CHAPTER 2

Purification and identification of the antifungal principle isolated from Clitoria ternatea L. plant

3,7-dihydroxy 3′,4′ orthodihydroxy flavone
Chapter 1 incorporated screening of antimicrobial activity of some members of family Leguminosae viz. *Clitoria ternatea* L., *Crotalaria pallida*, *Cassia siamea* L., *Cassia alata* L., *Acacia arabica* Willd. Antifungal activity was found in 50% aqueous ethanolic leaf extract of *C. ternatea* L. This active principle was subjected to further procedures for purification and identification of the sample.

After column chromatographic separation, the bioactive antifungal principle was found to be present in chloroform : carbinol (2:1), fraction 4.

**Chemical characterization of antifungal principle:**

Purification of chloroform:carbinol (2:1) fraction no.4 obtained from column chromatography vide page. 66.

**Purification of chloroform:carbinol (2:1) fraction 4. and isolation of the sample:**

Chloroform : carbinol (2:1) fraction 4. was subjected to preparative thin layer chromatography for further purification. Glass plates (20cm×14 cm) were coated with silica gel G layers, 0.25 mm thick, with slurry of silica gel G. Chloroform : carbinol (2:1) fraction 4 was concentrated and spotted on the silica coated plates with the help of thin capillary tubes and chromatograms were developed using various solvent systems. The developed chromatograms were air dried.

Chromatogram spots developed having Rf values of 0.994 in TBA (butanol: glacial acetic acid: water = 3:1:1) and 0.882 in HOAc (glacial acetic acid: water = 3:17). This sample had melting point at 245.0°C. The spot appeared with a violet fluorescence in UV light and dark mauve when treated with NH₃ under UV light.

The spots were scraped out and purified sample thus collected was then subjected to UV spectroscopic analysis.

**UV Spectral Analysis of the Bioactive Compound:**

(a) A stock solution was prepared by dissolving a small amount of the compound (about 0.1 mg) in about 10 ml of spectroscopic methanol. The concentration was then adjusted so that the optical density of the major absorption peak between 250 nm and 400 nm gave an optical density (O.D.) reading in the region 0.6-0.8.

(b) The methanol spectrum was measured at normal scan speed (about 50 nm/min) using 2-3 ml of the stock solution of the compound.
(c) The methanol spectrum was measured at slow scan speed (about 10 nm/min) in the
regions of the peak maxima in order to determine the wavelength of each maximum more
accurately.

(d) The NaOMe spectrum was measured immediately after the addition of three drops of
the NaOMe stock solution to the solution used for steps b and c. After 5 mins, the spectrum
was rerun to check for flavonoid decomposition. The solution was then discarded.

(e) The AlCl₃ spectrum was measured immediately after the addition of six drops of the
AlCl₃ stock solution to 2-3 ml of fresh stock solution of the compound.

(f) The AlCl₃/HCl spectrum was recorded immediately after the addition of three drops
of the stock HCl solution to the cuvette containing the AlCl₃ (from step e). The solution was
then discarded.

(g) The NaOAc spectrum of the flavonoid was determined as follows: Excess coarsely
powdered anhydrous reagent grade NaOAc was added with shaking to a cuvette containing
2-3 ml of fresh stock solution of the compound. About a 2 mm layer of NaOAc remained on
the bottom of the cuvette. All the NaOAc spectra presented in this volume were recorded
within 2 mins after the addition of the NaOAc to the solution. A second spectrum was run
after 5-10 mins. to check for decomposition.

(h) The NaOAc/H₃BO₃ spectrum was determined as follows. As no decomposition was
observed when NaOAc spectrum was rerun after 5 min. sufficient powdered anhydrous
reagent grade H₃BO₃ to give a saturated solution was added with shaking to the cuvette
(from step g) which contained the NaOAc. The solution was discarded after the spectrum
was recorded.
Results and Discussion:

Chemical characterization of the bioactive compound by UV spectral analysis:
The methanol spectra of flavones and flavonoids exhibit two major absorption peaks in the region of 240-400 nm (Mebry et al., 1970). These two peaks are commonly referred to as Band I (usually 300-380 nm) and Band II (usually 240-280 nm) Band I is considered to be associated with the absorption due to B-ring cinnamoyl system and Band II with absorption involving A-ring benzoyl system.

UV spectrum of the sample in Methanol (Fig. 2.1.a):
The spectrum shows absorption peaks (\(\lambda_{\text{max}}, \text{nm}\)) at 400, 240, 258sh, 223sh, which clearly indicates that the sample is flavonoid in nature.

UV spectral analysis of the sample with NaOMe (Fig. 2.1.a):
Use has been made of the effect of NaOMe on the UV spectrum of flavone and flavonols for the detection of free 3 and/or 4’-OH group. In this case, addition of NaOMe to the methanolic solution of the sample produced a bathochromic shift of 63 nm with decrease in intensity of the peak which suggests presence of free 3-hydroxyl group. This bathochromic shift with decrease in intensity nullified the presence of free 4’-OH group. However, other hydroxylation patterns in flavones notably 5,6,7; 5,7,8 and 3,4’,5’ may also cause alkali sensitivity.

UV spectral analysis of sample in presence of NaOAc (Fig. 2.1.b):
Na-acetate is a weaker base than NaOMe and is particularly a useful diagnostic reagent for the specific detection of 7-OH group. In this case bathochromic shift of 20 nm in the band II of the spectrum clearly indicates the presence of free 7-OH group. Also such small shift denotes presence of 3’, 4’-dioxygenated derivatives.

UV spectral Analysis of sample in presence of NaOAc/Boric acid: (Fig. 2.1.c)
Flavones and flavonols containing B-ring orthodihydroxyl group show bathochromic shift of Band I in presence of NaOAc/H\(_3\)BO\(_3\). In this case, Band I shift of 30 nm indicates presence of B-ring orthodihydroxyl group.

UV spectral analysis of the sample in presence of AlCl\(_3\)/HCl: (Fig. 2.1.d):
For some time, AlCl\(_3\) has been used as a diagnostic reagent for the detection of orthodihydroxyl group. The presence of an orthohydroxyl group in the B-ring of flavones and flavonols can be detected by comparison of the spectrum of the flavonoid in presence of AlCl\(_3\) with that obtained in presence of AlCl\(_3\)/HCl. In this case, the hypsochromic shift of 30 nm absorbed in the Band I of the aluminium spectrum on the addition of acid results from the decomposition of the complex of AlCl\(_3\) with orthohydroxy group. Hence, the structure of the flavonol can be depicted as shown in fig.1d that is 3-7 dihydroxy 3’,4’ orthodihydroxy flavone.
Fig. 2.1.a: Spectrum of sample in presence of NaOAc:
Fig. 2.1.b: Spectrum of sample in presence of NaOAc/Boric acid:
Fig. 2.1.c: Spectrum of sample in presence of AlCl₃/HCl:

Fig. 2.1.d: Structure of the compound identified as 3,7-dihydroxy 3',4' orthodihydroxy flavone: