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ANTIMICROBIAL ACTIVITIES OF CONSTITUENTS FROM *ISOLONA CAULIFLORA* VERDC AND *CLEISTOCHLAMYS KRIKII* BENTH (OLIV.) (ANNONACEAE)

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Abstract: Antimicrobial activities of crude extract, Caulindole D, a mixture of Caulindole E and F, Pinocembrin and an Oxyheptanoid (Clestochlamic acid) from stem bark of *Isolona cauliflora* and *Cleistochlamys krikii* on *Pseudomonas phaseolicola, Fusarium solani, Botryodiploida theobromae, Aspergillus niger* and *Aspergillus flavus* have been investigated. An in vitro bioassay test showed that the crude dichloro-methane extract from *C. krikii* and a very strong antimicrobial property.

The pure compound had strong to moderate inhibitory effect on *Pseudomonas syringae* pv. *phaseolicola* and *Botryodiplodia theobromae*.

The pure compounds from *Cleistochlamus krikii* had more pronounced inhibitory activities than the pure compounds from *Isola cauliflora*.

At lower concentration of 100-200 ppm, the crude extract of *Caulindole*, mixture of *Caulindole* E and F, *Pinocembrion* and *Oxyheptanoid* had effect on most of the investigated plant pathogens.

Higher concentration of 500-1000 ppm had moderate to weak effect on the *Aspergillus* spp.

I n t r o d u c t i o n

The increasing trend in environmental awareness has prompted efforts to search for environmentally and toxicologically safe and efficacious crop and animal protection agents (pesticides). Likewise, the increasing incidence of pests

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evolving pesticide resistance has contributed to enhance efforts to develop alternative for pests control. In this respect, natural product is considered to be potential sources for development of biodegradable pesticides. Plants are known to produce a veriety of secondary metabolites, which are bioactive and thus may have inhibitory effects on bacteria, fungi, insects and other microorganisms. (Owolade, et al, 2002) reported the control of brown blotch of cowpea with extracts from the leaves of *Carica papaya, Tithonia diversifolia* and *Acalypha ciliata*, as potential biofungicide against *Collectotrichum capsii*. The plant extracts reduced disease incidence and severity with performances comparable to those of benlate. Similar result was obtained by Tangian et al. 2002 with aquareous extracts of *Lantana camara, C. papaya, Zingiber officinale, Allium sativum* and *Azadirachta indica* on *Cercospora sojina*, Hara in vitro. This paper reports on the antimicrobial activities of crude extracts, alkaloids, flavonoids and other compounds isolated from Annonaceous plants on plant pathogenic organisms.

Materials and Methods

Test Microorganisms

Pure culture of *Pseudomonas syringae* pv. *phaseolicola* isolated from bean leaf was obtained from Agricultural Research Institute Uyole, Mbeya region. The culture was sub-cultured several times on nutrient agar and sabrose agar and kept on agar slant and 30^{o}C until use. The fungi were Isolated from rotted carrot roots, pawpaw, plantain banana and tomato brought from markets around the University of Dares Salaam. The fruits were washed with tap water and surface sterilized with 70% alcohol for 60s and washed with sterile distilled water several times, blotted dry with filter paper. The infected parts were sliced into small cubes and plated on potato dextrose agar (PDA) in 9cm Petri-dishes. The plates were incubated at 28^{o}C for three days, subculturing was done until pure cultures of the isolate were obtained and maintained on PDA slants in Macartney bottles. The Aspergili were identified with reference to Raper and Fennel (1965), and the rest with reference to Barnett and Hunter (1973), Nelson et al. (1983).

Plant crude extract and pure compounds

The stem bark of *I. cauliflora* was collected from Namikwe island near Kiwanda village in the zigi valley, Muheza district in Tanzania. The plant material was pulverized and extracted by soaking in consecutively (2x48h) in pet ether, dichloromethane, and ethanol at room temperature (ca 30^{o}C). The concentrated extracts obtained after evaporation of the solvents were fractionated

by vacuum liquid chromatography (VLC) and the fractions were separated further by column chromatography on silica gel or Sephadex LH20. Repeated column chromatography of VLC fraction, six of the dichloromethane extracts, yielded caulindole D as well as a mixture of caulindole E and F as yellow gums.

The stem bark of *C. krikii* was air dried, pulverized and extracted by soaking consecutively (2x48h) in petroleum ether, dichloromethane and enthanol. The concentrated extracts were fractionated by vacuum liquid crhomatography (VLC) eluting with pet ether containing increasing amounts of ethyl acetate. The VLC fractions were further separated by column chromatography in silica gel, followed by purification either by recrystallization or by gel filtration on Sephadex LH20. Repeated column crhomatography of VLC fraction, five of the dichloromethane extract, yielded pinocenmbrin as with needle like crystals with MP of 182-184^{o}C. This compound was also obtained from VLC fraction, six of the ethanol extract. The oxyheptanoid (cleistochlamic acid) was obtained from re-crystalization in methanol of VLC fraction, eight of the pet ether extract VLC, six of the dichloro methane extract also yielded this compound.

Anti-microbial assay (in vitro)

For the bacterial pathogen the anti-microbial assay was determined in vitro by paper disc method samples of plant crude extract and isolated pure compounds were dissolved in DMSO and appropriate calculations were carried out to give concentrations ranging from 100-1000 ppm.

Paper Disc Method

This was according to P1att (1986). Discs of 7 mm diameter were made from Whatman No1 filter papers. Disc was soaked into different concentrations of the dissolved samples of pure compounds and extracts for 5 minutes, and another disc was soaked in DMSO as control and different concentrations of streptomycin as standard antibiotic. The treated paper discs were left to dry on a sterile plates in the laminar flow for 5 mins before transferring them into separate pair of inoculated plates, and incubated at 30^{o}C for 48 hrs. Means and standard deviation (+SD) of the diameter of zones growth inhibition around the disc for 5 treatments were determined.

Effect of the isolated compounds on the radial growth of the fungal pathogens

Potato dextrose agar was used. The medium was prepared by dissolving 39.5 g of Oxoid PDA powder in 1 liter of distilled water, boiled and sterilized at 1.2 bar and 121^{o}C in an autoclave for 15 mins. Aseptically, 1 ml of each test compound solution at different concentration was pipetted into sterilized Petri

dish and overlaid with 10 ml of molten potato dextrose agar, and mixed thoroughly. After the agar solidified a 3 mm mycelia plug of 7 day old cultures of the fungal pathogen was placed in an inverted position at the center of the 9 cm Petri dish for the fungus to be in direct contact with the test compound. The plate was incubated at 28 ± 2^{o}C in an incubator for 3 to 5 days. After the incubation period, the diameter of growth of the pathogen was measured, the mean of the measurements for three replicates were recorded as the growth of the fungus for each concentration. Sterile distilled water and different concentrations of copper sulphate were used both as control and standard fungicide in the determination of radial growth of the fungal pathogen. Percentage inhibition on the diameter of growth was calculated as follows

$$\% \text{ growth inhibition} = (D_o - D_t) \times 100;$$

where: D_o= diameter of growth in the control
D_t = diameter of growth in the treatment

Effect of isolated compounds on mycelia growth of the fungal pathogens in liquid medium

The mycelia growth measurement was determined using a liquid medium, which consisted of the following composition; $NaNO_3$, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4$, 0.5 g; $FeCl_3$, 0,02 g; and dextrose 20 g, dissolved in 1000 ml of sterile distilled water in a conical flask. The growth medium was dispensed in 18 ml aliquots into 100 ml conical flask and covered with aluminium foil. These were sterilized by autoclaving at 121^{o}C for 15 min at 1.2 bar. After allowing the medium to cool down to room temperature, 2 ml of each test sample of the isolated compound at different concentration were aseptically pipetted into the each flask. A 5 mm mycelia disc of 5 day old fungal isolate was cut by sterilized 5 mm-cork borer and inoculated into each flask. The flasks were incubated at 30^{o}C for 7 days. On the 7th day the mycelia mats of the fungal isolate were harvested by filtering on a pre-weighed filter paper. Both filter paper and mycelia mat were then oven dried at 80^{o}C for 24 h. After this the filter paper with the mycelia mat was allowed to cool in a decisscator. The filter paper was re-weighed. The weight of the mycelia was determined by subtracting the weight of the filter paper from the total weight of filter paper plus mycelia. The control is obtained by substituting the isolated compounds with 2 ml of sterile distilled water and benomyl for the standard fungicide. There were 3 replicates for each sample, percentage growth inhibition was calculated as follows.

$$\% \text{ growth inhibition} = (W_o - W_t) \times 100;$$

where: W_o= weight of mycelia in the control
W_t = weight of mycelia in the treatment

The data generated from these studies were subjected to the Analysis of variance (ANOVA) and tests of significance were determined by Duncan's multiple range test.

Results

The paper disc method results for caulindole D and mixture of caulindole E and F from *I. cauliflora* showe low diameter of inhibition zones for *P. phaseolicola* at 300, 500 and 1000 ppm, but no inhibition zone at lower concentration (Table 1). The inhibition of Cleistochamic acid to *P. phaseolicola* was also moderate at 150 ppm and strong at 200, 250 and 300 ppm, but very strong at 500 and 1000 ppm (Table 1). Streptomycin at 1/μg per ml had a pronounced inhibitory effect on *P. phaseolicola* than any of the pure compounds of *I. cauliflora* and *C. krikii* at 1000 ppm (Table 1).

T a b. 1. – In vitro growth inhibition zones of *Pseudomonas syringae pv, phaseolicola*, in crude extract and isolated pure compounds obtained from *Isolona cauliflora* nad *Cleistochlamys kirkii*

Compound	Concentration ppm					
	150	200	250	300	500	1000
	Diametar of inhibition zones (mm)					
Crude extract	12.5[a]					
Streptomycin	17.5[b]					
Caulindole D	0	0	7.0±0.2[a]	8.6±0.2[a]	9,0[a]	10.5[a]
Caulindole E & F	0	0	8.5±0.4[a]	8.0[a]	9.0[a]	11.8±0.6[a]
Cleistochlamic acid	6.2.04[c]	8.0[a]	11.2±1.0[b]	12.0[b]	13.0[b]	15.0[b]
Pinocembrin	8.0±0.1[c]	5.3±0.1[a]	9.2±0.3[b]	9.6[b]	10.0[a]	11.0[a]
Control	0	0	0	0	0	0

Data are presented as mean for three replicates. Values followed by the same letters are not significantly different according to Duncan's multiple range test (P=0.05).

For the fungal pathogens, caulindole D and the mixture of caulindole E and F had weak to moderate inhibitory effect on the rot pathogens as shown by the % inhibition of the radial growth on agar and mycelia dry weight in liquid medium (Tables 2 and 3). The flavonoid (pinocembrin) and the oxyheptanoid (Cleistochlamic acid) inhibitory activities, in both radial growth on agar and mycelia dry weight, were higher than the results obtained for the compounds from *I. cauliflora*, showing moderate to strong inhibition (Tables 2 and 3). The minimum inhibition concentration for all the tested fungi, except *Fusarium solani*, was 250 ppm (Tables 2 and 3). The percent inhibition of benomyl at 0.5 ug/l was about 1 to 3 higher than the highest concentration of the pure compounds from the plants (Table 3). The results also showed that *B. theobromae* was more sensitive to the compounds than the other fungi pathogens.

T a b. 2. – Effect of compounds isolated from *I. Cauliflora* and *C Krikii* on radial growth of rot fungi (first repetition)

Compound	Concentration (ppm)	Inhibition of the diameter of growth of fungi (%)			
		Fusarium solani	*Botryodiplodia theobromae*	*Aspergillus niger*	*Aspergillus flavus*
Control	0.0	0	0	0	0
Benomyl	0.5	100	100[d]	100[d]	100[d]
Caulindole D	250	0	5.1[a]	0	0
	500	0	11.1[a]	10.0[a]	15.0
	1000	0	15.5[a]	15.5[a]	20.0
Caulindole E and F	0.0	0	0	0	0
	250	0	10.5[a]	5.0	0.0
	500	0	11.1[a]	16.5[a]	18.0
	1000	0	16.0[a]	18.8[a]	27.7
Pinocembrin	0.0	0	0	0	0
	250	0	16.6[a]	10.0	10.0[a]
	500	0	20.0[a]	18.8[a]	22.5[b]
	1000	0	30.0[b]	25.0[b]	30.3[c]
Cleistochlamic acid	0.0	0	0	0	0
	250	0	18.8[a]	15.6[a]	10.0
	500	0	25.2[b]	25.0[b]	22.5[b]
	1000	0	44.4[c]	30.3[c]	35.7[c]

% growth inhibition = (D_o-D_T) x 100; Where D_o= Diameter of Growth of Control and D_T= Diameter of Growth of Treatment.

D_o

Data are presented as mean for three duplicates. Values followed by the same letters are not significantly different according to Duncan's multiple range test (P=0.05).

T a b. 3. – Effect of compounds isolated from *I. Cauliflora* and *C Krikii* on mycelia growth of rot fungi (second repetition)

Compound	Concentration (ppm)	Inhibition of the mucelia growth of fungi (%)			
		Fusarium solani	*Botryodiplodia theobromae*	*Aspergillus niger*	*Aspergillus flavus*
Control	0.0	0	0	0	0
Benomyl	0.5	100	100[a]	100[a]	100[a]
Caulindole D	250	0	10.0[b]	5.0[b]	10[b]
	500	0	15.0[b]	13.5[c]	20.4[c]
	1000	0	25.0[c]	20.0[d]	28.0[c]
Caulindole E and F	0.0	0	0	0	0
	250	0	15.3[b]	15.0[c]	10.0[b]
	500	0	25.0[c]	24.0[d]	22.16[c]
	1000	0	30.0[d]	32.0[d]	30.1[c]
Pinocembrin	0.0	0	0	0	0
	250	0	18.6[b]	10[c]	12.5[b]
	500	0	26.5[c]	30.0[d]	24.1[c]
	1000	0	35.2[d]	38.1[d]	42.4[d]
Cleistochlamic acid	0.0	0	0	0	0
	250	0	26.5[c]	26.0[a]	10.5[b]
	500	0	37.6[d]	35.2[d]	28.5[c]
	1000	0	48.5[d]	45.3[d]	46.1[d]

% growth inhibition = (W_o-W_T) x 100; Where W_o= Weight of mycelia in the Control

W_o W_T= Weight of Mycelia in the Treatment

Date are presented as mean for three duplicates. Values followed by the same letters are not significantly different according to Duncan's multiple range test (P=0.05).

Discussion

The results obtained from the bioassay test suggested that the two annonaceous plants *Isolona cauliflora* and *Cleistochamys krikii* are sources of bioactive naturally occurring compounds that have antimicrobial properties. Both the bacterial and fungal tested pathogens were inhibited by the crude extract and pure compounds isolated from the two plants. Low concentrations of the crude extracts, streptomycin and benomyl produced pronounced inhibitory effects on the pathogens, whereas it required higher concentrations of the isolated pure compounds to produce similar effects. This suggested that a combination of two or more compounds were present in the crude extract and that the active ingredients in both standards were more active than the plant extracts; this does not say that the plant extracts are not effective. The high concentrations required for the pure compounds may be due to the lack of synergism or it may be due to the fact that less active compounds were isolated from the crude extracts.

Comparing the chemical nature of the compounds from the two annoaceous plants, one sees that the compounds from *I. Cauliflora* are basic (indoles), whereas the ones from *C. krikii*, are acidic. Of the two acidic compounds from *C. krikii* the oxyheptanoid (cleistochlamic acid) exhibited higher activity than the flavonoid (pinocembrin) on all the rot microorganisms. This trend in which the most acidic compound is also the most active followed by the second most acidic compound (the phenolic flavonoid) may suggest that the inhibitory activity may be dependent on the acidic nature of the compounds.

While there are still more grounds to be covered in order to reach a good conclusion on the possible use of these isolated compounds as biofungicides, the present preliminary bioassay results suggest that natural products from the annonaceus plants investigated have potential of being used as plant and or animal protection agents against pathogens without any environmental hazard.

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ANTIMIKROBNA AKTIVNOST EKSTRAKTA IZ IZOLA *CAULIFLORA* I *CHLEISTOCHLAMYS KRIKII* (ANNONACEAE)

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R e z i m e

Ispitivana je antimikrobna aktivnost ekstrakta iz kore stabla *I. coliflora* i *C. krikii*, Kaulindole D mešavina Caulindole E i F, Pinocembrina i Oksiheptanoida (kelistoclaminska kiselina) na *Pseudomonas syrinage* pv. *phaseolicola*, *Fusarium solani, Botryodiplodia theobromae, Aspergillus niger i Aspergillus flavus*. Ispitivanjima u in vitro uslovima utvrdjeno je da sirov dihlor-metan ekstrat iz C. kirkii je ispoljio veoma jaku antimikrobnu aktivnost.

Prečišćena komponenta ekstrakta je ispoljila jak ili umereni uticaj na *P. syrinagae* pv. *phaseolicola* i *B. theobromae*. Pričišćena komponenta iz *C. krikii* je ispoljila značajniju inhibitornu aktivnost nego prečišćena komponenta iz *I. cauliflora*. Niža koncentracija od 100-200 ppm, sirov ekstrakt Caulindole, mešavina Caulindole E i F, Pinocembrin i Oksiheptanoid su ispoljili uticaj na porast većine ispitivanih patogena. Viša koncentracija od 500-1000 ppm je ispoljila umeren do slab uticaj na *Aspergillus* spp.

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