



In-vitro anticandidial efficacy of tick egg wax from *Hyalomma marginatum*, *Rhipicephalus bursa* and *Dermacentor marginatus*

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ABSTRACT

Objective: In the previous studies, the antibacterial, antifungal, and antiviral efficacy of the tick egg wax-coating of certain tick species were examined and some significant results were obtained. However, related researches and studied tick species are limited. There are hundreds of tick species, and it is well known that the antimicrobial efficacy of the wax is closely related to the species. The aim of this study was to investigate the in-vitro anticandidial efficacy of the egg waxes belonging to three tick species, which have not been studied before and have quite different biological and ecological differences.

Methods: In the study, the egg waxes of the tick species, *Hyalomma marginatum*, *Rhipicephalus bursa*, and *Dermacentor marginatus*, were used on *Candida albicans* ATCC10231, *Candida parapsilosis* ATCC 22019, and *Candida tropicalis* ATCC 750. Antimycotic susceptibility test was carried out in accordance with the Clinical and Laboratory Standards Institute (CLSI) recommendations using the M27-A3 microdilution method.

Results: It was determined that the wax of *Rhipicephalus bursa* has inhibitory effect on *Candida tropicalis* ATCC 750 in a particular concentration, and no significant effects were observed in other trials.

Conclusion: Anticandidial effect obtained from the egg wax of *R. bursa* can be associated with some distinctive biological characteristics, and it was concluded that the detailed studies with different tick species might yield significant results for the discovery of new generation antifungals.

Keywords: Antifungals, tick egg wax, *Rhipicephalus bursa*, *Candida*

1. INTRODUCTION

It has been reported that there are about 20 *Candida* species among the clinically important fungi seen worldwide, and of those, *Candida albicans*, the most common fungal pathogen in humans, has been reported to cause 250,000-400,000 deaths annually (1). Both cutaneous and systemic forms of candidiasis can be encountered, and the mortality can reach up to 40% in systemic *C. albicans* infections (2). Although *C. albicans* is the main agent of candidiasis, other pathogenic species such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. lusitanae* (non-*albicans* candida/ NAC) are frequently isolated from the cases. Furthermore, NAC species are more common in some regions in the world, and a steady increase has been observed in NAC-related cases in the last decades (e.g., 2-10 times in the cases of systemic candidiasis). This increase in the prevalence has been reported to be associated with high antifungal resistance (e.g., specific azole resistance in *C. glabrata* and *C. krusei*) seen in this group, and this problem has been indicated to be exacerbated by

the irregular antifungal use (3-5). In *C. albicans* and NAC species, as well as many other fungi, significant antimycotic resistance, which has intrinsic or microbiological character, has been well documented (6, 7). For example, single or multiple resistances in various *Candida* species against different antifungals, such as fluconazole, voriconazole, amphotericin B, and caspofungin, have been reported from different parts of the world (6-10). The antifungal diversity in the clinical use is reported to be relatively low compared to the causative agent and disease variety in fungal infections. The widespread, repetitive, and irregular use of antifungal drugs are possibly the main reasons of the resistances. It was anticipated that this situation requires the discovery of new drugs, and moreover, this need will increase over time (6-7). At this point, due to the problems in the medical area such as the resistances and side-effects of the current chemical therapies, the researches on natural substances, which may ensure to overcome these problems, have particular

importance. The current status of the researches in this area is expressed as “a New Golden Age of natural products drug discovery is dawning” (11). On the other hand, most of the related studies are at the beginning level. The applications are mostly at the stage of using total extracts or selected fractions and the results are given as effective or non-effective (12, 13).

In order to determine the natural alternatives that can be used in the treatment of fungal infections, various substances from different origins have been investigated such as microorganismal substances (14), sea sponge (15), the poison of honey bee (*Apis mellifera*) (16), the larval secrets of a lepidopteran parasitoid, *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae) (17), and the total isolates or some specific fractions of many kind of plant species (13, 18).

Tick egg wax is one of the sources investigated in the process of determining natural antimycotics. In ixodid ticks, the biological process includes eggs, larvae, nymphs, and adults (male and female). After detachment from the host, engorged adult female ticks hide in a suitable area on the ground and then they deposit up to thousands of eggs in a period that can last for several days or weeks before they die. The eggs, each 50-100 µg in weight and 0.5-1 mm, are laid as a batch. The surfaces of the eggs were covered with the wax, which consists of the secretions of the epithelial cells and accessory glands of the reproductive tract, the porose area, and principally the Gené organ which is a female-specific gland (19, 20). It has been reported that the wax, which thickness varies between 0.5-2.0 µm (21), has an average amount of 24 mg per gram eggs mainly according to the tick species (22). The wax is known to have some vital missions for the eggs such as holding the egg mass together, protecting the eggs against drying, environmental physical, chemical or microbiological factors, and ensuring the proper gas exchange between eggs and air (20, 21, 23, 24).

In the previous studies, the egg waxes of *Amblyomma habreum* (25, 26) *A. cajennense* (27-29), *A. aureolatum* (29), *Boophilus (Rhipicephalus) microplus* (29-32), *Rhipicephalus sanguineus* (29), *Haemaphysalis longicornis*, *H. doenitzii*, *Dermacentor silvarum* and *Hyalomma asiaticum* (22) have been tested against various bacteria, fungi, and viruses, and some considerable results were obtained. However, these studies have also revealed that the antimicrobial efficacy of the wax varies dramatically related to the tick species. In this study, the egg waxes of three tick species (*H. marginatum*, *R. bursa*, and *D. marginatus*), which have not been studied before and have quite different biological and ecological features, were investigated in terms of in-vitro anticandidial efficacy on three *Candida* species.

2. METHODS

2.1. Tick eggs

Non-infected laboratory colonies of *H. marginatum*, *D. marginatus* and *R. bursa* were used in the study. The maintenance of the colonies was carried out at Tekirdag Namik Kemal University Experimental Animal Application and Research Center, within the framework of the permissions received from the Tekirdag Namik Kemal University Animal Experiments Local Ethics Committee (2014/08-04). New Zealand rabbits (*Oryctolagus cuniculus*) were used to feed all of the developmental stages (larvae, nymphs, and adults). After the detachment from the host, the engorged females were washed with distilled water, dried, taken into sterile tubes individually and placed in the incubator. The conditions of the incubator were set at 25-27 °C and 70-80% humidity for *H. marginatum* and *R. bursa*, and at 20-22 °C and 80-90% humidity for *D. marginatus*. The process of the egg laying was regularly monitored, and the eggs were examined under a stereomicroscope. Incubation was continued until the first laid eggs reached the advanced stages (just before the larva began to hatch) of embryogenesis. Thus the wax extract was intended to include some possible changes which can occur in the egg wax during and after the laying, e.g., hardening in the texture, increase in the adhesive properties, and reduction in the levels of unsaturated fatty acids caused by oxidation (21, 23, 33). After this incubation egg batches were taken from the tubes and weighed, taken into sterile tubes and kept at – 80°C until the extraction of the wax.

2.2. Extraction of the tick egg wax

In the wax extraction process, previously described method was used (25). The following procedure has been carried out to include all the fractions of the wax to the extract as much as possible: just before the procedure, a chloroform: methanol solution (≥99.8%, LiChrosolv®, Merck, Germany / 24216-2-2.5L-R, 99-99.4%, 1% ethanol, Sigma-Aldrich, Germany) was prepared in a 2:1 (v/v) ratio. Of this solution, 40 ml was added to each of the 50 ml tubes containing 4 g eggs. The tubes were gently shaken for 1 minute and put on hold for 1 minute, and then vortexed for 1 minute. The vortexed suspension was separated from the eggs by filtration, and the filtrate was centrifuged at 1,000 rpm for 10 minutes. Subsequently, the supernatant transferred to a new tube was lyophilized, and the lyophilisate was kept at – 20 °C until antimycotic susceptibility assay.

2.3. Determination of antifungal activity

In order to prepare Roswell Park Memorial Institute-1640 (RPMI-1640) medium, 10.4 g RPMI-1640 (with L-glutamine, without sodium bicarbonate, Sigma, USA) and 0.165 M 34.53 g MOPS (3-N-morpholinopropanesulfonic acid, Sigma, USA) were dissolved in dH₂O. 18g glucose was weighed and added with a glucose concentration of 2%. The pH was adjusted to 6.9-7.0 with 1N NaOH. After the final volume was completed

to 1,000 ml, it was sterilized under aseptic conditions by filtering with a 0.22 µm pore-diameter filter (Millipore). The prepared medium was used in experiments after keeping overnight in a 37 ° C and the sterilization controls were performed.

In antifungal susceptibility test, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, and *Candida tropicalis* ATCC 750 standard strains were used. For the preparation of inoculums, 24-hour fresh cultures of all isolates in the Sabauroud Dextrose Agar (SDA) medium were used in the preparation of yeast suspensions. Five colonies with a ≥1 mm diameter size were taken and suspended in 5 ml of 0.85% sterile saline. The yeast suspensions were homogenized by vortexing them for 15 seconds and then adjusted to 0.5 McFarland turbidity. With this process, stock yeasts containing 1-5x10⁶ cells per milliliter were obtained. Stock yeast suspensions were diluted with RPMI-1640 medium first at the rate of 1/50 and later at 1/20, and 1-5x10³ cell/ml concentration to be used in the study was reached. The appropriate dilutions were performed using these suspensions and planted in SDA, and the correct concentration was determined by counting the colonies formed at the next day (CLSI M27-A3) (34).

In order to prepare antifungal stock solutions, the tubes containing two-fold drug concentrations between 32-0.06 µg/ml for amphotericin B were prepared using a pre-prepared antifungal stock suspension and diluted to the degree that was twice as much as the final antifungal concentration. To prepare the egg wax solutions, stock solutions prepared from the waxes dissolved in 10 ml Dimethyl sulfoxide (DMSO) arranged at a concentration of 10,000 µg/ml.

In the study, the determination process of the minimal inhibitory concentration (MIC) value was performed as follows: In accordance with antifungal susceptibility test CLSI M27-A3 standards (34), the micro dilution method was employed. Amphotericin B (32-0.06 µg/ml) was used as a control group, and DMSO was used as the negative control in the study. Serial dilutions (5,000-9.76 µg/ml) of the wax stock solutions were performed. The first well of the microplate in the horizontal row was determined as the sterility control, and the last well (12th well) as the reproduction control well. 100 µl RPM-1640 and 50 µl RPMI-1640 was added to the sterility well and reproduction control well, respectively. 50 µl of the dilutions were distributed to each column of the microplate as one waxy cover and drug concentration per column. It was dispensed with a pipette, keeping the highest concentration in the second well and the lowest concentration in the 11th well. After the preparation of the microplates, 50 µl of yeast suspensions arranged as one isolate per row was added. Yeast suspension was not added into the sterility control well. At the end of this process, both yeast and drug concentrations were diluted by ½, and study concentrations were reached. After the microplates were covered and incubated at 35 °C for 48 hours, the susceptibility results were visually evaluated. The lowest concentration without reproduction after incubation was determined as MIC (CLSI M27-A3) (34). The procedures

were repeated 3 times using waxy cover obtained from different egg groups of the same species.

3. RESULTS

In this study, it was seen that the amount of egg batches obtained from 10 fully satiated females of each tick species would be sufficient to determine the repeated MIC value to be tested against at least three agents. Due to the solvents benefited, it was understood that the use of glass material was ideal at almost all steps of the study. One of the biggest problems that may be encountered in the process is the calculation of the amount of obtained lyophilisate, and at this point, it may be more accurate to try to determine the weight of the waxy cover, which is resistant to repeated dissolution, by dissolving it in a certain amount of solvent after lyophilization. At least, with such an approach, it was seen that it is necessary to confirm the calculations made.

In the trials, no antifungal effect was observed in any of the serial waxy cover concentrations (5.000-9.76 µg/ml) belonging to *H. marginatum* and *D. marginatus*. This result did not change in repetitions. On the other hand, in the serial waxy cover concentrations (5.000-9.76 µg/ml) belonging to *R. bursa*, an inhibition against *C. tropicalis* was identified, the MIC value was found to be 625 µg/ml, and the same results were obtained in repeated trials (Table 1).

Table 1. Minimum inhibitory concentration (MIC) values of the tick egg waxes and reference substance, Amphotericin B (µg/ml).

Substances	Microorganisms		
	<i>Candida albicans</i> ATCC 10231	<i>Candida tropicalis</i> ATCC 750	<i>Candida parapsilosis</i> ATCC 22019
<i>Hyalomma marginatum</i>	-	-	-
<i>Dermacentor marginatus</i>	-	-	-
<i>Rhipicephalus bursa</i>	-	625	-
Amphotericin B	1	2	1

(-) No antimycotic effects were observed at the concentrations of ≤5000 µg/ml.

4. DISCUSSION

Within the life cycle in nature, one of the most critical problems of the ticks is to protect the eggs until the larval hatching and maintain their vitality. The engorged female ticks that detached from the host prefer shady and humid areas such as clefts or cracks on the ground for laying eggs. However, such sites are the places where many pathogens, saprophyte bacteria and fungi are also found. This coexistence

especially possesses a potential problem for the tick species which have a long period of larval hatching (23). It is known that the egg wax is essential in terms of protecting the eggs from the microbiological, chemical, or physical factors (25, 26). In the studies carried out on some tick species, different type of molecules have been determined in the wax such as long-chain hydrocarbons, branched and unbranched alkanes, fatty acids, cholesterol, and some other steroid types, alcohols (20, 24, 26), and some specific proteins and lipoproteins (23, 25). It was stated that the wax is resistant to some physical and chemical factors such as cold, heat, proteinase K, and pronase (25). However detailed data have not yet been obtained at the point of the determination of the wax content, especially its bioactive substances (20, 26).

In the studies carried out to determine the antimicrobial activities of the wax, successful results have been obtained in various degrees against different agents such as *Escherichia coli* (22, 29), *Staphylococcus epidermidis* (25-27, 32), *Staphylococcus aureus* (22, 26, 29), *Serratia marcescens* (25), *Bacillus cereus* (26), *Bacillus subtilis* (25-27), *Micrococcus luteus* (27, 29, 30), *Pseudomonas aeruginosa* (22, 31), *Enterococcus faecalis*, *Enterococcus faecium*, *Nocardia asteroides*, *Salmonella enteritidis*, *Klebsiella pneumonia* (22), picornavirus (28), and influenza virus (28, 29). Furthermore, the wax of *Boophilus microplus* was reported to be effective on *C. albicans* to some extent (30), and the wax of *Rhipicephalus sanguineus* was found to be effective on the same agent (29). In our study, no effect was observed related to the wax of *H. marginatum* and *D. marginatus* on examined *Candida* species. Although similar results were detected from the wax of *R. bursa* against *C. albicans* and *C. parapsilosis*, some concentration of the wax of this tick species inhibited *C. tropicalis* (MIC: 625 µg/ml).

Related studies have obviously shown that the antimicrobial efficacy of tick egg wax is directly correlated to some factors such as the extraction methods and application dose of the wax, and the species of the ticks and microorganisms (22, 25, 29). The feature of the wax is well known to vary significantly according to the tick species, and it was indicated that this is an expected result, since the sites of egg-laying and habitat preferences of tick species are more or less different from each other (21, 33, 35). It was also clearly observed in our study, that the differences in the species of ticks and *Candida* directly affect the results. In accordance with the related information mentioned by the researchers (20, 36), our laboratory and field studies conducted in Thrace, Turkey, have revealed that *H. marginatum* and *R. bursa* lay eggs in hot months and in the relatively dry sites and *D. marginatus* during colder months and in more humid area. This difference creates the expectation that the physical and chemical properties of the egg wax of *D. marginatus* can differ from those of other tick species. However, our related studies have shown that the laying and larval hatching procedures of *R. bursa* exhibit significant differences compared to *H. marginatum* and *D. marginatus*. While the whole process of the egg-laying, larval hatching, and larval activation usually take a couple of weeks in the last two species, it can take months in *R. bursa*. As a

result of this delayed process, although the adult stages of this species feed on the host mostly in and around June, the larvae are found on the host in the late autumn or winter (detailed data not presented). In this context, it seems like a natural consequence that the eggs of *R. bursa* are supported by a more featured wax, which ensure adequate protection to the eggs during the relatively long hatching period.

5. CONCLUSION

The results obtained from the egg wax of *R. bursa* was interpreted as it could be associated with its some distinctive biological characteristics, and it was concluded that the detailed studies with this and some other tick species that share similar biological and ecological features might yield significant results for the discovery of the new antifungals.

CONFLICT OF INTEREST

Authors declare no scientific and financial interest.

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