CHAPTER - III

A NEW SPECTROPHOTOMETRIC METHOD IN CONNECTION WITH QUANTITATIVE ESTIMATION OF ROTENONE AND ROTENOIDS IN TEPHROSIA CANDIDA DC.

INTRODUCTION

It has been established earlier that T. candida contains a fair amount of rotenone and rotenoids that can be utilised commercially due to their potent insecticidal value without any residual toxicity (Krishna & Ghose 1938). Irvine and Freyre (1959) observed that content of rotenoid may vary in different plant parts, under various conditions. During the study on growth and development of plants a large number of samples are accumulated which needs the estimation of rotenoid for their purposeful utilisation. The methods so far utilised in connection with estimation of the chemical compound can be grouped into gravimetric method (Subba Rao, 1945), Colorimetric method (Jones, 1945,1946; Mayer & Rachmad, 1947; Milton & Waters, 1949) and the utilisation of HPLC (Bowman et al, 1978). But all these are noted to be time consuming and required large amount of sample. Moreover sometimes sophisticated instruments are not available in the laboratory. Thus, attempt has been made to establish a new and easy colorimetric method for quantitative determination of rotenoid compounds.

MATERIALS AND METHODS

Materials :

Different parts of T. candida were collected from the experimental plot of the Centre for Life Sciences, North Bengal University and utilized for this part of work.

Methods :

Quantitative Determination of rotenoid by optical density method proposed by Jones (1946)

Different plant parts of T. candida DC were dried in hot air oven at 60°C for 2 days and kept in dessicator until assayed. These parts (500mg each) were powdered and soaked in acetone (25ml) for over night after preliminary warming in water bath and filtered. 2 ml of the filtrate was taken and total relenoid was measured at 450 nm according to the method described earlier (Chapter-II, p.62), following the standard curve (10^3 to 10^{-1} ppm) of rotenone (Fig.17).

Quantitative Determination of Rotenone by the application of a new method proposed after the modification of Vanillin test (Haussler, 1946, 1947a)

100 mg dried plant part was taken and extracted with 5 ml ethanol (10 minutes). 0.5 ml was spotted in thin layer chromatography plate along with the authentic rotenone with the solvent mixture of chloroform:Benzene:Acetone (90:8:2; V/V/V). The plate was removed and air dried. The zone corresponding to rotenone was taken out and eluted with 1 ml ethanol. It was heated over a water bath at 70°C for 1 minute. 1 ml alcoholic vanillin (1%) was added to the warm alcoholic solution and the mixture was again heated over a boiling water bath (70°C) for 1 minute. It was allowed to cool at room temperature (5 minutes) and 0.5 ml conc. H_2SO_4 was

added to the mixture. This was again warmed at 70°C for 1 minute. The blue-violet colour, developed, was measured in spectrocolorimeter at the obsorption maxima of 601 nm (Fig.18). Percentage of rotenone was calculated from the standard curve (10^{3} to 10^{-1}ppm) prepared with authentic rotenone (Fig.19).

Estimation of rotenoid and rotenone from leaves

The leaves were collected according to the age (15, 30, 60, 90, 120, and 150 days old). As soon as the leaves were collected, these were kept in hot air oven at the temperature of 60°C for 2 days. The materials were powdered. Rotenoids and rotenone were estimated following the method described earlier.

Estimation of rotenoid and rotenone from root

Young and mature roots (age 30 and 180 days) were collected, powdered and rotenone and rotenoius were estimated following the methods, described earlier.

Estimation of rotenoid and rotenone in fruits and seeds

The age of the fertilized ovary having dried petals was considered as zero day for developing fruits. The estimation of rotenoid and rotenone in developing fruits and seeds were estimated according to the procedure mentioned earlier.

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Effect of temperature for the development of colour during the estimation of rotenone in presence of alcoholic Vanillin (1%) and Conc. H_2SO_4

No. of observations	Ethanolic soln. of plant sample (1 ml)	1% alcoholic Vanillin (1ml)	Conc.H_2SO_4 (0.5 ml)	Colouration	Optical Density
1	Cold	Cold	Cold	blue-violet	0.08
2	Warmed for 1 min.at 70°C	"	"	"	0.15
3	"	Warmed for 1 min. at 70°C	"	"	0.28
4	"	"	Warmed for 1 min. at 70°C	"	0.34
5	Warmed for 2 min. at 70°C	Warmed for 2 min. at 70°C	"	"	0.34
6	"	"	Warmed for 2 min. at 70°C	black	0.0

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Total Rotenoid and Rotenone contents in different plant parts of *T. candida* DC.

Plant parts	Age (days)	Rotenoid (%)	Rotenone (%)
Seedling	3	0.58	0.40
Leaf	15	0.80	0.50
	30	0.78	0.67
	60	0.87	0.74
	90	0.67	0.55
	120	0.56	0.43
	150	0.47	0.30
Root	30	1.25	1.00
	180	0.90	0.69
Whole fruit	15	1.02	0.76
Seed	60	1.40	0.90
	120	1.62	0.83
	365	1.23	0.78
	2190	0.70	0.35
Mean		0.86	0.63
S.E. (±)		0.34	0.21
C.D. (5% level)		0.54	0.43

RESULTS AND DISCUSSION

It has been noted that different workers proposed various methods for the quantitative estimation of rotenone. A gravimetric estimation of rotenone from air dried roots was performed earlier by Subba Rao (1945). Mayer & Rachmad (1947) proposed a colorimetric method for estimation of total rotenone content from finely ground root. The method needed 1 gm of plant sample and involved much time (2½ to 3 hrs) to complete the work. Spectrocolorimetric estimation of total rotenoids and rotenone was developed by Milton and Waters (1949). But, Jones (1945,1946) developed the spectrophotometric method and used 500 mg of dried plant sample for the estimation of rotenoids and rotenone and the method took about 1 hour. The light absorption (360 nm) by Dersis root extract with acetone was used for the estimation to total rotenoids by Pagan and Loustalot (1948). In this connection, 1 gm plant part was extracted with acetone for an hour. Total duration of method was about 2 hrs.

The method that has been proposed here in connection with the estimation of rotenone from plant parts of T. candida is based on the chemical reaction reported earlier by Haussler (1946,1947a). In the modified method, ethanolic solution of rotenone is treated with alcoholic Vanillin (1%) and heated over a boiling water bath. Blue-violet colour develops when the dried part was treated with conc. H_2SO_4 . The colouration was noted to be stable for half an hour and the solution had the absorption maxima at 601 nm (Fig.18). The standard curve was prepared and was noted to follow Beer's law

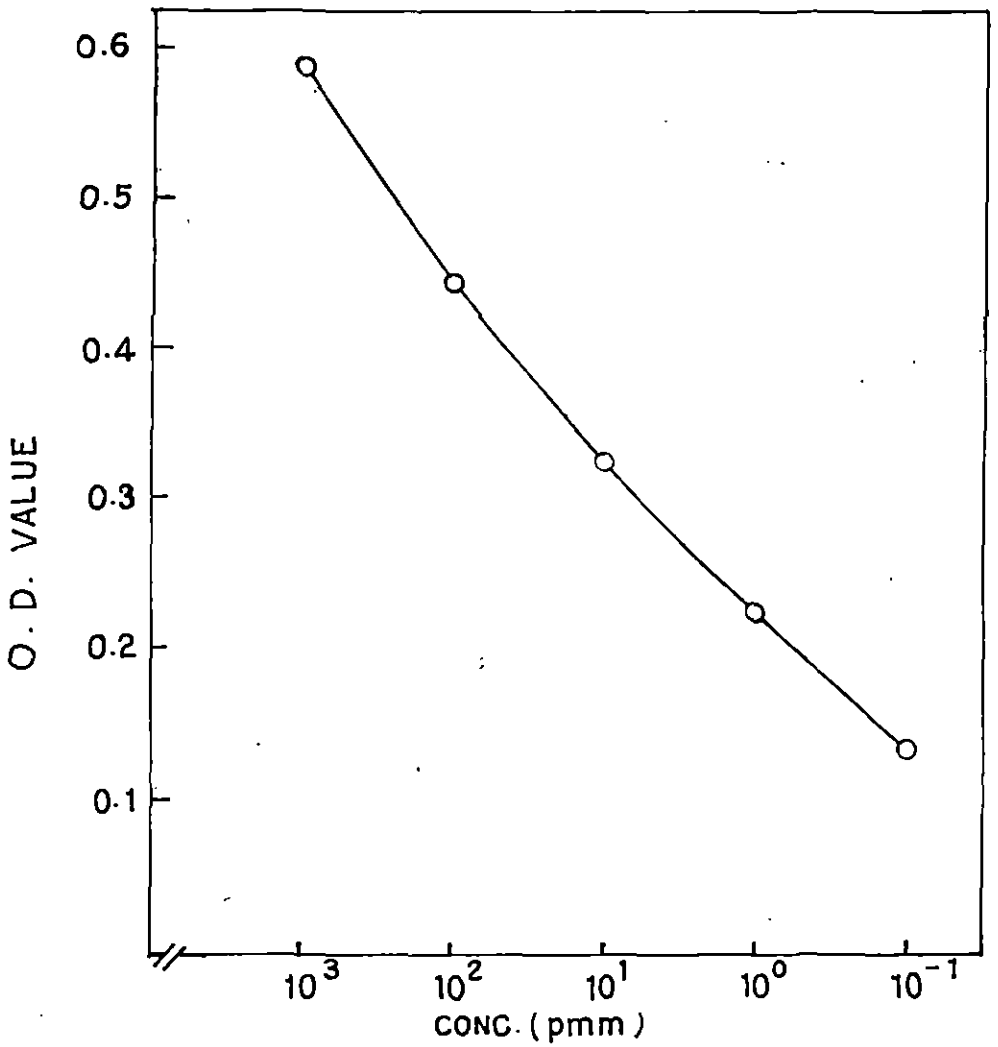

Fig. 17. STANDARD CURVE OF ROTENONE IN PRESENCE OF $NaNO_2$ AND CONC. H_2SO_4 .

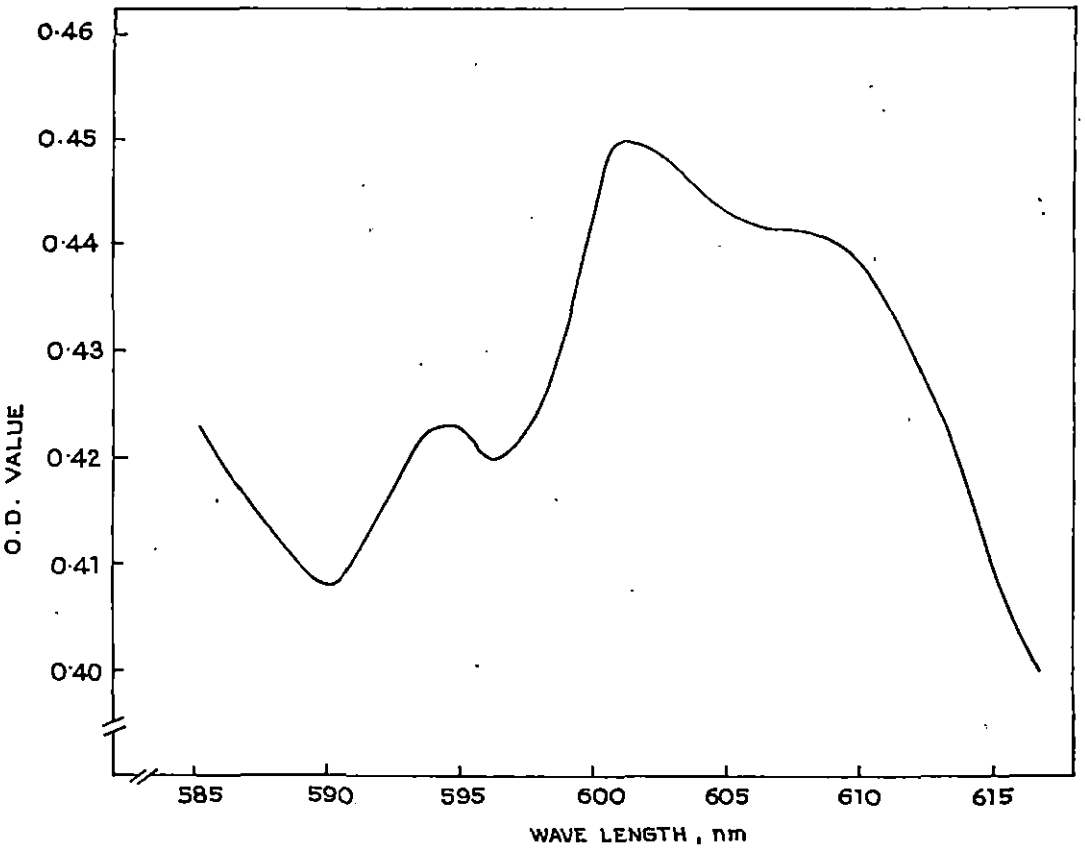

Fig. 18. Absorption maxima of Rotenone in presence of alcoholic Vanillin (1%) and conc. H_2SO_4.

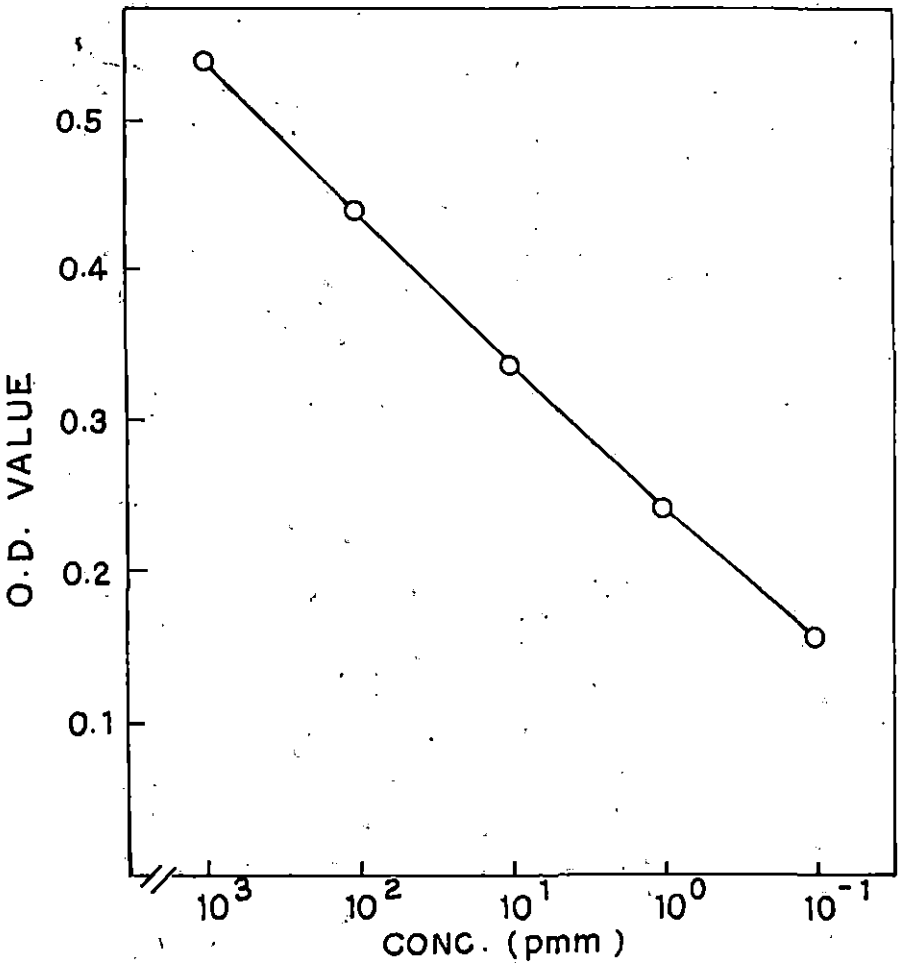

FIG. 19. STANDARD CURVE OF ROTENONE IN PRESENCE OF ALCOHOLIC VANILLIN (1%) AND CONC. H_2SO_4.

(Fig.19). It has also been observed that rotenone can be very easily estimated from only 100 mg of dry sample in contrast with the method proposed earlier by Jones (1946) where minimum 500 mg of dry sample was required. This new method is supposed to be very much sensitive for the estimation rotenone as low as 10^{-1}ppm of the active constituent and can be determined very easily (Fig.19). Moreover, the method is less time consuming as total duration for the estimation is supposed to cover only 25 minutes instead of 45 to 60 minutes for the earlier one and purification of rotenone from the crude extract can easily be obtained following thin layer chromatography.

Temperature requirement for the development of colour during the estimation of rotenone has been represented in Table-14. It has been observed that the characteristic blue-violet colour developed after heating the mixtures and O.D. value gradually increases as the mixture was warmed after the addition of concentrated H_2SO_4 (0.5ml) for 1 minute (Table-14). Moreover, 2 minutes heating turned the colour black showing the transmittance nil (Table-14). Thus, one minute heat treatment has been observed to be the optimum for the development of characteristic colour of rotenone with Vanillin (Table-14).

The accumulation of rotenoids and rotenone in the developing plant parts of T. candida was studied. Table-15 shows the accumulation of total rotenoids and rotenone in leaves according to the age. The maximum accumulation of rotenoids (0.87%) and rotenone (0.74%) was noted in 60 days old green leaf. Leaf of 15 and 30 days shows

0.80 and 0.78% total rotenoid, 0.50 and 0.67% rotenone respectively. Though younger leaf shows higher accumulation of active constituents in comparison to those of older leaf but much accumulation of the same has been observed in the leaves of the plant growing older. Though information on the rotenoid content according to the maturity of leaf is lacking but this observation confirm the result obtained in connection with other flavonoids (Rodwell, 1950; Humphrey, 1953; Krewson et al. 1953; and Basu, 1982) and polyphenols (Das et al. 1965). Though it has been noted by various authors that younger leaves contain more flavonoids and phenolics than that of older one it is not clear why such a high accumulation occur at the younger tissue. Recent progress in these investigation has largely been stimulated by the observation that the rates of synthesis and accumulation of flavonoids are higher in rapidly growing and differentiating tissues (Hahl brock et al. 1971; Barz and Hosel, 1975). It is, therefore, expected that the younger leaves which are also at the growing and differentiating stage, justify the high accumulation of rotenoid content. Table-15 shows that young root has accumulated greater amount of rotenoids and rotenone, (1.25 and 1.0% respectively) than the mature one i.e. 0.90 and 0.69% respectively. The values have been found to be higher than those obtained from leaf of any age (Table-15). Table-15 also shows the accumulation of rotenoids and rotenone in developing fruit. It has been noted that very immature fruit i.e. on "zero" day when pollination has already been completed as indicated by the withering of petals accumulates total rotenoids and rotenone in higher amounts (1.02 and 0.76% respectively). Mature

seed (120 days old) shows a fair amount of 0.83% rotenone and 1.62% total rotenoid as compared to younger seed (60 days old) showing high amount of 0.90 and 1.40% respectively (Table-15). However, during storage, the amount of rotenone & rotenoid in seed has been found to decrease within the period of one year i.e. 0.78% rotenone and 1.23% total rotenoid in old T. candida seeds (Table-15). In this connection, more significant decrease has been observed (0.35 and 0.70% respectively) in 6 years old non-viable seed of the plant (Table-15).

S U M M A R Y

A new and easy colorimetric method for the quantitative estimation of rotenone based on Vanillin Test has been proposed.

The content of total rotenoid and rotenone in different plant parts of T. candida has been determined.

The rotenone accumulation is observed to be the highest in young root (1.00%) and young seed (0.9%).

60 days old green leaf shows the highest accumulation of total rotenoid and rotenone, 0.87 and 0.74% respectively than those of other leaves.

Senescent and decomposed leaves have been noted to accumulate very low amount of both rotenoids & rotenone.

The highest amount of total rotenoid and rotenone have been noted to be accumulated in seed (1.62% and 0.83%).

One year storage of T. candida seeds shows a decrease in rotenoid and rotenone content.

A significant decrease has been observed in rotenoid and rotenone contents of 6 years old non-viable seeds of the plant.