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PRODUCTION OF AMYGDALIN BY MEANS OF CALLUS CULTURE IN SOME *PRUNUS* SPECIES

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Abstract

Amygdalin is a glycoside which commonly found in the seeds of stone fruits. This active ingredient has a great importance in drug industry. Amygdalin is commonly obtained from the seeds of three different *Prunus* species (almond, cherry laurel and apricot) by producing *in vitro* callus cultures. In this study, analyses of amygdalin was performed with HPLC from the explants, calluses and kernels of these three species. According to the results obtained, the maximum amygdalin was noted from the whole kernel of the apricot fruits as 5888.45 mg per 100 gwhereas the maximum amygdalin content of cotyledons was 213.47 mg per 100 g which was measured from the almond explants. Amygdalin content of the *Prunus* calluses were then noted as 1376.13 mg, 140.74 mg and 74.08 mg in 100 g at cherry laurel, apricot and almond, respectively. Present results suggested that the callus cultures of *Prunus* species can be used in order to obtain amygdalin.

Key word: *Prunus*, Callus, Amygdalin, Secondary Metabolite, HPLC.

Introduction

People have benefited from the plants for centuries, because of their diverse and abundant biologically active metabolites (secondary metabolites) in many fields, including pharmacy, textiles, nutraceutical, and cosmetics (Gundesli *et al.,* 2019; Okatan, 2020; Simsek *et al.,* 2010). Approximately 25% of prescription drugs used in the world is of plant origin and this ratio is continuously increasing. This had lead an increase in the demand for these valuable metabolites. Thus, to meet this demand, researchers have started to use innovative biotechnological methods to produce and/or obtain more active metabolites (Mohammed *et al.,* 2019; Khan *et al.,* 2019; Polat *et al*., 2020). Amygdalin is one of the most important plant originated secondary metabolites with a complex chemical composition. This metabolite is a naturally occurring glycoside, and is particularly used as an active substance of chemotherapy drugs in the treatment of cancer disease (Newmark *et al.,* 1981; Rauws *et al.,* 1982). Newmark *et al*., (1981) reported that the use of amygdalin was legally allowed for oral chemotherapeutic purposes in 23 states in the United States of America (USA). It has been also reported that amygdalin exists especially in the seeds and the other plant organs belonging to the Rosaceae family (Nahrstedt *et al.,* 1989; Güleryüz & Altıntaş, 1997; Gómez *et al.,* 1998; Santamour, 1998; Du *et al.,* 2005; Dursun, 2010). Some of the agents gave bitterness and cause a decrease in the consumer attractiveness of fruits. An example to them is glycosides, which are carbohydrate derivatives and the bitter-producing agent are commonly located in the seeds of almond, apricot, plum, peach and cherry (Haisman *et al.,* 1967; Gómez *et al.,* 1998; Tatsuma, 2000). The majority of amygdalin is known to be obtained from the seeds of these species and the other plant parts. On the other hand, obtaining amygdalin from these plants by conventional method is very costly. Therefore, several studies are being conducted with an aim to reduce the costs of amygdalin production and most of those studies are focusing in *in vitro* conditionswith cell and organ cultures (Cüce *et al.,* 2017; Cüce *et al.,* 2019; Kahramanoglu *et al.,* 2020). Diosgenin, codeine, morphine, atropine, hyoscyamine, scopolamine, digoxin, digitoxin, quinine, reserpine, artemisinin, reserpine, quinine, kinedin, aymalisin, vincristine, ephedrine and taxol are well-known pharmaceutical raw materials produced in cell and/or organ cultures (Sökmen & Gürel, 2001; Mulabagal & Shengsay, 2004; Çetin *et al.,* 2011). However, the biggest disadvantage of cell and organ cultures is that some of the secondary metabolites are either not producible or can be produced in very low quantities (Mulabagal & Shengsay, 2004; Cüce *et al.,* 2018). In line with this information, present study was conducted with a purpose to search the possibility of obtaining amygdalin in callus cultures derived from seeds of bitter almond (*Prunus dulcis*), bitter apricot (*Prunus armenica*), and cherry laurel (*Prunus laurocerasus*).

Material and Method

The seeds of the bitter almond, bitter apricot and cherry laurel were used as plant material in present study. Almond (*Prunus dulcis* Mill) seeds were obtained from a naturally grown tree with bitter taste grown in Arapgil district of Malatya province (39° 04′ 28″ N, 38° 48′ 84″ E). Apricot (*Prunus armenica* L.) seeds of Hacikiz variety and cherry laurel (*Prunus laurocerasus*) seeds of genotype no 52-06 were provided by Apricot Research Institute (38° 32′ 38″ N, 38° 28′ 47″ E) in Malatya and Hazelnut Research Institute (40° 91′ 01″ N, 38° 35′ 08″ E) in Giresun, respectively. After the harvest the seeds were extracted from the fruits by hand, washed with tap water, dried and stored at room temperature (20 ºC) in the dark until they were used.

Callus cultures: The cotyledons of the seeds were used as explant source for callus induction. For surface sterilization shells were cracked and the kernels were extracted. The kernels were shaken in 3% sodium hypochlorite (NaOCl) solution with 1 drop of Tween 20 for 20 min, and then were washed in sterile distilled water 3 times for 5 min (Aygün & Dumanoğlu, 1998). For callus formation experiments, the explants were planted in MS (Murashige and Skoog, 1962) basal medium containing 3% (w/v) sucrose and 0.7% (w/v) agar (Difco Bacto), and supplemented with 6-benzyladenine (BA; 0.0, 1.0, and 2.0 mg/L), in combination with Naphthalene acetic acid (NAA; 0.0, 0.5, 1.0, 2.0 and 5.0 mg/L) and 2,4-dichlorophenoxyacetic (2,4- D; 0.0, 0.5, 1.0, 2.0 and 5.0 mg/L) (Table 1). All of the growth regulators were added to the media before autoclaving at 121 °C for 20 min. The pH was adjusted to 5.7 before adding agar and autoclaving. All cultures were incubated at 24 ± 1°C in the dark. Initially, the explants were cultured for 4 weeks and then subcultured three times at 4 week intervals.

Table 1. The rate of callus formation in *Prunus* species of different BA + Auxin combinations.

BA (mg/L)	NAA (mg/L)	Callus formation rate (%)		
		Almond	Cherry laurel	Apricot
0.0	0.0	*	*	+
	0.5	*	*	*
	1.0	+	*	+
	2.0	+	*	+
	5.0	+	*	+
1.0	0.0	*	*	*
	0.5	+	*	+
	1.0	+	*	++
	2.0	+	+	+
	5.0	+	*	+
2.0	0.0	*	*	*
	0.5	+	*	+
	1.0	++	*	+
	2.0	++	*	++
	5.0	++	*	+
	2,4-D (mg/L)			
0.0	0.0	+	*	+
	0.5	+	*	+++
	1.0	*	*	+++
	2.0	++	*	+
	5.0	+++	+	+++
1.0	0.0	*	*	+
	0.5	+	*	*
	1.0	+++	*	+
	2.0	+++	+	+
	5.0	+++	+	++
2.0	0.0	+	*	*
	0.5	++++	*	++
	1.0	+++	+	+++
	2.0	+	*	++
	5.0	+	*	+

Data recorded on the 4 weeks after the culture and a total of three replicates of 25 explants per treatment for callus formation

* = No callus formation, + = 1-20%, ++ = 21-40%, +++ = 41-75% callus formation

Determination of amygdalin in plant tissues: For amygdalin extraction 1.0 g tissue samples well-crushed in porcelain mortar were used. Callus tissues were removed from the cotyledons, and callus and cotyledons were analyzed separately as well as whole kernels. The samples were extracted in the solvent of 10 ml methanol (MeOH)-chloroform (1:1) in the shaker at room temperature for 24 hours. Plant sediments filtered through coarse filter paper, and later through 0.45 µm filters. For amygdalin analysis the filtrate of 20 µl was given directly to the HPLC (High-performance liquid chromatography). Acetonitrile (Solvent A) (Merck, HPLC grade) and deionized water (solvent B) were used as mobile phase. For separation, C18 (4.6 x 150 mm, particle size 0.5 µm) column and UV detector were used. Scanning was made at different wavelengths (Berenguer *et al.,* 2002; Keskin & Kunter, 2007; Dursun, 2010). Standard stock solutions of 1000 ppm in MeOH were prepared. From this stock solution, concentrations of 500, 250, 100 and 25 ppm were prepared by diluting (with MeOH)) and given to the device with 20 ml-volume syringe and then the chromatograms were obtained. The results were calculated as mg/100g wet weight.

Statistical analysis

The experiments were conducted according to completely randomized design with five replications and five explants in each replication for callus cultures, and three replications for amygdalin analyses. The data was subjected to Analysis of variance (ANOVA) using SPSS (version 21.0) software, and the comparison of the means (± SE) was performed by Duncan's multiple range test ($p<0.05$).

Results

Callus formation from cotyledon tissue: The cotyledons of *Prunus* species used in the experiment formed callus *in vitro* at the end of the 3 subcultures. In the combinations used in the experiment, almond cotyledons formed the highest rate of callus, whereas the least callus formation was noted from the Cherry Laurel (Table 1; Fig. 1).

The results of amygdalin obtained from plant tissues: Amount of amygdalin was determined by HPLC in calluses, callus forming cotyledons and whole kernels. Figure 2 shows the HPLC chromatogram of amygdalin standard.

The amount of amygdalin was determined from different tissues of seeds in three of the *Prunus* species are given in Table 2 and Figures 3 and 4. Results suggested that the whole kernels yielded the highest amount of amygdalin among the materials used across the species. Almond kernels had the highest amount of amygdalin as 5888.45 mg/100g (Table 2; Fig. 4), and was followed by cherry laurel (3507.27 mg/100g) and apricot (2596.77 mg/100g). Considering cotyledons, almond yielded the highest amount of (213.47 mg/l00g) amygdalin (Table 2), whereas cherry laurel yielded the least (10.53 mg/100g). The amount of amygdalin in the cotyledon derived calluses was 1376.13 mg/100g, 140.74 mg/100g and 74.08 mg/100g in cherry laurel, apricot and almond species, respectively.

Table 2. Amygdalin amount in plant tissues (mg/100 g).

Plant tissue	Species		
	Almond	Cherry laurel	Apricot
Callus	74.08 ± 4.61c	1376.13 ± 24.38a	140.74 ± 5.12b
Cotyledons	213.47 ± 10.52a	10.53 ± 0.48c	48.53 ± 2.76b
Whole kernel	5888.45 ± 29.64a	3507.27 ± 14.54b	2596.77 ± 24.92b

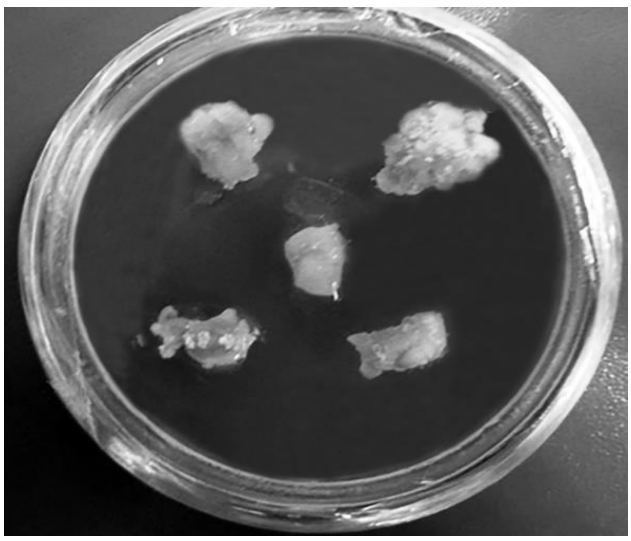

Fig. 1. The appearance of callus formation of apricot cotyledons in MS medium supplemented with 5.0 mg/L1 2,4-D.

Discussion

The use of secondary metabolites obtained from plant species has gained increasing importance in the medical field in recent years. The main objective of this study was to search the possibility of obtaining amygdalin glycoside, used as a medicine in treating cancer in recent years, through callus cultures. There have been previous reports describing the determination of amygdalin in plant tissues (Femenia *et al.,* 1995; Gómez *et al.,* 1998; Haque & Bradbury, 2002; Yıldırım & Askın, 2010; Lee *et al.,* 2013), but no reports exist on quantification of amygdalin in calluses produced in callus culture method. In the study, different levels of callus formation were observed in the same plant growth regulator combinations of cotyledons of 3 different Prunus species. In the combinations used in the experiment, almond cotyledons formed a higher rate of callus. As a matter of fact, callus formation was observed at the level of 65.00% 57.90% 7.50% in cotyledons of almond, apricot and cherry laurel, respectively. The researchers obtained 83.3-100.0% callus from immature embryos of peach, nectarine and flat Saturn peaches, and 97% from immature plum cotyledons (Yao *et al.,* 1990; Ning & Bao, 2007). In the study of Yang *et al.,* (2009) plant regeneration and 100% callus formation was obtained from three explants of stem, leaves and anthers in almonds. These results are higher than our findings. The difference could be due to the differences in genotypes and tissues used as an explant. On the other hand, there is a big difference between immature or mature explants in terms of callus formation. Since mitotic activity continues in explants taken from immature cotyledons, callus formation and regeneration is expected to be more.

The amount of amygdalin determined in the whole kernels in almond in this study was 5888.45 mg/100g. Amygdalin content in kernels of standard almond species of Italian, American and Russian origin was found to be 0.22-1.95% (22-195 mg / 100g) on dry weight basis (Kester & Asay, 1975; Simsek, 2011). On the other hand, Arrazola *et al.,* (2012), in a study using 29 sweet, slightly bitter, and bitter species grown in Spain, determined that the amount of amygdalin was 2.4-5.96 mg/100g in 6 bitter species. The results obtained from these studies remained far beyond of our study.

For instance, Lee *et al.,* (2013) determined the amount of amygdalin in non-bitter, slightly bitter, and bitter almond varieties, and reported that the amount of amygdalin varied between 3300.66 and 5399.83 mg/100g. It is thought that this genotype can also be considered in this aspect.

The amount of amygdalin obtained from cherry laurel seeds was 350.27 mg/100g. Genç (2009), in his study for the determination of amygdalin content in cherry laurel seeds, reported that the amount of amygdaline was 93.06 g/kg (9306 mg/100g). The study of Dursun (2010) revealed that the highest amount of amygdalin 103.36 g/kg (10336 mg/100g) was in the seeds of cherry laurel harvested on July the 12th. Our results are 1/3 of the results reported by the researchers. It may be said that this difference is caused by the period in which explants are taken as well as by the genotypes. Dursun (2010) determined 85.27 g/kg (8527 mg/100g) amygdalin from the seeds picked up on July 27. In cherry laurel, a decrease in the amount of amygdalin is reported with maturity. It is said that the seeds of Hacikiz apricot variety used in the study has a bitter taste. In the seeds of this variety, 2596.77 mg/100g of amygdalin has been determined. Yıldırım and Askın (2010) determined the highest amount of amygdalin as 6.35 g/100g in bitter variety Paviot and 4.41 g/100g in sweet variety Aprikoz. In comparison with our findings, these results were considered as very high. We thought that this discrepancy was initially caused by the genotypes. Additionally, since the amygdalin value rises in parallel with the bitterness (Zhao, 2012), we think that Pavilot variety, with higher bitterness than our own cultivar Hacikiz, has a higher ratio of amygdalin value. On the other hand, it is possible that the same amount of amygdalin may be obtained from genotypes showing differences in terms of bitterness (Arrazola *et al.,* 2012; Lee *et al.,* 2013).

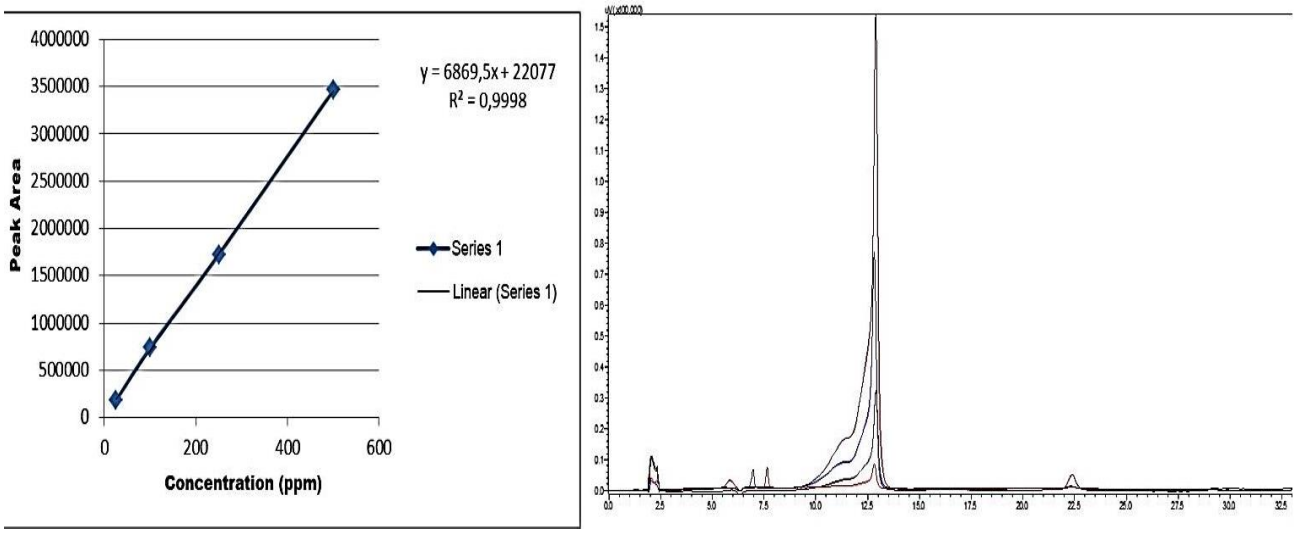

Fig. 2. HPLC chromatogram of amygdalin standard.

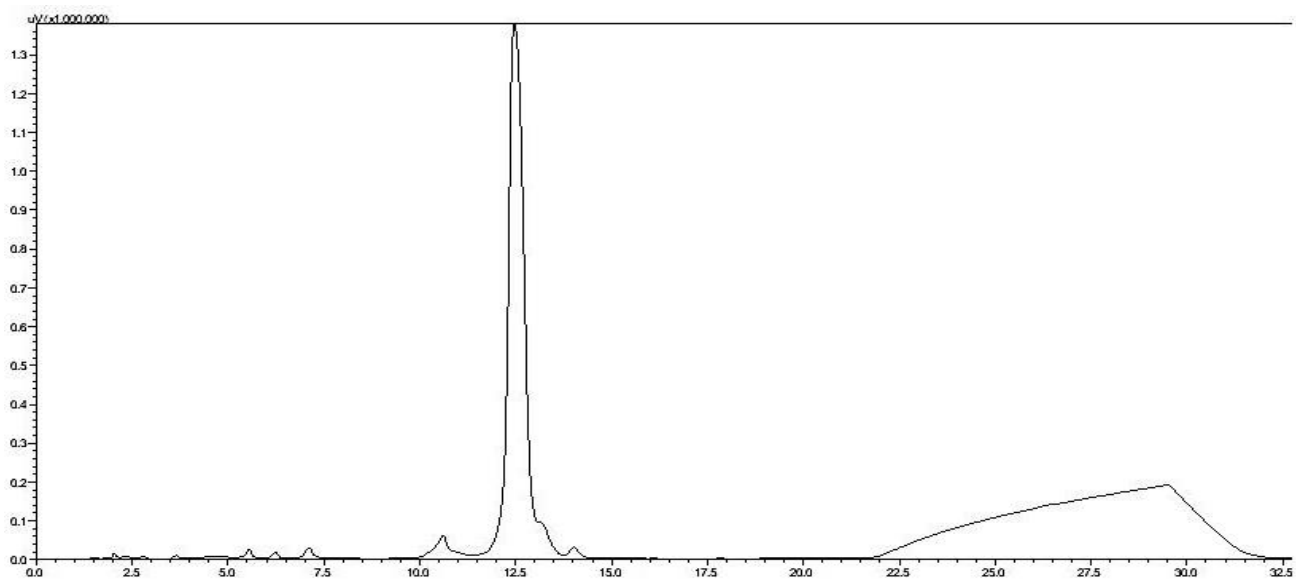

Fig. 3. An example of HPLC chromatogram of whole kernels in almond.

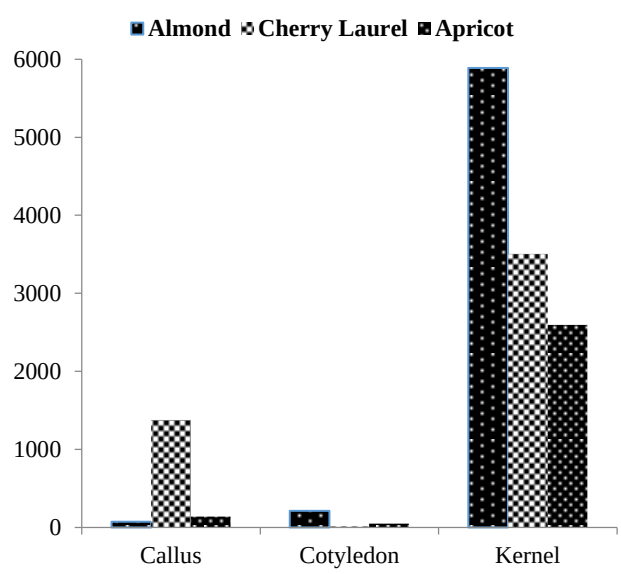

Fig. 4. Amount of amygdalin in *Prunus* species (mg/100g).

Amygdalin amounts of the cotyledon explants of *Prunus* species that formed callus have also been determined. Amygdaline content was found to be as 213.47 mg/100g in almond, 10.53 mg/100g in cherry laurel and 48.53 mg/100g in apricot explants. These amounts are too low to be compared to the amount found in the seeds. It is believed that the reason for this is that callus formation from explants takes a period of time and increasing of water content in explants along with their growth (Rao & Ravishankar, 2002). The amount of amygdalin in the callus obtained in this study was found to be 74.08 mg/100g in almond, 1376.13 mg/100g in cherry laurel and 140.74 mg/100g in apricot. These results are the first to *Prunus* species in the literature. The amount of amygdalin obtained from callus is very low compared to the amount determined in the seeds. However, considering the amount and the size of the callus produced per explants that amount corresponds to the amount determined in the seed. The results obtained from this study reveal that it is possible to obtain amygdalin continuously by callus cultures in *Prunus* species.

Conclusions

As a consequence of the present study, the callus production from almond, cherry laurel and apricot cotyledons has been achieved to some extent. According to the results obtained, some of these genotypes are found to have the ability to be used for obtaining amygdalin. On the other hand, since obtaining amygdalin from the calluses of *Prunus* species is the first attempt; this study fills a huge gap in the literature. Results also made it possible to do some recommendations for the further studies. Thus, the amount of amygdalin in callus is estimated to be increased especially by using abiotic stress conditions for plant growing.

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