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Bioautography as a search tool to identify the allelopathic compounds in *Virola sebifera*

F. M. P. TONELLI[1,*], J. M. DE SIQUEIRA[1], G. A. S. MAIA[1], L. F. SOARES[1], D. B. DA SILVA[2], C. A. CAROLLO[3] and A. L. B. SARTORI[3]

Campus Centro-Oeste Dona Lindu, Universidade Federal de São João del Rei, Rua Sebastião Gonçalves Coelho, 400, 35501-296, Divinópolis, MG, Brazil.
E. Mail: tonellinanda@gmail.com

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ABSTRACT

Using bioautography, phytotoxic compounds in hydromethanolic leaf extracts of *Virola sebifera* were identified. It was possible to identify the allelochemicals such as sesamin, kobusin and quercetrin in hexane and ethyl acetate fractions of the leaves. The results were similar to those obtained through traditional methods of extraction and fractionation such as preparative TLC and column chromatography. Thus bioautography can be an inexpensive, quick and reliable method to identify the allelochemicals in plants.

Key words: Allelochemicals localization, allelopathathy, bioautography, identification, kobusin, quercetin-3-*O*-α-rhamnopyranoside, sesamin, *Virola sebifera*.

INTRODUCTION

Current interest in allelopathy is due to the importance of natural plant chemicals for maximizing agricultural productivity, while preserving the environment. Allelochemicals have therefore relevance as alternative to pesticides and herbicides, which cause environmental damage (15). To isolate active phytochemicals, precise bioassays are essential (18,23). In testing for allelopathic chemicals, preliminary bioassays are done in Petri dishes using either monocot or dicot crops seeds (for example: tomatoes, onions and lettuce). After this preliminary step, the bioassays of higher complexity are required to establish the allelopathic nature of substances using pot or field culture (15).

Virola sebifera (Family Myristicaceae) grows in savannas of Central and South America. Its high drought resistance seeds, allows it to colonize extensively (13). Substances with allelopathic activity (such as neolignans, lignans and glycoside flavonoids) have been found in the *Virola* species leaves (*V. surinamensis* and *V. oleifera*) and in *V. sebifera* seeds (5,13).

[*]Correspondence author, [1]Campus Centro-Oeste Dona Lindu, Universidade Federal de São João del Rei, Rua Sebastião Gonçalves Coelho, 400, 35501-296, Divinópolis, MG, Brazil; E. Mail: tonellinanda@gmail.com; [2]Núcleo de Pesquisa em Produtos Naturais e Sintéticos (NPPNS), Departamento de Física e Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café s/n, 14040-903, Ribeirão Preto, SP, Brazil; [3]Centro de Ciências Biológicas e da Saúde, UFMS, CP 549, CEP79070-900, Universidade Federal de Mato Grosso do Sul, Brazil.

Using bioautography, a technique that combines bioassay and thin layer chromatography (TLC) and is commonly used to search for antimicrobial substances [with activities against bacteria and fungi (25)], it is possible to search for bioactive compounds from natural sources. We assessed the phytotoxic potential of aqueous methanolic crude extract of *V. sebifera* leaves on the germination of onion (*Allium cepa*, monocot) and lettuce (*Lactuca sativa*, dicot) first in Petri lates. Thereafter, we identified the allelochemicals using bioautography (15).

MATERIALS AND METHODS

I. Samples Collection

Virola sebifera samples (leaves, fruits and stems) were collected in Divinópolis (20°10´38.53" S and 44°55´06.03" W) in August 2008, and the voucher specimen was identified by Dr. Arnildo Pott and deposited in the CG/MS herbarium under the number 22882. Onion and lettuce seeds used in these studies were local varieties.

II. Preparation of hydromethanolic extract and its fractionation

Virola sebifera dry leaves (113 g) were subjected to extensive extraction by percolation in Soxhlet apparatus with a methanol and water mixture (7:3 v/v), yielding 39.4 g of crude extract (30.9% w/w). The crude extract was then solubilized and subjected to partitioning using separatory funnel (liquid-liquid extraction) and the hexane and ethyl acetate as the first and second solvents, respectively. It yielded 4.78 g, 1.86 g and 28.96 g in the hexane fraction, in ethyl acetate fraction and in marc (residual material after extraction of soluble components), respectively.

III. Allelopathic activity

Bioassays of hydromethanolic extract and fractions: Aqueous extract (E) and its fractions (F) [in hexane, ethyl acetate, and the marc] were tested at concentrations of 1000, 500 and 250 µg/mL (ppm), in triplicate, for allelopathic activity by Petri dish bioassay. Briefly, the samples were solubilized in two drops of DMSO and then added to MES [2-(N-morpholino) ethanesulfonic acid] buffer pH 6.0 (15). Allelopathy tests were conducted in Petri dishes (cleaned with a 70% ethanol solution) and lined with Whatman No. 1 filter paper. For seed germination assay, each plate contained 25 seeds, 5 mL of MES buffer (pH 6.0) and the crude extract or fraction dissolved in DMSO. The plates were incubated for one week in dark at a temperature of 25ºC (5,15). They were then cooled to -10°C for one day to stop the growth. The hypocotyl and radicle size was measured (15). The control consisted of seeds exposed only to two drops of DMSO and the 5 mL of MES buffer - pH 6.0); the data were analyzed by Student's t test in relation to the blank ($p < 0.05$).

IV. Phytotoxic activity test by bioautography

The phytotoxicity tests were done using only the fractions that strongly inhibited the seed germination in Petri dish. Chromatographic plates [silica gel matrix (5.0 x 8.0 cm)] with fluorescent indicator staining (Sigma Aldrich) containing hexane and ethyl acetate fractions were eluted (into chromatography chamber) using, respectively, chloroform

(eluted twice) and chloroform:methanol 7:3 (eluted once) as eluents, and then placed into Petri dishes (10.0 Ø). A piece of filter paper was placed over the fraction-containing chromatographic plates and seeds were placed on its top, as per TLC profiles visualized under UV light and previously highlighted in the fraction-containing chromatographic plates; 10 mL of MES buffer (pH 6.0) was added. *Allium cepa* seeds were used on the hexane fraction-containing chromatographic plate and the *Lactucca sativa* seeds on the ethyl acetate chromatographic plate: because these were the species for which it was perceived greater inhibition of seed development in the allelopathic activity test in Petri dishes performed previously. The negative control was made by adding the same amount of buffer to a Petri dish containing a blank chromatographic plate and the same amount of seeds.

After incubation for a week in dark, the Petri dishes were frozen at -10°C for 24 h to stop plants growth. The size of hypocotyl and radicle in germinated seeds of *Allium cepa* were measured as a function of the chromatographic profile of the hexane fraction in comparison to blank. The same procedure was adopted for *Lactucca sativa* seeds as a function of the chromatographic profile of ethyl acetate fraction. Results analysis was done by Student's t test in relation to blank ($p < 0.05$).

V. Phytotoxic activity of fractions (obtained by traditional extraction and fractionation methods)

The traditional extraction and fractionation methods, TLC and column chromatography, were performed for the hexane and ethyl acetate fractions, respectively. This difference in methodology was due to the amount of material available. Aiming to validate the results obtained by bioautography, the preparative plate chromatography facilitates the comparison of results obtained for active spots in relation to bioautography (which also involves a chromatography in plate); the comparison is most easily demonstrated (as for the column, it must be made a TLC with the active subfractions to compare with the bioautography; so it is required one more step). However, to perform the preparative chromatographic plate itself, it is necessary more material. It was obtained 4.78 g of hexane fraction and only 1.86 g of ethyl acetate fraction; so the latter had to be evaluated by column chromatography.

Preparative chromatographic plates (60 GF254 silica gel, Merck) were used to elute the hexane fraction (eluent chloroform, eluted twice as described in **IV**) and by using silica gel column chromatography (silica gel 60, 70-230 mesh, Sigma Aldrich), the ethyl acetate fraction was eluted once (eluent chloroform:methanol 7:3, as in item **IV**). The profiles from the preparative TLC plate were compared with those obtained in bioautography experiment for the hexane fraction, and the regions in which inhibition was observed (active regions) were extracted with methanol, for future GC-MS analysis. The subfractions of ethyl acetate fraction, obtained from the chromatographic column, were evaluated for allelopathic potential through a Petri dish assay (as described in item **III**), and the active fractions were compared by TLC to the bioautography assay results to confirm if they corresponded to those active regions.

VI. Analysis of the active subfractions/spots

To identify the allelopathic substances present in the active fractions of the crude extract, the active spots on the hexane fraction on TLC plate was extracted with methanol (as described in **V**). These were subjected to analysis by gas chromatography-mass spectrometry (GC-MS). GC-MS analysis was performed using the Shimadzu GC-MS QP-2010 instrument equipped with a DB5-MS (30 m x 0.25 mm x 0.25 mm) capillary column, and helium (He) was used as the carrier gas at a flow rate of 1.3 mL/min. The split ratio was 1/30, the injector temperature was 250˚C, and the injected volume was 1 µL. The temperature program was 100-290˚C with a gradient of 3˚C/min followed by 290˚C for 20 min.

The active sub-fractions from ethyl acetate fraction obtained through column chromatography, were subjected to liquid chromatography-mass spectrometry (LC-MS). LC-DAD-MS and LC-DAD-MS/MS were performed on a Shimadzu LC-20A instrument with a diode array detector (SPD-M20AV, Shimadzu) coupled to an UltrOTOFq (Bruker Daltonics) ESI-qTOF mass spectrometer in positive ion mode. Isolated compounds were diluted more than 10-fold and directly infused into the ionization source at a flow rate of 10 µL min^{-1}. In a secondary analysis, the standard Quercetrin (Sigma Aldrich) was co-injected with the active subfractions to confirm the presence of this substance.

All active subfractions/spots were also analyzed by nuclear magnetic resonance (NMR) imaging. ^{1}H NMR experiments were performed on a Bruker-Avance DRX-500 MHz instrument with $CDCl_3$ as a solvent and TMS as an internal standard. The chemical shifts were reported in δ units, and the coupling constants (*J*) were reported in Hz.

RESULTS AND DISCUSSION

LABORATORY BIOASSAYS

I. Allelopathic activity of extract and fractions in Petri dishes: Allelopathic evaluation of aqueous methanolic extract (E) and its fractions (F) from the partitioned extract (fractions in hexane, ethyl acetate and the marc) was first done by Petri dish bioassay (Fig. 1). The crude extract inhibited the lettuce hypocotyls and onion radicle and hypocotyl development in all concentrations tested, but the results were significant only on onion seeds. The ethyl acetate fraction significantly inhibited the development of both lettuce and onion radicle and hypocotyls. The hexane fraction negatively affected the development of lettuce hypocotyls and onion radicle and hypocotyls. However, each fraction preferentially affected one type of seed. The hexane fraction was more inhibitory to monocots; it inhibited onion seed radicle development by 73.91% and hypocotyl development by 72.73% at 1000 ppm. The ethyl acetate fraction caused the highest inhibition on dicots: 37.28% in lettuce seeds radicle development at 1000 ppm, and 30.94% in lettuce seeds hypocotyls development at 250 ppm.

II. Bioautography: Through bioautography it was found that the radicle lengths of onion seeds were significantly inhibited (96.8%) by the a TLC spot with an *Rf* of approximately 0.47, from the hexane fraction (Fig. 2).

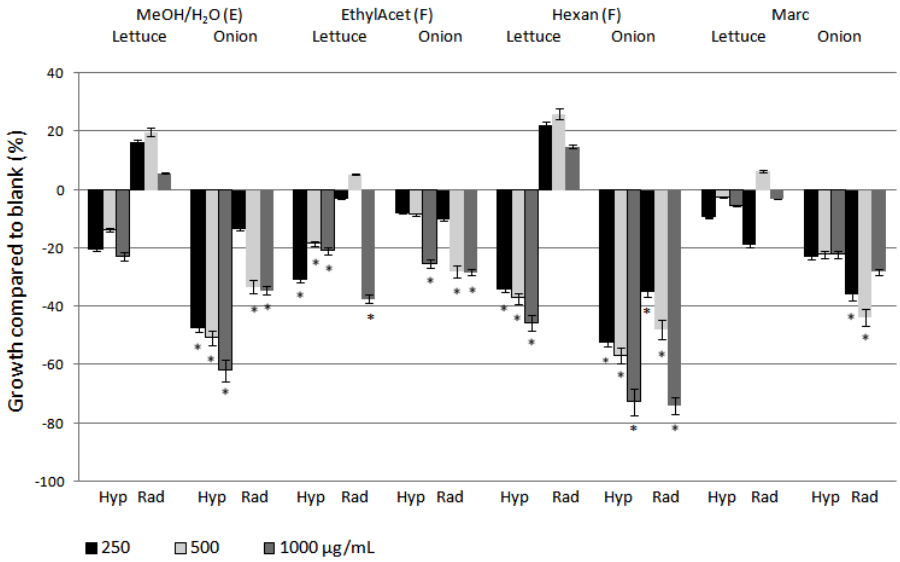


Figure 1. Phytotoxic effects of extract (E) and fractions (F) -in ethyl acetate, hexane and the marc- obtained from the leaves of *Virola sebifera* on lettuce and onion seeds. The growth inhibition or stimulation effect was determined by measuring lengths of radicle (Rad) and hypocotyl (Hyp) in relation to blank (control defined as 100%); * Significant inhibition at $p < 0.05$.

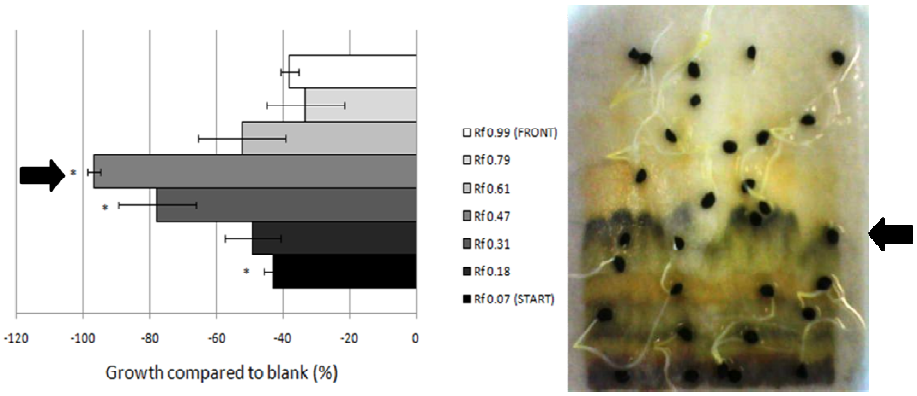


Figure 2. A. The relationship between the percentage of phytotoxic effect of hexane fraction (obtained from the leaves of *Virola sebifera*) on radicle of the standard target species *Allium cepa,* and the chromatographic profile (represented by the retention factor) of the hexane fraction on the TLC plate; *significant result at $p < 0.05$. B. Photo of the corresponding bioautography assay.

The development of lettuce seeds was more affected by substances found in the front part (or superior part, where more apolar substances are located) of the bioautography TLC plate containing the ethyl acetate fraction. There was significant reduction in the average size of the radicles (91.4%) (Fig. 3).

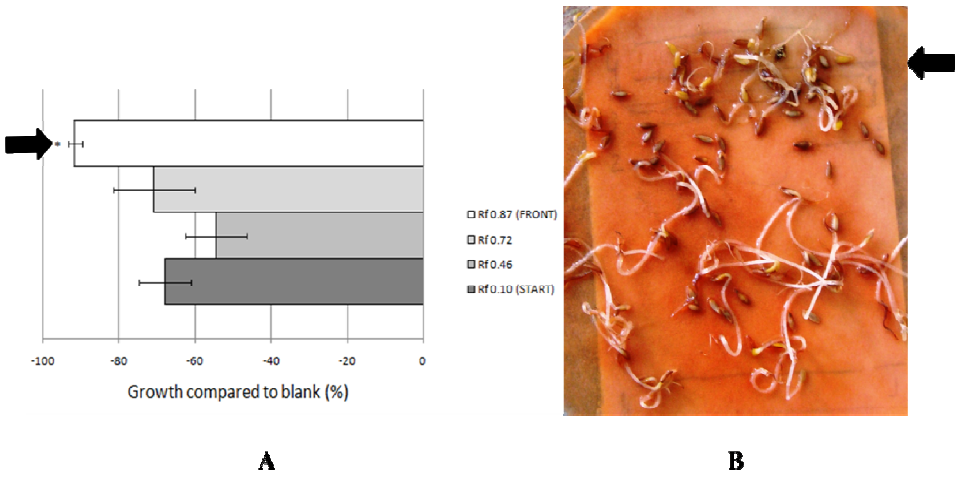


Figure 3. A. The relationship between the percentage of the phytotoxic effect of the ethyl acetate fraction (obtained from the leaves of *Virola sebifera*) on the radicle of standard target *Lactuca sativa*, and the chromatographic profile (represented by the retention factor) of the ethyl acetate fraction on the TLC plate; *significant result at $p < 0.05$. B. Photo of the corresponding bioautography assay.

III. Analysis of active sub-fractions/spots

To verify the effectiveness of bioautography methodology in comparison to established procedures in phytochemistry (extraction and fractionation methodologies such as TLC and chromatography column), the active spots from hexane fraction (R*f* 0.47, Fig 2) and the active subfractions from ethyl acetate fraction were isolated and analysed.

The active spot from hexane fraction, corresponding to inhibitory effects in the bioautography assay (*Rf* = 0.47) (Fig. 2) was removed with methanol from the preparative chromatography plate (as previously described in **V**) and analyzed by GC-MS (as previously described in **VI**). It was found to contain the lignans sesamin (I) and kobusin (II) (Fig. 4). Their MS and ^{1}H NMR spectra were in agreement with those described in other studies as the ones corresponding to the tetrahydrofurfuran lignans sesamin (I) and kobusin (II) (4, 11, 24):

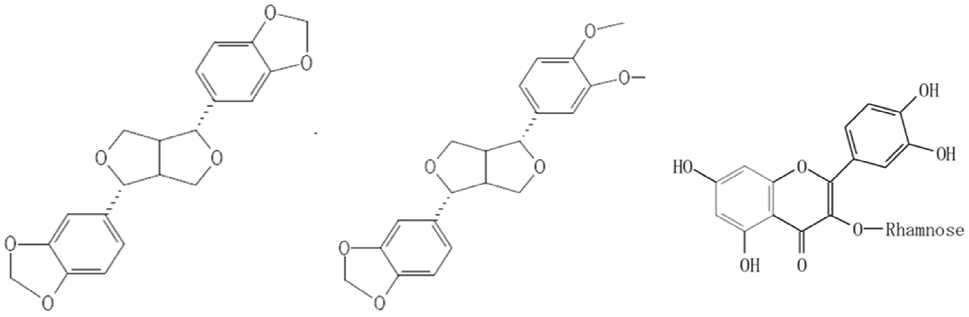
Sesamin (I): MS (Relative intensity): m/z 354 [M^+, $C_{20}H_{18}O_6$, (32)], 323 [M-OCH_3, (8)], 203 [$C_{12}H_{11}O_3$, (15)], 161 [$C_{10}H_9O_2$, (37)], 149 [$C_8H_5O_3$, (100)], 135 [$C_8H_7O_2$, (50)], 122 [$C_7H_6O_2$, (38)].

^{1}H NMR (500 MHz, $CDCl_3$) of **1**: δ 2.98 (m, 2H); 3.92 (dd, J 1.0, 2.4 and 9.6 Hz); 4.02 (td, J 1.3 and 10.9 Hz); 4.2 (m); 4.67 (brt, 6.4 Hz); 5.88 (s, 2H, OCH_2O); 6

aromatic H with δ 7.73 (brt, 3 Hz); 6.79 (brt, 4 Hz) and 6.84 (brd, 4 Hz). The NMR spectral data correspond to those described in the literature.

Kobusin (II): MS (Relative intensity): m/z 370 [M^+, $C_{21}H_{22}O_6$, (45)], 339 [M-OCH_3, (12)], 219 [$C_{13}H_{15}O_3$, (12)], 203 [$C_{12}H_{11}O_3$, (25)], 177 [$C_{11}H_{13}O_2$, (42)], 165 [$C_6H_9O_3$, (80)], 149 [$C_8H_5O_3$, (100)], 135 [$C_8H_7O_2$, (76)], 122 [$C_7H_6O_2$, (35)].

^{1}H NMR (500 MHz, $CDCl_3$) of **2**: δ 3.02 (m, 2H); 3.81 and 3.83 (s, 2 x OCH_3); 3.92 (dd, J 1.0, 2.4 and 9.6 Hz); 4.02 (td, J 1.3 and 10.9 Hz); 4.2 (m); 4.67 (brt, 6.4 Hz); 5.88 (s, 2H, OCH_2O); 6 aromatic H with δ 7.73 (brt, 3 Hz); 6.79 (brt, 4 Hz) and 6.84 (brd, 4 Hz).)



I II III

Figure 4. Active compounds found in *V. sebifera* extract fractions. Sesamin (I) and Kobusin (II) on hexane fraction and quercetin-3-*O*-α-rhamnopyranoside (III) on ethyl acetate fraction.

By GC-MS, lignan I showed a molecular ion peak at *m/z* 354 in the MS spectrum and a fragment peak at *m/z* 149 (base peak) (Fig. 5).

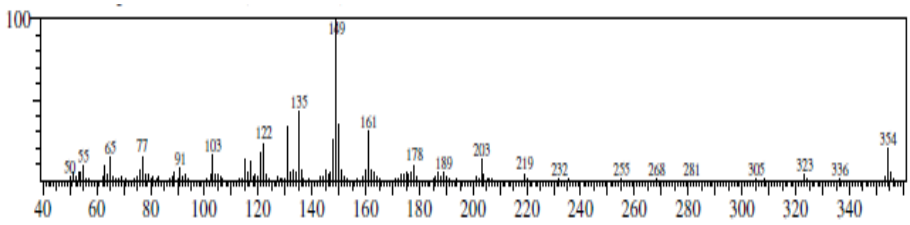


Figure 5. Gas chromatography-mass spectrometry results of I.

The MS spectrum of Kobusin was slightly different from lignin I because it showed a molecular ion peak at *m/z* 370 (an additional 16 amu (M+CH_3+H) compared to the mass of I) (Fig. 6); however, the same fragment at *m/z* 149 was also found for II. In addition to the molecular ion peak, another fragment of II was observed at *m/z* 165 (85%), which may correspond to a dimethoxy substituent rather than the methylenedioxy substituent found in I (Fig. 7 and Fig. 8) (4,11,24).

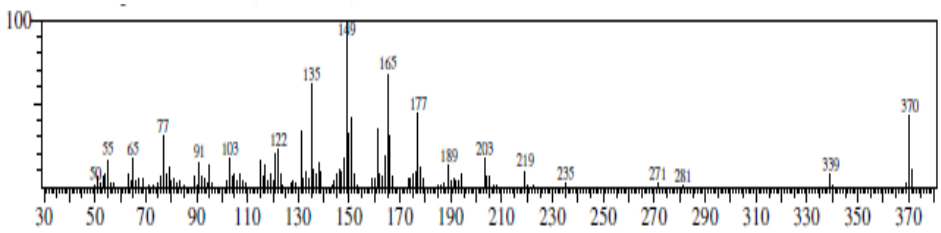


Figure 6. Gas chromatography-mass spectrometry results of II.

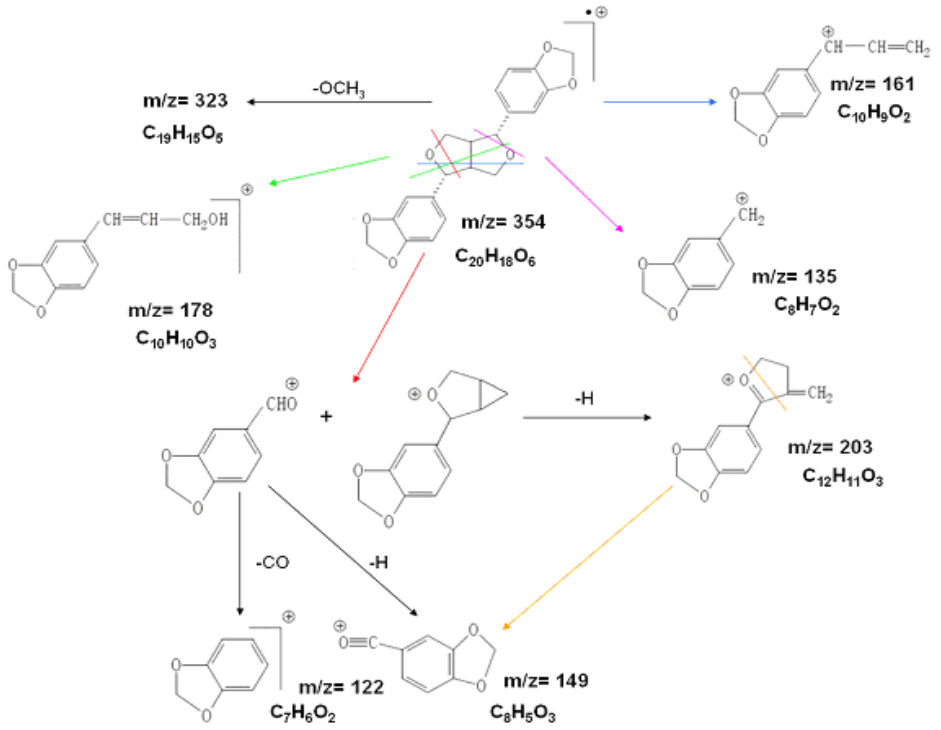


Figure 7. The MS fragmentation pathways of I.

Sesamin and other tetrahydrofurfuran lignans have been isolated earlier from the *Virola* genus (4,22). Some of these lignans are very inhibitory in wheat coleoptile bioassays but have low activities in standard target species (STS) bioassays (16). Sesamin is also present in the *n*-hexane fraction of the methanolic extract from the aerial parts of *Artemisia arborescens* L. and this fraction exhibited phytotoxicity *in vitro* (2). Besides being able to damage development of target species seeds, sesamin and kobusin can also be used to enhance the toxicity of insecticides, since they are able to inhibit the growth of silkworm (*Bombyx mori*) larvae (1). Kobusin has also antifungal activity (12).

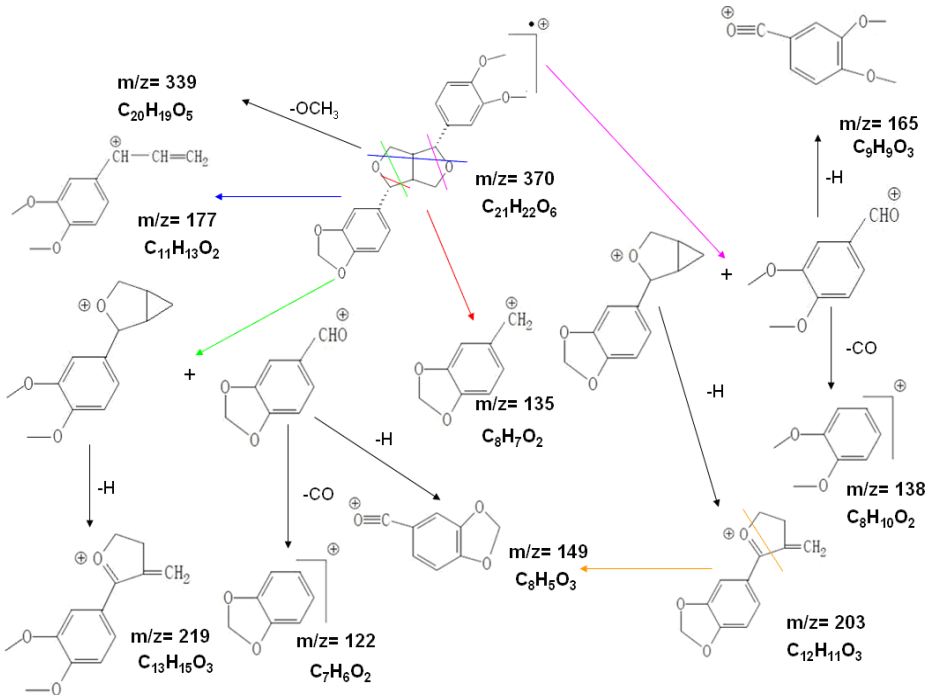


Figure 8. The MS fragmentation pathways of II.

The ethyl acetate fraction of crude methanolic extract was chromatographed on a silica gel column to yield 11 sub-fractions. The early fractions were the most active in seed germination. Subfractions 2 and 4 inhibited the lettuce seed radicle development by 22.7% and 31.8%: these were analyzed through LC-MS methodology to elucidate the structure of the active compounds.

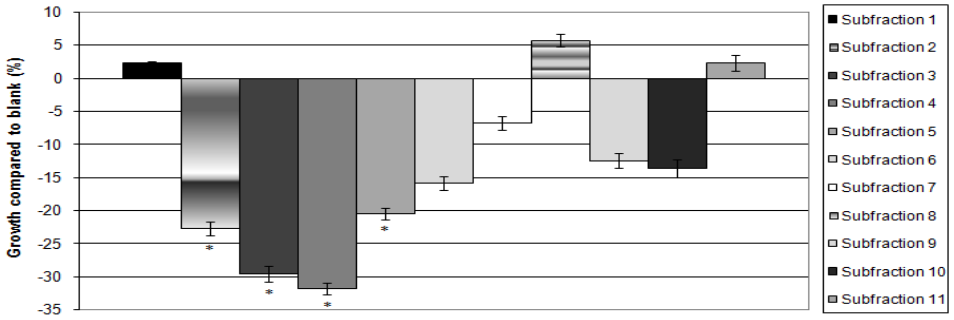
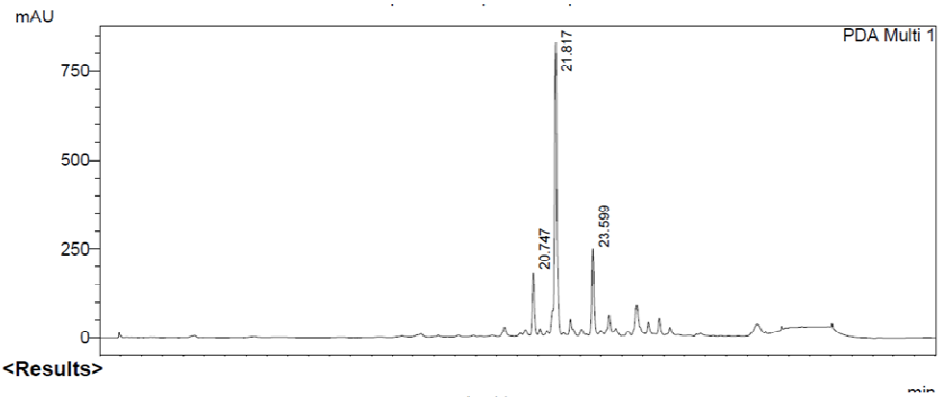


Figure 9. The percentage of phytotoxic effect on Petri dish assay of the ethyl acetate fraction's subfractions on the radicle of the standard target *Lactuca sativa*; * significant result at p< 0.05.

The active subfractions 2 and 4, corresponding to the leading part of solvent in bioautography assay (*Rf* = 0.87), contained quercetin-3-*O*-α-rhamnopyranoside (III) as the major constituent (Fig. 4). The LC retention time (Fig. 10), mass spectrum (Fig. 11) and UV profile were consistent with those obtained by co-injection of a standard of (III).



PeakTable

PDA Ch1 254nm 4nm

Peak#	Name	Ret. Time	Area	Height	Area %	Height %	Conc.
1		20.747	1250692	173318	15.293	14.588	15.293
2		21.817	5298687	775761	64.791	65.297	64.791
3		23.599	1628773	238967	19.916	20.114	19.916
Total			8178151	1188045	100.000	100.000	

Figure 10. LC chromatogram of the subfractions 2 and 4 obtained by column chromatography of the fractionated ethyl acetate fraction; the major product is quercetin-3-O-α-rhamnopyranoside, III.

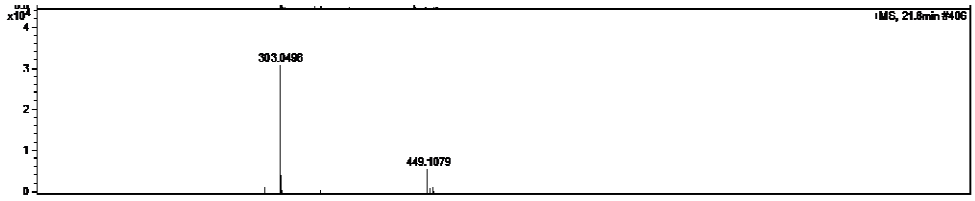


Figure 11. ESI-HRMS spectra of the major product quercetin-3-O-α-rhamnopyranoside III, found in subfractions 2 and 4, obtained by column chromatography from the ethyl acetate fraction.

MS spectra showed an *m/z* value of 449.1033 $[M+H]^+$, corresponding to quercetin-3-*O*-α-rhamnopyranoside, and a fragment with an *m/z* value of 303.0462, corresponding to the loss of the rhamnose unit. Quercetin-3-*O*-α-rhamnopyranoside is a known flavonoid with allelopathic activity (10,19), and the tetrahydrofuranic ring in the flavonoid structure (also called B-ring) is presumed to be the responsible for its allelopathic activity (6,19). Quercetin-3-*O*-α-rhamnopyranoside presented a MS profile as follows:

Quercetin-3-O-α-rhamnopyranoside (III): MS: m/z 449.1084 $[M+H]^+$ calculated for $C_{21}H_{21}O_{11}^+$, found 449.1079; also a fragment observed at m/z 303.0496, $[C_{15}H_{11}O_7]^+$.

In this study, bioautography proved to be an efficient method to localise a constituent in the allelopathic fractions of plant extracts. The phytotoxic effects observed in the bioautography test reproduced the results obtained by preparative plate chromatography of the hexane fraction and those obtained by column chromatography of the ethyl acetate fraction. Thus, bioautography can be adopted as a low-cost technique for identifying the substances with phytotoxic potential. The location of these substances can be obtained quickly by relating the observed phytotoxic effect to the chromatographic profile of the fraction in which these substances are present.

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