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Callus Production and Analysis of Isoflavonoids and Some Terpenes from *Bituminaria bituminosa*

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ABSTRACT

Background: *Bituminaria bituminosa* is a long-day plant. The plant has a high pastoral value and palatability. It is grazed by cattle, goats and sheep. Leaves and stems contain four furanocoumarins' psoralen, angelicin, xanthotoxin and bergapten. The seed is covered by the fruit. Germination was not observed in beaked fruits. A cut with a scalpel can increase the germination rate. According to the literature there are not enough studies about the culture media and convenient conditions.

Methods: In our study, we tried six medium for callus production with different hormone concentrations including 2,4-D, BAP and kinetin. In this study, a protocol for callus induction and analysis of isoflavonoids and terpenes of *Bituminaria bituminosa* has been developed.

Result: Maximum callus induction frequency was observed on Murashige and Skoog (MS) medium supplemented with 0.75 mg/L 2,4-D + 2.5 mg/L kinetin in young primary leaves. 0.75 mg/L 2,4-D+2.5 mg/L kinetin medium is more successful in the production ofses of flavonoids that can be produced in vitro and is more successful in producing phenols. In terpene production, it is seen that 1.25 mg/L 2,4-D+3.5 mg/L BAP, which is the best callus producing medium containing BAP, is the most successful medium among all tested mediums.

Key words: *Bituminaria bituminosa,* Callus, Isoflavonoids, Terpens.

INTRODUCTION

Bituminaria bituminosa is traditionally used for feeding goats in the Canary Islands (Spain) (Davis 1970; Stirton 1981; Sternberg *et al.,* 2006). It tolerates drought conditions and is known for its heavy metal-hytostabilization capacity in contaminated or degraded soils (Walker *et al.,* 2014; Tava *et al.,* 2007). Thus, the presence of toxic compounds in this plant can limit its use and must be considered. In Madeira Island, it is commonly used in folk medicine as a decoction with alcohol and iodine and applied externally for hair restoration. The infusion from fresh leaves is also used to treat fever and urinary infections (Llorent-Martínez *et al.,* 2015).

B.bituminosa have nearly 150 secondary metabolite. These bioactive compounds were flavonoids, coumarins, furanocoumarins, chalcones, quinines, terpenoids. These species exhibit significant anti-oxidant, anti-bacterial, anti-fungal, anti-viral, anti-helmintic, anti-diabetic, diuretic, hepatoprotective, anti-cancer and anti-tumor activities (Bouque *et al.,* 1998; Tava *et al.,* 2007; Azzouzi *et al.,* 2014; Llorent-Martínez *et al.,* 2015; Koul *et al.,* 2019; Tram *et al.,* 2021; Florvil *et al.,* 2022). Angelicin and psoralen protect against infection and herbivory and phototoxic subtance in *B.bituminosa* (Foresto *et al.,* 2021). The effects on angelicin and psoralen accumulation of the exposure of two populations of *B. bituminosa* to abiotic stress (cold, heat and drought) under field conditions were determined in the leaf and fruits (Walker *et al.,* 2010; Walker *et al.,* 2012).

The clastogenic activity of Mitomycin C (MMC) was significantly reduced by both erybraedin C and bitucarpin A. They were obtained from *B.bituminosa* and chemically characterised, they were used either a pre-incubation schedule or simultaneous treatment. These results suggest that the protective effects displayed by the two anti-clastogenic compounds against MMC could be due to the induction or inhibition of cellular reductive metabolic enzymes (Maurich *et al.,* 2004; Maurich *et al.,* 2006). Juan *et al.* (2003, 2004) has confirmed the self-pollinated breeding system of *B. bituminosa*. The pterocarpans bitucarpin A and B can be isolated from the aerial parts of *B. bituminosa* (Pistelli 2003). It has several potential uses such as; forage crop, phytostabilisation of heavy metal contaminated or degraded soils, synthesis of furanocoumarins (psoralen, angelicin, xanthotoxin and bergapten), compounds of broad

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pharmaceutical interest. Angelicin and psoralenused in suntan products and for photochemotherapy of vitiligo and psoriasis (Tava *et al*., 2007). The furanocoumarins psoralen and angelicin which act as repellents and feding deterrents and also are commercial interest due to the iruse in cosmetics and photochemotherapy. Terpens have similar properties, too (Atar and Çölgeçen 2013; Çölgeçen *et al.,* 2018).

Aucubin, catalpol and verbascoside a glycoside iridoid, is a class of monoterpenoids and the most common iridoid glycoside is aucubin. It has been revealed through different studies that aucubin could be a useful agent in the prevention of pancreatic cancer. In our literature search, aucubin, catalpol and verbascoside have never been studied before in *B.bituminosa*. The aim of the present study was to investigate and analysis the production of callus and secondary metabolites from *B. bituminosa* herba and *in vitro* culture.

MATERIALS AND METHODS

Plant material

The seed is covered by the fruit (fruits beaked). Fruits of *B. bituminosa* were collected from Zonguldak Bulent Ecevit University, Science and Art Faculty, Department of Biology experiment garden in August 2020. Fruits were sterilizated with 10% sodium hypochlorite solution for 20 minutes. Then fruits were rinsed three times with distile water. Beaked fruits and cut-beaked fruits were germinated in glass jars (100 mm × 200 mm) on hormone-free MS medium (Murashige and Skoog 1962) (Fig 1). Samples were incubated at 22-24°C with a 16/8 hour photoperiod.

In vitro callus culture

In vitro aseptically 15-day-old seedling were excised into hypocotyl (0.5-1 cm), cotyledon (0.5-1 cm), apical meristem (1-2 mm), epicotyl (0.5-1 cm) and young primary leaves (0.5-1 cm). The explants were cultured in Petri dishes (100×200×20 mm) (Fig 1). MS media containing 3% sucrose and 0.8% agar was used for *in vitro* germination. Different concentrations and combination of 2,4-dichlorophenoxyacetic acid (2,4-D), benzylaminopurine (BAP) and Kinetin were used for callus induction (Parameswari and Srimathi 2008); 2,4-D 0.75 mg/L + BAP 1.5 mg/L, 2,4-D 1 mg/L+ BAP 2.5 mg/L, 2,4-D 1.25 mg/L+ BAP 3.5 mg/L, 2,4-D 0.5 mg/L+Kinetin 2 mg/L, 2,4-D 0.75 mg/L+Kinetin 2.5 mg/L, 2,4-D 1.25 mg/L+Kinetin 3 mg/L. All media were adjusted pH 5.80 before autoclaving. Samples were incubated at 22-24°C in dark under a 16 h light /8 h dark photoperiod. Due to the darkening of calli after the third week, the shooted calli were subcultured onto the same media. Callus growth index (GI) was calculated by using the following formula:

$$GI = \frac{[\text{Final fresh weight (FW)} - \text{Initial FW}]}{\text{Initial FW}}$$

(Sánchez-Sampedro *et al.,* 2005).

Callus obtaining from all *B. bituminosa* explants were used for analysis of isoflavanoids and terpens.

Chemicals and equipment

Chemicals and solvents were purchased from Sigma or Merck and were of the highest analytical grade. For isoflavonoid analysis, schimic acid, gallic acid, caffeic acid, p-coumaric acid, taxifolin, rosmarinic acid, kaempferol, genistein, quercetin, biochanin A, formononetin, daidzein standards (>98% purity) were purchased from Sigma or Merck. For terpen analysis catalpol (50839, Sigma), aucubin (5561, Sigma) and verbascoside (V4015, Sigma) were obtained as standard compounds. Chromatographic grade-double ultrapure water; gradient grade for liquid chromatography methanol (Merck-1.06007) were used.

Thermo Scientific Dionex Ultimate 3000 variable Wavelength Detector, WPS-3000SL Analytical system was used for all HPLC experiments.

Phytochemical analysis of isoflavonoids

Phenolic extraction was done according to the modified procedure of Kiselev 2007. The freeze-dried and powdered 100 mg of callus culture sample were shaken twice in 25 ml of 80% MeOH at 180 rpm on a rotary shaker for 30 min and 24 hours. And all materials were filtered and 80% aqueous MeOH were evaporated under vacuum in a rotary evaporator at 45°C to dryness (Buchi Rotavapor). The dry residue was dissolved in 2 mL of pure methanol. The sample solution was membrane filtered (0.45 µm) and 20 µL aliquots were used for HPLC analysis.

The instrument used in the present study were Shimadzu 1200 series. Liquid chromatographic system equipped with UV-Vis detector and analysed by using Shimadzu CLASS-VP software. Chromatographic separation was performed on a C18 (5 µm 4.6 mm × 250 mm) column. Working standard solutions, 2,5-250 µg/ml, were prepared by diluting the stock standard solutions with methanol/water (30:70, v/v). A good linear correlation was obtained between the gradient retention time values. Retention times of standard in the examined plant extracts are presented in Table 1.

The analysis of gallic acid, caffeic acid, p-coumaric acid, taxifolin, rosmarinic acid, kaempferol, genistein, quercetin

Table 1: The retention times of isoflavonoid standards standard.

Isoflavonoid standards											
GA	CA	PCA	TA	RA	KA	GE	QU	BA	FO	DA	SA
2.54	8.23	11.95	13.63	16.43	23.73	30.15	31.37	39.90	5.49	4.16	1.21

(GA: Gallic acid, CA: Caffeic acid, PCA: P-coumaric acid, TA: Taxifolin, RA: Rosmarinic acid, KA: Kaempferol, GE: Genistein, QU: Quercetin, BA: Biochanin A, FO: Formononetin, DA: Daidzein, SA: Shikimic acid).

and biochanin A were resolved by using a mobile phase (aseto nitrile) and B (ultrapure water+1% formic acid) at a flow rate of 0.9 ml/min for gradient elution program shown in Table 2. The detection UV wavelength was set at 254 nm. The analysis of were resolved by using a mobile phase [A (MeOH) and B (ultrapure water)] at a formononetin and daidzein flow rate of 0.5 ml/min for the gradient elution program shown in Table 3. The detection UV wavelength was set at 250 nm. The analysis of shikimic acid were resolved by using a mobile phase [A (MeOH) and B (ultrapure water)] at a flow rate of 0.5 ml/min for the gradient elution program shown in Table 4. The detection UV wavelength was set at 230 nm.

Phytochemical analysis of terpenes

B. bituminaria dried calli were weighed of one hundred milligrams and extracted with 10 mL of methanol (MeOH) on a shaker for 48 h (Alipieva *et al.,* 2007). The extract was filtred and then evaporated with a rotary evaporator at 40°C (Crişan *et al.,* 2010). The sample was dissolved by adding 10 ml of methanol. Finally, all samples were filtrated by 0.45 µm filter. HPLC analyses were performed by using a sample injection volume of 20 µL (Alipieva *et al.,* 2007; Crişan *et al.,* 2010).

In the HPLC system (Thermo Scientific Dionex UltiMate 3000), a Thermo Scientific-Acclaim TM 120-C18, 3 µM, 4.6 × 150 mM Dionex Bonded Silica Products column was used for catalpol, aucubin and verbascoside. The sample injection volume was 20 µL. For data acquisition, the flow rate was adjusted to 0.5 mL min^{-1} for catalpol and aucubin and 0.8 mL min^{-1} for verbascoside (Table 5-6).

A gradient elution of A (methanol) and B (ultrapure water) was used as in the following Table 5 and Table 6. On-line UV spectra were recorded with a diode array detector as 200 nm for catalpol and aucubin and 330 nm for verbascoside (Wang *et al.,* 2010; Xie *et al.,* 2012). Standard solutions: catalpol 200, 100, 50, 25, 15, 10 and 5 ppm; aucubin 10, 5, 2.5, 1 and 0.5 ppm; verbascoside 15, 10, 5 and 2.5 ppm concentrations were prepared with methanol. Primary testing showed that these compounds corresponded to peaks with retention times of 2.307 for catalpol, 3.420 for aucubin and 27.04 for verbascoside.

Data analysis

The data were analysed using a one-way analysis of variance (One-way ANOVA) and the means were compared using the Duncan's multiple range test at 5% level of significance ($p<0.05$). All obtained data are average of the three measurements.

RESULTS AND DISCUSSION

The seed is covered by the fruit (fruits beaked). About 70% of seeds are hard and do not imbibe. No germination was observed in beaked fruits and cut beaked fruit. A small scalpel cut that can reach the seed coat improved imbibition and a germination rate of 85% was obtained (Fig 1). In some studies *B. bituminosa* seeds were scarified with pure H_2SO_4

Table 2: HPLC-UV gradient elution program of gallic acid, caffeic acid, p-coumaric acid, taxifolin, rosmarinic acid, kaempferol, genistein, quercetin and biochanin A.

Flow rate (ml/min)	Time (min)	%A (aseto nitrile)	%B (ultrapure water +1% formic acid) (V/V)
0.9	0	95	5
	3	85	15
	13	75	25
	25	70	30
	35	65	35
	39	60	40
	42	55	45
	44	50	50
	47	45	55
	50	30	70
	56	25	75
	60	20	80
	70	10	90

Table 3: HPLC-UV gradient elution program of formononetin and daidzein.

Flow rate (ml/min)	Time (min)	%A (MeOH)	%B (ultrapure water)
0.5	0	20	5
	0	20	15
	20	20	25

Table 4: HPLC-UV gradient elution program of shikimic acid.

Flow rate (ml/min)	Time (min)	%A (aseto nitrile)	%B (ultrapure water)
0.5	0	95	5
	3	85	15
	13	75	25
	25	70	30

Table 5: HPLC-UV gradient elution program of catalpol and aucubin.

Flow rate (ml/min)	Time (min)	%A (aseto nitrile)	%B (ultrapure water)
0.5	0-10	5	35
	10-15	35	45
	15-20	45	51
	20-40	51	61
	40-45	61	80
	45-60	80	80

Table 6: HPLC-UV gradient elution program of verbascoside.

Flow rate (ml/min)	Time (min)	%A (aseto nitrile)	%B (ultrapure water)
0.8	0-20	5	40
	20-40	40	60
	40-45	60	5
	45-60	5	40

for remove external coats. In our studies seeds were cut by scalpel because *B. bituminosa* seeds can taken with hands. Germinations were conducted in hormone-free MS medium different from others (D'Angiolillo *et al.,* 2014).

In our study, 15-day-old *in vitro* seedlings are the most suitable explant sources. *B. bituminosa* aseptic seedlings used different ages as 25-day-old explant sources. However, the studies display variations in the ages of the seedlings. The apical meristem, epicotyls, young primary leaves, cotyledon, hypocotyl were used in experiments for *B. bituminosa.* An apical tip and hypocotyl explants have been used commonly in almost other the studies and our study (D'Angiolillo *et al.,* 2014). Young seedlings are prefered generally due to their totipotency properties.

The first callus formation started on the first leaf in 2 days. Callus growth was observed within 3-5 days on of the hypocotyl, cotyledon, apical meristem, epicotyl andyoung primary leaves. Callus is friable and yellow. The highest callus production was in 0.75 mg/L 2,4-D+2.5 mg/L kinetin medium (Table 7). 2,4-D and kinetin hormones were used at a ratio of 1:3. It was observed that the media using 2,4-D and BAP were not very successful in terms of callus production. In one study, unlike our data, the auxin ratio was 4 times the cytokinin ratio. They were able to produce callus slowly with NAA (mature leaves) and fast and well with 2,4-D (young leaves) (in young and mature leaves). They obtained 4-week growth curves similar to our study. However, the production of green and compact callus obtained after 3 weeks by exposure to light is different from our study (D'Angiolillo *et al.,* 2014). *In vitro* regeneration protocol was developed from hypocotyl-derived callus of *Psoralea corylifolia* L. green compact nodular calli were induced from 3 day-old hypocotyl explants on Phillips and Collins (L2) medium containing 25 g/L sucrose, 7 g/L agar and supplemented with 10 mM NAA and 2 mM TDZ. Higher shoot regeneration (89.5 %) was achieved in enriched L2 medium supplemented with 2 mM BA, 4 m M TDZ and 50 mg/L BVN (Baskarana and Jayabalan 2009). In our study, the calli produced by subculture in 3-4 weeks were yellow and friable. To assess the production of isoflavonoids, the best callus producing different concentration of 2,4-D+Kinetin media and 1.25 mg/L 2,4-D+3.5 mg/L BAP medium were selected for analysis.

HPLC analysis

In some studies, *B.bituminosa* extracts of *in vitro* shoots and in wild shoots contained the flavonoid daidzein, while plicatin B, erybraedin C and bitucarpin A were found. The furanocoumarins angelicin and psoralen were found in *in vivo* and *in vitro* plants, but in the callus were not

Table 7: Callus growth index of *B. bituminosa* from hypocotyl, cotyledon, apical meristem, epicotyls and young primary leaves explants in MS.

Callus growth index		Apical meristem	Epicotyls	Young primary leaves	Cotyledon	Hypocotyl
(Auxin-cytokinin)	(mg/L)					
2,4-DBAP	0.751.5	0.34	0.67	72.84	16.02	8.09
2,4-DBAP	12.5	0.89	0.54	62.07	2.66	4.69
2,4-DBAP	1.253.5	1.37	0.61	68.28	22.70	0.53
2,4-DKinetin	0.52	2.59	3.12	175.92	13.00	8.38
2,4-DKinetin	0.752.5	1.07	11.88	228.23	16.67	4.26
2,4-DKinetin	1.253	5.04	3.96	171.30	34.34	7.81

Table 8: HPLC quantification of flavanoids and terpenes dried callus of *B. bituminosa* (mg kg^{-1}).

	0.5 mg/L 2,4-D+ 2 mg/L kinetin	0.75 mg/L 2,4-D+ 2.5 mg/L kinetin	1.25 mg/L 2,4-D+ 3 mg/L kinetin	1.25 mg/L 2,4-D+ 3.5 mg/L BAP
Schimic acid	182.1727±1.14	1106.6449±4.83	819.8954±4.22	673.0777±3.17
Gallic acid	0.0934±7.18	0.7106±4.72	3.7424±3.92	4.9105±5.12
Caffeic acid	0.1022±8.56	17.9953±5.36	2.7966±2.15	5.3430±3.43
p-Coumaric acid	0.1402±2.36	16.7468±2.12	2.0133±3.49	6.8820±1.73
Taxifolin	0.1877±1.14	3.9199±4.22	2.3119±3.17	7.7796±4.83
Rosmarinic acid	0.0991±3.18	1.8292±3.72	1.0017±1.92	0.9180±2.12
Kaempferol	0.0900±1.14	5.4217±4.22	0.2459±3.17	0.2529±4.83
Genistein	0.0176±0.56	6.1682±2.11	0.0272±1.16	0.0963±2.03
Quercetin	0.0033±3.61	3.8700±4.22	0.0318±2.01	0.0089±5.13
Biochanin A	7.7767±4.56	10.0862±7.03	2.4854±3.5	2.4054±5.16
Formononetin	1.2680±3.12	3.0010±4.15	0.1784±6.10	1.9350±2.18
Daidzein	1.4165±1.54	2.1763±4.22	2.4453±3.12	3.3270±3.71
Aucubin	6.55±1.18	6.25±1.15	6.22±1.75	8.15±1.40
Catalpol	29.10±2.12	30.72±5.10	28.56±1.28	31.69±3.45
Verbascoside	0.0425±1.58	0.174±1.45	0.075±1.32	0.08±1.18

ANOVA statistical analysis ($P \leq 0.05$) was performed (mean±SE).

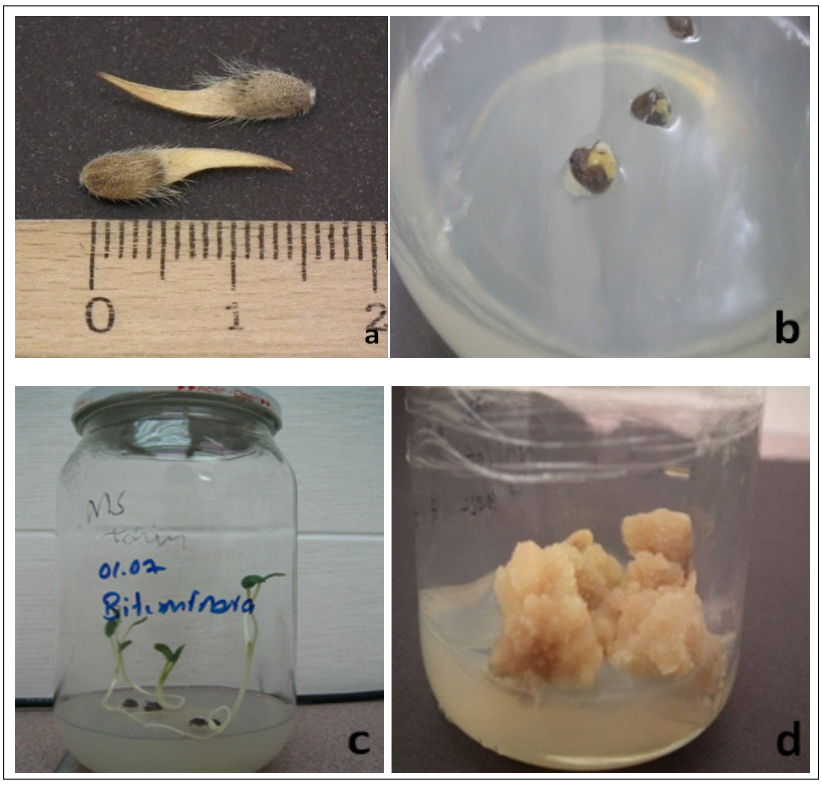

Fig 1: *Bituminaria bituminosa.* a. The seeds of *B. bituminosa*, b. first germination of seeds, c. aseptic seedling 15-day-old, d. callus formation.

detectable. The highest level of cytotoxicity in the extracts was found in *in vitro* shoot extracts in HeLa cell culture (D'Angiolillo *et al.,* 2014). The aeral part and *in vitro* products of *B. bituminosa* were analysed for different phenol and terpene group secondary metabolites. In general, it is seen that furanocoumarins, pterocarpans and additionally flavonoids have been detected in studies. The main furanocoumarins studied are angelicin and psoralen (Pistelli *et al.,* 2003; D'Angiolillo *et al.,* 2014).

Considering the analyses of flavonoids that can be produced *in vitro*, it is seen that 0.75 mg/L 2,4-D+2.5 mg/L kinetin medium is more successful in the production ofses of flavonoids that can be produced *in vitro*, it is seen that 0.75 mg/L 2,4-D+2.5 mg/L kinetin medium is more successful in producing these phenols. The very high production of Schimic acid must be due to the fact that it is one of the first synthesised products in the phenyl propanoid pathway. Using kinetin as three times of 2,4-D produced positive results in *in vitro* isoflavonoid production. The use of auxin/cytokinin ratio of 1/3 provided efficient production from this plant *in vitro*. Extracts from callus cultures showed especially the presence of daidzein (2.88 mg/g FW), even though its glucoside, daidzein, was found only in young leaf (D'Angiolillo *et al.,* 2014). Our results detected daidzein in a similar quantity with previous studies on *in vitro* cultures in *Bituminaria sp.* Bouque *et al.* (1998) found more lower amount daidzein.

In terpene production, it is seen that 1.25 mg/L 2,4-D+3.5 mg/L BAP, which is the best callus producing medium containing BAP, is the most successful medium among all tested mediums (Table 8). In the literature review, no terpene measurement has been made in *B. bituminosa* before and no data has been found. Therefore, our study is the first in this regard. In some family, terpenoid analysis similar results have Çölgeçen *et al.* (2018) catalpol, aucubin and verbascoside content were analysed in *Globularia trichosantha* subsp. *trichosantha* callus.

Conclusion

The limited consideration of the peculiar ecological role and of the biological accumulation of flavonoids and terpenoids in herba surfaces is often overlooked in the development of extraction and analytical methods, thus leading to an underestimation of their presence in natural products, resulting in a difficult evaluation of their risk assessment. In conclusion, a flavonoid and terpenoid analysis method was developed for *B. bituminosa*. With this study, we done first time terpenoid analysis for *B. bituminosa*. In particular, the highest amount terpen in the callus is catalpol. This result revealed that *B. bituminosa* may be an essential plant for catalpol production.

Conflict of interest: None.

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