PERGAMON

Phytochemistry 50 (1999) 607–614

PHYTOCHEMISTRY

Comparative phytotoxicity of artemisinin and several sesquiterpene analogues

Franck E. Dayan[a,*], Antonio Hernández[a,1], Stacy N. Allen[a], Rita M. Moraes[b], Jeffrey A. Vroman[c], Mitchell A. Avery[b,c], Stephen O. Duke[a]

[a]*United States Department of Agriculture, Agricultural Research Service, Natural Products Utilization Research Unit, P.O. Box 8048, University of Mississippi, Columbus, MS 38677, USA*

[b]*National Center for the Development of Natural Products, School of Pharmacy, University of Mississippi, Columbus, MS 38677, USA*

[c]*Department of Medicinal Chemistry and Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, Columbus, MS 38677, USA*

Received 20 July 1998

1. Introduction

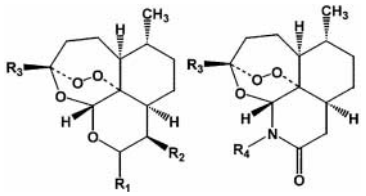
Plant-derived secondary compounds have great potential in the development of environmentally safe herbicides with novel molecular sites of action (Duke, Dayan, Hernández, Duke & Abbas, 1997). Artemisinin is a naturally occurring antimalarial sesquiterpene endoperoxide lactone isolated from the shoots of *Artemisia annua* (Klayman, 1985). Artemisinin and several of its structural analogues are also potent plant growth inhibitors (Duke, Vaughn, Croom & Elsohly, 1987; Duke, Paul & Lee, 1988; Chen & Leather, 1990; DiTomaso & Duke, 1991).

Several research groups (i.e. Brossi, Venugopalan, Dominguez Gerpe, Yeh, Flippen-Anderson et al., 1988; Avery, Gao, Chong, Mehrotra & Milhous, 1993; Posner, Oh, Wang, Gerena, Milhous et al., 1994; Avery, Bonk, Chong, Mehrotra, Miller et al., 1995; Jefford, Kohmoto, Jaggi, Timari, Rossier et al., 1995; Avery, Mehrotra, Johnson, Bonk, Vroman et al., 1996; Casteel, 1991), have generated a series of synthetic artemisinin analogues in order to address solubility and persistence problems associated with the use of these compounds in malaria treatment. Dihydroartemisinin and other derivatives including ethers and esters (arteether and artesunate, respectively) are more potent drugs than artemisinin and are available as commercial drugs in China and other countries. In spite of numerous studies, the mode of action of artemisinin remains controversial (Cumming, Ploypradith & Posner, 1997; Kamchonwongpaisan & Meshnick, 1996). In this paper, our group compared the phytotoxicity of a series of sesquiterpene analogues (Table 1), with respect to artemisinin. In an effort to discover environmentally and toxicologically safe herbicides with possible new sites of action, we also investigated the physiological responses of plants treated these compounds.

2. Results

2.1. Phytotoxicity of endoperoxide sesquiterpene lactone analogues

Most artemisinin analogues inhibited seed germination of monocots and root growth of dicots with the exception of the biologically inactive **6** (Table 2). Among the synthetic analogues, **4**, **7**, **8** and **10** were more potent than **1**. Other analogues tested (**11**, **12**) were not as active but were selective, being more toxic against *Lolium* than to *Lactuca* or *Arabidopsis*. However, a wider range of dosages are needed to con-



* Corresponding author. Tel.: 001 601 232 1039; Fax: 001 601 232 1035; E-mail: fdayan@ag.gov

[1] Permanent address: Universidad del Pais Vasco/EHU, Facultad de Ciencias, Dep. Biologia Vegetal y Ecologia. Apartado 644, 48080-Bilbao, Spain.

0031-9422/98 $ - see front matter
PII: S0031-9422(98)00568-8

Table 1
Structural characteristics of compounds used in this study

i.d.	Name	R_1	R_2	R_3	R_4
1	Artemisinin	O	CH_3	CH_3	
2	Dihydro	OH	CH_3	CH_3	
3	Arteether	OEt	CH_3	CH_3	
4	JAV-29	H,H	$CH_2CH_2CH_2CH_3$	CH_3	
5	JAV-126	H,H	$CH_2CH_2CH_2$-*p*-Cl-phenyl	CH_3	
6	JAV-146	O	$CH_2CH_2CH_2$-phenyl	CH_3	
7	SMUND-270	H,H	H	$CH_2CH_2CH_3$	
8	SMUND-283	H,H	H	CH_2 CH_2COOH	
9	JBUND-2			CH_3	$CH_2CH_2CH_2CH_2CH_3$
10	JBUND-8			CH_3	$CH_2CH(CH_3)_2$
11	JBUND-10			CH_3	$CH_2CH_2CH_2$-phenyl
12	JBUND-12			CH_3	CH_2CH_2-phenyl

firm selectivity. The most active compounds tested (**2**, **3** and **7**) were active at concentrations as low as 0.001 mg/ml (approximately 3 μM). All the other compounds required at least 10 times that concentration (Table 2).

2.2. Physiological responses to selected endoperoxide sesquiterpene lactones

Compounds **1**–**3** were selected as representative active compounds, and compound **6** was selected as an inactive derivative. Growth of roots was inhibited by the three active sesquiterpenes (Fig. 1A). Root growth inhibition was proportional to concentration and at higher concentrations, the compounds ranked, **2** > **3** > **1** > > > **6**, in order of decreasing activity.

The three active compounds also reduced chlorophyll content of lettuce seedlings, and the trend was similar to that observed with inhibition of root growth (Fig. 1B). High concentrations of **2** and **3** caused bleaching of cotyledonary tissues. Carotenoid concentrations were also reduced in tissues treated with the active analogues (data not shown). Some of these compounds demonstrated bleaching of photosynthetic tissues which is often associated with cellular and subcellular membrane degradation. None of the compounds tested demonstrated loss of membrane integrity as measured by monitoring electrolyte leakage from tissues (Fig. 2) (Duke & Kenyon, 1993).

Many phytotoxins inhibit plant growth and development by directly affecting cell division (Hess, 1987). Compound **1** reduced mitotis at the lowest concentration (10 μM), relative to untreated cells (Fig. 3). Mitotic inhibition was more pronounced at the highest concentration (100 μM), where few cells were actively dividing and all stages of mitosis were reduced (Fig. 3), suggesting that **1** prevents induction of the cell cycle. Some cells treated with **1** contained aberrant mitotic phases (Fig. 3). Chromosomes were well defined but were not correctly arranged within the cell (Fig. 4). Control treatments contained no aberrant mitotic forms.

As previously reported (Stiles, Leather & Chen, 1994), **1** increased oxygen uptake in lettuce root tips. Increase in oxygen consumption was proportional to

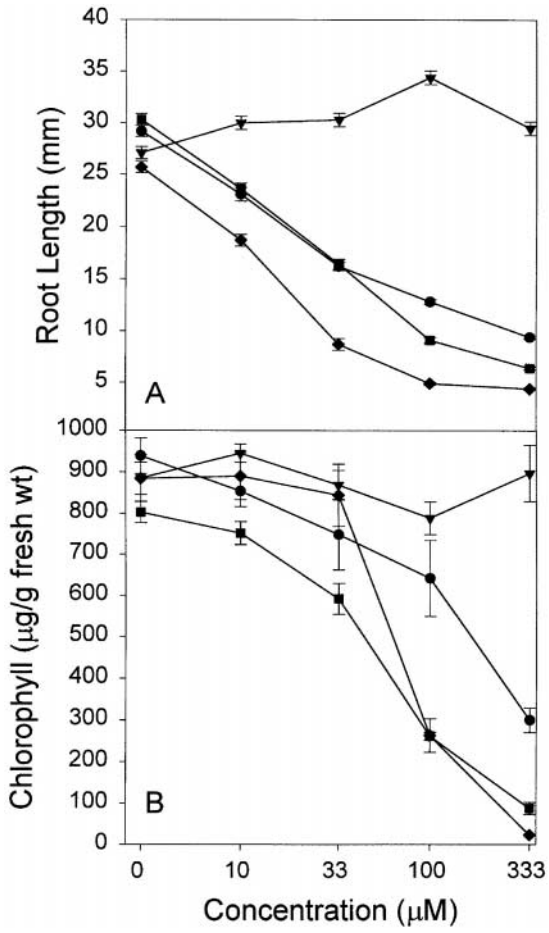


Fig. 1. Effects of artemisinin and its analogues on A) root length and B) chlorophyll content of 4 d of growth: (●) artemisinin (1), (■) arteether (3), (◆) dihydro (2), and (▼) JAV-146 (6). Error bar = ± 1 SD.

Table 2
Pre-emergence herbicidal activity of artemisinin and various analogues

Compound	Concentration		Root inhibition %[a]		Seed germination score[a]		Herbicidal activity	
	(mg/ml)	(μM)[b]	*Lactuca*	*Lolium*	*Arabidopsis*	*Lactuca*[c]	*Lolium*[c]	*Arabidopsis*[d]
Control	0	0	0	0	5.0	−	−	−
1	0.001	(3.5)	0	42	5.0	−	−	−
	0.01	(35)	0	47	5.0	−	−	−
	0.1	(350)	30	100	0	−	+	+
2	0.001	(3.5)	47	45	1.0	−	−	+
	0.01	(35)	100	100	0.0	+	+	+
	0.1	(350)	100	100	0.0	+	+	+
3	0.001	(3.2)	0	0	3.0	−	−	±
	0.01	(32)	100	100	0.0	+	+	+
	0.1	(320)	100	100	0.0	+	+	+
4	0.001	(3)	0	0	5.0	−	−	−
	0.01	(30)	100	100	0.0	+	+	+
	0.1	(300)	100	100	0.0	+	+	+
5	0.001	(3)	0	31	5.0	−	−	−
	0.01	(30)	30	72	0.0	−	±	+
	0.1	(300)	80	94	0.0	±	+	+
6	0.001	(3)	0	0	5.0	−	−	−
	0.01	(30)	0	13	5.0	−	−	−
	0.1	(300)	0	30	5.0	−	−	−
7	0.001	(3.5)	28	73	5.0	−	±	−
	0.01	(35)	100	100	0.0	+	+	+
	0.1	(350)	100	100	0.0	+	+	+
8	0.001	(3)	0	41	5.0	−	−	−
	0.01	(30	80	100	5.0	+	+	−
	0.1	(300)	100	100	2.0	+	+	±
9	0.001	(3)	0	15	5.0	−	−	−
	0.01	(30)	31	62	5.0	−	−	−
	0.1	(300)	33	100	0.0	−	+	+
10	0.001	(3)	68	81	4.5	−	−	−
	0.01	(30)	100	100	0.0	+	+	+
	0.1	(300)	100	100	0.0	+	+	+
11	0.001	(2.6)	0	0	5.0	−	−	−
	0.01	(26)	0	32	4.0	−	−	−
	0.1	(260)	0	90	0.0	−	+	+
12	0.001	(2.7)	0	43	5.0	−	−	−
	0.01	(27)	0	100	5.0	−	−	−
	0.1	(270)	16	100	2.0	−	+	±

[a] Each value corresponds to the average of 4 replications.
[b] Initial study was performed as a 'blind' screen for herbicidal activity and the compounds were tested on a mg/ml basis. The table also shows the actual μM concentration in parenthesis.
[c] Activity based upon *Lactuca* and *Lolium* root inhibition: high activity (+) 85–100%, moderate activity (±) 70–85%, below 70% no activity.
[d] Activity based upon *Arabidopsis* seed germination score: high activity (+) 0–1 (0–20% germination), moderate activity (±) 1–2 (20–40% germination), no activity (–) 2–5 (40–100% germination).

the concentration of **1** (Fig. 5A). When effects of other artemisinin analogues were tested along with **1** (Fig. 5B), a positive relationship was found between oxygen consumption levels and biological activity. The three active sesquiterpenes (**1**, **2** and **3**) caused greater respiratory rates than in untreated tissues, whereas **6** had no effect. However, the effect of **1** on respiration was not as clear when expressed on a root dry-weight basis (data not shown). This is probably due to the fact that artemisinin-treated roots were thicker than control roots. Thus, the apparent increase in respiratory rate observed in artemisinin-treated roots appears to be due to indirect developmental effects. Similar results have been reported by Duke et al. (1987) in lettuce cotyledons exposed to **1**. Nonetheless, the indirect effects of these compounds are consistent with the biological activity observed in our earlier screening experiment (Table 2) and the dose-response of selected compounds (Fig. 1A and B). This result suggests that, although unknown, the mechanism(s) of action of **1** and the active analogues on plants are probably the same.

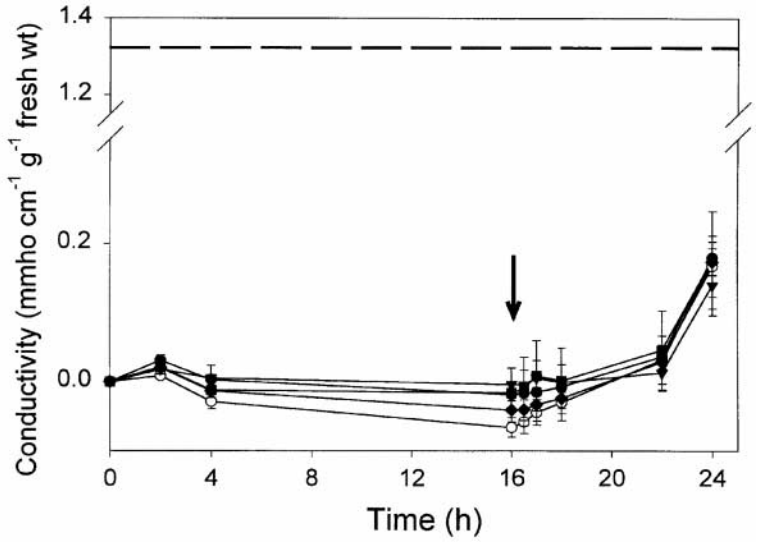


Fig. 2. Electrolyte leakage as measured by change in electrical conductivity of the bathing medium: (○) control, (●) artemisinin (1), (▼) arteether (3), (◆) dihydro (2), (■) JAV-146 (6). The dashed line represents maximum leakage obtained from boiled leaf discs. The arrow indicates the beginning of light exposure. Error bar = ± 1 SD.

2.3. Complementation experiments

The mechanism of action of **1** on the antimalarial parasite *Plasmodium* spp. has been associated with its interaction with hemes (Kamchonwongpaisan & Meshnick, 1996). Consequently, we attempted to reverse the inhibitory effect of **1** with various natural substrates. A high concentration of **1** (333 μM) caused a decrease in chlorophyll and carotenoid contents that δ-aminolevulinic acid (ALA) at 100 or 1000 μM could not revert (data not shown). ALA treatments failed to reverse the phytotoxicity of **1** and increased the inhibition of root growth relative to control (data not shown). Neither glutathione nor any of the organic acids tested (ascorbic, citric, oxalacetic, α-keto glutaric, malic, pyruvic, succinic and acetic acids) significantly diminished the phytotoxicity of **1** on root growth and chlorophyll content (data not shown).

None of the treatments with the amino acids, reducing agents, purines, and pyrimidines reversed the phytotoxic effects of artemisinin. Some of the compounds (e.g. pyruvate, oxaloacetate, and glutamate) increased the damage observed on the seedlings (data not shown).

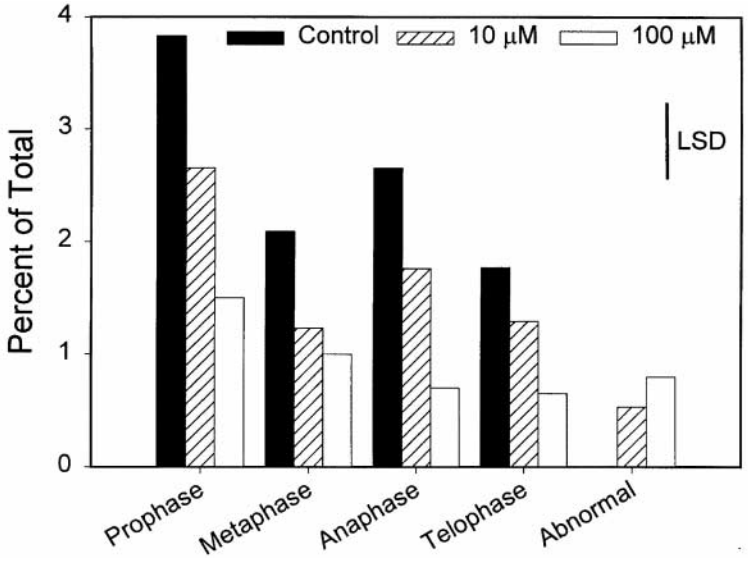


Fig. 3. Distribution of phases of mitotis in onion root tips treated with artemisinin. LSD at $p = 0.05$.

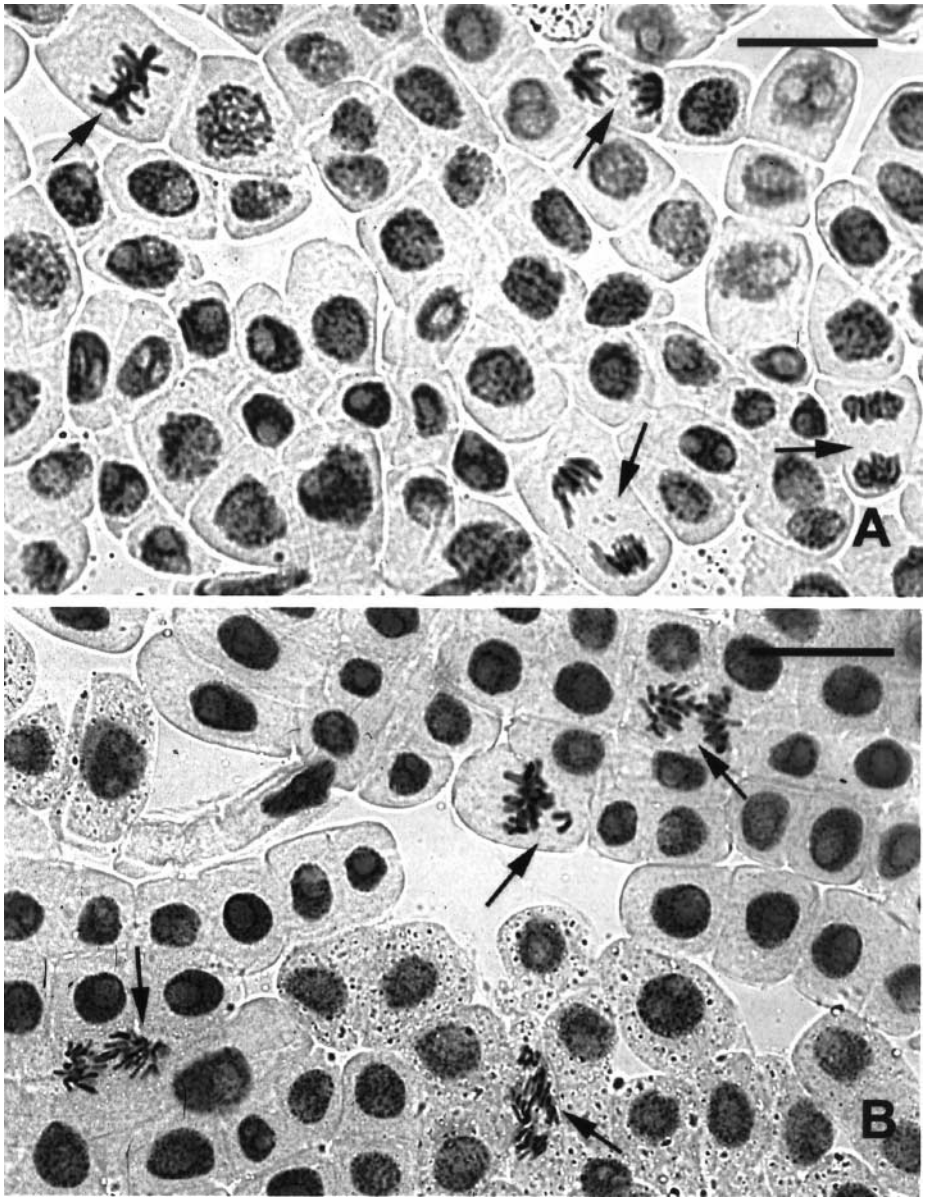


Fig. 4. Micrographs of A) control and B) treated-cells (100 μM artemisinin (1)) of onion root tip. Cells at various stages of mitosis are indicated by arrows. Notice the abnormal mitotic stage in artemisinin-treated cells shown in micrograph B. Bar represents 100 μm.

3. Discussion

Duke et al. (1987) first reported the phytotoxic properties of **1**. More recently, the herbicidal activity of **1** and various structural analogues has been the subject of numerous studies (Duke et al., 1988; Chen & Leather, 1990; Slater, Swiggard, Orton, Flitter, Goldberg et al., 1991; Bagghi, Jain & Kumar, 1997; Lydon, Teasdale & Chen, 1997). Our study sought to broaden the understanding of the structural requirement for phytotoxic activity of sesquiterpene endoperoxide lactone and investigate the mechanism of action of these compounds.

Compound **1** and most of the synthetic derivatives were phytotoxic and their effect was most evident on root growth and chlorophyll content. Inhibition of root growth may be associated with inhibition of cell division. Inhibition of mitosis is commensurate to the concentration of **1** applied. Abnormal mitotic stages observed at the highest concentration tested suggests that **1** may interfere with mitosis. However, secondary effects of phytotoxins can also result in interference with mitosis (Hess, 1987). It is unclear whether the inhibition of cell division is a direct effect of the mechanism of action of these sesquiterpene lactones. However, we observed several cells with multipolar configurations (arrows in Fig. 4B), which suggests that artemisinin may disrupt the formation of microtubule organizing centers. Similar observations of onion root cells treated with terbutol and sindone B indicated

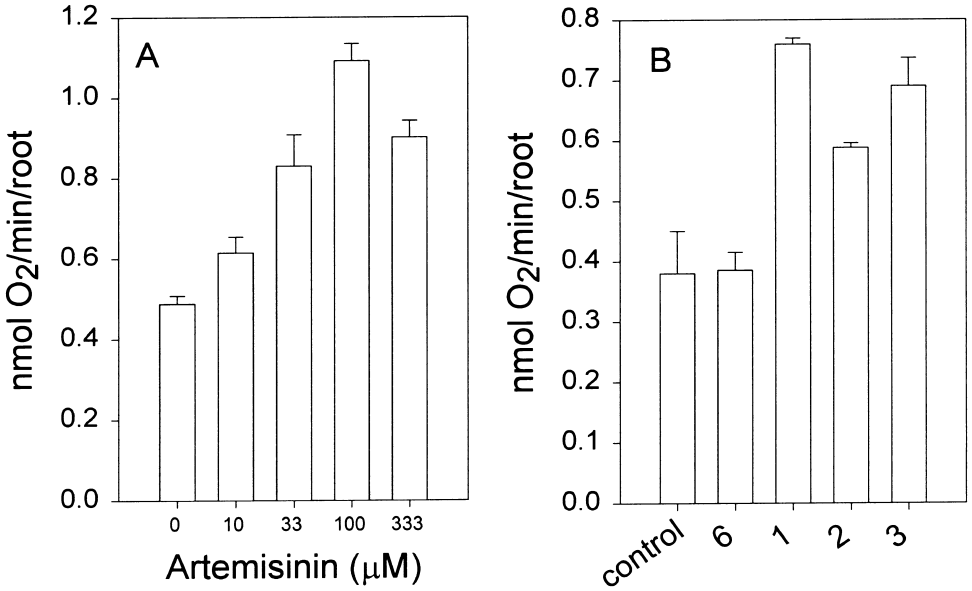


Fig. 5. Effect of artemisinin (1) on respiration of lettuce root tips (A), and comparison between various sequiterpene endoperoxide lactones at 33 μM (B). Error bar = ± 1 SD.

impaired microtubule development (Lehnen, Vaughan & Vaughn, 1990; Lehnen & Vaughn, 1992).

Similarly, the decrease in chlorophyll content does not appear to be through direct inhibition of the chlorophyll biosynthetic pathway. Addition of ALA did not restore chlorophyll synthesis, indicating that **1** does not cause inhibition of early steps of porphyrin biosynthesis. None of the complementation experiments demonstrated a reversal of root growth inhibition, suggesting that the phytotoxic effect of **1** on lettuce is not associated with impaired amino acid or nucleic acid pathways.

Stiles et al. (1994) suggested that the symptoms observed may be attributed to loss of membrane integrity. Our study indicates that none of the sesquiterpene analogues tested caused plasma membrane leakage. Therefore, it is not likely that these sequiterpene lactones damage plant membranes.

All of the active compounds caused similar symptoms, suggesting that they all act through the same mechanism(s) of action. The least phytotoxic compounds consisted of derivatives with large hydrophobic moieties (**6**,**9**,**11**,**12**). The lack of activity of such compounds may be due to poor uptake by plants. Finally, there was no correlation between the phytotoxicity of these compounds on plant and their biological activity on *Plasmodium* spp. Compounds **4** and **5** were as much as 60 times more active against *Plasmodium* than artemisinin (Avery et al., 1996) whereas they were only about 10 times more phytotoxic than artemisinin. As well, **6** was active against *Plasmodium* (Avery et al., 1996), while being inactive on plants. Conversely, **8** was totally inactive against *Plasmodium* but was more phytotoxic than artemisinin. This suggests that the mode of action of sesquiterpenes differs between *Plasmodium* and plants. However, differences in uptake and metabolism between plants and *Plasmodium* spp. may confound such comparisons.

4. Experimental

4.1. Phytotoxicity of natural and synthetic endoperoxide sesquiterpene lactone analogues

Natural products **1**–**3**, and synthetic analogues (**4**–**12**) were tested for herbicidal activity (Table 1). All compounds used in this study were synthesized and characterized according to published methods (Brossi et al., 1988; Avery et al., 1995; Avery et al., 1996). The synthetic compounds representing two different substitution patterns were C3 substituted (**7** and **8**) and C9 substituted (**4**, **5** and **6**). Compounds **4** and **5** are reduced forms of **1** at C10 position. Four amide analogues produced by ring modification (**9**, **10**, **11** and **12**) were also tested. Biological activity of these compounds were tested in 24 well plates. Each treatment consisted of 4 replicates and 2 controls. All compounds were dissolved in 10 μl of acetone and diluted with water containing 1 ml/L Tween 20 and tested at final concentrations of 0.1, 0.01, and 0.001 mg/ml. Controls included consisted of seeds germinated with similar amount of solvent without test compound. Species tested were lettuce (*Lactuca* cv. Iceberg), rye (*Lolium multiflorum* cv. Gulf), and mouseearcress (*Arabidopsis thaliana*). A 200 μl volume of each test

solutions was applied to each well. Plates were incubated at $25 \pm 2^{\circ}C$ under fluorescent lights maintaining a 16 h photoperiod at 400 μmol m^{-2} s^{-1}. Germination rates of Arabidopsis and root length of lettuce and rye were measured on 7 d old seedlings. Germination was rated on a 0 to 5 scale (no effect to 100% inhibition).

4.2. Growth of plant material

Lettuce seeds (0.100 g) were germinated in 9 cm disposable petri dishes lined with #1 Whatman filter paper in the presence of 3 ml of water with or without test compounds. Dishes were maintained in complete darkness for 24 h before placing under light (400 μmol m^{-2} s^{-1}) in a growth chamber at 25°C.

4.3. Dose-response of selected compounds

Dose-response curves for concentrations ranging from 0 to 300 μM were obtained for artemisinin (**1**), dihydroartemisinin (**2**), arteether (**3**), and JAV-146 (**6**). Plant responses to the test compounds included chlorophyll content and root length 4 d after treatment. Chlorophyll was extracted from 15 pairs of cotyledons per treatment in 3 mL dimethyl sulfoxide (Hiscox & Israelstam, 1979) and chlorophyll concentrations determined spectrophotometrically according to Arnon (1949).

4.4. Effect of artemisinin and analogues on onion root cell division (mitotic index)

Onion (*Allium cepa* L. cv. Evergreen bunching) seeds were germinated as previously described in the presence of 10 and 100 μM artemisinin at 25°C under a 14 h photoperiod. Root tips were prepared according to Armbruster, Molin and Bugg (1991) and mitotic analysis was performed on 1000 cells per slide (3000 cells per treatment).

4.5. Effect on oxygen consumption

Twenty lettuce root tips (6 mm) were cut and placed into the thermostabilized electrode chamber (25°C) with 2 ml of O_2 saturated water. Oxygen uptake was measured polarographically for 1 min using a computer-controlled Hansatech DW1 oxygen probe. A dose-response was measured for artemisinin in the following concentrations (0, 10, 33, 100 and 333 μM). Activity of artemisinin was compared to the 3 selected derivatives at 33 μM as described above.

4.6. Electrolyte leakage

Fifty 4 mm cotyledon discs of 7–10 d old cucumbers were placed on a 1% sucrose/1 mM Mes-NaOH buffer (pH 6.8) containing 0 or 333 μM of test compounds. Plates were incubated in darkness for 20 h prior to exposure to light (400 μmol m^{-2} s^{-1}). Conductivity of the bathing solutions was measured at regular intervals for 8 h. The experiment consisted of 3 replicates and the experiment was repeated.

4.7. Complementation experiments

We attempted to reverse the phytotoxic effects of artemisinin by complemention studies with amino acids, purines, pyrimidines, reductants and organic acids. All 20 essential amino acids, and purines and pyrimidines (adenine, hypoxanthine, uracil, methylcytosine, thymine, and cytosine) were individually tested at 100 μM in the presence or absence of 100 μM artemisinin. The organic acids (ascorbic, citric, oxalacetic, α-keto glutaric, malic, pyruvic, succinic, and acetic acids), and an antioxidant (glutathione) were tested at 333 μM in the presence of 333 μM artemisinin. ALA was tested at 100 and 1000 μM under the same condition as described above.

Acknowledgements

This research was supported, in part, by a fellowship from the Department of Education, Universities and Research of the Basque Government.

References

Armbruster, B. L., Molin, W. T., & Bugg, M. W. (1991). *Pestic. Biochem. Physiol., 39,* 110.

Arnon, D. I. (1949). *Plant Phys., 24,* 1.

Avery, M. A., Bonk, J. D., Chong, W. K. M., Mehrotra, S., Miller, R., Milhous, W. K., Goins, D. K., Venkatesan, S., Wyandt, C., Khan, I., & Avery, B. A. (1995). *J. Med. Chem., 38,* 5038.

Avery, M. A., Gao, F., Chong, W. K. M., Mehrotra, S., & Milhous, W. K. (1993). *Trends Org. Chem., 4,* 413.

Avery, M. A., Mehrotra, S., Johnson, T. L., Bonk, J. D., Vroman, J. A., & Miller, R. (1996). *J. Med. Chem., 39,* 4149.

Bagghi, G. D., Jain, D. C., & Kumar, S. (1997). *Phytochemistry, 45,* 1131.

Brossi, A., Venugopalan, B., Dominguez Gerpe, L., Yeh, H. J. C., Flippen-Anderson, J. L., Buchs, P., Luo, X. D., Milhous, W., & Peters, W. (1988). *J. Med. Chem., 31,* 646.

Casteel, D. A. (1991). In: M. E. Wolff, *Burger's medicinal chemistry and drug discovery, vol. 5* (p. 3). John Wiley & Sons.

Chen, P. K., & Leather, G. R. (1990). *J. Chem. Ecol., 16,* 1867.

Cumming, J. N., Ploypradith, P., & Posner, G. H. (1997). *Adv. Pharmacol., 37,* 253.

DiTomaso, J. M., & Duke, S. O. (1991). *Pestic. Biochem. Physiol., 39,* 158.

Duke, S. O., Dayan, F. E., Hernández, A., Duke, M. V., & Abbas, H. K. (1997). *Proc. Brighton Crop Prot. Conf., 00,* 579.

Duke, S. O., & Kenyon, W. H. (1993). In: P. Böger, & G. Sandmann, *Target assays for modern herbicides and related phytotoxic compounds* (p. 61). Lewis Publishers.

Duke, S. O., Paul, R. N., & Lee, S. M. (1988). *Am. Chem. Soc. Symp. Ser., 380,* 318.

Duke, S. O., Vaughn, K. C., Croom, E. M., & Elsohly, H. N. (1987). *Weed Sci., 35,* 499.

Hess, F. D. (1987). *Rev. Weed Sci., 3,* 183.

Hiscox, J. D., & Israelstam, G. F. (1979). *Can. J. Bot., 57,* 1332.

Jefford, C. W., Kohmoto, S., Jaggi, D., Timari, G., Rossier, J. C., Rudaz, M., Barbuzzi, O., Gerard, Burger, U., Kamalprija, P., Mareda, J., Bernardinelli, G., Manzanares, I., Canfield, C. J., Fleck, S. L., Robinson, B. L., & Peters, W. (1995). *Helv. Chim. Acta, 78,* 647.

Kamchonwongpaisan, S., & Meshnick, S. R. (1996). *Gen. Pharmac., 27,* 587.

Klayman, D. L. (1985). *Science, 228,* 1049.

Lehnen, L. P., Jr., & Vaughn, K. C. (1992). *Pestic. Biochem. Physiol., 44,* 50.

Lehnen, L. P., Jr., Vaughan, M. A., & Vaughn, K. C. (1990). *J. Exp. Bot., 41,* 537.

Lydon, J., Teasdale, J. R., & Chen, P. K. (1997). *Weed Sci., 45,* 807.

Posner, G. H., Oh, C. H., Wang, D., Gerena, L., Milhous, W. K., Meshnick, S. R., & Asawamahasakda, W. (1994). *J. Med. Chem., 92,* 811.

Slater, A. F. G., Swiggard, W. J., Orton, B. R., Flitter, W. D., Goldberg, D. E., Cerami, A., & Henderson, G. B. (1991). *Proc. Natl. Acad. Sci. U.S.A., 88,* 325.

Stiles, L. H., Leather, G. R., & Chen, P. K. (1994). *J. Chem. Ecol., 20,* 969.