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Synergistic Combinations of Flower and Leaf Extract Activities of *C. cinerariifolium* , *E. camaldulensis* and *N. tabaccum* Outweigh the Activities of Antagonistic Combinations

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Abstract: ***Crude leaf extracts of C. cinerariifolium(Cc), Eucalyptus camaldulensis (Ec) and Nicotiana tabaccum (Nt) were individually and in combination tested for their synergistic and antagonistic activities against third instar larvae of Anopheles gambiae s.s. Giles. Six different solvents were used for the extraction of oils from the flowers and leaves of the three plants namely ethanol, methanol, Dicholoromethane (DCM), hexane, ethyl acetate and aqueous. The larval mortality of the third instar larvae of An. gambiae s.s. Giles was observed on 24 hours exposure separately in control using 50, 100, 150, 200, 250, and 300 ppm of crude flower and leaf extract concentrations. For the individual tests, all extracts of the plants showed good larvicidal activity. Thirty treatments were carried out to determine synergism and antagonism on extract combination ratio of 1:1 (v/v) (i.e 100 ppm: 100 ppm) and it was observed that 16 extract combinations exhibited synergistic activities (SF>1) and 10 activities indicated antagonistic activities (SF<1) while 3 combinations were neither antagonistic nor synergistic (SF >1<).***

Keywords: Crude extracts, synergistic. antagonistic, solvents, mortality, individual activities, combination activities, *Cc cinerariifolium, E. camal;dulensis, N. tabaccum*, 3rd instar larvae, standard stock solution, concentrations.

1. Introduction

Milugo *et al*., (2013) describes the antagonistic effect of alkaloids and saponins on bioactivity in quinine tree from tissue samples of quinine tree (*Rauvolfia caffra* Sond) from a remnant forest in Kuria county of Western Kenya. This plant was found to contain phytochemicals including alkaloids, terpenoids, saponin, cardiac glycosides and steroids, important as antioxidant and antagonistic interaction hence lowering their activity as antioxidants.

The co-toxicity factor (CTF) calculated as COF = (O-E/E) x 100, where O is observed % mortality and E is expressed as % mortality categorizes result into three: A positive factor of ≥ - 20 indicating potentiation, a negative factor of ≤ - 20 indicates antagonism and the intermediate values of > - 20 to < 20 indicate an additive effect (synergism). For a mixture (50%) the resultant synergistic/antagonistic factor (SF) could give an indication to the nature of the effect; where SF > 1 means synergism, SF < 1 means antagonism; SF=1 means no obvious effect) (Rodnguez *et al.* (2013). WHO (2013) method of determining RR at the prescribed diagnostic dose and diagnostic time was: 100 – 98% mortality, indicated Susceptible; 98 – 90% mortality meant possibly Resistant and < 90% = confirmed Resistant (more testing required was suggested).

Synergy can result when the synergist (i) inhibits the detoxification of a toxin. Acute oral toxicity was analyzed through Probit-log to obtain percent mortality. (ii) modifies an inactive compound rendering it toxic (iii) enhances the penetration, transport or accessibility of a toxin to its target or (iv) attacks two independent steps in a process, such as two stages in development or two steps in a biosynthetic pathway. Antagonism can result when the reverse occurs. For example, an antagonism might modify a toxin rendering it inactive, or decrease the accessibility of a toxin to its target. Diawara *et al.* (1993) used x^2 analysis to test for antagonistic effects of chemical combinations on larval mortality for which the formula : E = Oa + Ob (1-Oa) was used to test mortality while larval weight, and development time were analyzed by ANOVA to interpret synergy or antagonism.

Greco *et al.* (1995) have come up with isobolographic analysis method of determining synergistic and antagonistic interactions in which combinations A and B act in additive manner for two independent with the additive effects. The combinations of A and B giving the same effect as A or B alone (the isoeffective dose combination) are represented by a straight, dashed line on an isobolograph that connects the effective doses of A and B when alone. This line is called the zero –interaction isobole (Tallarida 2012). The dosages for A and B producing the same level of effect are plotted as the intercepts for each axis. The straight, dashed line represents the isoeffective dose combinations, or the combinations of A and B giving the same effect as either A or B alone. The straight, dashed line between the intercepts represents the isobole, or the null model: Loewe additivity with no interaction between the compounds.

On the isobolograph the LD_{50} values for each compound alone are plotted as the intercepts for each axis. Thus, a dose response curve resulting from various isoeffective combinations of A and B for a synergistic interaction will be concave up and down the zero-interaction line. Conversely, an antagonistic interaction will be represented by a concave down curve above the line. The null model for the Loewe additivity is also represented by the equation: $d_a /D_a + d_b/D_b = 1$ (Tallarida 2012; (Al-Hussaini and Mahasnehy 2009) where d_a and d_b are the concentrations of A and B, respectively, used in combination and D_a and D_b are the individual concentrations of A and B producing a specific level of effect ($LD_{50,}$ for example). If the sum of the two

ratios equals one, no interaction, or Loewe additivity, exists. If the sum is less than one, synergy is said to occur. If the sum is greater than one, antagonism is said to occur.

2. Materials and Methods

2.1 Sample collection and extraction of essential oils from the plants

Collection and preparation of vegetative material

Mature vegetative material 20 kg of *each Cc, Ec* were obtained from Kiambereria location, Molo , a distance of 110 km east of Eldoret, and 20 kg of *Nt* from Malakisi , Bungoma County a distance of 126.2 km East of Eldoret and 22.2 km from Malaba, the Kenya-Uganda boarder. All the collected material were placed in clean gunny bags separately and transported to an airy open storage facility in Eldoret for drying. Twenty kg of green flowers of *Cc* weigh one quarter of the green flowers when dry (Gachie, 2018). The vegetative materials were washed thoroughly with distilled water and they were hanged away from the sun in an airy store well exposed for 3 weeks to dry naturally.

The vegetative materials were ground to powder using a blender mortar and pestle and powders labeled according to their plants and solvents.

Extraction of the oils from the plants were carried out exhaustively plant after plant and solvent after solvent by taking one hundred grams of each plant powder soaked into 200ml of each solvent in separate bottles with stoppers for 3 days at room temperature. These were placed on electric shaker one at a time (Uthayarasa *et al.* 2010) to promote complete dissolution. The mixtures were filtered using Whatman No. 1 filter paper (M/s Glassil Scientific Industries, India) and collected into conical flasks. From the six solvents and three plants, a total of 18 extracts were obtained and were placed in amber or blue bottles labeled indicating plant species, date of collection, by whom plants were collected, date of collection and place of plant origin. The products were then stored at 4^0C in air tight amber or blue bottles until use. Oil extraction was carried as shown in the flow diagram below:

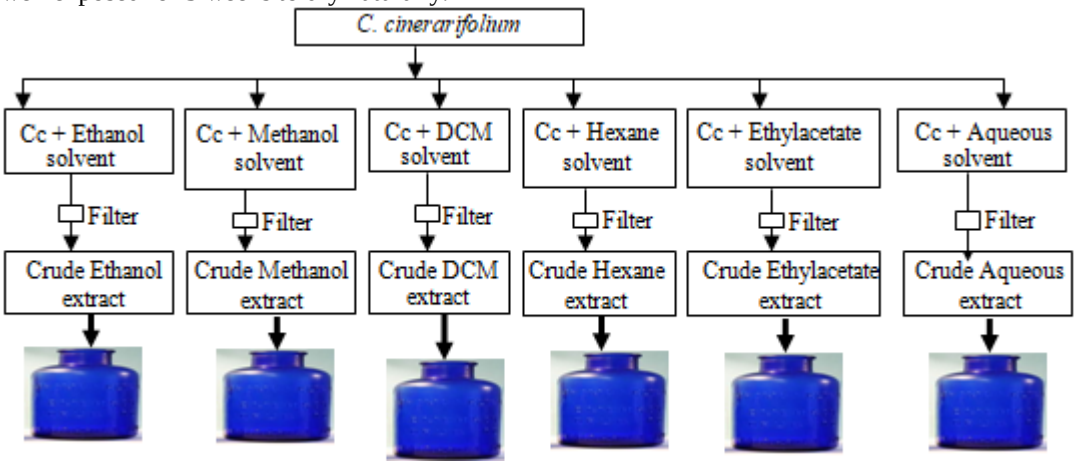

Figure 1: Flow diagram of extraction process of the essential oils

Amber bottles for extract storage 4^0C.

For better and longer keeping the extracts were concentrated to powder. The extracts were dried under reduced pressure using a rotary evaporator (Uthayarasa *et al.* (2010). All extracts were stored at 4^0C in air tight amber or blue bottles not to be accessed by light throughout the study period. These dried filtrates (solutes) were labeled: collector, date of collection, plant species, time and place of origin and purpose were shown.

From each powder (solute), stock standard solution (SSS) were prepared by weighing 100g on paper or small container and then transferred to a volumetric flask (''vol flask'') into which 200 ml of distilled water as solvent was added and then contents swirled gently until the solute was completely dissolved. More solvent was added until the meniscus of the liquid reached the calibration mark on the neck of the vol flask (a process called ''diluting to volume'' or ''adding to the mark''). The vol flask was then capped and inverted several times until the contents were mixed and completely dissolved. In this way, 1 litre (1,000 ppm) SSS of each extract was made. A total of 18 SSS were made which were accordingly labelled and stored as described in extraction.

From the SSS test concentrations of 50,100,150,200,250 and 300 ppm of the crude extracts were prepared in distilled water for use in larval bioassays Zuhara *et al.* (2014) and Nganjuwa *et al.* (2015). By use of micropipettes Gilson P-10, P-20, P-100 and P-200, concentrations of 50, 100, 150, 200 and 300 ppm of each extract solvent and of each plants were separately drawn from the 1,000 ppm concentrate of stock solution and placed into 1 litre volumetric flask. De-ionized water was used to add up to the mark. Mixtures were shaken gently side by side and were placed in air tight amber or blue bottles, labelled and stored as described in extraction.

2.3 Field sampling of adult male and female *Anopheles gambiae* mosquitoes for laboratory rearing

General distinguishing features of *Anopheles* male and female mosquitoes were used to identify the species for collection (White and Kaufman 2014) and Gillies and Coetzee 1987). In identification a magnifying glass of the power x 100 at a distance of 0.46 m (1ft 6 inches) from the mosquito landing surface was used and using backpack battery-powered hand-held aspirator or a hand-held mouth aspirator (as an alternative equipment) (Maia *et al.* 2011;

Olayemi *et al*. 2011; Pimid *et al.* 2015; and WA Nazni *et al*. 2009), adult male and female *Anopheles gambiae* mosquitoes in the ratio of 3:1 (male:female) (450 male and 150 female) were collected from their various resting places in Langas area of Eldoret municiality. Sucking of the mosquito was done at a much closer distance of half to one inch. In one year 4500 male and 1500 female mosquitoes were collected. Collection of the mosquitoes was done once a day and once in a month between the months of January and December 2009. Collection was executed within the first three days of the month to allow ample time for eggs oviposition and larvae development. The mosquitoes were collected into separate glass jars capped with a net cloth to allow oxygen for the mosquitoes. These were transported to the laboratory for rearing. The purpose of the adult mosquito collection was to enable grow pure colonies of mosquitoes for bioassays. Mosquitoes whose spermathecae appeared enlarged (engorged with eggs) were not collected.

Previous researchers have stated that there is no first hand rule for ratio of male and female mosquitoes for mating. Various ratios have been suggested: Gleiser and Zygadlo (2009), 1:1; Kweka (2012) 1:3 ; Pimid *et al.* (2015), Szekely*et al*. (2014) 4:3; (Diabet and Tripet, 2015), Olayeni *et al*. (2011) and Oliva *et al.* (2011) 1:1; Chang *et al*. (2003) 3:1 for *A.* arabiensis, (Michelle *et al*, 2011) applied a ratio of 1:1:1 (irradiated males : wild males : wild virgin females); Darmagadda *et al.* (2005) and Yankanchi et al. (2014) 2:1; Rajkumar and Jebanesan (2010) 1:1; and WHO (2013); WHO, (2005) recommends a ratio of 3:1 (male: female); Adelman Baughman et al (2017) and Ethiopian Public Health Institute (2017) a ratio of 3:1 (male:female), Galizi *et al*. (2014), Dao et al (2008), used a ratio of 3:1 (male:female) and Madakachery *et al* (2013) had a ratio of 3:1 (male : female). In this research a ratio of 3:1 (male:female) was used for mosquito rearing.

The mosquitoes were distributed into 3 cages in a combination of 150: 50 (males: females) per cage. Laboratory conditions were well maintained (Temperature, 28 ± 2^{o} C, Relative Humidity (RH) 75 ± 5^{0} and 12:12 dark: photo periods). The mosquitoes were continuously fed with 10% sucrose solution with 0.2% methylparapen socked in cotton wool. For the mosquitoes oviposition, a small filter paper wrapped in a conical shape was placed in a small beaker containing distilled water, and filter made moist. The purpose of the filter paper was to prevent eggs from sticking to the walls of the beaker. The sides of beakers were lined with black paper suitable for mosquito laying. The beaker was kept inside the cage overnight for the mosquitoes to lay eggs. The mosquitoes were fed with blood and were able to lay eggs 2 days after blood feed. Into tray with 300 ml distilled water filter paper containing eggs was placed and a pinch of brewer's yeast was added to the tray and eggs were allowed to hatch to larvae during the next days.

Larvae were carefully fed for their growth and development by adding a pinch of ground fish food meal (0.1 g for 1st and 2nd instar larvae) to each tray, and 0.3 g of brewer's yiest for 3rd instar larvae at 8:00 and 16:00 hrs each day to each tray (Damdangdee *et al*., (2013). They were also fed every day with two tablets of finely ground brewer's yeast, and ground fish meal and monitored for density and population. By the 8th day the larvae had fully developed into 3rd instar and they had to be tested quickly before day 10 when they were to be in their 4th instar.

The larval bioassays were conducted in accordance with WHO, (2013) and as also conducted by Srinivasan et al (2014), Uthayarasa *et al.* (2010), and Singh *et al.* (2014). The 25, 3rd instar larvae picked at random for use was in accordance with the previous workers WHO, (2013); Govindarajan and Karuppannan, (2011); Abdullahi and Singh (2014) Bossou *et al.* (2013); Jayakumar *et al.* (2014); Borah *et al*. (2010); and Sankaran *et al*. (2012). Six 500 ml capacity beakers were prepared and concentrations of 50, 100, 150, 200, 250 and 300 ppm were placed into the beakers , each concentration in separate beaker. Into these concentrations 200ml of distilled water was added. These beakers then represented six different concentrations for one solvent eg. ethanol solvent. Other concentrations were made using methanol, DCM, hexane, ethyl acetate and aqueous and this completed the total 36 concentrations for *Cc*. Similarly, other 36 concentrations were made for each of the other two plants, *Ec* and *N*t. In all 108 concentrations were prepared for the three plants. Control experiment was prepared and consisted of a mixture of acetone and DMSO (1:1v/v) while alternatively 200 ml of dechlorinated water with 2 ml of acetone and another set of beakers with dechlorinated water only served as a complementary control. For each concentration three replicates were run at the same time.

By use of a mouth aspirator, a batch of 25 3rd instar larvae were collected at random and immersed into the beaker of the concentration as done by previous workers (WHO, (2013); Govindarajan and Karuppannan, (2011); Abdullahi and Singh (2014) Bossou *et al.* (2013); Jayakumar *et al.* (2014); Borah *et al*. (2010); and Sankaran *et al*. (2012).Each experiment set-up was maintained at optimum conditions (temperature 28±2^{0}C, Relative Humidity 75±5%, and a photo period of 12:12h (light : dark). Since a dehumidifier was not available wet towels were hang on three sides of cages to control humidity (Wijegunawardana, 2015). The larvae were fed with dry baker's yeast powder (50 mg/L) on the water surface throughout the experiment periods. The larvae were exposed to different concentrations of the oils and mortality was observed in 24h post-treatment.

Mortality of larvae was monitored by tapping side of the beaker ; prodding larvae with a wooden applicator; stimulating with a Pasteur pipette; prodding with a needle at their cervical region. Dead larvae did not show wiggling movement, move away, respond, and did not rise to the surface. Moribund larvae were identified , counted and added to the dead ones for the calculation of the percentage mortality. Mortality data was subjected to Probit regression analyses (Finney 1971; Norusis, 2008) to determine lethal concentrations 50% (LC_{50}) and 90% (LC_{90}) and other statistics at 95% fiducial limits of upper confidence limits (UCL) ad lower confidence limit (LCL), and chi-square lethal concentration. values were calculated using the SPSS 18.0 (Statistical Package of Social Sciences) software. The percentage mortality was calculated using Dawider (2013) formula.

3. Experimental Procedures

3.1 Effects of individual crude leaf extracts.

Three experiments were performed under individual crude leaf extracts activities to ascertain their effect on the larvae.

1) Experiment 1: The effect of *Cc* flower extract on the third instar larvae of *Anopheles gambiae*
2) Experiment 2: The effect of *Ec* leaf extract on the third instar larvae of *Anopheles gambiae*
3) Experiment 3: The effect of *Nt* leaf extract on the third instar of *Anopheles gambiae*

In the above experiments each extract was tested individually to 25 3rd instar larvae and exposed for 24 hrs to determine the effect of each extract to the larvae.

The results obtained from experiments 1,2 and 3 above were used to test for the synergistic and antagonistic activities of the combined extracts in the ratio of 1:1 (100 ppm: 100 ppm) against the larvae as shown below:

Synergistic experiments

Experiment (i): The effect of *Cc* flower extract in combination with *Ec* leaf extract on third instar larvae of *Anopheles gambiae*

Experiment (ii): The effect of *Cc* flower extract in combination with *Nt* leaf extract on the third instar larvae of *Anopheles gambiae*

Experiment (iii): The effect of *Ec* leaf extract, in combination with *Nt* leaf extract on the third instar larvae of *Anopheles gambiae*

Antagonistic experiments

These were carried out as in synergistic experiments (i), (ii) and (ii) above.

4. Results

The results for individual flower and leaf extracts activities recorded in Table 1.

Table 1: Individual flower and leaf extracts activities

Name of plant	*Extract solvent*	*LC_{50} (ppm)*	*Fiducial limits*		*Regression equation*	*Chi-square value (x2)*
			Upper	Lower		
C. cinerariifolium	13Ethanol	187.78	179.78	196.53	0.7470+1.7758x	4.5217
	Methanol	222.45	209.85	238.71	0.4684+1.9089x	10.6452
	DCM	164.86	161.57	176.28	0.7336+1.7893x	14.2584
	Hexane	230.66	214.79	252.67	0.5566+1.9098x	19.5759
	Ethylacetate	227.56	219.77	269.96	0.4899+1.9078x	19.5759
	Aqueous	247.84	233.37	267.72	0.6758+1.2123x	18.6202
E. camaldulensis	Ethanol	210.15	193.88	232.07	0.7686+1.9694x	4.6621
	Methanol	197.46	189.61	208.69	0.4868+1.9227x	13.256
	DCM	168.65	152.44	176.95	0.6975+1.9567x	10.4532
	Hexane	198.56	181.66	220.45	0.7086+1.8685x	9.5033
	Ethylacetate	260.56	240.77	289.96	0.05886+1.7825x	14.0773
	Aqueous	259.58	239.87	288.87	0.05977+1.8365x	6.7556
N. tabaccum	Ethanol	189.58	181.50	298.42	0.7376+1.8898x	3.5463
	Methanol	224.35	211.73	240.86	0.4988+1.8985x	3.8642
	DCM	229.72	216.80	246.63	0.5878+1.9874x	15.5740
	Hexane	235.85	221.25	240.53	0.5663+1.9096x	4.6542
	Ethylacetate	201.52	191.00	213.84	0.6455+1.8990x	14.0773
	Aqueous	258.42	238.79	287.58	0.0608+2.1015x	6.4444

Key: A – Antagonistic; SA- neither Synergistic nor Antagonistic

The results for synergistic and antagonistic activities recorded in Table 2

Table 2: The effect of overall crude leaf extract combination of the plants *C. cinerariifolium* (pyrethrum), *Eucalyptus camaldulensis (Eucalyptus)* and *Nicotiana tabaccum* (Tobacco) in the ratio 1:1 (100 ppm : 100 ppm) for comparison of synergistic and antagonistic activities derived from 30 treatments.

Plant	Ratio	Combination solvent extracts	Individual LC_{50}ppm		Combination LC_{50}ppm	SF		effect
Cc + *Ec*	1:1(100:100ppm)	Ethanol +Aqueous Methanol	187.78	210.15	152.85	1.2285	1.3749	S
		+Ethyl acetate	224.45	260.56	189.54	1.736	1.3747	S
		DCM + Hexane	164.86	168.65	127.85	1.2894	1.3191	S
Cc+ *Nt*	1:1(100:100ppm)	Ethanol +Aqueous Methanol	187.78	189.58	146.64	1.2855	1.2928	S
		+Ethyl acetate	224.45	224.35	232.66	0.9561	0.9643	A
		DCM + Hexane	164.86	229.72	130.78	1.2606	1.7565	S
Ec. + *Nt*	1:1(100:100ppm)	Ethanol +Aqueous Methanol	210.15	258.42	242.80	0.8655	1.0643	SA
		+Ethyl acetate	197.46	201.52	219.45	0.8998	0.9182	A
		DCM + Hexane	168.65	235.85	135.55	1.2442	1.7399	S
Pyr. + *Ec*	1:1(100:100ppm)	Methanol + Aqueous	224.45	259.58	201.55	1.1136	1.2879	S
		DCM + Aqueous	164.86	259.58	151.75	1.0864	1.7106	S
		DCM +Methanol	164.86	197.46	118.65	1.3895	1.6642	S
		DCM +Ethyl acetate	164.86	260.56	121.45	1.3574	2.1454	S
		Methanol +Ethyl acetate	224.45	260.56	230.50	0.9651	1.1304	SA

		Methanol + Hexane	197.46	198.56	147.46	1.3391	1.3465	S
		Ethylacetate+ Hexane	227.56	198.56	236.65	0.9616	0.8390	A
Cc + *Nt*	1:1(100:100ppm)	DCM +Methanol	164.86	224.35	225.80	0.7301	0.9936	A
		DCM +Ethyl acetate	164.86	201.52	160.75	1.0256	1.2536	S
		Methanol + Aqueous	224.45	259.58	263.15	0.8453	0.9864	A
		DCM + Aqueous	164.86	259.58	155.80	1.0582	2.6164	S
		Methanol +Ethyl acetate	224.45	201.52	160.75	1.0256	1.2536	S
		Methanol + Hexane	224.45	235.85	231.65	0.9360	0.9924	A
		Ethylacetate+ Hexane	227.56	235.85	241.45	0.9425	0.9768	A
Ec + *Nt*	1:1(100:100ppm)	DCM +Methanol	164.65	224.35	149.35	1.1292	1.5022	S
		DCM +Ethyl acetate	164.65	201.52	211.55	0.7972	0.9526	A
		DCM + Aqueous	164.65	259.58	158.43	1.0406	1.6385	S
		Methanol + Aqueous	222.45	259.58	264.65	0.8483	.9808	A
		Methanol + Hexane	197.46	235.85	220.75	0.8945	1.0684	SA
		Ethylacetate+ Hexane	260.56	235.85	262.60	0.9922	0.8981	A

Key: S- Synergistic; A – Antagonistic; SA- neither Synergistic nor Antagonistic
The results of synergism and antagonism explained in Tables 3 and below.

Table 3: Synergistic activities explained

Synergistic flower and leaf extract	Improved concentration
Ethanol of *Cc*	Improved from 187.78 ppm to 152.85 ppm by aqueous of *Ec*
Methanol of *Cc*	Improved from 224.45 ppm to 189.54 ppm by ethyl acetate of *Ec*
DCM of *Cc*	Improved from 164.86 ppm to 127.85 ppm by hexane of *Ec*
Ethanol of *Cc*	Improved from 187.78 ppm to 146.64 ppm by aqueous of *Nt*
Methanol of Cc	Improved from 224.45 ppm to 201.55 ppm by aqueous of Ec
DCM of *Cc*	Improved from 164.86 ppm to 151.75 ppm by aqueous of *Ec*
DCM of Cc	Improved from 164.86 ppm to 118.65 ppm by methanol of *Ec*
DCM of *Cc*	Improved from 164.86 ppm to 121.45 ppm by ethyl acetate of *Ec*
Methanol of *Cc*	Improved from 197.46 ppm to 147.46 ppm by hexane of *Ec*
DCM of *Cc*	Improved from 164.86 ppm to 155.80 ppm by aqueous of *Nt*
DCM of *Ec*	Improved from 164.65 ppm to 149.35 ppm by methanol of *Nt*
DCM of *Ec*	Improved from 164.65 ppm to 158.43 ppm by aqueous of *Nt*
DCM of *Cc*	Improved from 164.86 ppm to 127.85 ppm by hexane of *Ec*
DCM of *Ec*	Improved from 164.65 ppm to 135.55 ppm by hexane of *Nt*
DCM of *Cc*	Improved from 164.86 ppm to 160.75 ppm by ethyl acetate of *Nt*
Methanol of *Cc*	Improved from 224.45 ppm to 160.75 ppm by ethyl acetate of *Nt*

Table 4: Antagonistic activities explained

Antagonistic flower and leaf extracts	Improved concentration
Methanol of *Cc* and ethyl acetate of *Nt*	Both methanol of *Cc* and ethyl acetate of *Nt* were reduced from 224.45 ppm and 201.52 ppm respectively to 232 ppm. Hence double antagonism i.e. were antagonistic to each other.
Ethyl acetate of *Nt* Hexane of Ec	Reduced from 201.52 ppm to 219.45 ppm by methanol of *Cc* *Reduced from 198.56 ppm to 236.65 by methanol of Cc*
DCM of *Cc* and Methanol of *Nt*	They were antagonistic to each other reduced from 164.86 ppm and 224.35 ppm respectively to 225.80 ppm
Hexane of *Ec*	Reduced from 198.56 ppm to 236.65 ppm by ethyl acetate of *Cc*
DCM of *Ec*	Reduced from 164.86 ppm to 225.80 ppm by methanol of *Nt*
Methanol of *Cc*	Reduced from 224.45ppm to 263.15 ppm by aqueous of *Nt*
Methanol of *Cc*	Reduced from 224.45 ppm to 231.65 ppm by hexane of *Nt*
Ethyl acetate of *Cc*	Reduced from 227.56 ppm to 241.45 ppm by hexane of *Nt*
DCM of *Ec*	Reduced from 164.65 ppm to 211.55 ppm by ethyl acetate of *Nt*
Methanol of *Ec*	Reduced from 222.45 ppm to 264.65 ppm by aqueous of *Nt*
Hexane of *Nt*	Reduced from 260.56 ppm to 262 ppm by ethyl acetate of *Ec*.

5. Discussion

5.1 Synergistic activities of the crude extracts when they acted in combination.

From synergistic combinations, it was observed that crude flower and leaf extracts with the highest activity (Cc) were easily antagonized. Synergistic activities (those with F < 1), antagonistic activities (i.e those with SF > 1) and those activities neither synergistic nor antagonistic (> 1 <) were indicated in Tables 2, 3 and 4. The results identified 16 synergistic and 10 antagonistic of the combining extracts, giving a ratio of 16 : 10 (8 : 5) or (61.54% : 38.46%) respectively. Hence, it was observed that synergistic activities were more than antagonistic activities. They were more by a factor of 1.6 (2) hence outweighing the antagonistic activities and were the cause of the use of less concentrations and therefore economical. From tables 2 and 4 it was observed that DCM extracts of the three plants dominated in being synergized 10 times by other extracts compared to methanol 4 times and ethanol 2 times. This showed that DCM indicated flexibility to combine and being synergized by a range of extracts. Hexane, ethyl acetate and aqueous were not synergized although they were synergists themselves. In its flexibility to be synergized, DCM extracts became the most suitable extracts to be used in synergism. This was viewed from the fact that DCM extract of Cc was synergized by various extracts of other

plants (*Nt* hexane, *Nt* aqueous, *Ec* hexane, *Nt* ethyl acetate, and *Ec* methanol) 10 times against synergy of other plants extracts which were far bellow. *Nt* various extracts became preferred synergist as they became positively operational six times, a majority of them synergizing *Cc* DCM. Methanol of *Cc* and DCM of *Ec* were both synergized 4 times by *Nt* extracts and *Cc* ethanol 2 tmes by the same extracts. This showed the superiority of DCM in extract combination. Simply, then concluded that DCM extract was the best extract in synergistic activities because it accepted a range of extracts for synergism.

From the results obtained, four lessons were learnt:- (i) Two highest combining extracts e.g. *Cc* DCM (164.86 ppm) + *Ec* hexane (168.65 ppm) still yielded to the highest concentrations (127.85 ppm) for the mortality of the larvae. The resultant 127.85 ppm described as highest concentration was better than the two combining extracts; (ii) The synergistic result formed by two low concentrations e.g. *Cc* methanol (224.45 ppm) + *Ec* aqueous (259.58 ppm) still yielded to low concentrations (201.55 ppm), much closer to each of the two combining extracts. The resultant 201.55 ppm described as low concentration was just slightly better or no better than the concentrations of the two combining concentrations; (iii) When a high concentration was combined with a low concentration e.g. Cc DCM (164.86 ppm) + *Ec* ethyl acetate (260.56 ppm), a high concentration 121.45 ppm resulted for the mortality of the larvae. The resultant 121.45 ppm described as high was a creation of the low *Ec* ethyl acetate which improved on the high concentration of *Cc* DCM and (iv) Synergistic combinations yield to smaller concentrations and therefore this would be economical. Smaller concentrations when used appropriately will disintegrate faster than large doses which will tend to persist in the environment. In this view, persistence of chemical insecticides for example, will render the untargeted organisms likely to die.

Some other extracts were noted to antagonize other extracts marginally. For example, Methanol of *Cc c* achieved 98% mortality of LC_{50} by 224.45ppm. When it combined with ethyl acetate of *Nt* (224.35 ppm) it exhibited 96% larval mortality at 232.66 ppm. This translated that ethyl acetate at 224.35 ppm was higher than methanol at 224.45 ppm and the resultant of these two was 232.66ppm meaning that methanol of *Cc* lowered the effect of ethyl acetate of *Nt* by 3.7%, i.e. 232.66 – 224.35 = 8.31; 8.31 ÷ 224.35 x 100 = 3.7 %).

From the synergistic combinations it was observed that some extracts have compounds that render other extracts weaker in conversion and others have strong compounds not easily convertible. There will be need to investigate which compounds these are.

Documented evidence on synergism tests based on the plants of this study is limited. Despite this the very few documented are mainly outside the plants of study and may not form good comparison. This also applies to antagonism.

5.2 Antagonistic activities of the crude extracts when they acted in combination.

Antagonistic is the opposite of synergistic i.e. opposing, when it is combined. The best that will be expected of antagonism is reduced performance. In this study there were ten antagonistic activities. The highest to lowest crude leaf extract combination concentrations occurred within a difference of 0.10 ppm i.e.*Cc* (methanol extract -224.45 ppm) and *N*t *(* ethyl acetate extract – 224.35 ppm) and 59.70 ppm (*Ec* DCM extract – 164.65 ppm ; *Nt* ethyl acetate extract – 260.56 ppm).

The most important principle observed in antagonism was that a high concentration (indicated by less ppm) was converted to a weaker concentration (indicated by more ppm). The more the concentration applied the more it indicated the extract's weakness or more mosquito resistance to the extract. It was also observed that every antagonistic extract rendered the corresponding combining extract weaker such that the concentration could not achieve 100% larval mortality. From the combinations, it was noted that crude flower and leaf extracts with low (more ppm) concentrations were easily antagonized. However, for reasons not known, two extracts with high concentrations (shown by less concentrations) namely both DCM and methanol Ec. (164.65 ppm and 197.46 ppm, respectively) were lowered to 211.55 ppm and 219.45 ppm respectively. It was also observed that four of *Nt* extracts (aqueous , ethyl acetate, hexane, methanol), acted as antagonists in various combinations while two of *Ec* (ethyl acetate and hexane) and one extract of *Cc* (methanol) as well acted as antagonists. Extracts of *Nt* therefore dominated in rendering other plants extracts inactive with an exception of ethanol and DCM.

As the activities of the antagonized extracts continued to be lowered, larval mortality percent also reduced . Therefore a decrease in larval mortality became an indicator of antagonism. For example, individual extracts of *Ec* methanol and DCM had achieved 100% larval mortality but when ethyl acetate of *Nt* combined with these two extracts larval mortality declined to 91% and 89% respectively. Methanol of *Nt* reduced larval mortality of DCM *Cc* from 100% to 90%. Even in the lower larval mortality cases the individual extracts which attained average larval mortality were seen to tremendously running low in larvae mortality. This is in reference to extract hexane of *Ec* which antagonized ethyl acetate of *Ec* to have mortality decreased from 80% to 68% and ethyl acetate of *Ec* acting against hexane of *Nt* pushing down larval mortality from 84% to 60%.

Two important issues were observed in the antagonistic combinations: i) Crude leaf extract with low concentration in combination reduced the crude leaf extract with higher concentration. The resultant effect was that the concentration of the two combining extracts was lowered below the concentration of each of the combing extracts. This trend would complicate the efforts for the malaria vector control particularly when the accompanying antagonism in biopesticides is not well understood ; ii) Amongst the combinations four different solvents of *Nt* i.e. ethyl acetate, methanol, aqueous and hexane appeared commonly and

overwhelmed other solvents which simply implies that the resultant solvent extracts had a major role in antagonism.

Following this discussion, it was thus concluded that a majority of *Nt* extracts (9 *extracts)* were antagonists and it will be important to avoid the use of *Nt* extracts in combination with other extracts in mosquito control programmes in the field.

6. Conclusion

Synergists should be encouraged for use in mosquito control programmes as they would act efficiently and will render biopesticides cheap and affordable and importantly due to their less amounts in concentrations the bio-pesticides will disintegrate quickly as they are applied and pause no threat to non-target organisms. Compounds in synergists that synergize other extracts need further study.

Antagonists act in opposition of synergistic. Antagonistic exhibit toxification of a toxin, demodify an active compound rendering it nontoxic, hinders the penetration, transport or accessibility of a toxin to its target, and do not attack two independent steps in a process e.g stages in development or biosynthetic pathways. In this respect antagonists can render mosquito control programme difficult and unsuccessful. It is important one to have prior knowledge on antagonistic activities before any crude leaf extract combinations is attempted to be used in mosquito control strategies. However, there is need for further studies in antagonism to come out clearly which compound(s) in each crude leaf extract is antagonistic to other compound(s) of the combining extract. Antagonistic compounds can render mosquito control programmes expensive since there will be unanticipated purchasing and repeated use of insecticides thus incurring large financial expenditure and importantly a waste of time.

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