

THE EFFECT OF TERMITES EXTRACT ON INHIBITION OF GROWTH OF SOME PATHOGENIC BACTERIA AND SYNTHESIS OF BIOFILM

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ABSTRACT

This study was aimed to extract the effective material from the dry nests of termites and detect its antibacterial activity against some pathogenic bacterial isolates and inhibit synthesis of its biofilm. Termites dry nests were collected and the effective material was extracted then the antibacterial activity was detected using the disc diffusion assay. Results were showed that the extract have antibacterial material from the Termites dry nests, this extract showed antibacterial activity against Gram positive bacteria (*Staphylococcus aureus*) at (21.5mm) and Gram negative bacteria (*Enterobacter sp.* and *Pseudomonas aeruginosa*) at (26 mm and 20 mm) respectively by inhibiting their growth, as well as its effect on biofilm production of pathogenic bacteria. *Staphylococcus aureus*, *Enterobacter sp.* and *Pseudomonas aeruginosa* revealed a significant decrease ($P<0.01$) in biofilm synthesis as the concentration of the extract increased.

Keywords: insects, microbes, antibacterial activity, antibiofilm, antibacterial.

صالح وآخرون

مجلة العلوم الزراعية العراقية - 2020: 51(عدد خاص): 176-183

تأثير مستخلص حشرة الارضة *Termites* في تثبيط نمو بعض الاجناس البكتيرية الممرضة و تكوين غشاءها الحيوي

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المستخلص

هدفت الدراسة الحالية الى استخلاص المادة الفعالة من اعشاش الارضة الجافة واختبار قابليتها في تثبيط نمو بعض الاجناس البكتيرية الممرضة وتكوين الغشاء الحيوي فيها حيث تم جمع حشرة الارضة مع اعشاشها الجافة وتم استخلاص المادة الفعالة منها ثم اختبار فعاليتها ضد البكتيريا بطريقة انتشار الاقراص. بينت النتائج ان المادة الفعالة كانت لها القابلية على تثبيط كل من البكتيريا الموجبة (*Staphylococcus aureus*) بقطر 21.5 ملم والبكتيريا السالبة (*Enterobacter* و *Pseudomonas aeruginosa*) بقطر 26 و 20 ملم على التوالي , بالاضافة الى تأثيرها على انتاج الغشاء الحيوي للبكتيريا المرضية حيث اظهرت كلا من *Staphylococcus aureus* و *Enterobacter* و *Pseudomonas aeruginosa* انخفاضاً معنوياً عند ($P<0.01$) بانتاج الغشاء الحيوي كلما زاد تركيز المستخلص.

الكلمات المفتاحية: الحشرات، الميكروبات، الفعالية ضد البكتيرية، الفعالية ضد الغشاء الحيوي، الفعالية ضد البكتيرية.

INTRODUCTION

In the last decade, insects have been recognized for having strong immune defences by producing different kinds of antimicrobial compounds that effect and inhibit various pathogens (13). Thus, they have been targeted as a significant source of antimicrobial compounds (10, 28). Insects have excellent immune responses that act synergistically to protect themselves against microbial infections (33). When pathogens break through morphological barriers, insects start innate immune responses comprised of cellular and humoral reactions. Phagocytosis and encapsulation represent hemocyte mediated cellular reactions, while humoral reactions depend on the production of antimicrobial molecules and activation of enzymatic reactions (20). Over the final few decades, more than a hundred of insect antimicrobial

peptides/proteins (AMPs) have been recognized from different kinds of insects (34). Lysozymes, cecropins, spinigerin, attacins, defensins, and proline rich peptides have been reported as insect AMPs. Most of these molecules have antifungal activities and only a few showed a detectable antibacterial activity (3). Studies showed that South Indian tribes used termites to treat diseases related to asthma or microbes like, *Heamophilus influenza* bacteria and even viruses (29). Not so far, few antimicrobials have been identified from three termite families: Termopsidae (27), Rhinotermitidae (4), and Termitidae (5). The termites kingdom known as Animalia, phylum: Arthropoda, class: Insect, order: Isoptere, (Fig 1). They are found in large group or colonies in the soils of the earth diffuse significantly and feed on cellulose in the wood, trees and plant (19).



Figure 1. Magnified picture of a termite (30).

Termites have occurred a long time ago, over 55 million years and are successfully good in keeping their species. An important reason behind their success can be ascribed to their cooperative behaviour (22). They are social insects. This means that they live in groups called colonies. Social insects are distinctive from other insects (beetles, cockroaches, or grasshoppers) because each termite in the colony performs a particular work that benefits the colony as a whole. Most other insects work to benefit themselves only (25). Termites have three main body parts: head, thorax and abdomen, termites head have a distinctive pair of straight antennae that look like small beads connected together. Thorax subdivided into three segments and is the part of the body where movement takes place mainly. They have three pairs of legs one joined to each segment on the thorax [8]. Workers termites do the digestion of cellulose in food, the process of worker termites feeding other nest mates is known as trophallaxis, which is the

exchange of food or other fluids between members of the colony through mouth-to-mouth (stomodeal) or anus-to-mouth (proctodeal) feeding. It can involve the transfer of different items such as pheromones molecules, organisms such as symbionts, and others to serve as a form of communication (21, 18). The aim of this study was to find alternative (suitable and benefits solution from termites) to antibiotics that cause mutations of pathogenic bacteria and work as detergents at least.

MATERIALS AND METHODS

Sample collection

The termites dry nests (10gm) were collected from the walls of different parts of houses in Baghdad city and was identified by entomologist specialist at Biology Department –College of Science/University of Baghdad, during the spring season then placed in sterile container and transformed to laboratory.

Extraction of antibacterial material

Termites dry nests were placed in a flask and (100ml) of 90% alcohol was added to extract the antibacterial material in dry form then the flasks were placed in a shaker incubator for one week. Thereafter, the solution was placed in the centrifuge to obtain supernatant that contained the active component which was

then added in a pre-weighed petri dish for desiccation for two days. Subsequently the material was collected again by adding absolute alcohol to a final concentration (100mg/ml), finally the extract was placed in tubes and kept in freezer for further antimicrobial experiments, Fig (2a,b).

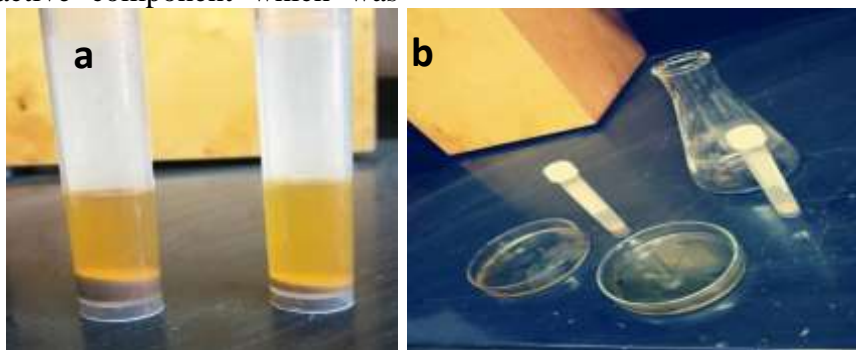


Figure 2. a) Termites dry nest extract after centrifugation; b) Extract dried in petri dishes.

Preparation of bacterial culture solution

Six pathogenic bacterial strains, obtained from Biology department – College of science /University of Baghdad. were included (*Staphylococcus aureus* ; *Streptococcus pyogens* ; *Pseudomonas sp.* , *Escherichia coli*, *Klebsiella sp.* , and *Enterobacter sp.* which previously identified). Each strain was sub-cultured in Nutrient broth and incubated for 18 hr. at 37 °C, then used as bacterial stock culture for further experiments (12).

Disk diffusion assay: This method was used to detect the antibacterial effect of termites extract. Hundred µl of the overnight bacterial stock culture was inoculated in 10 ml fresh nutrient broth and grown for 18 hr.. The culture of each bacteria was subjected to McFarland tube (0.5 turbidity) to obtain 1.5×10^8 (CFU/ml). Nutrient agar plates were prepared previously then 10 µl of each broth culture transferred separately to agar plates and spread with a sterilized glass rod spreader. A six mm sterile (Whatman number 1) filter paper disc that was cut out and sterilized by U.V were impregnated with Dilution of extract. Dilutions were (20mg/ml , 10mg/ml and 5mg/ml) of termites extract to obtain a final disk load of each concentration (200µg/disk, 100µg/disk and 50µg/ml), thereafter the disks were evaporated and placed on agar plate previously seeded with respective bacterial strains. Negative control (disc was soaked with 10 µl alcohol and

Positive control disc contained 30 µg/disc chloramphenicol) were used. Then plates were incubated at 37C° for 24hr. Inhibition zones were measured (mm). Results were expressed as mean ± SE and SD and subjected to the student's t-test, P< 0.01 and P<0.05 was considered as significant.

Detection of antibiofilm activity of Termites extract: The ability of termites dry nests extract to inhibit biofilm production was evaluated by using crystal violet staining technique in polystyrene microtiter plat. The optical density (O.D) was measured at 490 nm (31). Overnight cultures of (pathogenic bacteria mentioned previously) were cultured in tryptic soy broth (TSB) supplemented with (1% glucose) and diluted to 1.5×10^8 CFU/ml. Individual wells of flat-bottomed 96 well polystyrene plates were filled with 100 µl aliquots of each culture separately then 100µl of each termites extract at concentrations (20, 10, 5, 2.5, and 1.25 mg/ml were added to reach the final concentration 200, 100, 50, 25, and 12.5 µg /ml) and incubated for 24 hr. at 37C°. Then the wells were washed 3 times with 200 µl of sterile phosphate buffer saline (pH: 7.2). Biofilms were fixed with heating at 60 C° for 15 min. Crystal violet solution (0.1% wt. /vol.) (200 µl) was added to all wells and left for 15 min. Excess crystal violet was removed with distilled water and the plate was air dried overnight. The bounded crystal violet was released by adding 200 µl of 96% ethanol.

Thereafter absorbance was measured spectrophotometrically at 490 nm (A_{490}). The test was performed in duplicates; negative control wells contained TBS only. The results were calculated according to (24,11), where absorbance was proportional to biofilm formation.

RESULTS AND DISCUSSION

Antibacterial activity of Termitis extract

Termitis extract was used to inhibit the growth

of both Gram positive and Gram negative bacteria. Results showed that the antibacterial activity was increased with increasing the concentration of the extract against both Gram positive and Gram negative bacteria. The highest inhibition zone for Gram positive bacteria was (21.5mm) against *S.aureus*, (Fig. 3a, Table 1).

Table 1. Inhibition zones of termitis extract against pathogenic bacteria.

Pathogenic bacteria	Inhibition zone (mm)			Mean	SD	SE	t-test	P-value
	200µg/disk conc.	100µg/disk conc.	50µg/disk conc.					
<i>S.aureus</i>	21.5	19.5	16	19.0	2.78	1.6	11.8	0.04*
<i>Streptococcus</i>	9.5	7.0	7.0	7.8	3.32	1.9	2.1	0.09NS
<i>E.coli</i>	17.5	15.5	7.5	13.5	7.07	4.1	3.3	0.08NS
<i>Klebsiella sp.</i>	10.5	8.5	7.5	8.8	3.21	1.9	3.2	0.08NS
<i>P. aeruginosa</i>	20	13.5	11.5	15.0	4.44	2.6	5.8	0.02*
<i>Enterobacter sp.</i>	26	22	17.0	21.6	9.45	5.5	4.8	0.04*
LSD	78.36	154.3	90.5		-			0.000**

** $P<0.01$; * $P<0.05$; NS:Non-significant

On the other hand, the highest inhibition zone for Gram negative bacteria was (26mm) against *Enterobacter*, (Fig.3b, Table1). All concentrations showed significant differences for each isolate at $P<0.05$, except

Streptococcus, *E.coli* and *Klebsiella sp.* which were least affected by the different concentrations of the termites extract, which indicate a specific effect of the extract toward specific kinds of bacteria.

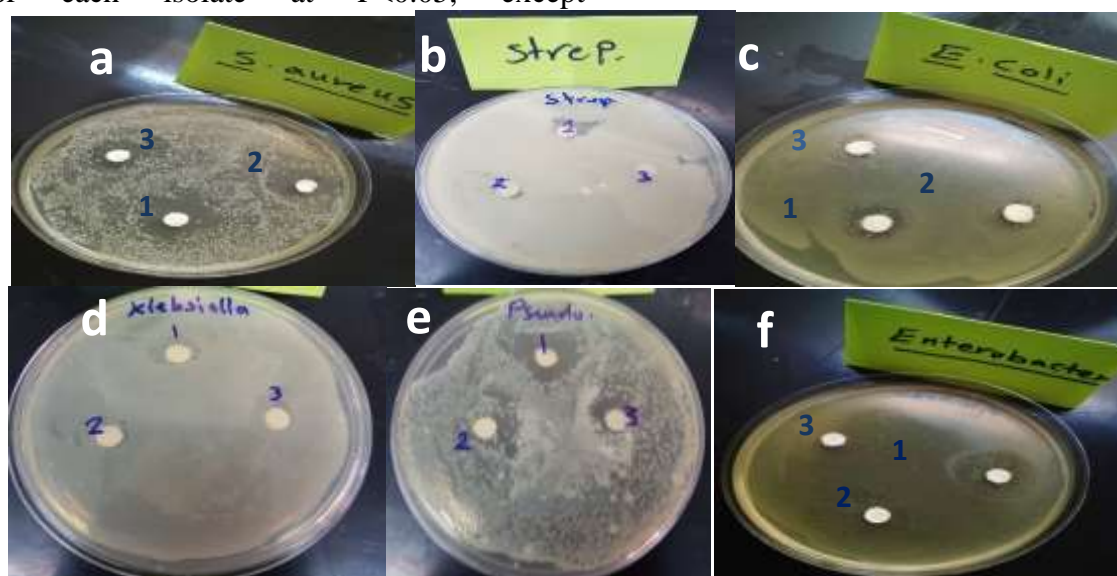


Figure 3 Inhibition zones against pathogenic bacteria.1: 200µg/disc; 2: 100µg/disc and 3: 50µg/disc.

A major problem that faces humans nowadays are diseases caused by multi drug resistant (MDR) bacteria, for example wound and burn infections caused by *P.aeruginosa* (32), that may be very dangerous and fatal. Studies on this issue are focused on how to find alternatives to solve this problem and efforts have been made in different fields and directions using extracts from animals, plants,

and bacteria itself to combat pathogenic microbes (23). Termites from order Isoptera. Revealed a novel antimicrobial molecule that is a cysteine-rich 36 residue peptide, with antifungal and to a less extent antibacterial that weakly affects several gram+ve bacteria. Furthermore it has been shown that the termite antimicrobial peptides present in the hemocyte granules and in the salivary glands of

Pseudocanthotermes spiniger (9). Reports mentioned induction of antibacterial activity from the whole body homogenates of *Coptotermes formosanus* Shirak upon exposure with various bacteria, including a human pathogen *Staphylococcus aureus* (16). These termites have developed disease resistance mechanisms that facilitated their survival and propagation they nest and forage in soil (6). Termite-produced AMPs, termicin (initially isolated from a fungus-growing termite) and tGNBPs (termite gram-negative binding proteins), have been described in the eastern subterranean termite *Reticulitermes flavipes* and the dark southern subterranean termite *R. virginicus* (14). GNB2 has β -1,3-glucanase activity in termites and contributes to external antifungal defense. We previously reported the discovery of constitutive antibacterial activity from the cell free extract (CFE) of *R. flavipes* against a common gram-positive soil-borne entomopathogenic bacterium, *Bacillus subtilis* determined the

presence, characteristics, and levels of constitutive and inducible antibacterial activities in *R. flavipes* against a panel of human bacterial pathogens including three common multidrug resistant nosocomial pathogens and five non-MDR pathogens (35). **Antibiofilm activity of Termites extract** Antibiofilm activity of termites extract was detected in this study by using the crystal violet microtiter plate method, the results were showed the ability of bacterial biofilm production was decreased with the increasing of termites extract concentration, the O.D value decreased significantly when termites extract reaches 100 $\mu\text{g/ml}$ and more in the Gram positive bacteria group ($P \leq 0.01$) specially for *S. aureus* bacteria, Fig.4a; whereas in the Gram negative bacteria it decreased when the concentration of termites extract reaches 50 $\mu\text{g/ml}$ and more ($P \leq 0.01$), except for *E. coli* and *Klebsiella* sp. that showed resistance to the extract and continuous production of biofilm (Fig.4b).

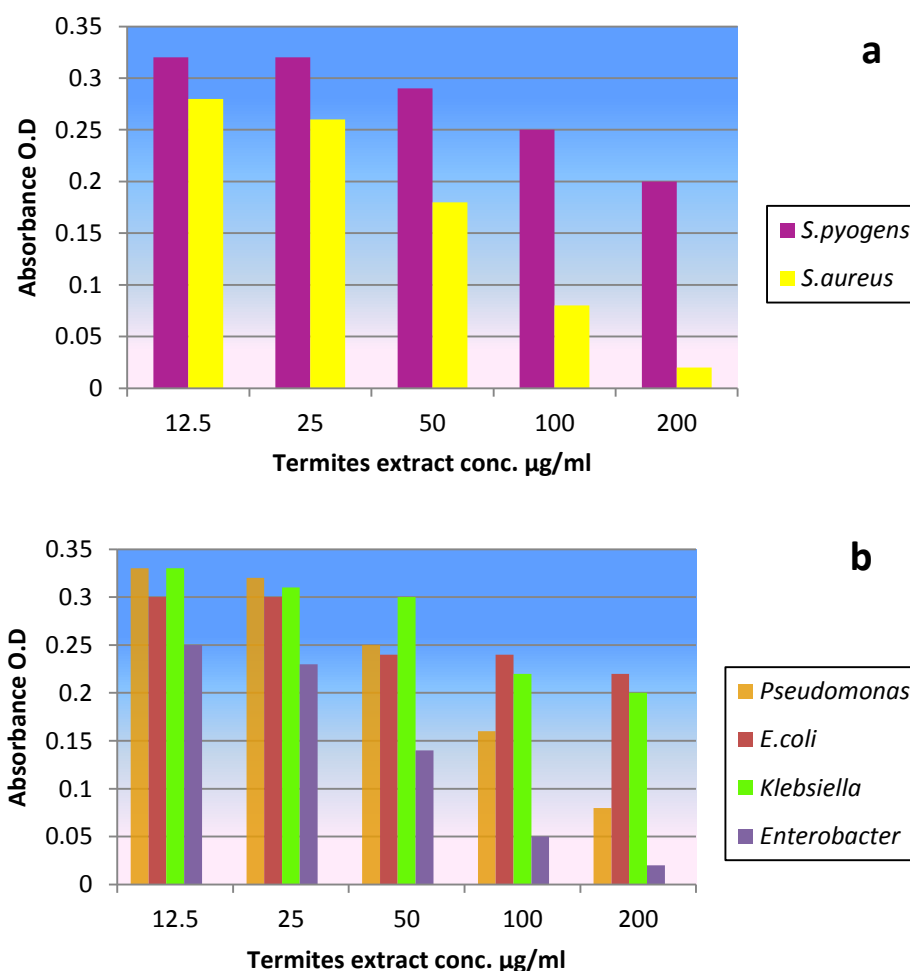


Figure 4 .Absorbance (O.D) values of crystal violet in gram positive bacteria (a); and gram negative bacteria (b) grown in tryptic soy broth at different concentrations of termites extract.

Biofilms are extracellular polymeric matrix which is produced by bacterial communities; these biofilms can be found on a variety of surfaces such as living tissues, medical devices, water pipes and others (15). Biofilms are known to increase pathogenicity and antibiotic resistance in different kinds of pathogenic bacteria such as *P.aeruginosa*, *E.coli*, *Klebsiella*, And others. Immune response can be activated by the components of biofilm which are recognized by the pattern recognition receptors of the immune system, this kind of immune response start the activation of IFN-1 by the host (2). Many biofilm inhibitors have been investigated including plant extracts (1), biosynthesized nanoparticles (17) and others. The idea of using termites extract as an antibiofilm was not described. Therefore these results investigated that the termites extract possess an antibiofilm activity. Some methodologies can clarify this activity in different organisms; some demonstrate the interference with cell to cell communication which is called quorum sensing inhibitors (7) that effect biofilm production. Others obtain antibiofilm polysaccharides produced by bacteria that demonstrated great promise as inhibitors of biofilm production (26). Another way to control biofilm synthesis was the utilization of bacteriophage by entering biofilm and kill host bacteria or produce enzymes that lysis the exopolysaccharide matrix resulting in destruction of the structure of the biofilm (35). Termitis extrat have a good ability to inhibit the growth of pathogenic bacteria. The study provides information on natural biological resources for disinfectants and new pharmaceutical, the results show significant importance and shed light on benefits of these arthropods in the industry of modern medicine which open a new field for study of substances produced by insects that have many benefits for human kind.

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