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(54) Title: FIELD APPLICATION FOR MALARIA VECTOR CONTROL USING SUNLIGHT ACTIVE FORMULATED EXTRACT

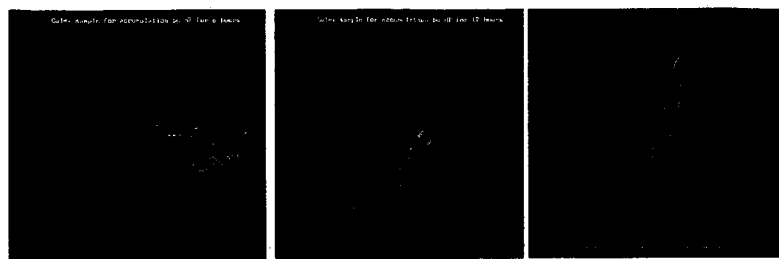


Fig. (4)

(57) Abstract: This invention introduces an innovative modality for malaria vector control that combines both effectiveness and efficiency with the highest levels of human safety and environmental friendliness. The novelty of this patent lies in developing a new ecologically safe technology using a natural plant extract (photoactive porphyrine derivative) and mosquito larvae attractant (autolized yeast) as Sunlight- Active Formulated Extract (SAFE) against the three different species of mosquito larvae (Anopheles, Aedes and Culex). In this context, the accumulated photoactive compound (photosensitizer) in the larvae body induces, upon sunlight exposure, an oxidation stress that results in organism death. The obtained results revealed that SAFE is highly effective as it ensures 100% mortality of mosquitoes larvae. The effectiveness of SAFE accumulation in the larvae bodies was qualitatively and quantitatively investigated using the Confocal Laser Scanning Microscopy (CLSM) technique.

**FIELD APPLICATION FOR MALARIA VECTOR
CONTROL USING SUNLIGHT ACTIVE FORMULATED
EXTRACT**

Technical Field :

Pharmacy

Background Art :

One of the greatest dilemmas that face Malaria epidemic countries is the use of DDT for Indoor Residual Spraying (IRS) as an essential way to control the disease (WHO Report 2006).

On one hand, Malaria is an extremely serious disease threatening more than 3.2 billion people in more than 107 countries. It remains one of the major causes of poverty and underdevelopment, and one of the main obstacles in front of economic growth. Despite the World Health Organization (WHO) efforts since the 1950s to eradicate this disease, it still results in massive economic and human losses, especially in sub Saharan Africa and in some rural areas of Asia and Latin America. Each year, more than 350 million people get this disease and more than one million people die from it (WHO Report 2007).

On the other hand, DDT, with its acute toxicity and chemical stability impose another significant risk. It was proven that DDT accumulates over the long run causing the threat of toxicity to human beings and wildlife. DDT is one of the 12 chemicals that are identified as persistent organic pollutants. Therefore, its production and use is strictly restricted by an international agreement known as the Stockholm Convention on Persistent Organic Pollutants. It is already totally prohibited by many industrialized countries, and the United Nations Environmental program is now negotiating for the global ban of DDT (WHO Report 2007).

While it was not possible to resolve this dilemma, and until it is well proven that the risks outweigh the benefits, the WHO declared a cautious position from DDT. It stated that "It is expected that there will be a continued role for DDT in malaria control until equally cost effective alternatives are developed". In its report, the WHO confirmed that there is an urgent need to develop alternative products and methods to both eradicate malaria and to eliminate the use of DDT. Hence, the WHO urged governments to provide the financial resources and technical support to help accelerate the development of this alternative (WHO Report 2006, 2007).

This invention presents the long awaited answer for the puzzling dilemma. It introduces a new method that combines both the effectiveness and the efficiency of DDT but with the highest levels of human safety and environmental friendliness. The new method is designed for the outdoors malaria control by using the SAFE as an environmental friendly photolarvicide with sunlight to control the larvae of *Anopheles*, *Culex* and *Aedes*.

The photosensitizer used in the formula is approved by Food and Drug Administration (FDA) and exhibits several advantages; it can be extracted as a natural product from green plants. It is rapidly photodegraded upon exposure to sunlight, it is used in very low concentrations (ppm) and it is easy to be applied in the field by dissolving it in an aquatic environment and it is highly effective as it ensures 100% mortality rate of the mosquito larvae.

Disclosure of Invention:

Methodology:

Before carrying the field applications, laboratory experiments and semi-field investigation has been done as follows:

Laboratory experiments:

The third instars mosquito larvae of lab experiment (*Culex pipiens*, *Aedes aegypti* and *Anopheles sp.*) was obtained from the Medical Insects Institute, Health Ministry, Cairo, Egypt.

Optical absorption and emission spectra were recorded by means of a Perkin Elmer Lambda 40 double beam spectrophotometer. A Confocal Laser Scanning Microscope (LSM 410, Zeiss, Germany) was used for fluorescence and lifetime measurements in the larvae tissues.

Preparation of SAFE stock solution:

The 100 ml stock solution of SAFE was prepared by dissolving 1 gm of the formula in 10 ml of 0.1 M NaOH and 90 ml distilled water, and was adjusted to pH = 6.5 by using 0.1 M HCl. The concentration of active ingredient (photosensitizer) was obtained by measuring the optical absorption density at the wavelength of 401 nm, using $\epsilon=423000 \text{ M}^{-1}\text{cm}^{-1}$. Keeping stock solution of SAFE at 4°C in the dark made it stable for at least four weeks.

Different concentrations were prepared by appropriate dilution with distilled water.

Irradiance measurement:

The values of irradiance of natural sun or solar simulator were measured on three Channels Eldonet Dosimeter (Real Time Co., Germany). This instrument has an ability to measure the irradiance of UV-B (280-315 nm), UV-A (315-400 nm) and Visible light (400-700 nm). These can be measured on the terrestrial surface or under water. The resolution of the instrument is:

$< 0.1 \text{ W/m}^2$ (visible), $< 0.01 \text{ W/m}^2$ (UV-A) and $< 0.0005 \text{ W/m}^2$ (UV-B).

The analog data are converted (12 Bit A/D conversion) and processed by the digital output (RS232) for automatic computer measurements, compatible with any PC computer with serial port.

The source of artificial light was represented by an ORIEL CORPORATION Solar simulator equipped with a 1000-Watt Xenon lamp, which has nearly the same light spectrum of sunlight.

Dark experiments:

In the dark experiment, the larvae were left in the medium, which contains the SAFE and they were not exposed to light.

Semi-field and Field Investigations:

For the semi-field studies the three different types of mosquito larvae were obtained from the infected swamps in Kampala, Uganda.

Field investigations were carried out in sewage routes as well as in infected swamps in Kampala, Uganda.

The exposure tanks containing the larvae were treated by using SAFE under the direct sunlight exposure, the irradiance (I) were measured by the ELDONET dosimeter. A value of $I = 400 \text{ W/m}^2$ was measured as an average of different sun irradiances during the whole exposure time.

The results of all exposure studies were monitored at time intervals along a period of 48 hours and the relation between the time intervals and the percentage of survival is determined.

For field investigation, the required concentration of SAFE in the treated site was prepared by adding the appropriate amount of the stock solution to the treated site according to the volume of the water in this site. Treated swamps were exposed to direct sunlight all over the day time in the actual natural conditions.

Estimation of SAFE concentrations in vivo:***By Spectrofluorimeter analysis:***

After feeding the larvae with the used formula, they were frozen and then homogenized with a polythron in 3-ml of 2% SDS (Sodium dodecyl Sulphate). The suspension obtained was centrifuged for 10 minutes at room temperature and at 3000 rpm. The pellet was discarded, while known aliquots of supernatant were 10-fold diluted by means of SDS. The photosensitizer concentration was determined by measuring the fluorescence intensity at λ_{ex} 401 nm, and λ_{em} = 460-660 nm using Perkin Elmer Spectrofluorimeter.

By Laser Scanning Microscopy:

After feeding with the SAFE, the larvae were frozen. Some samples were mounted on microscopic slides using Aquatex, as a mounting medium. Other samples of mosquitoes larvae were cut under cooling using Tissue-Tek, as a cutting medium and a microtome of type Cryostate, Cryo-Cut E, (Leica Mikrosystem Vertrieb GmbH). All samples were investigated using the LSM 410 invert laser scanning microscope.

The concentrations of the photosensitizer inside the tissues of larvae were determined by measuring the fluorescence intensity after excitation with 488 nm, emitted by the internal argon laser for LSM 410. The emission signal was detected using an external spectrometer coupled to the LSM 410. The lifetime of the photosensitizer inside the tissues of larvae was measured after excitation with 398 nm using 70 ps LDH-C 400 short Pulsed Diode Laser, PicoQuant, Germany. The fluorescence emission detection was carried out using Czerny Tuner spectrometer (SP150, Acton Research Corp., USA), grating 150 1/mm and Blaze wavelength = 500 nm and a micro Channel-plate intensified, cooled CCD detector (ICCD-MCP100, Princeton Instruments INC., USA) minimum gate width = 3 ns.

Brief Description of Drawings:

Photosensitization treatment processes:

The effect of SAFE formula concentration, total light dose and irradiation time were monitored on the larvae of mosquitoes by measuring the survival percent at selected time intervals after the insects were fed in the specific medium and then were exposed to light. The results were compared with a control group subjected to the same treatment protocol except that the feeding medium did not contain the SAFE.

Fig. (1):- shows the effect of different concentrations from 10^{-5} up to $9 \times 10^{-5} \text{ mol dm}^{-3}$ on the larvae of *Culex pipiens* after 30 minutes exposure to sunlight (for each concentration a group of 20 larvae per replicate was used). No survival was observed during exposure, when the concentration of $9 \times 10^{-5} \text{ mol dm}^{-3}$ was used. On the other hand, the other concentrations caused less than 20% survival up to 60 hours after irradiation. The control group, which was fed in a normal feeding medium (without SAFE), showed 100% survival even after three days.

Fig. (2):- shows that the photosensitizer accumulation by larvae gradually increases with increasing SAFE concentration in the feeding medium. The accumulation showed a particularly steep increase in the concentration range of 5 to $7 \times 10^{-5} \text{ mol dm}^{-3}$, and underwent a slower increase at higher concentrations, at least up to $9 \times 10^{-5} \text{ mol dm}^{-3}$.

Fig. (3):- illustrates the effect of different irradiances from the solar simulator on the survival on *Culex pipiens* larvae in the presence of $5 \times 10^{-5} \text{ mol dm}^{-3}$ photosensitizer. The difference in survival between the highest and lowest irradiance were less than 20% after 60 hours. This result can be attributed to the higher sensitivity toward the light in the photosensitization process.

Confocal Laser Scanning Microscopy (CLSM):

CLSM provided three sources of information: fluorescence images, fluorescence spectra and fluorescence lifetimes of the inside the organs and tissues of mosquitoes larvae. Fluorescence images shown in (Fig. 4a-c) demonstrate the extent of photosensitizer accumulation as a function of incubation time.

In (**Fig. 4a**) the incubation time 6 hours of SAFE accumulation seemed to be very high and initiated blocking and swelling of the alimentary canal. The accumulation caused stress on the walls of the alimentary canal. Some amount of the photosensitizer diffused outside to the hemolymph. After an incubation period of 17 hours, large amounts of photosensitizer disappeared (Fig. 4b) and the alimentary canal volume decreased. (Fig. 4c) shows a larva incubated in SAFE free medium.

Fluorescence images of *Culex pipiens* larva microtome sections demonstrate the extent of damage by singlet oxygen.

Fig.(5)A: illustrates a transversal section of *Culex pipiens* larva incubated for 6 hours with photosensitizer and left in the dark until sections were prepared and CLSM was performed. SAFE fluorescence appeared inside and outside the alimentary canal. A similar transversal section in another larva incubated for 6 hours and exposed to 350 W/m² for a period of 20 minutes as shown in (Fig. 5b). No specific fluorescence accumulation could still be detected.

Fig. (6): shows CLSM optical slices for the foregut of *Culex pipiens* larva.

The location of SAFE distribution both inside and outside the alimentary canal could be easily identified.

Fluorescence images of Anopheles larva transverse section, shown in (**Fig. 7 and 8**):-

demonstrate the extent of SAFE accumulation as a function of incubation time (3 and 9 hours respectively). In (Fig. 7), SAFE accumulation seemed to be very high. The accumulation of SAFE is everywhere in the transverse section of Anopheles larva which was incubated with SAFE for 3 hours in the dark. On the other hand the amount of SAFE in the tissue of the larvae incubated for 9 hours is less than the SAFE amount accumulated in the tissue of those incubated for three hours. This means that the release process of SAFE by the larvae may affect the proper time of photosensitizer application in the field.

Fluorescence spectra measurement inside the tissues of *Anopheles* larvae is a quantitative method for estimating the accumulated SAFE at any time of incubation and after the light exposure. These are very important measurements for field application to optimize the parameters of the photochemical processes for our own target and to save the non-targets which are living in the same infected swamp (target selectivity) (**Fig 9 & 10**). Fluorescence lifetime measurements at definite spots inside the tissues of *Culex pipiens*, *Anopheles sp.*, and *Aedes aegypti* larvae provided a good estimation of the distribution ratio of aggregated and non-aggregated forms of the photosensitizer. Lifetime measurements of spots inside the larvae were determined to be 1.5 ± 1.04 ns for the 6 hours incubation period and 11.9 ± 3.3 ns for 17 hours incubation period. The data were obtained by monoexponential fitting. The differences may be attributed to the different ratio of aggregated and non-aggregated forms of SAFE.

4.3. Results of SAFE Field Application

The field investigation was implemented on the different species of mosquito's larvae.

This study was designed to evaluate the sensitivity of the Ugandan mosquito larvae stages to the photosensitizer formula post their exposure to sunlight in their breeding swamp.

Fig. 11:- shows the effect of photosensitizer on the mosquito larvae (*Culex*, *Anopheles* and *Aedes*) which was exposed to sunlight and the results were collected after 2 hours from sunlight exposure onset.

It was observed that from 85% to 100% mortality of larvae population was obtained as a result of the different concentrations of SAFE used ranging from 10^{-7} mol.dm⁻³ up to 10^{-4} mol.dm⁻³

The other biological beneficiary organisms, such as the dragon fly larvae and mosquito predator larvae which were present in the same treated swamps were not affected (target selectivity). Both the control light group aquaria (larvae without SAFE treatment) and control dark group aquaria (larvae treated with SAFE and were not exposed to light) showed only maximum mortality of 2%.

Fig. (12, a-d):- shows the site of the field application in Kampala, Uganda

Claims

- 1- A method for out door field application for malaria vector control
- 2- The method according to claim 1, providing a novel formula, consists of photoactive porphyrine derivative as an active ingredient photosensitizer and autolized yeast as a larva feeding attractant material.
- 3- The practical procedures according to claim 1, was optimized to achieve a maximum efficiency of malaria vector control via adjusting the following parameters:
 - a- application time
 - b- light exposure dose
 - c- proper concentration in different environmental conditions
 - d- target selectivity
 - e- data monitoring

AMENDED CLAIMS

received by the International Bureau on 24 March 2009 (24.03. 2009)

1. *a method for outdoor field application for Malaria, Filaria and Yellow fever vectors control using environmentally friendly formulated extract from Plant origin (SAFE).*
2. *The formula (SAFE) according to claim 1, consisting of novel Ingredients including one of photo-active prophyrin derivatives as an active ingredient and autolysed yeast as a larvae feeding attractant and natural material as a carrier.*
3. *The practical procedures for field application were performed by the folloeing steps:*
 - *Testing different times for implication at the sites of larvae breeding*
 - *Measuring the efflux rate of solar intensities during the day time.*
 - *Applying different concentrations of SAFE in the implemented swamps.*
 - *Testing the target selectivity in presence of the other organisms.*

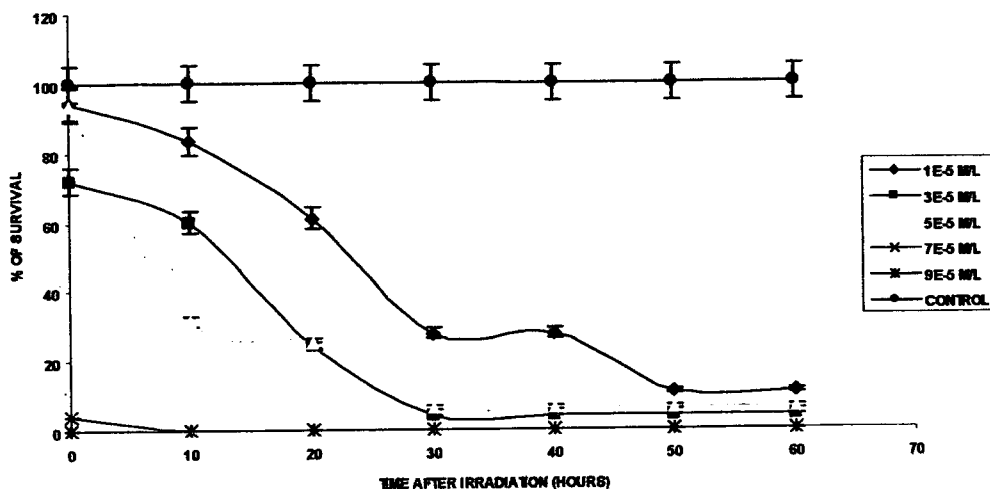


Fig. (1)

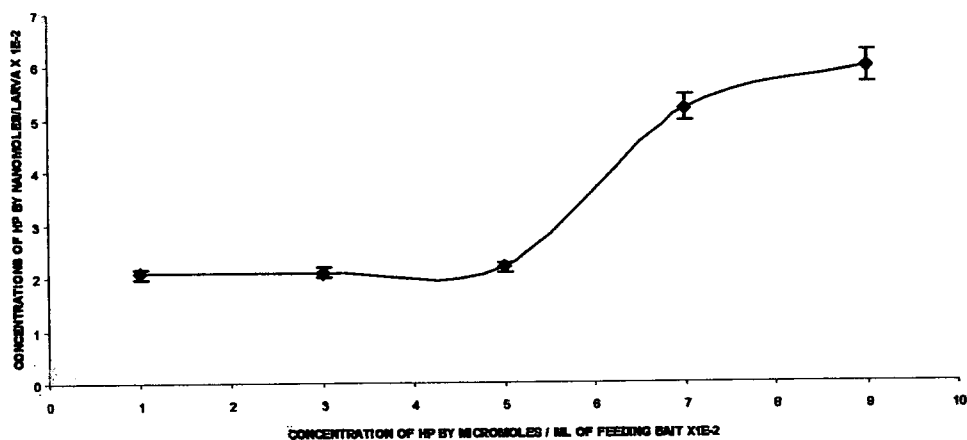


Fig. (2)

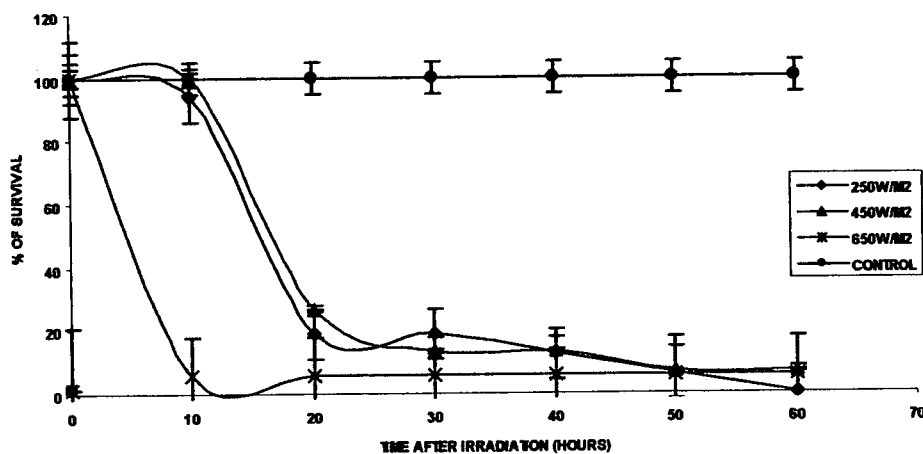


Fig. (3)

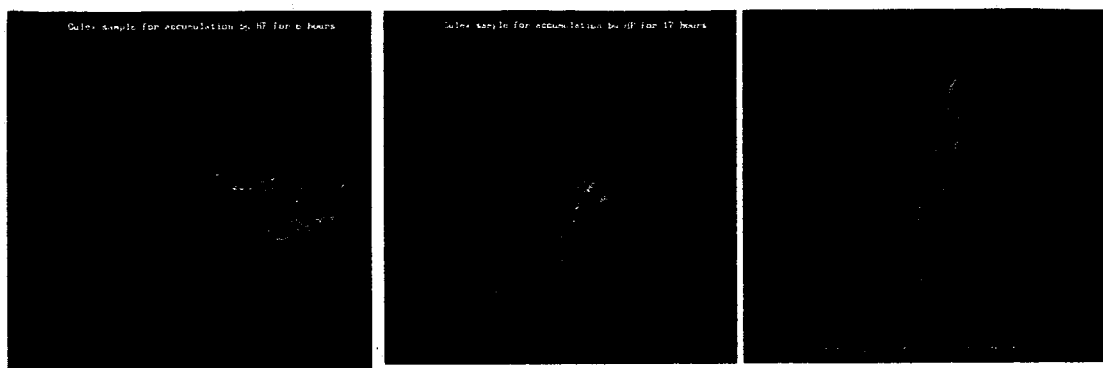


Fig. (4)

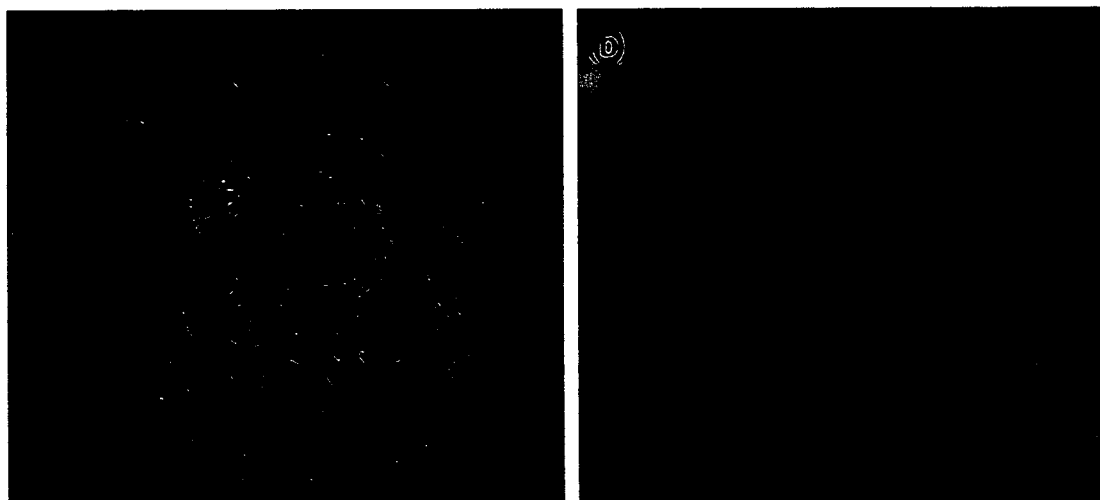
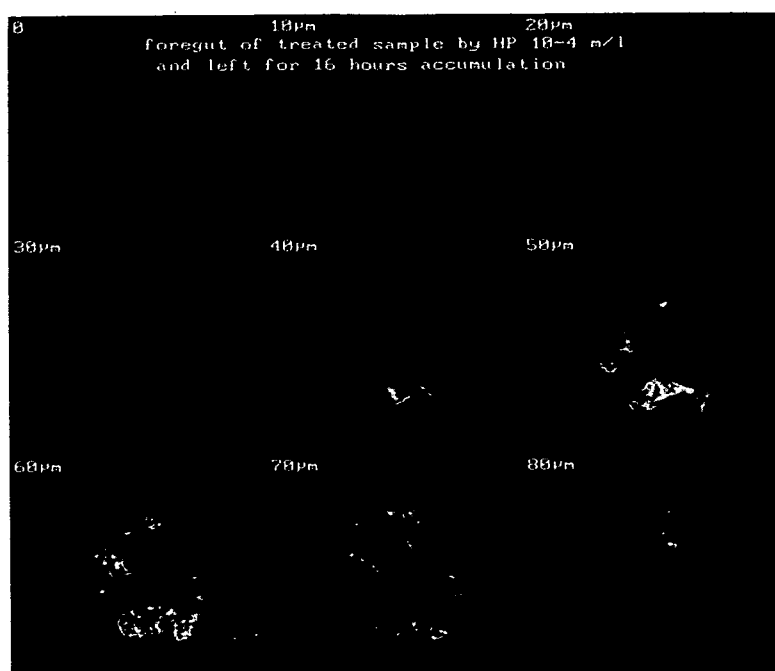


Fig. (5)

**Fig. (6)**

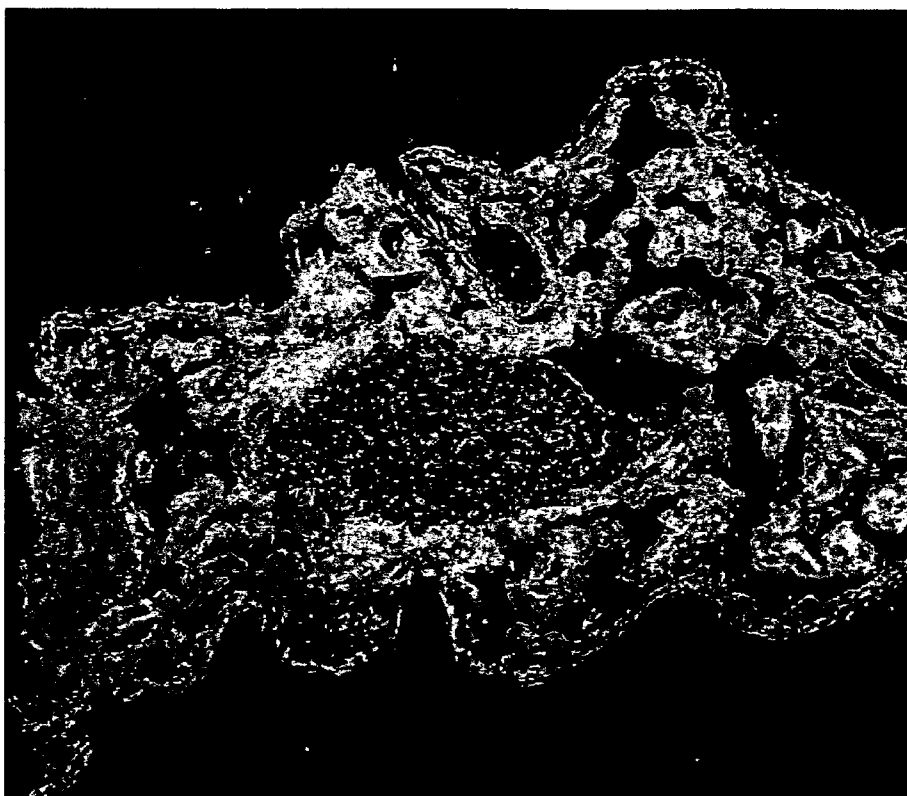


Fig. (7)

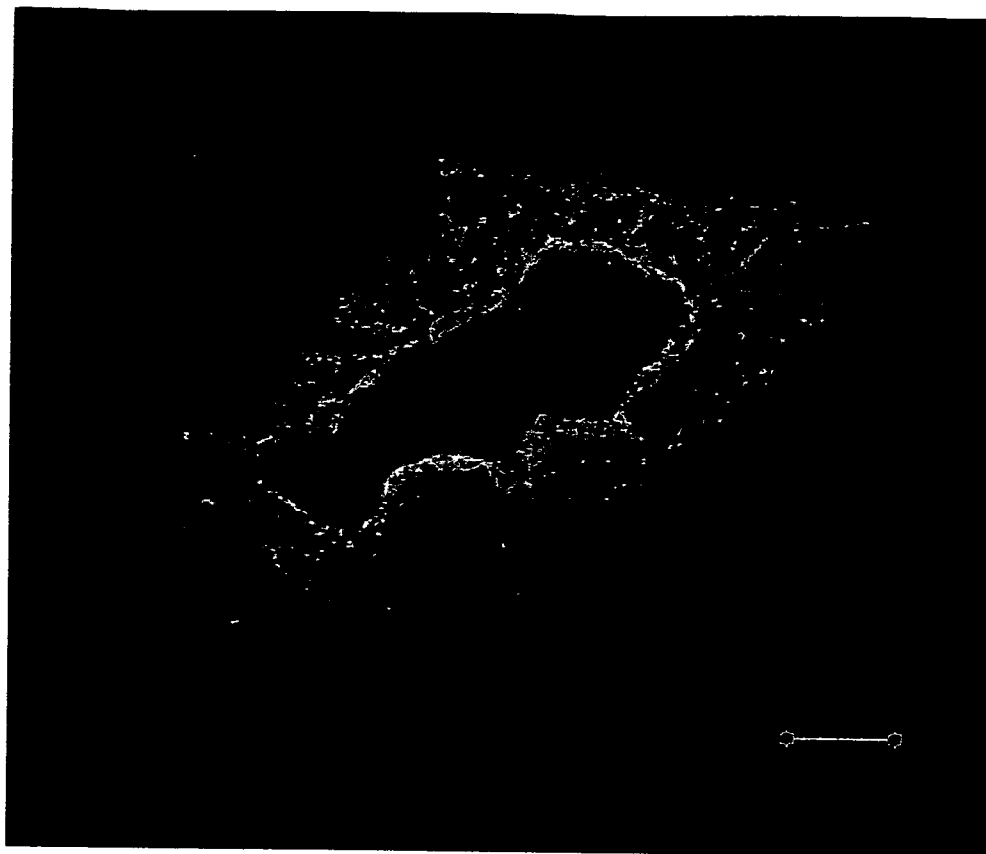
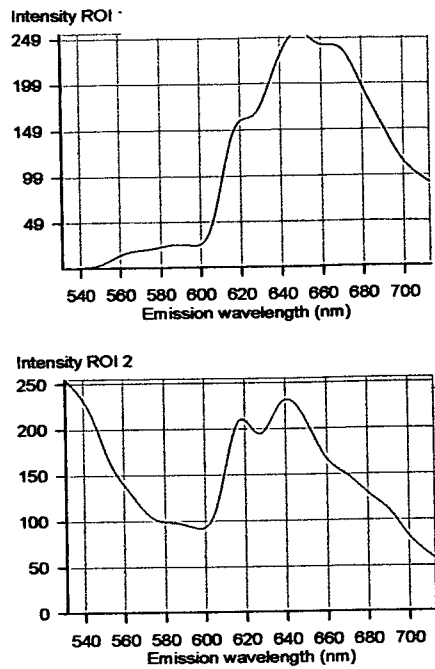
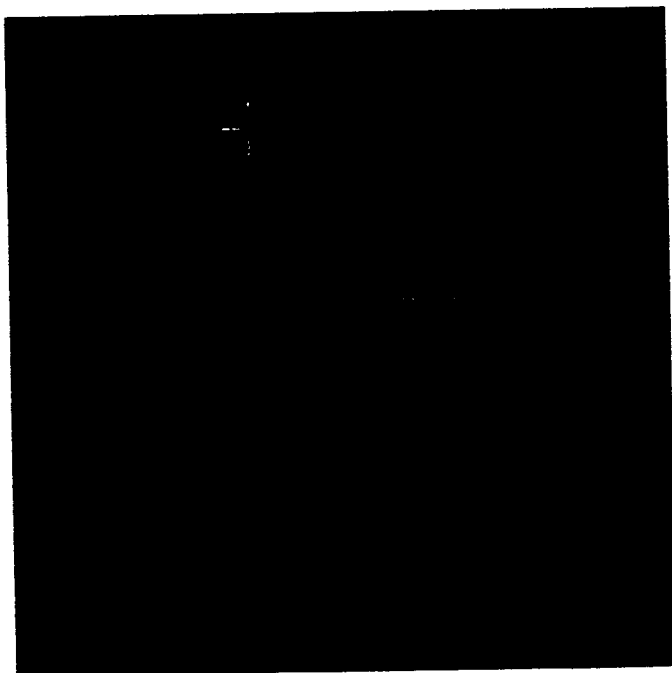
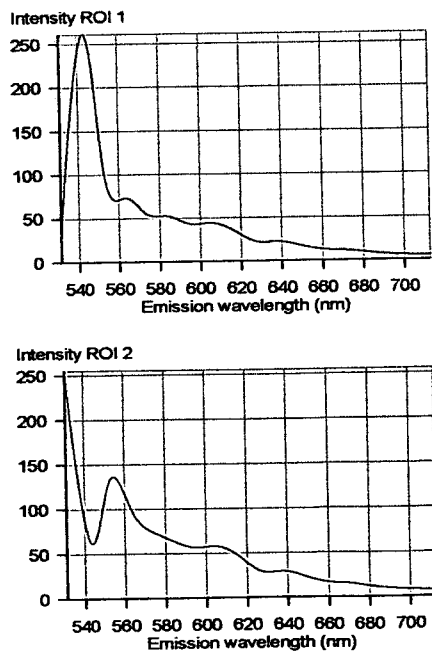


Fig. (8).

**Fig. (9)****Fig. (10)**

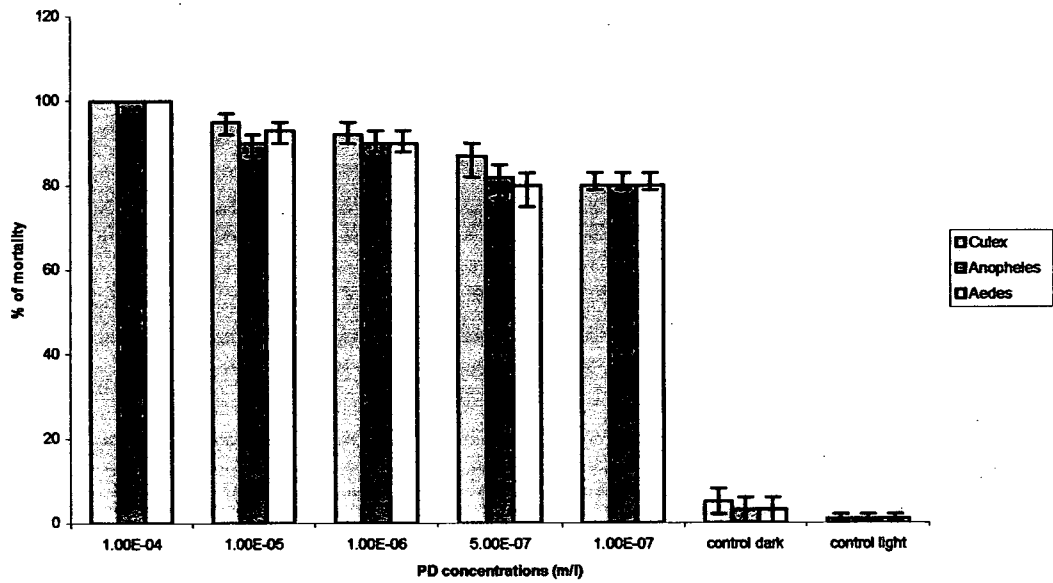


Fig. (11)

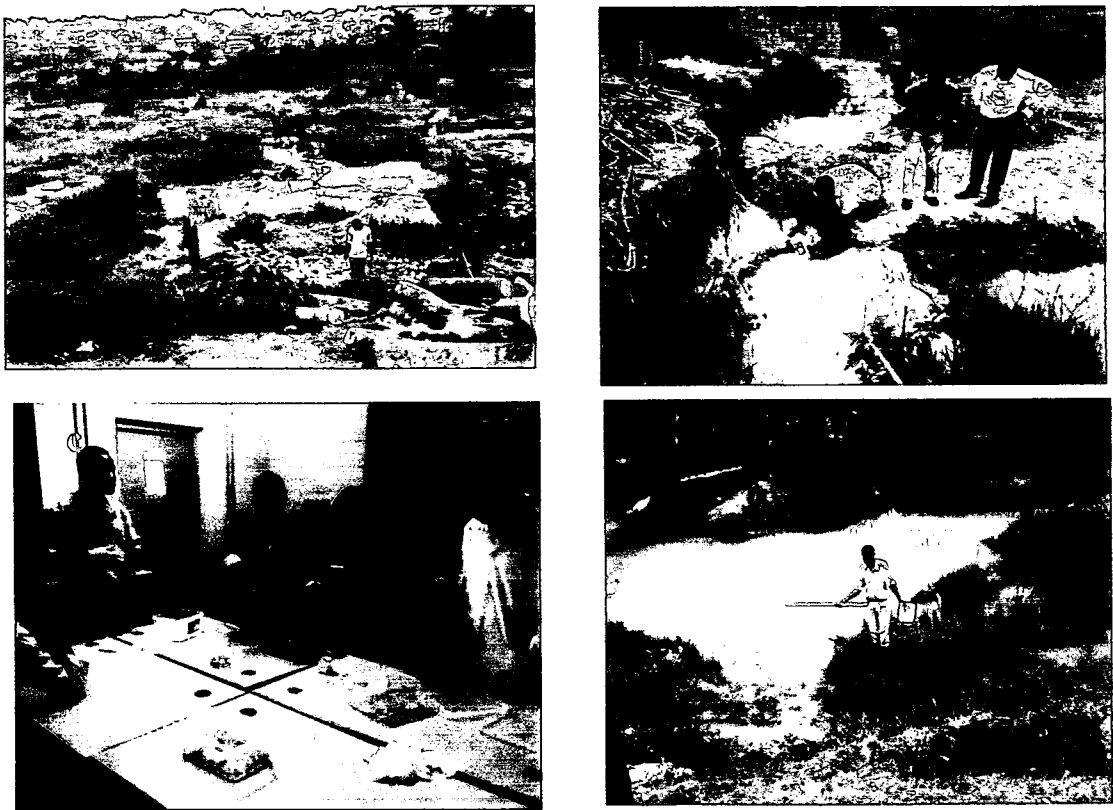


Fig. (12, a-d)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EG 2008/000019

A. CLASSIFICATION OF SUBJECT MATTER

IPC⁸: **A01N 43/90** (2006.01); **A01N 65/00** (2009.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC⁸: A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPI, TXTE, TXTG, NPL, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Thameur et al; "Sunlight-activated insecticides: historical background and mechanisms of phototoxic activity" Insect Biochemistry and Molecular Biology; Volume 30, Issue 10, Pages 915-925; October 2000 <i>Abstract; Historical background Page 916</i>	2
A	US 5 091 385 A (Gulliya et al.) 25 February 1992 (25.02.1992) <i>Description Column 1 Line 24-37, Column 7 Line 49-62</i>	2

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search
26 January 2009 (26.01.2009)Date of mailing of the international search report
6 February 2009 (06.02.2009)Name and mailing address of the ISA/ AT
Austrian Patent Office
Dresdner Straße 87, A-1200 ViennaAuthorized officer
HUNGER U.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EG 2008/000019

Continuation of first sheet

Continuation No. II:

Observations where certain claims were found unsearchable

(Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

Claims Nos.: 1, 3 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 1 characterizes a method exclusively in a function-oriented manner without presenting a solution, which is not allowed. Concerning claim 3, a method cannot be characterized by an itemization of parameters for maximum efficiency.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/EG 2008/000019

Patent document cited in search report			Publication date			Patent family member(s)			Publication date		
US	A	5091385				US	A	5489590			1996-02-06
						US	A	5177073			1993-01-05
						WO	A1	9003187			1990-04-05
						JP	T	4501717T			1992-03-26
						EP	A1	0436642			1991-07-17
						DE	T2	68922399T			1995-10-05