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Anti-*Salmonella* activity of metabolites from African soldier termites, *Macrotermes bellicosus*

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Abstract:

Background: The global emergence and rapid dissemination of multidrug resistant *Salmonella* strains necessitate research to find new antimicrobials that will effectively be used against these pathogens. In the present study, anti-*Salmonella* activity of metabolites from African Soldier Termites, *Macrotermes bellicosus* was demonstrated and subsequently compared with a potent antibiotic, ciprofloxacin.
Materials and Methods: N-hexane, ethylacetate, methanol and aqueous extracts of metabolites from the *M. bellicosus* were assayed for anti-*Salmonella* activity using the agar dilution method in the determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The inhibitory activities of the extracts were compared to ciprofloxacin (256µg/ml). Also, the bioactive components of the extracts were determined using standard techniques.
Results: At 4000 µg/ml, N-hexane extract inhibited the growth of *Salmonella* Typhi, *S.* Paratyphi A, B and C while ethylacetate extract was able to inhibit *S.* Paratyphi A and C. Methanolic and aqueous extracts at the same concentration were unable to inhibit these strains of *Salmonella*. Furthermore, our findings revealed that the MIC of ethylacetate extract was 2000µg/ml for *S.* Paratyphi A and B, 250µg/ml for *S.* Typhi, and 125µg/ml for *S.* Paratyphi C. Also, the MIC of hexane extract was 4000µg/ml for *S.* Paratyphi B, 2000 µg/ml for *S.* Paratyphi C, 500µg/ml for *S.* Typhi and 250µg/ml for *S.* Paratyphi A respectively. The screening of bioactive components revealed the presence of cardiac glycosides and alkaloids.
Conclusion: Our results provide evidence of anti-*Salmonella* action of metabolites from African Soldier Termites, *M. bellicosus*. N-hexane and ethylacetate extracts of *M. bellicosus* may be explored as novel antimicrobials for the treatment of typhoid and paratyphoid fevers thereby reducing the pressure exerted on available antibiotics.

Keywords: *Salmonella*, antimicrobials, insects, extracts

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Activité anti-*Salmonella* des métabolites de termites soldats africains, *Macrotermes bellicosus*

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Abstrait:

Contexte: L'émergence et la dissémination rapide de souches de *Salmonella* multirésistantes nécessitent des recherches pour trouver de nouveaux antimicrobiens qui seront utilisés efficacement contre ces agents pathogènes. Dans la présente étude, l'activité anti-*Salmonella* de métabolites de *Macrotermes bellicosus*, African Soldier Termites, a été démontrée et comparée par la suite à un antibiotique puissant, la ciprofloxacine.
Matériels et méthodes: L'activité anti-*Salmonella* a été dosée avec du N-hexane, de l'acétate d'éthyle, du méthanol et des extraits aqueux de métabolites provenant de *M. bellicosus* en utilisant la méthode de dilution en gélose dans la détermination de la concentration minimale inhibitrice (CMI) et de la concentration bactéricide (MBC). Les activités inhibitrices des extraits ont été comparées à la ciprofloxacine (256µg/ml). En outre, les composants bioactifs des extraits ont été déterminés à l'aide de techniques classiques.
Résultats: À 4000µg/ml, l'extrait de N-hexane inhibe la croissance de *Salmonella* Typhi, *S*. Paratyphi A, B et C alors que l'extrait d'acétate d'éthyle est capable d'inhiber *S.* Paratyphi A et C. Les extraits méthaniques et aqueux à la même concentration ne peuvent inhiber ces souches de *Salmonella*. En outre, nos résultats ont révélé que la CMI de l'extrait d'acétate d'éthyle était de 2000µg/ml pour S. Paratyphi A et B, de 250 µg/ml pour *S.* Typhi et de 125µg/ml pour *S.* Paratyphi C. De plus, la CMI de l'extrait d'hexane était de 4000µg/ml pour *S*. Paratyphi B, 2000µg/ml pour S. Paratyphi C, 500µg/ml pour *S.* Typhi et 250µg/ml pour *S.* Paratyphi A respectivement. Le dépistage des composants bioactifs a révélé la présence de glucosides et d'alcaloïdes cardiaques.
Conclusion: Nos résultats fournissent des preuves de l'action anti-*Salmonella* des métabolites de termites de soldat africains, *M. bellicosus*. Des extraits d'N-hexane et d'acétate d'éthyle de *M. bellicosus* peuvent être explorés comme nouveaux antimicrobiens pour le traitement des fièvres typhoïde et paratyphoïde, réduisant ainsi la pression exercée sur les antibiotiques disponibles

Mots-clés: *Salmonella*, antimicrobiens, insectes, extraits

Introduction:

Typhoid and paratyphoid fevers are a severe, life-threatening disease caused primarily by serovars Typhi and Paratyphi A, B, or C of *Salmonella enterica* subspecies enterica. Typhoid accounts for approximately 90% of enteric fevers in Africa, Asia, and Latin America, mostly regarded as endemic, with high morbidities and mortalities (1). It is transmitted by the faecal-oral route via contaminated food and water and is therefore common where sanitary conditions are inadequate and access to clean water is limited (2).

Although, antibiotics have been used for the treatment and control of *Salmonella* infections in human and animals, recent studies show increase resistance to conventional drugs, including fluoroquinolones and third generation cephalosporins which are the drugs of choice for infections caused by *Salmonella* and other members of the Enterobacteriaceae family (3, 4). The emergence of *Salmonella* strains with multidrug-resistant (MDR) genes has constituted a serious public health problem, resulting in higher treatment cost, longer stay in the hospital, and increase morbidity and mortality especially in developing countries (5). Therefore, an urgent search for novel anti-*Salmonella* agents with high potency which will be used as alternatives to conventional drugs is imperative.

Insects have been recognized recently to possess highly potent immune defenses that synthesize constitutive and inducible antimicrobial compounds capable of combating a wide spectrum of pathogens (6). Consequently, insects are now the major target as the abundant potential source of different antimicrobial compounds (7, 8). Although insects make up 90% of the total number of animals on earth, insect-derived antimicrobial peptides (AMPs) only account for approximately 10% of more than 2,830 AMPs listed in the Antimicrobial Peptide Database; thus there are more AMPs with activity just waiting to be discovered (9).

African Soldier Termites, *Macrotermes bellicosus* are well distributed in Nigeria and other African countries. These termites can survive and propagate in diverse ecological environments due to their ability to develop resistance mechanisms against different diseases (10). This study, therefore, investigated the *In vitro* anti-*Salmonella* activity of different extracts from African soldier termite, *M. bellicous* against strains of *Salmonella*.

Materials and Methods:

Zoological Method

Termites were sampled form the mound using a hoe in Minna, North-Central Nigeria by methods previously described (11). The samples were put in plastic bags with mound soil and immediately transported to the laboratory. The termites were further picked using forceps and transferred to a bottle containing methanol, and were identified as African soldier termites, *M. bellicous*, by the Entomology section of the Department of Biological Sciences, Federal University of Technology, Minna, Nigeria.

Preparation and Screening of Extracts for Bioactive Components

The head of the termite which is distinct from the body was carefully removed using a sterile scalpel, which was air-dried for two weeks and pounded using clean mortar and pestle. Successive reflux extraction of the powdered termites' heads (80g) was carried out using solvents ranging from non-polar to polar to obtain various soluble portions. The resulting mixtures were filtered using Whatman filter paper No. 1 while the soluble portion was evaporated to dryness using a steam bath. At the end of the extraction protocol, four extracts of N-hexane, ethylacetate, methanol, and aqueous extracts were obtained and stored in sterile universal bottles at 4^{o}C until further processing. The extracts obtained were screened for the presence of bioactive components as previously described (12). Different bioactive components including saponins, tannins, phlobatannin, cardiac glycosides, alkaloids and reducing sugars were sought for.

Bacterial Strains

Pure clinical strains of *Salmonella* Typhi and *S*. Paratyphi A, B and C were collected from the Vaccine Laboratory, Department of Microbiology, Federal University of Technology Minna, Nigeria. The strains were further confirmed by biotyping using the Microbact^{TM} 12A (12 E) kit.

Anti-*Salmonella* Activity

The extracts were reconstituted in DMSO and distilled water as 0.4g of the different extract was dissolved in 1ml of DMSO plus 4ml of distilled water. The anti-*Salmonella* activities of all the extracts were determined using agar dilution method (13). Under aseptic conditions, 1 ml of each reconstituted extract was dispensed into sterile Petri plates, and 19 ml of sterilized nutrient agar was added to make a final concentration of 2000 µg/ml, after which the plates were swirled for homogeneity. The plates were prepared in duplicates and control plates consisted of organism viability control (OVC), extract sterility control (ESC), medium sterility control and DMSO control plate was also prepared. The activities of extracts were compared with that of ciprofloxacin (256µg/ml). Ciprofloxacin, which is a fluoroquinolone known to be potent against *Salmonella*, was obtained from General Hospital, Minna, Nigeria and dissolved in sterile water before use.

Minimum Inhibitory Concentration Determination

The broth dilution method of Cheesbrough (13) was employed for MIC determination. The hexane and ethylacetate extracts were diluted with nutrient broth to obtain 4000µg/ml, 2000 µg/ml, 500µg/ml, 250µg/ml and 150µg/ml respectively. A loopful of each *Salmonella* strain was added to the diluents and incubated at 37°C for 24hr. The tubes with the least concentration of the extracts that showed no turbidity were recorded as the MIC.

Minimum Bactericidal Concentration Determination

The MBC of the extracts were determined as earlier described (13). Briefly, the MIC tubes were sub-cultured on sterile nutrient agar and incubated at 37°C for 24hr. The plates with the least concentration of the extracts that showed no growth after sub-culturing was recorded as the MBC.

Results:

The anti-*Salmonella* activity of crude extracts of *M. bellicous* are presented in Table 1. The N-hexane exracts showed activity against the four isolates, *S.* Typhi and *S*. Paratyphi A, B and C screened while activity was only recorded against *S*. Paratyphi A and C from the Ethylacetate extract respectively. There were no anti-*Salmonella* activities recorded for both methanol and aqueous extracts.

The minimum inhibitory concentration (MIC) of N-hexane extract showed activity ranged between 4000 to 250µg/ml only for *S.* Typhi and *S*. Paratyphi A, B and C while that of Ethylacetate extract ranged between 2000 to 125µg/ml only for *S.* Typhi and *S*. Paratyphi A, B and C (Tables 2 and 3). The presence of bioactive components as obtained from the N-hexane and Ethylacetate extracts indicated the presence of cardiac glycosides and alkaloids (Table 4).

Table 1: Anti-*Salmonella* activity of crude extracts of *Macrotermes bellicous*

Test organisms	Crude Extract (4000 µg/ml)			
	ST^{SH}	ST^{SE}	ST^{SM}	ST^{SW}
S. Typhi	+	-	-	-
S. Paratyphi A	+	+	-	-
S. Paratyphi B	+	-	-	-
S. Paratyphi C	+	+	-	-

+ = activity; - = no activity; ST^{SH} = N-hexane extract; ST^{SE}= Ethylacetate extract; ST^{SM} = Methanol extract; ST^{SW}= Aqueous extract

Table 2: Minimum Inhibitory Concentration (MIC) of N-Hexane Extract Crude Extract of *Macrotermes bellicous*

Test organisms	N-hexane (µg/ml)					
	4000	2000	1000	500	250	125
S. Typhi	-	-	-	+	-	-
S. Paratyphi A	-	-	-	-	+	-
S. Paratyphi B	+	-	-	-	-	-
S. Paratyphi C	-	+	-	-	-	-

Activity = +; - = No activity

Table 3: Minimum Inhibitory Concentration (MIC) of Ethylacetate Extract Crude Extract of *Macrotermes bellicous*

Test organisms	Ethylacetate (µg/ml)					
	4000	2000	1000	500	250	125
S. Typhi	-	-	-	-	+	-
S. Paratyphi A	-	+	-	-	-	-
S. Paratyphi B	-	+	-	-	-	-
S. Paratyphi C	-	-	-	-	-	+

+ = activity: - = no activity

Table 4: Bioactive components of the N-hexane and Ethylacetate extracts

Bioactive components	Extract	
	N-hexane	Ethylacetate
Tannin	-	-
Phlobatannin	-	-
Reducing sugar	-	-
Alkaloids	-	+
Steroids	-	-
Saponin	-	-
Cardiac glycosides	+	+

+ = Present; - = Absent

Discussion:

The study demonstrates that metabolites from the head of African soldier termites have antibacterial activities that are effective against some human pathogens. The pathogens tested were found to show different degree of susceptibility to various extracts obtained from the head of African soldier termites. In this study, N-hexane extract inhibited the growth of typhoid and paratyphoid bacilli while the Ethylacetate extract inhibited *S.* Paratyphi A and C only. Termites have developed the ability to deal with a rich microbial community inhabiting their nests and feeding sites. One of the ways which they do this is by the synthesis of antimicrobial peptides, among other defense mechanisms (14). These antimicrobial peptides may explain the reason for the anti-*Salmonella* activity observed in this study. This claim was supported by the observation of Zeng (15), who reported the antibacterial activity of some peptides, spinigerin and termicin obtained from termites against pathogenic organisms. Furthermore, spinigerin and termicin peptides that are rich in cysteine and contained α-helical properties have high ability to permeate microbial cytoplasmic membranes (16). This was further corroborated by Lee (17), who studied the antimicrobial properties of spinigerin.

Although extracts used in this study were not obtained from plants, it has been suggested that for plants to be used for medicinal purposes, their extracts should possess antimicrobial activity with MIC<1000µg/mL (18). With reference to their report, results of the MIC obtained from this study indicated that N-hexane extracts possess strong antimicrobial activity for *S.* Typhi and *S.* Paratyphi A while Ethylacetate extracts possess antimicrobial activity against *S*. Typhi and *S.* Paratyphi C since their MICs were less than 1000µg/mL. The evaluation of the MBC to ascertain the bactericidal effect of the N-hexane and Ethylacetate extracts on the *Salmonella* strains examined at various concentrations did not showed any bactericidal activity (results not presented). This further shows that a higher concentration above the MIC is required for bactericidal activity.

Phytochemicals are known to have complementary and overlapping mechanisms of action in the body including modulation of hormone metabolism and enzyme detoxification, antioxidant effects, stimulation of the immune system and antibacterial activity (19, 20). The qualitative screening of bioactive components from the head of African soldier termites shows the presence of some plant secondary metabolites; alkaloids and cardiac glycosides. The presence of these components in termites could undoubtedly arise from their host plants. This is because this species of termites is mainly xylophage. They are herbivorous whose diets consist primarily of wood. As such, Phyto-compounds present in the plants consumed may still be in circulation within the insects in relatively high amounts (21, 22). The presence of these bioactive components may also be the reason for the antimicrobial activity recorded in this study.

It was observed that alkaloids and cardiac glycosides also significantly decreased the growth and proliferation of pathogenic *Klebsiella pneumoniae* and *Staphylococcus aureus* (23). Similarly, Fernandez-Melendez (24) observed that the alkaloids in fire ant, *Solenopsis invicta* inhibited the growth of Gram-positive and Gram-negative bacteria acting as a broad-spectrum antimicrobial agent. Their findings are in consonance with the results obtained from this current study.

The extracts of *M. bellicosus* at 4000µg/ml displayed similar bacteriostatic effects on the test organisms when compared with ciprofloxacin (265µg/ml). It is noteworthy to further state that such results as obtained from this study can be attributed to the crude state of the extract used. Furthermore, upon purification and quantification of the extracts used in this study, their activities may be more potent and biocidal even at lower concentrations.

Conclusion:

The findings of the current study show that metabolites from the head of solider termites can inhibit the growth and survival of some pathogenic bacteria. However, further studies are needed to standardize potential use of *M. bellicosus* as complementary agent for eliminating pathogenic bacteria. Also, synergistic effect of the use of metabolites of *M. bellicosus* and conventional antibiotics can also be examined.

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