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ACTIVATION OF THE IMMUNE SYSTEM OF *ANOPHELES GAMBIAE* AGAINST MALARIA PARASITE: A COMPARISON BETWEEN BACTERIAL INJECTION AND A BOTANICAL EXTRACT

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ABSTRACT

The mosquito *Anopheles gambiae*, the major vector of malaria in Africa, was used to test the hypothesis that the utilization of immune system against malaria parasites could be possible. Two immune elicitors, the bacteria, *Micrococcus luteus* and the black-seed (BS) oil, *Nigella sativa*, were used to stimulate the immune system of this vector against the rodent malaria parasite, *Plasmodium yoelii nigeriensis*. Mosquitoes were inoculated with bacteria or allowed to orally feed on glucose-oil mixture both before and after malaria infection. The resultant antimicrobial activities and the impact on the number of oocysts successfully formed were then monitored. Although infection of mosquitoes with malaria resulted in a significant antimicrobial activity, inoculation with *M. luteus* has induced a significantly higher antimicrobial activity. Thus, when mosquitoes injected with *M. luteus* immediately before malaria infection, the number of the successfully formed oocysts was significantly reduced by 46.5%. This in fact indicates that *M. luteus* has partially activated the immune system against the malaria parasite. On the other hand, a well-known immune activator, the BS oil has significantly reduced the number of oocysts by 90% only when fed to mosquitoes from 6 days before malaria infection and until completion of oocysts formation. This indicates that BS oil could be more effective than bacterial inoculation. This, in fact, may help in improving the recent strategies of blocking the malaria life cycle in the gut of its mosquito vector using natural products.

Key words: Mosquito; malaria; *M. luteus*; black-seed oil; immune system; antimicrobial activities

INTRODUCTION

Insect immunity is the innate or acquired resistance to particular pathogens or parasites. Protein molecules and haemocytes are parts of insect immune system and involved in non-self recognition. This system contributes to inactivation of poisons, elimination of pathogens and blocking parasite development in its vector. This, in fact, facilitates the survival of insects even in habitats that are highly contaminated with microorganisms (Townson & Chaithong 1991 and Dimopoulos *et al*.; 2001). Humoral immune responses (reviewed by Brey & Hultmark, 1998) and the

coagulation and melanization responses, activated by proteolytic cascades, are essential immune responses in insects (Richman & Kafatos, 1996). Phagocytosis or nodule formation of prokaryotic organisms and encapsulation of eukaryotic organisms, such as parasites, constitute the main cellular response mechanisms mediated by haemocytes (Ratcliffe & Rowley, 1979 and Lackie, 1988).

Utilizing the immune system of insect vectors against disease causing parasites has recently gained the attention of the global health organizations. One of these parasitic diseases is malaria which threatening large parts of the world and transmitted by the anopheline mosquito, *An. gambiae*, in Africa (Coluzzi, 1992). Based on the ability of mosquitoes to transmit malaria, they are categorized into two groups. The first group is the refractory mosquito, which has innate immune responses that able to block the parasite development inside its gut. This takes place either by encapsulating the ookinetes in the midgut (Christensen *et al*.; 1984; Collins, *et al*, 1986; Paskewitz *et al*, 1988 and Somboon *et al*.; 1999), by preventing sporozoites from penetrating the salivary gland (Rosenberg, 1985) or by physiological incompatibility between the mosquito and the parasite (Thathy *et al*.; 1994 and Severson *et al*.; 1995). The second group is the susceptible mosquito (the malaria vector), in which the malaria parasite succeeds to complete its life cycle and making it infective to a new vertebrate host.

The idea of targeting the immune system of mosquito vector to control malaria disease came as a result of pesticide resistance of the mosquito vector and the rapid spread of multiple anti-malarial drug-resistance of malaria parasite. Therefore, alternative novel control strategies aiming at blocking the mosquito stages in the susceptible mosquito midgut are progressing nowadays to reduce the competence of the vector to transmit malaria. This prime objective includes the recent rapidly evolved area of research on malaria, “the transmission-blocking immunity strategy” (Gwadz *et al.;* 1989; Shahabuddin *et al*.; 1998 Yoshida *et al*.; 2001) including genetic selection of malaria-refractory vector (Collins *et al.;* 1986) and genetically modifying incompetent vector (Kohoza *et al.;* 2000).

Although there have been no malaria cases reported in Egypt since 1989 (www.who.int/ith/english/egypt.htm) and no *An. gambiae* exist in Egypt since the mid 1940s (Kenawy, 1990), we must expect re-emergence of this disease as long as the vector is spread near the borders of Egypt

(Kenawy, 1990). Thus, one previously proved as Egyptian malaria vector, the *An. gambiae* (Farid, 1984 and Kenawy, 1988 & 1990) have been selected for the present study to test the hypothesis of enhancing the immune system of this vector against the rodent malaria parasite, *P. y. nigeriensis*. This took place by comparing between

inoculation (injection) of the bacteria, *Micrococcus luteus*, and administration of the botanical extract, the black-seed (BS) oil, *Nigella sativa,* a well-known immune activator in animals (El-Dakhakhny, *et al*.,2002 and Abdel-Ghaffar, *et al*.; 2003) and insects (Abu El-Magd, 1995). This, in fact, may help in improving the recent strategies of blocking the malaria life cycle in the gut of its mosquito vector.

MATERIALS AND METHODS

A)- Maintenance of *An. gambiae* and malaria parasite

Mosquitoes and malaria parasite were maintained in Vector Biology and Parasitology Laboratory at Keele University, United Kingdom. Hatched larvae of *An. gambiae* KIL (Liverpool School of Tropical Medicine and Hygene, UK) were reared under standardized conditions to produce adults of uniform size as detailed in Ahmed *et al.*, (1999). Access to glucose solution was denied 12 h prior to blood feeding. *P. y. nigeriensis* Killick-Kendrick (N67), was maintained in CD male mice, as described in Ahmed *et al.*, (1999). Blood packed-cell volume (PCV) for mice was determined immediately prior to mosquito feeding and parasitaemia was assessed in thin blood smears. Only infected mice containing exflagellating microgametocytes (seen in thick blood smears) were used in the study.

B)-Infection of mosquitoes

Prior to vector feeding, a gametocytaemic mouse was anaesthetised by intraperitoneal injection of Hypnorm and Diazepam (dose adjusted according to the weight of mice). Nulliparous (not blood-fed) 6 day-old mosquitoes were starved for 12h then allowed to feed on an anaesthetised secondary infected CD mouse for 10 minutes. Fully engorged females were randomly selected and assigned to their experimental groups as detailed in the experimental design (see below). Twenty infected mosquitoes were kept separately for 8 days to test the prevalence of infection, which was more than 90% of all infected mosquito groups used in this study.

C)- Counting ookinetes in mosquito midgut

Fully engorged malaria infected mosquitoes were used to dissect their midguts. Each blood meal was individually expelled gently from the dissected gut onto a clean microscopic slide, dissolved in 5 µl *Aedes*-physiological saline (APS) and smeared as a monolayer of cells. Slide was air dried for few minutes, fixed in 90% (v/v) methanol for 1 min, rinsed with tap water and air-dried before staining by Giemsa's stain for 15 min. Excess stain was rinsed from the slides with tap water then, dried before observing under phase contrast oil immersion (x1000 magnification). Ookinetes were counted in all the viewing fields across the slide. Any slide not showing ookinetes was excluded from the study.

D)- Bacterial inoculation and oocysts counting

Micrococcus luteus (strain NCTC 2665) (Sigma, UK) was prepared for inoculation as detailed in Ahmed *et al*.; (2001). Mosquitoes were immobilized on ice and bacterial infection was performed by pricking (injection) with a fine capillary needle dipped in a concentrated suspension of *M. luteus* according to Dimopoulos *et al*.; (1997). Any mosquito that was severely bled after inoculation was discarded from the study. After pricking, the mosquitoes were allowed to recover and used for the experiment as detailed below. Midguts of experimental mosquitoes were dissected in 5% saline solution and then socked in mercurochrome for 2 minutes to visualize the oocysts (Fig. 2) for counting the oocysts under the microscope. The mean number of oocysts was calculated from 10 replicates (10 mosquitoes), and uninfected midguts were discarded.

F)- Haemolymph collection and humoral antibacterial activity assay

Haemolymph was collected from the thorax of experimental mosquitoes using a pre-calibrated capillary needle as described in Ahmed *et al*.; (2001). Antibacterial activity, seen as a clear inhibition zones as a result of the existence of antibacterial peptides (Fig 2) has been done as detailed in Ahmed *et al*.; (2002).

G)- Administration of BS oil and counting of oocysts

BS oil was mixed with glucose solution 10% (w/v) in three different concentrations, 0.1%, 0.3% and 0.5%. The oil was mixed with the glucose solution very well by shacking vigorously several times a day to insure a homogenous mixture. Mosquitoes were allowed to feed on the sugar-oil

mixture according to the experimental design illustrated in Fig. 6. Midguts of experimental mosquitoes were dissected for counting the oocysts as described above.

H)- Experimental designs

I)- Time profile for ookinete invasion of mosquito midgut

For obtaining a time course profile for ookinete development and invasion of the mosquito midgut, a group of 50 infected fully engorged mosquitoes (of similar body sizes) were randomly selected immediately after malaria infective blood meal. Five midguts were dissected at each time point studied (14, 16, 18, 20, 22 and 24 h post-infection). Ookinetes were counted for each slide as detailed above. The mean number of ookinetes from 5 different mosquitoes at each time point of the experiment was calculated. A group of 20 infected mosquitoes were kept for 8-10 days for dissecting their midguts to assess the prevalence of infection, which was 90% and the intensity of infection which was approximately > 50 oocysts/midgut.

II) Humoral antibacterial assay

To study the humoral antibacterial activity, six days-old mosquitoes were divided, randomly, into 4 groups (50 mosquitoes each). The first group was pricked with a needle dipped in sterile APS (control), the second group was pricked with a needle dipped in a concentrated *M. luteus* bacterial suspension, the third group was allowed to feed on un-infected mouse (control 1) and the forth group was infected with malaria (control 2). After treating and blood feeding, treated and fully engorged females assigned to each group were randomly selected and moved into new small cages (6 x 6 x 6 cm each) as described before. To measure humoral antibacterial activity, haemolymphs were collected from mosquitoes 24h post-treatment as described before and used to perform inhibition zone assay as detailed in Ahmed *et al.*; (2002).

III)- Activation of mosquito immune system against malaria by bacterial inoculation:

Mosquitoes (6 days old) were randomly divided into 7 groups (~ 50 female mosquitoes each). The first, second and third groups were pricked with *M. luteus* in different times (one day before, immediately after and 24h post-malaria infection respectively). The fourth, fifth and sixth groups were

pricked with APS (sham) in different times by the same way of the first three groups in response to malaria infection. The seventh group was infected with malaria and left un-treated (control group). Treated mosquitoes assigned to each group were randomly selected and transferred into seven small cages (6 x 6 x 6 cm each), 25 mosquitoes/cage, and supplied with 10% glucose solution until dissection of midguts for oocysts counting 8 days post-infection as described before.

IV)- Activation of mosquito immune system against malaria by BS oil feeding:

As illustrated in figure 6, newly emerged adults (step 1) were randomly divided into five groups, A, B, C1, C2 & C3 (step 2). Groups A (≈ 150 female mosquitoes) and B (50 female mosquitoes) were allowed to feed on 10% glucose solution (w/v) until malaria infection took place 6 days later. Groups C1, C2 & C3 (50 female mosquitoes each) were allowed to feed on 10% glucose solution mixed with 0.1%, 0.3% & 0.5% (v/v) BS oil respectively until malaria infection took place 6 days later. All mosquito groups were then allowed to feed on one gametocytemic mouse (to ensure the identity of infection conditions) (step 3). The mouse was used to feed all groups one by one (for 10 minutes each) by moving it from one group to another until most of female mosquitoes of all groups become fully engorged. After feeding on malaria infective blood meal, and as shown in step 4, 30 fully engorged females from each of the three Cs groups were randomly selected and allowed to feed on the same sugar-oil mixture of the same mixing % as before malaria infection. Fully engorged females of the group A were selected and randomly divided into 3 subgroups A1, A2 & A3 (30 female mosquitoes each) and allowed to feed on 10% sugar solution mixed with 0.1%, 0.3% and 0.5% (v/v) BS oil respectively. Thirty fully engorged females were randomly selected from group B and allowed to feed on 10% glucose solution only like before malaria infection (control group). All mosquito groups were kept in their cages until dissecting their midgets (8 days post-infection), as described before, for counting oocysts.

V)- Statistical analysis

All statistical analyses were undertaken using MINITAB software (MINITAB, State College, PA). Data were tested for normality using Anderson Darling test, and variances were tested for homogeneity prior to further analysis. To determine overall effects of bacterial inoculation on the number of oocysts formed, antibacterial activity and comparisons between

treatments were made using one-way ANOVA and the Kruskal-Wallis test. The Mann-Whitney *U* test (for individual comparison) was used for analysing the non-parametric data resulting from the mosquito groups fed on black- seed oil.

RESULTS

A)- Time profile for ookinete invasion of mosquito midgut

The development and invasion profile of ookinetes in the mosquito midgut was demonstrated after ingestion of a malaria infective blood meal. Ookinetes were first detected in the midgut at 14 h post-infection, their number peaked between 18 and 20 h after which, it declined until they became hardly detectable at 24 h post-infection (Fig. 1). This clearly shows that the peak time of ookinetes invasion of *An. gambiae* midgut is between 18 and 22 h post-infection. Oocysts (Fig. 4) were observed from 6-8 days post-infection and disappeared (burst) 10-12 days after infection as the sporozoites migrate to the salivary glands.

B)- Anti-*M. luteus* activity in haemolymph from bacteria-injected mosquitoes

Lysozyme equivalent humoral activity against *M. luteus* was detected in haemolymphs from mosquitoes at 24h post-treatment (Fig. 2 and 3). In untreated mosquitoes fed on malaria infective blood meal (control 2), it was 356.56 ± 0.016 ng/µl haemolymph. Whereas, in untreated mosquitoes fed on un-infective blood meal (control 1), it was 254.6 ± 32.5 ng/µl haemolymph, which is significantly lower (Tukey's pairwise comparison; $P < 0.05$) (Fig. 3). This indicates that malaria infection caused a significant mean increase of 28.6% in the anti- *M. luteus* activity. Furthermore, pricking mosquitoes with bacteria immediately before malaria infection caused an overall significant effect (One-way ANOVA; Tukey's pairwise comparison; $P < 0.05$, n = 5 per treatment). Thus, means significant increases in anti-*M. luteus* activity by 51.25%, 31.72% and 46.37% in bacterial inoculated group compared with those of control 1, control 2 and sham injected groups respectively (One-way ANOVA; Tukey's pairwise comparison; $P < 0.05$). This increase in antibacterial response coincides with the peak of ookinetes invasion to the midgut (Fig 1). Sham injected females showed non-significant difference in anti-*M. luteus* activity compared with those of the two control groups (Tukey's pairwise comparison; $P > 0.05$).

C)- The effect of bacterial inoculation on the number of oocysts

The number of successfully formed oocysts (Fig 4) was counted 8 days post-malaria infection in all mosquito groups regardless the times of different treatments. This number was significantly reduced in malaria infected females pricked with bacteria immediately before malaria infection (day 0). This reduction was by 67.2% and 67.5% compared with those pricked with bacteria one day before malaria infection (day –1) and one day after malaria infection (day 1) (One-way ANOVA; Tukey's pairwise comparison; $P < 0.05$, n = 10 per treatment) (Fig. 5). Furthermore, the number of oocysts was significantly reduced by 55.3%, 46.5%, 53.0% and 52.0% compared with those pricked with sterile saline at day -1, day 0 and day 1 respectively (One-way ANOVA; Tukey's pairwise comparison; $P < 0.05$, n = 10 per treatment) (Fig. 5). In addition, this number was significantly reduced by 52.0% compared with control group (malaria infected un-treated) (One-way ANOVA; Tukey's pairwise comparison; $P < 0.05$, n = 10 per treatment). These reductions coincide with the significant increase of antibacterial response 24h post-bacterial inoculation (Fig. 3), which may affect ookinetes invasion to the midgut (Fig. 1).

The One-way ANOVA test showed no difference in oocyst numbers between all other groups (Tukey's pairwise comparison; $P > 0.05$, n = 10 per treatment) (Fig. 5). This indicates that pricking mosquitoes with bacteria immediately before malaria infection has significantly reduced the number of successfully formed oocysts (Fig. 5).

D)- The effect of BS oil administration on the number of oocyst

For this nonparametric data, Kruskal-Wallis test showed an overall significant difference in the number of oocysts between oil-treated and untreated females. Individual analysis using Mann-Whitney *U* (a non-parametric test) showed that sugar solution mixed with 0.1, 0.3 and 0.5% (v/v) oil significantly reduced the oocyst number by 51.2, 76.5 and 90.2% respectively when fed to mosquitoes continuously from 6 days before malaria infection and until oocyst counting 8 days post-infection ($P < 0.05$) compared with control (infected untreated) (Fig. 7). Moreover, there were significant reductions in oocysts numbers by 34.8, 71.2 and 40.8% respectively when mosquitoes were fed on the same mixtures post-malaria infection and until gut dissection 8 days post-infection ($P < 0.05$) compared with control (Fig. 7).

A significant 83.4% reduction ($P < 0.05$) was noticed in oocysts number in mosquitoes fed on sugar mixed with 0.5% oil (v/v) 6 days before infection compared to that in mosquitoes fed on the same mixture but only after infection. However, no significant differences ($P > 0.05$) were detected in oocysts number in the midguts of mosquitoes fed on sugar solution mixed with a concentration of either 0.2 or 0.3% oil before infection compared with those fed on the same mixtures but only after infection (Fig. 7). Although, they showed significant fewer numbers off oocysts compared with control group.

The overall results indicate that 0.5% BS oil mixed with sugar solution (v/v) has significantly reduced the number of oocysts formed in mosquito vector compared to all treatment either before or after infection (Fig. 7). It also resulted in 1.7% more reduction in oocysts number compared with that of bacterial pricking (Fig. 5).

DISCUSSION

Malaria remains the most important of the vector-borne disease resulting in approximately more clinical cases and fatalities worldwide than any other parasitic disease (WHO, 1998). Ookinete is one of the most important stages during *Plasmodium* development, which is morphologically distinct from the earlier sexual stages, gametocytes and zygotes. In its mosquito vector, the parasite has to develop to an ookinete to escape from the blood cells and to cross the peritrophic membrane (PM) in order to protect itself from the digestive environment of the midgut. Therefore, the developmental time period of the parasite from a young zygote to a motile mature ookinete, that is able to invade the gut epithelium, plays a crucial role in the success of the parasite to invade the midgut (Mehlhorn, *et al*.; 1980). After being mature, approximately 15h after zygote formation, ookinetes are able to secrete chitinase, which is needed for crossing the chitinuous PM (Shahabuddin *et al*.; 1993).

Previous studies showed differences exist in the kinetics of ookinete development among mosquito species (Vaughan, *et al.;* 1994). Thus, it was important for the current study to know both the start and peak times of invasion of *P. y. nigeriensis* ookinetes in *An. gambiae* under the experimental conditions of this study. Results revealed that *P. y. nigeriensis* ookinetes were first detected in the midgut of infected *An. gambiae* at 14h post-infection, which might be the start time of invasion. Their number

peaked at 18-22h post-infection after which, it was undetectable. The period from 14 to 22h post-infection is therefore the suitable time to attack this weakest malaria stage via the vector immune system as happening in most of the refractory vectors (Collins, *et al*.; 1986 and Paskewitz, *et al*.; 1988). Thus, the malaria blocking strategy should be effective during this time period.

Recently, two strategies are aiming at blocking malaria development in its mosquito vector. The first is the genetic selection of malaria-refractory vector (Collins *et al.;* 1986, Paskewitz *et al*, 1988 and Somboon *et al*.; 1999) and genetically modifying vectors by driving parasite-inhibiting genes from refractory into susceptible mosquito populations (Curtis, 1994, James *et al*.; 1999 and Kohoza, 2000). These two genetic strategies are aiming to produce genetically defined lines of mosquito vector to be incompetent to the development of malaria parasite in its gut. The second strategy is based on using transmission-blocking targets in mosquito midgut. For example, the use of polyclonal sera that raised against mosquito midguts. This target blocks the passage of malaria ookinetes across the midgut, leading to a significant reduction of infection in mosquitoes (Lal, *et al*.; 2001). Thus, these two strategies, and as in the current study, consider the vector immune system the prime target to be utilized to block the development of *Plasmodium* inside its mosquito vector.

Results of the current study showed a partial activation of the immune system, which was shown as a significant increase in the humoral antibacterial activity as a result of malaria infection. However, it seems not sufficient for complete blocking of the parasite development. Hence, if this humoral activity is enhanced, it would be effective against *Plasmodium* development as antiparasitic

response. This is based on previous studies that proved antiparasitic effects for the humoral antimicrobial peptides against disease causing parasites including malaria (Gwadz *et al*.; 1989; Beerntsen & Christensen, 1990; Ham *et al*., 1994*a*&*b* and Shahaboddine *et al*.; 1998). Thus, inoculation of *M. luteus* into malaria infected mosquitoes took place in this study to enhance the production of antibacterial peptides, during the period of ookinetes development and midgut invasion, for causing maximum effects on this stage. The outcome of this activity has been shown clearly as a significant 46.5% reduction in the number of formed oocysts compared to infected control (saline injected) mosquitoes. This indicates that antimicrobial peptides could have antimalarial activity which could promise as an effective control method.

Feeding mosquitoes with BS oil, mixed with glucose by 0.5% (v/v), both before and after malaria infection has significantly reduced the number of oocysts by 90%. This extent of reduction is 1.9 fold of the reduction in oocyst number resulted from bacterial inoculation. This indicates that BS oil could be a better immune elicitor than *M. luteus*. The effect of BS oil is probably the result of stimulating the melanotic encapsulation response (Abu El-Magd, 1995) as occurred in the antimicrobial activity against ookinetes, which resulted in that much reduction comparing to bacterial inoculation.

Generally, BS oil has been proven to enhance the immune system of vertebrate animals. This may take place via stimulating different immune responses (Swamy & Tan, 2000 and Abdel-Ghaffar, *et al*.; 2003) which could have been occurred by the active compounds in this oil. Two active compounds have been proven to stimulate the immune responses in vertebrates, the thymoquinone (the active principle of BS oil) and nigellone (the carbonyl polymer of thymoquinone) (El-Dakhakhny *et al.*, 2002). It is not known whether this is the case in insects or not. However, BS oil might have stimulated one or different immune responses against ookinetes while they are invading and developing in the mosquito gut. It is still however to be investigated which of the immune responses have been stimulated against this parasite in the future work. This may lead up a better understanding of utilizing the immune system of the vector to block the human malaria transmission.

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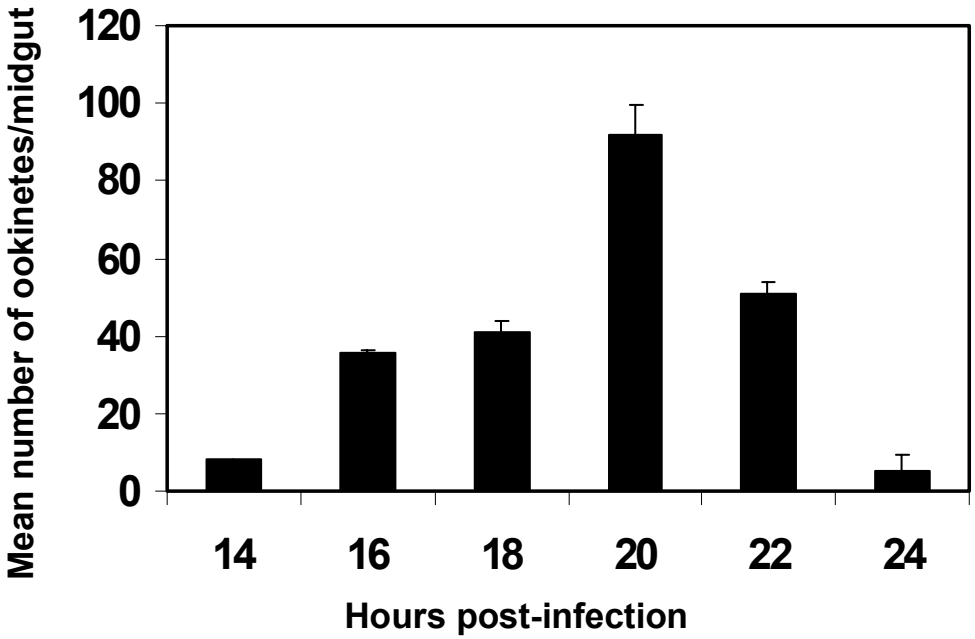

Fig. 1: Time profile for the development of the ookinetes of *P. y. nigeriensis* in *An. gambiae* midgut post-infective blood meal. Ookinetes were counted in smeared blood meals on slides under the microscope. Error bares represent the mean numbers of ookinetes/midgut of 5 replicates at each time point.

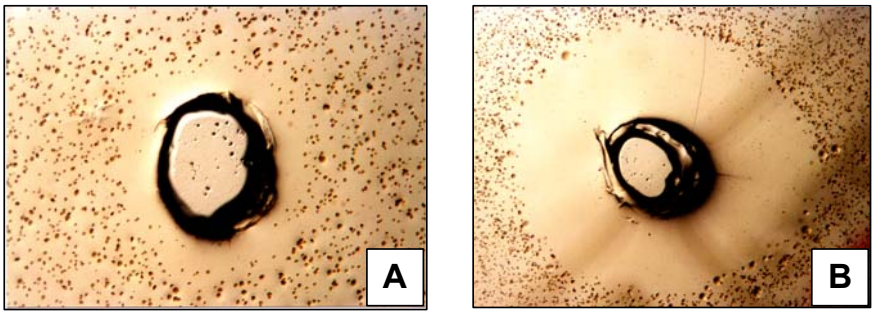

Fig. 2: Antibacterial activity assay 24 h post-treatment in *An. gambiae.* Inhibition of bacterial growth appears as a clear zone which contains the antibacterial peptides. The diameter of the zone around the well depends on the concentration of antibacterial peptides exist in the haemolymph loaded into it as shown from saline injected (**A**) and bacterial injected (**B**) mosquitoes (microscopic photograph x100 magnification). Diameters were measured directly by naked eyes using ruler.

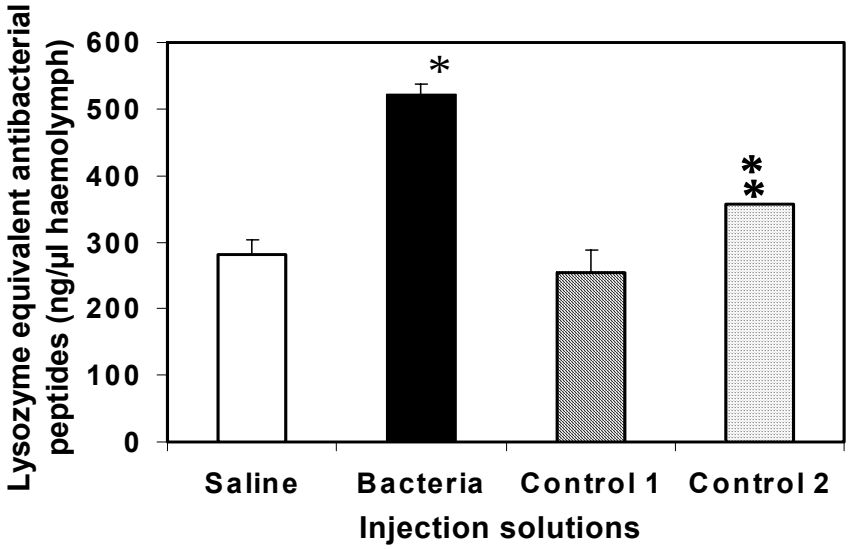

Fig. 3: Humoral Antibacterial activity (µg peptides/µl haemolymph) equivalent to a standard lysozyme activity. The histogram represents antibacterial activity in mosquitoes injected with sterile saline post-feeding on un-infective blood meal (**white bar**) and in those injected with bacteria post-malaria infection (**black bar**). Uninjected mosquitoes fed on un-infective blood meal (**control 1**) on malaria infective blood meal (**control 2**) have also shown antibacterial activity. Data were first tested for normality using Anderson-Darling test prior to One-way ANOVA. Error bars represent the standard errors of means of 5 replicates. *Significant higher compared to all control groups, and **significant higher compared to control 1 and saline injected ($P < 0.05$).

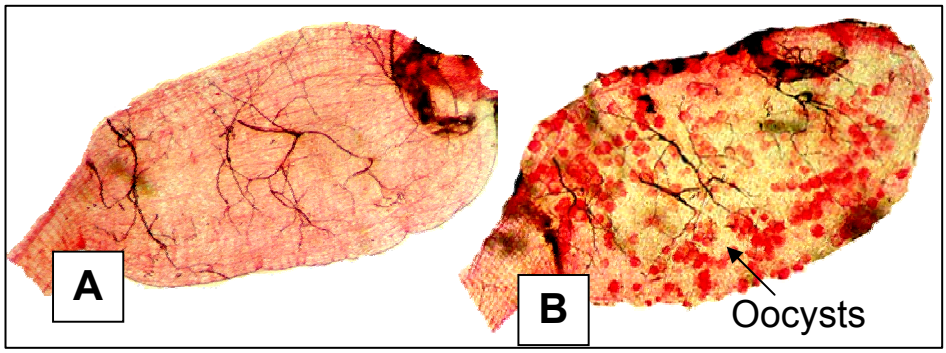

Fig. 4: Dissected midguts from un-infected (**A**) and malaria infected (**B**) mosquitoes 8 days post-blood feeding. Midguts were dissected in 5% saline solution and then socked in mercurochrome for 2 minutes to visualize the oocysts. Oocysts were then counted under microscope (x200 magnification).

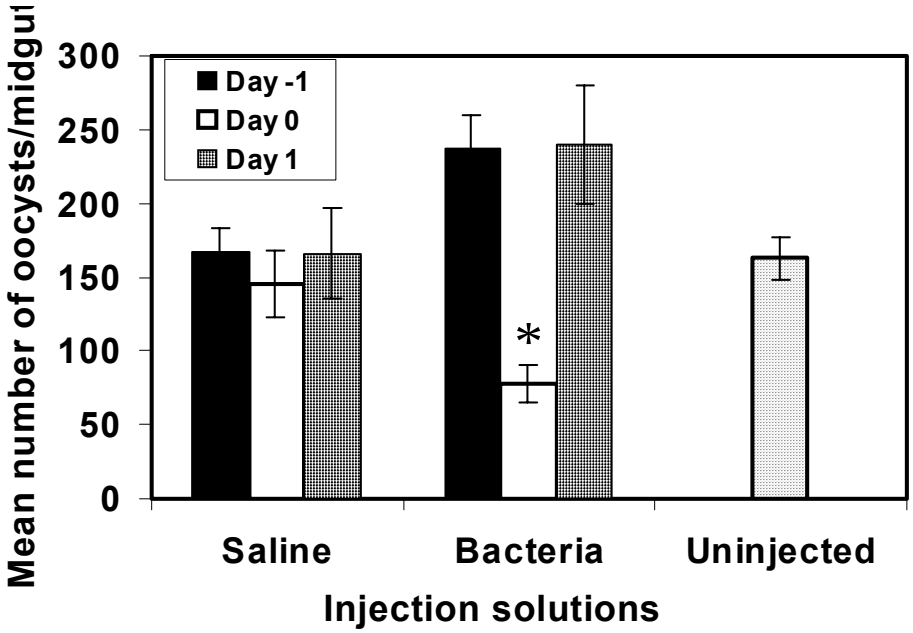

Fig. 5: The effect of *Micrococcus luteus* injection on the number of oocysts/midgut at three different times in relation to malaria infection; one day before infection (black bars **"day -1"**), immediately before infection (white bars **"day 0"**) and one day after infection (dotted bars **"day 1"**). Means of oocysts numbers were tested for normality using Anderson–Darling test prior to One-way ANOVA. Tukey's test was used to compare differences between treated groups at all time points. Each error bar represents the standard errors of 10 replicates. *Significant lower oocysts number compared to all other groups.

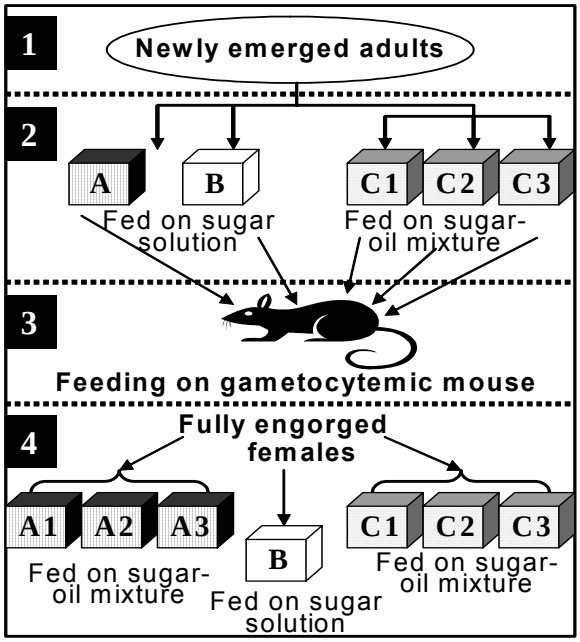

Fig. 6: A diagram illustrates the experimental design of studying the effect of oral feeding of black-seed oil on the development of oocysts in the midgut of infected *An. gambiae.* The first set of groups (**A**s) were allowed to feed on oil-sugar mixture after malaria infection only. The second set of groups (**C**s) were allowed to feed on oil-sugar mixture starting from 6 days before malaria infection and continuously until dissection of midguts 8 days post-infection.

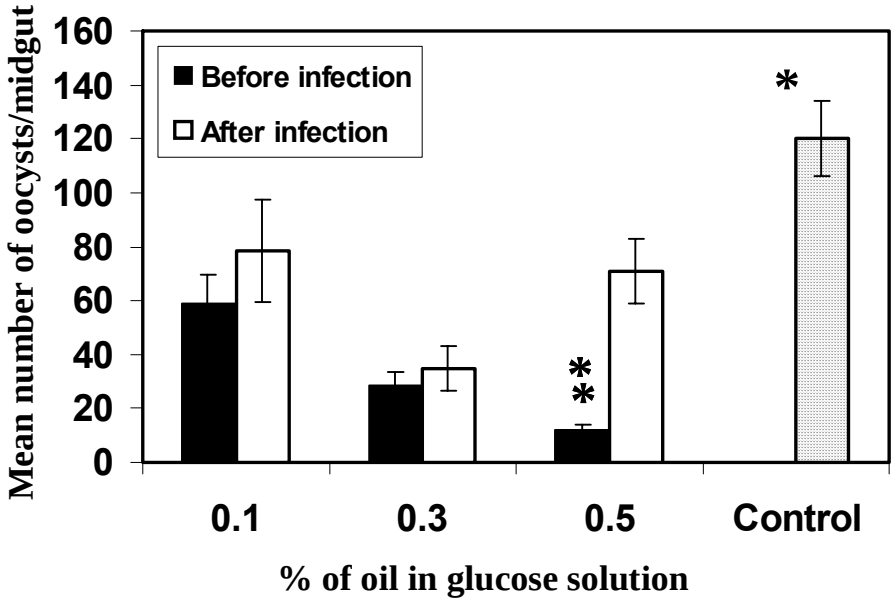

Fig. 7: The effect of different concentrations of black seed oil, mixed by 0.1, 0.3 or 0.5% with glucose solution (v/v) on the number of oocysts/midgut. Mosquitoes were allowed to feed on glucose-oil mixture before (**black bars**) and after malaria infection (**white bars**). Control group (**dotted bar**) is malaria infected group fed on oil-free glucose solution throughout the experiment. Means of oocysts numbers were tested for normality using Anderson–Darling test prior to further statistical analysis using Mann-Whitney *U* test, as they were nonparametric. Each error bar represents the standard errors of the means of 10 replicates. *Significant higher oocyst numbers and ** significant fewer oocyst numbers compared to all groups.

تنشيط الجهاز المناعى للبعوضة *أنوفيليس جامبيا* ضد طفيل الملاريا:
مقارنة بين التطعيم بالبكتريا وإطعام مستخلص نباتى

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لقد استُخدمت البعوضة *أنوفيليس جامبيا*، الناقل الأساسى للملاريا فى إفريقيا، فى هذه الدراسة لاختبار إمكانية تحفيزجهازها المناعى ضد طفيل الملاريا. ولقد استُخدم اثنين من محفزات الجهاز المناعى فى الحشرات وهما البكتريا، *مَيكروكوكَاس لوتِيَسْ*، و زيت حبة البَرَكة *ناجِيلا ساتيفا*، وذلك لتحفيز الجهاز المناعى لهذه الحشرة ضد طفيل ملاريا الفئران *بلازموديام يُوويليای نَيْجيريئْسِيس*. ولقد استُخدمت طريقة تطعيم البكتريا عن طريق الوخز بإبرة ملوثة، و وطريقة الإطعام الفَمِى لمخلوط الجلوكوز مع تركيزات مختلفة من زيت حبة البَرَكة، وذلك قبل وبعد الإصابة بالملاريا. وتم بعد ذلك تسجيل النشاط المناعى المضاد للميكروبات وتأثيره على عدد الحويصلات الجرثومية (أُوأُوسِيسْت) المتكونة فى النهاية. وعلى الرغم من أن مجرد الإصابه بالملاريا قد أدى إلى زيادة واضحة فى النشاط المضاد للميكروبات، فإنه بالمقارنة، قد أدى التطعيم بالبكتريا إلى زيادة أكبر فى هذا النوع من النشاط. و لهذا، عندما تم تطعيم البعوض بالبكتريا، بعد الإصابة بالملاريا مباشرة، لوحظ إضطراداً واضحاً فى إنخفاض عدد الحويصلات الجرثومية (أُوأُوسِيسْت) المتكونة بنسبة ٤٦,٥%. وهذا، فى الحقيقة، يدل على أن البكتريا *مَيكروكوكَاس لوتِيَسْ* قد ساهمت بوضوح فى تنشيط جزئى للجهاز المناعى ضد طفيل الملاريا. ومن ناحية أخرى، فإن المحفز المناعى المعروف، زيت حبة البركة، قد أدى إلى انخفاض أكبر فى عدد الحويصلات الجرثومية بنسبة وصلت إلى ٩٠% عندما أُطْعِم للبعوض بتركيز ٠,٥% فى محلول السكر (حجم/حجم) بدءا من ٦ أيام قبل الإصابة بالملاريا وحتى وقت عد الحويصلات عند ٨ أيام بعد الإصابة بالملاريا. هذا فى الحقيقة قد يساهم فى تطوير الإستراتيجيات الحديثة والهادفة إلى قَمْع النمو الطُوْرِى لطفيل الملاريا داخل البعوضة الناقلة له باستخدام مستخلصات طبيعية، وذلك كبديل للمقاومة الكيميائية واللتى أصبحت غير فعالة.