https://doi.org/10.26651/allelo.j/2018-45-2-1191 0971-4693/94 Euro 20.00
Allelopathy Journal 45 (2):255-262 (November, 2018) *International Allelopathy Foundation* 2018
Table: 1, Figs : 3

The natural compound podophyllotoxin induces growth inhibition and microtubule condensation on *Arabidopsis* roots

A. Costas-Gil, M.J. Reigosa and A.M. Sánchez-Moreiras*
Department of Plant Biology and Soil Science, Faculty of Biology,
University of Vigo, Campus Lagoas-Marcosende s/n, 36310, Vigo, Spain
E-mail: adela@uvigo.es

(Received in revised form: October 1, 2018)

ABSTRACT

We determined the phytotoxicity of podophyllotoxin on *Arabidopsis thaliana* (L.) Col-0. Seedlings treated with the natural compound podophyllotoxin were severely affected, showing left-handed growth and strong reduction of development. In this study we also analyzed, whether podophyllotoxin can interfere with microtubule arrangement and induce structural malformations on *Arabidopsis* roots. The results showed that the severe inhibitory effects of podophyllotoxin on *Arabidopsis* root cells altered the cortical microtubules, resulting in complete stoppage of root growth.

Key words: *Arabidopsis thaliana*, growth inhibition, lignan lactone, microtubules, phytotoxicity, secondary metabolite, podophyllotoxin, root.

INTRODUCTION

Modern agriculture uses synthetic herbicides, which are simple, cheap and easy to control weeds (7). However, these have limited number of molecular targets in plant metabolism, leading to the appearance of resistant species. The use of natural products has been proposed as an alternative to synthetic compounds, their mechanisms of action differ from synthetic herbicides (9). The synthetic herbicides are harmful to the environment and human health, while natural products usually degrade rapidly in the environment and are therefore *a priori* less harmful (7).

The search for new natural compound with herbicide potential is complex task. The most common ways to find the natural herbicides are either to test compounds that are used for other purposes due to their biological activity (used in pharmaceutical industry) or to isolate plant compounds with known allelopathic properties (9). Although compounds with phytotoxic activity can be extracted from various natural sources (bacteria, fungi, lichens, insects or plants) but most herbicides are obtained from microorganisms (6). It is thus important to find new sites of activity. Secondary metabolites produced by plants and macro- or microscopic algae are possible sources of new compounds with herbicidal potential but has not been explored.

Podophyllotoxin is a lactone commonly found in land plants of the family Berberidaceae. Although the antifungal, antiviral, anti-rheumatic and anti-carcinogenic activities of this secondary metabolite have been broadly demonstrated, its phytotoxic activity has been poorly investigated. Podophyllotoxin is natural compound of ayltetralin lignan lactones. It is found in Berberidaceae family plants [*Podophyllum peltatum* and *Podophyllum emodi* (Bruschi)], besides it is also found in brown alga *Cytoseira tamariscifolia* (1). This compound is anti-mitotic to animal cells (18,19) and blocks their

*Correspondence author

cell division through the inhibition of tubulin assembly to microtubules, in particular at the tubulin binding to the colchicine site (20). Therefore, since 1950s, different analogues of podophyllotoxin (etoposide or teniposide) are used as chemotherapeutics for tumours (20). Regarding phytotoxic activity, podophyllotoxin inhibited 94% growth in *Lolium* sp. at 250 µM concentration (21). However, little is known about its effects on roots and on microtubules of plant cells.

This study aims to analyze the phytotoxic potential of podophyllotoxin on lettuce and *Arabidopsis* roots and its effects on microtubule assembly.

MATERIAL AND METHODS

Germination and growth bioassays were done to qualitatively and quantitatively determine the phytotoxic effects of podophyllotoxin on the target species. All the experiments were done in a controlled growth chamber located at the University of Vigo (Spain). Germination and growth dose-response curves allowed calculation of IC_{50} and IC_{80} values (concentrations of the compound that induce 50% and 80% inhibition compared to the control), and LCIC (lowest concentration of compound at which inhibition is complete) (9).

Germination bioassays

(i). ***Arabidopsis thaliana* :** Its seeds of ecotype Columbia (Col-0) were sterilized in 50% EtOH and 0.5% NaOCl with 0.01% Triton for 3 min and washed thrice with autoclaved miliQ water. After sterilization, the seeds were maintained in 0.1% agar at 4 °C for 72 h for vernalization and synchronize germination. Seeds were then sown in square Petri dishes (100 x 100 x 15 mm) in Plant Agar medium mixed with macro- and micronutrients (w/v; Murashige-Skoog, Sigma-Aldrich, St Louis, MO, USA) supplemented with 1% sucrose. Except control 50, 100, 200, 400, 800, 1200 µM concentrations of podophyllotoxin (Sigma-Aldrich, St Louis, MO, USA), dissolved in EtOH (0.1%), were added to the agar. Twenty-four seeds were sown in each dish (5 dishes per treatment) and the dishes were placed vertically in growth chamber [temperature of 22 ± 2 °C, a photoperiod of 8 h light (175 µmol m^{-2} s^{-1}) and 16 h darkness for delayed flowering, and 55% relative humidity]. The number of germinated seeds was recorded 14 days after sowing.

(ii). ***Lactuca sativa*** : The bioassays of cv. Great Lakes (Phyto) were done in 9 cm dia Petri dishes on Wathman 3MM paper disks moistened with 4 mL of each podophyllotoxin solution, prepared as previously explained for *Arabidopsis* bioassays. Podophyllotoxin was prepared in ethanol (EtOH) and diluted in distilled water to 0, 50, 100, 200, 400, 800 and 1200 µM concentrations to a final concentration of EtOH of 0.1 %. Petri dishes (5 per treatment) were sown with 25 seeds per dish and placed in a growth chamber [27 °C in dark for 24 h]. Different germination indices were recorded every 3 h for 24 h to calculate both speed and rate of germination (2,4,8).

Total germination $G_T = 100 \cdot N_T/N$,

Where T : Last observation and N : Total number of seeds sown.

Germination Speed $S = n_1 + (½)n_2 + (⅓)n_3 + \ldots + (1/T)n_T$,

Where $n_t = (N_t\text{-}N_{t\text{-}1})/N_T$ (with $N_0 = 0$) is number of seeds that germinated between observations t-1 and t, expressed as a proportion of all germinated seeds.

Speed of Accumulated Germination $AS = N_1 + (½)N_2 + (⅓)N_3 + \ldots + (1/T)N_T$, which can be seen proportional to a weighted average speed in which the weights increase with time since sowing.

Growth bioassays

Arabidopsis thaliana seeds were grown as per the method described in germination bioassay, because after podophyllotoxin treatment no differences were detected in total germination or germination speed. Root length was measured after 15 days of vertical growth and these data were used to calculate dose-response curves.

For *Lactuca sativa* growth bioassays, pre-germinated seeds in water were used to independently analyze the processes of germination and growth and to prevent any bias in measurement of phytotoxic effects (14). Five replicates were used per concentration. For each treatment, 20 pre-germinated seeds (approx 2 mm radicle), were placed in 9 cm petri dishes with Whatman 3MM paper disks moistened with 4 mL water. These dishes were then kept for 48 h in growth chamber under the same conditions as for germination. The 0, 50, 100, 200, 400, 800 and 1200 µM podophyllotoxin concentrations were tested on both, *A. thaliana* and *L. sativa*. The mean data obtained from the root length were used to make a dose-response curve, from which IC_{50} and IC_{80} values were obtained.

Additionally, root structure, thickness, growth direction, presence or absence of root hairs were studied using a Nikon SMZ 1500 stereomicroscope with a CCD camera.

Microtubule fluorescence

Microtubules were detected by immunofluorescence according to the method of Holzinger *et al*. (15) with some modifications. Briefly, 70 seedlings of *Arabidopsis thaliana* were treated with IC_{50} podophyllotoxin for 7 and 14 days. Then, root meristems were cut and fixed with 0.5% glutaraldehyde solution and 1.5% formaldehyde in 50 mM PIPES buffer at pH 7.2 with 2 mM EGTA, 2 mM manganese sulfate and 0.1% Triton X-100 for 45 min. After 3-4 washings, roots were chopped and digested by incubation in PIPES buffer pH 5.5 containing 1% cellulase (cellulase from *Aspergillus* sp, Sigma-Aldrich) and 1% pectolyase Y-23 (pectolyase from *Aspergillus japonicus*, Sigma-Aldrich) for 30 minutes. Subsequently, samples were washed with PIPES buffer pH 7.2, incubated in methanol for 10 min at -20 °C, washed in PBS and incubated for 20 min in PBS with 1 mg mL^{-1} sodium borate. Roots were then incubated at room temperature in PBS with 1% BSA and 50 mM Glycine for 20 min. After this incubation, roots were incubated again at 4 °C with the primary antibody 1:1000 in PBS (Monoclonal Anti-α-Tubulin antibody produced in mouse, Sigma-Aldrich) overnight. The next day, the samples were washed with PBS to remove the primary antibody and were incubated at 37 °C with the secondary antibody 1:200 in PBS (Alexa Fluor 488F (ab')2 fragment of goat anti-mouse IgG (H+L), Molecular Probes, NY, USA) for 3 h. Samples were then mounted on Citifluor AF1 antifade agent and visualized by fluorescence microscopy using a Leica TCS SP5 microscope (Wetzlar, Germany) with a 63X oil immersion objective and a 496 nm excitation wavelength (argón laser); and photographed with a LAS-AF software.

Statistical analyses

The normality of data was verified by Kolmogorov-Smirnov tests and then statistical test of Levene was done to verify the homogeneity of variances. In

homoscedastic data, one ANOVA was performed with DMS, while T2 of Tamhane was performed for the heteroscedastic data.

RESULTS

The podophyllotoxin treatment did not affect the germination of *Lactuca sativa*, as slightly decreased the total germination index at 800 µM concentration (Table 1). This decrease was also observed in germination speed (shown by S and AS indexes), indicating slight delay in germination process. A similar trend was observed in root growth assay, since although significant reductions of 20% compared to the control were observed at the lowest podophyllotoxin concentration (50 µM) tested, this inhibition remained constant. The effect was so slight that the IC_{50} (2.5 mM) and IC_{80} (5 mM) concentrations were very high for phytotoxic studies (Table 1).

Table 1. Percentage values compared to the control of the Total Germination (GT), Germination Rate (S) and Cumulative Germination Rate (AS) calculated for *Lactuca sativa* seedlings at different times during 24 h treatment with different concentrations of podophyllotoxin in a controlled growth chamber. IC_{50} and IC_{80} values for radicle growth were also calculated after 24 h podophyllotoxin treatment.

Podophyllotoxin concentrations	**Germination Index**		
	GT	**S**	**AS**
Control	100.00^{ab}	100.00^{a}	100.00^{a}
50 µM	101.23^{b}	99.14^{ab}	98.68^{ab}
100 µM	107.62^{b}	98.01^{abc}	96.02^{ab}
200 µM	105.65^{b}	97.52^{abc}	71.71^{ab}
400 µM	104.39^{b}	96.77^{abc}	92.45^{ab}
800 µM	85.68^{a}	95.19^{c}	88.13^{b}
1200 µM	100.18^{ab}	95.80^{bc}	89.53^{ab}
	Radicle growth		
IC_{50}	2.5 mM		
IC_{80}	5 mM		

Superscripts indicate statistically significant differences between the different podophyllotoxin concentrations. Shaded boxes indicate significant differences compared to the control ($p \leq 0.05$). The IC_{50} and IC_{80} values for radicle growth inhibition are also shown in the table.

On the contrary, although germination of *Arabidopsis thaliana* was neither affected after podophyllotoxin treatment, but the root length of *Arabidopsis* treated seedlings was strongly affected (Fig. 1), with IC_{50} and IC_{80} values as low as 111 and 294 µM, respectively (Fig. 1A).

The growth bioassay showed that podophyllotoxin adversely affected the seedlings growth, reduced not only the root length but also the shoot growth (Fig. 1B) in treated seedlings, with smaller cotyledons and absence of true leaves than control. In contrast, no differences were observed in the number of secondary roots in the treated seedlings.

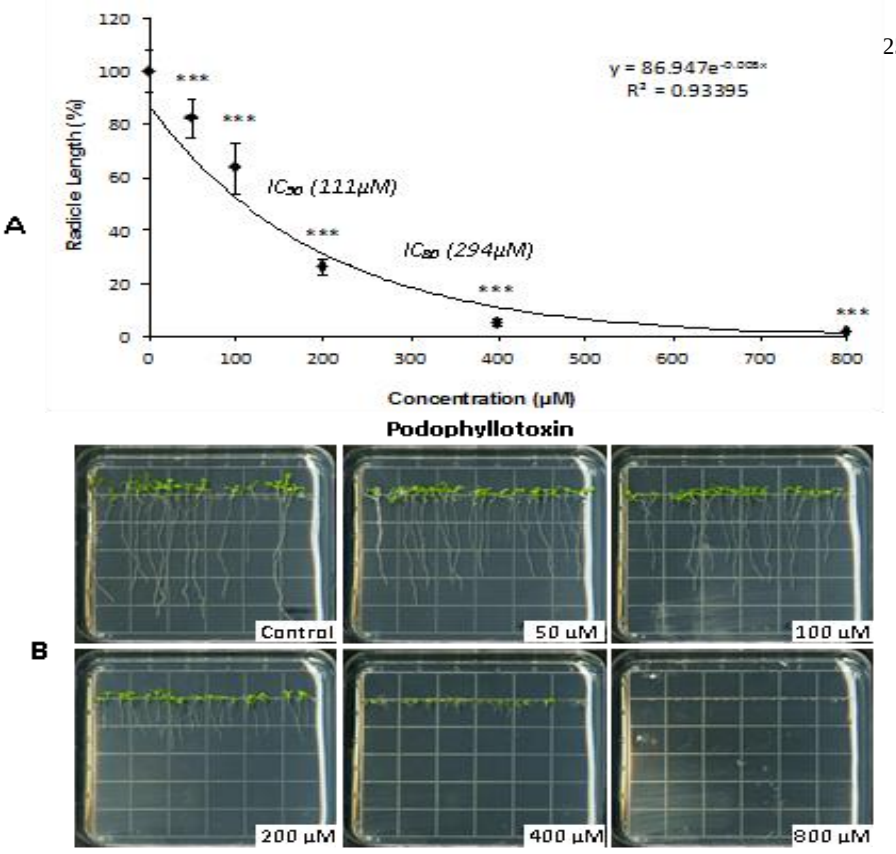

Figure 1. **(**A) Radicle length dose-response curve (R^2 = 0.9339) for podophyllotoxin-treated *Arabidopsis* seedlings, as a percentage of the control, where *** indicates highly significant differences compared to the control ($p \leq 0.001$). The IC_{50} and IC_{80} values are shown in the graph. A Kolmogorov-Smirnov test was performed to verify the normality of the data. The data did not follow a normal distribution and were analyzed by Kruskal-Wallis. (B) *Arabidopsis* seedlings after 14 days of podophyllotoxin treatment.

The results presented here confirm the phytotoxicity of podophyllotoxin to the test species *Arabidopsis thaliana*, which showed a strong inhibition of root growth accompanied by root twisting and deformations in the cell wall after the treatment. These results were different from those of lettuce, where root length showed just a slight significant decrease after podophyllotoxin treatment. This weak phytotoxic effect is in agreement with the results obtained by Oliva et al. (21), who found an almost complete inhibition of the root elongation of *Lolium* root growth after treatment with 250 µM podophyllotoxin but no inhibition of lettuce growth. In addition, our work shows that no relevant effects on lettuce germination are observed after podophyllotoxin treatment, as only a slight delay in the germination process could be detected at the stronger concentrations tested (800 and 1200 µM).

When analysed under magnifier, the control *Arabidopsis* roots showed symmetric rows of cells in the elongation and differentiation zones, while podophyllotoxin-treated roots showed very irregular cell rows either in the division or elongation zone and a reduction in the number of root hairs at the higher tested concentrations (Fig. 2). Besides,

treated roots showed left-handed deviation that resulted from apparent loss of gravitropic response with horizontal growth and occasionally upward growth of roots for seedlings treated with higher concentrations of podophyllotoxin (i.e. 400 μM; Fig. 1B).

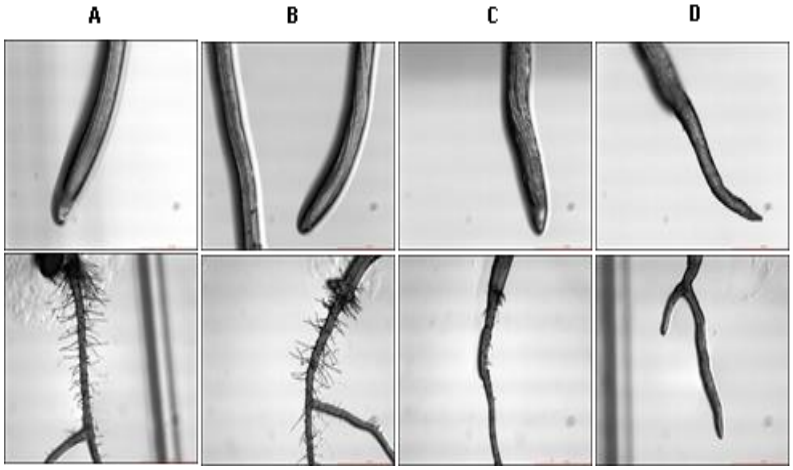

Figure 2. Apical region of *Arabidopsis thaliana* radicles seen under magnifier after 14 days growth on A) 0 μM, B) 100 μM, C) 200 μM, D) 400 μM, podophyllotoxin concentrations. Upper pictures show apical meristems and lower pictures show transition zone between rot and shoot zones.

The inhibitory effects induced by podophyllotoxin on *Arabidopsis* root growth, as well as on cotyledon size, may be related to the effect of this compound on the mitotic process, previously demonstrated for other natural compounds as mevalonic acid on tobacco cells (13), *Leucophyllum* lignans on onion roots (22); 2-benzoxazolinone on lettuce roots (23), or citral on *Arabidopsis* cells (11); but also for podophyllotoxin on *Allium cepa* (21). In fact, podophyllotoxin has been found to have antimitotic properties on animal cells, inhibiting the assembly of tubulin to microtubules (3, 5, 12).

The observation of microtubules by immunofluorescence revealed the regular arrangement of cortical microtubules in cells of control roots, which were transverse to the longitudinal axis of the cell (Fig. 3A). In contrast, root cells treated with podophyllotoxin showed clear changes in the arrangement of microtubules, which were strongly condensed. After 7 days of treatment with IC_{50} podophyllotoxin, the microtubules were not only shortened and thicker, clear symptom of their condensation, but their distribution also became totally erratic (Fig. 3B,C,D). In addition, treated cells showed highly thickened irregular walls with zigzag appearance (Fig. 3D). At 14 days of treatment, condensation and microtubule disorganization were still evident (Fig. 3E), although some cells showed less deformation and condensation, but also reduced the number of microtubules (Fig. 3F).

The alteration caused by podophyllotoxin on microtubule arrangement of *Arabidopsis* roots could be due to the podophyllotoxin-induced inhibition of tubulin assembly on the treated cells, in a way similar to that demonstrated for animal cells. This microtubular alteration may be the reason of the torsion observed in roots treated with 50 μM concentration (10, 16) as well as of the malformations observed in the cell walls, since cortical microtubules affect the deposition of cellulose microfibrils (17).

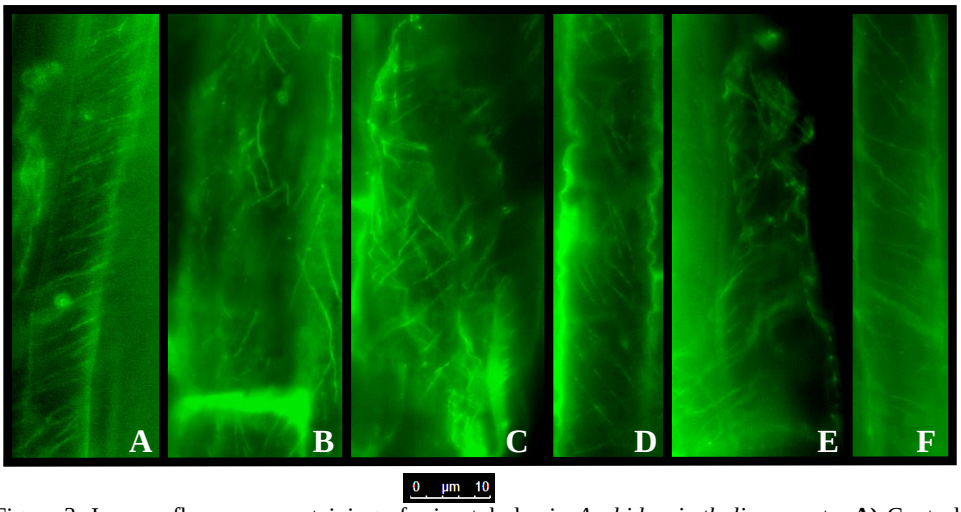

Figure 3. Immunofluorescence staining of microtubules in *Arabidopsis thaliana* roots. **A)** Control; **B), C)** and **D)** after 7 days of IC_{50} podophyllotoxin treatment; **E)** and **F)** after 14 days of IC_{50} podophyllotoxin treatment.

In summary, the great inhibitory effect of podophyllotoxin on *Arabidopsis* root cells was associated to the alteration of cortical microtubules, resulting in a complete block of root growth, which makes this compound a promising candidate for weed management.

ACKNOWLEDGEMENTS

Authors would like to thank Inés Pazos and Suso Méndez from the Central Research Services, University of Vigo for their invaluable assistance on confocal microscopy. This research was supported by the Spanish Ministry of Economy and Competitiveness, through the project number AGL2013-41281-R.

REFERENCES

1. Abourriche, A., Charrouf, M., Berrada, M., Bennamara, A., Chaib, N. and Francisco, C. (1999). Antimicrobial activities and cytotoxicity of the brown alga *Cytoseira tamariscifolia*. *Fitoterapia* **70:** 611-614.
2. Anjum, T. and Bajwa, R. (2005). Importance of germination indices in interpretation of allelochemical effects. *International Journal of Agriculture and Biology* **7:** 417–419.
3. Brewer, C.F., Loike, J.D. and Horwitz, S.B. (1979). Conformational analysis of podophyllotoxin and its congeners. Structure-activity relationship in microtubule assembly. *Journal of Medicinal Chemistry* **22:** 215-221.
4. Chiapusio, G., Sánchez, A.M., Reigosa, M.J., González, L. and Pellisier, F. (1997). Do germination indices adequately reflect allelochemical effects on the germination process? *Journal of Chemical Ecology* **23:** 2445–2453.
5. Cortese, F., Bhattacharyya, B. and Wolff, J. (1977). Podophyllotoxin as a probe for the colchicine binding site of tubulin. *The Journal of Biological Chemistry* **4:** 1134-1140.
6. Dayan, F.E., Cantrell, C.L. and Duke, S.O. (2009). Natural products in crop protection. *Bioorganic and Medicinal Chemistry* **17:** 4022-4034.

7. Dayan, F.E., Hernández, A., Allen, S.N., Moraes, R.M., Vroman, J.A., Avery, M.A. and Duke, S.O. (1999). Comparative phytotoxicity of artemisin and several sesquiterpene analogues. *Phytochemistry* **50:** 607–614.
8. Dias, L.S. (2001). Describing phytotoxic effects on cumulative germination. *Journal of Chemical Ecology* **27:** 411-418.
9. Duke, S.O., Dayan, F.E. and Romagni, J.G. (2000). Investigating the mode of action of natural phytotoxins. *Journal of Chemical Ecology* **26:** 2079-2094.
10. Furutani, I., Watanabe, Y., Prieto, R., Masukawa, M., Suzuki, K., Naoi, K., Thitamadee, S., Shikanai, T. and Hashimoto, T. (2000). The SPIRAL genes are required for directional control of cell elongation in *Arabidopsis thaliana*. *Development* **127:** 4443-4453.
11. Graña, E., Sotelo, T., Díaz-Tielas, C., Araniti, F., Krasuska, U., Bogatek, R., Reigosa, M.J. and Sánchez-Moreiras, A.M. (2013). Citral induces auxin-mediated malformations and arrests cell division in *Arabidopsis thaliana* roots. *Journal of Chemical Ecology* **39:** 271-282.
12. Haar, E., Rosenkranz, H.S., Hamel, E. and Day, B.W. (1996). Computational and molecular modeling evaluation of the structural basis for tubulin polymerization inhibition by colchicine site agents. *Bioorganic and Medicinal Chemistry* **10:** 1659-1671.
13. Hemmerlin, A. and Bach, T.J. (1998). Effects of mevinolin on cell cycle progression and viability of tobacco BY-2 cells. *Plant Journal* **14:** 65-74.
14. Hoagland, R.E. and Williams, R.D. (2004). Bioassays-useful tools for the study of allelopathy. In : *Allelopathy: Chemistry and Mode of Action of Allelochemicals* (Eds., F.A. Macías, J.C. Galindo, J.M.G. Molinillo, H.G. Cutler). pp. 315-351 Boca Raton, FL: USA.
15. Holzinger, A., Kawamura, E. and Wasteneys, G.O. (2010). Strategies for imaging microtubules in plant cells. *Cytoskeleton Methods and Protocols* **2010:** 243-262.
16. Hussey, P.J. (2004). Cytoskeleton: Microtubules do the twist. *Nature* **417:** 128-129.
17. Ishida, T., Thitamadee, S. and Hahimoto, T. (2007). Twisted growth and organization of cortical microtubules. *Journal of Plant Research* **120:** 61-70.
18. Jardine, I. (1980). Podophyllotoxins. In: *Anticancer Agents Based on Natural Products Models* (Eds., J.M. Casady, J. Douros), pp. 319-351. Academic Press, New York.
19. Keller-Juslen, C., Kuhn, M., Stahelin, H. and von Wartburg, A. (1971). Mitosis-inhibiting natural products. Synthesis and antimitotic activity of glycosidic lignan derivatives related to podophyllotoxin. *Journal of Medicinal Chemistry* **10:** 936-940.
20. Meresse, P., Monneret, C. and Bertounesque, E. (2004). Synthesis of podophyllotoxin analogues: δ-lactone-containing picropodophyllin, podophyllotoxin and 4'-demethyl-epipodophyllotoxin derivatives. *Tetrahedron* **60:** 2657-2671.
21. Oliva, A., Moraes, R.M., Watson, S.B., Duke, S.O. and Dayan, F.E. (2002). Aryltetralin lignans inhibit plant growth by affecting the formation of mitotic microtubular organizing centers. *Pesticide Biochemistry and Physiology* **72:** 45-54.
22. Rimando, A.M., Dayan, F.E., Mikell, J.R. and Moraes, R.M. (1999). Phytotoxic lignans of *Leucophyllum frutescens*. *Journal of Natural Toxins* **7:** 1-5.
23. Sánchez-Moreiras, A.M., Coba de la Peña, T. and Reigosa, M.J. (2008). The natural compound benzoxazolin-2(3*H*)-one selectively retards cell cycle in lettuce root meristems. *Phytochemistry* **69:** 2172-2179.