



ISOLATION AND SYNTHESIS OF BIOLOGICAL ACTIVE NAPHTHOQUINONE DERIVATIVE FROM A PLANT SOURCE

Banasri HAZRA, S. PAL and A. BANERJEE

Departments of Pharmacy and Chemistry, Jadavpur University, Calcutta - 700 032, India

Background

The indigenous plant *Diospyros montana* Roxb. is known for its piscicidal nature and folklore use as a remedy for tumours; crushed leaves and fruits of the tree used by tribal fishermen for stupefying fish, while the fruit is reported to be used externally to treat boils and tumours. The stem-bark of the plant contains a bis-naphthoquinone compound, diospyrin (D ; I), the isolation and structure elucidation of which have been reported from various laboratories in India, Japan, Scotland and Portugal (1-7). However, there was no scientific report on its biological activity whatsoever, when we observed that a crude ethanolic extract of the powdered stem-bark could inhibit the *in vivo* growth of Ehrlich Ascites Carcinoma (E.A.C.) in Swiss A mice (8). An extensive fractionation of the plant extract, followed by systematic bio-assay of each component culminated in the identification of diospyrin to be the biologically active principle showing antitumor activity against Ehrlich Ascites Carcinoma and Sarcoma 180 in Swiss A mice (9). Subsequently, it was also observed and reported by us that diospyrin shows significant anti-protozoal activity as well towards *Leishmania donovani* promastigotes *in vitro* (10, 11).

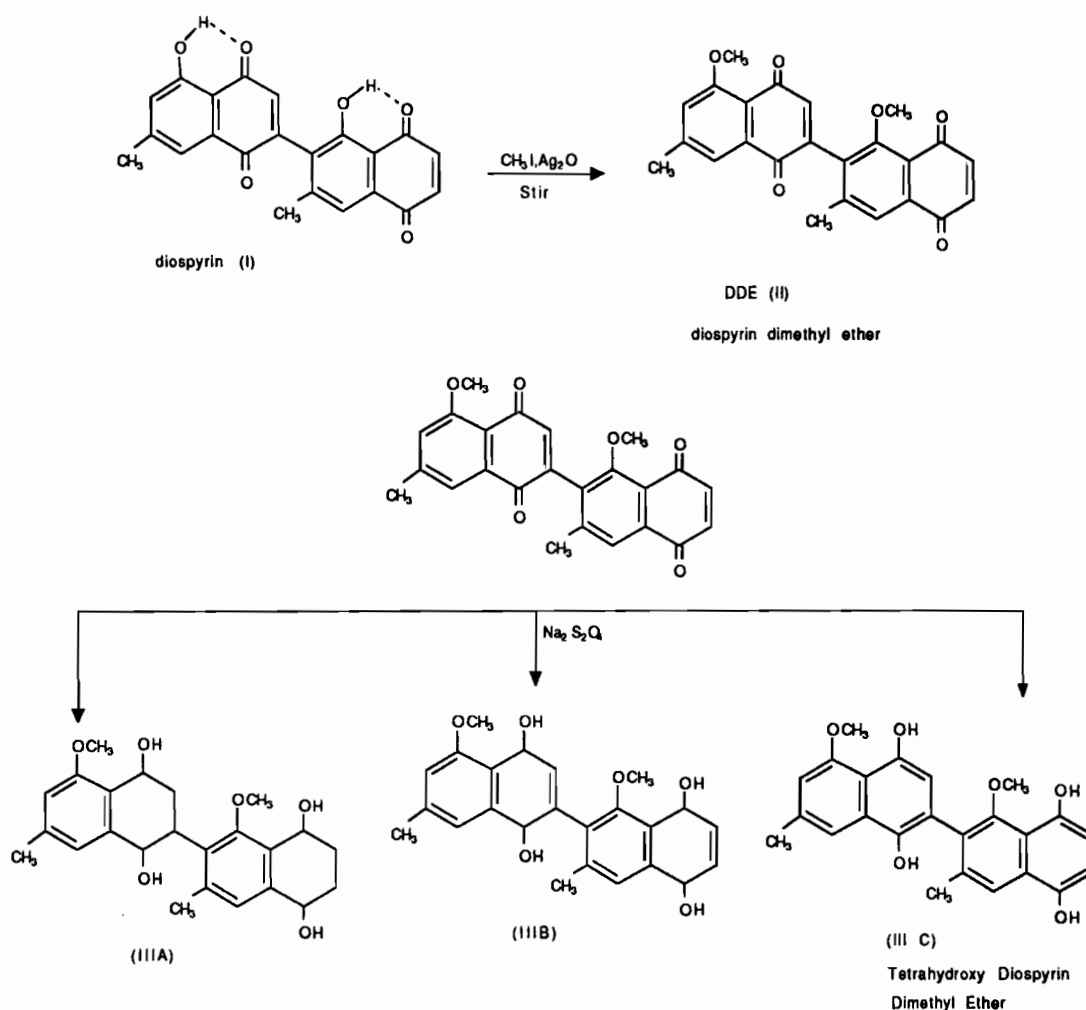
Chemistry

Once it was established that this naturally occurring naphthoquinone compound is a potential bio-active material, it was our endeavour to improve the chemotherapeutic activity of diospyrin by synthesising suitable derivatives of the same so that the cytotoxicity shown by diospyrin toward host cells could be minimised without reducing its *in vivo* activity towards E.A.C. An encouraging development towards this objective was the synthesis of diospyrin dimethyl ether (DDE ; II), a new compound with less toxicity, showing better *in vivo* activity against E.A.C. in Swiss A mice, as well as significant antileishmanial activity.

Diospyrin itself resists conventional chemical transformations due to the presence of strong chelation through hydrogen-bonding between the phenolic -OH and quinone groups. Hence, DDE has to be utilised as a prospective synthon for preparing other derivatives in this novel series of bio-active compounds, so far mostly unexplored for its interesting chemotherapeutic potential. Now, reduction of DDE with sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) gave a new compound 7,7'-dimethyl 1,4,1',4'-tetrahydroxy dimethyl ether of diospyrin (TDDE ; III). The structural identity of this new compound was established by the usual analytical and spectroscopic methods.

Effect on E. A. C.

Respiration (*in vitro*): The significant toxicity of this new compound towards E.A.C. cells *in vitro* was demonstrated by measuring the respiratory inhibition of the tumour cells in a Warburg respirometer. The data represent the amount of oxygen uptake by 4×10^7 E.A.C. cells in course of 45 minutes in absence and in presence of the drug in two different concentrations. In presence of 0.02 and 0.06 mg of TDDE, the inhibition of respiration were 21% and 69%, respectively (Fig. 1).



Bio-assay (*in vivo*): for carrying out *in vivo* bio-assay of this new compound, E.A.C. cells were harvested from an eight-day old transplant mouse, and a challenge of approximately 2×10^5 cells per mouse was given intraperitoneally to two groups of mice : "treated" (T) and "control" (C). The "T" group was treated with a suspension of the compound in DMSO after maceration with a drop of Tween 80. The dose-regimen was standardised by repeated trial and error. The data obtained represent the average from 3 sets of bio-assay experiments. It was found (Fig. 2) that the inhibition of *in vivo* growth of E.A.C. by the drug was quite significant. Two out of six mice in a "treated" group survived beyond 60 days, representing 33% cure, while the mean life span of the rest of the "T" mice was 33 ± 5.0 days and that of the "C" mice was 16 ± 2.2 days, representing an increase in life span of more than 100%. Another significant study was the measurement of "Packed Cell Volume" obtained by centrifugation of the total ascitic fluid aspirated out of the experimental mice around day "15". The mean volume of packed ascites cells (Table 1) was more than 5 ml for the "C"-group, while that for the "T"-group it was only about 0.2 ml, since no fluid was obtained from most of the "T"-group.

Group	P.C.V. (ml \pm S.E.)
Control (C)	5.48 - 0.48
Treated (T)	0.17 - 1.68

Table 1 : Effects of TDDE on packed cell volume of ascites fluid (collected Around Day 15)



The results of the bio-assay of TDDE are now compared with those obtained with the precursor DDE, as well as the natural product diospyrin. It is obvious that the synthetic derivatives of diospyrin could bring about an increase in life span of more than 100%, while it was about 50% with diospyrin itself.

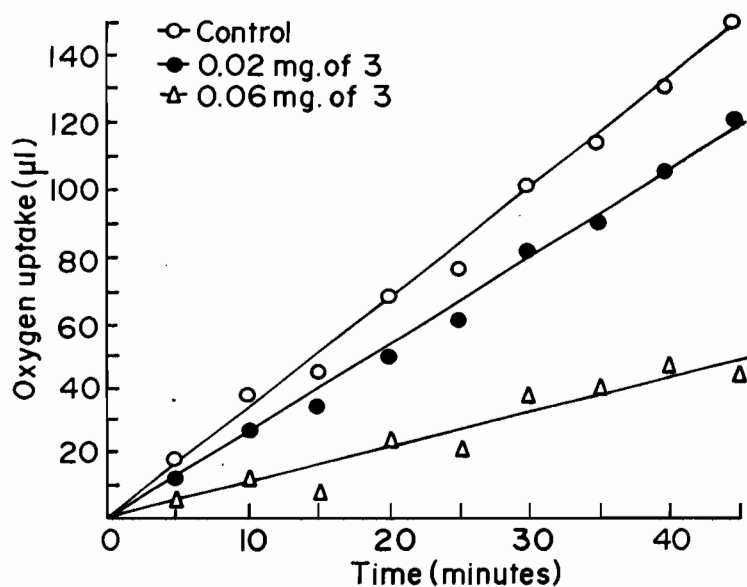


Fig.1 : Inhibition of respiration of E.A.C. cells (4×10^7) in presence of TDDE (III) at different concentrations.

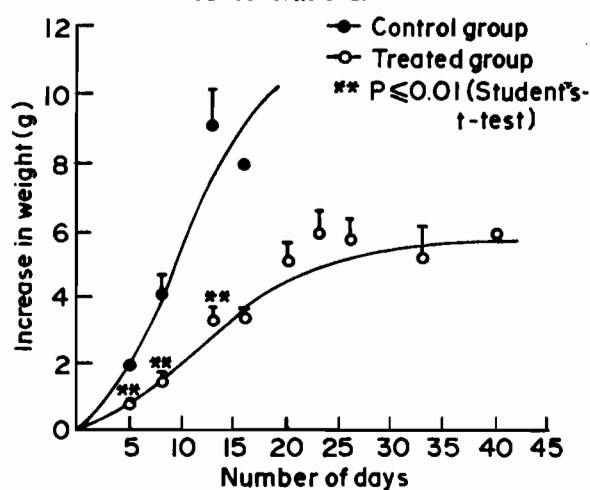


Fig.2 : Bio-assay of TDDE on E.A.C. in Swiss A mice. Each point with vertical bar represents the mean increase in weight of 6 mice \pm S.E.

Again, TDDE was as effective as DDE at about half the dose as required for DDE to bring about a similar chemotherapeutic effect (Table 2).



Drug used	Dose regiment (i.p.)	'Cure' (%) [>60 days]	Mean life Span 'T'[days]	Mean life Span 'C' [days]	Increase in life Span of 'T' w.r.t.'C' (%)
Diospyrin	Days 1-10 : 9 doses of 1.5 mg/kg/day each daily. Total : 13.5 mg/kg	20	28 ± 3.7	19 ± 0.8	47
DDE	Day 1 : 3 mg/kg/day Days 3-9 : 4 doses of 1.5 mg/kg/day each, on alternate days. Total : 9 mg/kg	33	4 ± 2.8	19 ± 0.6	110
TDDE	7 doses of 0.75 mg/kg/day each on days : 1,2,4,5,7,8 & 10. Total : 5.25 mg/kg	33	33 ± 5.0	16 ± 2.2	106

'T' : Treated Group

'C' : Control Group

Table 2 : Bio-Assay of Diospyrin and its Derivatives against E.A.C. in Swiss a Mice

Haematology

The process of tumour progression causes a multitude of pathological and bio-chemical changes in the host system, some of which are reflected in the haematological parameters, biochemical composition of body fluids and histopathological status of the vital organs. Hence, the analysis of peripheral blood drawn from the tail-veins of normal, cancerous and TDDE-treated mice were performed around day "15". The data show a remarkable increase in the total count of leucocytes in the "C"-group amounting to about 235% with respect to the "N"-group, while the increase in T.C. for the "T"-group was only about 29%. This is also reflected in the differential count (D.C.) of W.B.C., showing an abnormal rise in the neutrophil count for the "C" group, with a concomitant decrease in lymphocyte numbers, while almost total restoration was observed in the "T"-group (Table 3).



Group	Haemoglobin (G%±S.E.)	T.C. of W.B.C (x10 ⁶ /ml±S.E)	D.C.of W.B.C. Neutrophil	(% ± S.E.) Lymphocyte	T.C.of R.B.C. (x10 ⁹ /ml±S.E)
Normal (N)	10.20 ± 0.40	10.4 ± 0.6	35 ± 0.7	62 ± 0.9	8.8 ± 0.3
Control (C)	8.72 ± 0.33	34.8 ± 2.6	69 ± 1.7	31 ± 1.7	4.7 ± 0.2
Treated (T)	9.62 ± 0.25	13.4 ± 1.4	39 ± 0.4	61 ± 0.5	6.1 ± 0.3

Table 3 : TDDE : Haematological studies

Challenge : 1.05 x 10⁵ cells of Ehrlich Ascites Carcinoma

Drug Dose : 0.75 mg/kg/day

Treatment Schedule : Days 1,2,4,5,7,8 and 10.

Significant observations:

- Increase in T.C. of W.B.C. w.r.t. "N" group : 'C' group ~ 235 %
'T' group ~ 29%
- Increase in neutrophil count abnormal in 'C' group.
- Decrease in haemoglobin content and R.B.C. more in 'C' group than in 'T' group.

Biochemistry

A growing tumour is known to develop by using the resources of the host ; this causes unfavourable changes in nitrogen metabolism followed by a gradual depletion of the protein-reserve of the normal tissues of the host. In our experiments we also observed a substantial decrease in the protein content of the blood serum of the "C" group with respect to the "N" group, while a significant restoration is observed in the "T"-group (Table 4).

Serum sample collected from	Protein content of serum (µg/µl)	Protein content of serum (g/100 ml)
Group 'N'	111.33 ± 1.96	11.13 ± 1.96
Group 'C'	48.50 ± 2.47	4.85 ± 2.47
Group 'T'	71.40 ± 0.61	7.14 ± 0.61

- Each group contains 6 mice.; 2. Serum collected around day '15'.

Table 4 : Effect of TDDE- treatment on the total protein content in blood-serum of mice

Histopathology

All the experiments described so far could certainly establish the fact that the new synthetic derivative TDDE can not only effect a positive and significant inhibition of E.A.C. tumour growth *in vivo*, but also helps to restore the haematological and biochemical parameters of the treated mice. This was further established by studying the histopathological status of the vital organs of the "treated" mice as compared to the "control". The liver, kidney and spleen of "T" and "C"-group animals sacrificed around day "15" were preserved for histopathological examinations. The organs of mice surviving for more than 60 days were also studied to observe the long-term effect of the treatment with all the drugs. The photomicrograph of a liver section of a mouse on 65th day after the treatment with TDDE exhibits a normal architecture and hardly any damage, while liver sections of a control mouse on the 15th day after challenge show massive necrosis, hydropic degeneration and venous congestion. The kidney and spleen sections of a treated mouse show definite signs of recovery of normalcy.



Effect on leishmaniasis

In addition to the inhibitory activity towards E.A.C. exhibited by diospyrin and its synthetic derivatives, this interesting naphthoquinone compound has also been shown to possess significant antiprotozoal activity against *Leishmania donovani* promastigotes, the effect being stronger than that of a standard clinical agent, pentamidine. Since visceral leishmaniasis or *Kala-azar* is one of the major public health problems in many third-world countries, including India, it was interesting to investigate this property of diospyrin and its derivatives. It was found that DDE is more effective than D in this regard (Fig. 3). Growth inhibition of *Leishmania donovani* cells in liquid culture media is approximately ten times greater with DDE than with D. Scanning Electron Microscopic studies of *L. donovani* promastigotes (7×10^6 cells/ml) treated with $1 \mu\text{g/ml}$ of D and $0.1 \mu\text{g/ml}$ of DDE show extensive damage of flagella, as well as cytoskeletal structures of the treated cells with respect to the control. Now, similar investigations with TDDE are being undertaken and preliminary results show it to be equally promising.

Comparative cytotoxicities

Finally, the *in vivo* toxicities of diospyrin and its two synthetic derivatives have been studied on Swiss A mice by intraperitoneal inoculation with equal amounts of these drugs. It was found that with a dose of 40 mg/kg the mortality was 67% with diospyrin, 50% with DDE and 38% with TDDE.

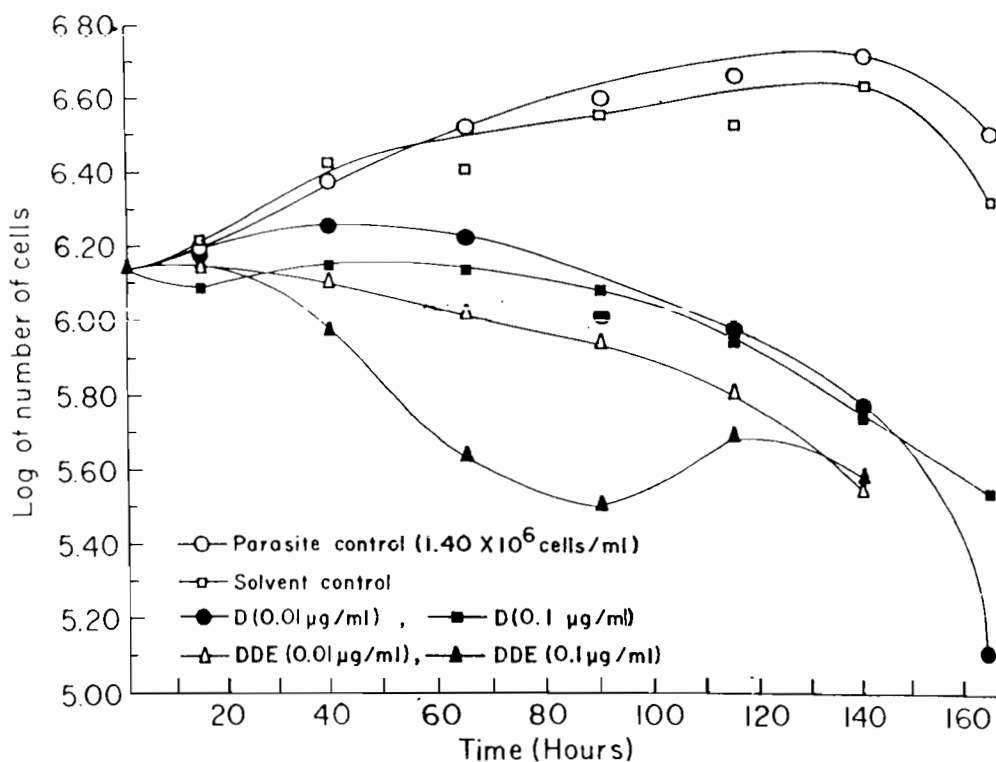


Fig. 3 : Inhibition of growth of *L. donovani* cells in liquid culture media containing diospyrin and DDE.

In conclusion, we must say that the synthesis of appropriate derivatives of a natural product was found to be a quite rewarding experience. Physicochemical studies incorporating cyclic voltametry on D and DDE vis-à-vis other anticancer agents also furnished encouraging



results. The electrochemical characteristics of these quinones indicate the feasibility of *in vivo* electron - transfer, the toxicity of the compounds resulting from an oxygen-dependent redox cycling mechanism (12).

Acknowledgement: this work is supported by the University Grants Commission, India and the International Foundation for Science (Grant No. F/1836-1), Sweden.

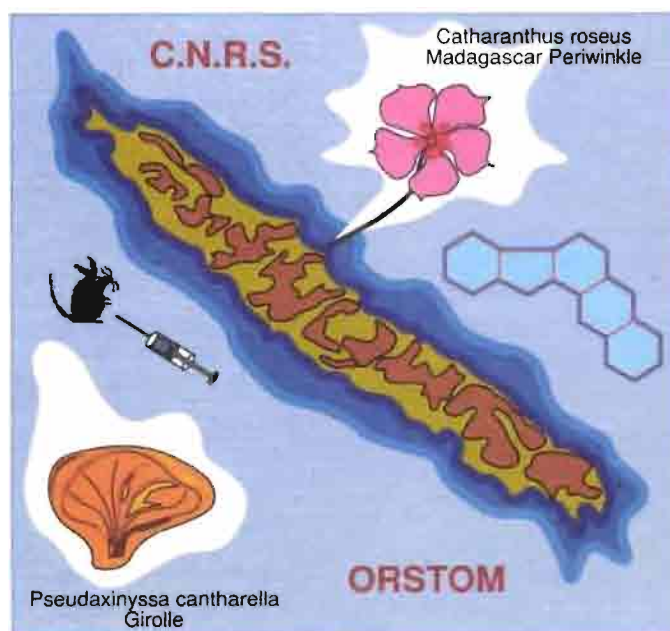
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Troisième Symposium sur les substances naturelles d'intérêt biologique de la région Pacifique-Asie

Nouméa, Nouvelle-Calédonie, 26-30 Août 1991

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Editeurs : Cécile DEBITUS, Philippe AMADE,
Dominique LAURENT, Jean-Pierre COSSON