ANTIBACTERIAL ACTIVITIES OF CONSTITUENTS FROM *ISOLONA CAULIFLORA* AND *CLEISTOCHLAMYS KIRKII*

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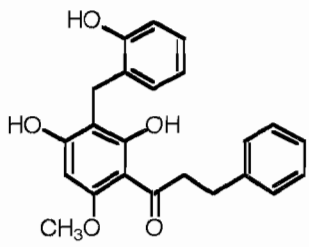
ABSTRACT

Antibacterial activities of caulindole D, a mixture of caulindole E and F, pinocembrin and an oxyheptanoid (cleistenolide), obtained from stem barks of Isolona cauliflora *and* Cleistochlamys kirkii, *on* Staphylococcus aureus *and* Pseudomonas phaseolicola *were determined. An* in vitro *assay test by paper disc method showed that the caulindole D, E and F from* Isolona cauliflora *produced no inhibition zone on both bacteria pathogens at 100-200 ppm while cleistochlamic acid from* C. kirkii *showed weak to moderate inhibition zone at 100-200 ppm. At concentration above 200 ppm, the bacteria pathogens were inhibited by all the compounds isolated from the plant extracts. Furthermore, there was an influence of bioassay method on the sensitivity of extracts on both test bacteria.*

INTRODUCTION

The family Annonaceae is known to be a rich source of alkaloidal constituents, which are generally characterized by the presence of an isoquinoline or an indole moiety, and flavonoids. A number of compounds belonging to both phytochemical classes have been shown to exhibit cytotoxicity, anti-tumor and anti-microbial activities (Hufford & Lasswell 1976). Ethanolic extracts of *Uvaria chamae* and C-benzylated flavonoids isolated from the same plant have been shown to demonstrate inhibitory activity against lymphocytic leukemia and anti-microbial activity at low concentration on *Staphylococcus aureus, Bacillus subtils, and* Mycobacterium *senegmatis* (Lasswell & Hufford 1977, Hufford & Lasswell 1978). Uvaretin (**1**) and diuvaretin (**2**), which are also flavonoids from Annonaceae, have been found to be active against *Plasmodium falciparum* malaria parasite with IC_{50} values of 3.49 and 4.20 mg/ml, respectively (Nkunya *et al.* 1991). Isoquinoline alkaloids such as (**3**) from the stem bark of *Annickia (Enantia) kummariae* have also been shown to have strong inhibitory activity against the sleeping sickness parasite *Trypanosoma brucei in vitro* and against the malaria parasite *Plasmodium falciparum* (Pavanand *et al.* 1989, Freiburghaus *et al.* 1997, Muhie 1996, Landolt *et al.* 1995 and Momburi 1998)



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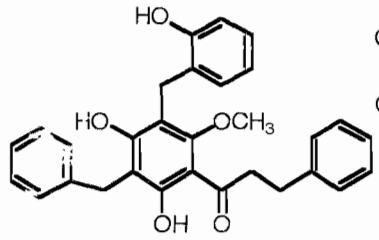

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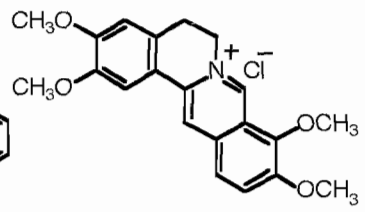

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Investigations into these bioactive natural products are now taking new dimensions because of increased environmental awareness as well as anticipated side effects of some synthetic drugs. These factors have prompted efforts in the search for environmentally and toxicologically safe and efficacious pesticides and drugs. The present study examined anti-bacterial activities of crude extracts, alkaloids, flavonoids and other compounds isolated from two Annonaceous plants on both human and plant pathogenic bacteria.

METHODS

Test microorganisms

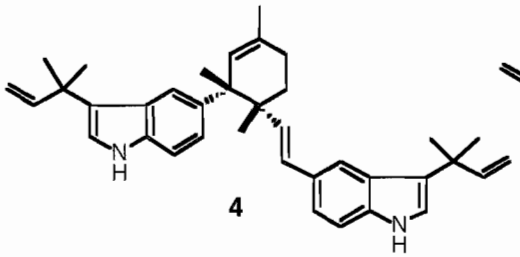
Pure cultures of *Staphylococcus aureus* were obtained from the Applied Microbiology Unit of the Department of Botany University of Dar es Salaam, and the culture of *Psuedomonas phaseolicola* isolated from diseased bean leaf was obtained from Uyole Agricultural Research Institute, Mbeya region, Tanzania. Both cultures were sub-cultured several times on nutrient agar and sabrouse agar and kept on agar slant at 30°C until use.

Media for antimicrobial assay

The solid medium used during antimicrobial testing of bacteria was Nutrient Agar (NA), containing 2% agar as a solidifying agent. Liquid medium used for the growth of bacteria was Nutrient Broth (NB). Both media were prepared according to a manufacturer's instructions (SIGMA).

Crude plant extracts and pure compounds

The stem bark of *Isolona 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 consecutively (2 x 48 hours) in pet ether, dichloromethane, and ethanol at room temperature (*ca.* 30°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' LH 20. Repeated column chromatography of VLC fraction six of the dichloromethane extract yielded caulindole D (**4**) as well as a mixture of caulindole E (**5**) and F (**6**) as yellow gums. Structural determination of compounds studied has been published elsewhere (Irungu 2001).



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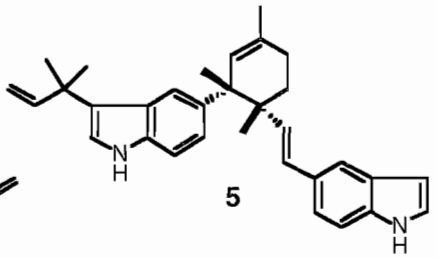

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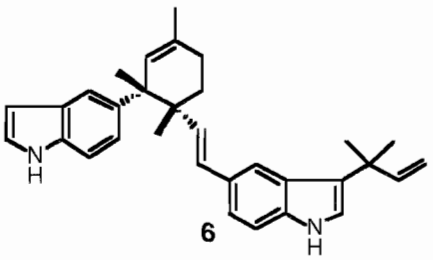

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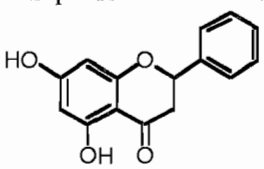
The stem bark of *Cleistochlamys kirkii* was air dried, pulverized and extracted by soaking consecutively (2 x 48 hours) in petroleum ether, dichloromethane, and ethanol. The concentrated extracts were fractionated by vacuum liquid chromatography (VLC) eluting with petroleum ether containing increasing 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' LH 20.

Repeated column chromatography of VLC fraction five of the dichloromethane extract yielded pinocembrin (7) as white needles with m.p. range of 182-184^{O}C. This compound was also obtained from VLC fraction six of the ethanol extract. Cleistenolide (**8**) was obtained from recrystallization in methanol of VLC fraction 8 of the pet ether extract. VLC fraction six of the dichloromethane extract also yielded this compound.



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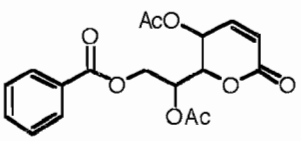

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Anti-microbial assay (*in vitro*)

The microbial assay was determined *in vitro* by paper disc methods. Samples of the plant extracts and pure compounds were dissolved in dimethyl sulfoxide (DMSO) and appropriate calculations were carried out to give concentrations ranging from 100 - 1000 ppm.

Paper Disc Method

This was carried out according to Platt (1986). Discs of 7 mm diameter made from Whatman No. 1 filter paper were used. A disc was soaked into the dissolved samples of either the pure compounds or extracts for 5 minutes and another disc was soaked in DMSO as control. Both discs were left to dry on a sterile plate in the laminar flow hood for 5 minutes after which they were

transferring onto separate pairs of inoculated plates. The plates were stored in a refrigerator for 6 hrs prior to incubation at 30°C for 48 hrs. The means and standard deviation (± SD) of the diameter of zones of growth inhibition around the disc for 5 treatments were determined.

Streak Method

Aseptically, 1 ml of each sample at different concentrations were added into sterile petri-dish and overlaid with mixing, 20 ml of molten nutrient agar to achieve a an end concentration of 2% agar. The medium was allowed to solidify and then a loopful of test bacteria was inoculated using the streaking method before the inoculated plates were incubated at 30°C for 24-48 hrs after which the plates were observed for antimicrobial activity as inhibition of bacterial growth. The streak method was used a preliminary indicator of antimicrobial activity before the activity was quantification through determination of inhibition zones by the Paper Disc method. In a previous study (Lyantagaye 1999), the lower sensitivity of the agar streak method was also observed and its limited use as a preliminary screen of antimicrobial activity was reported.

Paper Disc Method

Innoculation and Testing

Each time 0.5 ml of an overnight culture of test bacteria in Nutrient Broth (SIGMA) was added on solid medium (NA) on plates and spread evenly by using a drigalsky spatula.The paper disks, prepared as described above were then laid on the inoculated plate as source of the antimicrobial extract. The plates were then incubated at the appropriate temperature.

RESULTS

Data from the paper disc showed that caulindole D (**4**) and a mixture of caulindole E (**5**) and F (**6**) from *I. cauliflora* had no inhibitory erffect on both *S. aureus* and *P. phaseolicola* at 100-250 ppm and a weak inhibition at 300 ppm. These compounds exhibited moderate inhibitory effects on *P. phaseolicola* at 500-1000 ppm and strong inhibition on *S. aureus* at 1000 ppm (Table 1).

The inhibitory effect of cleistenolide (**8**) *P. phaseolicola* was moderate at 100-300 ppm and very strong at 500-1000 ppm (Table 1). Pinocembrin (7) showed no inhibitory zones against *P. phaseolicola* at 100-250 ppm, a weak effect at 300 ppm and a moderate effect at 500-1000 ppm. The compound also showed weak inhibition against *S. aureus* at 500 and 1000 ppm (Table 1).

The paper disc method results for caulindole D (**4**) and a mixture of caulindole E (**5**) and F (**6**) showed small diameters of inhibition zones for both *S. aureus* and *P. phaseolicola* at 300, 500 and 1000 ppm, but no inhibition zone at lower concentrations (Table 3). Pinocembrin (7) and cleistenolide (**8**) showed moderate inhibitory zones against *S. aureus* at 100, 150, 200 and 250 ppm and strong inhibition zone at 300, 500 and 1000 ppm (Table 3). The inhibition of cleistenolide (**8**) against *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 3).

The crude dichloromethane extract of *C. kirkii* showed a very strong antibacterial activity on *P. phaseolicola* at all concentrations; while its effect on *S. aureus* was moderate at all concentrations (Table 2).

Table 1. *In vitro* Growth Inhibition of Isolated Bacteria by the Pure Compounds Isolated from *Isolona cauliflora* and *Cleistochlamys kirkii* (Annonaceae) by the Streak Method.

Conc. (ppm)	*Staphyllococcus aureus*		*Pseudomonas phaseolicoi*		*Staphyllococcus aureus*		*Pseudomonas phaseolicola*	
	Caulindole D	Caulindole E and F	Caulindole D	Caulindole E and F	Cleistenolide (**8**)	Pinocembrin (7)	Cleistenolide (**8**)	Pinocembrin (7
100	-	-	-	-	-	-	++	-
150	-	-	-	-	-	-	++	-
200	-	-	-	-	-	-	++	-
250	-	-	-	-	+	-	++	-
300	+	+	+	+	+	-	++	+
500	++	++	++	++	++	+	+++	++
1000	+++	+++	++	++	++	+	+++	++
Contl.	-	-	-	-	-	-	-	-

Key:

-	=	No inhibition
+	=	Weak inhibition
++	=	Moderate inhibition
+++	=	Strong inhibition
Contl.	=	Control

Table 2. *In vitro* Growth Inhibition of Isolated Bacteria by the Crude Dichloromethane Extracts from *Cleistochlamys kirkii* (Annonaceae) by the Streak Method

Concentration (ppm)	*Pseudomonas phaseolicola*	*Staphyllococcus aureus*
100	+++	++
150	+++	++
200	+++	++
250	+++	++
300	+++	++
500	+++	++
100	+++	++
Control	-	-

Key:

-	=	No inhibition
++	=	Moderate inhibition
+++	=	Strong inhibition

Table 3. *In vitro* Growth Inhibition zones (diameter of zone in mm) of Isolated Bacteria by the Isolated Pure compounds obtained from *Isolona cauliflora* and *Cleistochlamys kirkii* (Annonaceae) by the Paper Disc Method

Conc. (ppm)	*Staphyllococcus aureus*		*Pseudomonas phaseolicola*		*Staphyllococcus aureus*		*Pseudomonas phaseolicola*	
	Caulindole D	Caulindole E and F	Caulindole D	Caulindole E and F	Cleistenolide (**8**)	Pinocembrin (7)	Cleistenolide (**8**)	Pinocembrin (7
100	0	0	0	0	3.0 ± 0.3	0	0	0
150	0	0	0	0	5.0 ± 1.0	0	6.2 ± 0.4	3.0 ± 0.1
200	0	0	0	0	6.0	3.5 ± 0.1	8.0	5.3 ± 0.1
250	0	6.0	7.0 ± 0.2	8.5 ± 0.4	6.2 ± 0.4	7.3 ± 0.3	11.2 ± 1.0	9.2 ± 0.3
300	5.0	5.5 ± 0.9	8.6 ± 0.2	8.0	8.5 ± 1.2	7.5	12.0	9.6 ± 0.5
500	8.5	10.0	9.0	9.0	10.0	8.2 ± 1	13.0	10.0
1000	12.4	12.6 ± 1.6	10.5	11.8 ± 0.9	13.5	10.5	15.0	11.0
Contl.	0	0	0	0	0	0	0	0

Key:

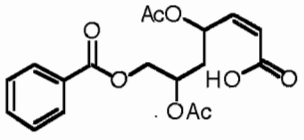
0 = No inhibition zone

Contl. = Control

DISCUSSION

The results obtained from this bio-assay test suggest that there are bio-active constituents in the stem and root barks of *Isolona cauliflora* and *Cleistochlamys kirkii*. The constituents appear to have anti-microbial/anti-bacterial properties because they exhibited inhibitory effects against the test organisms. Low concentrations of crude extracts produced pronounced inhibitory effects on both organisms whereas it required a high concentration of the isolated pure compounds to produce similar effects. This may suggest that a combination of two or more compounds, present in the crude extracts, may have acted synergistically to produce the observed strong inhibitory effects. The high concentrations required for the pure compounds may be due to lack of the aforementioned synergism or it may be due to the fact that less active compounds were isolated from the crude extracts as the main constituents, while the more active compounds could not be isolated due to either their small concentrations or because they may have decomposed during the isolation process. Moreover, the weak to moderate inhibitory effects in some of the samples may suggest that their potency/activity were limited by the solid medium in which they were incorporated. We strongly suggest that the solid medium used may have limited the potency of some samples, especially when we consider the activity of pinocembrin (7); because Lasswell and Hufford (1977, 1978) found it to have high inhibitory effects on *Staphylococcus aureus*, *Bacillus subtilis* and *Mycobacterium senegmatis* at 50 mg/ml.

The better inhibitory effects of cleistenolide (**8**) to both pathogens may be accounted for from the opening of the pyranyl ring to form an acidic metabolite, chleistochlamic acid (**9**). This would suggest a tendency for acidic compounds to be more active than the flavanoids and the alkaloidal components. Jung *et al.* (1990) have isolated compounds having the heptene (C_7) substructure analogous to that of cleistochlamic acid. These compounds, isolated from *Melodorum fruticosum* Lour. (Annonaceae), were shown to exhibit cytotoxic activities against human tumour cell lines.



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The C_7 (heptene) moieties are reminiscent of the C_7N units of antibiotics of *Streptomyces* (pactamycin, geldanamycin, streptonigrin, validamycin, etc) and a C_7 unit is omnipresent in several of the aromatic compounds of Annonaceae (altholactone, goniofufurone, goniopyrone, senepoxide, pipoxide, etc). Hence common biosynthetic origins of these C_7 units of Annonaceae aromatic compounds via pathways similar to that of C_7N units could be suspected.

The observed poor sensitity shown by the steak method agrees with the observation by Platt (1986). This observation implies that sensitivity of a test is proportional to the amount of an anti-microbial agent applied to the agar plate. This points to the dependency on concentration for the anti-microbial diffusion rate and the bacteria killing process. Following the potential displayed by the extracts in this study, a follow-up study is being planned in our laboratory in which the activities of the best extracts will be compared to known (standard) antimicrobial agents such as antibiotics.

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