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Effect of Concentration on the Rate of Killing of Some Microorganisms and Haemolytic Activity of Two Varieties of *Acalypha wilkesiana*

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Abstract: The effect of concentration change on the rate of killing of some selected microorganisms by ethanolic extracts from two varieties of *Acalypha wilkesiana* was carried out using the plate count technique. The haemolytic activities by the agar diffusion method was investigated. The killing rate was found to increase as concentration increases. This was shown by reduction in the amount of survivors in cfu mL^{-1} as the exposure time progresses. The rate of killing of the microbial population by the extract was also found to be concentration dependent as increase in concentration lead to reduction in microbial loads. The relationship was established to be exponential one as revealed by the concentration queficent The two varieties of the *Acalypha wilkesiana* also differ in the ability to kill the different bacteria species and fungus (*Candida albicans*) with the macrophylla showing higher degree of killing than the Hoffmanin. Generally, the rate of killing was found to vary among the different bacteria species with gram negative ones like *Escherichia coli*, *Klebsiella* and *Pseudomonas* being killed at a lower rate than the gram positive organism like *Bacillus* and *Staphylococcus. Candida albicans* being a fungus was killed at extremely lower rate than the bacteria. The haemolytic activity was found to be higher in Macrophylla than Hoffmanin and the values increase as the concentration increases.

Key words: *Acalypha wilkesiana*, killing rate, haemolysis

Introduction

Ethno-pharmacology is the study of plant used in traditional medicine. Plants had a long history of uses in the treatment of diseases (Cragg and Neuman, 2005). More than 60% of currently used drugs in the treatment of diseases of microbial origin are derived in one way or the other from natural sources including plants, marine organisms and microorganisms (Nueman *et al*., 2002). Many of these traditional medicines are still included as part of the habitual treatment of various maladies; such as using *Vaccinium macrocarpon* to treat urinary tract infections.

The fact that traditional knowledge systems are largely oral and not written, accentuates the fragility of this type of indigenous knowledge. The changes in socio-political climate in last few years have resulted in increased awareness of use of herbs in therapy like *Acalypha wilkesiana* (Adesina *et al*., 2000).

Acalypha wilkesiana whose common names include copper leaf, beaf steak or Jacob's coat belong to the family Euphorbiaceae. The plant has been found to possess antimicrobial activity due to the presence of gallic acid, corilagin and geranin as the bioactive components (Adesina *et al*., 2002). However there have not been reports on the evaluation of the effects of concentration on the rate of killing of microorganisms by the extract of the two most popular varieties of the *Acalypha wilkesiana*; Macrophylla and Hoffmanni whose leaves are reddish brown and bright green respectively (Fauan, 2005). The current research focus on effects of concentration on antimicrobial efficacy of the two varieties as well as the haemolytic activities of the ethanolic extracts from the two *Acalypha wilkesiana*.

Materials and Methods

Plant Sample, Extraction and Purification

Plant sample, extraction and fractionating, The two variety *Alcalypha wilkesiana* L. (Euphobiaceae) leaves were obtained from Orchid of the Federal University of Technology, Akure Nigeria. Leaves dried at 40°C were pulverized and extracted with 70% ethanol. The extract was then concentrated in a vacuum using rotary evaporator.

Exactly 5 g of the crude extract was adsorbed on silical gel of 60-120 mesh (BDH) and chromatographed on a column of silical gel-60 slurry packed in petroleum ether. The column was gradient eluted with petroleum ether and then with ethyl acetate: ethanol 40:1 and finally with 100% ethanol.

A 100 mL of the fraction was collected and analyzed by thin layer chromatography (TLC) on a pre-coated plated Merck, silica gel 60 254, 0.2 mm thickness. The fractions collected were numbered fraction showing the same TLC characteristics were bulked together. This was also confirmed by measuring their absorbance with the aid of spectrophotometer. Visualization of the spots on plates were by observing under ultra-violet light and by spraying separately with vanillin sulphuric acid reagent followed by heating at 100°C for 5 min.

Rate of Killing

The method of Khan *et al*. (2006) was used to determine the rate of killing of the microorganisms by the active fractions. The number of the organisms to be used was first determined. A 0.5 mL/volume of known concentration by viable count from each 18 h old culture suspension was added to 4.5 mL of the test fractions such that the final concentration gave 25, 50 and 100% w/v. The suspension was thoroughly mixed and held at room temperature (28-30°C) and the killing rate affect 1, 2, 3, 4 and 5 h interaction was determined. Exactly 0.5 mL volume of each suspension was withdrawn at the appropriate time intervals and transferred into 4.5 mL of nutrient broth (oxoid) recovery medium containing 3% tween 80 to neutralize the effect of any antimicrobial component carry over from the suspension. The mixture was shaken properly and diluted serially up to 10-fold in sterile distilled water and exactly 0.5 mL of the final dilution was transferred into pre sterilized nutrient agar at 45%. The plates were allowed to set and incubated upside down at 37°C for 72 h. Control well was set up.

Haemolytic Assay

The haemolytic activity of the extract was determined using agar diffusion technique on blood agar plate (Ahmed *et al*., 2006). Blood agar was prepared and well measuring 5.00 mm were made on the agar using cork borer. The wells were filled with 0.5 mL of 10% w/v of the extracts solutions. The plates were then incubated at 37°C for 4 h. Clear zones of haemolysis indicated positive results.

Results and Discussion

The rate of killing of the organisms by the extracts of the varieties *Acalypha wilkesiana* (Macrophylla and Hoffmanin) was found to be concentration dependent as well as the contact time (Table 1-8). The rate was found to increase as the concentration increase and vice versa. Tadeg *et al*. (2005) why working on the antimicrobial activities of some Ethiopian medicinal plants reported that the potency increase as the concentration increases. The reason for this may be that at higher concentration, the extracts was able to induce higher cellular damage to the organisms by inactivation of metabolic enzymes, disrupting the cell wall, leakage of nuclear materials like proteins, nucleic acid, sodium and potassium ions (Totora *et al*., 2002). The relationship between concentration and antimicrobial activity has been shown to be an exponential one.

Table 1: Rate of killing of *Bacillus cereus* by ethanolic extracts from two varieties of *Acalypha wilkesiana* at 20, 50 and 100 mg mL^{-1}

	Macrophylla			Hoffmannin		
Time (h)	25	50	100	25	50	100
0	700	680	710	650	720	600
1	430	200	140	520	314	100
2	180	60	10	250	178	46
3	55	11	0	106	63	08
4	60	8	0	49	14	0
5	42	5	0	24	06	0

Table 2: Rate of killing of *Klebsiella Pneumonae* by ethanolic extracts from two varieties of *Acalypha wilkesiana* at 20, 50 and 100 mg mL^{-1}

	Macrophylla			Hoffmannin		
Time (h)	25	50	100	25	50	100
0	690	710	645	720	714	760
1	300	120	85	450	270	130
2	163	70	18	298	111	60
3	97	10	0	200	115	71
4	82	4	0	165	95	48
5	54	0	0	108	74	32

Table 3: Rate of killing of *Escherhia coli* by ethanolic extracts from two varieties of *Acalypha wilkesiana* at 20, 50 and 100 mg mL^{-1}

	Macrophylla			Hoffmannin		
Time (h)	25	50	100	25	50	100
0	705	638	692	670	630	642
1	610	524	414	526	600	310
2	518	295	148	301	214	106
3	312	91	44	316	26	86
4	180	20	36	198	14	60
5	39	46	40	64	10	11

Table 4: Rate of killing of *Staphylococcus epidemidis* by ethanolic extracts from two varieties of *Acalypha wilkesiana* at 20, 50 and 100 mg mL^{-1}

	Macrophylla			Hoffmannin		
Time (h)	25	50	100	25	50	100
0	658	706	690	720	714	688
1	210	100	75	445	190	200
2	70	80	0	300	100	38
3	66	0	0	90	41	0
4	60	0	0	60	0	0
5	60	0	0	24	0	0

Table 5: Rate of killing of *Bacillus cereus* as by ethanolic extracts from two varieties of *Acalypha wilkesiana* 20,50 and 100 mg mL^{-1}

	Macrophylla			Hoffmannin		
Time (h)	25	50	100	25	50	100
0	645	670	607	701	730	690
1	376	134	75	408	247	134
2	214	59	41	377	215	88
3	199	27	0	125	92	14
4	64	0	0	74	85	0
5	71	0	0	61	34	0

The contact time was also found to have effect on the rate of killing; the longer the exposure time, the higher the degree of killing. This may result from increase in the amount of the extract that interacted with the organelles and inclusion bodies in the organism which led to the damage of the latter and consequent death of the entire cell of the organisms.

Table 6: Rate of killing of *Clostridium sporogenes* as by ethanolic extracts from two varieties of *Acalypha wilkesiana* 20, 50 and 100 mg mL^{-1}

	Macrophylla			Hoffmannin		
Time (h)	25	50	100	25	50	100
0	650	635	690	628	645	670
1	600	250	400	610	42	215
2	610	230	130	420	410	158
3	540	300	175	330	175	19
4	480	214	67	310	94	06
5	100	120	19	180	76	06

Table 7: Rate of killing of *Staphylococus aureus* as by ethanolic extracts from two varieties of *Acalypha wilkesiana* 20, 50 and 100 mg mL^{-1}

	Macrophylla			Hoffmannin		
Time (h)	25	50	100	25	50	100
0	713	656	704	703	731	659
1	660	430	241	545	316	194
2	180	74	68	215	117	97
3	56	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0

Table 8: Rate of killing of *Pseudomona saeruginosa* as by ethanolic extracts from two varieties of *Acalypha wilkesiana* 20, 50 and 100 mg mL^{-1}

	Macrophylla			Hoffmannin		
Time (h)	25	50	100	25	50	100
0	665	714	634	625	670	600
1	414	172	95	377	145	99
2	386	131	16	122	54	71
3	110	86	0	81	61	70
4	38	14	0	77	64	72
5	04	0	0	54	70	75

Table 9: Rate of killing of *Candida albicans* by ethanolic extracts from two varieties of *Acalypha wilkesiana* 20, 50 and 100 mg mL^{-1}

	Macrophylla			Hoffmannin		
Time (h)	25	50	100	25	50	100
0	700	730	645	635	701	600
1	540	600	320	600	400	355
2	510	600	315	590	380	355
3	480	580	300	500	400	370
4	490	410	298	500	420	210
5	500	450	300	510	310	218

Table 10: Zones of Haemolysis (mm) of ethanolic extracts from two varieties of *Acalypha wilkesiana* at different conclusions

	Concentration (mg mL^{-1})		
Variety	25	50	100
Macrophylla	5.00	7.5	12.5
Hoffmanin	3.5	6.5	11.5

The two *Acalypha wilkesiana* varieties differ in the ability to kill organism at a given concentration and exposure time. The Macrophylla capability for killing was found to be higher than Hoffamanin. This might be due to presence of different bioactive molecules of pharmacological importances in the two varieties with varied potency to induce killing of microbial population.

Generally, the rate of killing of the Gram negative organisms like *Escherichia coli*, *Klebsiella* and *P. aeruginosa* was found to be lower than for Gram positive like *Bacillus* and *Staphylococcus*; which

possess an outer peptidoglycan layer which is not an effective permeability barrier. The reason for this may lie in the complex natures of the cell wall of Gram negative organism over the Gram positive. Gram negative bacteria are frequently reported to have developed multi drug resistance (Sadder *et al*., 2002). Another factor that may be responsible for the observed trend in the rate of killing among the different bacteria may be due to variation in the genetic composition of the organisms tested. Susceptibility and resistance of bacteria to a given antimicrobial agent are largely dependent on the nature, structure and complexity of the cell wall and genetic constitution of the organisms which may be chromosomal or plasmid coded (Madigan *et al*., 2002).

The fungus *Candidda albicans* was shown to be killed at a lower rate then the bacteria (Table 9). The effect of the extract on the fungus was found to be fungistatic rather that the bactericidal activity on some of the bacteria like *Staphylococcus aureus*. The eukaryotic nature of *Candida* as well as it ability to secrete extra-cellular enzyme that can degrade the medium of suspension may be responsible for this observation.

In this study, the extracts from the two varieties possess haemolytic activities (Table 10). The zones of haemolysis was directly proportional to the concentration of the extracts used. Macrophylla was also shown to possess higher haemolytic activity than Hoffmannin. The ability of the extract to lyse the blood cell can be linked with the antimicrobial factors like Saponin, Tanin, Anllinquinine which has been shown to be widely distributed in *Acalypha wilkesiana* (Adesina *et al*., 2000).

Conclusions

The ethanolic extract was found to kill the pathogenic organisms at different rate and was found to be concentration and exposure time dependent. Both Macrophylla and Hoffmanin also possess haemolytic activities and can thus be used in treatment of infectious disease that is cause by intracellular parasite in blood. However, when such extract are used in phyto-medicine there is a need for the patient to be taken along with it blood builders and vitamin supplements.

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