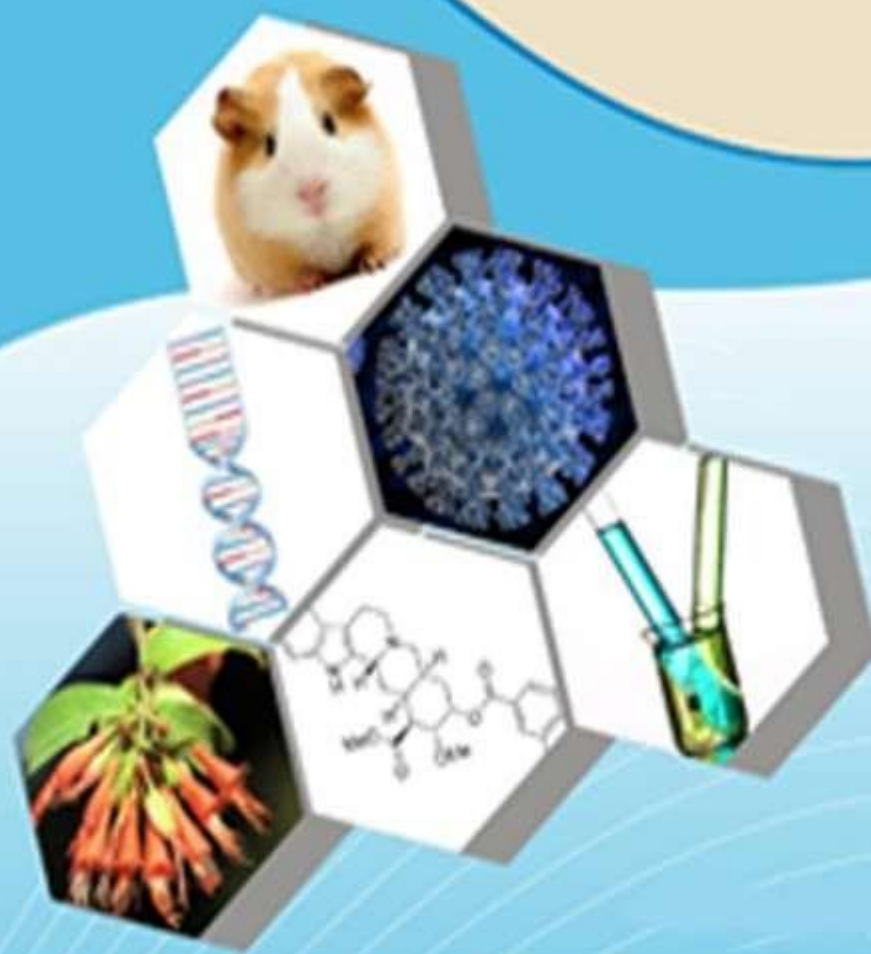




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***In vitro* Inhibition of Candida Species by Aqueous Garlic Extract in Gel and Lotion: Problem of Stability**

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ABSTRACT

Aqueous garlic extract (AGE) has been shown to suppress the growth of several therapeutically relevant microbes, including Candida species. Not much is known about this extract's stability and effectiveness in topical medicinal compositions. The anticandidal activity and stability of AGE in lotion and gel formulations are detailed in this communication. The physical and biological stability of gels and lotions containing 50, 100, and 200 mg garlic/ml were tested at 40°C. Using Muller Hinton agar and the well diffusion technique, we assessed the anticandidal impact of each formulation. The newly made lotion did not show any anticandidal action, however the results showed that both formulations were physically stable. Gels inhibited effectively, however their efficacy was lost after 9 days of storage at 40°C. Since the lotion lost its activity while being prepared, it may be argued that it was not an appropriate delivery vehicle for AGE. While gels were effective inhibitors when made immediately, they lost some of their efficacy after being stored. Stabilizing the AGE gel composition for the anticipated shelf life requires comprehensive investigations. The anticandidal action, formulation, gel, lotion, and biological stability of an aqueous garlic extract are all pertinent terms.

INTRODUCTION

There is a copious amount of scientific literature that deals with garlic (*Allium sativum* L.) as an effective agent for the treatment of various illnesses. [1-2] Of special interest is the antimicrobial effect of garlic extract or garlic components and again this subject has been extensively investigated. [3-5] In fact there is enough evidence which indicates that garlic exhibits antimicrobial activity against a wide range of microorganisms comparable to or exceeds that of expensive antibiotics. [6] Even multi antibiotic resistant organisms were found to be susceptible to garlic. [7] This particular observation is important as it places cheep plants in the forefront as a possible alternative to the "by many" unaffordable modern antimicrobial drugs. Garlic contains at least 33 sulfur compounds, several enzymes, minerals, vitamins, fiber and water. [8] The most abundant sulfur compound in garlic is alliin (Sallylcysteine sulfoxide), which is present at 10 and 30 mg/g in fresh and dry garlic, respectively. [9] Allicin which is the major antimicrobial component isolated from garlic, is formed when the garlic clove is crushed and the enzyme alliin lyase comes into contact with alliin. The main inhibitory

effect of allicin may be due to its chemical reaction with thiol groups of various enzymes, e.g. alcohol dehydrogenase, thioredoxin reductase, and RNA polymerase. [10-11] The antimicrobial effect of garlic extract incorporated into pharmaceutical formulations particularly those applied topically was the subject of few publications. Most important topical preparations are gels, lotions and creams. The addition of garlic volatile oil in the formulation of gels was tried by Bodhankar and Patil [12], whereas the addition of allicin into a readymade gel preparation was also reported. [11] Garlic extract or allicin were added to commercially available cream to establish the suitability of this formulation as a carrier of the antimicrobial activity exhibited by garlic and results proved encouraging. [13] The approach of triturating any component or extract with commercially available creams poses questions regarding the industrial feasibility of this practice and casts doubt about the long term physical stability of the preparation. It must be emphasized that as far as is known there has been no attempt to study the

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biological stability of garlic or allicin in formulated topical products. No formulation can be proposed for commercial use without providing evidence to its physical and biological stability. The proposed preparation should be applicable for industrial scale production and the manufactured product should maintain its antimicrobial effectiveness over the expected shelf life. The objectives of this investigation were first to establish the *in vitro* activity of a simple gel and lotion formulations "with AGE" against clinical isolates of *Candida* species, second to determine the biological stability of these preparations over a period of time.

MATERIALS AND METHODS

Garlic aqueous extract preparation

Fresh garlic bulbs (*Allium sativum* L.) were purchased from a local supermarket in the city of Zarqa, Jordan. The cloves were peeled and 500 g of the edible portion was homogenized in 1 liter of sterile distilled water using pestil and mortar. The homogenate was allowed to stand for 2 hours then filtered under vacuum through a 25 mm pore-size filter. The filtrate was collected in a flask and then stored at 4°C until used. This aqueous extract contained 500 mg of garlic/ml.

Gel formulation

The gel formulation was prepared by mixing 200 ml of aqueous garlic extract in 764 ml of sterile distilled water. To this mix 20 g of Carbomer 934 fine powder was added slowly while mixing and the gel was formed after the addition of 16 g Triethanolamine to the preparation while being agitated. This formulation contained 100 mg/ml aqueous garlic extract. Three other preparations were also processed in the same manner to contain zero, 50 and 200 mg/ml garlic extract.

Lotion Formulation

A simple lotion was prepared as follows: to a mix containing 763 ml sterile distilled water and 200 ml of garlic extract, 18 ml of triethanolamine was added, mixed, heated and maintained at 80°C. In another vessel 20 g cetyl alcohol, 10 g stearic acid and 10 g of beeswax were heated to 80°C. The prepared aqueous mix was added to the oily preparation while mixing at 80°C. Two other similar formulations were prepared; one with 200 g/ml and the other devoid of garlic extract.

Inoculums Preparation

A total of 6 *Candida* isolates were obtained from the laboratories of Jordan University hospital. Each isolate was grown on Sabouraud dextrose agar for overnight at 37°C before colonies were harvested and suspended in phosphate buffer pH 7.0. The turbidity of the suspension was adjusted to that of 0.5 MacFarland standards and thus contained 10⁸ CFU/ml. [7] This suspension was further diluted with the same buffer so that the final concentration of cells would be 10⁶ CFU/ml or as required.

Inhibition studies

To establish the anti candidal effect of aqueous garlic extract in formulation, Sabouraud dextrose agar plates were seeded with each clinical isolate under test from the dilution that contained 10⁶ CFU/ml. After drying, 4 wells in each plate were bored using a sterile cork borer of 6 mm in diameter. One hundred micro liters of each gel or lotion was separately dispensed into a well and plates were then incubated at 37°C for 24 hours before the zone of inhibition around each well was measured. For each AGE concentration and each test organism triplicate plates were prepared. The difference between the mean values was analyzed by Student's t test and *p* value of < 0.05 was considered as significant.

Time-kill investigation

In vitro kill rate of garlic extract against 2 clinical isolates of *Candida* species was performed following the technique described by Yen *et al.*

[14] but with some modifications. A cell suspension of the *Candida* species under test was prepared as described above. One ml aliquot of this suspension was inoculated into 9 ml of the gel formulations prior to the addition of few drops of triethanolamine to ensure even distribution of the inoculum. At intervals, 0.5 ml quantity was withdrawn and transferred to a tube containing 4.5 ml sterile phosphate buffer pH 5.5, mixed and the number of surviving *Candida* was determined by plating 100 micro liters aliquot onto SDA.

Stability Study

Two gel formulations were made; one with 100 mg/ml AGE and the other with 200 mg/ml. Both preparations were incubated at 40°C and at intervals the persistence of the inhibitory effect of these formulations against *C. albicans* was determined as described above.

RESULTS AND DISCUSSION

This investigation was primarily concerned with the formulation of topical preparations for carrying AGE in presentable pharmaceutical form which can be manufactured at an industrial scale and this was achieved. The second concern was to determine if AGE in formulation can maintain its inhibitory effect against clinical isolates of *Candida* species and for how long. Soon after preparation, lotion formulation exhibited no antagonistic effect against *Candida* species tested in this study whereas; gel formulations were inhibitory to all isolates at 100 and 200 mg AGE/ml. This is clearly demonstrated in Table 1 which shows the zone of inhibition measured to various AGE concentrations incorporated into the gel preparation. The same table demonstrates that the gel prepared without any AGE exhibited no anticandidal effect. Therefore, any inhibition obtained must have been solely due to the added AGE.

Table 1: Susceptibility of *Candida* species to various concentrations of AGE in gel

formulation (inhibition zone is the mean of 3 readings measured in mm)

Candida spp.	Zone of inhibition in mm.			
	200 mg/ml	100 mg/ml	50 mg/ml	0 mg/ml
<i>C. albicans</i>	23	18	12	Zero
<i>C. tropicalis</i>	26	16	15	Zero
<i>C. glabrata</i>	19	13	10	Zero
<i>C. parapsilosis</i>	14	9	Zero	Zero
<i>C. krisei</i>	12	8	Zero	Zero

The oil and water phases of lotion were mixed after heating each portion at about 80°C and this might explain why this formulation demonstrated no inhibitory effect against the *Candida* species tested. Sah *et al* [15] reported that aqueous extract derived from garlic cloves boiled in water at 100°C for 30 minutes, exhibited reduced antimicrobial properties as compared to that obtained by cold extraction. Dababneh and Al-Delaimy [16] indicated that heating garlic extract at 80°C to 90°C for 5 minutes completely destroyed its antimicrobial activity. Therefore, the heat treatment employed in the preparation of lotion was probably behind the lack of anticandidal activity in our preparation. Perhaps, this is why Cutler and Wilson [14] preferred to use a readymade cream for testing the antimicrobial effect of AGE rather than to prepare a cream formulation in the laboratory; rightly so to avoid heat treatment. When the gel contained 50 mg/ml concentration of AGE, no inhibitory effect against *C. parapsilosis* or *C. krisei* was observed. It is important to note that the strength of garlic extract used in the formulations studied did not rely on the minimum inhibitory concentration of AGE reported in literature but rather depended on pragmatic data. Cutler *et al*, [11] used 500 mg Allicin/100 ml of gel to test for the antimicrobial effect whereas, Adejare *et al* [17] found that their clinical isolates of *Candida* species required as high as 200 mg/ml of AGE for effective inhibition. Therefore, these concentrations were used in this investigation.

Kill time of *C. albicans* and *C. parapsilosis* inoculated separately into gels with 50, 100, and 200 AGE is shown in Figure 1 (A & B). It is evident from figure 1A that complete inhibition of *C. albicans* was achieved in less than 4 hours regardless of the AGE concentration used. Figure 1B shows that *C. parapsilosis* was killed by the gels containing 100 mg/ml and 200 mg/ml in less than 5 hours. Thus, kill time is dependent on the AGE concentration used in formulation and the microbial strain tested. However, this particular observation is in agreement with those reported by others for plain AGE, the kill time for *C. albicans* was almost half the time reported by Iwalokun *et al* [7]. This difference could be attributed to the higher concentration of AGE used in the gel formulation employed in this study.

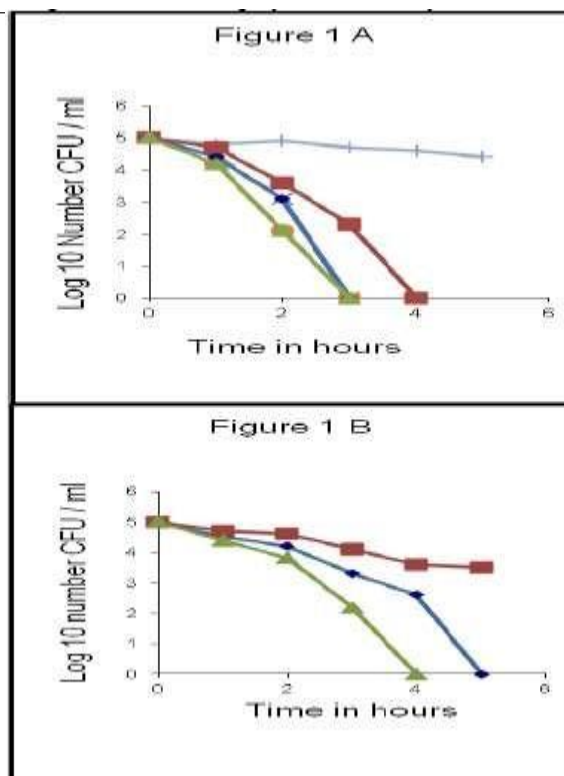


Fig. 1: Effect of different AGE concentrations in a freshly prepared gel formulation on the survival of *C. albicans* (Fig. 1A) and *C. parapsilosis* (Fig. 1B). ▲ 200 mg/ml, ■ = 100 mg/ml, □ = 50 mg/ml, x = Plain gel with no AGE

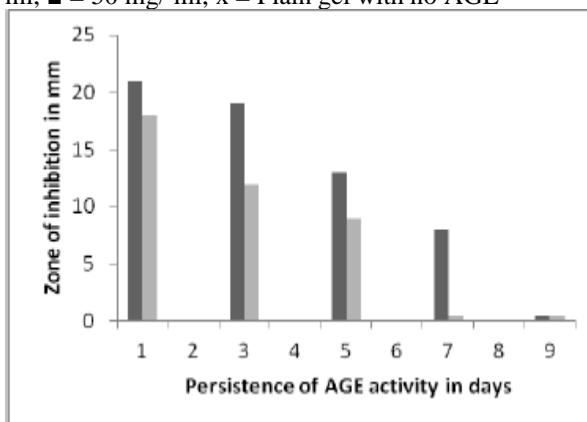


Fig. 2: Persistence of inhibitory effect in mm versus concentration of AGE in gel formulations (Dark column= 200 mg/ml, Light column= 100 mg/ml)

Stability study of any pharmaceutical product is performed to ensure the maintenance of product quality, safety and efficacy throughout the shelf life. [18] Figure 2 demonstrates that freshly prepared gels with 2 different concentrations of AGE was active inhibitor to the *C. albicans* studied, but this activity was reduced by almost 50% after 5 days of incubation at 40°C and completely lost after 9 days. This means that the formulated gel was not biologically stable as the AGE antimicrobial activity disappeared from the preparation. Belguith *et al* [19] found that while

AGE stored at 4°C retained at least 90% of its original inhibitory potential for more than 10 days, the extract solution stored at 22°C lost the antibacterial activity slowly over a period of 6 to 10 days. Chong *et al* [20] investigated the Allicin stability in aqueous garlic extract by high performance liquid chromatography and demonstrated that when the garlic extract was incubated at 37°C, there was more than 50% loss of allicin over 5- day period. These findings are very close to those reported herein. Because Allicin plays a vital role in the antimicrobial activity of garlic, its degradation means loss of this activity. This is probably the case with our gel preparation which acted as growth inhibitor to all *Candida* species when it was just prepared but with the passage of time allicin was degraded and thus this activity was diminished. Block [21] attributed the loss of allicin biological activity to its transformation with time into more stable components: polysulfides and thiosulphonates. The biological half-life of allicin in water was determined by Fujisawa *et al* [22]; they found that the generated antibacterial activity and the detectable Allicin in crushed fresh garlic cloves declined in aqueous and ethanolic solutions upon storage at room temperature. The same authors calculated the biological and chemical half-lives to be 6 and 11 days, respectively. Gel formulations are aqueous preparations and therefore, the biological activity of garlic in such a formulation would be expected to follow the same trend as observed for aqueous garlic extract. This is exactly what was observed in this investigation. Formulation of active substance into a suitable dosage form in addition to its stability and efficacy are components of the lengthy process of product development. It is concluded that lotion is not a good choice as a delivery system for carrying the anticandidal activity of AGE due the inactivation of garlic active molecules by heat. Formulation of AGE into a gel which can be produced at a commercial scale is presented but a long time might elapse before the problem of its biological stability can be resolved.

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