BULL. PHIL. BIOCHEM. SOC. 4(1 & 2): 8 – 14 (1981)

ANTIMUTAGENIC EFFECTS OF *CYANOTIS VAGA* LOUR. (SCHULTES) EXTRACTS

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ABSTRACT

Ecdysterone was isolated from the leaves of *Cyanotis vaga* Lour. (Schultes). This was shown to reduce the revertant colonies of Salmonella typhimurium TA 1535 and TA 1537 caused by two mutagens, sodium nitrite and quinacrine.

The same observation was made on the crude extracts from the leaves and stems of *Cyanotis vaga,* Lour. (Schultes).

Thus, both ecdysterone and the crude extracts possess antimutagenic effects.

INTRODUCTION

In 1965, it was reported (1) that water extracts from an herb known scientifically as *Cyanotis vaga* Lour. (Schultes) could effectively cure breast cancer. Subsequent studies done by Santos (2) using various crude extracts on *Erlich ascites* tumor cells (EATC) and leukemic cells showed that these fractions could control lymphoid leukemia. In a later communication (3) the National Institute of Science and Technology reported the isolation of a substance, a steroid, from the acetone-benzene portion of the *Cyanotis vaga* extracts. Spectrometric properties of the substance named commisterone, and further chemical tests on its stereochemistry have shown that it is the insect-moulting hormone 20-hydroxy-ecdysone (variously named crystecdysone, ecdysterone and polypodine A).

Another isolate from the benzene-ethyl acetate-acetone fraction yielded CV-3 of molecular formula $C_{21}H_{30}O_5$ and melting at 245-255°C. Spectrometric properties revealed that it is closely related in structure to ecdysterone (3).

The literature on *Cyanotis vaga* and its activities and components is meager, and further research must be done to test the validity of the above report. Meanwhile, investigations are being done to test its anti-mutagenic effects on mutant strains of *Salmonella typhimurium.*

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MATERIALS AND METHODS

Reagents and Equipments. All reagents used were of analytical g Silica gel (70-325 mesh) was from E. Merck, Harnwell, Makati. *Cyanotis v* Lour. (Schultes) leaves were obtained from the farm of Mr. Marceli Punzalan in Canaman, Camarines Sur, Philippines. Standard ecdysteron isolated from *Cyanotis vaga* leaves were given by Dr. Modesto Chua from the Department of Chemistry, Ateneo University. Mutant strains of *Salmonella* typhimurium, coded TAI535 and TAI537 were obtained from the laboratory of Dr. Ames, University of California, Berkeley.

Preparation of *Cyanotis vaga* Lour. (Schultes) Extracts. The general methods used for the preparation of the hexane and acetone extracts and the isolation of ecdysterone followed that used by Santos (3) and Chua (4)'

A. Hexane Extract.

Shredded dried *Cyanotis vaga* leaves were wrapped in filter paper and extracted in a soxhlet using hexane as solvent. The crystals which precipitated out of the yellow-colored extract were dissolved in hot hexane then re-crystallized by cooling.

B. Crude Acetone Extract.

After extraction with hexane, the leaves were reextracted with acetone as solvent in soxhlet, producing a green solution. The solvent (acetone) was distilled off until a brown oil tar remained.

C. Isolation of Ecdysterone crystals.

The brown oil tar was washed with methanol and chromatographed in silica gel (70-325 mesh), eluted with absolute methanol and the middle fractions taken. The solvent was removed in a vacuum evaporator, yielding colorless prisms. The melting point was determined after recrystallization.

D. Water Extract.

Two methods were employed in the preparation of the water extract. The first method followed that used by Masilungan *et al* (5) as follows: Fresh *Cyanotis vaga* leaves were macerated in acetone at a concentration of 5 g/ml. This preparation was shaken for 24 hours, the acetone was allowed to evaporate until only a brown H_2O extract remained. This was filtered 2-3 times then sterilized. The second method is that used by provincial folks taking the extract as cure for breast cancer and other ailments. The extract was made by directly pounding the fresh *Cyanotis vaga* leaves to squeeze the juice out.

Organism. The strains used were histidine mutants of *Salmonella typhimurium.* TAI535 contains his G46 mutation which is an alteration of

one codon in the mRNA from the gene coding for the first enzyme of histidine biosynthesis, and a single deletion through the galactose operon, biotin operon, and excision repair system for DNA (uvrB gene) and chlorate-resistant genes. TAI537 is a strain containing his C3076 mutation, which is an addition of a base-pair in the amino transferase gene in Salmonella typhimurium, and a galactose-biotin uvrB deletion.

The bacteria was grown in 10 ml nutrient broth for 5-8 hours at 37°C. A 0.10 ml sample was taken and added to a nutrient broth-dimethyl-sulfoxide (DMSO) solution (2:1) From this stock, another 0.10 ml sample is taken and added to a 5-10 ml nutrient broth and grown for another 5-8 hours at 37°C. This culture is used for plating.

Direct test for Anti-mutagenicity (Pour Plate Method). All media used was prepared according to the method described by Ames (6, 7, 8). Sterile pour plates containing a minimum amount of bottom agar are prepared for the tests. A 2.0 ml sample of the top agar (0.6% agar containing 0.5% NaCl) containing 0.5 mM L-histidine. Hcl - 0.5 mM Biotin was added to a sterile test tube. Then 0.1 ml ecdysterone solution or 0.02, 0.05, 0.08 or 0.10 ml crude extracts was added and the mixture allowed to equilibrate to 45°C. A 0.10 ml aliquot of the mutagen solution (5 mg/ml $NaNO_2$ for TAI535 or 5 ug/ml quinacrine for TAI537) and 0.01 ml Salmonella typhimurium tester strain (1/100 inoculum) culture was added to the tube and mixed The mixture was poured immediately into the plate containing the bottom agar.

For positive controls, the *Cyanotis vaga* extracts or isolate was omitted. Instead 0.10 ml distilled H_2O was added to the top agar. For a "blank", the *Cyanotis vaga* extracts or isolate and mutagen were replaced by distilled H_2O. To determine whether the extracts or isolate are, by themselves, mutagenic, the mutagen was replaced by 0.10 ml distilled H_2O.

The plates were incubated at 37°C for 5-8 hours and the number of revertants determined using a Coulter counter.

RESULTS

Preparation of Crude Extracts and Isolates from *Cyanotis vaga* Lour. (Schultes). Table 1 shows the results of the extraction methods using organic solvents for the isolation of ecdysterone and other possible anti-mutagenic substances from the leaves and stems of *Cyanotis vaga* Lour. (Schultes). At least two kinds of crystals were isolated: 1) the powdery white crystals which precipitated out of the solvent hexane appears to be a low melting wax. The anti-mutagenic properties of these cyrstals, i.e. the ability to inhibit growth of revertant colonies due to $NaNO_2$ or quinacrine, were not tested. 2) Colorless prisms isolated from the middle fractions of the chromatographic run gave a melting point of 146-151°C corresponding to the ecdysterone

crystals isolated from the same leaves *(Cyanotis vaga)* by Chua (4). Test for anti-mutagenicity of the crystals showed that up to a certain limit of concentration, the crystals could inhibit growth of revertant colonies due to $NaNO_2$ and quinacrine.

The brown oily tar residue of the acetone extract which was subsequently chromatographed showed that this crude extract could also inhibit growth of revertant colonies due to the mutagens.

Table 1. Some properties of extracts and crystals from *Cyanotis vaga* Lour. (Schultes).

	Extraction Method	PROPERTIES		
		Appearance	Melting Pt.	inhibition of revertant growth due to mutagens
I.	Soxhlet extraction using hexane as solvent	white, powdery crystals	82-84°C	not tested
II.	Soxhlet extraction of residues of I using acetone as solvent	brown oily tar		+
III.	Chromatography of brown tar from II on silica gel using methanol as eluant; middle fractions taken and solvent evaporated	colorless prisms	146-151°C*	+

**Colorless prisms isolated by Chua (4) using the same procedure gave the same melting point fo 146-151°C and appearance of the crystals. Spectrophotometric properties of their crystal isolate showed that it is the insect-moulting hormone, ecdysterone.*

Anti-mutagenicity Testing of *Cyanotis vaga* Lour. (Schultes) Extracts and Isolates. Mutant strains, coded TAI535 and TAI537, of Salmonella typhimurium were grown in agar containing Vogel Bonner E medium, histidine and biotin. The culture was characterized by a clear bacterial lawn. Upon addition of $NaNO_2$ to TAI535 and quinacrine (atabrine) to TAI537, revertant colonies were produced indicating that these substances are direct mutagens, i.e. the previous mutation which caused inhibition of growth of

the wild type of *Salmonella typhimurium* was negated by another mutation (This time due to $NaNO_2$ and quinacrine) enabling the wild or native revertant bacteria to grow. The addition of Cyanotis vaga isolate (ecdysterone) and the crude extracts to the culture containing mutagen caused a decrease in the number of revertants produced. As the concentration of crude extract increased, the number of revertants due to the mutagens decreased correspondingly. Likewise, addition of increasing concentration of ecdysterone

Table 2. Pour plate method for determining the anti-mutagenicity of *Cyanotis vaga* Lour. (Schultes) Extracts on a mutant strain, TAI535, of *Salmonella typhimurium.*

A 0.01 ml aliquot of Salmonella typhimurium mutant, TAI535, 0.10 ml $NaNO_2$ and crude extracts or ecdysterone were mixed with top agar then poured into a plate containing bottom agar. The plate was incubated at 37°C for 5-8 hours and the number of revertant colonies determined thereafter.

Test Plates	*Average Number of Revertant Colonies
TAI535	8.5
TAI535 + 0.50 mg $NaNO_2$	73.0
TAI535 + 10 ug ecdysterone	19.3
TAI535 + 0.10 ml crude extract	8.7
TAI535 + 0.50 mg $NaNO_2$ + 0.10 ug ecdysterone	64.5
TAI535 + 0.50 mg $NaNO_2$ + 2.0 ug ecdysterone	67.0
TAI535 + 0.50 mg $NaNO_2$ + 2.0 ug ecdysterone	46.0
TAI535 + 0.50 mg $NaNO_2$ + 100 ug ecdysterone	48.4
TAI535 + 0.50 mg $NaNO_2$ + 0.02 ml crude extract	120.0
TAI535 + 0.50 mg $NaNO_2$ + 0.05 ml crude extract	63.7
TAI535 + 0.50 mg $NaNO_2$ + 0.08 ml crude extract	61.6
TAI535 + 0.50 mg $NaNO_2$ + 0.10 ml crude extract	37.7

*Average of 6 trials.

correspondingly decreased the number of revertant colonies growing, except for a concentration of about 0.10 mg where there is a drastic increase in the number of revertants produced. These findings indicate that the extracts and isolate from Cyanotis vaga Lour. (Schultes) could reverse or counter the mutation due to $NaNO_2$ and quinacrine. The results are shown in Tables 2 and 3.

Table 3. Pour plate method for determining the anti-mutagenicity of *Cyanotis vaga* Lour. (Schultes) Extracts on a mutant strain, TAI537, of *Salmonella typhimurium.*

A 0.01 ml aliquot of *Salmonella typhimurium* mutant, TAI537, 0.10 ml quinacrine and crude extracts or ecdysterone were mixed with top agar then poured into a plate containing bottom agar. The plate was incubated at 37°C for 5-8 hours and the number of revertant colonies determined thereafter.

Test Plates	*Average Number of Revertant Colonies
TAI537	3.2
TAI537 + 0.5 ug quinacrine	262.4
TAI537 + 10.0 ug ecdysterone	3.2
TAI537 + 0.10 ml crude extract	7.4
TAI537 + 0.5 ug quinacrine + 0.10 ug ecdysterone	300.6
TAI537 + 0.5 ug quinacrine + 1.0 ug ecdysterone	204.0
TAI537 + 0.5 ug quinacrine + 2.0 ug ecdysterone	121.0
TAI537 + 0.5 ug quinacrine + 5.0 ug ecdysterone	62.6
TAI537 + 0.5 ug quinacrine + 0.02 ml crude extract	178.8
TAI537 + 0.5 ug quinacrine + 0.05 ml crude extract	90.0
TAI537 + 0.5 ug quinacrine + 0.08 ml crude extract	60.6
TAI537 + 0.5 ug quinacrine + 0.10 ml crude extract	32.4

**Average of 6 trials.*

DISCUSSION

A study was done using crude extracts and pure ecdysterone a steroid isolated from the acetone extract of *Cyanotis vaga* Lour. (Schultes), on *Salmonella typhimurium* mutants. It is known that mutagens or substances which cause base alterations of cellular DNA and RNA, are potential carcinogens when they exert their effect on somatic cells. Thus, if a substance, e.g. ecdysterone or "unknowns" from the crude extracts is shown to be anti-mutagenic, then it is possible that it can also be anti-carcinogenic.

The results of the tests which showed inhibition of revertant growth directly due to $NaNO_2$ and quinacrine by extracts from *Cyanotis vaga* indicate that the extracts are anti-mutagenic. Inhibition by pure ecdysterone prior to stimulation would indicate that there is an upper limit of concentration that would effect anti-mutagenicity. This is probably due to a competition by excess anti-mutagen with mutagens for gene site since both mutagen and anti-mutagen could cause mutational changes in the bases of cellular DNA and RNA and consequent copy errors.

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