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Insecticidal activity of *Artemisia annua L.* (CBGE/CHNA/09/ LTNGS/G) ethanolic leaf and seed extracts on *Anopheles gambiae*

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Abstract

The dry leaves (500g) and dry seeds (500g) of *Artemisia annua L.* were separately extracted with 2 litres of ethanol for a period of 48 hrs at room temperature (25^{o}C). The phytochemical constituents of the plant were determined. The extracts (leaves and seeds) were tested singly and in combinations for their insecticidal activities at different concentrations (1.0mg/litre, 2.0mg/litre, 4.0 mg/litre and 5.0mg/litre) on *Anopheles gambiae* 4th instar larvae, pupae and adult females. The effects of the smoke from burning dry leaves of *A. annua* on *A. gambiae* (larvae, pupae and female adults) were also determined. The plant was found to contain alkaloids, flavonoids, glycosides, phenols, resins and tannins. Artemisinin, a secondary metabolite which is essential for the production of antimalaria drugs was extracted from the plant. The plant was also found to contain oil (essential oil). The extracts were found to have lethal effects on the larvae, pupae and adult females of *A. gambiae.* A combination of the leaf and seed extracts was found to be more lethal than the singler effects of either of the extracts. The smoke from the burning dry leaves of the plant was found to have lethal and repellent effects on the mosquitoes. The volatile nature of the constituents of the leaf and seed extracts may have been responsible for their insecticidal actions. The implications of the results obtained have been discussed.

Keywords: Artemia annua L., Anopheles gambiae, ethanolic, mosquitoes, extracts, anti-malaria

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Introduction:

Artemisia annua L. (annual wormwood, sweet wormwood, sweet annie) is a highly aromatic annual herb of Asiatic and Eastern European origin. It is widely distributed throughout the temperate region. The plant has become naturalized in many countries including Argentina, Bulgaria, France, Hungary, Italy, Spain and Yogoslavia (Gray, 1884, Klayman, 1989, 1993). The plant has recently been introduced in Kenya and Nigeria where it is known to be doing very well and under commercial cultivations.

Simon *et al.*(1990) reported that the species has naturalized in the United States and is sold on a limited scale as a dried herb for the floral and craft trade where it is used as an aromatic wreath. The same authors reported that the plant has traditionally been grown in China as a medicinal and more recently in Europe for its aromatic leaves which are used in flavouring beverages.

A. annua is a source of qinghaosu (artemisinin) which is an antimalarial agent. Artemisinin has been reported to be a potent plant inhibitor with natural herbicidal potential (Chen *et al*. 1987; Duke *et al*., 1987). Artemisinin and its derivatives, artemether and artesunate, have been studied for their efficacy as antimalaria agents. In vitro trials conducted in China in 1981 (WHO cited by Simon *et al.,* 1990) showed that all the three compounds were effective against the erythrocytic stages of two chloroquine-resistant Hainan strains of *Plasmodium falciparum*, the malaria parasite, at lower minimum effective concentrations as compared to chloroquine, the most commonly used malaria drug. Artemisinin and its derivatives have been used effectively for the treatment of malaria including cerebral malaria in humans with no apparent adverse reactions or side effects.

Malaria is a mosquito- borne infectious disease caused by a eutkaryotic protist of the genus *Plasmodium.* It is widespread in tropical and sub-tropical regions including parts of the Americas, Asia and Africa. Approximately 350-500 million global cases of malaria are recorded annually with approximately one to three million deaths, majority of whom are young children in sub-Saharan Africa (Snow *et al.*, 2005). In 2008, malaria caused nearly one million deaths, mostly among African children (WHO, 2010). WHO, 2010 also reported that malaria can decrease the gross domestic product as much as 1.3% in countries with high disease rates. Non- immune travelers from malaria-free areas are very vulnerable to the disease when they get infected. In African a child dies every 45 seconds of malaria and the disease accounts for 20% of all childhood deaths (WHO, 2010).

Malaria is naturally transmitted by the bite of a female Anopheles mosquito. During the bite a small amount of infected blood is taken which contains malaria parasites. The parasites develop within the mosquito and about one week later, when the mosquito takes its next blood meal, the parasites are injected with the mosquito's saliva into the person being bitten. After a period of between two weeks and several months (occasionally years) spent in the liver, the malaria parasites start to multiply within red blood cells, causing symptoms that include fever and headache. In severe cases, the disease worsens leading to hallucinations, coma and death.

About 20 different Anopheles species are locally important around the world (WHO, 2010). All of the important vector species bite at night. They breed in shallow collections of fresh water like puddles, ricefields, marshy areas and hoofprints. Transmission is more intense in places where the mosquito is relatively long-lived (so that the parasite has time to complete its development inside the mosquito) and where it prefers to bite humans rather than other animals. The long lifespan and strong human-biting habit of the African vector species is the underlying reason why more than 85% of the world's malaria deaths are in Africa (WHO, 2010).

Vector control is the primary public health intervention method for reducing malaria transmission at the community level. Many people are now becoming allergic to synthetic insecticides used for the control of mosquitoes especially asthma patients. Chemical insecticides pose a lot of problem. They tend to harm non-target organisms such as man, domestic animals, beneficial insects and wildlife (Aizawa, 1982). Microbial preparations and formulations in the insect pest control programme are known as "microbial insecticides." Preparations of *Bacillus thuringiensis* showed effectiveness on the following insect pests in Southeast Asia: Common cabbageworm, diamondback moth, fall webworm, oriental tobacco budworm, persimmon fruit moth, rice skipper and apple tortrix (Aizawa, 1982). However there is no existing report on the effects of existing microbial insecticides on Anopheles mosquitoes.

Tripathi *et al.* (2000) tested the essential oil of *A. annua* for its toxic repellent and development inhibitory activities against 2 economically important stored product insects, *Tribolium castaneum* (Herbst) and *Callosobruchus maculatus* L. Adult beetles of *T. castaneum* were repelled significantly by oil of *A. annua* at 1% concentration (vol: vol) and above in filter paper arena test. Dose-response relationship of *A. annua* oil revealed that there was a significant negative correlation between larval survival, pupal survival and adult emergence of *T. castaneum.*

Shaalan *et al.* (2005) reported that synthetic and botanical insecticides can have a profound effect on the developmental period, growth, adult emergence, fecundity, fertility and egg hatch, resulting in effective control at sub-lethal concentrations. They found that the lethal concentration (LC_{75}) of *Callitris glaucophylla* outperformed synthetics by completely prohibiting adult *Aedes aegypti* emergence. Consequently, they recommended it for field application either in combination with synthetic or natural insecticides or alone.

Umar *et al.* (2007) evaluated the effects of solvent extracted neem seed kernel on *Aedes aegypti* 4th instar larvae using WHO standard procedures. They found that the most potent larvicides were hexane, 2 propanol, ethanol, acetone, methanol and DMSO extracted neem seed kernels which after one hour of exposure gave the lowest LC_{50} values of 0.71%, 1.26% and 2.2%, 2.27% and 3.39% (v/v)ml respectively. The neem seed kernel extracted with benzene, ethyl acetate and distilled water had no larvicidal activity after one hour exposure.

Efforts to eradicate malaria by eliminating mosquitoes have been successful in some areas. Malaria was once common in the United States and Southern Europe but vector control programmes in conjunction with the monitoring and treatment of infected individuals eliminated it from those regions.

The present study was designed in order to find out whether the extract of *A. annua* will have any insecticidal effect on mosquitoes. It is hoped that this method of vector control will complement the Roll Back Malaria partnership, a global partnership of malaria donors, which recommends a comprehensive strategy that includes improving diagnosis, getting highly effective anti-malarial drugs quickly to all who need them, spraying interior walls of houses with long-lasting insecticides so that mosquitoes can be killed when they land there to rest and giving pregnant women two doses of an anti malaria to prevent them from getting malaria.

Materials and Methods

A. annua leaves employed for the study were obtained from University of Jos Centre for Biotechnology and Genetic Engineering Agricultural Biotechnology Field Research Station, Gangnum, Plateau State. The plant variety was CBGE/CHNA/09/LTGS/G. The leaves were obtained from the plants that were about to flower because earlier studies by the Research Group had shown that artermisinin (the active ingredient of the plant extract) has its optimum level of production at this stage. A good quantity of the mature dried viable seeds of the plant were also collected.

Extraction of experimental leaves and seeds: The leaves were dried aseptically in the laboratory at room temperature (25^{o}C) for a period of 10 days. The seeds were further dried under the same environmental conditions. The dried leaves and seeds were separately ground into powders with the aid of electric blender and sieved with the aid of fine-mesh plastic sieve. The processes led to the production of fine powders of the leaves and seeds respectively.

A weight of 500g of the leaf powder was extracted with 2 litres of ethanol for a period of 48 hours at room temperature. The same weight of the seed powder was subjected to the same treatment. Distilled water was not used because that process was discouraged by Umar *et al* (2007). Each extract was then filtered with the aid of Whatman No. 1 filter paper. The extracts were evaporated to dryness by placing the containers in rattling water bath (40^{o}C). the extracts were further dried by placement near an electric fan (Ascher, 1981). The extract were then stored in labeled specimen bottles for bioassays.

Assessment of Phytochemical constuents of the leaves and seeds: Samples of the test plant materials were analysed by the National Institute of Phamaceutical Research, Abuja for their biochemical constituents using standard methods described by Harbone (1984) and Evans (2002).

Source of test mosquitoes employed for the study: Anopheles gambiae females were obtained from the National Vector and Malaria Control Unit of Federal Ministry of Health, Yaba, Lagos and subsequently maintained at 25 ± 2^{o}C and 70% ± 5 R.H. *A. gambiae* 4th instar larvae were also collected and kept in plastic buckets half-filled with tap water and fed on goldfish flakes as reported by Shaalan *et at.* (2005). The flakes were collected from a commercial goldfish farmer in Jos, Plateau State. The water used in rearing the mosquitoes and the larvae was changed on two-day basis. The Adult female mosquitoes were fed on 10% sugar solution in a sterilized bowl placed in the container that housed the mosquitoes as described by Shaalan et al. (2005). The said adult female mosquitoes were also provided with opportunity to feed on rat blood. The experimental rat was put in well aerated rat cage and then carefully placed in the same container that housed the experimental female *A. gambiae*.

The larvae were fed *ad labitum* on 0.5g powdered liver and glucose at a ratio of 3:2 (wt:wt) as an enhanced method of Roberts and as described by Umar *et al.* (2007). The larvae were also provided with wine yeasts (*Saccharomyces cerevisiae)* as supplementary feeding.

The experiments were repeated when percentage population was found to be greater than 10.

Bioassays: Each of the following weights of the leaf extract: 1.0mg, 2.0mg, 4.0mg and 5.0mg was dissolved in one litre of ethanol as reported by Umar *et al.* (2007) and then used to impregnate 4cm x 4cm Whatman filter paper strips. Ethanol was used for reconstitution because preliminary studies showed that ethanol had no effect on the mosquito larvae, pupae or the adult female anopheles. The experiment was repeated using the seed extract and 50/50 concentrations of a combination of the leaf and seed extracts. Another batch of 4cm x 4cm filter paper strips was impregnated with sterile distilled water for the control experiments.

Four sets of sterile 500ml plastic cups were set up. Each set contained 3 of such sterile cups. A total of 100 adult female Anopheles mosquitoes were introduced into each sterile cup of the first set. The second set of sterile plastic cups had 100 each of Anopheles mosquito pupae which had

developed from the initial larvae collected from the National Malaria control Unit of Federal Ministry of Health, Lagos. The third set of sterile plastic cups contained mosquito larvae. Each cup had a total of 100 4^{th} instar larvae. The larvae and pupae were maintained in the same water medium in the experimental cups. The environment was same as in the Insectary. On the whole 300 adult female anopheles mosquitoes, 300 anopheles pupae and 300 anopheles larvae were employed for the bioassays.

One strip of the extract-impregnated 4cm x 4cm Whatman filter paper was aseptically inserted into each cup. Such cup was quickly covered with a piece of cotton mosquito net in order to safeguard the cup contents. The control experiments contained the said mosquito forms and inserted sterile water treated strips. Each set of such cups was then put in well- aerated plastic mosquito cage at room temperature and left for hourly observations. Adult, pupal and larval mosquito mortalities were recorded using the insect growth regulator testing instructions of (WHO, 1996). The effective concentration (EC 50) when the F_1 progeny was reduced by 50% was then determined. The experiments were repeated using the seed extract and a 50/50 concentration combination of the leaf and seed extracts. The widely used Abbott (1925) method of computing the effectiveness of an insecticide was adopted for the analysis of the insecticidal effects of the extracts. The experiment was repeated for each extract concentration and the average mortalities of each experiment were recorded.

Larval mortality was assessed after 48hrs of exposure by probing the larvae with needle and moribund larvae were counted as dead (Azmi *et al.,* 1998). The inability of pupae to emerge into mosquito adults after a normal pupae to adult cycle was used to assess pupae mortality while the dead female adult mosquitoes were assessed by the way they lied on their backs, sideways, their dry nature and their inability to resurrect and fly away after the opening of the mosquito cage and the 250ml experimental plastic cups.

Repellency and toxicity of smoke from burning dry leaves of A. annua on female anopheles mosquitoes: Two rooms in a recently completed house which had their walls and ceilings painted white were used for this experiment. Clean white bed covers were used to cover the floors of the rooms. The white colour was used for easy identification of the mosquitoes. All the windows and doors of each room were then closed. The lights were put off and a total of 50 female *Anopheles gambiae* adult mosquitoes were released into each room. A weight of 500g of the dry leaves of *A. annua* was then lighted in an open ceramic bowl in one of the rooms in order to find out the repellence effects of the smoke from the burning leaves on the test mosquitoes. The second room was free from this treatment and so served as the control. The two rooms were then closed for a period of one hour and then opened in order simulate what happens during the use of synthetic insecticides. The white bed covers were inspected for the presence of dead and immobilized test mosquitoes. The white painted walls were also inspected for the presence and behaviour of some of the mosquitoes.

Results

The phytochemical constituents of the plant included alkaloids, flavonoids, glycosides, phenols, Resins and Tannins. Artemisinin which is a secondary metabolite was also extracted from the plant. Artemisinin is a sesquiterpene lactone endoperoxide. Essential oil was also extracted from the plant.

The results obtained revealed that the leaf and seed extracts were lethal to 4^{th} instars of *A. gambiae* larvae, pupae and adult females. The effective concentration (EC 50) for the reduction of F_1 progeny by 50% was found to be 5.0mg/litre for the larvae, pupal and the adult females for the leaf extract (Table 1). As for the seed extract, a similar result was obtained (Table 2). The combined action of the leaf and seed extract appeared to be more lethal to the adult females (Table 3). The combined extracts of the leaves and seeds of the *A. annua* resulted in faster mortality of the larvae and pupae as shown in Table 3. A total of 164 larvae were killed by all the concentrations of the leaf extract (Table 1) while a total of 146 larvae were killed by the same extract concentrations used for the experiments (Table 2). The highest level of larval mortality (224) was observed in combined action of the leaf and seed extracts (Table 3). Similar results were obtained for the pupae and adult females. The increased levels of the extracts concentrations led to corresponding mortality rates on the different forms of the *A. gambiae* employed for the study.

The smoke from the burnt dry leaves of the *A. annua* led to the death of 22 adult female mosquitoes which were found to lie on their backs, side-ways and life-less on the clean white bed covers. The remaining mosquitoes were found immobilized, very

weak and unable to fly away or fly about when the door was opened. In the control room, the test adult mosquitoes were found very active, and were seen flying about when the door was opened. No deaths were recorded. The volatile components of the extracts (essential or volatile oils which are mostly associations of terpenes with alcohol, phenols, ketones, aldehydes or esters in the plant), must have been responsible for the insecticidal actions observed.

Table 1: The effects of *A. annua* leaf extract on the test *Anopheles gambiae* forms.

Concentration of leaf Extract in mg/litre	Number of dead Mosquito forms after 48 hours Larvae	Pupae	Adults	Total
1.0	27 (0)*	20 (0)*	10 (0)*	57
2.0	40 (0)	38 (0)	18 (0)	96
4.0	47 (0)	42 (0)	32 (0)	121
5.0	50 (0)	51 (2)	50 (0)	151
Total	**164**	**151**	**110**	**425**

*Number in bracket = Mortality in control experiment.

Table 2. The effects of *A. annual* extract on the test *Anopheles gambiae* forms.

Concentration of seed Extract in mg/litre	Number of dead Mosquitoe forms after 48 hours Larvae	Pupae	Adults	Total
1.0	2.2 (0)*	16 (0) *	7 (0)*	45
2.0	32 (0)	20 (0)	12 (0)	64
4.0	44 (0)	30 (0)	32 (0)	106
5.0	48 (0)	50 (0)	50 (0)	148
Total	**146**	**116 (0)**	**101 (0)**	**363**

* Number in bracket = Mortality in control experiment.

Table 3. The combined effects of *A. annua* leaf and seed extract on test *Anopheles gambiae* forms.

Equal Concentrations of leaf and seed Extracts in mg/litre	Number of dead Mosquito forms after 48 hours Larvae	Pupae	Adults	Total
1.0	30 (0)*	32 (0)*	35 (0)*	97
2.0	44 (0)	42 (0)	48 (0)	134
4.0	50	49(0)	65 (0)	164
5.0	100 (0)	100 (0)	100 (0)	300
Total	**224 (0)**	**223 (0)**	**248 (0)**	**695**

* Number in bracket = Mortality in control experiment.

Discussion

The *A. annua* ethanolic extracts were found to be lethal to the A. gambiae larvae, pupae and adult females. The total number of Mosquitoes killed increased with increase in the concentration of the extracts. Marcard *et al*. (1986) obtained similar results. Essential oil was one of the components extracted from the *A. annua.* Tripathi *et al*. (2000), worked on the repellency and toxicity of oil from *A. annua* to certain stored-product beetles. They found that oil from the plant was largely responsible for both repellent (behavioral) and toxic (physiological) actions on 2 species of insect tested. Essential oil was extracted from both the leaves of the plant and the seeds through steam distillation.

Several factors may have been responsible for the mortality of the plant extracts on the mosquito forms. The main effect must have been on the respiratory system. Corbet *et al.* (1995) attributed the susceptibility of mosquito larvae to essential oils to tracheal flooding and chemical toxicity while Rey *et al* (1999a) and David *et al*. (2000) separately showed that they primarily affected the midgut epithelium and secondarily affected the gastric caeca together with the malpighian tubules in mosquito larvae. David *et al.* (2000) reported that the histopathological effects differed qualitatively according to their

location along the midgut and quantitatively according to the concentration assayed, duration of the treatment and mosquito species used. In the present study it was found that a combination of the leaf and seed extracts proved more lethal to the mosquito forms. This could have stemmed from the increased oil content of the mixture which had a corresponding effect on the respiratory or physiological systems of the mosquito forms. Both the leaves and seeds of the plant were found to contain essential oil. The combination of the two was found to contain more essential oil than when they were used separately. Tripathi *et al.* (2000) reported that there was a significant negative correlation between larval survival, pupal survival and adult emergenct of *Tribolium castaneum* (Herbst) and *Callosobruchus maculatus* (L.) during their study on the repellency and toxicity of oil from *A. annua* to certain stored- product beetles. It appears that the active principle that is responsible for the mortality of the female Anopheles Mosquito larvae, pupae and adults is contained in the oil component of the plants extracts. Essential or volatile oils are mostly associations of terpenes with alcohols, phenols, ketones, aldehydes or esters found in plants. Cases of allergy to synthetic insecticides is now on the increase. This is where bioinsecticides become very handy. The present study has shown the effectiveness of bioinsecticide in the control of mosquitoes. Shaalan *et al* (2005) reported that botanic extract of *Callitris glaucophylla* induced a wide range of sub-lethal effects on *Aedes aegypti* larval mortality.

The leaf extract was found to contain artemisinin which is a secondary or natural plant metabolite which has been identified as a sesquiterpene lactone endoperoxide (Simon, et al., 1990). The Artemisinin has been synthesized and its structure and absolute stereochemistry established by Simon *et al* (1990), but the biosynthetic pathways and the mechanisms and regulation processes are unknown. The analysis of artemisinin is difficult because the compound is unstable and the concentration in the plant is low apart from the fact that the intact molecule stains poorly and other compounds in the crude plant extracts interfere in its detection. An offshoot of this study is looking at the direct effect of artemisinin on female anopheles mosquito apart from the effects of the compound on *Plasmodium* species.

The plant was found to contain alkaloids among other biochemicals. Many vegetable drugs owe their action to alkaloids. These are basic nitrogenous compounds of heterocyclic structure such as derivatives of pyridine, quinoline, isoquinoline and indole. Most alkaloids are bitter in taste and that could account for the bitter taste of the leaf *A. annua* when it is taken as herbal tea.

The *A. annua* dry leaves burning experiment simulated the use of mosquito coils. The smoke from the burning leaves killed some mosquitoes and seemed to have immobilized the rest. In view of the widespread poverty in the land, people employ this principle in controlling the menace of mosquitoes in their residential houses. However, what they burn or use are dry leaves of *Eucalyptus* species and the peels of Citrus fruits. It will be interesting to investigate the combined effects of these leaves or peels and the dry leaves of *A. annua* on the females of *A. gambiae.*

The pant is safe to humans as it is used in the production of malaria drugs. Artemisinin-based products are relatively safe towards non-target biota and the Chinese have successfully used it for more than one hundred years in the control of malaria. The biodegradability of the plant process wastes and the environmental impact assessment are being looked at and the results will soon be published.

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