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Catalytic degradation of amygdalin by extracellular enzymes from *Aspergillus niger*

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ABSTRACT

Amygdalin is a controversial anti-tumor natural product that has been used as an alternative cancer drug for many years. The anti-tumor mechanism and metabolism of amygdalin have been the focus of many studies. However, previous studies by our group demonstrated that amygdalin itself has no anti-tumor activity, but rather the active ingredients were determined to be amygdalin degradation products. To screen novel drugs with anti-tumor activity, the extracellular enzymes from *Aspergillus niger* were used to degrade amygdalin. Within 4 h of the catalytic reaction at 37°, amygdalin was rapidly degraded into four products. The products were then extracted and purified by column chromatography. By comparing the HPLC chromatograms, ^{1}H NMR, ^{13}C NMR and MS data, the products were identified as mandelonitrile, prunasin, benzaldehyde and phenyl-(3,4,5-trihydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-acetonitrile (PTMT), a novel hydroxyl derivative of prunasin. Furthermore, pharmacology studies of these compounds demonstrated that 10 mg/kg of PTMT significantly suppressed the growth of S-18 tumor cells within 11 days in a concentration-dependent manner.

1. Introduction

Amygdalin (Fig. 1) is a cyanogenic glucoside [1] found in plants of the *Rosaceae* family, particularly of the *Prunus* genus and other food plants, including linseed and manioc [2]. This natural product has been reported to effectively prevent and treat cancer in humans [3–6]. Previous studies on amygdalin have focused on its purification [7], toxicity related to the liberation of cyanide, identification of its metabolites in plasma [8] or herbs [9,10] and pharmacology to cancers [11]. When co-cultured with microorganisms, amygdalin readily decomposes or is converted into benzaldehyde and hydrocyanic acid via catalysis by nitrile lyase [12]. Hydrocyanic acid has been reported to be one of key compounds involved in killing tumor cells. Amygdalin is unstable in the cultures of microorganisms, and pH has a great influence on the degradation of amygdalin. It is known that *Aspergillus niger* produces different enzymes [13,14]. To screen new anti-tumor drugs, amygdalin was degraded by extracellular enzyme mixtures isolated from *A. niger* to avoid pH interference in the amygdalin degradation process. Four products were determined by an HPLC assay and identified as mandelonitrile, prunasin, benzaldehyde and phenyl-(3,4,5-trihydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-acetonitrile, a novel hydroxyl derivative of prunasin.

2. Materials and methods

2.1. Chemicals

Amygdalin (90%) was extracted and purified from seeds of *Prunus armeniaca* L. var. ansu Maxim. HPLC grade methanol was purchased from Honeywell International Inc., USA. Standards of amygdalin, mandelonitrile, prunasin and benzaldehyde were all greater than 98% in purity (Sigma–Aldrich Corp.). Other chemicals used in this study were of analytical grade.

2.2. Microorganism, tumor cell line and animals

A. niger was purified from soil and preserved in our laboratory. The culture was maintained on PDA slants at 4 °C and transferred every 2 months.

S-18 cell line and C57BL/6 mice were obtained from the Type Culture Collection of the Chinese Academy of Science Cell Bank and preserved in our library.

2.3. Preparation of extracellular enzyme mixtures of A. niger

The medium used for *A. niger* consisted of 8 g/l glucose, 5 g/l peptone, 5 g/l yeast extract, 5 g/l KH_2PO_4, 5 g/l NaCl, 1 g/l $MgSO_4{\cdot}7H_2O$, 1 g/l $MnSO_4{\cdot}4H_2O$ and the pH was adjusted to 6.5 with 1 mol/l NaOH prior to sterilization. A volume of 1 ml of *A. niger* spores was transferred into 40 shake-flasks (250 ml flask containing 50 ml of medium) and cultured at 28 °C while being agitated at 160 rpm for 5 d. The cultures were collected and filtered. Subsequently, salt precipitation was performed by adding saturated $(NH_4)_2SO_4$ into the supernatant and incubated at 4 °C for 12 h. The solution was centrifuged, and the precipitate was collected, desalted (at 4 °C

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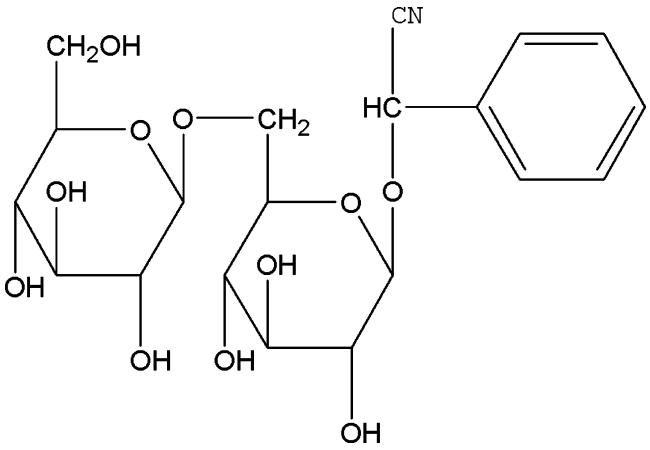

Fig. 1. Structure of amygdalin.

for 12 h), lyophilized and stored at 4 °C for future use as a mixture of extracellular enzymes.

2.4. Catalysis of amygdalin and HPLC assay

Amygdalin (0.5 ml of a 10 mg/ml stock solution) was added into 2 ml of a mixture of extracellular enzymes (82 U/ml, where U refers to a unit of β-glucosidase activity) and incubated at 37 °C for 4 h. Aliquots of the assay were analyzed by HPLC every 0.5 h. Chromatographic separation (with an injection volume of 10 μl) was performed on an Agilent HPLC system equipped with a C_{18} ODS column (250 × 4.6 mm i.d.) by elution with 35% (v/v) methanol in water at a flow rate of 1 ml/min. The detection wavelength was 210 nm, and the column temperature was maintained at 25 °C.

2.5. Separation and purification of products

The reaction solution was extracted three times with an equal volume of petroleum (60–90 °C) and subsequently extracted three times with an equal volume of chloroform. The chloroform layers were pooled, dried over sodium sulfate and evaporated to dryness under vacuum. The residue was then separated on a macroporous resin column using chloroform and methanol as the gradient elution followed by a silica gel column using methanol and water as the gradient elution (30–60%).

2.6. Structure determination

High resolution mass spectrum (MS) was performed on a Bruker Apex II FI-ICR mass spectrometer with ESI (Agilent Corp., Palo Alto, USA) as the ion source. Ions were monitored in the positive ion mode. The purified products were dissolved in $CDCOCD_3$ for NMR analysis. The ^{1}H- and ^{13}C NMR were performed on a Bruker AV400 (Bruker BioSpin Group, Faellanden, Switzerland). Chemical shifts were reported as δ values relative to the TMS internal standard. The melting point was determined using a Yanaco MP-3 micro melting point apparatus (Yanaco Corporation, Kyoto, Japan) and uncorrected. IR spectra were acquired on a FT-IR spectrophotometer (PerkinElmer, Altham, USA) using KBr disks.

2.7. Assay of anti-tumor activity

S-18 cells were cultured in 1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Gibco) and maintained at 37 °C in a humidified chamber with 5% CO_2 for 7 d. The tumor cells (10^7/ml) were then transferred into mice (45 males and 45 females). The tested compound was orally administered at 5, 10 and 20 mg/kg doses once every day for 10 d. Water and cyclophosphamide were used as negative and positive controls, respectively. On day 11, all mice were killed, the weights of tumors were measured, and the inhibition rates were calculated.

Data obtained from animal experiments were expressed as mean ± standard error (SEM). Statistical differences between the treatments and the controls were tested by a one-way analysis of variance (ANOVA) and Student–Newman–Keuls post hoc test. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Degradation of amygdalin

Amygdalin was fully degraded into four products within 4 h, as observed by HPLC. To identify the amygdalin degradation products, chromatographic characteristics of standards of mandelonitrile, prunasin and benzaldehyde were determined under the same HPLC conditions. The HPLC retention times of peaks **1**, **3** and **4** were 3.3, 16.37 and 22.75 min, respectively (Fig. 2), which were the same as the retention times of the mandelonitrile, prunasin and benzaldehyde standards. Thus, peaks **1**, **3** and **4** were identified as mandelonitrile, prunasin and benzaldehyde.

The time course of the catalytic degradation of amygdalin (Fig. 3) demonstrated that amygdalin rapidly degraded into prunasin, an unknown peak **2,** mandelonitrile and benzaldehyde. The concentration of prunasin dramatically increased to 0.7 mg/ml from 0 to 1.5 h and then slowly decreased to 0.3 mg/ml from 1.5 to 4 h. The concentrations of the unknown peak **2**, mandelonitrile and benzaldehyde steadily increased to 0.346, 0.251 and 0.520 mg/ml, respectively, within a time range of 0 to 4 h. After 4 h, no amygdalin was observed, indicating that amygdalin was fully degraded.

3.2. Separation and purification

To purify the unknown peak **2**, we scaled up the degradation of amygdalin such that the reaction was carried out in a 1000 ml reaction bulb containing 600 ml of a mixture of extracellular enzymes (82 U/ml). After 4 h, the reaction mixture was separated and purified as mentioned above. The results of purification are shown in Table 1. After the pooled chloroform layer was evaporated to dryness under vacuum, 2.3 g of residue was obtained and the purity of peak **2** was estimated to be 50% by HPLC. The elution of the dissolved residue on the macroporous resin column with chloroform and methanol as gradient mixture yielded 1.6 g of residue containing 85% of peak **2**. The eluate was dried, purified on silica gel column, and 1.1 g of peak **2** (98% in purity) was obtained.

3.3. Structure elucidation of compound *2*

Peak **2** was isolated as white needles ($CHCl_3$–MeOH); mp: 137–140 °C; $[\alpha]_D^{20}$ −37.0 (CH_3COCH_3); ν_{max}: 3470 cm^{-1}; ESI-MS *m/z*: 341.2986 $[M+H]^+$; ^{13}C NMR ($CDCOCD_3$, 125 MHz): δ_C 106.7 (C-1′), 69.6 (C-2′), 118.4 (C-3′), 125.3 (C-4′), 108.9 (C-5′), 154.4 (C-6′); ^{1}H NMR ($CDCOCD_3$, 500 MHz): δ_H 6.57 (2H, m, H-5, 7), 7.05 (2H, m, H-4, 8), δ_H 5.91 (1H, s, H-2), δ_H 4.30 (1H, d, J = 7.3 Hz, H-1′); element analysis: *Anal*. C 49.32%, H 5.36%, O 41.21%, N 4.14%, *calcd* for $C_{14}H_{18}O_7N$, C 49.41%, H 5.33%, O 41.13%, N 4.11%.

On the base of the elemental analysis (C 49.32%, H 5.36%, O 41.21% and N 4.14%), the molecular formula of **2** was established to be $C_{14}H_{18}O_7N$, which is consistent with the high resolution ESI-MS ($[M+H]^+$ at *m/z* 341.2986) results. In the IR spectra, one hydroxyl group was observed. The ^{1}H NMR data exhibited one glycogenic proton at δ_H 4.30 and four olefinic protons at δ_H 6.57 (2H, m, H-5, 7) and 7.05 (2H, m, H-4, 8). The ^{13}C NMR data on δ_C 106.7, δ_C 69.6, δ_C 118.4, δ_C 125.3, δ_C 108.9 and δ_C 154.4 suggest that there is one glucopyranosyl unit in compound **2**. Based on the ^{1}H NMR and ^{13}C NMR data of compound **2** and prunasin [4], we concluded that the 6-H of prunasin is substituted by one hydroxyl group in compound **2**. Thus, the unknown peak **2** was identified as phenyl-(3,4,5-trihydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-acetonitrile, a novel hydroxyl derivative of prunasin. The structures of phenyl-(3,4,5-trihydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-acetonitrile (compound **2**), mandelonitrile, prunasin and benzaldehyde are shown in Fig. 4.

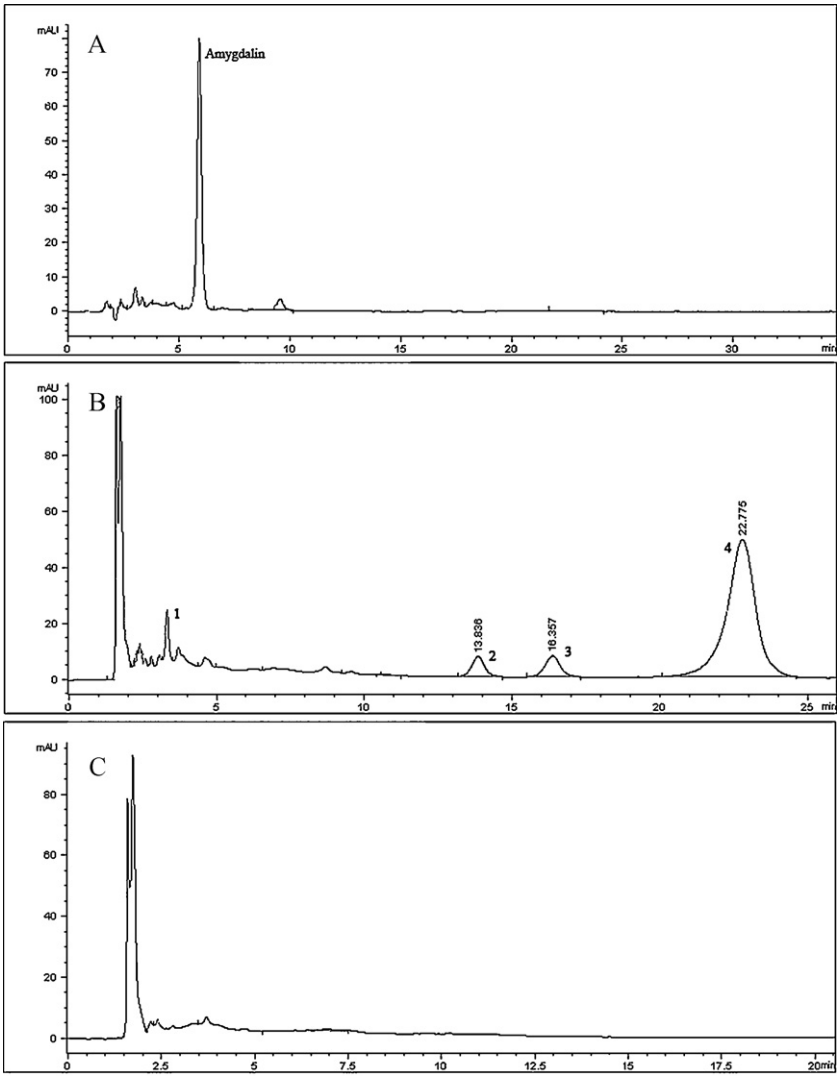

Fig. 2. HPLC chromatograms of the amygdalin degradation products catalyzed by a mixture of extracellular enzymes. The sample was taken out at 4 h. (A) HPLC chromatogram of amygdalin (95%, 2 mg/ml); (B) HPLC chromatogram of amygdalin degradation products; (C) HPLC chromatogram of the mixture of extracellular enzymes (82 U/ml); compound **1**, compound **2**, compound **3** and compound **4** are amygdalin degradation products.

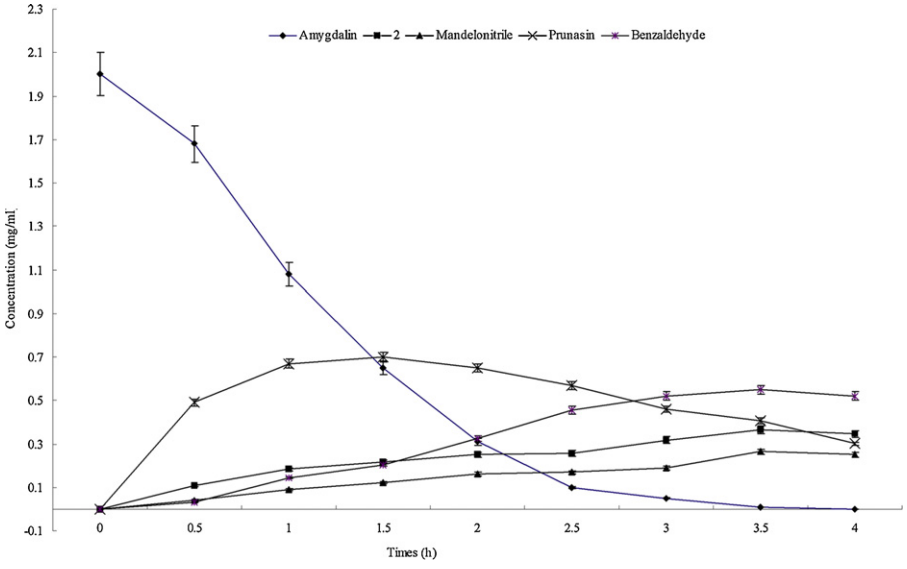

Fig. 3. The time course of the catalytic degradation of amygdalin.

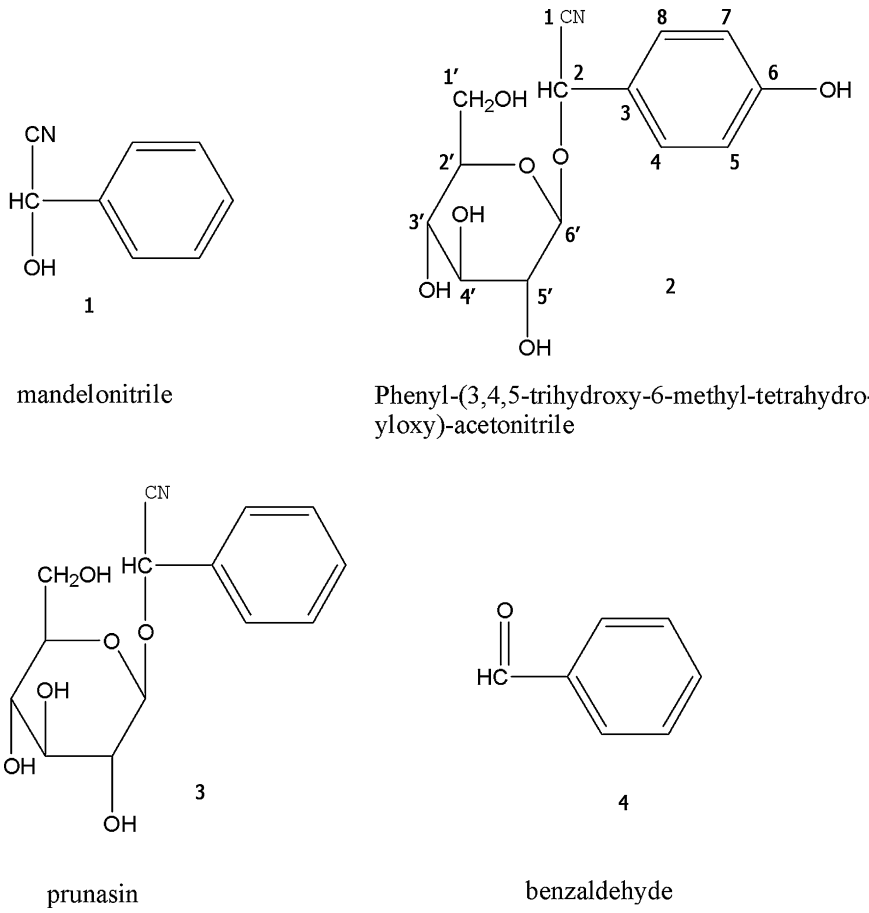

Fig. 4. The structures of amygdalin degradation products mandelonitrile, phenyl-(3,4,5-trihydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-acetonitrile, prinasin and benzaldehyde.

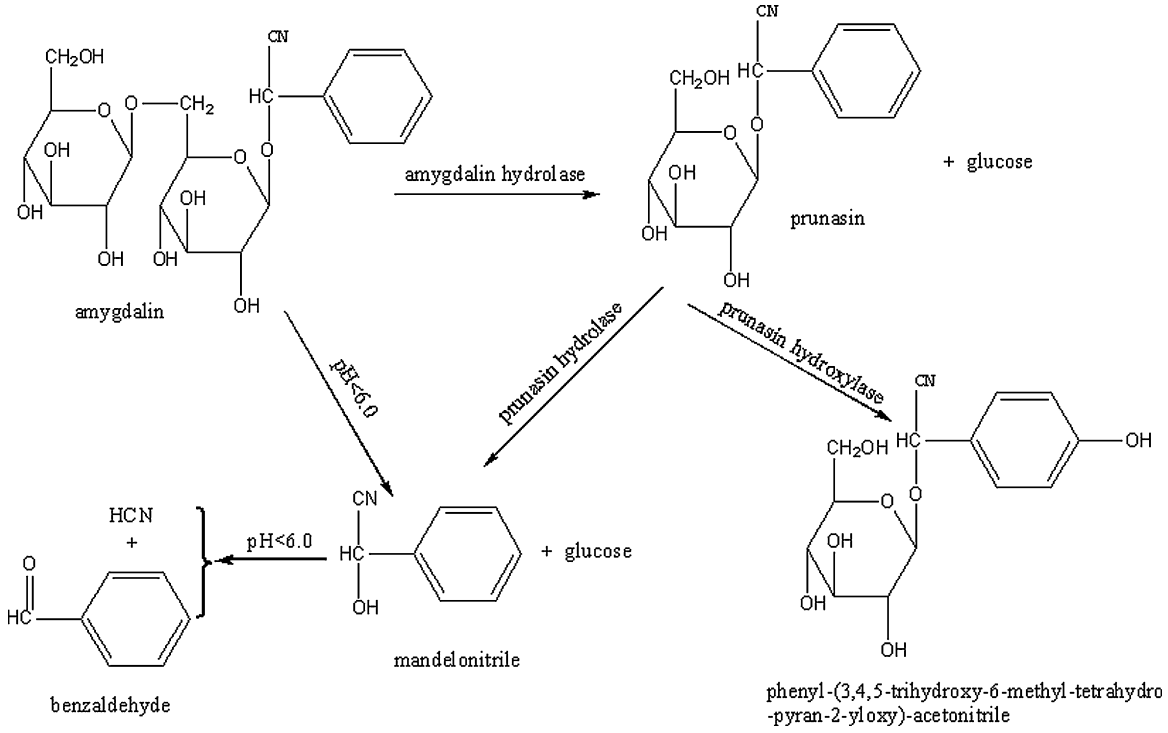

Fig. 5. The degradation route of amygdalin.

Table 1
Purification of peak **2**.

	Steps			
	Extraction with petroleum	Extraction with chloroform	Macroporous resins column elution	Silica gel column elution
Purification (%)[a]	3%	50%	85%[b]	98%[c]
Total weight (g)	5.7	2.3	1.6	1.1

[a] The purity of compound **2** was determined by HPLC and calculated using the area moralization method.
[b] The elution solution was 45% chloroform and 55% methanol.
[c] The elution solution was 35% methanol in water.

Table 2
The anti-tumor effect of compound **2** on S-18 tumors ($n = 10$).

Tested compounds	Doses (mg/kg)	Tumor weight (g)	Inhibition (%)	Survivors on day 11
Control	0	2.03 ± 0.22	–	10/10
Product **2**	20	0.95 ± 0.27**	53%*	10/10
	10	0.95 ± 0.13**	52%*	10/10
	5	1.34 ± 0.19*	34%	10/10
Cyclophosphamide	20	0.95 ± 0.15**	68%to 0pt**	10/10
	10	0.77 ± 0.12**	62% to 0pt**	10/10
	5	1.03 ± 0.21*	49%	10/10

* $P < 0.05$ significant from control.
** $P < 0.01$ significant from control.

3.4. Anti-tumor activity

To test the anti-tumor activity of compound **2**, 7-d-old S-18 tumor cells were transferred into mice. The inhibition rate of compound **2** on the growth of the lymphosarcoma caused by S-18 cells was determined (Table 2), demonstrating that both cyclophosphamide (positive control) and compound **2** greatly suppressed the growth of the S-18 cells over 10 days of the experiment. On day 11, the inhibition rates of compound **2** at 5, 10 and 20 mg/ml doses were 34%, 53% and 52%, respectively. There was no significant difference between the inhibition rates between 20 mg/kg and 10 mg/kg doses, indicating that compound **2** significantly ($P < 0.01$) suppressed the growth of S-18 tumor and its anti-tumor effect was concentration-dependent. The inhibition rates of compound **2** at 5, 10 and 20 mg/kg doses were significantly lower than those of cyclophosphamide at 5, 10 and 20 mg/kg doses, respectively.

4. Discussion

Amygdalin, also called laetrile, has been used as a cytotoxic agent to treat cancer, though its use is now restricted. In small quantities, glycosides such as amygdalin do exhibit expectorant, sedative and digestive properties [2]. Cancer patients treated with conventional therapies have used amygdalin as an alternative cancer drug for about 40 years [15]. Since its discovery in 1845, many researchers have reported the anti-tumor activity of amygdalin[16]. However, some researchers reported that this "alternative cancer cure" was not supported by encouraging evidence as some of these alternative cures are associated with considerable risks [17]. Amygdalin was reported to degrade into benzaldehyde and hydrocyanic acid [12], a key ingredient in killing tumor cells but one that is also toxic to patients. In this experiment, mandelonitrile, prunasin, benzaldehyde and a novel product, phenyl-(3,4,5-trihydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-acetonitrile (compound **2**), were found in the reaction mixtures by an HPLC assay. As shown in Fig. 5, we hypothesize that the potential mechanism of degradation of amygdalin may occur such that amygdalin is sequentially hydrolyzed to prunasin and then mandelonitrile, a reaction that is catalyzed by a β-glucosidase-type enzyme [18,19]. Mandelonitrile is then hydrolyzed to benzaldehyde and toxic HCN by the action of another enzyme. In this study, the 6-H of prunasin was determined to be substituted by a hydroxyl group to produce compound **2**. At a pH lower than 6.0, prunasin can also be degraded into benzaldehyde and hydrocyanic acid. To verify the hydroxylation at 6-C of prunasin, prunasin (1 mg/ml) was catalyzed by a hydroxylase (70 U/ml) at 37 °C for 2 h and the products were determined by HPLC. The result showed that 30% of prunasin was transformed into compound **2**.

Comparing the inhibition rates of compound **2** with that of water (negative control) and cyclophosphamide (positive control), this novel product significantly suppressed ($P < 0.01$) the growth of S-18 tumor at dose of 10 mg/kg, suggesting that compound **2** is an effective anti-tumor compound.

Kwon et al. [20] reported that the dextrorotatory (R) configuration is the active form of natural amygdalin and Park et al. [21] reported that amygdalin have an anticancer effect via downregulation of cell cycle-related genes in SNU-C4 human colon cancer cells, but they did not study the stability and metabolism in tumor cells. Our previous work demonstrated that amygdalin itself has no anti-tumor effects and the active ingredients are its degradation products [22], including hydrocyanic acid. And β-glucosidase could greatly promoting the anti-tumor activity of amygdalin. Stock [23] also reported that, except for oral administration, hight dose of amygdalin was ineffective against the DMBA-induced rat mammary carcinoma and experimental tumors such as Sarcoma 180, plasma cell tumor LPC-1, leukemia L1210 and Mecca lymphosarcoma, while amygdalin derivatives, such as amygdalinic acid, benzyl β-gentiobioside and benzyl β-D-glucopyranoside were reported to remarkably inhibit the activation of EBV-EA [24]. The anti-tumor mechanism of degradation products of amygdaline were reported to the toxicity produced during the degradation process of amygdaline. The previously reported metabolites with anti-tumor activity are all hydrolysis products from amygdaline or its metabolites. The compound 2, however, is a hydroxylation product of prunasin which meant that the anti-tumor mechanism of this novel product is different from those reported products and should be further investigated.

5. Conclusion

Amygdalin was catalytically degraded into four products by extracellular enzymes from *A. niger*, and the products were identified as phenyl-(3,4,5-trihydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-acetonitrile, mandelonitrile, prunasin and benzaldehyde.

Product **2** was shown to be a novel hydroxylated derivative of prunasin with high anti-tumor activity.

Conflict of interest

None of the authors have declared a conflict of interest.

Acknowledgement

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