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Colour Cues: Effects of *Ipomoea* Plant Extract on *Culex quinquefasciatus* Say Gravid Females in Choosing Oviposition Site

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Abstrak: Interaksi antara tumbuhan dan serangga adalah dinamik, dan memberikan kebaikan kepada samada tumbuhan ataupun serangga. Bahan kimia tumbuhan adalah terlibat secara mendalam dalam hubungan ini dan mempengaruhi kelakuan serangga. Di sini, kami mengkaji kelakuan tindak balas oviposisi nyamuk *Culex quinquefasciatus* berdasarkan isyarat warna yang terhasil daripada ekstrak tumbuhan *Ipomoea cairica*. Dalam kajian ini, dua kumpulan eksperimen untuk pilihan oviposisi telah dijalankan: (1) satu konsentrasi larutan di dalam satu sangkar; dan (2) pelbagai konsentrasi larutan di dalam satu sangkar. Dalam kajian satu konsentrasi larutan, terdapat cuma satu sahaja tempat oviposisi yang disediakan dan ditawarkan kepada lima nyamuk betina gravid. Manakala dalam kajian pelbagai konsentrasi larutan, 4 tempat oviposisi disediakan dan ditawarkan kepada 20 betina gravid. Konsentrasi yang diuji pada 100 mL adalah: (1) kawalan (air suling sahaja); (2) 50 ppm, (3) 150 ppm; dan (4) 300 ppm ekstrak tumbuhan *I. cairica*. Konsentrasi tertinggi pada 300 ppm menunjukkan intensiti tertinggi dengan warna tergelap diikuti dengan konsentrasi 150 ppm dan 50 ppm. Lebih banyak betina gravid didapati lemas dalam konsentrasi tertinggi iaitu 300 ppm berbanding 150 ppm dan 50 ppm ekstrak yang diuji. Tiada telur dijumpai dalam semua konsentrasi. Ekstrak yang dikaji didapati berkesan untuk menarik nyamuk *Cx. quinquefasciatus* betina gravid untuk bertelur dan seterusnya menyebabkan kematiannya dan menghalang aktiviti peneluran. Gangguan yang disebabkan oleh ekstrak aseton *I. cairica* terhadap aktiviti oviposisi *Cx. quinquefasciatus* akan memberikan kesan kawalan yang lebih baik kepada serangga vektor.

Kata kunci: *Culex*, Isyarat Warna, Nyamuk, Oviposisi, Ekstrak Tumbuhan

Abstract: The interaction between plants and insects is dynamic, and may favour either the plant or the insect. Plant chemicals are deeply implicated in this relationship and influence insect behaviour. Here, we investigated the oviposition behaviour response of *Culex quinquefasciatus* mosquitoes based on the colour cues produced by *Ipomoea cairica* leaves extract. In this study, two sets of oviposition choice experiments were conducted: (1) single solution in a cage; and (2) multiple concentration solutions in a cage.

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In the single solution experiment, only 1 available oviposition site was offered to 5 gravid females and in the multiple concentration tests, 4 available oviposition sites were offered to 20 gravid females. The tested concentrations were set up at 100 mL of: (1) control (distilled water only); (2) 50 ppm; (3) 150 ppm; and (4) 300 ppm of *I. cairica* plant extracts. The highest concentration of 300 ppm appeared to show the highest intensity with the darkest colour followed by 150 ppm and 50 ppm concentrations. More gravid females were found drowned in the highest concentration, 300 ppm of acethonilic leaves extract, compared to 150 ppm and 50 ppm of the tested extract. No eggs were found in all tested solutions. The studied extract was found to effectively attract gravid *Cx. quinquefasciatus* females and subsequently cause mortality and inhibit egg deposition. The interference caused by the acethonilic extract of *I. cairica* on the oviposition activity of *Cx. quinquefasciatus* can result in better control of the vector insect.

Keywords: *Culex*, Colour Cues, Mosquito, Oviposition, Plant Extract

INTRODUCTION

There has been an increased interest in the manipulation of pest behaviour in order to achieve better pest management and reduced dependency on broad spectrum insecticides (Foster & Harris 1997). Using plant resources for deliberate manipulation of pest behaviour can be applied in pest management programs. Plants have coevolved with insects and have produced secondary compounds to deter phytophagous insects. Secondary compounds from plants have been identified to be toxic to arthropod pests. Although the primary function of these compounds is for defence against phytophagous insects, many of them are also effective against mosquitoes and other biting Dipterans (Pichersky & Gershenzon 2002) as repellent, larvicide or adulticide.

Phytochemicals from plants are important mosquito oviposition attractants and stimulants. However, they can also have a negative or deterrent effect on oviposition (Bentley & Day 1989). According to Waliwitiya *et al*. (2009), the phytochemicals tested, thymol, pulegone, citronellal and eugenol, showed strong repellent/deterrent activity, whereas β-pinene, borneol acetate, borneol, and camphor acted as strong oviposition attractants. The leaf extract of *Solanum trilobatum* at 0.1% concentration greatly reduced the number of eggs deposited by gravid *Anopheles stephensi* by 90%–99% (Rajkumar & Jebanesan 2005). The essential oil of *Cinnamomum zeylanicum* was found to have oviposition-deterrent potential against *An. stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus* (Prajapati *et al.* 2005). Rajkumar and Jebanesan (2009) have reported on the oviposition deterrence effects of ethanolic leaf extract of *Cassia obtusifolia.*

Various other cues are also involved in mediating a wide range of mosquito behaviours, which includes the recognition of specific features of the oviposition sites (Mboera *et al.* 2000). The location and selection of an oviposition site involves visual, olfactory, and tactile responses. The ovipositional flight is triggered by environmental factors, especially rainfall, relative humidity, temperature, and wind speed (Bentley & Day 1989). Oviposition sites selection by gravid female mosquito is very important to determine the resources available for the aquatic immature stages to develop into adulthood. The selection of an appropriate oviposition site is particularly important in mosquito species, since the juveniles are unable to move to a better habitat if the condition becomes unfavourable (Spencer *et al.* 2002). It is essential throughout the ontogeny of all mosquito species for continuous offspring survival in specific locations.

Multiple researches had been conducted concerning mosquito ovipositional behaviour caused by different possible stimuli. Based on a study done by Chadee *et al.* (1990), it was reported that *Ae. aegypti* mosquitoes avoided sites containing eggs laid by themselves or by conspecifics. Bacteria present in the organic-rich water were found to produce chemicals that are highly attractive to gravid mosquitoes (Reiter *et al.* 1991; Copeland & Craig 1992; Allan & Kline 1995). The presence of predatory tadpole shrimp (*Triops*) deterred oviposition by the mosquito *Cx. quinquefasciatus* due to the physical disturbance created by predator movement (Tietze & Mulla 1991). The chemicals released from discarded cigarette butt microcosms tended to cause more egg deposition by *Aedes albopictus* females as decomposition progressed but somehow killed the newly hatched larvae in the oviposition site (Dieng *et al.* 2011). Gravid *Cx. quinquefasciatus* females were found to locate suitable oviposition sites by odour orientation associated with high organic content and lay their eggs in clusters, called egg rafts, on the water surface (McCall & Eaton 2001). Their preference for moderately aerated, polluted water which is rich in decaying organic matter for oviposition sites have made *Cx. quinquefasciatus* a dangerous vector because these sites are usually prevalent near human habitation (Blackwell *et al.* 1993).

Ipomoea L. from the family Convolvulaceae, chosen for this study, comprises of about 650 species and is distributed all over the world (Austin & Hua´man 1996; Mabberley 2008). *Ipomoea* (commonly known as “morning glory”) is a cosmopolitan climbing genus from warm and pan tropical regions. Its members have large showy trumpet shaped flowers in different colours mainly white, purple, blue, pink, and red (Cronquist 1981). *Ipomoea cairica* is commonly known as the “railway creeper”, and being ornamental in nature it is widely used in fencing in domestic and peri-domestic situations (Thomas *et al.* 2004). *I. cairica* reproduces easily through both sexual and asexual propagation (Liu *et al.* 2006). This plant is known for its medicinal properties, in which a drink made from the plant’s crushed leaves is used for the treatment of body rashes, especially those accompanied by fever (Watt & Brandwijik 1962). Topical repellent effects of the essential oil extracted by steam distillation from the leaves of *I. cairica* were evaluated and found to be highly toxic to the larvae of *Culex tritaeniorhynchus* followed by *Ae. aegypti*, *An. stephensi,* and *Cx. quinquefasciatus* (Thomas *et al.* 2004; Rajkumar & Jebanesan 2007).

In the current study, we tested the effects of acethonilic extract of *I. cairica* leaves on the oviposition behaviour response of the urban vector for bancroftian filariasis, *Cx. quinquefasciatus* gravid female mosquitoes. We hypothesised that the highly concentrated plant extract solution will attract more *Cx. quinquefasciatus* gravid female to oviposit their eggs and also cause mortality to the gravid females. We also examined the interactive component of reception visual stimuli caused by the *I. cairica* plant that may become an attractant to *Cx. quinquefasciatus* gravid females in choosing an oviposition site.

MATERIALS AND METHODS

Mosquito Colonies

Wild strain *Cx. quinquefasciatus* larvae were collected from drains containing stagnant water in Bagan Dalam area, Pulau Pinang, Malaysia (5°24′00″N, 100°23′00″E). Collected larvae were reared in enamel trays containing dechlorinated tap water and fed with fine powder mixture consisting of dog biscuit, beef liver, yeast, and milk powder at a ratio of 2:1:1:1 by weight. The pupae were transferred into plastic containers and placed in screened cages (23 × 23 × 32 cm) to allow emergence of adults. Adult mosquitoes were continuously provided with 10% sucrose solution in a vial with a cotton wick. On day 5 after adult emergence, females were allowed to feed overnight on a restrained laboratory mouse. Three days after blood feeding, gravid females from these colonies were used as subjects in oviposition assay experiments. The mosquito colonies were maintained under laboratory condition at a temperature of 28±2°C and 80±10% relative humidity (RH).

Preparation of Plant Extracts

Samples of *I. cairica* leaves were collected from a residential area in Relau, Pulau Pinang, Malaysia (5°25′00″N, 100°19′00″E) and were identified by the Botanical Department of Universiti Sains Malaysia. A crude extract was made by grinding the air dried leaves using electrical stainless steel blender and extracted with acetone using the Soxhlet apparatus. Extracts were then removed of the excess solvent using the rotary vacuum evaporator. The solvent from the concentrated crude extract was further removed by placing the extract in an electrical oven at 37ºC for a week. Stock solution was prepared by dissolving 1 g of crude extract in 100 mL of solvent and subsequently test concentrations of 50 ppm, 150 ppm, and 300 ppm were prepared using the serial dilution technique.

Colour Cues Experiment

To investigate the colour cues from the plant extract, we used spectrographic analysis. We prepared different concentrations of the tested plant extract solution comprising of 50, 150, and 300 ppm, and compared with the control (distilled water) as reference in order to test the reflectance properties of *I. cairica* colour solutions. By using a USB Ocean Optics 2000 spectroradiometer (Ocean Optics Inc., Dunedin, USA) and Xenon Pulse X2 lamp (Ocean Optics Inc., Dunedin, USA) as the light source, we compared the plant colour solution based on the reflectance and whether this affected the choice of oviposition site by *Cx. quinquefasciatus* gravid females. The fibre optics probe was mounted inside a matte black plastic tube to exclude ambient light. The distance between each object and the probe was fixed at 1 cm. The angle of illumination and reflection was fixed at 45° to minimise glare. Spectra were calculated at 5 nm intervals from 300 to 700 nm with the SpectraSuite software (Ocean Optics Inc., Dunedin, USA).

Oviposition Experiments

In this study, two different sets of oviposition choice experiments were conducted: (1) single solution in a cage; and (2) multiple concentration solutions in one cage. Single and multiple concentrations of acetone extract of *I. cairica* leaves were assessed for their effects on the selection of oviposition site by *Cx. quinquefasciatus* gravid females. Both experiments were designed in order to understand the possible effects caused by the acethonilic extract of *I. cairica* leaves on oviposition site selection by gravid *Cx. quinquefasciatus* females. Tests were carried out under laboratory conditions at 28±2°C and 80±10% RH.

In the single concentration solution experiment, five gravid females were transferred each to a separate screened mosquito cage (23 × 23 × 32 cm). Each cage was offered with only one available oviposition site and was ran separately. Ceramic bowls were used as the oviposition site. Each oviposition site consisted 100 mL of: (1) control (distilled water without any plant extract solution); (2) 50 ppm; (3) 150 ppm; or (4) 300 ppm of *I. cairica* plant extract. Three replicates for each concentration were conducted simultaneously. The cages containing different test concentrations were placed side by side with 0.5 m distance between each other. Data of drowned gravid female were recorded.

In the multiple concentration solutions test, 20 gravid females were transferred into a screened cage and 4 ceramic bowls with 100 mL of: (1) control (distilled water only); (2) 50 ppm; (3) 150 ppm; and (4) 300 ppm of *I. cairica* plant extracts were placed in the same cage. Bowls were positioned in a square shape and the position of the bowls was alternated between the different replicates to nullify any bias for site preferences. The experiment was replicated three times. The analysis for measuring the oviposition response in this experiment was based on the number of drowned females rather than egg rafts. This is because no eggs rafts were laid by *Cx. quinquefasciatus* mosquitoes in all treatments.

Statistical Analysis

Analysis of variance (ANOVA) was conducted separately using SPSS program version 20.0 (IBM Computing, Illinois, USA) for the number of drowned females in single and multiple concentration tests to determine significant differences of the extract solution concentration. Data were tested for normality using Kolmogorov-Smirnov test prior to analysis. Results with $p<0.05$ were considered to be statistically significant. The effects of visual stimuli on *Cx. quinquefasciatus* in choosing the oviposition site were represented by the mean number of gravid *Cx. quinquefasciatus* drowned in the given oviposition site.

RESULTS

Spectrographic results showed that the acethonilic extracts of *I. cairica* leaves at different concentrations have reflectance intensity peaking around 500–600 nm. The spectral curve corresponds to the colour of the extract, which is yellowish green. Highest intensity counts were given by the tested extract at 300 ppm concentration followed by 150 ppm and 50 ppm (Fig. 1). The reflectance curves given by the plant extract at the tested concentrations clearly showed difference in the colour intensity level of the plant extract. The highest concentration, 300 ppm, of the studied plant extract appeared to show the highest intensity with a darker colour followed by 150 ppm and 50 ppm concentrations.

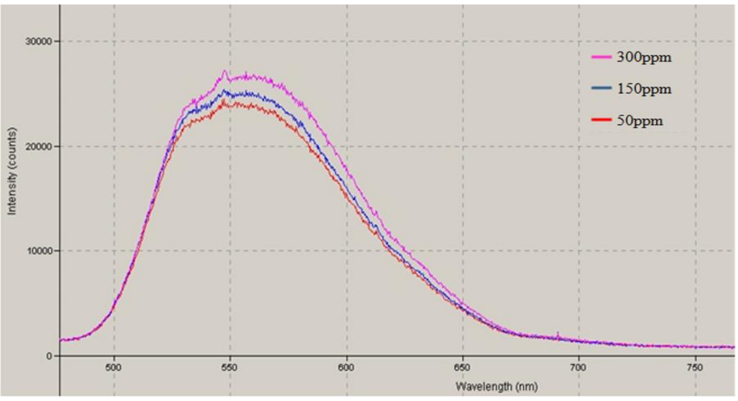

Figure 1: Reflectance spectra of *I. cairica* plant extract at different concentrations of 50, 150, and 300 ppm.

High numbers of gravid *Cx. quinquefasciatus* females were found in the oviposition site with a highly concentrated and dark coloured plant extract solution, for both single and multiple concentration solutions tests (Fig. 2). In multiple concentration solutions tests, gravid females were shown to be attracted to oviposit in the plant extract solution, causing death to the females. Similar conditions occurred even when only one solution was made available. No eggs were found in the tested solutions except in the control.

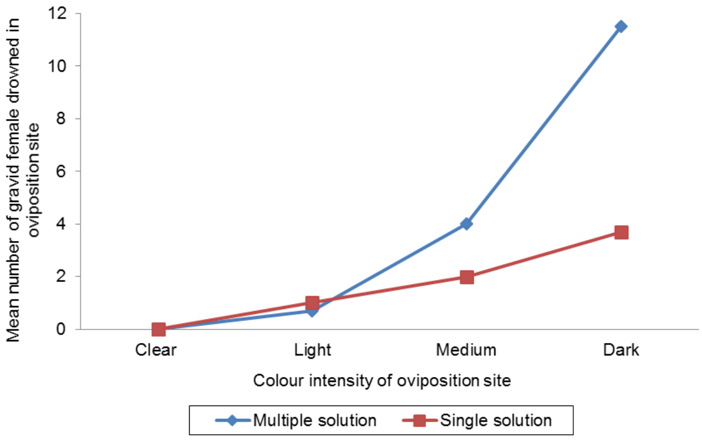

Figure 2: Effects of visual stimuli on oviposition site selection by *Cx. quinquefasciatus* gravid females.

A greater number of gravid females were found significantly drowned in the oviposition site with the highest concentration, 300 ppm, of the tested extract for single solution (df = 3, F = 12.571, p<0.05; Table 1) and multiple solutions (df = 3, F = 88.242, p<0.05; Table 1) compared to the lower concentrations. Drowned gravid females were found even at the lowest concentration but at a reduced proportion. No drowned gravid females were observed in the control solution (Table 1). The acethonilic extract of *I. cairica* leaves were found to inhibit egg deposition as no egg rafts were found laid in the treated oviposition sites.

Table 1: Effects of different concentrations of acethonilic extract of *I. cairica* leaves extract on gravid *Cx. quinquefasciatus* females' mortality in single and multiple solutions tests.

Treatment	Mean no. of drowned gravid females			
	Control	50 ppm	150 ppm	300 ppm
Single	0.00 ± 0.00^{a}	1.00 ± 0.58^{ab}	2.00 ± 0.58^{bc}	3.67 ± 0.33^{c}
Multiple	0.00 ± 0.00^{a}	0.67 ± 0.33^{a}	4.00 ± 0.58^{b}	11.33 ± 0.88^{c}

Note: *At the horizontal line, the mean value followed by different letters are significantly different (Tukey test, p>0.05).

DISCUSSION

Our study revealed that gravid *Cx. quinquefasciatus* females were attracted to the oviposition sites with high intensity and darker colour of *I. cairica* plant extract compared to control (distilled water). However, no eggs were found in any of the plant extract solutions. Interestingly, more *Cx. quinquefascitaus* gravid females were found dead due to drowning in the highest concentration of *I. cairica* plant extract (300 ppm). The 300 ppm concentration had the darkest colour and the highest intensity among all treatments. The colour of the water or water clarity is an important factor influencing the oviposition behaviour of female mosquitoes (Li *et al.* 2009). The reflection colour (Barbosa *et al.* 2007) caused by the container can also attract mosquitoes to oviposit. Li *et al.* (2009) found that *Culex pipiens pallens* females laid significantly more eggs in dark blue water (50 mg/L of methylene blue solution, or 50 mg/L and 200 mg/L of brilliant blue solution) than in distilled water. *Anopheles quadramaculatus* also oviposited preferentially in oviposition waters that appeared darker than controls in a laboratory experiment (Lund 1942). Black and red were the most preferred colours for oviposition of *Cx. annulirostris* and *Cx. molestus*, respectively, whereas, yellow and green were the least preferred colours for both species under laboratory condition (Dhileepan 1997). Oviposition waters dyed with ink were detected to be significantly attractive to ovipositing *Cx. quinquefasciatus* when compared to distilled water controls in a laboratory evaluation. The study also suggested that dark colours attract more gravid *Cx. quinquefasciatus* because females respond to the increased optical density of the dyed water rather than to the volatile components of the dye (Beehler *et al.* 1993).

The reflected colour of yellowish green at 500–600 nm of the acethonilic extract of *I. cairica* leaves may have been detected effectively by *Cx. quinquefasciatus* gravid females. Based on a previous study, electroretinographs revealed spectral sensitivity of the eye of the female *Ae. aegypti* ranging from ultraviolet (323 nm) to orange-red (621 nm), with sensitivity peaks in the ultraviolet (323−345 nm) and green (523 nm) wavelengths. It was also reported that the eye of the female *Ae. aegypti* had relatively poor acuity and is capable of some wavelength discrimination but has high overall sensitivity to light (Muir *et al.* 1992). Since *Ae. aegypti* and *Cx. quinquefasciatus* are from the same subfamily (Culicinae) there could be a similarity in the eye sensitivity peak. We proposed that the yellowish green colour of the tested extract can be effectively detected by *Cx. quinquefasciatus* gravid females. We however acknowledge that further comparative studies needs to be conducted, for example by comparing the extract concentration against the dyed concentration. We believe that our preliminary result is sufficient to suggest such colour specific preference for oviposition by *Cx. quinquefasciatus*.

Additionally, the organic content of the plant extract solution might have also attracted *Cx. quinquefasciatus* gravid females to oviposit. Previous studies have reported that high organic content in surveillance traps attracted more gravid female mosquitoes. Ovitraps baited with grass infusions, *Panicum maximum* (Jacq.) evaluated in the field were reported to collect significantly more *Aedes e*ggs than controls containing tap water (Santana *et al.* 2006). Bermuda grass (*Cynodon dactylon*), oak leaf (*Quercus virginiana*), acacia leaf (*Acacia schaffneri*), and algae (*Spirogyra* sp.) infusions were found to be effective in collecting *Cx.quinquefasciatus*, *Culex nigripalpus*, and *Culex erraticus* (McPhatter & Debboun 2009), whereas, bioassays with *Spirogyra majuscula* organic extracts indicated that these extracts attracted more *Anopheles pseudopunctipennis* gravid females under laboratory conditions (Torres-Estrada *et al.* 2007).

Even though the acethonilic extract of *I. cairica* leaves attracted more gravid females, no eggs rafts were laid in the treated oviposition site. The tested plant extract solution caused mortality of the gravid *Cx. quinquefasciatus* females used in our study, thus inhibiting the oviposition activity. The different concentrations of the studied plant extract may have reduced the water surface tension to a certain extent, which prevented proper landing and egg deposition by gravid females. Similar observations were found in a study by Kassir *et al.* (1989) on limonene, a major constituent of citrus essential oil isolated from abraded fresh peels of bitter orange *Citrus aurantium* L. against *Cx. quinquefasciatus* under laboratory conditions. Limonene was reported to demonstrate interference with oviposition as indicated by the zero egg rafts deposited in 1 and 50 ppm concentrations compared with control water. All of the adults which were used in the oviposition experiments in the study died after drowning within 2 weeks. Inspection of the water surface tension (by ring method) revealed that the tension was reduced in limonene-treated water compared to control water.

The toxicity of the acethonilic *I. cairica* leaves extract may also be a possible factor for the mortality of gravid *Cx. quinquefasciatus* females during alighting in the treated oviposition site. Pre-oviposition drinking by *Culex* mosquitoes has been reported by Weber and Tipping (1993). It has been found that since drinking occurs before oviposition began, contact chemoreceptors on the mouthparts could be involved in the final stages of ovisite acceptance. Time spent for drinking exceeded that necessary for mere tasting, and drinking may also serve to distend the abdomen and assist in movement of eggs through the oviduct (Weber & Tipping 1990). Therefore, there is a possibility that this behaviour may have caused poisonous interference in biochemical and physiological functions of the gravid female mosquitoes in the current study due to the active compounds in the tested plant extract solution. The physiological constraints during oviposition might have caused less resource for egg deposition and eventually led gravid *Cx. quinquefasciatus* females to death.

The ability of the acethonilic extract of *I. cairica* leaves to attract and kill gravid *Cx. quinquefasciatus* female mosquitoes can be a very useful tool in mosquito control programs and hopefully this study can assist in further analysis on the interference caused by the tested plant extract on the oviposition activity of *Cx. quinquefasciatus* mosquitoes.

On whole, the acethonilic extract of *I. cairica* leaves were found to effectively attract gravid *Cx. quinquefasciatus* females and subsequently cause mortality of the gravid females, inhibiting egg deposition. The difference in colour intensity also influences the oviposition site selection by the gravid females, whereby oviposition sites with tested plant extract of a darker colour intensity attracted more gravid females. However, conclusion from this study was deduced from relatively short-run laboratory experiments; thus longer and detailed studies as well as field evaluations are required. Isolation and identification of specific phytochemicals responsible as oviposition attractants, and further investigations on the factors causing the mortality of the gravid females are required for better understanding of *I. cairica* plant extract interference in the oviposition activity.

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