxins (WaKTs) is the interaction with cell wall carbohydrates, where glucan is hydrolyzed to glucose [39]. Also, the effective enzymatic activity of WaKTs, marine yeast *Wickerhamomyces*

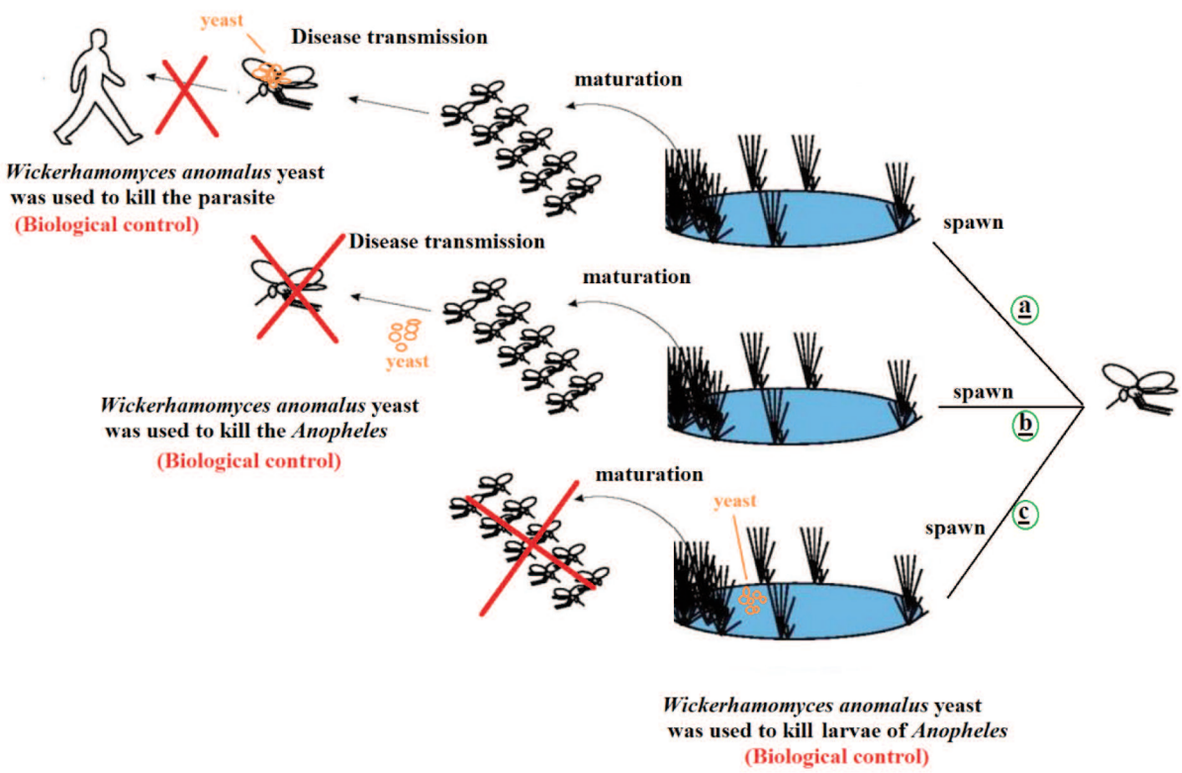

Figure 1. Different mechanisms of the *Wickerhamomyces anomalous* to biological control of malaria. a. the fungus can prevent the transmission of malaria to humans by killing the *Plasmodium* parasite in mosquitoes; b. the fungus can prevent the transmission of malaria to humans by killing *Anopheles* mosquitoes; c. the fungus can reduce the human transmission of malaria by killing the larvae of the *Anopheles* mosquito

anomalous YF07b, against fungal pathogens in crabs has been reported [40]. On the other hand, studies have shown a mutualistic relationship between beetles and yeast due to a nutritional dependence on the coleopteran *Doubledaya bucculenta* [41].

So surprisingly, the symbiotic fungus *Wickerhamomyces anomalous* has both protective and nutritional functions. The results from previous studies have shown that among insects, *Wickerhamomyces anomalous* inhabits the inner body of *Drosophila* sp., and malaria vector *Anopheles* mosquitoes, which is very important in terms of public health. Although the presence of the yeast (strain WaF17.12) in the midgut and reproductive system of *Anopheles stephensi* has been confirmed, the mutualistic relationships between *Anopheles* mosquitoes and *Wickerhamomyces anomalous* certainly need further studies, to investigate the possibility of the notable importance of the yeast in controlling malaria, a symbiotic control [17].

Anti-plasmodial activities of WaF17.12

Following the discovery of the *in vivo* anti-plasmodial activity of WaF17.12 killer toxin against various developmental stages of *Plasmodium berghei*, especially sporogonic stages, there was a question of whether *Wickerhamomyces anomalous* could directly affect the parasite development or not. Therefore, to investigate direct interactions between WaF17.12 and the murine *Plasmodium berghei*, *in vitro* and *in vivo* experiments were done by Cappelli [42]. Immunofluorescent assay (IFA), chromatography, and western blot using the yeast killer toxin specific monoclonal antibodies (mAbKT4) were used to confirm the presence of activated yeasts, under stimulating conditions to produce the killer toxin, in the mosquito gut and also to prove killer toxin secretion from the yeast *Wickerhamomyces anomalus* [17,43].

Furthermore, the MTT colorimetric assay was carried out to assess the cell viability of murine cell lines treated with WaF17.12-KT+ (killer toxin producer). Since treatment with WaF17.12 led to a reduction in the number of parasites (about 37.1%) and there was no significant difference between WaUM3 (an environmental strain of *Wickerhamomyces anomalous* that is not able to produce KT) and control, the effect of *Wickerhamomyces anomalous* on *Plasmodium berghei* sporogonic stages development was deduced to be strain-dependent and correlated to the KT content in the medium [17].

Treatment with WaF17.12-KT+ caused a change in the morphology of ookinetes (serrated and bigger shapes) as well as cell damage and death which was due to the direct effect of KT on *Plasmodium berghei* sporogonic stages (Fig.1). It is interesting to note that the anti-plasmodial activity of the WaF17.12-KT+ was specific. The findings of this study brought about supplementary *in vivo* investigations on anti-plasmodial activities of WaF17.12 strain in *Anopheles stephensi*. Furthermore, it was found that the WaF17.12 strain surely resides in the gut of *Anopheles stephensi*, as a symbiont.

For this reason, no signals were detected in the mosquitoes treated with WaUM3 or in the control group. It is worth noting that the development of mosquitoes containing WaF17.12 was 65.2% less than the control group. Studies have also shown that a mosquito diet supplemented with activated WaF17.12 cells affects the development of ookinete in the *Anopheles stephensi* midgut. Besides the anti-sporogonic function, the inhibitory action of purified WaF17.12-KT on the erythrocytic stages of *Plasmodium berghei*, led to a decrease in parasitemia in mice (without any side effects on murine cell lines) and was also discovered [17,44].

Effect of Purified KT on parasite morphology

To determine the effects of KTs produced by *Wickerhamomyces anomalus* on the sporogonic stages of *Plasmodium berghei in vivo*; these toxins were purified by using various methods such as gel filtration chromatography and HPLC. To evaluate the results, ion-exchange chromatography analysis was used, and finally, it was found that KTs secreted by both strains WaF17.12 and WaATCC 96603 of *Wickerhamomyces anomalous* have anti-plasmodial activities against the sporogonic development of *Plasmodium berghei*. In fact, a decrease in the growth of the ookinetes was observed after their exposure to the yeast killer toxin. It is noteworthy that, this anti-plasmodial activity of the KTs was dose-dependent, and as soon as the *Plasmodium berghei* parasites were exposed to lethal toxins, lower fluorescence signals were detected in the sporogonic stages, indicating morphological changes in parasites [17,45–47]. These morphological changes including irregular cell shapes and toothed cell boundaries, pale cytoplasmic areas, discontinuity of crystalloids, pale and unspecified cytoplasmic granules, were not

observed in the control group parasites (non-exposure to TK).

Optimal conditions for killing activity of KT

The yeast *Wickerhamomyces anomalous* secretes various KTs which are characterized by their molecular weights. The production of various toxins leads to the expansion of the optimal pH and temperature ranges as well as the development of the broad-spectrum anti-microbial activity. An optimal temperature and pH of 16°C and 3.5 were reported for the killing activity of the purified killer toxin of *Wickerhamomyces anomalous* YF07b (a marine-derived yeast) with a molecular mass of 67.0 kDa [47].

The toxin was stable at temperatures less than 40°C and pH less than 6.5, but its lethal activity decreased rapidly above 40°C and disappeared completely at 50°C. Also, it was found that the incubating of the killer toxin in the presence of 4.0% (w/v) NaCl can significantly increase its killing activity [48]. It is worth underlining that another killer toxin of strain YF07b (47.0 kDa) showed the most action at pH 4.5 and a temperature of 40°C [40]. On the other hand, the high stability of the killer toxin of *Wickerhamomyces anomalous* NCYC 432 (47.0 kDa) at pH values between 3 and 5.5 and up to 37°C was revealed [48].

KT mechanism of action

WaKTs via their β-glucanase-mediated mechanism of action exerts extensive anti-microbial activities by targeting the cell-wall glucans of bacteria, yeasts, and protozoa or in other words, by detecting specific membrane receptors on target cells [49]. The killer toxins cause cell death by using a two-step mechanism. Initially, these protein molecules bind to primary receptors, cell-wall glucans in target cells, then move to secondary receptors on the plasma membrane, resulting in osmotic lysis and finally cell death [50].

Results from studies revealed a reduction in the killing activity of killer toxins of WaF17.12 and WaATCC 96603 treated with castanospermine and/or Ni2+ (two β-glucanase inhibitors), indicating the effective and important role of β-glucanase in surface membrane damage of *Plasmodium berghei* parasite in sporogonic stages [42,49,50].

As a matter of fact, the interaction between WaKTs and β-glucans located on the surface of *Plasmodium berghei* cells results in the strong inhibition of *Plasmodium berghei* development from gametocytes to ookinetes. Additionally, as a result of the killer toxins binding to specific receptors, channels are created on the cell membrane, which causes the leakage of cellular contents [49–51].

In conclusion, the killer toxins produced by *Wickerhamomyces anomalous* and their lethal effect on malaria parasites within vector mosquitoes were verified by western blot and IFA analyses using the monoclonal antibody (mAbKT4), and killing activity tests. The obtained results could be a major step in the interruption of the life cycle of malaria parasites within vector mosquitoes. Western blot analysis showed that as a result of *Wickerhamomyces anomalus* attack on malaria vector *Anopheles stephensi,* a protein toxin is released by the yeast (strain WaF17.12 from *Anopheles stephensi*) in the culture medium that is detected by the monoclonal antibodies directed against WaKT. Interestingly, the WaKTs signals were detected even in the mosquito larvae fed with yeast, indicating the vertical transfer of yeasts and the long-term effect of induction of killer toxin secretion. On the other hand, the most anti-microbial activities of secreted toxins were at an optimal pH of 4.5. Therefore, using the information obtained from subsequent studies, we can hope to use *Wickerhamomyces anomalous* yeast in interrupting the malaria transmission cycle and thus controlling and eliminating this disease.

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791
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