CHAPTER - V

ISOLATION AND CHARACTERISATION OF ANTIFUNGAL CONSTITUENT IN THE YAM OF CULTIVATED SPECIES OF *Dioscorea alata* L.

Introduction

While working on isolation of steroidal constituents from the yam of different species of *Dioscorea* it was observed that even in rainy season, the water extract of the yam of *D. alata* did not show any contamination of micro organism though other extracted material kept in a container showed severe contamination with a fungal strain. The fungus was identified as *Aspergillus niger.* No report is available in connection with the antifungal activity of water extract of *D. alata* with special emphasis on characterisation of chemical constituent.

Recently pthenanthrene derivative from the tuber of *D. delicata* has been ovserved to show antifungal activity against *Ciadosporium cladosporoides* (Haragachi et at. 1999). Hu *et. al.* (1999) studied bioactivity of traditional Chinese herval medicine, *D. composita* against pyricularia oryzae.

In order to understand the nature of chemical inhibitar in the yam of *D. alata* to serve as antifungal agent, investigation has been carried out following conventional phytochemical method.

Materials and Methods

Materials: Yam of *D. alata.*

Methods :

Collection of preparation yam of *D. alata*

Yam of *D. alata* was taken out from the soil and washed in water to free it from soil debris. 500 gms of freshly cut yam was crushed in an electrically operated mixer to form a paste.

Extraction and purification of isolated product (Brian *et al.* 1968)

The pasted material was mixed with 250 ml water and boiled at 100°C for one hour under reflux condition. It was filtered to obtain water extract of the yam. The extract was concentrated and evaporated more or less to dryness. The solid mass left was treated with methanol and refluxed for 30 min. It was filtered to obtain methanolic extract. The methanol extract was concentrated to small volume. Chloroform was added dropwise to produce turbidity in the solution. It was kept overnight in cold condition to obtain crystals.

Thin layer chromatography and Paper cromatography (PC) for identification of the isolated product

Petroleum ether, (b) Butanol : acetic acid : water (4 : 1 : 5, lower fraction) and Methanol : Chloroform (9 : 1), d) phenol saturated with water have been used during paper chromatography. Chloroform : acetone (4 : 1, V/V) and Hexane : acetone (4 : 1, V/V) have been used for TLC.

The isolated product was run with the authentic sample of saponin. The dried paper and TLC plate were placed in iodine chamber to locate the position of the spot for determination of the Rf value.

Preparation of P.D.A.

Composition :	Peeled potato	-	40.00 gm.
	Dextrose	-	2.00 gm
	Agar-agar	-	2.00 gm
	Distilled water	-	100 ml

Small blocks were made from peeled potatoes. All ingredients were weighed according to the composition. Potato blocks were taken in a conical flask placed on a heater to get decoction. It was boiled till the smell of boiled potatoes came out. The resultant decoction was filtered out using strainer. To the filtered solution dextrose was added and stirred well until the dextrose dissolved. Finally the agar was added and again heated on a water bath to melt the agar completely. The medium was poured in sterile culture tubes and immediately plugged using sterile cotton. For slant preparation 5-6 ml of medium was poured

where as for stab preparation 20 ml of medium was poured in each culture tubes. All culture tubes were placed in an autoclave to make it free from contamination at 120°C, 15 lb pressure for 15 min. All culture tubes were taken out of the autoclave. Stabs were placed in a test tube stand in a vertical position and slants were placed in a slanting position.

Inocculation of *A. niger* in to culture medium

Two types of slants were used. In one type *A. niger* was inocculated to freshly prepared P.D.A. in the test tube. In another tube the culture medium was previously mixed with 100 ppm of the isolated product in water and the tube was inocculated with the same fungus. The observations were noted after seven days, keeping both the culture tubes at room temperature (25°C).

Result and Discussion :

The crystals that were obtained after extraction of yam of *D. alata* with water and subsequent Methanol chloroform treatment, were filtered off and recrystallised from methanol chloroform mixture, when 30 mg of crystals having m.p. 287°C was obtained. That the isolated product was a saponin was confirmed by the produciton of heavy frothing while boiling the crystals dissolved in water.

Table 25 :Paper chromatographic behaviour of Dioscin and the saponin like isolated natural product inthe water extract of the yam of *D. alata.*

Chemicals	**Petroleum ether Rf.**	**Butanol : Acetic acid : water (4:1:5 V/V/V) Rf.**	**Methanol : Chloroform (9 : 1,V/V) Rf.**
Dioscin	0	0.25	0.45
Saponin like isolated natural product	0	0.25	0.45

The IR spectrum of the isolated saponin showed characteristic peaks λmax 3350 (Broad) 1640, 1375, 1175, 1050, 850, 821, 720 cm^{-1} (Fig. 42) and which are superimpossible with those of authentic dioscin. It was further confirmed

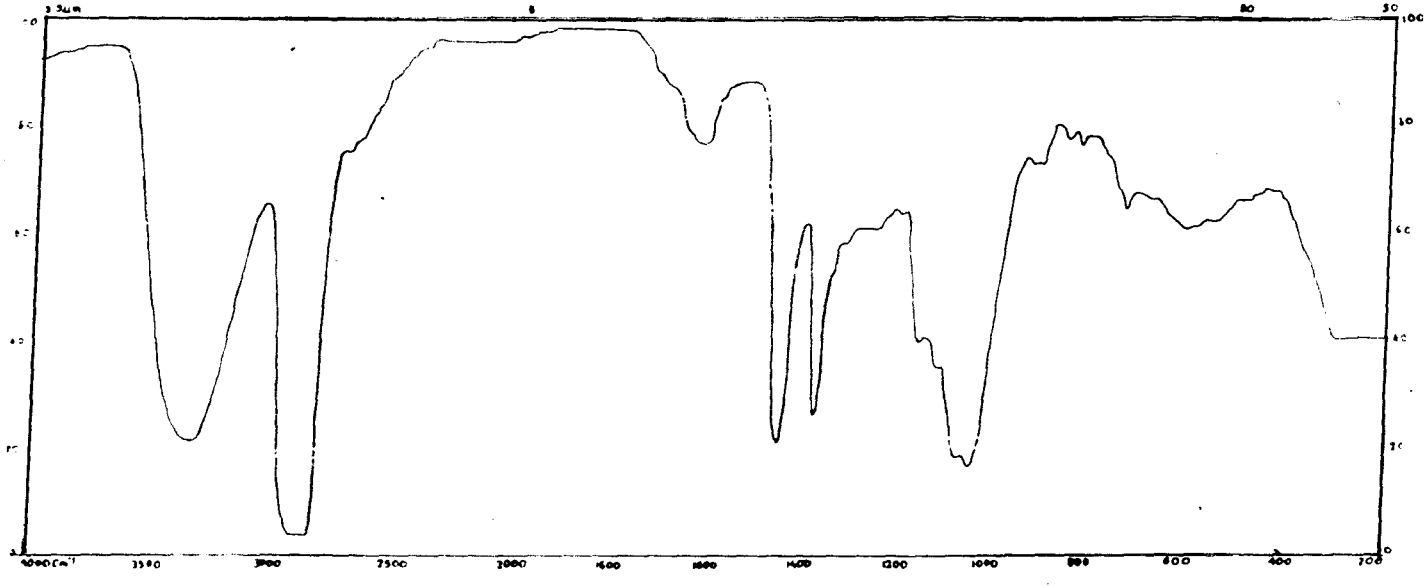

Fig. 42. IR spectrum of dioscin.

with the chromatographic behaviour of the isolated saponin with that of Dioscin (Table 25). The sapogenin was derived after following the procedure adopted by Brain *et al.* (1968). The residue was hydrolysed with molar HCl for 6 hrs. It was neutralised and was shaken with chloroform. The chloroform part was evaporated to dry mess and fine crystals were obtained from chloroform, methanol mixture. The crystal showed m.p. 206-208°C. It was subjected Rf. 0.58, Hexane : Acetone (4 : 1, V/V) Rf. 0.32. The plate showed purple spots after spraying with Antimony chloride in conc. HCl and subsequent heating at 110°C for 10 min. The physical and chemical properties including IR spectrum analysis were the same with those obtained in connection with the authentic diosgenin (Fig. 39) and that has been represented in the chapter dealing with pharmacognosy work.

After removal of diosgenin from the acid hydrolysed product of saponin, the filtrate was neutralised with barium carbonate. After filtration the filtrate was concentrated under reduced pressure and spotted on paper for chromatography in solvent mixture of Butanol : acetic acid : water (4:1:5V/V/V, upper layer) and phenol saturated with water, sugars from the hydrolysed product were identified to be glucose and Rhammose Rfs of different sugars in various solvent mixture during paper chromatography have been represented in the table26.

Table 26.: Identification of sugars after acid hydrolysis of saponin by paper chromatography.

Chemicals	**Butanol : acetic acid : water (4:1:5, V/V/V) Rf.**	**Phenol Saturated with water Rf.**	**Colour of the spot due to aniline hydrogen pthalate.**
Sugars obtained from acid hydrolysed saponin			
Sugar I	0.13	0.24	Brown
Sugar II	0.34	0.59	Yellow brown
Authentic			
Glucose	0.13	0.34	Brown
Rham nose	0.34	0.59	Yellow brown

Fig. 43 A shows that the slant without dioscin of 100 ppm served medium for good growth of *A. niger* as reflected fromt he very good formation of white mat of mycellium. Whereas Fig. 43 B shows the dead mycellium which became black due to degeneration of hyphal mat due to lysis caused by dioscin (100 ppm) in the medium. It is very interesting to note that the centrally placed small part of agar without dioscin to serve inoculum shows white mat of hyphae still in living condition. Antifungal activity of dioscin against *A. niger* has not been reported earlier though Roddick *et al.* (1990) showed antifungal activity of solamargine. While working with antimicrobial tests of natural product of plant origin Imai *et al.* (1967) mentioned that *D. tokoro* gave dioscin as active agent. Recently Haraguchi *et al.* (1999) has isolated a phenanthrene derivative from *D. delicata* showing antifungal activity against *Cladosporium cladospodoides*. Solamargine may be considered as the nitrogen analogue of rhamnose-glucose-rhanmose bearing Dioscin (Fig. 44). Because both of them have got the same sugar moiety i.e. rhamnose, glucose-ramnose being attached to solasodine, a steroidal alkaloid to form solamargine and also to diosgenin, a sapogenin to form dioscin. As the nitrogen in the chemical structure of solasodine is replaced by oxygen in diosgenin, the former is considered nitrogen analogue of the latter. According to Roddick et al. (1990) rhamnose-glucose-rhamnose in solamargine cause significant disruption of membrane in biological materials due to lysis. It is expectedthat the same type of bioactivity may be claimed to be due to rhamnose-glucose-rhamnose in Dioscin having the same structural configuration as that of solamargine. The accumulation of dioscin in the yam of *D. alata* may be claimed to help the underground part of the plant to become free from the attack of microbes.

SUMMARY

The water extract of the yam of *Dioscorea alata* has been observed to contain a chemical constituent having the antifungal property against *Aspergillus niger*.

After purification following conventional method of fractional crystallisation from methanol chloroform mixture, crystals having m.p. 287°C has been obtained.

Following comparative behaviour and I.R. spectrum of isolated product with those of authentic sample, it was identified as dioscin, a saponin. The glycoside nature of it has been verified after identification of diosgenin and the sugar components of rhammose and glucose, during acid hydrolysis of saponin.

The antifungal activity of dioscin against *A. niger* has been verified during the culture of the fungus in P.D.A. medium mixed with dioscin as compared to the control showing good growth of the fungus in a medium free from dioscin.

The antifungal activition of dioscin is being claimed to be due to chacotriose i.e. rhamnos-glucose-rhamnose combination of sugar in dioscin.

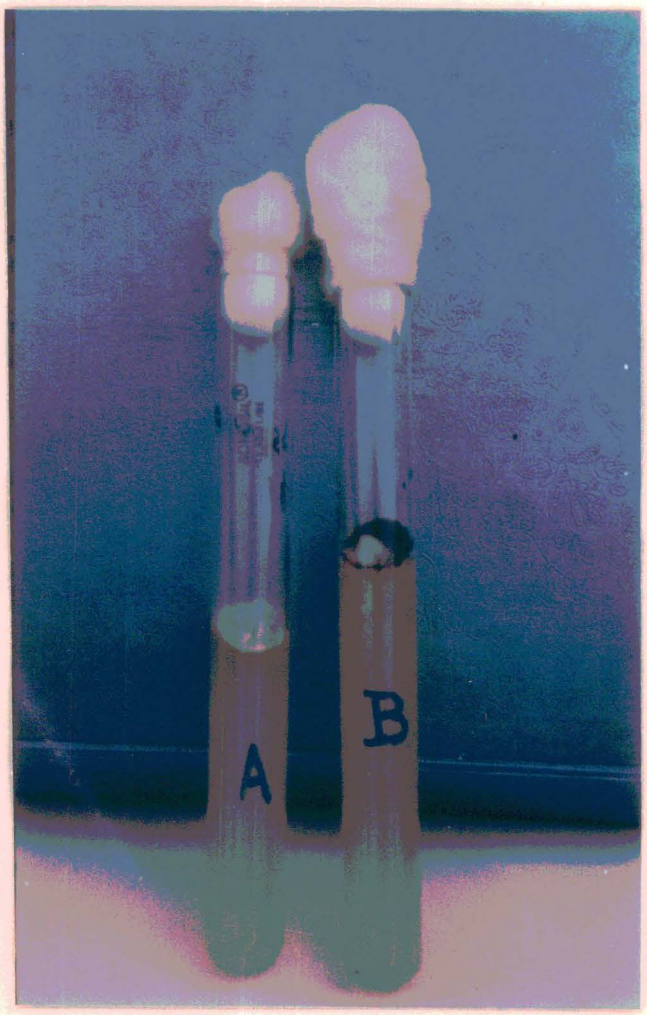

Fig. 43 Antifungal activity of dioscin against Aspergillus niger.
(A). Slant without dioscin showing good growth of fungal hyphae.
(B). Slant with dioscin showing black mass of lytic hyphae surrounding dioscin free agar bearing in occulum.