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ARTICLES

Chemical composition, antioxidant and topical anti-inflammatory activities of *Croton cordiifolius* Baill. (Euphorbiaceae) 22

Iasmine Andreza Basilio dos Santos Alves, Simone Maria dos Santos, Raudiney Frankilin Vasconcelos Mendes, José Wellington da Silva, Maria de Fátima Rodrigues, Bárbara de Azevedo Ramos, Márcia Vanusa da Silva, Maria Tereza dos Santos Correia, Fernanda das Chagas Angelo Mendes Tenório, Roberta Jeane Bezerra Jorge, René Duarte Martins, Julianna Ferreira Cavalcanti de Albuquerque, Karina Perrelli Randau and Rafael Matos Ximenes

Induction of biochemical active constituents of Jojoba (*Simmondsia chinensis* (Link) Schneider) callus affected by hormones 34

Mohei El-Din Solliman, Wael F. Shehata, Heba Allah A. Mohasseb, Mohammed I. Aldaej, Abdullatif A. Al-Khateeb, Solliman A. Al-Khateeb, Adel E. A. Hegazy and Hesham M. Abdel-Moneim

Full Length Research Paper

Chemical composition, antioxidant and topical anti-inflammatory activities of *Croton cordiifolius* Baill. (Euphorbiaceae)

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Croton cordiifolius is widely used in Brazilian Caatinga folk medicine to treat general inflammation, pain, and gastrointestinal disturbances. Currently, its medicinal properties are not well understood, owing to the absence of chemical and pharmacological studies. The aims of this work were to analyze the chemical composition of *C. cordiifolius* stem bark and evaluate its *in vitro* antioxidant and *in vivo* anti-inflammatory activities. *C. cordiifolius* ethanolic extract (CcEE) was obtained by maceration, while essential oil (CcEO) was extracted by hydrodistillation in a Clevenger-type apparatus. The chemical composition was evaluated by thin-layer chromatography and GCMS. Total phenolics, flavonoids, and antioxidant activity were quantitated by spectrophotometry. Topical anti-inflammatory activity was evaluated by different ear edema models in mice. The major compounds in CcEO were α -pinene (51.76%) and β -pinene (19.08%). CcEE analysis indicated the presence of alkaloids, mono- and sesquiterpenes, flavonoids, phenylpropanoids, triterpenes, steroids, and coumarins. CcEE showed antioxidant activity *in vitro*. In a topical anti-inflammatory assay, CcEO showed no activity. On the contrary, CcEE inhibited ear edema induced by phorbol 12-myristate 13-acetate (PMA), arachidonic acid (AA), ethyl phenylpropiolate (EPP), and phenol. Probable mechanisms include inhibition of AA metabolite biosynthesis, vasoactive amine activity, and cytokine release/activity. These results corroborate the popular reputation of *C. cordiifolius* as an anti-inflammatory remedy.

Key words: *Croton cordiifolius*, Euphorbiaceae, caatinga, topical anti-inflammatory activity, ear edema.

INTRODUCTION

The Euphorbiaceae family comprises 228 genera and more than 6,500 species, including trees, shrubs, herbs and creepers (The Plant List, 2013). This family is,

quantitatively, the second most representative of Brazilian Caatinga, with about 60 species only of *Croton* genus (Souza et al., 2012). This genus, the second

largest of the family, consists of about 1,200 species, of which 350 are distributed in Brazil (Silva et al., 2010). Species of *Croton*, the predominant genus in the Caatinga, attract interest owing to their diverse array of chemical compounds, ethnopharmacological uses, and proven biological activities. Various compounds have been isolated from many species of the genus *Croton*. These include mono- and sesquiterpenes, diterpenes, flavonoids, tannins, and alkaloids, suggesting that this genus can be a promising source of bioactive molecules with remarkable research potential (Randau et al. 2004). Compounds extracted from several species to date display anti-inflammatory, antinociceptive (Falcão et al., 2005; Lima et al., 2015; Rocha et al., 2008), antioxidant (Shukla et al., 2009; Rocha et al., 2008), antiulcer (Almeida et al., 2003), antidiabetic (Torricco et al., 2007), healing, anticancer, antimycotic, antibacterial, and antiviral activity (Pieters and de Bruyne, 1995; Melo et al., 2013; Rodrigues et al., 2012).

Croton cordiifolius, popularly known as “quebra-faca” in the Brazilian Northeast, is used to treat general inflammation, pain, and gastrointestinal disturbances (Cartaxo et al., 2010; Monteiro et al., 2011). Nogueira et al. (2015) described the antinociceptive activity and chemical composition of its leaf’s essential oil.

Although the *Croton* genus is widely studied, including some species found in the caatinga, no studies characterizing the chemistry and pharmacology of *C. cordiifolius* stem bark have been published. The present study analyzed the chemical composition of the extract and essential oil of the stem bark of *C. cordiifolius* and evaluated its *in vitro* antioxidant and *in vivo* topical anti-inflammatory activities.

MATERIALS AND METHODS

Chemicals and drugs

Ethanol, phorbol 12-myristate 13-acetate (PMA), arachidonic acid (AA), ethyl phenylpropiolate (EPP), phenol, capsaicin, Folin-Ciocalteu reagent, phosphomolibdenum, DPPH, and ABTS were purchased from Sigma-Aldrich (USA, St. Louis). All other reagents and substances were of analytical grade.

Plant material and extraction

Stem bark fragments from *C. cordiifolius* were collected (April/2014) in the morning in the rural area of Salgueiro, Pernambuco, Brazil (-8° 04' 27" S, -39° 07' 09" W, 420 m). The botanical material was authenticated by Maria Olívia de Oliveira Cano. A voucher specimen was deposited in the Herbarium of the Instituto Agrônomico de Pernambuco (IPA 89,210).

For the essential oil extraction, fresh stem bark fragments were immediately submitted to hydrodistillation for 2 h in a Clevenger-

type apparatus, yielding 1.56% of *C. cordiifolius* essential oil (CcEO). The essential oil was subsequently dried over anhydrous sodium sulfate, protected from light and frozen under -20°C until use. Fresh plant material was also extracted with ethanol (1:10, w/v) by dynamic maceration for 3 h. After filtration, the solvent was evaporated using a rotary evaporator (RV10, IKA, Germany), yielding 6.1% of *C. cordiifolius* ethanolic extract (CcEE).

Essential oil composition

Gas chromatography and mass spectrometry were carried out using a Shimadzu™ model 7A/QP 5050A equipped with a capillary non-polar DB-5 column (30 m x 0.25 mm x 0.25 μm). The oven temperature was programmed at 70°C with an increase of 4°C/min until 280°C, and maintained for 15 min. The carrier gas was helium, with a constant flow of 1.4 ml/min. The temperature of the ionization source was maintained at 280°C, the ionization energy at 70 eV, and the ionization current at 0.7 kV. Mass spectra were recorded from 30 to 450 m/z. Individual components were identified by matching their 70 eV mass spectra with those of the spectrometer data base using the Wiley L-Built and NIST libraries and by comparing their retention indices with those reported in the literature (Adams, 2001). The retention indices were compared with those of obtained by Craveiro et al. (1981) for other Euphorbiaceae species.

Phytochemical screening, total phenolic and flavonoid content

The ethanolic extract of *C. cordiifolius* stem bark was analyzed by thin-layer chromatography (TLC) using Si gel F₂₅₄ plates (ALUGRAM® 818131, Macherey-Nagel, Germany) and different solvent systems. It was verified the presence or absence of flavonoids, phenylpropanoids, terpenoids, steroids, saponins, alkaloids, coumarins, proanthocyanidins and quinones as described in Table 1.

Total phenolic content was determined by the Folin-Ciocalteu method (Li et al., 2008). Briefly, an aliquot of 200 μL of the extracts diluted in methanol (1 mg/mL) was added to 1 mL of the 10-fold diluted Folin-Ciocalteu reagent. After 4 min, 2.5 mL of sodium carbonate solution (0.7 M) was added. The test tubes were incubated for 30 min at room temperature, protected from light, after which the absorbance was read at 765 nm. Gallic acid was used for standard curve calibration (0 – 100 mg/L). The results were obtained from the regression equation: $y = 0.0121x + 0.032$, $R^2 = 0.9967$ and expressed as milligram of gallic acid equivalent (mgGAE)/g of extract. The experiments were performed in triplicate.

The determination of flavonoids was made by the method described by Woisky and Salatino (1998). An aliquot of 0.5 mL of the extracts diluted in methanol (1 mg/mL) was added to 0.5 mL of 2% AlCl₃ in methanol. The test tubes were incubated for 30 min at room temperature, protected from light, after which the absorbance was read at 420 nm. Quercetin was used for standard curve calibration (0 – 100 mg/L). The results were obtained from the regression equation: $y = 0.0262x - 0.0892$, $R^2 = 0.9982$ and expressed as milligram of quercetin equivalent (mgQE)/g of extract. The experiments were performed in triplicate.

Total antioxidant activity by phosphomolybdate assay (P-Mo)

Total antioxidant capacity of CcEE was determined by the

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phosphomolybdate method using ascorbic acid as standard (PIETRO et al., 1999). A 0.1 mL aliquot of extract diluted to 1 mg/mL in methanol was added to 1 mL of solution containing 600 mM sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. Test tubes were capped and incubated in a boiling water bath at 95°C for 90 min. Samples were then cooled to 25°C at room temperature and absorbance relative to a blank (1 ml of reagent solution and 0.1 mL of methanol) was measured at 695 nm. Total antioxidant activity (TAC) was expressed relative to ascorbic acid and calculated by the following formula,

$$\%TAC = \frac{Sabs - Babs}{AAabs - Babs} \times 100$$

Where Babs is the absorbance of the blank, Sabs is the absorbance of the sample (extracts) and AAabs is the absorbance of ascorbic acid.

1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity

DPPH free radical-scavenging activity of CcEE was determined using 96-well microplates as described by Brand-Williams et al. (1995). Briefly, 0.04 mL of CcEE or gallic acid diluted in methanol (31.25, 62.5, 125, 250, 500, 1000 µg/ml) and 0.25 ml of 1 mM DPPH in methanol was added to each well. Microplate was kept for 30 min at 25°C, protected from light. After this time, absorbance was measured at 517 nm. The free radical-scavenging activity of CcEE was expressed as percentage of inhibition of DPPH, calculated by the following formula,

$$DPPH \text{ inhibition } (\%) = \frac{(Sabs - Babs)}{Babs} \times 100$$

Where Babs is the absorbance of the blank (0.04 mL of methanol and 0.25 mL of 1 mM DPPH) and Sabs is the absorbance of the sample (CcEE or gallic acid).

2,2-azino-bis-(3 ethylbenzothiazoline)-6-sufonic acid (ABTS) radical cation scavenging activity

ABTS (7 mM) was oxidized by the addition of 140 Mm potassium persulphate. The solution was kept at 25°C for 16 h, in the dark. Before use, the ABTS+ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 units at 734 nm. For the determination of ABTS+ radical scavenging activity of CcEE, 0.01 mL of CcEE (31.25, 62.5, 125, 250, 500, 1000 µg/ml) diluted in methanol was added to 1.0 mL of ABTS+ solution. After 6 min, the absorbance was measured at 734 nm. ABTS radical cation scavenging activity of CcEE was expressed as percentage of inhibition of ABTS+, calculated by the following formula,

$$ABTS \text{ inhibition } (\%) = \frac{(Sabs - Babs)}{Babs} \times 100$$

Where Babs is the absorbance of the blank (0.01 mL of methanol and 1.0 mL of ABTS+) and Sabs is the absorbance of CcEE.

Animals

Male Swiss or BALB/c mice (20–30, n = 6) were provided by the Animal Facility of the Universidade Federal de Pernambuco. The animals were housed in a room with controlled temperature ($23 \pm 2^\circ\text{C}$) under a 12/12 h light/dark cycle with food and water *ad libitum*. Experiments were carried out according to the Guide for the Care

and Use of Laboratory Animals of the US Department of Health and Human Services (NIH publication number 85-23, revised in 1985). The project received prior approval from the Animal Ethics Committee of Universidade Federal de Pernambuco (protocol number 23076.016724/2016-62).

Topical anti-inflammatory activity

Ear edema measurement

For evaluation of ear weight, animals were euthanized and samples of 6 mm diameter were taken from both ears using a biopsy punch (Richter®, Brazil). Each biopsy was weighed on a semi-micro analytical balance (AUW-D 220, Shimadzu, Japan). Ear edema (EE) was expressed as the increase in ear sample weight, using the following formula:

$$EE (mg) = wRE - wLE$$

Where wRE is the weight obtained from the right ear sample (inflamed ear) and wLE is the weight obtained from the left ear sample (noninflamed ear).

For all treatments, animals were anesthetized with 1% halothane. Right ears were then challenged with different phlogistic agents diluted in acetone (20 µL). CcEE and CcEO were applied topically in 20 µL acetone. Dexamethasone or indomethacin (0.1 and 0.5 mg/ear, respectively) was used topically as a positive control. Ruthenium red (3 mg/kg, s.c.) was used as a positive control for capsaicin-induced ear edema.

PMA-induced ear edema

Ear edema was induced by topical application of 20 µL of PMA (2.5 µg/ear) in acetone on both sides of the right ear. Immediately after, CcEE (0.1, 0.3, and 1.0 mg/ear), CcEO (1, 3 and 10 µL/ear) or dexamethasone (0.1 mg/ear) was topically applied on both sides of the right ear, while the left ear received 20 µL of acetone. After 6 h, animals were euthanized for ear edema measurement (Carlson et al., 1985).

Histological analysis

After weighing, ear samples were fixed in buffered formalin for 24 h. Fixed tissues were then dehydrated by adding increasing concentrations of ethanol (70-100%), and processed for embedding in paraffin. The resulting blocks were sliced into 4 µm thick sections, stained with hematoxylin and eosin and observed under a light microscope (Nikon, Japan).

Investigation of the mode of action of CcEE on ear edema

The mechanisms of topical anti-inflammatory activity of CcEE on PMA-induced ear edema were evaluated using different phlogistic agents to induce ear edema: arachidonic acid (2 mg/ear), ethyl phenylpropionate 5% (20 µL/ear), phenol 10% (20 µL/ear), and capsaicin (0.25 mg/ear). Immediately, CcEE (1.0 mg/ear) or indomethacin (0.5 mg/ear)/dexamethasone (0.1 mg/ear) was topically applied on both sides of the right ear, while the left ear received 20 µL of the vehicle. Ruthenium red (3 mg/kg, s.c.) was used as positive control for capsaicin-induced ear edema. After 1 h, animals were euthanized for ear edema measurement. Animals challenged with capsaicin were euthanized after 30 min of exposure (Brattsand et al., 1982; Carlson et al., 1985; Gábor and Rázga, 1992).

Table 1. Solvent systems and spray reagents used for thin-layer chromatography analysis of *Croton cordiifolius* Baill. (Euphorbiaceae) stem bark ethanolic extract.

Secondary metabolite classes	Standard	Solvent system	Spray reagent	References
Flavonoids and phenylpropanoids	Quercetin, rutin and chlorogenic acid	EtOAc-HCOOH-AcOH-H ₂ O (100:11:11:26 v/v)	Natural products-polyethylene glycol reagent	Wagner and Bladt (1996); Brasseur and Angenot (1986)
Triterpenes and steroids	β -sitosterol	Toluene:EtOAc (90:10 v/v)	Lieberman-Burchard reagent	Harborne (1998)
Saponins	-	EtOAc-HCOOH-AcOH-H ₂ O (100:11:11:26 v/v)	Lieberman-Burchard reagent	Harborne (1998)
Mono and sesquiterpenes	Thymol	Toluene: EtOAc (97:3 v/v)	Anisaldehyde-sulphuric acid reagent	Harborne (1998)
Coumarins and quinones	Coumarin and lapachol	CHCl ₃ -MeOH (98:2 v/v)	Potassium hydroxide reagent	Wagner and Bladt (1996)
Alkaloids	Pilocarpine	EtOAc-HCOOH-AcOH-H ₂ O (100:11:11:26 v/v)	Dragendorff reagent	Wagner and Bladt (1996)
Proanthocyanidins	Catechin	EtOAc-HCOOH-AcOH-H ₂ O (100:11:11:26 v/v)	Vanillin-hydrochloric acid reagent	Roberts et al. (1957)
Hydrolysable tannins	Gallic acid	n-BuOH-H ₂ O-AcOH (40:50:10 v/v)	Ferric ammonium sulfate 1%	Sena Filho et al. (2008)

Statistical analysis

Data are expressed as mean \pm S.E.M. and analyzed by ANOVA followed by Bonferroni post-test using GraphPad Prism 5.0 with significance * set at $p < 0.05$.

RESULTS

Chemical composition and antioxidant activity

Phytochemical screening of CcEE showed the presence of flavonoids (aglycones, mono and diglycosides of 3',4'-OH flavonoids), phenylpropanoids, mono- and sesquiterpenes, triterpenes and steroids, alkaloids, and coumarins. The predominant compounds were terpenes and steroids. There were no hydrolyzable tannins, quinones, saponins, proanthocyanidins, or leucoanthocyanidins. The total phenolic and flavonoid contents of CcEE were determined by spectrophotometry to be 135.8 ± 7.62 EAG/g extract and 21.24 ± 0.37 EQ/g extract. GCMS analysis of CcEO identified 97.92% of the compounds, revealing a monoterpenoid rich essential oil (91.13%), with only 6.79% of sesquiterpenoids. The major compounds identified were α -pinene (51.76%), β -pinene (19.08%), camphene (9.71%), borneol (4.52%), camphor (4.37%), and β -caryophyllene (4.31%) are listed in Table 2.

Total antioxidant activity was determined with the phosphomolybdenum method, which is based on reduction of molybdenum VI to molybdenum V, in the presence of antioxidants. This reduction results in the formation of a green complex. According to this assay, total antioxidant activity in CcEE was $26.88 \pm 2.83\%$ of the activity of the ascorbic acid control. Free radical scavenging activity by DPPH and ABTS+ assays were also measured, with EC₅₀ values $> 1,000$ μ g/mL of CcEE for both assays.

Anti-inflammatory activity

PMA-induced ear edema

CcEE (0.1, 0.3 and 1 mg/ear) significantly inhibited PMA-induced ear edema at all tested doses as seen in Figure 1A. By contrast, CcEO (1, 3 and 10 μ L/ear) showed less promising results, with significant activity only at the highest dose (Figure 1B). Histological analysis of ear samples aimed to evaluate the following parameters: congested vessels (CV); polymorphonuclear cells infiltration (PCI) and/or edema (E). Total ear and epidermis thickness was also evaluated relative to the negative control group. PMA-induced ear edema produced CV filled with white blood cells, intense PCI and edema. Dexamethasone (0.1 mg/ear) and CcEE at 0.3 and 1 mg/ear yielded similar results. CV were present and filled with red blood cells and discrete PCI and E. Ears treated with CcEE at 0.1 mg/ear and CcEO at all doses tested (1, 3 and 10 μ L/ear) had CV filled with red and white blood cells and moderate PCI and E (Figure 2A-H). Based on these results, CcEE at 1.0 mg/ear was chosen for elucidation of CcEE's mechanism of topical anti-inflammatory activity using other phlogist agents.

Investigation of the anti-inflammatory mechanism of CcEE

CcEE (1 mg/ear) and indomethacin (0.5 mg/ear) demonstrated significant reductions of 57.7 and 53.2% respectively in AA-induced ear edema relative to the negative control (Figure 3A). In EPP-induced ear edema, CcEE (1.0 mg/ear) inhibited ear edema by 42%. This was less effective than the positive control drug dexamethasone (0.1 mg/ear), which inhibited ear edema by 75.2% (Figure 3B). CcEE (1.0 mg/ear) reduced phenol-

Table 2. Chemical composition of the essential oil of *Croton cordiifolius* Baill. (Euphorbiaceae) stem bark.

Peak	Compound	Retention time (min)	RI ^a	RI ^b	Relative area (%)
1	NI	5.092	-	-	0.51
2	α -pinene	5.405	938	932	51.76
3	Camphene	5.781	943	946	9.71
4	β -pinene	6.573	968	974	19.08
5	Myrcene	6.917	978	988	0.39
6	Cymene	7.992	1011	1020	0.29
7	Limonene	8.158	1022	1024	1.01
8	NI	8.242	-	-	0.21
9	NI	10.192	-	-	0.23
10	NI	10.583	-	-	0.58
11	Camphor	12.203	1121	1141	4.37
12	Borneol	13.017	1148	1165	4.52
13	NI	13.425	-	-	0.26
14	NI	13.908	-	-	0.28
15	β -caryophyllene	22.075	1434	1417	4.31
16	β -selinene	23.175	1479	1489	0.50
17	α -muurolene	24.675	1480	1500	1.08
18	Caryophyllene oxide	27.267	1567	1582	0.90
Total identified					97.92
Monoterpene hydrocarbons					82.24
Oxygenated monoterpenes					8.89
Sesquiterpene hydrocarbons					5.89
Oxygenated sesquiterpenes					0.90

^aKovats retention index according to n-alkanes (C8–C26); ^bAccording to Adams (2009).

induced ear edema by 33.9%, while dexamethasone (0.1 mg/ear) reduced this edema by 91.6% (Figure 3C). In capsaicin-induced ear edema, CcEE (1.0 mg/ear) was not able to inhibit ear edema. Ruthenium red (3 mg/kg), a standard positive control drug, when given 30 min prior to capsaicin administration, inhibited ear edema by 73.5% (results not shown).

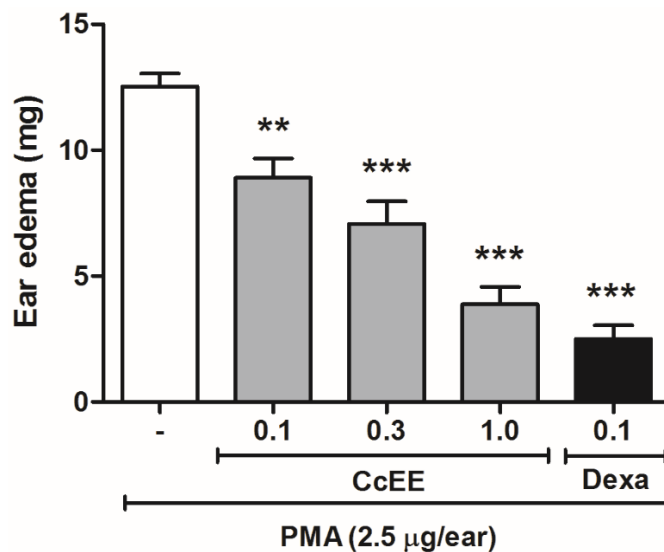
DISCUSSION

The *Croton* genus is characterized by a large chemical diversity. Based on the existing descriptions of chemical composition of many *Croton* species, this genus can be characterized as a promising source of bioactive molecules with a remarkable potential for discovery of novel biological activities. Growing interest in studying *Croton* species has resulted in isolation and identification of more than 100 currently known chemical compounds in Brazil (Angélico, 2011; Song et al., 2015).

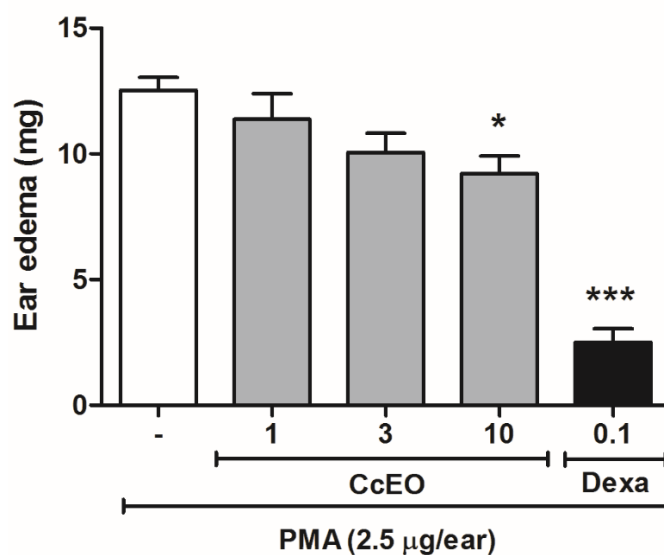
Although not common in the Euphorbiaceae family, some *Croton* species should be highlighted for the production of different alkaloid classes (Salatino et al., 2007). Identification of this group of secondary metabolites by TLC in *C. cordiifolius* corroborates several

studies in other species of the same genus. Salatino et al. (2007) described 35 alkaloid types from 14 *Croton* species. Among the species present in Brazilian caatinga, β -carboline alkaloids 2-ethoxycarbonyltetrahydroharman and 6-hydroxy-2-methyltetrahydroharman have been isolated from *C. moritibensis* (Araújo-Júnior et al., 2004); anabasine and the novel guaiane-type alkaloids muscicapines A, B and C were isolated from roots of *C. muscicapa* (Araújo-Júnior et al., 2005). Alkaloids were also identified in *C. campestris* (Brito-Júnior et al., 2014) and *C. rhamnifolius* roots, and *C. rhamnifolioides* leaves, stem and roots (Randau et al., 2004); however, no compounds have been isolated.

Flavonoids are a class of polyphenols widely distributed in plants, including the genus *Croton*. Different studies have reported antioxidant, analgesic, antibacterial, antifungal, anti-inflammatory, antiviral, antitumor, antiallergic, and antiparasitic activities for these metabolites (Coelho et al., 2016). Among *Croton* species, it has not yet been possible to establish a standard for flavonoids. Although some compounds have already been isolated from hexanic, ethanolic and methanolic extracts of *Croton* spp., most investigations are not directed at the characterization of the flavonoid



A.



B.

Figure 1. **A.** Effect of *C. cordiifolius* ethanolic extract (CcEE, 0.1, 0.3 and 1 mg/ear), and **B.** essential oil (CcEO, 1, 3 and 10 µL/ear) on PMA-induced ear edema in mice. Dexamethasone (0.1 mg/ear) was used as positive control. Results are expressed as mean \pm S.E.M. (n = 6) and analyzed by ANOVA with Bonferroni post test. * p <0.05, ** P <0.01, and *** p <0.001 compared to negative control group.

profile presented by the plant. A standardized analysis of the flavonoids present in different species of *Croton* may be an auxiliary tool in the differentiation of this genus subgroups.

In this context, the presence of flavonoids and phenylpropanoids in *C. cordiifolius* stem bark is

consistent with the findings of Palmeira Júnior and collaborators (2005, 2006) for *C. sellowii* leaves and stem, containing the flavonoids artemetin, crysoplenetin, casticin, penduletin, tilioside, and the phenylpropanoid threo-7-ethoxy-8-hydroxy-dihydroanol; and *C. brasiliensis* leaves and stem, containing casticin, penduletin,

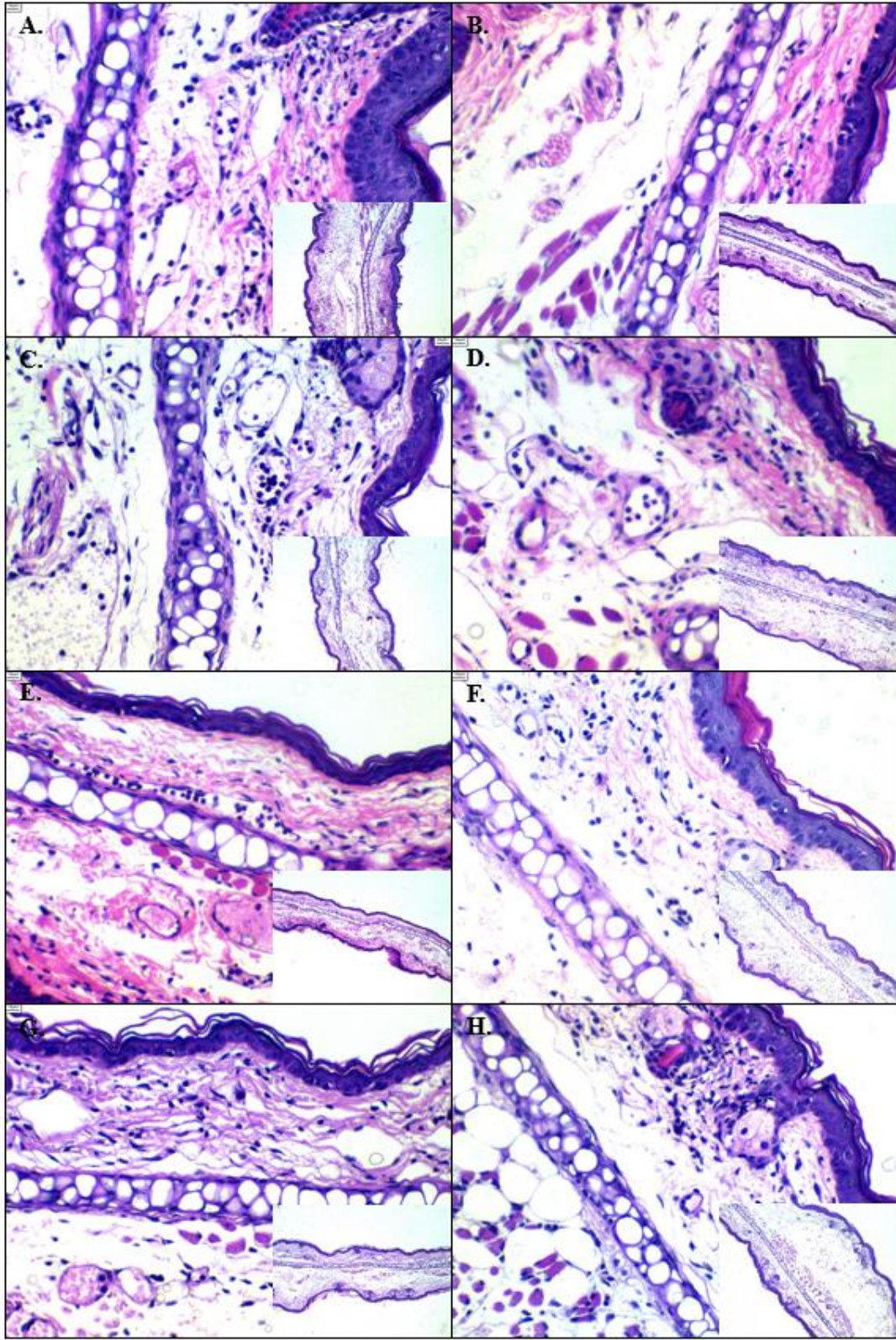
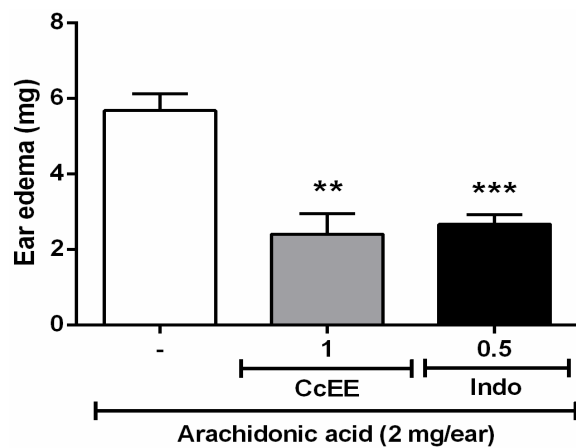
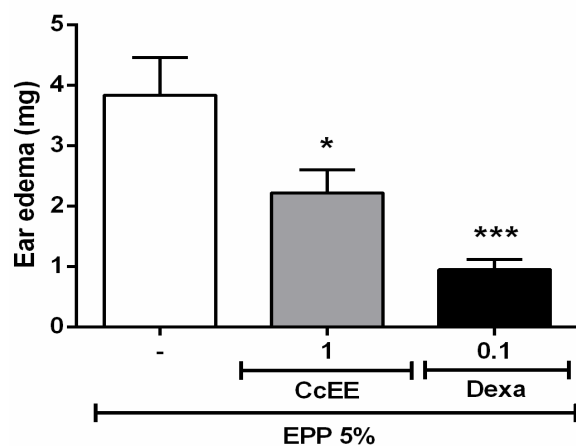


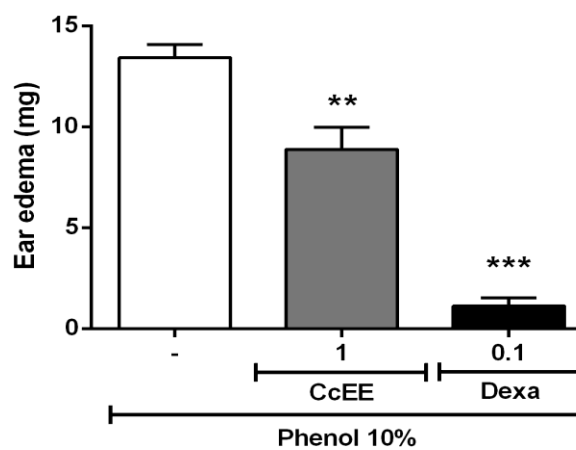
Figure 2. Histological analyses of PMA-induced ear edema samples. **A.** Negative control group, **B.** Dexamethasone (0.1 mg/ear); **C.**, **E.**, and **G.** *C. cordifolius* ethanolic extract (CcEE, 0.1, 0.3 and 1 mg/ear, respectively). **D.**, **F.**, and **H.** *C. cordifolius* essential oil (CcEO, 1, 3 and 10 µL/ear, respectively). The ears were stained with hematoxylin and eosin. The tissue sections were observed under light microscope at 400x and 100x (detail). Scale bars represent 10 µm.



A.



B.



C.

Figure 3. Effects of *C. cordifolius* ethanolic extract (CcEE, 1 mg/ear) on **A.** arachidonic acid-, **B.** ethyl phenylpropionate, and **C.** phenol-induced ear edema in mice. Indomethacin (Indo, 0.5 mg/ear) and dexamethasone (Dexa, 0.1 mg/ear) were used as positive controls. Results are expressed as mean \pm S.E.M. ($n = 6$) and analyzed by ANOVA with Bonferroni post test. * $p < 0.05$, ** $P < 0.01$, and *** $p < 0.001$ compared to negative control group.

chrysofenol-D and artemetin; of Morais and collaborators (1988) for *C. mucronifolius* aerial parts, who found polyhydroxylated flavonoids; of Barreto and collaborators (2013) for *C. muscicarpa* leaves, stem and roots, which contains the flavonoids retusin, 3,7,4'-trimethoxy kaempferol, ombuine, pachipodol, kaempferol, casticin, 5-hydroxy-3,6,7,4'-tetramethoxyflavone and artemetin; of Coelho and collaborators (2016) for *C. betulaster* leaves, which isolated 5-hydroxy-7,4'-dimethoxyflavone, casticin, and penduletin and of Lopes and collaborators (2012) for *C. pedicellatus* leaves, who found seven flavonoids: tiliroside, 6"-O-*p*-coumaroyl- β -galactopyranosyl-kaempferol, 6"-O-*p*-coumaroyl- β -glucopyranosyl-3"-methoxy-kaempferol, kaempferol, 3-glucopyranosyl-quercetin and alpinumisoflavone, as well as 4-hydroxy-3,5-dimethoxybenzoic acid.

Although tannins are ubiquitous polyphenols in plants, only proanthocyanidins have been reported for *Croton* species, specifically those containing red latex such as *C. urucurana*, *C. lechleri* and *C. panamensis*. These metabolites were not found in Caatinga species (Salatino et al., 2007).

Terpenes and steroids are chemical constituents of interest owing to their anti-inflammatory, analgesic, antimicrobial, antiviral, hepatoprotective, and hormonal activities (Gupta et al., 1969; Mahato et al., 1988). Clerodane diterpenes are frequently found in the stem bark of *Croton* species, such as *C. cajucara*, *C. urucurana*, *C. lechleri*, but few were found in caatinga species, such as *C. brasiliensis*, from which clerodanes diterpenes crotobrasilins A and B were isolated from leaves and stem bark (Palmeira-Júnior et al., 2005). Diterpenes bearing the novel skeleton sarcopetalane, besides labdane, trachylobane, cembranoid, and halimane skeletons are not found in species from Caatinga.

The presence of triterpenes and steroids in *C. cordiifolius* agrees with the findings for *C. muscicarpa* leaves, stem, and roots, from which were isolated 6 α -methoxy-cyperene, dammaradienol, squalene, acetyl aleuritic acid and spathulenol (Barreto et al., 2013); *C. regelianus* leaves and stem, from which were found the bisnorditerpene rel-(5b,8a,10a)-8-hydroxy-13-methylpodocarpa-9(11),13-diene-3,12-dione and the guaiane sesquiterpene rel-(1R,4S,6R,7S,8aR)-decahydro-1-(hydroxymethyl)-4,9,9-trimethyl-4,7-(epoxymethano)azulen-6-ol (Torres et al., 2010); and *C. sellowii* leaves and stem, from which were isolated sitosterol, stigmasterol, cycloart-25-ene-3 β ,24 β -diol, cycloart-25-ene-3 β ,24 α -diol and betulonic acid (Palmeira-Júnior et al., 2006).

Coumarins, which are rare in the Euphorbiaceae family and *Croton* genus, were found in *C. cordiifolius* in this study. This result concurs with those reported by Lima and collaborators (2010), who related the presence of a coumarin in *C. adenocalyx*, representing an important role in the chemotaxonomy differentiation of these species.

Essential oils have been described for various species of this genus (Almeida et al., 2015; LIMA et al., 2010; LEITE et al., 2015; Melo et al., 2013; Neves and Camara, 2012; Ramos et al., 2013; Turiel et al., 2016). Nogueira et al. (2015) described the constituents of the essential oil of *C. cordiifolius* leaves, which is rich in mono- and sesquiterpenes and whose main constituents were the monoterpenes 1,8-cineole and α -phellandrene. By contrast, the major constituents of the essential oil of *Croton cordiifolius* stem bark were the monoterpenes α - and β -pinene. α -pinene was also a major compound of the essential oil of *C. argyrophylloides* aerial parts (Morais et al., 2006), *C. conduplicatus* stem bark (Almeida et al., 2015), and *C. adenocalyx* leaves (Lima et al., 2010). Some authors have speculated that the co-occurrence of α - and β -pinene is a characteristic of the genus *Croton*. Others claim that β -caryophyllene and linalool are equally frequent major constituents (Almeida et al., 2015).

In addition to differences in essential oil constitution among distinct species of the genus *Croton*, several authors have demonstrated distinct essential oil compositions of different tissues from the same species (Brasil et al., 2009; Lima et al., 2010; Morais et al., 2006; Neves and Camara, 2012). In general, the essential oils of Brazilian *Croton* species present mono and sesquiterpenes as major components, with the exception of *C. zehntneri*, in which the phenylpropanoids eugenol and anetol predominate (Craveiro et al., 1981).

Most studies of the chemical constitution of the essential oil of *Croton* species refer to the leaves or aerial parts. Among the studies that report the constitution of the stem essential oil, it can be highlighted the one realized by Neves and Camara (2012) for *C. pulegioides*, with α -calacorene as the major compound, and for *C. heliotropiifolius*, with guaial as the major compound; by Almeida et al. (2015) for *C. conduplicatus*, with α -pinene as the major compound; and by Suarez et al. (2005) for *C. molambo* with methyleugenol as the major compound.

The chemical composition of *Croton* species may be correlated with their geographical distribution. An example of this is the labdane and caurane diterpenes, which have not yet been reported for New World species; alkaloids, which were only found in American species; and the aromatic species, which are also prevalent in America (Salatino et al., 2007). Moreover, the presence of compounds found only in Brazilian caatinga species could indicate the existence of unique chemotypes within species of *Croton* found only in that biome (Angélico, 2011).

C. cordiifolius, *C. heliotropiifolius*, and *C. conduplicatus* are popularly known as "quebra-faca" due to the rigidity of their stem (Randau et al., 2004). These species also present similar popular uses: *C. cordiifolius* is used to treat general inflammation, pain, and gastrointestinal disturbances (Nogueira et al., 2015); *C. heliotropiifolius* is used as folk remedy for the treatment of wounds,

inflammation, fever, cancer, stomach pain, vomiting, bloody diarrhea and to alleviate fever (Neves and Camara, 2012; Randau et al., 2004); the decoction of *C. conduplicatus* leaves and stem bark is used in folk medicine to treat influenza, headache, indigestion, stomach problems and stomachache (Almeida et al., 2015).

In this context, the differentiation of their chemical composition and pharmacological activities, together with their botanical characterization, constitute a diagnostic tool for these species. Neves and Camara (2012) studied the chemical composition of the essential oil of *C. heliotropiifolius* stem bark, observing a characteristic profile of the genus by the presence of β -caryophyllene as one of the major constituents. It differs from *C. cordiifolius* stem bark essential oil by the presence of sesquiterpenes and phenylpropanoid derivatives. Almeida et al. (2015) described for the first time the chemical constitution of the essential oil of *C. conduplicatus* stem bark, whose main compounds were α -pinene, β -pinene, camphor and (E)-caryophyllene. The chemical composition of *C. cordiifolius* stem bark essential oil is very similar, with α - and β -pinene as major constituents. The major difference is the presence of the monoterpene camphene as the third major constituent in *C. cordiifolius*, which can be converted into camphor *in vivo*. Randau et al. (2004) performed a phytochemical study of the extract of different parts of *C. rhamnifolioides*. It was observed the presence of alkaloids, triterpenes and steroids, sugars, flavonoids and phenylpropanoglycosides. Therefore, the presence of coumarins in *C. cordiifolius* bark extract can be considered a useful marker in the differentiation of these species. Despite the existence of some studies that point to chemical patterns in the genus, given its variability and the small sampling of species chemically studied, definitive conclusions can not be drawn about chemical relationships among *Croton* species (Salatino et al., 2007).

Many *Croton* species studies have reported outstanding antioxidant activities, which set a precedent for the results of this study of *C. cordiifolius*. Morais and collaborators (2006) evaluated the antioxidant activities of *C. zenhtneri*, *C. nepeatefolius*, and *C. argyrophyloides* essential oils. This activity is probably due to the presence of E-anethole in *C. zenhtneri*, methyleugenol in *C. nepetaefolius*, and α -pinene, E-caryophyllene, and 1,8-cineole in *C. argyrophyloides*. Angélico (2011) reported antioxidant activities in *C. blanchetianus* and *C. heliotropiifolius* extracts, attributing it to the possible presence of phenolic compounds belonging to the tannin and flavonoid classes.

Chronic inflammation mediates the development of various diseases, and increased levels of free radicals have been found in many pathological conditions other than inflammation, including cancer, ischemic disorders, and dementia (Ramos et al., 2013). The search for

medicinal plants with anti-inflammatory and antioxidant activities is fundamental to direct the development of pharmaceutical products designed to treat major diseases.

Mechanisms of anti-inflammatory activity can be elucidated through research with animal models in which edema is induced by different phlogistic agents with different well-understood mechanisms. To identify topical anti-inflammatory mechanisms of *C. cordiifolius*, we used five phlogistic agents with distinct mechanisms in a mouse model of topical inflammation.

Ear edema induced by PMA is primarily mediated by prostaglandin E₂ and protein kinase C. This model is useful for screening topical active extracts and/or compounds, and anti-inflammatory efficacy implies the presence of prostaglandin and leukotriene biosynthesis inhibitors (Silva et al., 2005). In this model, CcEE probably inhibited edema by directly interfering with prostaglandins' action or by inhibiting enzymes such as cyclooxygenase or phospholipase A₂ (PLA₂). According to Nair and collaborators (2006), the flavonoid quercetin decreased production and expression of proinflammatory cytokine TNF- α in human mononuclear cell cultures stimulated by phorbol myristate acetate (PMA). This effect was associated with inhibition of NF- κ B. Other flavonoids, such as santin and ermanin, also inhibited PMA-induced ear edema in mice, being more active than the positive control indomethacin at identical concentrations (3 mg/ear) (Martinez et al., 1997). Thus, the presence of flavonoids demonstrated in phytochemical screening and quantified by spectrophotometry could contribute to this activity.

The inflammatory process instigated by AA is mediated by production of metabolites such as prostaglandin E₂ (PGE₂), leukotriene C₄ (LTC₄), and leukotriene D₄ (LTD₄) (Humes et al., 1986). The ear edema induced by AA is characterized by sensitivity to inhibitors of cyclooxygenase and lipoxygenase enzymes. Therefore, AA is commonly used to validate substances capable of inhibiting the action of mediators of edema (Crummey et al., 1987; Opas et al., 1986; Humes et al., 1986). This model is not sensitive to PLA₂ inhibitors such as glucocorticoids. CcEE probably inhibit edema in this model by preventing the metabolism of arachidonic acid to prostaglandins and leukotrienes or by inhibiting the action of its metabolites. In the literature, there is no evidence of topical anti-inflammatory activity in that model from species of the genus *Croton*.

Because ethyl phenylpropionate (EPP) is responsible for release of inflammatory mediators such as histamine and serotonin (Nualkaew et al., 2009), CcEE may interfere with the release of these inflammatory mediators. Phenol-induced ear edema mimics contact dermatitis processes visualized in humans (Lim et al., 2004). The intense irritation resulting from application of this phlogistic agent is a consequence of disruption of keratinocytes plasma membranes, with release of

preformed IL-1 α , IL-8, TNF- α , free radicals, and inflammatory mediators such as AA downstream metabolites.

Although this process occurs independently of AA metabolites, the mentioned cytokines (IL-1 α , IL-8, TNF- α) can induce production and release of AA and reactive oxygen species (Lim et al., 2004; Murray et al., 2007; Wilmer et al. 1994). In contrast to PMA, phenol activates nuclear transcription factors through protein kinase C dependent pathways to induce inflammatory cytokines. Despite using different routes, both models produce arachidonic acid metabolites and reactive oxygen species in the inflammatory response (Wilmer et al., 1994). In this context, the performance of CcEE in reducing production of inflammatory mediators and cytokines through protein kinase C dependent pathways can be highlighted.

Capsaicin, an alkaloid obtained from species of the genus *Capsicum*, mediates a neurogenic inflammatory response characterized by vasodilation-induced edema with increased blood flow, and plasma extravasation, in addition to sensitized nociceptors responsible for contact induced-pain sensation in skin or mucous membranes (Zegarska et al., 2006). In this process, TRPV-1 receptors are activated, resulting in the release of neuropeptides, such as substance P, which are responsible for erythema and papule formation and macrophage degranulation, followed by release of substances such as histamine and serotonin (Inoue et al., 1993).

Topical application of capsaicin promotes the release of pro-inflammatory mediators which result in an immediate vasodilation and erythema response followed by edema, which peaks within 30 minutes after administration (Gabor, 2000; Zegarska et al., 2006). CcEE did not reduce capsaicin-induced edema, suggesting that it does not act on TRPV-1 receptors, and therefore does not inhibit mast cell degranulation or release of histamine and serotonin, mediators responsible for increasing cell permeability and edema (Inoue et al., 1993).

The anti-inflammatory activity of *C. cordiifolius* found in this study corroborates the ethnobotanical survey conducted by Nogueira and collaborators (2015), which showed the highest number of “quebra-faca” citations for inflammation and pain treatment.

Conclusions

The first of its kind phytochemical survey of *Croton cordiifolius* stem bark extract and the characterization of its essential oil provide important information for directing future research aimed at isolating chemical constituents of this species, which is widely used in Northeast Brazilian folk medicine.

Our *in vivo* anti-inflammatory activity tests showed that *C. cordiifolius* essential oil is not effective at the doses tested. In contrast, the extract contained anti-inflammatory compounds with different mechanisms of action because it showed significant activity in four of the five assays. Our results suggest that the extract can interfere with AA

metabolism and prostaglandin biosynthesis, release of inflammatory mediators such as histamine and serotonin, and inhibition of preformed cytokine release. No inhibition of TRPV-1 receptors or mast cells degranulation was observed. Additionally, the observed antioxidant activity of *C. cordiifolius* stem bark extract could be, in part, responsible for its anti-inflammatory effects.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Induction of biochemical active constituents of Jojoba (*Simmondsia chinensis* (Link) Schneider) callus affected by hormones

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The present study aimed to identify some metabolites products obtained from different jojoba callus tissue extracts using gas chromatography/mass spectrometry analysis (GC-MS). It is known that Jojoba, a medicinal and oil-yielding, has multi-purpose uses. In addition, it produces toxins, fatty acids, phenolic compounds and other secondary metabolites from callus identified using GC-MS. Despite the direct effect of 2,4-D on directing the explant towards callus induction, it has interaction effect with Kin and achieves hormonal balance inside the explant. There is a significant interaction effect between Kin and 2,4-D. The increased concentration of both Kin and 2,4-D raises the extent of nodal segments response towards proliferation, development, and callus induction. Our results showed that the interaction effect between Kin and 2,4-D is the best results for phytochemical active constituents formation achieved from callus cultured. Qualitative results revealed that increasing cytokinins Kin concentration leads to slightly double increase in active constituents from the explants for Triodecanoic and Methoxyacetic acids. While, the gradual increase of 2,4-D to 2.0 mg l⁻¹ caused a single increase in active constituents for Triodecanoic, Methoxyacetic and Octadecanoic acid, respectively. On the other hand, Decanoic acid has no effect by adding both hormones (Kin and 2,4-D) and also Octadecanoic acid when kin is added. Also, there is a direct relation between the development of callus and reproduction of active phytochemical constituents, which increase qualitatively by increasing callus development and 2,4-D hormone concentration at 2 mg l⁻¹ for 1-Hexadecanol, Nonadecatriene, Aristolene and Tricosene, respectively compared to free hormone treatment.

Key words: Active constituents, callus, fatty acids, jojoba, *Simmondsia chinensis*.

INTRODUCTION

Jojoba [*Simmondsia chinensis*, family *Simmondsiaceae*] is a shrub with an oil seed. It has male and female sexual

organs, and grows in semi and desert regions. Its native country is Southwestern US. Jojoba is used for medicinal

purposes and is a folk medication for cold, obesity, wounds, sore throat warts, dysuria, parturition, and cancer (Kolodziejczyk et al., 2000; Mohasseb et al., 2009a). Jojoba belongs to order caryophyllales, which contains simmondsin and three non-ferulated compounds in different organs of tissue cultured plants which cause reduction in food intake.

Jojoba is a crop that bears harsh environment and thrives in areas where other crops cannot be cultivated. It consumes less water and therefore can be farmed in new areas. Conventional breeding, which aims to improve tolerance to drought and salinity, takes a long time and does not succeed in producing sustainable resistant plants. These circumstances necessitate the use of genetic engineering and biotechnology, as one of the most promising new technologies to improve jojoba (Mohasseb et al., 2009a, b).

Jojoba oil has many usages depending on the site where the modification is being done. Virtually, it has no traces of glycerine, making it a unique plant of oil along with the fact that it can be modified via hydrogenation, sulfurization, halogenation and many other techniques. With its uses in industries like cosmetic, pharmaceutical, lubricant and petrochemicals, the importance of jojoba oil in the market is high (Arya and Khan, 2016).

Jojoba seed has a fluid wax (jojoba oil) used in pharmaceutical products, cosmetics, and mechanical lubricants. Jojoba meal, a waste from jojoba seeds, is the remaining product after extracting of oil and constitutes over half of the seed. Jojoba meal composed of 25 to 30% protein is rich in dietary fiber and may be used in livestock feed fortification. The defatted meal holds sugars and 11 to 15% of natural products, constantly on structurally identified simmondsin. Jojoba is clonally propagated by nodes and the rate of propagation is very limited because the nodes are hard to roots, so that the only solution to solve this problem is through rapid mass production by tissue culture technique (Mohasseb et al., 2009b).

Jojoba plants have many woody stems that commonly grow between 0.6 and 2 meters in height, and over 3 m have been observed in the wild. In full light, it branches near the base. Plants can vary from almost prostrating, with the branches growing laterally. The wood from mature plants is hard and heavy, lemon yellow and without distinctive scent or taste. The natural life span appears to be over 100 years and may exceed 200 years (Mohasseb et al., 2009a).

This study aimed to produce more amounts of biochemically active constituents and simmondsin by increasing the cell numbers of seeds that produce biochemical active constituents in it. So, callus was produced from seeds or leaf and root by *in vitro* culture

technique. After that, a lot of biochemically active constituents used for many purposes can be extracted and also biochemical active constituents can be produced from callus and hairy roots of jojoba. Thus, there are two sources to produce commercial amounts of biochemical active constituents.

MATERIALS AND METHODS

This study was carried out at the Department of Agricultural Biotechnology, College of Agricultural and Food Sciences, King Faisal University, Al-Hassa, Saudi Arabia.

Explant preparation and sterilization process

Plant materials were taken from semi-hardwood stems of female jojoba adult shrub provided by Mohasseb et al. (2009a).

1. Each inter node (2 cm) explant was excised from a 5-years-old shrub.
2. The cuttings were thoroughly washed by 1% savlon solution for 20 min
3. They were rinsed twice in sterile distilled water (SDW).
4. All subsequent operations were carried out inside a laminar air-flow cabinet.
5. The clean cuttings were given a quick (30 s) rinse in 70% ethanol, followed by two washings in SDW.
6. These cuttings were then surface-sterilized in 0.15% mercuric chloride (HgCl₂) solution for 13 min and rinsed thrice with SDW.

Establishment stage and callus induction

1. The cuttings were slightly trimmed at both ends to expose the fresh.
 2. Tissues were exposed with inorganic salts, supplemented with (in mg l⁻¹): 100 Myoinositol; 30000 sucrose; 7000 agar (Sigma Chem. Co.)
 3. They were planted in MS culture medium (Murashige and Skoog, 1962).
 4. The pH value was adjusted at 5.7±0.1 before adding agar.
 5. The medium was autoclaved at 121°C for 15 min, while, the changes in the nutrient medium are as follows (Table 1).
- All the cultures were maintained in diffused light, 2000 lux and 16 h photoperiod at 25±2°C. The nodal segments of each treatment were repeatedly subcultured for three subcultures at four weeks intervals. For parameters of callus induction stage, data were recorded at the end of 3 months from initial culture. Each treatment had five replicates. Each replicate contained one jar, and each jar contained three nodal segments. The data were presented as the average per callus induction as follows: 1- (Pro) = proliferation (No.), 2- (CP) = callus induction (No.), 3- (FW) = fresh weight (g), and 4- (DW) = dry weight (g).

Phytochemical qualitative analysis by GC-MS

Preparation of callus ethanol extracts

1. Callus was dried at 40°C for 24 h in an oven using a vacuum.
2. Samples were grinded into powder using a mortar under steam

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Table 1. Composition of nutrient media for *in vitro* callus induction from jojoba.

Kin (mg l ⁻¹) \ 2,4-D (mg l ⁻¹)	0.0	0.5	1.0	2.0
	0.0	A*	B	C
0.5	E	F	G	H
1.0	I	J	K	L
2.0	M	N	O	P

*Symbol of media used in the form of letters A, B, C, ... P. (*media code).

Table 2. Specific effect of Kin on callus induction from nodal segments jojoba.

Media code	Kin (mg l ⁻¹)	Nodal segments			
		Pro ¹ (No)	CP ² (No)	FW ³ (g)	DW ⁴ (g)
A	0.0	0.9100 ^a	0.0000	3.2500 ^c	0.9615 ^b
B	0.5	0.8900 ^a	0.0000	1.8960 ^d	0.9116 ^b
C	1.0	0.9200 ^a	0.0000	4.1927 ^b	0.6754 ^c
D	2.0	0.8900 ^a	0.0000	4.3200 ^a	1.1666 ^a
Means		0.9025	0.0000	3.4147	0.9288

1- Pro = proliferation; 2- CP = callus Induction; 3- FW = fresh weight; 4- DW = dry weight.

of liquid nitrogen; after that it was extracted.

3. A quantity of 3.0 g dry base of callus powder was extracted with 15 ml of ethanol (70%) at room temperature, protected from sunlight and mixed several times with a sterile glass rod.

4. Mixture was then filtered through Wattman No.1 filter paper. The extracted liquid was subjected to rotary evaporation to dryness in order to remove the ethanol (Akueshi et al., 2002).

5. After that, the extract was stored at 4°C in refrigerator for future use (Harborne, 1984).

Gas chromatography/mass spectrometry analysis (GC-MS)

The adapted samples were chosen based on their total jojoba extract and those with high total biochemical active constituents in callus tissues, considered as promising treatments. They were subjected to GC-MS analysis after filtering with 0.22 µm pore size syringe filter.

GC-MS analysis was carried out in College of Agricultural and Food Sciences, King Faisal University, Al-Hassa, Saudi Arabia, using GC-MS apparatuses, under the following conditions: a HP-5MS capillary column (Agilent Technologies, Santa Clara, CA) operating at electron impact mode at 70 eV. Pure helium gas with built-in purifier was used at a constant flow rate of 1.0 ml/min employed in a split less mode with injector temperature of 250°C and ion source of 280°C. The stepped temperature program was as follows: initial temperature oven was started at 220°C and held for 5 minutes and followed by a ramp to 300°C at 5°C/min held for another 15 min. A post-run of 5 minutes at 300°C was sufficient for the next sample injection.

Biochemical active compounds identification

Mass analyzer was used in full scan mode scanning from 40 to 550 m/z and mass spectra were taken at 70 eV. For the compound

identification, manual spectral matching was ascertained by using the mass spectral library of National Institute Standard and Technology (NIST) version 2.0 and with the aid of Automated Mass Spectral Deconvolution and Identification (AMDIS) software version 2.70 by deconvoluting the chromatography peak at the corresponding retention time (Cheong et al., 2016).

It should be noted that the chromatograms not only belong to saponifiable but also some bioactive compounds in the unsaponifiable fraction in the jojoba callus extracts of each treatment.

Statistical analysis

Data were statistically analyzed by using a randomized complete block design (RCBD) (Snedecore and Cochran, 1990). Mean separations were done by using a MSTAT-C computer program v.4 (Duncan, 1955).

RESULTS AND DISCUSSION

The interaction effect of hormones and their concentrations on callus induction

Table 2 shows the significant effect of Kin on the extent of response of jojoba nodal segment to *in vitro* callus formation. These data show that the more Kin was used the more the explant responded to callus formation. Increased use of Kin concentration raises the extent of explants' response towards proliferation and development and the best results were achieved at the concentration of 2.00 mg l⁻¹. It can be noted that nodal segments

Table 3. Specific effect of 2,4-D on callus induction from nodal segments jojoba.

Media code	2,4-D (mg l ⁻¹)	Nodal segments			
		Pro ¹ (No)	CP ² (No)	FW ³ (g)	DW ⁴ (g)
A	0.0	0.9100 ^a	0.0000 ^c	3.2500 ^c	0.9615 ^c
E	0.5	0.9300 ^a	0.0000 ^c	2.7013 ^d	1.1212 ^b
I	1.0	0.8900 ^a	1.0000 ^b	4.2760 ^b	2.0017 ^a
M	2.0	1.0000 ^a	3.0000 ^a	5.2980 ^a	2.1492 ^a
Means		0.9325	1.0000	3.8813	1.5584

1- Pro = Proliferation; 2- CP = callus induction; 3- FW = fresh weight; 4- DW = dry weight.

Table 4. The interaction effect of 2,4-D, Kin and their concentrations on callus induction from nodal segments jojoba.

Media code	Treatments (mg l ⁻¹)		Nodal segments			
	2,4-D	Kin	Pro ¹ (No)	CP ² (No)	FW ³ (g)	DW ⁴ (g)
A	0.0	0.0	0.9100 ^a	0.0000 ^d	3.2500 ^c	0.9615 ^c
F		0.5	0.6700 ^b	0.6700 ^c	1.8960 ^f	0.9116 ^c
G	0.5	1.0	0.6700 ^b	2.0000 ^b	1.3580 ^g	0.7203 ^d
H		2.0	0.9300 ^a	2.0000 ^b	1.9033 ^f	1.0350 ^b
J		0.5	0.7600 ^b	1.0000 ^c	3.1627 ^c	1.2117 ^b
K	1.0	1.0	0.9000 ^a	1.0000 ^c	2.9073 ^d	1.2025 ^b
L		2.0	0.6700 ^b	2.0000 ^b	1.8320 ^f	0.9095 ^c
N		0.5	0.9200 ^a	2.0000 ^b	3.3067 ^b	0.9677 ^c
O	2.0	1.0	0.9000 ^a	2.0000 ^b	2.1953 ^e	1.0959 ^b
P		2.0	1.0000 ^a	3.0000 ^a	3.8573 ^a	2.1213 ^a
Means			0.8330	1.5670	2.5669	1.1137

1- Pro = Proliferation; 2- CP = callus induction; 3- FW = fresh weight; 4- DW = dry weight.

cultured on hormone free medium (control) showed slight response almost as weak as that achieved by those cultured on 0.5 mg l⁻¹ Kin medium. Thus, it can be concluded that Kin has indirect effect on callus formation. While, the interaction effect with 2,4-D auxin shows that it has indirect effect on the development and the proliferation of the explant for callus formation and production. These results are in agreement with those reported by Hamama et al. (2001) and Mohasseb et al. (2009b).

Results in Table 3 show that 2,4-D auxin has a clear effective role in the proliferation and development of the explant for callus induction, as it can be noted that 2,4-D containing media were better than auxin free control medium (A) for callus induction from the explant. It can be noted also, that increasing 2,4-D auxin concentration in the nutrient medium revises the extent of explant response towards proliferation and development for callus formation and production as the best results were achieved at the concentration of 2.00 mg l⁻¹ 2,4-D. This was confirmed by Gaber et al. 2007).

Despite the direct effect of 2,4-D on directing the explant towards callus induction, its interaction effect with

Kin (Table 4) shows the importance of adding cytokinins into the medium to achieve hormonal balance inside the explant. Table 4 shows that there is a significant interaction effect between Kin and 2,4-D, as the increased concentration of both Kin and 2,4-D raises the extent of nodal segments response towards proliferation, development, and callus induction. The data show that the best result for callus formation was achieved from the nutrient medium that contains the highest concentration of both 2,4-D (2.00 mg l⁻¹) and Kin (2.00 mg l⁻¹). The present results agreed with that of Abu (2000).

From the above, it can be concluded that hormonal balance between auxins and cytokinins supports the cultured explant for better development and growth than being cultured alone in one of them (Al-Ani et al., 2008; Mohasseb et al., 2009b).

Using balanced concentrations of auxins and cytokinins leads to achieving the best results and the most optimum growth rate of the explant, especially if the main goal of the research is to produce natural secondary products from the resultant callus. The interaction effect between Kin and 2,4-D, is the best results for phytochemical active constituents formation achieved from callus cultured on

medium (P). The balanced concentration of Kin and 2,4-D used for producing these natural products was 2.00 mg l^{-1} , as it produced triple the quantity produced from the callus cultured on control medium (A).

Phytochemical qualitative analysis by GC-MS

Induction of biochemical active constituents from jojoba callus

Data illustrated in Table 5a and b showed that there are two major functional divisions produced by induction of jojoba callus from different doses of plant growth regulator (PGR) [Kin and 2,4-D]. This can be called phytochemical active constituent.

Data presented in Table 5a revealed that, there is a direct relation between the development of callus and re-induction of phytochemical active constituents, which increase qualitatively by increasing callus development and 2,4-D hormone concentration at 2 mg l^{-1} for 1-hexadecanol, nonadecatriene, aristolene and tricosene, respectively as compared to free hormone treatment (Abu, 2000).

In addition, there is fluctuation in qualitative measurements in Kin treatment; it decreased under initial limit and then increased with increased concentration of Kin hormone in nonadecatriene and aristolene. There is a remarkable decrease in qualitative measurements of 7-hexadecenal and tricosene as compared to free hormone treatment (Agoramoorthy et al., 2007).

Both Kin and 2,4-D have the same significant effect on induced active constituents as they gave better results than those cultured on hormone free (control) medium (Table 6). It was noticed that increased Kin and 2,4-D concentration leads to increased active constituents induction from the explants at 2.00 mg l^{-1} , especially for 1-hexadecanol, nonadecatriene, aristolene and 7-hexadecenal. Also, the same trend of increasing value was observed in 1-hexadecanol constituent by increasing both hormones gradually from 0.5 mg l^{-1} (2,4-D) and 1.0 mg l^{-1} (Kin) up to 2.0 mg l^{-1} . On the other hand, the fraction of tricosane was not affected by increasing the concentration of both hormones. This was confirmed by Cheong et al. (2016) and Arya and Khan (2016).

From results in Table 5a, it can be concluded that there is a direct positive relationship between growth and development of callus and the resultant active constituents enhanced by PGR stress. These major active constituents are divided into five fractions: 1. 1-Hexadecanol which has the following biological activity: a composite medium used in the synthesis of other compounds (Turgumbayeva et al., 2015), has flavors and smells like composite simmondsin (Scrivner et al., 1984) and has luminous bacteria (Shimomura et al., 1974); 2. nonadecatriene compound acting as pheromone that attracts male insects (Nilsson, 2009); 3. Aristolene with


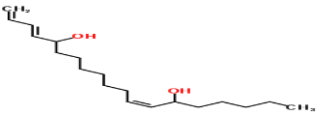
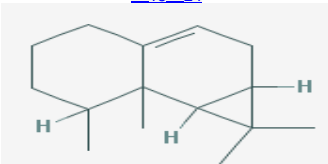
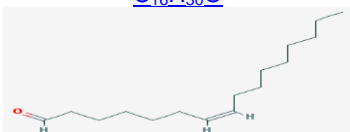
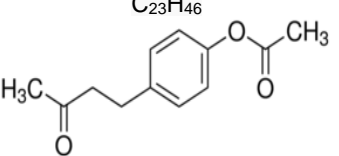
the following biological activity: has a smell that attracts predators and wolves (Scrivner et al., 1984), is an antibiotic bacterium, Bacillus, and is used for throat congestion (Zour), it is anti-inflammatory, the cradle of the nerves (Tian-Shung et al., 2004), pain reliever and has luminous bacteria (Shimomura et al., 1974); 4. 7-Hexadecenal with the following biological activity: used for treating patients with diabetes, by increasing the secretion of insulin in the body (Turgumbayeva et al., 2015), it is found in the liver of sheep and thus attractive to wolves and used in hunting wild animals (Scrivner et al., 1984), has Luminous Bacteria "Aldehydeless" Dark Mutant (Shimomura et al., 1974); 5. Tricosene which has kairomone effect (Ananthakrishnan et al., 1991).

From the data obtained in Table 5b and 6, it can be concluded that there is a direct positive relation between growth and development of callus and the resultant active constituents. Whereas using both Kin and 2,4-D has the same significant effect on active constituents induction as they gave better results than those cultured on hormone free (control medium).

Table 5b revealed that increasing cytokinins Kin concentration leads to slightly double increase in the active constituents from the explants clearly in case of triodecanoic acid and methoxyacetic. While, gradual increase of 2,4-D up to 2.0 mg l^{-1} caused increase in active constituents singly in case of triodecanoic acid, methoxyacetic and octadecanoic acid, respectively. On the other hand, decanoic acid has no effect by adding both plant growth regulators (Kin and 2,4-D, PGR) and also octadecanoic acid by adding Kin. While, data obtained from Table 6 showed that there is a significant effect in interaction between both PGR in growth medium from 0.5 up to 2.0 mg l^{-1} . Also, it can be mentioned from the data presented in Table 6, that all the fractionated active constituents decreased by half at 0.5 mg l^{-1} in both PGR.


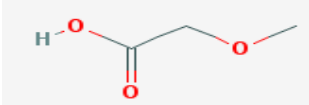


From results in Table 5b, it can be concluded that there is a direct positive relation between growth and development of callus and the resultant fatty acids activity enhanced by PGR stress. Therefore, we noticed that these major active constituents are divided into four fractions: 1. Triodecanoic acid, which has the following biological activity: it prevents the effect of bacteria and fungi fat, and has a role in getting rid of harmful pollutants, tolerant to salinity and so is grown on the banks of canals, used to get rid of contaminants (Agoramoorthy et al., 2007), used for the analysis of fat, and stimulates the working of lipase enzyme, which analyzes the cell walls of microbes (Enig, 2004); 2. Methoxyacetic acid with the following acid: it is used against harmful animals like mice (toxic baits), that inhibit fertilization processes and thus prevent the breeding and spread of these dangerous animals (Shannon et al., 1983); 3. Decanoic acid which is used in the manufacture of insect repellents; 4. Octadecanoic acid used for the treatment of bulges (Rampage stomach), aids digestion

Table 5a. Qualitative phytochemical analysis for active constituents form Jojoba (*Simmondsia chinensis* (Link) Schneider) callus under the specific effect of Kin and 2,4-D.

Active constituents	Hormone (mg l ⁻¹)	Media code				Chemical Structure	Properties of active constituents	
		0.0	0.5	1.0	2.0		Biological activities	Reference no.
1-Hexadecanol	Kin	++	++	++	++	$C_{16}H_{34}O$ 	<ul style="list-style-type: none"> - Composite medial enters in the synthesis of other compounds. - Enters the Flavors and smell like simmondsin Composite. - Luminous bacteria. 	Turgumbayeva et al., 2015 Scrivner et al., 1984 Shimomura et al., 1974
	2,4-D	++	++	++	+++			
Nonadecatriene	Kin	++	+	+	++	$C_{19}H_{34}O_2$ 	<ul style="list-style-type: none"> - Pheromone attracts male insects (Dynamic combat). 	Nilsson, 2009
	2,4-D	++	++	++	+++			
Aristolene	Kin	++	+	+	++	$C_{15}H_{24}$ 	<ul style="list-style-type: none"> - Has a smell attractant for predators, where the sheep so they are attractive to the wolves and predators. - Antibiotic bacteria Bacillus, and is used for congestion-Throat (Zour). - Anti-inflammatory, and the cradle of the nerves, and pain reliever. - Luminous bacteria. 	Scrivner et al., 1984 Tian-Shung et al., 2004) Tian-Shung et al., 2004 Shimomura et al., 1974
	2,4-D	++	++	++	+++			
7-Hexadecenal	Kin	++++	+	+	++	$C_{16}H_{30}O$ 	<ul style="list-style-type: none"> - The treatment of patients with diabetes, where increases the secretion of insulin in the body (Nutritional supplement). - There in the liver of sheep and attractive to the wolves, so it can be used in hunting wild animals. - Luminous Bacteria "Aldehydeless" Dark Mutant. 	Turgumbayeva et al., 2015 Scrivner et al., 1984 Shimomura et al., 1974
	2,4-D	++++	++	++	+++			
Tricosene	Kin	++++	+	+	++	$C_{23}H_{46}$ 	<ul style="list-style-type: none"> - Kairomone Effect. 	Ananthakrishnan et al., 1991
	2,4-D	++++	++++	++++	+++++			

- (+) = One portion, (++) = two portion, (+++++) = six portion of constituents.

Table 5b. Qualitative phytochemical analysis for active constituents from Jojoba (*S. chinensis* (Link) Schneider) callus under the specific effect of Kin and 2,4-D.

Active constituents	Hormone (mg l ⁻¹)	Media code				Chemical structure	Properties of active constituents		References
		0.0	0.5	1.0	2.0		Biological activities		
Triodecanoic acid	Kin	++	++	++	++++	 C ₁₃ H ₂₆ O ₂	<ul style="list-style-type: none"> - Saturated with him the perverse effect of bacteria and fungi fat. - Has a role in getting rid of harmful pollutants tolerant to salinity (found in mangrove) so is grown on the banks of canals and to get rid of contaminants. - Effective material for the analysis of fat. - Stimulate the working of lipase enzyme, which analyzes the cell walls of microbes. 	Agoramoorthy et al., 2007 Agoramoorthy et al., 2007 Enig, 2004 Enig, 2004	
	2,4-D	++	++	++	+++				 C ₃ H ₆ O ₃
Decanoic acid	Kin	++	+	+	++	 C ₁₀ H ₂₀ O ₂	<ul style="list-style-type: none"> - Used in the manufacture of materials insect repellents. - Used in organic synthesis and industrially in the manufacture of perfumes, lubricants, greases, rubber, dyes, plastics, food additives and pharmaceuticals. 		
	2,4-D	++	++	++	++				 C ₁₀ H ₂₀ O ₂
Octadecanoic acid	2,4-D	++	++	++	+++				

- (+) = One portion, (++) = two portion, (++++++) = six portion of constituents.

and prevents fermentation (Thrombosis), aids clots, does not lead to high cholesterol, and gets rid of (LDL) low molecular weight lipids that cause atherosclerosis (Tvrzicka et al., 2011).

Active constituents from jojoba (*Simmondsia chinensis* [Link] Schneider)

The physical properties of jojoba oil have high stability and low volatility. Its composition is less affected by temperatures up to 300°C. Jojoba oil

contains straight-chained C20 and C22 fatty acids and alcohols and two unsaturated bonds, which make the oil susceptible to many different types of chemical manipulations. Fatty Acids present in jojoba oil according to Busson-Breyse et al., (1994), is seen in this site https://en.wikipedia.org/wiki/Jojoba_oil. The fatty acids are Lignoceric acid (C24:0), and Nervonic acid (C24:1). The oil can be used as an antifoam agent in antibiotics production and as a treatment for skin disorders. Other proposed uses include

candles, plasticizers, detergents, fire retardants, transformer oil, and for the leather industry (Undersander et al., 1990).

Biogenetically, phenolic compounds proceed from two metabolic pathways: the shikimic acid pathway where, mainly, phenylpropanoids are formed and the acetic acid pathway, in which the main products are the simple phenol (Sánchez-Moreno, 2002). Most plants phenolic compounds are synthesized through the phenylpropanoid pathway (Hollman, 2001). The combination of

Table 6. Evaluate of phytochemical active constituents form by Kin and 2,4-D interaction up to 2 mg l⁻¹ stress in Jojoba (*S. chinensis* (Link) Schneider) callus.

Media code	Treatments (mg l ⁻¹)		Phytochemical active constituents								
	2,4-D	Kin	1-Hexadecanol	Nonadecatriene	Aristolene	7-Hexadecenal	Tricosene	Triodecanoic acid	Methoxyacetic acid	Decanoic acid	Octadecanoic acid
A	0.0	0.0	++	++	++	++++	++++	++	++	++	++
F		0.5	+	+	+	+	++	+	+	+	+
G	0.5	1.0	++++	++	++	++	++++	++	++	+++++	++
H		2.0	++++	++	++	++++	++++	++	++	+++++	++
J		0.5	+++	++	++	++++	+++	++	++	+++++	++
K	1.0	1.0	++++	++	++	++++	++++	++	++	+++++	++
L		2.0	++++	++	++	++++	++++	++	++	+++++	++
N		0.5	++++	++	++	++++	++++	++	++	+++++	++
O	2.0	1.0	++++	++	++	++++	++++	++	++	+++++	++
P		2.0	++++	+++	+++	+++++	++++	+++	+++	+++++	+++++

- (+) = One portion, (++) = two portion, (+++++) = six portion of constituents.

both pathways leads to the formation of flavonoids, the highest group of phenolic compounds in nature (Sánchez-Moreno, 2002). Additionally, through the biosynthetic pathways to the flavonoids synthesis, among the not well-elucidated condensation and polymerization phases, the condensed tannins or non-hydrolysable tannins and glycosides are formed. Hydrolysable tannins are derivatives of gallic acid or hexahydroxydiphenic acid (Stafford, 1983).

The result of the present study further revealed that jojoba simmondsin content increased quantitatively with the increase in callus from main to the fourth weeks of cultivation. The percentage of browning and necrosis is also in conformity with increase in fatty acids content in old culture. The increase in simmondsin content is normally associated with increase in the enzymes that regulate the synthesis of fatty acids compound, while the intensity of browning is related with the hyperactivity of oxidative enzymes (Cochrane, 1994). This compound has also shown a very

strong antibacterial activity against bacteria and was detected only in jojoba oil in the present study. The GC-MS analysis showed the presence of forty-eight compounds in the leaves of *B. Cylindrica* by comparing their retention times and by interpretation of their mass spectra. The compounds identified and their retention time, molecular formula, and concentration (peak area %) are presented in Table 5a and b.

It should be noted that the obtained chromatograms represent not only the fatty acids and oil but also all the compounds in the extracts of each treatment by gas chromatography-mass spectroscopy (GC-MS) analysis (Table 5a). In this study, various secondary metabolites were detected within jojoba callus. We have described the biological activity of certain metabolites and compared the callus based on the effect of different growth regulators on produced metabolites. Many of the important compounds were produced by jojoba callus. Some of them were not detected in any of the other species, for

instance 3-Triodecanoic acid. This compound is a derivative of jojoba oil octane, which has a wide variety of biological activities and has a substantial importance as a structural fragment of a synthetic pharmaceutical compound (Table 5b).

Eventually, it can be concluded that there is a direct proportion between callus growth and active constituents' induction in general as growth and development of cells and tissues contribute greatly to production, of the active constituents from the explant. In addition, auxins play more role than that of cytokinins for callus induction and subsequent active constituents production from the explants.

Conclusion

Some secondary metabolites are involved in the complex interactions between hormones added in tissue culture media and their growth conditions. Thus, analytical methods for the identification of

criteria like mass-spectral factor, the application of this method shows a wide variety of major metabolites in jojoba callus from different explants. According to the trait of the compounds described in this study, we suggest the usage of complementary methods for purification of jojoba callus compounds, due to their application in agriculture as biological control agents of plant pathogens, or in medicine as sources of antimicrobial substances.

Conflict of interests

The authors have not declared any conflict of interests.

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