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Short communication

Antitumour evaluation of di-(2-ethylhexyl) phthalate (DEHP) isolated from *Calotropis gigantea* L. flower

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The objective of the study is to explore the anticancer activity of di-(2-ethylhexyl) phthalate (DEHP) isolated from *Calotropis gigantea* flower against Ehrlich ascites carcinoma cells (EAC) in Swiss albino mice. The activity of DEHP was evaluated at doses of 10, 20 and 40 mg kg^{-1} body mass applied intraperitoneally. DEHP showed a significant decrease in viable cell count ($p < 0.05$), mass gain (due to tumour burden) and elevated the life span of EAC cell bearing mice. Altered hematological profiles such as RBC, hemoglobin, WBC and differential count were reverted to normal levels in DEHP-treated mice. DEHP also brought back altered biochemical parameters (glucose, cholesterol, triglycerides, blood urea, SALP and SGOT) to normal level. Results of this study indicate that DEHP show potent dose dependent antitumour activity against EAC *in vivo*.

Keywords: Calotropis gigantea (Asclepiadaceae), flower, di-(2-ethylhexyl) phthalate, antitumour activity

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Plants have a long history of use in the treatment of cancer. Over 60 % of currently used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and microorganisms (1). Hence the impact of medicinal plants in cancer treatment should be considered in order to discover new drug molecule for cancer research.

The genus *Calotropis* R. Br (*Asclepiadaceae*) comprises four species, but in Indian subcontinents including Bangladesh, it is mainly represented by two popular species *viz.*, *Calotropis procera* and *Calotropis gigantea* (2). *Calotropis gigantea* L. grows in the tropical region and is most abundant in Bangladesh, India, Burma, Pakistan and in the sub-Himalayan tract (3). In small doses, powdered flowers of *Calotropis gigantea* are useful in the treatment of colds, coughs, asthma, catarrh, indigestion and loss of appetite (4). Various parts of this plant are reported to possess multiple therapeutic properties, like antipyretic, analgesic, anticonvulsant, anxiolytic, sedative, hepatoprotective, wound healing,

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antidiabetic, larvicidal, anti-inflammatory and anti-diarrhoeal (4). Chemical investigations of *Calotropis gigantea* report isolation of different types of phytochemicals such as cardenolides, flavonoids, glycosides, triterpenoids, steroids, *etc.* (4). In our earlier screening, ethyl acetate extract of *Calotropis gigantea* flower was shown to possess potent antitumour activity against Ehrlich ascites carcinoma (EAC) cells in mice (5), but in order to explain this activity, it was necessary to isolate and identify the active chemicals of ethyl acetate extract that were involved in antitumour activity. In continuation, the present study demonstrates the antitumour effect of di-(2-ethylhexyl) phthalate (DEHP) isolated from *Calotropis gigantea* flower against EAC cells in mice.

EXPERIMENTAL

Plant material

The flowers (flower petals) of *Calotropis gigantea* (*Asclepiadaceae*) were collected in March, 2010 from the relevant area of the Rajshahi University campus, Bangladesh, and authenticated by Professor A. T. M. Naderuzzaman, Department of Botany, University of Rajshahi, Bangladesh. A voucher specimen was preserved in the Department of Botany, University of Rajshahi, Bangladesh.

Isolation and extraction

The collected flowers of *Calotropis gigantea* were shed-dried and powdered. Flower (1.0 kg) was kept immersed in 3.0 liters ethyl acetate (Merck, Germany) kept in an aspirator bottle at room temperature for 15 days with occasional shaking and stirring. Then, the contents were pressed through a tincture press (Karlkolb, Germany) and the extract was then filtered through filter paper (Whatman No. 1) and concentrated with a rotary evaporator under reduced pressure at 40 °C to obtain 38.6 g crude ethyl acetate extract. Crude ethyl acetate extract (15 g) was applied on silica gel (Merck, 0.15–0.3 mm) and chromatographied using *n*-hexane (Merck) with a gradient of ethyl acetate up to 100 %; sixty four fractions were collected. Among these fractions, fractions 40–48 were further subjected to preparative thin layer chromatography. The sample was dissolved in ethyl acetate and applied to the plates as a uniform band. The plates were developed with a mobile phase of *n*-hexane/methanol (20:0.1). After development, the plates were allowed to dry and under UV light (254 and 366 nm); the specific active band was scraped off from each plate. The compound was eluted from the collected silica matrix by dissolving it in ethyl acetate and removing from the silica gel by filtration. Fractions 40–48 afforded the pure compound (375 mg) as colorless oily liquid, which was designated as compound **1**. The purity of the isolated compound was checked on TLC plates.

^{1}H NMR (DMSO-d_6) δ 7.11 (dd, J = 6.3 ~ 2.2 Hz, 1H), 6.96 (dd, J = 6.3 ~ 2.2 Hz, 1H), 4.15 (m), 2.60 (m), 2.30 (dq, J = 4.3 Hz, 2H), 0.93 (t, J = 4.3 Hz, 3H), 1.23 ~ 1.40 (m), 0.84 (t, J = 5.3 Hz, 3H); ^{13}C NMR (DMSO-d_6) δ 171.10, 132.65, 124.75, 118.95, 65.21, 40.76, 29.67, 29.50, 24.80, 22.68, 20.79, 14.11.

General methods

IR-spectra were taken on an FTIR-8900 spectrophotometer (Shimadzu, Japan). High Resolution TOF mass spectra were obtained using a Waters LCT Premier mass spectrometer (USA) coupled with a Waters AQUITY HPLC system. Data acquisition was achieved using MassLynx software, version 4.0. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 MHz FT spectrometer (DPX-400, Switzerland).

Experimental animals

Male Swiss albino mice (6–8 weeks old, weighing 25 to 30 g) were procured from the Animal Research Branch of the International Centre for Diarrhoeal Diseases and Research, Dhaka, Bangladesh. The mice were grouped and housed in iron cages and maintained under standard laboratory conditions (temperature 25 ± 2 °C, humidity 55 ± 5 %) with 12 h dark/light cycles. The animals were fed on pellet food and tap water was available *ad libitum*. The animals were acclimatized to the laboratory for at least 5 days before testing. The experiments were carried out after approval of the protocol by the Institutional Ethics Committee for Experimentations on Animal, Human, Microbial and Living Natural Sources (225/320-IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Tumour cells

EAC cells were obtained by the courtesy of Indian Institute for Chemical Biology, Kolkata, India, and were maintained by weekly intraperitoneal inoculation of 10^5 cells per mouse in the laboratory.

Antitumour studies

In order to determine the effect of compound **1** on EAC cell growth, 30 mice were randomly divided into five groups (6 animals in each group). For therapeutic evaluation, the mice of all groups were inoculated with 1.5 x 10^5 cells per mouse on day 0. After 24 hours, the mice in group 1 received intraperitoneally 2 % (*V*/*V*) dimethyl sulfoxide (Sigma, USA) and the group was designated as EAC control. Compound **1** (dissolved in 2 % dimethyl sulfoxide) was administered *i.p.* into the mice of groups 2, 3 and 4 at doses of 10, 20 and 40 mg kg^{-1} body mass, respectively, whereas group 5 received the standard drug, bleomycin (Biochem Pharmaceutical, India) at the dose of 0.3 mg kg^{-1} body mass. Treatment was continued for 5 days. On day 6 after tumour inoculation, animals were sacrificed and tumour cells were collected by repeated washing with 0.9 % saline. Viable tumour cells per mouse of the treated groups were compared with untreated controls (5).

To study the effect of compound **1** on survival time, mice in five groups were inoculated with 1.3 x 10^5 cells per mouse on day 0 and a similar design (as described above) for treatment was followed. The treatment was continued for 10 days. The average body mass gain (after 12 days) and mean survival time (MST) of each group were noted. The mean survival time of the treated groups was then compared with the EAC control and the percentage increase in life span was also calculated (5).

Effect on haematological and biochemical parameters

Swiss albino mice were divided into six groups (n = 6). All animals except group 1, were injected with EAC cells (2 x 10^5 cells per mouse) intraperitoneally. This was taken as day 0. On the first day, normal saline (5 mL kg^{-1} per day) was administered in group 1 (normal control) whereas 2 % dimethyl sulfoxide (5 mL kg^{-1} per day) was administered in group 2 (untreated EAC control). Groups 3, 4 and 5 were treated with compound **1** at doses of 10, 20 and 40 mg kg^{-1} body mass, respectively. Group 6 received bleomycin 0.3 mg kg^{-1} body mass. All treatments were given 24 h after tumour inoculation, once daily for 10 days. Hemoglobin content (Hgb), red blood cell (RBC) and white blood cell (WBC) counts were taken from freely flowing tail vein blood (6) and differential count of WBC was carried out from Leishman stained blood smear (6) of each mouse of each group on day 12 after tumour inoculation. Then, all mice were sacrificed and blood was collected by cardiac puncture. Serum was separated by centrifugation at 4000 rpm for 10 minutes and analyzed for glucose, total cholesterol, urea, triglyceride, serum alkaline phosphatase (SALP), serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) in a Bioanalyzer (Microlab 200, USA) using commercial kits (Atlas Medica, UK).

Statistical analysis

All values were expressed as mean ± SEM. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Dunnett's t-test using SPSS statistical software, version 14.

RESULTS AND DISCUSSION

Isolated and purified compound **1** was characterized by IR, mass and NMR spectral data. Molecular formula for compound **1** was deduced as $C_{24}H_{38}O_4$ through EI-MS, which showed the molecular ion (M^+) peak at m/z 390.3617 (calcd for $C_{24}H_{38}O_4$). The presence of a phthalate was inferred from the EI-MS peaks at m/z 167 and m/z 149. The IR spectrum revealed a carbonyl band observed at 1739 cm^{-1} and strong C-O bands in the range 1047–1250 cm^{-1}.

Finally, by comparison of ^1H and ^{13}C NMR data with those published in literature (7), compound **1** was identified as di-(2-ethylhexyl) phthalate (DEHP) (Fig. 1). DEHP is

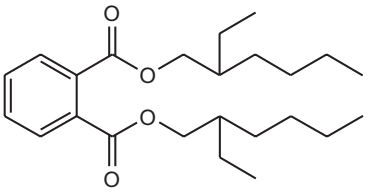

Fig. 1. Di-(2-ethylhexyl) phthalate (DEHP, compound **1**).

actually a well known synthetic plasticizer and has been already reported to be present in different plant species (8–10). But to the best of our knowledge, DEHP from *Calotropis gigantea* and also from *Calotropis* genus is reported for the first time here. In addition, DEHP has a low toxic potential in oral or intraperitoneal administration (LD_{50} > 25 g kg^{-1}) to mice and rats (11).

In antitumour evaluation, DEHP has been found to be capable of reducing viable EAC cells and mass gain of EAC cell bearing mice. The reduction ability of DEHP increases in a dose-dependent manner (Table II). With 40 mg kg^{-1} (*i.p.*), DEHP showed maximum reduction of viable EAC cells compared to that of the control (Table I).

The effect of DEHP on the survival of EAC bearing mice is shown in Table II. The MST of the control group was 21.5 ± 0.5 days, while it was 26.0 ± 1.9, 29.3 ± 1.1 ($p < 0.05$), 32.4 ± 1.1 ($p < 0.05$) and 39.2 ± 0.9 ($p < 0.05$) for the groups treated with DEHP (10, 20 and 40 mg kg^{-1}) and bleomycin (0.3 mg kg^{-1}), respectively. DEHP at doses of 20 and 40 mg kg^{-1} body mass, significantly elevated the MST and ILS of EAC tumor bearing mice (Table II). Prolongation of life span is a reliable criterion for judging the efficacy of any anticancer drug (12) and DEHP was able to meet this criterion.

In cancer chemotherapy, myelosuppression and anemia are the major problems (13). Anemia encountered in tumour bearing mice is mainly due to reduction in RBC or hemoglobin percentage, which may occur either due to iron deficiency or due to haemolytic or myelopathic conditions (13). In this study, haematological parameters of tumour bearing mice on day 12 showed significant changes compared to normal mice (Table II). On day 12 after tumour cell inoculation, treatment with DEHP (20 and 40 mg kg^{-1}) could significantly ($p < 0.05$) change these altered parameters more or less to normal (Table II). This indicates that DEHP possesses protective action on the haematopoietic system.

Biochemical parameters shown in Figs. 2 and 3 indicate the elevated level of cholesterol, triglycerides, urea, SALP and SGOT in serum in the EAC control group with respect to normal animals, while glucose level was significantly ($p < 0.05$) reduced from the normal value. Development of hypoglycemia and hyperlipidemia in experimental

Table I. Effect of DEHP on the viable cell count, survival time, life span and body mass gain in EAC bearing mice

Treatment	Viable tumour cells (x 10^7 cells mL^{-1} per mouse)[a]	Median survival time (day)	Increase of life span (%)	Body mass gain[b]
EAC control	4.05 ± 0.25	21.5 ± 0.5	–	54.23
EAC + DEHP (10 mg kg^{-1})	2.37 ± 0.19[c]	26.0 ± 1.9	21.02	37.61
EAC + DEHP (20 mg kg^{-1})	1.87 ± 0.17[c]	29.3 ± 1.1[c]	36.04	34.33
EAC + DEHP (40 mg kg^{-1})	1.27 ± 0.13[c]	32.4 ± 1.1[c]	50.55	25.15
EAC + bleomycin (0.3 mg kg^{-1})	0.26 ± 0.05[c]	39.2 ± 0.9[c]	82.32	15.45

Data are expressed as mean ± SEM ($n = 6$).
Significant difference with respect to EAC control: [c] $p < 0.05$.
[a] Day 6 after inoculation.
[b] After 12 days.

Table II. Effect of DEHP on haematological parameters of EAC cell bearing mice

Parameter	Treatment (mg kg^{-1} body weight)					
	Normal + salie	EAC + 2% DMSO	EAC + DEHP (10)	EAC + DEHP (20)	EAC + DEHP (40)	EAC + Bleomycin (0.3)
Hgb (g per 100 mL)	15.4 ± 0.22	7.35 ± 0.20[a]	7.76 ± 0.82	9.83 ± 0.68[b]	9.92 ± 0.58[b]	14.3 ± 0.25[b]
RBC(x 10^9 cells mL^{-1})	5.67 ± 0.10	2.27 ± 0.06[a]	2.69 ± 0.13	3.41 ± 0.27[b]	4.51 ± 0.42[b]	4.90 ± 0.09[b]
WBC(x 10^6 cells mL^{-1})	8.75 ± 0.53	25.3 ± 1.19[a]	16.1 ± 0.01[b]	17.1 ± 1.75[b]	15.7 ± 1.75[b]	9.37 ± 0.59[b]
Lymphocytes (%)	77.5 ± 1.36	33.8 ± 1.35[a]	34.5 ± 3.28	40.0 ± 2.29	49.6 ± 3.18[b]	68.2 ± 0.90[b]
Neutrophils (%)	19.6 ± 1.38	63.7 ± 1.04[a]	59.2 ± 3.47	53.0 ± 2.17[b]	44.8 ± 2.93[b]	28.8 ± 0.93[b]
Monocytes (%)	1.87 ± 0.40	1.50 ± 0.37	3.87 ± 0.71	4.75 ± 0.64[b]	3.75 ± 0.56[b]	2.00 ± 0.27

Data are expressed as mean ± SEM (n = 6).
Doses in mg kg^{-1} in parentheses.
Significant difference with respect to normal: [a] $p < 0.05$, with respect to EAC control: [b] $p < 0.05$.

animals with carcinoma has been previously reported (14). In this experiment, reduced glucose and elevated cholesterol and triglyceride levels were restored to more or less normal level in drug treated mice (Fig. 2). Inoculation and progression of EAC proliferation in mice are also associated with some extent of kidney damage and blood urea elevation (14). DEHP reduced the elevated level of blood urea.

Significant elevation in the SGOT and SALP levels reveals that, to some extent, cellular damages were associated with EAC 12 days after inoculation (15). Treatment with DEHP restored elevated SGOT and SALP to more or less normal range (Fig. 3), indicat-

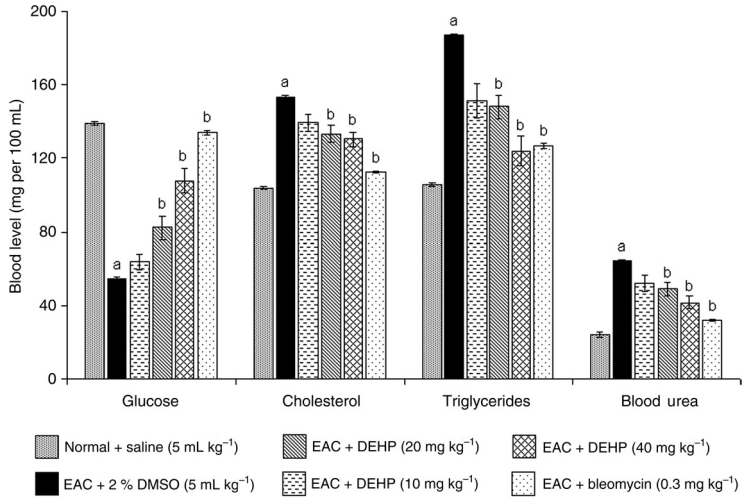

Fig. 2. Effect of DEHP on glucose, cholesterol, triglycrides and blood urea level of EAC cell bearing mice. Data are expressed as mean ± SEM (n = 6). Significant difference with respect to normal: [a] $p < 0.05$; with respect to EAC control: [b] $p < 0.05$.

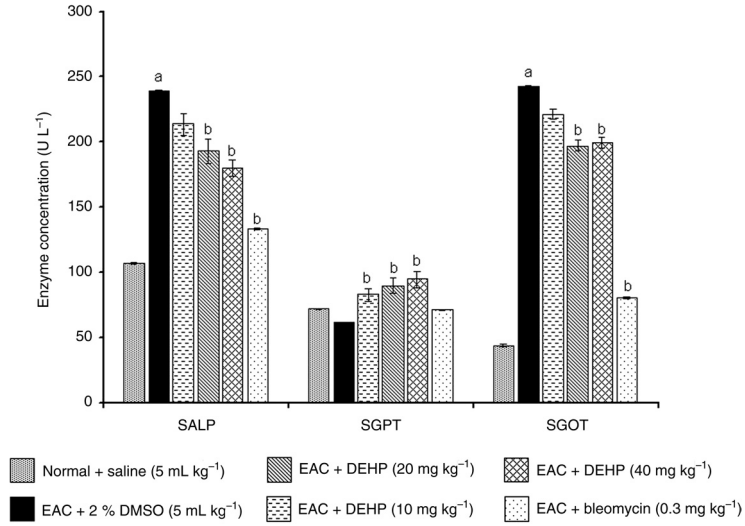

Fig. 3. Effect of DEHP on SALP, SGPT and SGOT of EAC cell bearing mice. Data are expressed as mean ± SEM (n = 6). Significant difference with respect to normal: [a] $p < 0.05$; with respect to EAC control: [b] $p < 0.05$.

ing protection of the tumour cell induced cellular damages by DEHP. However, significant elevation in SGPT activity was observed after DEHP treatment at 10, 20 and 40 mg kg^{-1} (Fig. 3).

In addition to our findings, it is necessary to mention that Lee *et al.* (10) also reported i*n vitro* anti-leukaemic and anti-mutagenic effects of DEHP isolated from *Aloe vera* against human tumour cell lines.

CONCLUSIONS

In this study, we have demonstrated *in vivo* the remarkable antitumour activity of di-(2-ethylhexyl) phthalate (DEHP) isolated from the ethyl acetate extract of *Calotropis gigantea* flower against EAC in mice and identified DEHP as one of the active constituents of ethyl acetate extract against EAC. The mechanisms of action should be further studied.

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REFERENCES

1. D. J. Newman, G. M. Cragg and K. M. Snader, Natural products as sources of new drugs over the period 1981–2002, *J. Nat. Prod.* **66** (2003) 1022–1037; DOI: 10.1021/np030096l.
2. Z. N. Wang, M. Y. Wang, W. L. Mei, Z. Han and H. F. Dai, A new cytotoxic pregnanone from *Calotropis gigantea*, *Molecules* **13** (2008) 3033–3039; DOI: 10.3390/molecules13123033.
3. A. K. Pathak and A. Argal, CNS activity of *Calotropis gigantea roots*, *J. Ethnopharmacol.* **106** (2006) 142–145; DOI: 10.1016/j.jep.2005.12.024.
4. A. K. K. Mueen, A. C. Rana and V. K. Kixit, *Calotropis* species (Asclepiadaceae): A comprehensive review, *Pharmacog. Mag.* **1** (2005) 48–52.
5. M. R. Habib, A. A. Aziz and M. R. Karim, Inhibition of Ehrlich's ascites carcinoma by ethyl acetate extract from the flower of *Calotropis gigantea* L. in mice, *J. Appl. Biomed.* **8** (2010) 47–54; DOI 10.2478/v10136-009-0007-7.
6. M. Jesmin, M. M. Ali and J. A. Khanam, Antitumour activities of some schiff bases derived from benzoin, salicylaldehyde, amino phenol and 2,4 dinitrophenyl hydrazine, *Thai J. Pharm. Sci.* **34** (2010) 20–31.
7. P. Amade, M. Mallea and N. Bouaicha, Isolation, structure identification and biological activity of two metabolites produced by *Penicillium olsonii* Bainier and Sartory, *J. Antibiot.* **47** (1994) 201–207.
8. G. N. Rao, P. M. Kumar, V. S. Dhandapani, T. R. Krishna and T. Hayashi, Constituents of *Cassia auriculata*, *Fitoterapia* **71** (2000) 82–83; DOI: 10.1016/S0367-326X(99)00108-2.
9. H. Mavar-Manga, M. Haddad, L. Pieters, C. Baccelli, A. Penge and J. Quetin-Leclercq, Anti-inflammatory compounds from leaves and root bark of *Alchornea cordifolia* (Schumach. & Thonn.) Müll. Arg., *J. Ethnopharmacol.* **115** (2008) 25–29; DOI: 10.1016/j.jep.2007.08.043.
10. K. H. Lee, J. H. Kim, D. S. Lim and C. H. Kim, Anti-leukaemic and anti-mutagenic effects of di-(2-ethylhexyl) phthalate isolated from *Aloe vera* Linn., *J. Pharm. Pharmacol.* **52** (2000) 593–598; DOI: 10.1211/0022357001774246.
11. R. M. David, M. R. Moore, D. C. Finney and D. Guest, Chronic toxicity of di-(2-ethylhexyl) phthalate in mice, *Toxicol. Sci.* **58** (2000) 377–385; DOI: 10.1093/toxsci/58.2.377.
12. A. Bala, B. Kar, P. K. Haldar, U. K. Mazumder and S. Bera, Evaluation of anticancer activity of Cleome gynandra on Ehrlich's ascites carcinoma treated mice, *J. Ethnopharmacol.* **129** (2010) 131–134; DOI: 10.1016/j.jep.2010.03.010.
13. M. Gupta, U. K. Mazumder, R. S. Kumar, T. Sivakumar and M. L. M. Vamsi, Antitumor activity and antioxidant status of Caesalpinia bonducella against Ehrlich ascites carcinoma in Swiss albino mice, *J. Pharmacol. Sci.* **94** (2004) 177–184, DOI: 10.1254/iphs.94.177.
14. R. A. Killington, A. E. Williams, N. A. Ratchffe, T. P. Whitehead and H. Smith, hypoglycaemia and malignancy differences of closely related sublines of a rat tumour, *Brit. J. Cancer.* **25** (1971) 93–102; DOI: 10.1038/bjc.1971.13.
15. P. Saha, U. K. Mazumder, P. K. Halder, S. Naskar, S. Kundu, A. Bala and B. Kar, Anticancer activity of methanol extract of *Cucurbita maxima* against Ehrlich ascites carcinoma, *Int. J. Res. Pharm. Sci.* **2** (2011) 52–59.

S A Ž E T A K

Evaluacija antitumorskog djelovanja di-(2-etilheksil)-ftalata (DEHP) izoliranog iz cvjetova *Calotropis gigantea* L.

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U radu je opisano ispitivanje antitumorskog djelovanja di-(2-etilheksil)-ftalata (DEHP) iz cvjetova *Calotropis gigantea* na Ehrlichove tumorske stanice (EAC) na švicarskim albino miševima. Aktivnost DEHP-a praćena je u koncentracijama od 10, 20 i 40 mg kg^{-1} tjelesne mase nakon intraperitonealne primjene. DEHP je pokazao značajno povećanje viabilnih stanica ($p < 0,05$), prirast mase (zbog tumora) te produljenje preživljavanja miševa s EAC karcinomom. Poremećeni hematološki profili kao što su RBC, hemoglobin, WBC i diferencijalna krvna slika vratili su se na normalne vrijednosti kod miševa tretiranih s DEHP-om. DEHP je također vratio u normalu promijenjene biokemijske parametre (glukozu, kolesterol, trigliceride, koncentraciju uree u plazmi, SALP i SGOT). Rezultati pokazuju da DEHP posjeduje snažno, o dozi ovisno antitumorsko djelovanje na EAC *in vivo*.

Ključne riječi: *Calotropis gigantea*, cvijet, di-(2-etilheksil)-ftalat, antitumorsko djelovanje

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