Determination and quantification of bonediol contend in callus and cell suspension cultures of Bonellia macrocarpa

Cecilia Castro-Martínez
Tecnologico Nacional de Mexico/Tuxtla Gutiérrez
Carlos Alberto Lecona Guzmán (leconaguzmancarlos@gmail.com)
Tecnológico Nacional de México/Tuxtla Gutiérrez https://orcid.org/0000-0003-3382-8181
María Celina Luján-Hidalgo
Tecnologico Nacional de Mexico/Tuxtla Gutiérrez
Nancy Ruiz-Lau
CONACYT/TECNM/Tuxtla Gutiérrez
Lucia M. C. Ventura-Canseco
Tecnologico Nacional de Mexico/Tuxtla Gutiérrez
María del Carmen Silverio Gómez
INIFAP CIRGOC CE Huimanguillo: Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias Campo Experimental Huimanguillo
Federico A. Gutiérrez-Miceli
Tecnológico Nacional de México/Tuxtla Gutiérrez

Research Article

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Abstract

Plant growth regulators naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-Benzylamino purine (BAP) were evaluated at different concentrations (0, 0.5, 1.0, and 2.0 mgL^{-l}) in three different explants (leaf, stem, and root) on the induction of responses morphogenic. In the suspension cell culture establishment, callus obtained from the stem were used and two concentrations of coconut water (20 and 40% v/v) were evaluated to increase the disintegration of the cell suspension. Bonediol was quantified using the HPLC technique in callus obtained from different explants (leaf, stem, and root) and suspension cell cultures. Higher callus formation was obtained in stem explants, with BAP at 1.0 mgL^{-l} indirect organogenesis was obtained and with 2,4-D at 0.5 and 1.0 mgL^{-l} somatic embryogenesis. Bonediol concentration detected was 49.57 mg/g callus, derived from stem explant. During the cultivation of cells in suspension, greater disintegration of the compact calli was observed with 20% coconut water. Bonediol concentration in suspension cell cultures was 24.44 mg/g dry cell. The objective of this work was to evaluate the effect of growth regulators on callus formation as well as the quantification and determination of bonediol content in callus and suspension cells of *Bonellia macrocarpa*.

Key Message

In the present work a quantitative estimation of the production of bonediol were carried out through callus and cells in suspension obtained from different explants of *Bonellia macrocarpa*.

Introduction

Bonellia macrocarpa (Cavanilles) Ståhl and Källersjö is a plant that has generated interest because of the cytotoxic activity connected with stem and root bark extracts (Caamal-Fuentes et al. 2011). Bonediol is a secondary metabolite extracted from *B. macrocarpa* root that induce inhibition of prostate cancer cells from humans. Therefore, bonediol could serve as a chemopreventive treatment with therapeutic potential against prostate cancer (Mooc-Puc et al. 2015). The use of wild plants causes a risk of overexploitation because of secondary metabolites of interest extraction. An alternative for producing secondary metabolites is the callus and suspension cell cultures because they can produce bioactive compounds (Chandran et al. 2020). To achieve callus inducted from different types of explants, the use of plant growth regulators is needed. Auxins and cytosines have been used to regulate the growth and organization of plant tissue cultures and consequently callus induction. The objective of this work was to evaluate the effect of growth regulators on callus formation as well as the quantification and determination of bonediol content in callus and suspension cells of *Bonellia macrocarpa*.

Methodology

Plant material

Bonellia macrocarpa plantlets were obtained after in vitro seed germination in MS medium (Murashige and Skoog 1962) supplemented with 2.0 mg/L of Gibberellic Acid (GA_3). Leaf, stem, and root explants were obtained from these plantlets and placed in a semi-solid MS medium supplemented with 30.0 g/L of sucrose and 2.5 g/L of phytagel.

Morphogenic Induction

Naphthaleneacetic Acid (NAA), 2,4-Dichlorophenoxyacetic Acid (2,4-D) and 6-Benzylamino Purine (BAP) were evaluated at different concentrations (0.5, 1.0 and 2.0 mgL^{-1}) for induction of morphogenic responses (Table 1). The culture conditions were a photoperiod of 16/8 h light/darkness in a growth chamber at 24 ± 2°C. The response variables were the percentage of callus formation, embryo numbers, and organogenesis evaluated during a period of 60 days. The callus formation index (CFI) was obtained with the following formula reported by Martínez-Silvestre et al. (2022).

$CFI=(nG)/N \times 100$

Where:

n = Total callus formed

G = Average callus score

N = Total number of cultured explants

For biomass proliferation, subcultures were performed in semi-solid MS medium supplemented with 2,4-D 1.0 mgL^{-1} and 1.0 mgL^{-1} TDZ (Thidiazuron), citric acid 150 mgL^{-1}, ascorbic acid 300 mgL^{-1} and activated carbon 0.25 gL^{-1}, sucrose 30 gL^{-1}, and phytagel 2.5 gL^{-1}. Subcultures were performed every 30 days until sufficient biomass was obtained for the later experiments.

Suspension cell culture

Suspension cell culture establishment was conducted using compact calli induced from stem explants, and two concentrations of coconut water (20 and 40% v/v) were evaluated for callus disintegration. Approximately 0.5 g of callus were inoculated in 50 mL of liquid MS medium added with 2,4-D (2.0 mgL^{-1}), 300 mgL^{-1} ascorbic acid, 300 mgL^{-1} of cysteine, and 150 mgL^{-1} citric acid in Erlenmeyer flasks 250 mL and maintained under orbital agitation of 110 rpm. The culture medium was renewed every 15 days for three months.

Quantification of bonediol by high-performance liquid chromatography (HPLC)

For bonediol quantification in HPLC, the methodology by Ruíz-Ramírez et al. (2018) was followed, making some modifications. Callus from different sources of explants (leaf, stem, and root) were lyophilized (LABCONCO freeze-dryer) at -40°C for 24 h. After 250 mg of each of the tissues lyophilized were weighed

and 37.5 mL of methanol were added respectively. The essays were conducted with three biological replicates. The cells in suspension were lyophilized (LABCONCO lyophilizer) at -40°C for 24 h. Then, 50 mg of lyophilized cells were weighed, and 7.5 mL of methanol was added. The procedure was in duplicate. Then, all the samples were sonicated (Cole-Parmer sonicator) for 5 minutes and macerated for 24 h. The samples were then filtered, and the rotary evaporated under vacuum at 45 ± 1°C. The dry extract was resuspended in HPLC-grade methanol (4 mL were used for callus extracts and 1 mL for cells in suspension) then all samples were refrigerated. Before secondary metabolites quantification, extracts were centrifuged at 10,000 rpm for 10 minutes at 4°C.

For quantification, a bonediol standard curve was used Ruíz-Ramírez et al. (2018). The Flexar model HPLC equipment was used with a Zorbax ODS column (5 µm X 250 mm X 4.6 mm). For the phases, deionized water was used adding 0.05% formic acid (A) and acetonitrile with 0.05% formic acid 60/40 (v/v) as mobile phases (B). It was started with 30% A, then changed from 30 to 5% A in 5 min and 5% A constantly for 15 min at a flow rate of 0.8 mL/min. Detection was carried out at 260 nm. 20 µL of the sample was injected and the column was stored at room temperature (23°C).

Statistic analysis

All experiments were done under a completely randomized experimental design and three replications for each experimental unit. Simple ANOVA analysis was carried out. Their means were compared using the LSD test ($p < 0.05$) with the help of the statistical software Statgraphics Centurion XVII.

Results And Discussion

Callus formation index, shoot, and embryo number obtained with different explant of Bonellia macrocarpa

The growth regulators used in different concentrations induced different morphogenic responses: callogenesis, organogenesis, and somatic embryogenesis depending on the explant used (root, stem, and leaf). 2,4-D at concentrations of 0.5 and 1.0 mg^{-l} L induced the highest callogenic response when root was used as explant, while at a concentration of 2.0 mgL^{-l} it promoted callogenesis when leaf was used as explant. Regarding the use of ANA and BAP at a concentration of 2.0 mgL^{-l}, both regulators promoted callus formation with a higher percentage when the stem was used as an explant (Table 1). At a concentration of 1.0 mgL^{-l} of BAP, an organogenic response was obtained with the highest number of shoots per explant at 60 days. Treatments with NAA and 2,4-D did not generate shoot formation. However, 2,4-D favored the formation of embryos at 60 days in the globular state in root explants (Table 1).

The response caused by BAP is a result that this regulator is a cytokinin and this type of hormone promotes cell division and differentiation, which is, it initiates directly process of bud formation and multiplication (Van Staden et al. 2008). The molecular mechanism of the cytokinin-induced (BAP)-

induced shoot generation process in *B. macrocarpa* is still unknown, but previous studies have reported results that could explain the genetic events involved in the formation of shoots in plant cultures *in vitro*. It has been reported that a key event for this generation of cytokinin-induced budding (BAP) is the activation of the WUS gene. However, cytokinin signaling activation alone is not sufficient to induce WUS (Zhang et al. 2017). Therefore, the participation of HD Zip III transcription factors phabulosa (PHB), phavoluta (PHV), and revoluta (REV) act as necessary regulators for the induction of WUS independent of cytokinins for the subsequent formation of buds (Zhang et al. 2017). In addition, HD ZIP III interacts with type B ARRs (Zhang et al. 2017), as well as other regulatory factors STM (Shi et al. 2016) and AP2/ERF RAP2.6L (Che et al. 2007; Yang et al. 2018) that are of importance for shoot formation.

The results obtained in the formation of callogenesis in the different types of explants in *Bonellia macrocarpa* could be explained by the fact that there are different concentrations of endogenous regulators in the different parts of the plant and the complementary effect with the exogenous regulators induces callogenesis. According to (Gaspar et al. 1996), they mention that endogenous regulators interact with exogenous regulators, so the biological activity of exogenous regulators can be equivalent or superior to endogenous regulators. Interaction with endogenous regulators is specific, and cell and tissue responses are highly-dependent on plant species and explant sources. In *B. macrocarpa* no endogenous quantification studies of regulators have been carried out, which warrants further studies. I could be a consequence of the use of NAA and 2,4-D auxins regulators that play the role of cell elongation, cell division, and callus formation in culture media. The molecular mechanism in callus induction in *B. macrocarpa* is still unknown, but previous studies carried out in *Arabidopsis* plants explain how auxins induce callogenesis. Auxin signaling is transduced by transcription factors (ARF 7 and 19) (Fukaki et al. 2005), to activate (LBD 16, 18, and 29) to promote callus formation (Okushima et al. 2007; Fan et al. 2012). LBDs activate transcription factors such as (E2FA) (Berckmans et al. 2011); (EXP14) (Lee et al. 2013); (FAD-BD) and (PME2) (Xu et al. 2018). These LBDs regulate cellular processes associated with root initiation or callus formation. This molecular mechanism could be occurring in the formation of callus in *B. macrocarpa*.

When root was used as an explant, cream-colored compact calli were obtained (Fig. 1A). While, in explants of stem and leaf, the callus was compact and yellow in color (Fig. 1B, C). Callogenesis formation was observed 30 days after the explants were placed in the culture medium. Oxidation was observed in the callus from the beginning of the induction. In Fig. 1, photographs with calluses of compact texture are observed. In stem explants BAP induced the formation of shoots while 2,4-D induced the formation of somatic embryo in the globular stage.

In *Bonellia macrocarpa* we observed that the degree of compaction of the calluses can vary depending on the type of plant used, the composition of the medium, growth regulators, and the environmental conditions of the crop. In addition, the compact texture of calluses is due to the ability of plant tissue to absorb nutrients and growth regulators in the culture medium. It was observed that the root calluses were white, while the leaf and stem explants had a yellow color. The coloration of the calluses can be due to

various factors, such as pigmentation, light intensity, and the explant source of the different parts of the plants.

The BAP regulator induced green organogenic callus after 30 days, this event occurs because the callus shows chlorophyll formation. According to Sugiyarto et al. (2014) and Sari et al. (2019) state that this color is the result of the influence of cytokinins on the formation of chlorophyll. In addition, the generation of shoots is observed 60 days after induction, using stems as a source of explant (Fig. 1D). With the 2,4-D regulator, using concentrations of 0.5 and 1.0 mg/L, the greatest formation of somatic embryogenesis was obtained, using root as an explant (Fig. 1E). While NAA and BAP did not favor responses of somatic embryogenesis (Table 1). The result obtained in the formation of embryogenesis is because the 2,4-D regulator is one of the exogenous auxins used in the induction of somatic embryogenesis (Jiménez 2001). *Arabidopsis thaliana* has been used as a model to understand the molecular mechanisms and genes involved in the process of somatic embryogenesis. According to Tsuwamoto et al. (2010), among the responsible genes related to the competition for embryogenesis, are the LEAFY COTYLEDON genes, (LEC1) (Lotan et al. 1998) and LEC2 (Stone et al. 2008), WUSCHEL (WUS) (Zuo et al. 2002), BABY BOOM (BBM) (Boutilier et al. 2002), AGAMOUS-LIKE-15 (AGL15) (Harding et al. 2003) and AINTEGUMENTA-LIKE5/PLETHORA 5/EMBRIOMAKER (AIL5/PTL5/ EMK). These are the genes that encode transcription factors, and when overexpressed they promote somatic embryogenesis. These genes could be participating in the somatic embryo formation response in root explants of *B. macrocarpa*; however, more specific studies are needed to confirm this. In addition, there are factors such as regulators (endogenous to the explant) that influence metabolic processes and genetic regulation that are involved in this morphogenic response.

The use of 2,4-D favored the formation of embryos in the globular stage when the root was used as an explant. The generation of the embryos occurred 60 days after the explants were placed in the culture medium (Fig. 1E).

Quantification of bonediol in callus from different explants (root, stem, and leaf)

Callus from explants (root, stem, and leaf) were analyzed for bonediol quantification in HPLC. The concentration of bonediol present in the callus, coming from the stem as an explant source, was 49.57 mg bonediol/g callus. However, in the leaf and root calluses, the presence of bonediol could not be confirmed (Table 2 and Fig. 2).

Similar studies regarding the quantification of bonediol have been carried out in *Bonellia* root in wild plants (Caamal-Fuentes et al. 2011), and recently in transformed roots (Ruíz-Ramírez et al. 2018). The presence of bonediol was confirmed only in callus from stem explants. It is important to perform further studies on the biosynthesis in each part of the *B. macrocarpa* plant and if there is the translocation of this secondary metabolite because there are no reports on the biosynthetic route of bonediol. One of the strategies for understanding the biosynthesis pathway is using proteomic techniques. These techniques allow investigating the genes and enzymes are involved in the biosynthesis of active compounds in

plants (Yang et al. 2021). On the other hand, the importance of this work was to verify the presence of bonediol in calluses. The concentration of bonediol in callus from stems was higher than in transformed root, obtaining a content which was 49.57 mg bonediol/g dry callus, a concentration 17.8 times higher, compared to the content of 2.78 mg bonediol/g dry weight of transformed roots.

Quantification of bonediol in suspension cell cultures

In cells cultured suspension (Fig. 1F), it was possible to detect the metabolite of interest with 24.44 mg bonediol/g dry cells in HPLC, being the first identification of bonediol in cells in suspension. These cell suspensions are made up of meristematic cells, which is, undifferentiated cells, which have the characteristic of not separating after division and form aggregates of different sizes and shapes (Meyer et al. 2002). The tendency of these cells to aggregate is regulated by the cohesiveness of the cell wall and allows cell-cell communication, which can favor the transport of intermediates, necessary for the biosynthesis of secondary metabolites. The content of bonediol in cells is important for the upscaling of this secondary metabolite that various strategies can be used for the increase of bonediol employing several approaches to develop the production of the desired natural products, such as the selection of high-producing cell lines, optimization of the conditions of the culture medium, addition of elicitors or precursors and metabolic engineering (Yue et al. 2014). Suspension cell culture remains one of the most widely used techniques for the production of secondary metabolites. Therefore, it is important to clarify the biosynthetic route of bonediol to regulate the biosynthetic process, as well as to combine strategies such as the addition of inducers, precursors to improve the content of secondary metabolites (bonediol), in addition to seeking an adequate selection in the use of bioreactors for the scale of this secondary metabolite (Yue et al. 2014).

Conclusion

Different responses were found concerning to the different types and concentrations of regulators in *Bonellia macrocarpa* explants. The highest percentage of callogenesis was obtained from stem explants with the regulator NAA and BAP at a concentration of 2.0 mgL^{-1}. BAP at a concentration of 1.0 mgL^{-1} induced the highest shoot response from stem explants. Somatic embryogenesis was obtained with 0.5 and 1.0 mgL^{-1} of 2,4-D using root explant. Bonediol was present in the callus from the stem as a source of explant with a concentration of 49.57 mg/g dry weight of callus. The establishment of suspension cell culture showed greater disintegration with 20% coconut water and bonediol concentration was 24.44 mg/g dry cells

Declarations

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Author Contributions: CCM, CALG designed and supervised the experiment. CCM and MCLH performed the experiments. NRL and LMCVC oversaw experiment, MCSG analyzed data. MCSG, FAGM and CALG revised and finalized the manuscript.

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Tables

Table 1 Callus formation index, shoot and embryo number obtained with different explant of *Bonellia macrocarpa* incubated in MS medium supplemented with Naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and Benzylaminopurine (BAP).

	Callus formation index			Shoot number	Embryo number
Plant growth regulator [mgL^{-1}]/explant	Root	Stem	Leaves	Stem explant	Root explant
Control	$0±0^{c}$	$0±0^{d}$	$0±0^{b}$	$0.0±0^{b}$	$0.0±0^{c}$
NAA 0.5	$0±0^{c}$	$33±8^{bc}$	$0±0^{b}$	$0.0±0^{b}$	$0.0±0^{c}$
1.0	$25±14^{abc}$	$33±8^{bc}$	$0±0^{b}$	$0.0±0^{b}$	$0.0±0^{c}$
2.0	$25±14^{abc}$	$75±14^{a}$	$0±0^{b}$	$0.0±0^{b}$	$0.0±0^{c}$
BAP 0.5	$25±14^{abc}$	$33±8^{bc}$	$0±0^{b}$	$5.0±2.5^{a}$	$0.0±0^{c}$
1.0	$33±22^{abc}$	$17±6^{cd}$	$0±0^{b}$	$6.3±0.8^{a}$	$0.0±0^{c}$
2.0	$17±8^{bc}$	$75±10^{a}$	$0±0^{b}$	$5.66±0.8^{a}$	$0.0±0^{c}$
2,4-D 0.5	$58±8^{a}$	$42±17^{bc}$	$0±0^{b}$	$0.0±0^{b}$	$28.33±5.36^{a}$
1.0	$50±14^{ab}$	$58±8^{ab}$	$0±0^{b}$	$0.0±0^{b}$	$33.33±4.97^{a}$
2.0	$25±14^{abc}$	$50±14^{ab}$	$50±10^{a}$	$0.0 ±0^{b}$	$17±2.08^{b}$
LSD ($P<0.05$)	26	28	15	2.9	13.2606
The same letters mean that there is no statistically significant difference $p<0.05$ according to the least significant difference (LSD).					

Table 2 Quantification of bonediol in callus of *Bonellia macrocarpa*.

Callus from explants	mg bonediol/g dry callus
Root	Not detected
Steam	49.57
Leaf	Not detected

Figures

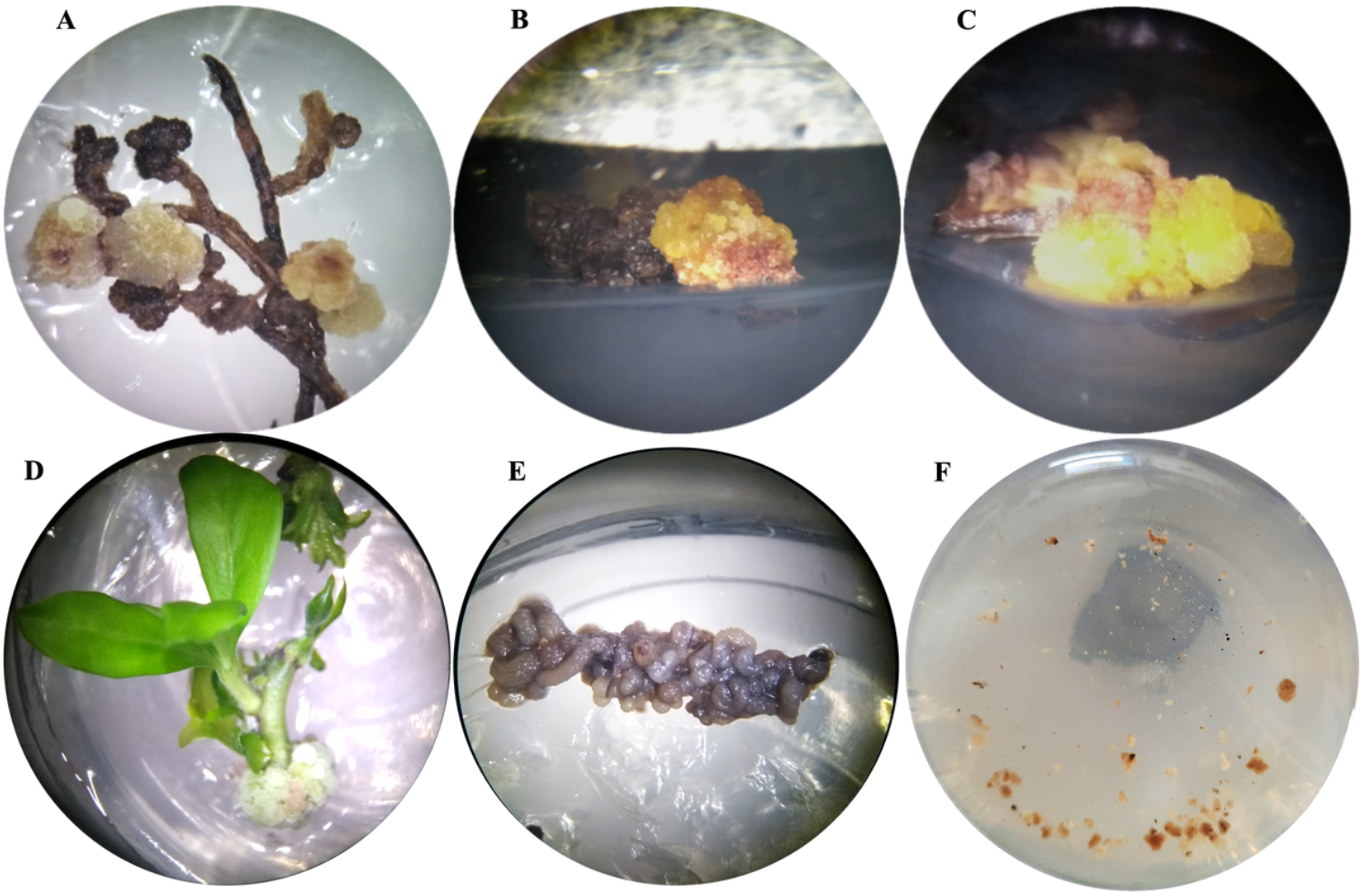

Figure 1

Effect of regulators 30 days after induction. A) 2,4-D effect of 1.0 mgL^{-1} in root explant, B) Callus formation in stem explant with NAA 2 mgL^{-1}, C) Effect of 2,4-D with 2.0 mgL^{-1} in leaf explant, D) Shoots of *Bonellia macrocarpa* in stem explant with 1.0 mgL^{-1} BAP, E) Embryos somatic in roots explant in medium MS supplemented with 1.0 mgL^{-1} 2,4-D, F) cell suspension from stem explant in liquid MS medium supplemented with 20% coconut water, 2.0 mgL^{-1} 2,4-D, 300 mgL^{-1} ascorbic acid, 300 mgL^{-1} cysteine and 150 mgL^{-1} citric acid.

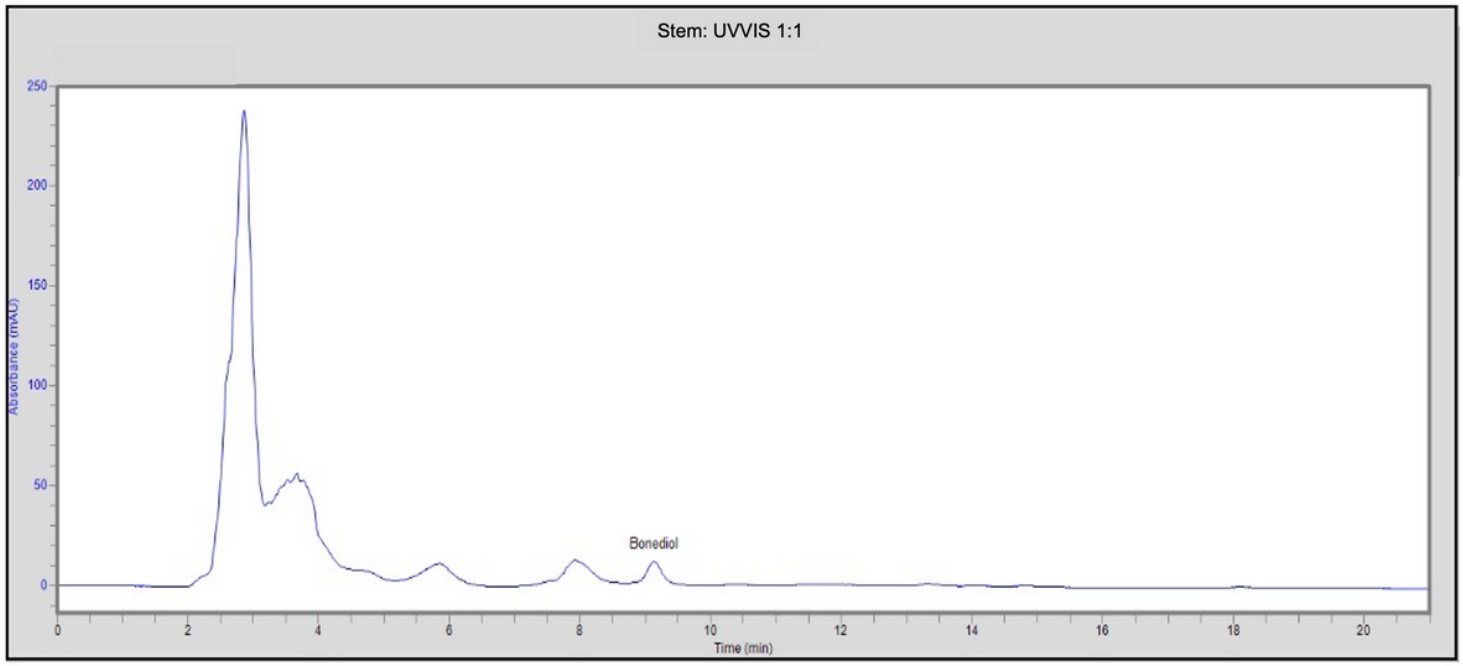

Figure 2

Chromatogram of callus extract obtained from *Bonellia macrocarp*a stem explants.