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Jatrophane Diterpenoids With Protective Effect on Human Lymphocytes DNA

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Abstract

Two sets of structurally different jatrophanes (**1-11** and **13-16**), jatrophane **12**, and latex extract of 2 *Euphorbia* species (**17** and **18**) were tested for in vitro protective effect against chromosome aberrations in peripheral human lymphocytes using the cytokinesis-block micronucleus (CBMN) assay. Jatrophanes **1-6** in minimal doses of 1 μg/mL prominently decreased micronuclei (MN) frequency in the range 44.86% to 34.29% and manifested considerable protective effect. From the other set of jatrophanes, **13** in the same minimal dose notably decreased MN frequency by 31.05%, while extracts **17** and **18** at a concentration of 4 μg/mL remarkably decreased the frequency of MN by 37.94% and 36.12%, respectively. Jatrophanes **12**, **14,** and **16** showed moderate protection, while **7-11** and **15** were less active than positive control. The structure-activity relationship (SAR) studies of the tested jatrophanes (**1-16**) indicated the favorable position of benzoate at C-8 or C-9 (**3**, **4,** and **13**) and a preference of isobutanoyloxy group at C-3 (**1-3**) rather than propanoyloxy at the same position (**4-6**) for pronounced protective effect on human lymphocytes DNA. In a previous SAR study on 11 jatrophanes (**1**, **3-8**, and **13-16**), the same structural features in **3**, **4,** and **13** influenced powerful inhibition of P-gp, while growth inhibition of cancer cells was more than doubled in **1** (isobutanoyloxy group at C-3) compared to **6** (propanoyloxy at C-3).

Keywords

CBMN assay, MN, human lymphocytes, jatrophanes, protective effect, SAR studies

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Some degenerative illnesses such as Alzheimer's and Parkinson's disease manifest in higher level of micronuclei (MN) as products of chromosome breakage or loss in peripheral blood lymphocytes.[1,2] The cytokinesis-block micronucleus (CBMN) assay, based on cytokinesis inhibition by cytochalasin B (Cyt-B), has facilitated MN analysis exclusively in binucleate (BN) cells that have completed their first in vitro division after treatment with the test agent or following culture initiation.[3-7] The CBMN assay in peripheral blood lymphocytes has been used to detect DNA and chromosome damage as a consequence of an autoimmune disease,[8] chronic kidney disease,[9] type 2 diabetes,[10] metabolic syndrome,[11] head and neck cancer,[12] and other illnesses, or to evaluate the presence and extent of chromosomal damage in human populations that are exposed to genotoxic agents.[13]

Natural phenolic compounds from medicinal herbs and dietary plants play an important role in cancer prevention and treatment.[14] CBMN assays have shown that treatment of human lymphocytes with natural phenolic compounds, such as flavonoids,[15,16] diarylheptanoids,[17,18] or seeds extracts from grape,[19] raspberry, blackberry, and currant,[20-22] induced decrease in the frequency of micronuclei and manifested protective effect on human lymphocytes DNA, compared to the action of alkylating agent mitomycin C (MMC) (negative control) and/or amifostine WR-2721 (positive control).

Genus *Euphorbia* (family Euphorbiaceae) is a rich source of jatrophanes, macrocyclic diterpenoids with *trans*-bicyclo[10.3.0.]pentadecane structure characterized by a flexible 12-membered ring. Jatrophanes may perform different biological activities such as cytotoxic,[23] antimicrobial,[24] antiviral,[25,26] and lipid-lowering activity,[27] or may modulate overexpression of P-glucoprotein (P-gp) in order to overcome multidrug resistance to anticancer drugs.[28,29] Several jatrophanes exhibit microtubule (MT) interacting activity

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and induce paclitaxel-like microtubules.[30,31] Compounds that stabilize MT represent potential therapeutic approach for the treatment of Alzheimer's disease and related tauopathies.[32]

In this study 16 jatrophane diterpenoids isolated from *Euphorbia dendroides*[33,34] and latex extracts from *E. dendroides* and *Euphorbia nicaeensis* induced decrease in the frequency of MN and manifested protective effect on human lymphocytes DNA.

Jatrophanes **1-16** and extracts of lyophilized latex of 2 *Euphorbia* species (**17** and **18**) were tested for in vitro protective effect against chromosome aberrations in peripheral human lymphocytes using cytochalasin-B blocked MN assay. The cell cultures were treated with amifostine WR-2721 (positive control) at a concentration of 1 μg/mL and gave a significant ($P < 0.01$) decrease in the MN frequency of 17.94% compared to control cell cultures. The treatment with alkylating agent MMC (negative control) at a concentration of 0.2 μg/mL gave a significant ($P < 0.01$) increase in the MN frequency of 36.46% compared to control cell cultures. After treatment of cell cultures with **1-18** at concentrations of 1.0, 2.0, and 4.0 μg/mL, the frequencies and distribution of MN in human lymphocytes were scored and the results are presented in Table 1.

In the course of a structure-activity relationship (SAR) study, jatrophanes were classified into 2 groups. The first group (**1-11**) are penta- or hexaesters with a carbonyl at C-14, an endocyclic 11(12)*E*-double bond, and an exomethylene 6,17-double bond. Jatrophane **12** stands apart as a single triester with an additional carbonyl at C-9, otherwise with the same structural features as **1-11**. The second group (**13-16**) are heptaesters that differ from **1** to **11** by an endocyclic-5,6-double bond, instead of an exomethylene 6,17-double bond in the latter (Figure 1). In the CBMN assay the increased concentration of applied jatrophane (from 1.0 to 4.0 μg/mL) decreased the level of MN in human lymphocytes and consequently afforded better protection.

Jatrophanes **1-3** have shown the prominent protective effect in minimal concentration of 1 μg/mL decreasing the frequency of MN by 44.86%, 43.53%, and 41.44%, respectively, compared to control human lymphocytes (Table 1). In the same concentration jatrophanes **4-6** decreased frequency of MN by 36.11%, 34.43%, and 34.29%, respectively. The chemical structures of **1-6** are rather uniform and only differ in an ester group at C-3 (either OiBu or OPr) and at C-8 position (OAc, OBz, or ONic), while the rest of the molecular structure of **1-6** are equal to each other. Higher protection of human lymphocyte DNA was observed when isobutanoyloxy is at C-3, rather than propanoyloxy at the same position (comparison of **1** to **6** and **3** to **4**).

Weaker protective effect but still higher than positive control was observed when the highest dose (4 μg/mL) of **7**, **8,** and **12** was applied, to decrease MN frequency by 23.18%, 21.09%, and 22.66%, respectively. In the same dose, jatrophanes **9-11** that structurally differ from **1** to **8** by 5-ONic (instead of OAc), 15-OAc (instead of OH), and 3-OAc (instead of OiBu or OPr), respectively, produced lower results than positive control decreasing MN frequency by 17.18%, 16.80%, and 8.42%, respectively (Figure 1, Table 1). On the other hand much weaker protective effect of **8** (with isobutanoyloxy at C-3), compared to structurally almost identical **5** (propanoyloxy at C-3), cannot be explained.

Among the tested jatrophanes of the second group the most effective was **13** with benzoyloxy at C-9 that in minimal concentration of 1 μg/mL gave 31.05% decrease in frequency of MN. Jatrophane **14** differing from **13** by a nicotinyloxy group at C-9, instead of benzoyloxy at the same position in the latter, decreased MN frequency by 20.31%. Jatrophane **15** with propanoyloxy at C-3 contrary to acetate at C-3 in **14** decreased the frequency of MN by 10.10%. In the same concentration **16** with nicotinyloxy at C-8, differing from **14** by an acetate at C-8, decreased MN frequency by 24.93%, respectively.

The tested hexane extracts of lyophilized latex of *E. dendroides* (**17**) and *E. nicaeensis* (**18**) exhibited prominent effect decreasing the frequency of MN by 37.94% and 36.12%, respectively, at a concentration of 4 μg/mL when compared to control human lymphocytes (Table 1).

Antioxidant potential of heterocyclic compounds using CBMN assay has been summarized in a review.[35] Polyphenols could reduce the incidence of single-strand breaks in double-stranded DNA, as well as radical-induced base damage.[36]

MT stabilizing drugs may be utilized in treatment of neurodegenerative diseases to compensate for the loss of tau function and to maintain or restore effective axonal transport. Although a growing number of MT stabilizing natural products continue to be discovered, only a few selected compounds, such as paclitaxel and epothilone, have been characterized as potential candidates for the treatment of neurodegenerative diseases.[32]

Jatrophanes isolated from *Euphorbia semiperfoliata* Viv. stimulated purified tubulin assembly in vitro. The rearrangement of MT was in contrast to the bundling produced by paclitaxel.[30] In another study, nontoxic jatrophane **6** that showed inhibitory effect on cancer cell was incubated with tubulin solution and MT assembly was examined. The IC_{50} value for **6** was 29 μM, compared to 0.7 μM for paclitaxel.[31] Despite their structural difference from paclitaxel and other MT-interacting agents, the potential of jatrophanes as MT stabilizing drugs has not been investigated thoroughly.

As revealed in a previous SAR study on P-gp inhibition of 11 jatrophanes (**1**, **3-8**, and **13-16**) in colorectal carcinoma,[34] **3**, **4,** and **13** happen to be the most powerful inhibitors of P-gp. In a SAR study on protective effect against chromosome aberrations in peripheral human lymphocytes the larger number of jatrophanes (**1-16**) was examined. The most remarkable protection was obtained with jatrophanes **1-6** and **13** at a concentration of 1 μg/mL. Both SAR studies of

Table 1. Incidence of MN, Distribution MN Per Cells, CBPI, and Frequency of MN Measurement in Cell Cultures of Human Lymphocytes Treated With Different Concentration of **1-18**.

Tested compounds and extracts	Conc., µg/mL	MN/1000 Bn cell	% Bn cell with MN	MN/Bn Cell	CBPI	Frequency of MN (%)
Control		27.62 ± 0.55	2.29 ± 0.08	1.21 ± 0.03	1.62 ± 0.04	100.00
Amifostine	1.0	22.64 ± 0.78[a]	1.89 ± 0.10	1.23 ± 0.06	1.65 ± 0.04	82.06
MMC	0.2	37.42 ± 1.78[a, b]	3.26 ± 0.15	1.12 ± 0.04	1.76 ± 0.08	136.46
1	1.0	14.74 ± 1.21[a, b, c]	1.18 ± 0.17	1.28 ± 0.09	1.73 ± 0.07	55.14
	2.0	14.03 ± 1.26[a, b, c]	1.25 ± 0.14	1.12 ± 0.03	1.77 ± 0.07	52.49
	4.0	13.69 ± 2.11[a, b, c]	1.25 ± 0.13	1.08 ± 0.06	1.58 ± 0.05	51.21
2	1.0	16.08 ± 0.57[a, b, c]	1.42 ± 0.11	1.21 ± 0.07	1.57 ± 0.02	56.47
	2.0	15.92 ± 0.61[a, b, c]	1.29 ± 0.08	1.19 ± 0.06	1.58 ± 0.03	55.78
	4.0	14.74 ± 0.39[a, b, c]	1.31 ± 0.08	1.22 ± 0.07	1.63 ± 0.04	51.64
3	1.0	16.73 ± 1.08[a, b, c]	1.41 ± 0.11	1.17 ± 0.07	1.66 ± 0.09	58.56
	2.0	16.28 ± 1.31[a, b, c]	1.42 ± 0.11	1.18 ± 0.06	1.67 ± 0.08	57.21
	4.0	15.64 ± 1.02[a, b, c]	1.42 ± 0.09	1.13 ± 0.02	1.67 ± 0.03	54.67
4	1.0	18.19 ± 0.72[a, b, c]	1.48 ± 0.05	1.12 ± 0.05	1.58 ± 0.01	63.89
	2.0	17.72 ± 0.59[a, b, c]	1.47 ± 0.04	1.18 ± 0.03	1.59 ± 0.01	62.13
	4.0	16.78 ± 0.87[a, b, c]	1.42 ± 0.04	1.17 ± 0.06	1.61 ± 0.03	58.89
5	1.0	18.57 ± 0.81[a, b, c]	1.59 ± 0.09	1.14 ± 0.09	1.61 ± 0.02	65.57
	2.0	18.23 ± 1.10[a, b*, c]	1.57 ± 0.09	1.18 ± 0.08	1.59 ± 0.02	63.89
	4.0	17.07 ± 1.29[a, b, c]	1.52 ± 0.10	1.21 ± 0.09	1.63 ± 0.04	60.02
6	1.0	17.57 ± 1.12[a, b*, c]	1.49 ± 0.12	1.19 ± 0.06	1.70 ± 0.05	65.71
	2.0	16.21 ± 1.67[a, b*, c]	1.38 ± 0.08	1.17 ± 0.06	2.02 ± 0.03	60.64
	4.0	15.56 ± 0.84[a, b, c]	1.27 ± 0.11	1.23 ± 0.05	1.61 ± 0.01	58.21
7	1.0	23.71 ± 0.26[a, c]	1.78 ± 0.04	1.31 ± 0.03	1.67 ± 0.09	83.21
	2.0	23.33 ± 0.74[a, c]	1.79 ± 0.03	1.34 ± 0.05	1.59 ± 0.03	81.78
	4.0	21.92 ± 0.65[a, c]	1.54 ± 0.08	1.41 ± 0.02	1.67 ± 0.09	76.82
8	1.0	24.44 ± 0.68[a, c]	1.98 ± 0.07	1.31 ± 0.06	1.71 ± 0.08	85.64
	2.0	24.01 ± 0.67[a, c]	1.92 ± 0.06	1.34 ± 0.06	1.63 ± 0.04	84.25
	4.0	22.49 ± 0.39[a, c]	1.77 ± 0.09	1.22 ± 0.05	1.67 ± 0.05	78.91
9	1.0	24.99 ± 0.82[a*, c]	1.88 ± 0.05	1.28 ± 0.02	1.71 ± 0.02	87.67
	2.0	24.51 ± 0.56[a, c]	1.86 ± 0.04	1.27 ± 0.03	1.64 ± 0.03	86.04
	4.0	23.57 ± 0.62[a, c]	1.76 ± 0.14	1.28 ± 0.04	1.76 ± 0.08	82.82
10	1.0	24.60 ± 0.86[c]	1.93 ± 0.08	1.28 ± 0.04	1.72 ± 0.04	92.03
	2.0	23.75 ± 0.39[a, c]	1.86 ± 0.09	1.23 ± 0.05	1.67 ± 0.02	88.85
	4.0	22.24 ± 0.63[a, c]	1.64 ± 0.13	1.32 ± 0.11	1.66 ± 0.01	83.20
11	1.0	26.30 ± 1.66[c]	2.17 ± 0.14	1.21 ± 0.02	1.66 ± 0.06	98.39
	2.0	25.25 ± 1.26[c]	2.09 ± 0.13	1.14 ± 0.04	1.64 ± 0.02	94.46
	4.0	24.48 ± 0.81[c]	1.94 ± 0.10	1.21 ± 0.03	1.67 ± 0.01	91.58
12	1.0	22.37 ± 0.42[a, c]	1.91 ± 0.04	1.13 ± 0.03	1.66 ± 0.05	78.62
	2.0	22.04 ± 0.48[a, c]	1.89 ± 0.02	1.18 ± 0.03	1.68 ± 0.05	77.18
	4.0	20.93 ± 0.89[a, c]	1.74 ± 0.06	1.14 ± 0.06	1.59 ± 0.02	77.34
13	1.0	18.43 ± 0.46[a, b, c]	1.56 ± 0.04	1.14 ± 0.05	1.64 ± 0.01	68.95
	2.0	17.69 ± 0.83[a, b, c]	1.56 ± 0,04	1.08 ± 0.02	1.61 ± 0.02	66.18
	4.0	16.40 ± 1.59[a, b*, c]	1.34 ± 0.17	1.32 ± 0.06	1.62 ± 0.02	61.35
14	1.0	21.30 ± 0.95[a, c]	1.71 ± 0.12	1.25 ± 0.05	1.71 ± 0.05	79.69
	2.0	20.70 ± 0.84[a, c]	1.68 ± 0.05	1.23 ± 0.06	1.68 ± 0.06	77.44
	4.0	19.37 ± 0.34[a, b*, c]	1.66 ± 0.04	1.12 ± 0.04	1.65 ± 0.01	72.46

(Continued)

Table 1. Continued

Tested compounds and extracts	Conc., μg/mL	MN/1000 Bn cell	% Bn cell with MN	MN/Bn Cell	CBPI	Frequency of MN (%)
15	1.0	24.03 ± 0.61 [a*, c]	1.92 ± 0.09	1.20 ± 0.03	1.68 ± 0.02	89.90
	2.0	23.43 ± 0.67 [a, c]	1,83 ± 0.08	1.24 ± 0.05	1.67 ± 0.02	87.65
	4.0	21.56 ± 0.71 [a, c]	1.79 ± 0.09	1.21 ± 0.06	1.70 ± 0.05	80.66
16	1.0	21.42 ± 0.54 [a, c]	1.74 ± 0.13	1.29 ± 0.07	1.67 ± 0.08	75.07
	2.0	20.97 ± 0.85 [a, c]	1.78 ± 0.12	1.24 ± 0.08	1.64 ± 0.08	73.73
	4.0	19.76 ± 0.93 [a, b*, c]	1.65 ± 0.06	1.31 ± 0.09	1.60 ± 0.03	69.52
Euphorbia dendroides extract **17**	1.0	18.89 ± 0.62 [a, b, c]	1.51 ± 0.06	1.18 ± 0.04	1.67 ± 0.02	66.27
	2.0	18.60 ± 0.38 [a, b, c]	1.58 ± 0.09	1.18 ± 0.05	1.68 ± 0.02	65.33
	4.0	17.67 ± 1.71 [a, b*, c]	1.59 ± 0.12	1.14 ± 0.03	1.70 ± 0.06	62.06
Euphorbia nicaeensis extract **18**	1.0	19.31 ± 1.07 [a, b*, c]	1.64 ± 0.09	1.18 ± 0.07	1.56 ± 0.02	67.71
	2.0	19.08 ± 1.19 [a, b*, c]	1.68 ± 0.09	1.21 ± 0.09	1.58 ± 0.02	67.04
	4.0	18.19 ± 0.56 [a, b, c]	1.52 ± 0.08	1.16 ± 0.06	1.60 ± 0.02	63.88

Abbreviations: CBPI, cytokinesis-block proliferation index; MMC, mitomycin C.
MN/Bn cells: incidence of micronuclei in binucleated cells.
% Bn cells with MN: binucleated cells with micronuclei.
MN/1000 Bn cells: incidence of micronuclei in 1000 binucleated cells (examined for each concentration).
Frequency of MN: incidence of MN present like % from control groups in cell cultures of human lymphocytes treated with different concentration of jatrophanes or plant extracts.
The statistical significance of difference between the data pairs was evaluated by one-way analysis of variance followed by the Tukey test. Statistical significance at $P < 0.01$.
[a]Compared with control groups, statistically significant at $P < 0.01$.
[a*]Compared with control groups, statistically significant at $P < 0.05$.
[b]Compared with amifostine WR 2721, statistically significant at $P < 0.01$.
[b*]Compared with amifostine WR 2721, statistically significant at $P < 0.05$.
[c]Compared with MMC, statistically significant at $P < 0.01$.
[c*]Compared with MMC, statistically significant difference $P < 0.05$.

the tested jatrophanes pointed out that benzoyloxy at C-8 or C-9 (in **3**, **4,** and **13**) is responsible for a prominent degree of both P-gp inhibition and DNA protection of human lymphocytes. On the other hand jatrophane **1**, although a moderate P-gp inhibitor, manifested high protective effect in human lymphocytes. Furthermore, somewhat higher protective effect was observed when isobutanoyloxy is at C-3 (**1-3**), rather than propanoyloxy at the same position (**4-6**). Previous studies demonstrated that **1** with isobutanoyloxy at C-3 exhibited more than 2 times stronger inhibitory effect on the sensitive NCI-H460 cell line and its resistant counterpart NCI-H460/R than **6** with propanoyloxy at the same position.[31]

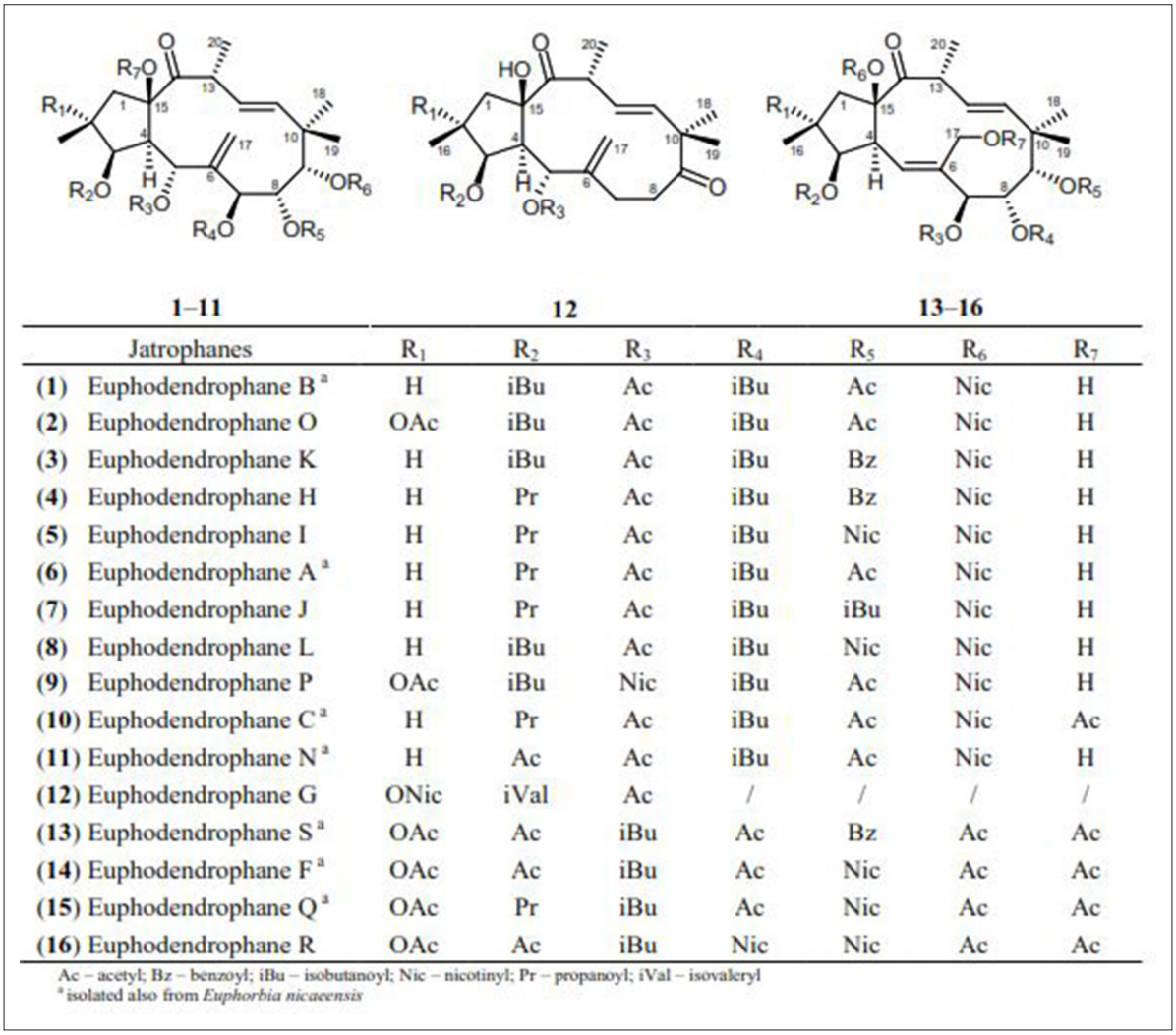
Compounds that stabilize MT may offer therapeutic approach for the treatment of Alzheimer's disease and related tauopathies. So far, several jatrophanes exhibited MT interacting activity and induced paclitaxel-like MTs. On the other hand detection of DNA and chromosomal damage through the elevated level of MN using in vitro CBMN assay in peripheral blood lymphocytes may be an indication of several diseases, such as Alzheimer's and Parkinson's.

Therefore a set of jatrophanes (**1-16**) and extracts of lyophilized latex of 2 *Euphorbia* species (**17** and **18**) were subjected to CBMN assay in peripheral blood lymphocytes. Jatrophanes **1-6** and **13** (in concentration of 1 μg/mL), and extracts **17** and **18** (in concentration of 4 μg/mL) prominently decreased MN frequency and manifested notable protective effect on human lymphocytes DNA. The potential of jatrophanes as MT stabilizing drugs needs further investigation.

Experimental

Subjects

Venous blood samples were withdrawn into heparinized sterile vacutainers (Becton Dickinson, Bradford, MA) from 6 healthy nonsmoking male volunteers who had not been exposed to chemicals, drugs, or other substances. A safety protocol concerning blood-borne pathogen/biohazard was taken. The volunteers consented to use their blood for the experiment. From each subject, 2 aliquots of blood, 5 mL each were obtained. The study complied with the code of ethics of the World Medical Association (Helsinki Declaration of 1964, as revised in 2002). The blood samples were obtained at the Medical Unit in accordance with current Health and Ethical regulations in Serbia, Law on Health Care (2005).



1–11			12			13–16	
Jatrophanes	R_1	R_2	R_3	R_4	R_5	R_6	R_7
(1) Euphodendrophane B [a]	H	iBu	Ac	iBu	Ac	Nic	H
(2) Euphodendrophane O	OAc	iBu	Ac	iBu	Ac	Nic	H
(3) Euphodendrophane K	H	iBu	Ac	iBu	Bz	Nic	H
(4) Euphodendrophane H	H	Pr	Ac	iBu	Bz	Nic	H
(5) Euphodendrophane I	H	Pr	Ac	iBu	Nic	Nic	H
(6) Euphodendrophane A [a]	H	Pr	Ac	iBu	Ac	Nic	H
(7) Euphodendrophane J	H	Pr	Ac	iBu	iBu	Nic	H
(8) Euphodendrophane L	H	iBu	Ac	iBu	Nic	Nic	H
(9) Euphodendrophane P	OAc	iBu	Nic	iBu	Ac	Nic	H
(10) Euphodendrophane C [a]	H	Pr	Ac	iBu	Ac	Nic	Ac
(11) Euphodendrophane N [a]	H	Ac	Ac	iBu	Ac	Nic	H
(12) Euphodendrophane G	ONic	iVal	Ac	/	/	/	/
(13) Euphodendrophane S [a]	OAc	Ac	iBu	Ac	Bz	Ac	Ac
(14) Euphodendrophane F [a]	OAc	Ac	iBu	Ac	Nic	Ac	Ac
(15) Euphodendrophane Q [a]	OAc	Pr	iBu	Ac	Nic	Ac	Ac
(16) Euphodendrophane R	OAc	Ac	iBu	Nic	Nic	Ac	Ac

Ac – acetyl; Bz – benzoyl; iBu – isobutanoyl; Nic – nicotinyl; Pr – propanoyl; iVal – isovaleryl
[a] isolated also from *Euphorbia nicaeensis*

Figure 1. Structures of the tested jatrophanes isolated from *Euphorbia dendroides* and *Euphorbia nicaeensis*.

Cytokinesis-Block Micronucleus Assay

Jatrophanes **1-16** were isolated and purified as described.[33,34] Lyophilized latex (200 mg) of *E. dendroides* and *E. nicaeensis* were extracted with petroleum ether (30 mL, 3 times) to obtain extracts of lyophilized latex **17** (170 mg) and **18** (155 mg). The culture lymphocytes were treated with 3 concentrations (1, 2, and 4 µg/mL) of jatrophanes **1-16** and latex extracts **17** and **18**. One cell culture served as the control and, to this, jatrophane or lyophilized latex extract was not added. Amifostine WR-2721 (Marligen-Biosciences, USA) (1 µg/mL) was used as a positive control and MMC (0.2 µg/mL, in phosphate buffer) as a negative control. All cultures were incubated in a thermostat at 37°C. Treatment with **1-18** lasted 19 hours, when after all cultures were rinsed with pure medium, transferred into 5 mL fresh RPMI 1640 medium (RPMI 1640 Medium +GlutaMAX + 25 mM HEPES; Invitrogen-Gibco-BRL, Vienna, Austria) and incubated for additional 72 hours. The incidence of spontaneously occurring MN in control samples was scored. The further treatment of all cultures was previously described.[20]

Statistical Analysis

The statistical analysis was performed using Origin software package version 7.0. The statistical significance of difference between the data pairs was evaluated by one-way analysis of variance followed by the Tukey test. Statistical difference was considered significant at $P < 0.01$. The index calculating is presented as the percent of change comparing different groups.

Declaration of Conflicting Interests

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