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In Vitro Modulation of P-Glycoprotein Activity by *Euphorbia intisy* Essential Oil on Acute Myeloid Leukemia Cell Line HL-60R

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Abstract: *Euphorbia* species have a large spectrum of traditional medicinal uses. We tested the biological activities of the essential oil (EO) of *Euphorbia intisy* Drake in an acquired multidrug resistance leukemia model to assess whether the EO obtained by hydrodistillation of stems was able to reverse the resistant phenotype. HL-60R cell lines are characterized by the overexpression of P-glycoprotein (P-gp), inhibitors of apoptosis proteins (IAPs) and constitutive expression of NF-κB. EO chemical composition was determined by GC/MS analysis; cytotoxic activity of EO by MTS assay alone or in combination with doxorubicin; pro-apoptotic effect and doxorubicin accumulation were analyzed by flow cytometry; P-gp ATPase activity was measured by P-gp-Glo™ assay systems kit. The ability to inhibit NF-κB and its target genes was also assessed. *E. intisy* EO exhibited a comparable cytotoxic effect and ability to block P-gp in both the HL-60 and its MDR variant HL-60R. In addition, EO suppressed P-gp protein expression and significantly downregulated MDR1 mRNA level, as well as some IAPs proteins, probably through the inhibition of NF-κB. Our results suggest that *E. intisy* EO could reverse P-gp-mediated drug resistance in tumor cells acting as a chemosensitizing agent.

Keywords: essential oil; cancer cell; P-glycoprotein; multidrug resistance; myeloid leukemia cell; NF-κB; inhibitors of apoptosis proteins

1. Introduction

Multidrug resistance (MDR), which is often of a multiple type, is a real obstacle to the pharmacological treatment of many types of cancer diseases and can be defined as the ability of cancer cells to acquire resistance to both conventional and novel chemotherapy agents [1]. In this paper we focus on drug resistance acquired after treatment with a chemotherapic drug which is a complex and multifactorial phenomenon.

1.1. P-Glycoprotein Mediated Drug Resistance

In the MDR process a key role is played by P-glycoprotein (P-gp), a drug efflux pump which is responsible for the decrease in intracellular drug accumulation. P-gp is the most studied drug efflux pumps, described for the first time by Juliano and Ling [2]. It is part of a vast superfamily of ATP-dependent transporters, a glycoprotein with a molecular weight of about 170 kDa encoded by human gene ATP-binding cassette sub-family B member 1 (ABCB1) or Multi Drug Resistance 1 (MDR1), consists of a dozen transmembrane domains of six extracellular loops and two adenosine triphosphate (ATP) binding sites. The physiological role of P-gp, present in different tissues such as the liver, blood–brain barrier, lymphocytes, testicles, placenta, small and large intestines, is to protect and regulate the absorption, distribution, and excretion of xenobiotics and drugs. Numerous molecules

have been proposed to bypass the multidrug resistance due to the overexpression of P-gp. The main pharmacological strategy of preclinical and clinical research to overcome MDR and revert the resistant phenotype is represented by the co-administration of anticancer chemotherapy drug and P-gp substrate-inhibitor compounds in order to restore the effective therapeutic concentration of the drug in the tumor resistant cells [3]. The P-gp inhibition can occur by molecules that compete for the binding site with the substrate, or by non-competitive or allosteric inhibition, blocking ATP hydrolysis and altering the lipid layer of cell membranes or affecting P-gp expression. In this context, natural products are considered as a new source of drugs to be used in multidrug resistant diseases and a valid alternative therapy to those currently in use. Some secondary metabolites isolated from different plants were reported as P-gp expression or activity inhibitors, such as flavonoids, coumarins, terpenoids, alkaloids, and steroidal saponins [4].

Previous works demonstrated that essential oils (EOs), from succulent plants, have antitumor activities on different types of tumors such as triple negative breast cancer and acute myeloid leukemia all characterized by scarce responsiveness to chemotherapeutic drugs [5–7]. The biological effects showed that the EOs extracted by the hydrodistillation of leaves seem to be due to the presence of terpene compounds such as phytol. A recent analysis suggests that we must diversify the species we utilize and/or substitute towards those better adapted to future climate [8]. Succulent plants could represent advantageous alternatives, thanks to their ability to sustain growth in arid environment [9]. Varying sources of bioactive compounds with drought-tolerant species appears to be a convenient option in drug discovery, as it links the usefulness with the resilience. Furthermore, pharmacological investigations on their medicinal properties are promising for several contemporary diseases [10–13].

1.2. The Genus Euphorbia Uses

The genus *Euphorbia* L. (Family: Euphorbiaceae) is distributed in different parts of the world, identifiable by the white latex and unique inflorescences [14,15]. However, species with succulent traits are mostly found in arid areas and represented in subgenera Athymalus and *Euphorbia* [16]. *Euphorbia* species have a large spectrum of traditional medicinal uses [17] ranging from digestive system disorders, skin aliments, infections/infestations, injuries/inflammations and respiratory complaints; some species are cited as vertebrate poisons [18]. These knowledges are supported by a broad range of biological properties and potential usages in derived natural products [19–21]. A considerable amount of literature has been also published on the anticancer activities of *Euphorbia*; these are mostly ascribed to a variety of terpenic compounds acting on different pathways of tumor formation [22–25].

Chemical composition of *Euphorbia* EOs is largely studied: e.g., *E. acanthothamnos*, *E. apios*, *E. characias*, *E. dendroides*, *E. helioscopia*, and *E. rigida* with nonanal, germacrene, phytol, β-caryophyllene, heneicosane, and heptacosane as major components [26], *E. teheranica* with elemol as major component [27], *E. caracasana* and *E. cotinifolia* with β-caryophyllene as a major component [28], and *E. heterophylla* with 1,8-cineole as major component [29].

However, there are few studies that have investigated their potential anticancer capacities. *Euphorbia* species occurring in Southern Africa and Madagascar produce a high diversity of diterpenoids possibly because of an adaptive pressure exerted by not yet described herbivores [16]. Indeed, the southern arid zone of Madagascar is a hotspot for *Euphorbia* with more than 170 native species [30]. *Euphorbia* is locally classified among the top 5 species-rich genera with medicinal properties [31]. *Euphorbia intisy* Drake (Figure 1) is a tree-like succulent of the dry spiny forests whose exploitation dates to the end of the 19th century, boosted by the strong European demand for latex [32]. *E. intisy* provides rubber with high quality latex and low resin contents from trunks and roots [33,34]. The latex itself is not toxic, however, the seeds are toxic and cause vomiting, severe bowel disturbance, and heartburn [35]. *E. intisy* was nearly made extinct by overexploitation, in the beginning of the 20th century [36]. It is now classified as Near Threatened according to the IUCN

red list [37]. *E. intisy* is cultivated in the open ground at the Botanical Garden of Palermo. It is easily propagated by cuttings and therefore enough material for experiments can be obtained with no damages to wild populations, showing to be a good candidate to investigations on its chemical composition and biological activities.

Figure 1. *Euphorbia intisy* cultivated at the Botanical Garden of the University of Palermo (photo M. Sajeva).

For all previously made assumptions we used a multidrug resistance leukemia model obtained by us by exposure of the sensitive cell line HL-60 with increasing doses of doxorubicin, characterized by overexpression of P-gp, inhibitor of apoptosis proteins (IAPs) and constitutive expression of the transcription factor NF-κB, to assess whether the EO extracted from *Euphorbia intisy* leaves was able to reverse the resistant phenotype.

2. Results and Discussion

2.1. Chemical Composition

The yield in essential was 0.0034%. The plants from which the stems were collected were not affected by the removal of the stems and they completed their vegetative cycle and flowered in the following years. In the essential oil of *E. intisy*, we detected 70 compounds

(Table 1 and Figure S1) accounting 68.79% of the whole composition. Only compounds that showed mass spectral similarities ≥80% in respect to our libraries were considered [38,39] and NIST11. The composition was dominated by terpenoids with 48 compounds covering 37.98% of the total EO composition. Sesquiterpene alcohols and sequiterpene hydrocarbons where the classes with the higher number of compounds. Phytol and isophytol are the only diterpene, and phytol alone covers 9.55% of the whole EO composition. Mammals are generally able to use phytol, as they would do with any fatty acid, as a source of energy through its beta-oxidation [40,41]. Recent studies on phytol have shown effects as a function of anxiolytic, metabolism modulator, cytotoxicity, antioxidant, autophagy, and induction to apoptosis, antinociceptive, anti-inflammatory, immunity modulator, and bactericide [42]. Recently Poma et al. [6] showed that the essential oils of two succulent plants, rich in phytol, interfere with NF-κB on human cancer cell lines. Aliphatic compounds were the second most abundant class (22.89%), and heptacosane with 15.73% was the most abundant aliphatic compound and the most abundant one of the whole EO. Heptacosane falls into the category of phytoecdysteroids that mimic the biological function of the insect mover hormone, interfering with the synthesis of chitin, thus not allowing induction to the moult and metamorphosis [43]. This also may play a role in plant defense against insects. Benzyl benzoate is the only aromatic compound found in the EO of *E. insity*. It is a powerful acaricide [44]. The ketone compounds found in *E. intisy* are the isomer (Z)-jasmone (0.23%) that is produced by some plants and can act both as an attractor and as a repellent for various insects [45], and hexahydrofarnesyl-acetone (0.35%) that appears to be an antimicrobial for bacteria and fungi [46,47]. Some of the compounds identified in *E. intisy* are found in the EOs of other *Euphorbia* species (as reported in the introduction): nonanal, phytol, heneicosane, heptacosane, and beta-elemol [27–29]. Three compounds, accounting for 7.60%, were not identified and are reported in Table 1 with their mass spectra.

Table 1. Essential oil composition of *E. intisy*. Compounds belonging to the same chemical class and functional group are arranged according to Kovats Retention Indices (KRI) of the Supelcowax 10 capillary column.

KRI	Compound	Relative Amount %	MS Similarity
	Aliphatic Acids		
2512	Dodecanoic acid	0.42	93
2720	Oleic acid	1.37	90
	Aliphatic Alcohols		
1354	1-Hexanol	0.05	92
1383	(Z)-3-Hexen-1-ol	0.05	97
1407	(E)-2-Hexen-1-ol	0.02	91
1557	(E)-3-Octen-1-ol	0.02	92
1968	1-Dodecanol	0.02	85
1557	(E)-3-Octen-1-ol	0.02	92
2447	1-Heptacosanol	0.85	96
	Aliphatic Aldheydes		
1081	Hexanal	0.08	91
1217	(E)-2-Hexenal	0.65	96
1389	Nonanal	0.06	90
	Aliphatic Alkanes		
1900	Nonadecane	0.05	H

Table 1. *Cont.*

KRI	Compound	Relative Amount %	MS Similarity
2200	Docosane	0.15	H
2300	Tricosane	0.33	H
2400	Tetracosane	0.44	H
2500	Pentacosane	1.53	H
2600	Hexacosane	0.31	H
2700	Heptacosane	15.73	H
Aliphatic Esters			
2550	Methyl linolenate	0.74	92
Aromatic Esters			
2606	Benzyl benzoate	0.13	96
Diterpene Alcohols			
2296	Isophytol	0.38	84
2615	Phytol	7.89	97
Irregular Terpene Ketones			
2124	Hexahydrofarnesyl acetone	0.29	90
Miscellaneous Ketones			
1932	(Z)-Jasmone	0.19	95
Monoterpene Alchols			
1550	Linalool	1.25	98
1612	Hotrienol	0.02	91
1849	Geraniol	0.22	95
Monoterpene Ethers			
1435	(Z)-Linalool oxide furanoid	0.42	97
Monoterpene Hydrocarbons			
1247	(E)-beta-Ocimene	1.50	97
Monoterpene Ketones			
1629	Pulegone	0.07	82
Sesquiterpene Alcohols			
1876	epi-Cubebol	0.01	81
2042	Nerolidol isomer	0.21	75
2073	beta-Elemol	0.06	88
2156	gamma-Eudesmol	3.06	95
2186	Pogostol	1.58	83
2206	alpha-Eudesmol	1.58	80
2213	beta-Eudesmol	1.76	82
2219	alpha-Cadinol	0.11	89
2231	(–)-Spathulenol	0.28	80
2262	Valerenol	0.46	84
2281	Juniper camphor	0.48	87

Table 1. *Cont.*

KRI	Compound	Relative Amount %	MS Similarity
2354	Farnesol	0.09	90
2492	gamma-Costol	0.09	86
	Sesquiterpene Ethers		
1464	(E)-Linalool oxide furanoid	0.26	92
1507	Vitispirane	0.03	93
1510	Vitispirane isomer	0.02	93
2023	Caryophyllene oxide	0.02	80
	Sequiterpene Hydrocarbons		
1441	alpha-Cubebene	0.05	93
1470	alpha-Copaene	2.94	97
1495	beta-Bourbonene	0.49	96
1571	beta-Caryophyllene	1.11	96
1573	(Z)-beta-Elemene	0.12	84
1622	gamma-Elemene	0.02	82
1644	alpha-Humulene	0.08	93
1666	alpha-Amorphene	1.85	94
1684	beta-Guaiene	2.64	91
1698	alpha-Selinene	0.06	89
1704	alpha-Muurolene	1.56	95
1736	delta-Cadinene	2.07	96
1742	(E,E)-alpha-Farnesene	0.14	86
1759	Cubenene	0.06	91
1811	Isocalamenene	0.10	87
1894	alpha-Calacorene	0.34	93
1926	Neophytadiene	0.38	94
1939	beta-Calacorene	0.03	84
1955	Neophytadiene isomer	0.12	87
1981	Neophytadiene isomer	0.27	96
1987	alpha-Gorgonene	0.40	87
	Sesquiterpene Ketones		
2234	Mustakone	1.01	92
	Unidentified		
2136	Unknown *m/z*: 161 (100), 105 (99), 43 (93), 121 (65), 93 (62), 81 (60), 95 (56), 119 (56), 79 (49), 91 (48)	4.58	90
2255	Unknown *m/z*: 107 (100), 93 (78), 105 (74), 119 (63), 69 (60), 41 (56), 81 (53), 91 (49), 79 (44), 161 (43)	1.35	NF
2329	Unknown *m/z*: 105 (100), 143 (94), 91 (80), 107 (67), 81 (60), 119 (57), 79 (54), 93 (50), 147 (50), 121 (46)	1.67	85
	Total	**68.84**	

2.2. In Vitro Cytotoxic and Cell Death Effects of E. intisy essential Oil

In this paper, we focus on drug resistance acquired after drug treatment with chemotherapy which is a complex and multifactorial phenomenon. The HL-60 is a chemosensitive cell line, whereas HL-60R cells is resistant to doxorubicin as well as other chemotherapeutic drugs [48]. The HL-60 cell line and its multidrug resistance variant HL-60R were treated with different concentration of *E. intisy* EO for 72 h. The essential oil induced cell growth inhibition in a concentration-dependent way in the two cell lines with IC_{50} values quite similar with low cytotoxicity in differentiated Caco-2 cells (Figure 2, Table 2). Interestingly, Poma et al. [6] found similar results with the EOs of two succulent plants rich in phytol.

Figure 2. Cytotoxic activity of *E. intisy* essential oil on leukemia HL-60, HL-60R, and differentiated Caco-2 cell lines. Cells were exposed to EO for 72 h, at different concentrations and viability was assessed by MTS assay. Data are expressed as mean $\pm$ standard error of three experiments performed in triplicate. Different letters represent significant differences (Tukey's test) in cytotoxic activity among the concentrations of each cell line ($p < 0.05$).

Table 2. IC_{50} values of the three cell lines treated with the essential oil of *E. intisy*.

Cell Line	IC_{50} (Mean $\pm$ SE)
HL-60	22.0 $\pm$ 0.7 µg/mL
HL-60R	24.5 $\pm$ 0.07 µg/mL
Caco-2	62.5 $\pm$ 0.35 µg/mL

The HL-60 and HL-60R cell lines were incubated with the essential oil (25 µg/mL) for 24 h, and cell death was evaluated by flow cytometry analysis of DNA stained with propidium iodide. *E. intisy* EO caused a comparable marked block in the $preG_0$-G_1 position in both cell lines by preventing the entry of cells to G2/M (Figure 3). These results confirm the interest in *Euphorbia* species as potential anticancer drugs [49].
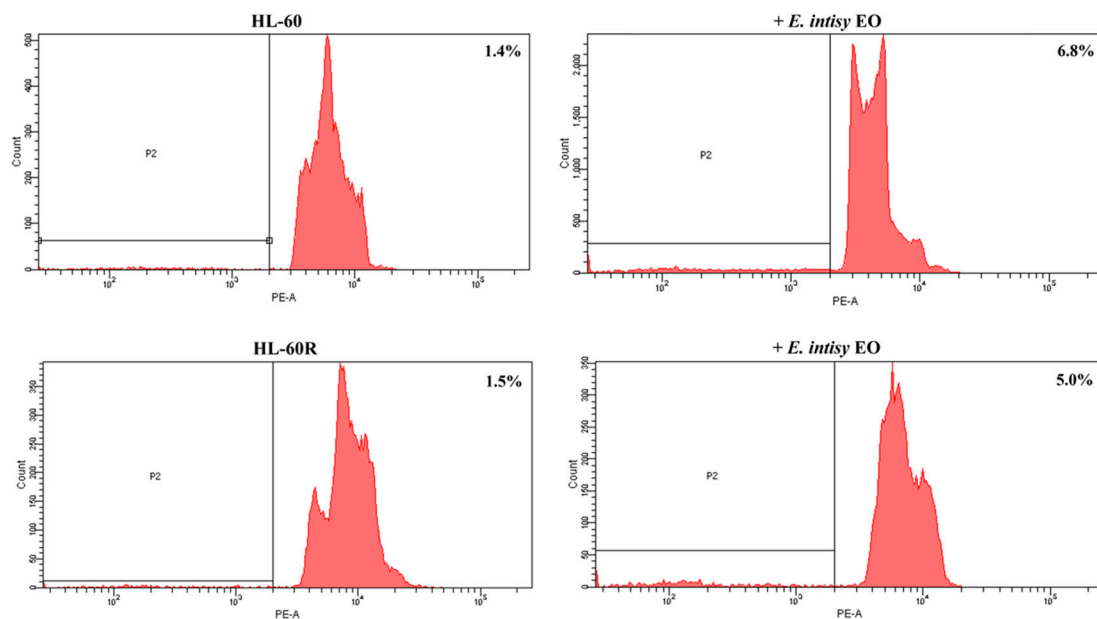
Figure 3. Induction of cell death by *E. intisy* essential oil in HL-60 and HL-60R cells. Representative example of flow cytometry analysis of cell death and cell cycle in the HL-60 and HL-60R cells treated with 25 µg/mL of *E. intisy* essential oil for 24 h. The profiles of propidium iodide-stained DNA are shown. Numbers in the panels indicate the % of the events in the preG$_0$-G$_1$ position.

2.3. E. intisy Essential Oil Treatment Inhibits the Activation of the NF-κB Signaling Pathway

The multidrug resistant variant HL-60R is characterized by constitutive expression of NF-κB in respect to HL-60 [50]. In order to evaluate if *E. intisy* EO could interfere in the NF-κB DNA binding capacity, HL-60 and HL-60R cell lines were treated with 25 µg/mL of essential oil for 24 h. We observed a strong reduction of NF-κB DNA-binding activity only in HL-60R cells (Figure 4). Previous papers [48,50] highlighted that HL-60R cells are characterized by an overexpression of P-gp, Bcl-2 and IAPs. Moreover, the treatment with the essential oil also caused a substantial mRNA and protein decrease of some NF-κB target genes, in particular antiapoptotic factors such as Survivin, XIAP, Bcl-2, and the multidrug efflux transporter P-glycoprotein (Figures 5 and 6). Previous works demonstrated that the antiproliferative and pro-apoptotic activities of essential oils extracted from some species of succulent plants can interfere with NF-κB pathway in the same cellular model [6]. Based on these results, we speculate that the transcriptional changes in P-gp and IAPs are primary effects and that these modifications induce cytotoxicity. These results corroborate the idea that EOs extracted from succulent plants rich in phytol interfere with NF-κB pathway in MDR models.
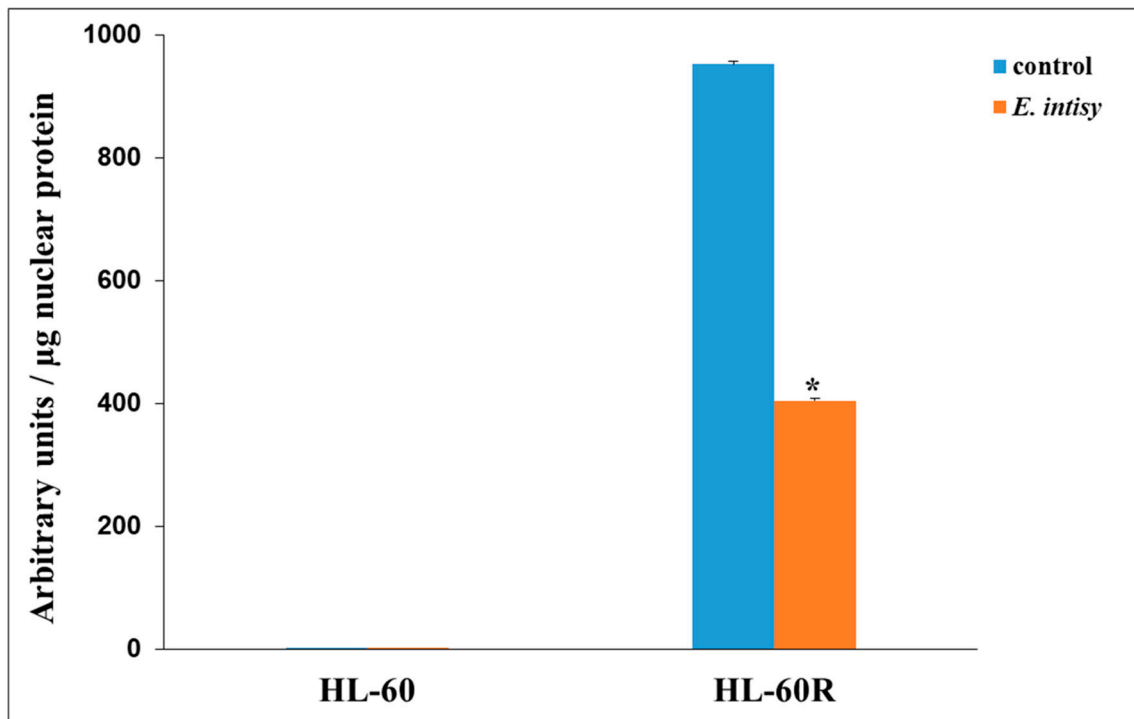
Figure 4. NF-κB (p65 subunit) DNA binding capacity in nuclear extracts of HL-60 and HL-60R cells. The cells were treated for 24 h with *E. intisy* essential oil (25 µg/mL). Results (mean ± standard error of three experiments carried out in duplicate) are expressed as arbitrary units/µg protein of cells nuclear extracts. Statistical differences are * $p < 0.01$ vs. control (Tukey's test).
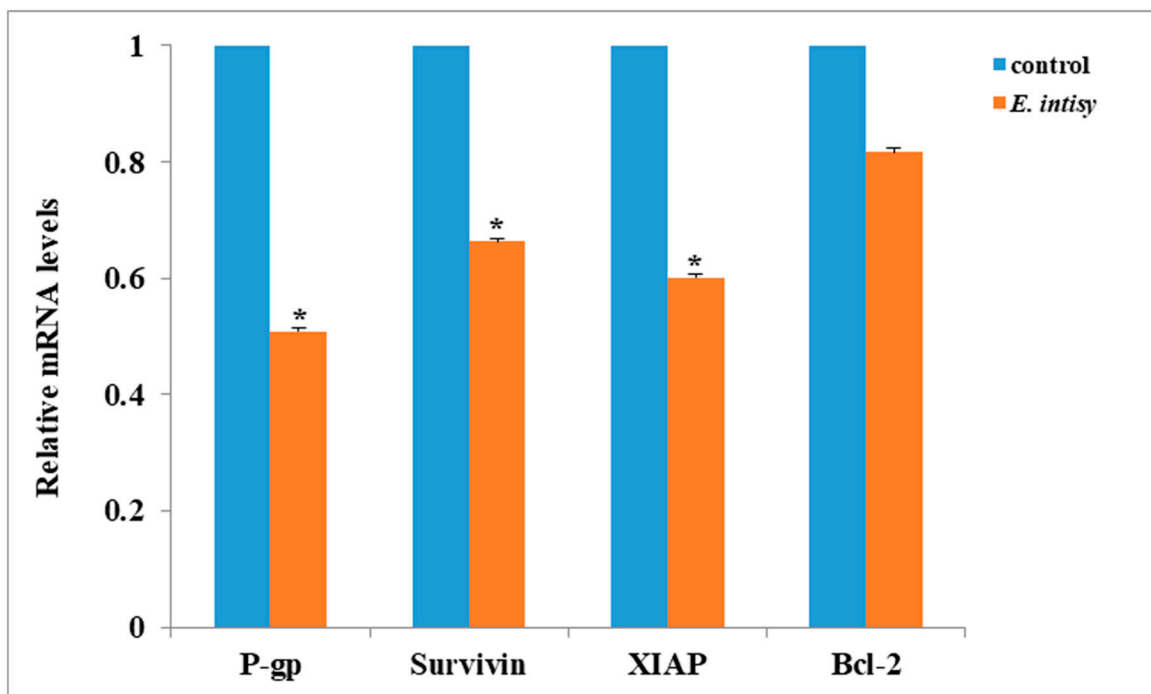

Figure 5. Genes mRNA expression levels by quantitative polymerase chain reaction in HL-60R cells. The cells were treated for 24 h with *E. intisy* essential oil (25 µg/mL). Data are expressed as mean ± standard error of three experiments. Differences when treatments are compared to the control * $p < 0.01$ (Tukey's test).

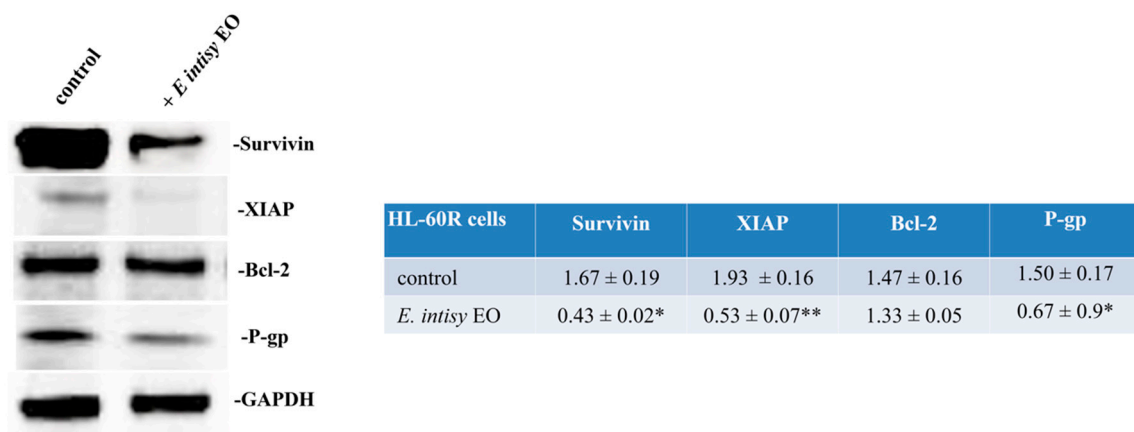| HL-60R cells	Survivin	XIAP	Bcl-2	P-gp
control	1.67 ± 0.19	1.93 ± 0.16	1.47 ± 0.16	1.50 ± 0.17
E. intisy EO	0.43 ± 0.02*	0.53 ± 0.07**	1.33 ± 0.05	0.67 ± 0.9*

Figure 6. Western blotting analysis of the levels of Survivin, XIAP, Bcl-2, and P-gp, in HL-60R cells. The cells were treated for 24 h with *E. intisy* essential oil (25 µg/mL). On the left, the results of a representative experiment; on the right, the results expressed as mean ± standard error of three experiments. Differences when treatments are compared to the control: ** $p < 0.01$, * $p < 0.05$ (Tukey's test).

2.4. Effects of E. intisy EO on Intracellular Accumulation of Doxorubicin in HL-60R Cell Line

Due to the strong reduction of mRNA and protein level of P-gp under treatment with essential oil in our cell lines, we investigated the possible involvement of the EO in the drug resistance mechanism. P-gp-overexpressing cells show significant decreases in drug accumulation compared to non-P-gp-expressing. In order to evaluate the effects of *E. intisy* EO on intracellular accumulation of doxorubicin, a P-gp substrate anticancer drug, HL-60 and HL-60R cells were treated with EO 15 µg/mL for 24 h. After this incubation, doxorubicin 1 µg/mL was added for different times (30 min, 1 h and 2 h). The intracellular accumulation of doxorubicin was measured by flow cytometric analysis. The results are presented as percentage of fluorescence intensity in HL-60 (Figure 7) and HL-60R (Figure 8).
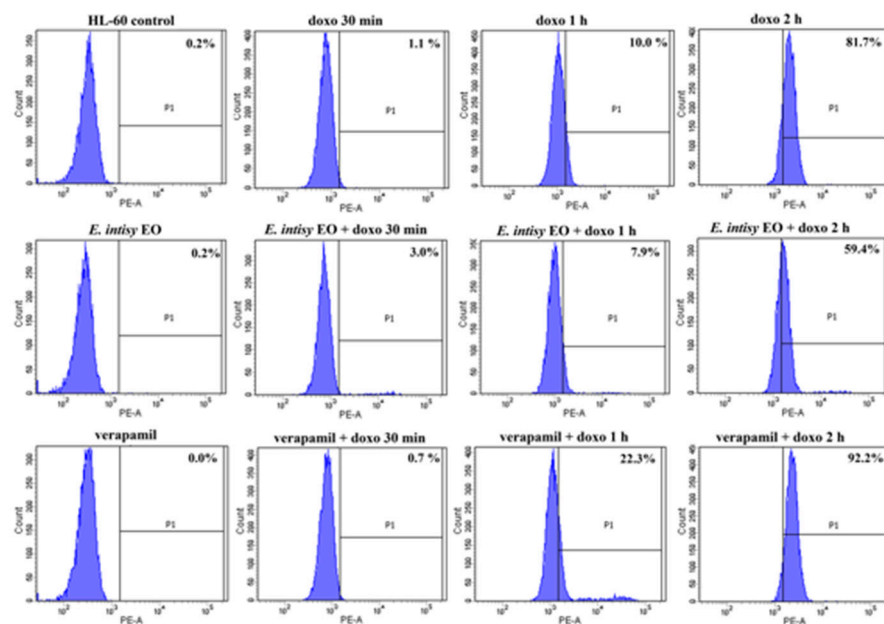

Figure 7. Effects of *E. intisy* EO on intracellular accumulation of doxorubicin in HL-60 cell line. Representative example of flow cytometry analysis of intracellular accumulation of doxorubicin (1 µg/mL) after different time of incubation (30 min, 1 h and 2 h) in HL-60 cells pre-treated with 15 µg/mL of *E. intisy* essential oil or verapamil 10 µM for 24 h.
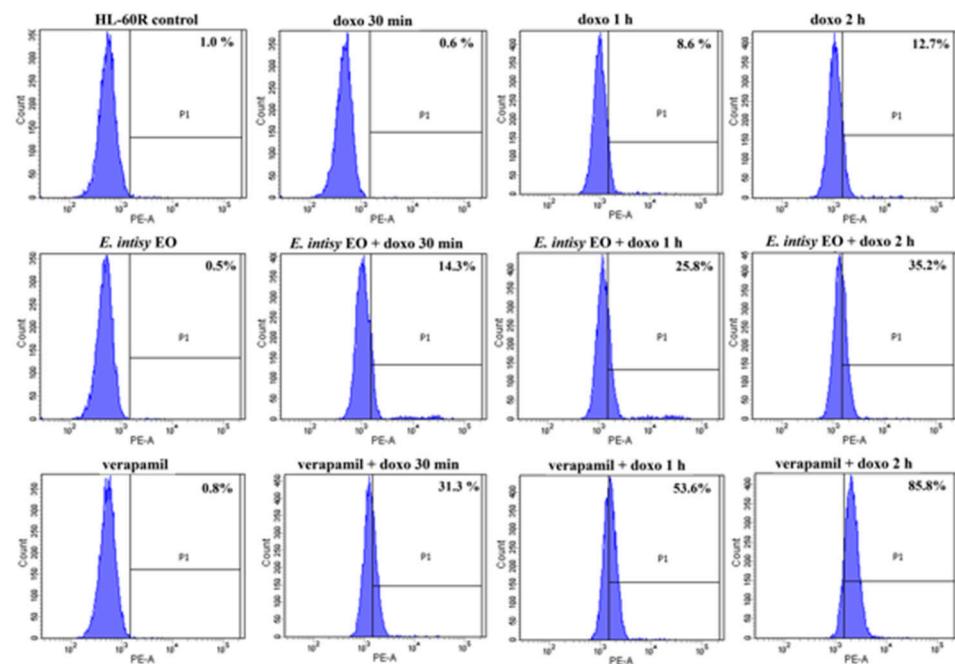
Figure 8. Effects of *E. intisy* EO on intracellular accumulation of doxorubicin in HL-60R cell line. Representative example of flow cytometry analysis of intracellular accumulation of doxorubicin (1 μg/mL) after different time of incubation (30 min, 1 h and 2 h) in HL-60R cells pre-treated with 15 μg/mL of *E. intisy* essential oil or verapamil 10 μM for 24 h.

The results indicate a significant increase in fluorescence in HL-60R of the samples pretreated with *E. intisy* essential oil, showing an intracellular accumulation of doxorubicin after exposure at different times. Unexpectedly, in HL-60 the pretreatment with EO reduces the accumulation of doxorubicin (only 2 h after treatment with doxorubicin) in respect to its control. These data could suggest that in HL-60R cells *E. intisy* EO acts like an inhibitor of P-gp function, as verapamil that produced similar results on doxorubicin accumulation (Figure 7, Figure 8 and Table 3). These results are in accordance with the finding of Fidyt et al. [49] who demonstrated that oxygenated terpenes potentiate the classical drug efficacy by augmenting their concentrations inside the cells. It is interesting to note that despite *E. intisy* EO shows a lower accumulation of doxorubicin in respect to verapamil, it has a low cytotoxic effect (Table 2).

2.5. Effects of E. intisy EO on P-gp Activity

P-gp functions as an ATP-dependent drug efflux pump. The compounds that are substrates for transport by P-gp typically stimulate its ATPase activity. We performed the P-gp-Glo™ Assay that detects a luminescent signal inversely proportional to the ATP consumption.

As shown in Figure 9, the essential oil of *E. intisy* increased basal P-gp ATPase activity in a dose-dependent way.

To further confirm these outcomes, we performed the same assay adding verapamil in the wells treated with EO and the results are shown in Figure 10. The data obtained demonstrated that EO not only stimulated basal P-gp ATPase activity, but also increased verapamil-stimulated P-gp ATPase activity. These results suggested that EO might be a stimulator of ATP-dependent drug efflux transporter, acting as substrate for transport by P-gp that stimulates P-gp ATPase activity, and lead to the inhibition of P-gp efflux function, in the same way verapamil acts.

Eid et al. [51] indicates that, among others, lipophilic terpenoids are probably competitive inhibitors of P-gp in cancer cells. Devanjee at al. [3] report that terpenoids possess significant P-gp inhibitory activity via different mechanisms. According to Wortelboer et al. [52] citral inhibits MRP1 and MRP2. In particular, Duarte et al. [22] identified latilagascene B, latilagascene

E, and latilagascene D as inhibitors of P-gp that directly blocking its active sites. Some terpenoids can inhibit P-gp activity via binding with its active sites [53–55]. It is interesting to note that in a recent review Kemboi et al. [17] described the effects of terpenoids from some *Euphorbia* species like a possible multi-drug resistance reverting agents in comparison to verapamil.

Table 3. Effects of *E. intisy* EO on intracellular accumulation of doxorubicin in HL-60 and HL-60R cell lines. The cells were treated with EO 15 µg/mL or verapamil 10 µM. After 24 h of incubation, doxorubicin 1 µg/mL has been added for different time: 30 min, 1 h and 2 h. The intracellular accumulation of doxorubicin was measured by flow cytometric analysis. The results are presented as percentage of fluorescence intensity (means ± standard error of three experiments).

Treatment	Fluorescence %	
	HL-60	**HL-60R**
control	0.2 ± 0.004	0.9 ± 0.004
doxo 1 µg/mL 30 min	1.0 ± 0.05	0.6 ± 0.09
doxo 1 µg/mL 1 h	9.7 ± 0.70 *	8.8 ± 0.09 *
doxo 1 µg/mL 2 h	80.2 ± 0.65 *	11.9 ± 0.79 *
E. intisy EO 15 µg/mL	0.12 ± 0.03	0.47 ± 0.07
verapamil 10 µM	0.03 ± 0.03	0.73 ± 0.10
E. intisy EO + doxo 30 min	3.0 ± 0.41 *	14.7 ± 0.18 *,a
E. intisy EO + doxo 1 h	7.97 ± 0.53 *	25.6 ± 0.25 *,a
E. intisy EO + doxo 2 h	59.8 ± 0.97 *	35.5 ± 0.19 *,a
verapamil + doxo 30 min	0.6 ± 0.05	31.1 ± 0.05 *,a
verapamil + doxo 1 h	21.8 ± 0.75 *,a	53.5 ± 0.24 *,a
verapamil + doxo 2 h	91.7 ± 0.70 *	85.6 ± 0.25 *,a

* $p < 0.01$, vs. the control. [a] $p < 0.01$ represent significant differences among the treatments in combination with essential oil or verapamil compared to doxorubicin alone for the same time (one-way ANOVA followed by Tukey's test).
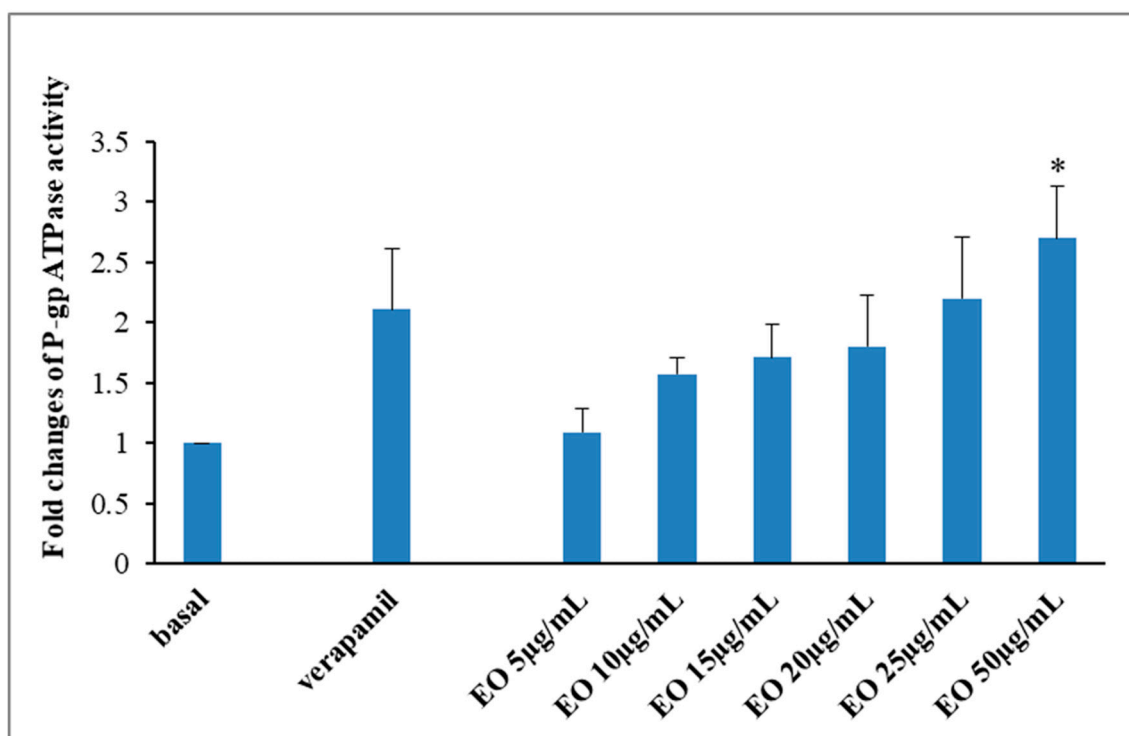

Figure 9. Effects of *E. intisy* EO (5–50 µg/mL) on P-gp basal ATPase activity. The data are expressed as fold changes of P-gp ATPase activity compared to basal one ($\Delta RLU_{TC} / \Delta RLU_{basal}$) and are presented as mean ± SE of three experiments, each in duplicate. * Differences when treatments are compared to the basal activity $p < 0.01$ (Tukey's test).
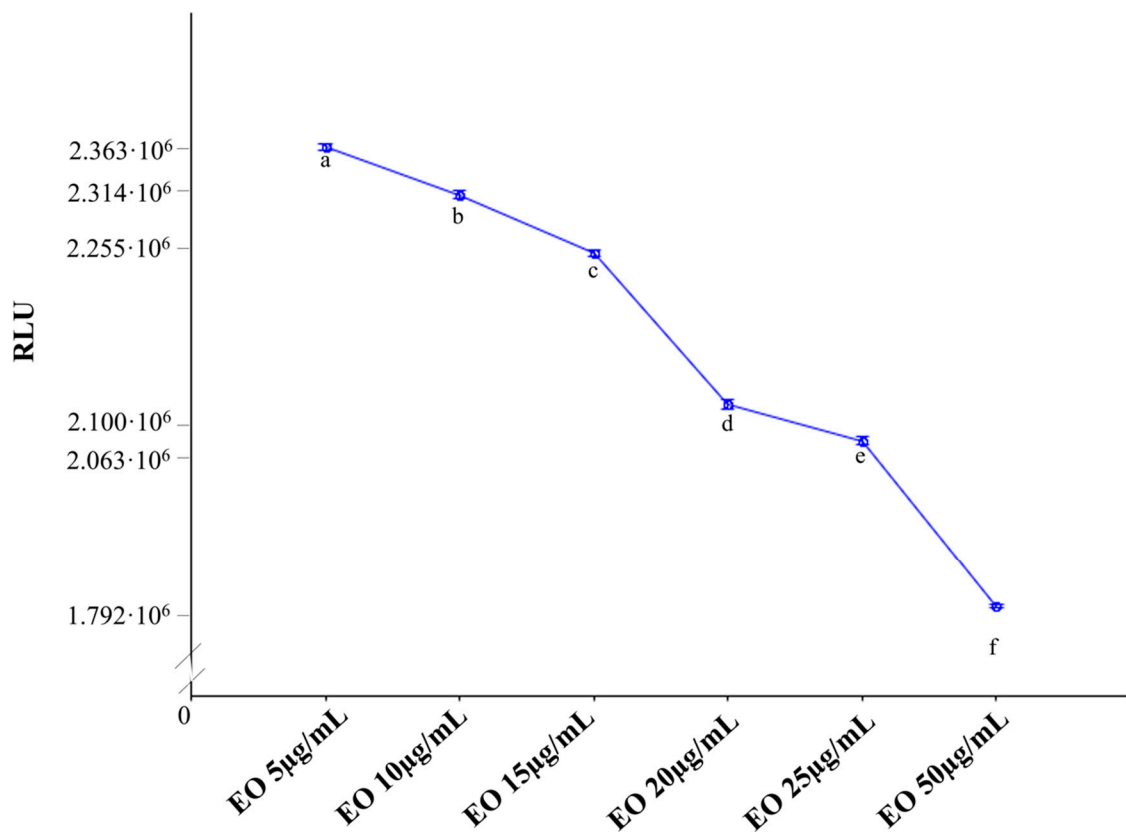
Figure 10. Effects of *E. intisy* EO (5–50 µg/mL on 0.5 mM verapamil-stimulated P-gp ATPase activity. The data are expressed as luminescence (RLU) and are presented as mean ± SE of three experiments, each in duplicate. Different letters represent significant differences (Tukey's test) in luminescence among the concentrations ($p < 0.01$).

2.6. Cytotoxic and Cell Death Effects of E. intisy EO in Combination with Doxorubicin

Given the results obtained, we wanted to verify whether the increasing intracellular accumulation of doxorubicin caused by *E. intisy* EO would lead to greater cytotoxic effects of doxorubicin in HL-60R. The effects of *E. intisy* EO and doxorubicin alone or in combination on the inhibition of cell growth were evaluated using MTS assays, while cell death was evaluated by flow cytometry analysis of DNA stained with propidium iodide. In Table 4, we report the percentages of cell growth inhibition by EO and doxorubicin treatments alone. As expected, *E. intisy* EO carried out a strong potentiation effects of doxorubicin in terms of cell growth inhibition in HL-60 R, as indicated by the combination indices (Table 5). The combination of the essential oil (25 µg/mL) and doxorubicin at different concentrations in HL-60R cells also caused a significant potentiation in the induction of cell death (Table 6 and Figure S2).

Table 4. Cell growth assays of HL-60R cells following treatment with various concentrations of *E.* ± standard error of three independent experiments.

Treatment	Cell Growth Inhibition %
E. intisy EO 15 µg/mL	16.0 ± 1.4 *
E. intisy EO 20 µg/mL	22.0 ± 0.7 *
E. intisy EO 30 µg/mL	56.0 ± 1.4 *
doxo 1 µg/mL	0.5 ± 0.3
doxo 5 µg/mL	5.7 ± 3.5
doxo 10 µg/mL	11.3 ± 3.8 *

* p < 0.01 vs. control (one-way ANOVA followed by Tukey's test). Data are expressed as the mean ± standard error of three independent experiments.

Table 5. Cell growth inhibition and combination indices in HL-60R cells following combination treatment with various concentrations of *E. intisy* EO and doxorubicin.

Treatment	Cell Growth Inhibition %	Combination Index
E. intisy EO (15 µg/mL) + doxo (1 µg/mL)	17.0 ± 0.5 *	0.80
E. intisy EO (15 µg/mL) + doxo (5 µg/mL)	40.0 ± 4.0 *	0.82
E. intisy EO (15 µg/mL) + doxo (10 µg/mL)	58.0 ± 3.4 *	0.83
E. intisy EO (20 µg/mL) + doxo (1 µg/mL)	24.0 ± 0.7 *	0.85
E. intisy EO (20 µg/mL) + doxo (5 µg/mL)	59.3 ± 1.8 *	0.88
E. intisy EO (20 µg/mL) + doxo (10 µg/mL)	64.3 ± 3.2 *	0.88
E. intisy EO (30 µg/mL) + doxo (1µg/mL)	49.0 ± 0.5 *	0.90
E. intisy EO (30 µg/mL) + doxo (5 µg/mL)	69.0 ± 6.3 *	0.91
E. intisy EO (30 µg/mL) + doxo (10 µg/mL)	81.6 ± 0.9 *	0.88

* $p < 0.01$ vs. control (one-way ANOVA followed by Tukey's test). Data are expressed as the mean ± standard error of three independent experiments.

Table 6. Cell death in HL-60R cells treated for 48 h with 25 µg/mL of *E. intsy* essential oil and different concentrations of doxorubicin, either alone or in combination.

HL-60R	*Cell Death %*	*Expected %*
	4.5 ± 0.25	
+ *E.intisy* EO 25 µg/mL	5.1 ± 0.10	
+ doxo 2 µg/mL	4.8 ± 0.20	
+ doxo 5 µg/mL	8.6 ± 0.10	
+ doxo 10 µg/mL	12.7 ± 0.30	
+ doxo 25 µg/mL	15.0 ± 0.15	
+ *E.intisy* EO + doxo 2 µg/mL	6.6 ± 0.10	5.3 ± 0.55
+ *E.intisy* EO + doxo 5 µg/mL	17.6 ± 0.35	9.1 ± 0.45 *
+ *E.intisy* EO + doxo 10 µg/mL	21.6 ± 0.20	13.2 ± 0.65 *
+ *E.intisy* EO + doxo 25 µg/mL	36.8 ± 0.15	15.6 ± 0.50 *

* $p < 0.01$ vs. observed (one-way ANOVA followed by Tukey's test). Data are expressed as the mean ± standard error of three independent experiments. Expected value: Sum of the effects of the agents alone minus that of the untreated cells.

Our results agree with those reported by Neto et al. [56] and Reies et al. [57] that show that *Euphorbia* secondary metabolites were more efficient than verapamil in reverting MDR phenotypes. For this reason, we hypothesize that *E. intisy* EO can represent an effective and non-toxic P-gp inhibitor to reverse P-gp-mediated multidrug resistance and improve the potency of anticancer drugs.

3. Material and Methods

3.1. Plant Species

Euphorbia intisy Drake, known locally as "Herotse" or "Horotsy", is a cactiform shrub of the Southern Malagasy dry forest attaining 4 to 7 m. It belongs to the section Pervilleanae of the Euphorbiaceae that groups together seven species which are strictly Malagasy [30]. Trunk is upright and branched at the base. Young stems are dichotomously branched with leaves reduced to scales (almost leafless). Root system are swollen displaying a strategy to store water in xeric conditions [33]. *E. intisy* was nearly made extinct by massive exploitation in the beginning of the 20th century [36]. It is now classified as Near Threatened according to the IUCN red list [37].

3.2. Plant Material

Unlignified stems of *Euphorbia intisy* were collected in September 2018 from plants cultivated in the open air at the Botanical Garden of the University of Palermo. The plants were raised from seeds in 1984 with reference code: Euphorbiaceae N.19.C. The matrices

were placed in paper bags and kept at −30 °C for 24 h before hydrodistillation. No specific permits were required for the collection of plant material because the plants are part of the living collection of the Botanical Garden of the University of Palermo and the authors have access to that. The plant species used in the present study is listed in CITES Appendix II together with all succulent Euphorbiae and therefore seeds are exempt from the provisions of CITES. The seeds were obtained, and the plants raised before the Convention on Biological Diversity (CBD) entered into force on 29 December 1993 and therefore are pre-CBD specimens.

3.3. Essential Oil

Stems (500 g) were hand-cut into small pieces (~2 cm) and hydrodistillated for 3 h in a Clevenger-type apparatus, using *n*-pentane as collection solvent [58]. The oil was dried by anhydrous sodium sulphate and stored at −30 °C until chemical analysis and pharmacological tests. The essential oil yield was 12.93 mg. To prepare the stock solution for biological studies 2 mg of essential oil were dissolved in 1 ml of dimethyl sulfoxide (DMSO).

3.4. Gas Chromatography-Mass Spectrometry

Essential oil of *E. intisy* was analyzed by GC-MS on a single quadrupole Shimadzu GC-MS-QP2010 (Kyoto, Japan) Plus equipped with an AOC-20i autoinjector (Shimadzu, Kyoto, Japan) and a Supelcowax 10 capillary column (30 m long, 0.25 mm i.d., 0.25 μm film thickness). One μL of diluted samples (1/100 *v/v*, in n-pentane) was injected at 250 °C in a split ratio of 1:1, and the column flow (carrier gas: helium) was set at 1 mL/min. The GC oven temperature was held for 5 min at 40 °C, then increased by 2 °C/min to 180 °C, held for 60 min, and finally raised to 240 °C at 10 °C/min. The MS interface worked at 280 °C, and the ion source at 250 °C. Mass spectra were taken at 70 eV (in EI mode) from *m/z* 30 to 600. The GC/MS data were analyzed using the GCMSolution package, Version 4.11.

3.5. Identification of Compounds

The identification of compounds was carried out using the mass spectral libraries in respect to our libraries were considered [38,39], and NIST11. We only considered compounds which showed mass spectral similarities > 80% in respect to our libraries. Kovats retention indices were calculated using a series of n-alkanes (C10-C30).

3.6. Cell Lines

The HL-60 cells were obtained from ATCC®(CCL-240, Rockville, MD, USA), while its variant HL-60R were cultured as previously described [6]. Caco-2 cells was kindly provided by Professor Mario Allegra (Department of Biological, Chemical and Pharmaceutical Science and Technology, University of Palermo, Italy). HL-60 and HL-60R cells were cultured in Roswell Park Memorial Institute (RPMI) 1640, while Caco-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (HyClone Europe Ltd., Cramlington, UK) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units /mL penicillin and 100 μg/mL streptomycin (all reagents were from HyClone Europe Ltd., Cramlington, UK) in a humidified atmosphere at 37 °C in 5% CO_2. Caco-2 cells were treated 15 days after confluence, at which time the cells are differentiated in normal intestinal-like cells.

3.7. Cell Growth Assays

The cells were seeded at 5×10^3 cells/well onto 96-well plates and incubated overnight at 37 °C. At time 0, the medium was replaced with fresh complete medium supplemented with the substances (*E. intisy* EO, doxorubicin, or their combinations) at the indicated concentrations. Following 72 h of treatment, 15 μL commercial solution obtained from Promega Corp. (Madison, WI, USA) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine etho-

sulfate was added. The plates were incubated in a humidified atmosphere at 37 °C in 5% CO_2 for 2 h, and the bioreduction of MTS dye was evaluated by measuring the absorbance of each well at 490 nm using a microplate absorbance reader (iMark Microplate Reader; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell growth inhibition was expressed as a percentage of the absorbance of the control cells. For the combinations, to evaluate the type of interaction between the agents, dose-effect curves were analyzed according to the Chou and Talalay method [59] using CalcuSyn®software (version 2.1; Biosoft, Cambridge, UK) as a non-constant ratio combination. The combination index (CI) indicates a quantitative measure of the degree of drug interaction in terms of synergistic (CI < 1), additive (CI = 1) or antagonistic (CI > 1) effects.

3.8. Evaluation of Cell Death by Flow Cytometry

The cells were collected and washed twice with ice-cold PBS and then resuspended in a hypotonic fluorochrome solution containing propidium iodide (PI) 50 μg/mL in 0.1% sodium citrate plus 0.03% (*v/v*) Nonidet P-40, at 1×10^6/mL. After incubation (at least 1 h at 4 °C) in this solution, the samples were filtered through nylon cloth, 40 μm mesh, and their fluorescence was analyzed using a FACSCanto instrument (Becton Dickinson, Montain View, CA, USA). The data were analyzed with BD FACSDiva software v.6.1.2. (Becton Dickinson). Cell death was determined by evaluating the percentage of events accumulated in the $preG_0$-G_1 position [48].

3.9. NF-κB Activation

The DNA binding capacity of NF-κB (p65 subunit) was measured in the nuclear extracts of cells treated using the TransAM NF-κB and Nuclear ExtractTM Kits (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. The results were expressed as arbitrary units: One unit is the DNA binding capacity shown by 2.5 μg of whole cell extract from Jurkat cells stimulated with 12-Otetradecanoylphorbol-13-acetate (TPA) + calcium ionophore (CI)/μg protein of HL-60/R nuclear extracts.

3.10. Extraction of Cellular RNA and Reverse Transcription-Quantitative PCR (RT-qPCR)

Total RNA was extracted from cell lines using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). For the evaluation of gene expression, RNA was reverse transcribed using a high-capacity complementary DNA (cDNA) reverse transcription kit (Applied Biosystems Life Technologies Inc., Foster City, CA, USA). The resulting cDNAs were subjected to real-time RT-PCR using the TaqMan Gene Expression Master Mix kit (Applied Biosystems Life Technologies Inc., Foster City, CA, USA) in triplicates. The PCR cycling conditions were as follows: Denaturation at 50 °C for 2 min, annealing at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and extension at 60 °C for 60 min. The running of the samples and data collection were performed on a StepOne AB Real Time PCR system (Applied Biosystems Life Technologies Inc., Foster City, CA, USA). β-actin was used as an internal standard. The specific TaqMan Assay used were: Survivin Hs00153353, XIAP Hs00236913, Bcl-2 Hs00236329, ABCB1 Hs00184005 (Applied Biosystems Life Technologies Inc., Foster City, CA, USA). Relative expression was calculated using the comparative Ct method [ΔCt = Ct(target gene)−Ct(housekeeping gene)]. Where Ct was the fractional cycle number at which the fluorescence of each sample passed the fixed threshold. Fluorescence was measured at 515–518 nm using StepOne AB Real Time PCR System software (Applied Biosystems Life Technologies Inc., Foster City, CA, USA). The ΔΔCt method was used to determine gene expression levels. ΔΔCt was calculated using the formula: $\Delta\Delta Ct = \Delta Ct_{(each\ sample)} - \Delta Ct_{(reference\ sample)}$. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ equation.

3.11. Western Blot Analysis

The whole-cell lysates were obtained from HL-60R cells using radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology Inc., Dallas, TX, USA) and 25 μg protein

was subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane (Amersham, Pharmacia Biotech, Milan, Italy) using a semi-dry fast blot apparatus (Bio-Rad, Milan, Italy). Membranes were blocked with 5% (*w/v*) BSA in PBS-0.1% (*v/v*) Tween 20 for 1 h and then filters were incubated with primary antibodies raised against GAPDH (1:20,000; Sigma-Aldrich Srl, Milan, Italy.), XIAP (1:500; Cell Signaling Technology, Inc. Danvers, MA, USA), survivin (1:2000, Abcam Limited, Cambridge, UK), Bcl-2 (1:1000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and P-gp (1:100, Invitrogen, Milan, Italy). The hybridization was visualized using an enhanced chemiluminescence detection kit (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific Life Technologies Italia, Monza, Italy) and the Versa DOC imaging system (BioRad Laboratories, Milan, Italy). Immunoblots were quantified by densitometry and results were expressed as arbitrary units (protein/GADPH).

3.12. Determination of Doxorubicin Accumulation

In order to evaluate the effects of *E. intisy* EO on intracellular accumulation of doxorubicin in HL-60R cell line, the cells were cultured in 24-well plates at a density of 1×10^5 cells per well. After 24 h, the cells were treated with *E. intisy* EO 15 μg/mL or verapamil 10 μM. After 24 h of incubation doxorubicin 1 μg/mL has been added for different time: 30min, 1h and 2h. Subsequently, the cells were washed twice with PBS and then resuspended in 400 μL PBS for doxorubicin fluorescence intensity measurement by flow cytometry using a FACSAria III instrument (Becton Dickinson, Mountain View, CA, USA). The results are presented as percentage of fluorescence intensity relative to control (means $\pm$ SE of three experiments).

3.13. P-gp ATPase Activity Determination

P-gp ATPase activity was performed with Pgp-Glo™ Assay Systems (Promega, Madison, WI, USA) following manufacturer's instructions. Different concentrations of *E. intisy* EO (test compound, TC) were added to a 96-well white plate in duplicate and incubated with recombinant human P-gp membranes. No treatment control (NT) with only Pgp-GLO assay buffer was used to provide a measure of unregulated ATPase activity, while Na_3VO_4 (0.25 mM) was used as the selective inhibitor of P-gp ATPase activity and provides a measure of P-gp-independent ATPase activity. Verapamil (0.5 mM) is a P-gp substrate that stimulates P-gp ATPase activity and serves as a positive control for drug stimulation of P-gp ATPase activity. We also performed an ATP Standards curve as internal control, to verify the correct execution of the assay, and another control with DMSO (equivalent amount of higher concentration of *E. intisy* EO). MgATP (5 mM) was added to initiate the ATPase activity. After 40 min incubation at 37 °C, the reaction was stopped with 50 μL ATPase Detection Reagent and then incubated for 20 min at room temperature. Luminescence was measured using a GLOMAX Multidetection System (Promega). We calculated ΔRLU_{basal} that reflects basal P-gp ATPase activity as the difference between the average luminescent signals from Na_3VO_4-treated samples (RLU_{Na3VO4}) and untreated (NT) samples (RLU_{NT}). After, we calculated ΔRLU_{TC} that reflects P-gp ATPase activity in the presence of a test compound, as the difference between the average luminescent signals from Na_3VO_4-treated samples (RLU_{Na3VO4}) and test compound-treated samples (RLU_{TC}). The data were presented as change in luminescence (ΔRLU). To confirm that the EO acts like the verapamil, we examined its inhibitory effects against a verapamil-stimulated P-gp ATPase activity; in this case verapamil is added in the TC wells, and the results presented as RLU produced by EO on stimulated P-gp.

3.14. Statistical Analysis

The results are given as means $\pm$ standard error (SE). Statistical analysis was carried out according to Poma et al. [7] by analysis of variance (one-way ANOVA) followed by Tukey's test. Statistica ver. 12 (StatSoft Inc., Oklahoma City, USA, 1984–2014) was used as software for the analyses.

4. Conclusions

Numerous pump inhibitors that have been produced (E.G. verapamil) are characterized by significant toxicities that reduce their use in therapy. *E. intisy* EO seems to have several potentialities with a very low toxicity. Our results suggest that *E. intisy* EO might; (i) be a stimulator of ATP-dependent drug efflux transporter; (ii) act as substrate for transport by P-gp that stimulates P-gp ATPase activity; (iii) lead to the inhibition of P-gp efflux function; (iv) reduce the expression of P-gp through NF-κB modulation. In fact, even though EO has a low effect on cell death, its multiple actions on P-gp enhance the antitumor effects of doxorubicin. EOs could be active in several types of cancer but their mechanism of action is complex due to the presence of several secondary metabolites. Further studies are planned to investigate the putative compounds involved in P-gp modulation among the most abundant in *E. intisy* EO. These compounds, or the whole EO, could be used as an adjuvant in anticancer therapy to overcome multidrug resistance. Further investigations are needed to study the pharmacokinetics of the EO.

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