

# Induction of flavonoid biosynthesis by *in vitro* cultivation of *Astragalus glycyphyllos* L.

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## Abstract

Establishment of *in vitro* cultures from *Astragalus glycyphyllos*, determination of biomass and analysis of total flavonoids, rutin and camelliaside A were performed. To increase flavonoid production various combinations of plant hormones and light/dark regimen were investigated. Suspension cultures with exogenous quercetin were evaluated for possible increase in flavonoid production. Shoots, calli and suspensions were successfully established. Rutin and camelliaside A were proved in highest amount in shoots. Calli, cultivated on modified G48 medium, with double amount of Ca<sup>2+</sup> and Mg<sup>2+</sup>, achieved higher total flavonoid content (2.37 and 2.03 mg/g DW). Suspensions cultures, cultivated on modified G48 medium with 10, 20 and 30 mg/mL quercetin achieved higher total flavonoid content (0.09, 0.10 and 0.13 mg/mg DW). Biotransformation of quercetin to isoquercitrin was achieved. The highest concentration of isoquercitrin (56.73 ng/mg DW) was observed on suspensions cultures cultivated on modified G48 medium, with 20 mg/mL quercetin.

## Keywords

*Astragalus glycyphyllos*, flavonoids, *in vitro* cultures

## Introduction

Plant biotechnology approaches could be a successful tool for increasing the yield of pharmaceutically important metabolites such as various flavonoids. *In vitro* derived cultures can be used as an alternative for meeting out the demand of secondary metabolites within reasonable time and obtain them in large amount.

*Astragalus glycyphyllos* L. (Fabaceae) is a perennial, herbaceous plant, native to Europe and extensively used in Bulgarian folk medicine (Shkondrov et al. 2019). The traditional therapeutic significance of *A. glycyphyllos* in a decoction form has been described: emollient and diuretic activities, in cases of gastroenteritis and hypertension; in urolithiasis, oliguria, scrofula, dermatitis, as a laxative, as an expectorant in acute respiratory diseases;

for the treatment of rheumatism, dermatitis, to stimulate labour and accelerate separation of the placenta in gynaecology (Belous 2005; Lysiuk and Darmohray 2016). Previous phytochemical research of the species revealed that it accumulates polysaccharides, saponins and flavonoids. It was proved that the aerial parts of the plant contained cosmosin, astragalinalin and isorhamnetin-3-*O*-glucoside (Krasteva et al. 2016) and the rare triglycoside camelliaside A (Shkondrov et al. 2018). Flavonoids isolated from genus *Astragalus* have been demonstrated to possess antioxidant, hepatoprotective, antimicrobial, antidiabetic, anti-inflammatory as well as other pharmacological activities (Bratkov et al. 2016).

Thus, the object of this study was to establish *in vitro* cultures (callus, suspensions and shoots) from *A. glycyphyllos* as well as to determine the influence of modified

culture medium on the production of the total flavonoids content and especially on rutin and camelliaside A by means of LC-MS analysis.

## Materials and methods

### Plant material and *in vitro* cultivation

*A. glycyphyllos* seeds were obtained from Vitosha Mountain in August 2018. The seeds were sterilized using standard procedure (Ionkova et al. 2010) and germinated on DoH medium (MS, supplemented with casein 1 g/L and sucrose 20 g/L). After 30 days of cultivation, the seeds were growing.

For establishment of shoots, seedlings were incubated under sterile conditions, in flasks with solid MS culture medium Murashige and Skoog (1962) on light regimen of cultivation. Every four weeks shoots were transferred on a fresh medium.

Callus cultures were obtained when shoot explants were cultivated on G48 medium (Ionkova et al. 2010) and G56 medium (MS, supplemented with casein 1g/L, kinetin 2 mg/L and indoleacetic acid 1 mg/L) in dark and light regimen. Every three weeks the calli were transferred in fresh medium.

Actively growing calli were transferred to G48 liquid medium (G48 medium without agar-agar) under light regimen of cultivation and transferred to fresh medium every two weeks. Thus, suspension cultures of *A. glycyphyllos* were obtained.

### Increased flavonoid production

Due to the low quantity of flavonoids in callus and suspension cultures, experiments were made in order to increase the production of flavonoids. For that reason callus cultures were cultivated on modified G48 medium in light regimen of cultivation supplemented with various concentrations of  $\text{Ca}^{2+}$  (G48 +  $\frac{1}{2}\text{Ca}^{2+}$  220 mg/L and G48 +  $2\text{Ca}^{2+}$  880 mg/L) and  $\text{Mg}^{2+}$  (G48 +  $\frac{1}{2}\text{Mg}^{2+}$  185 mg/L and G48 +  $2\text{Mg}^{2+}$  740 mg/L). For increasing the production of flavonoids within suspension cultures, at the first day of cultivation sterile quercetin solution was applied in three concentrations (10, 20 and 30 mg/mL) under aseptic conditions. Each concentration level consists of three randomized samples and the results are the average of three replicates. In addition, three controls were cultivated without addition of substrate.

### Extraction of *in vitro* cultures

The air-dried powdered plant material (0.20 g) was exhaustively extracted with 80% methanol under reflux ( $3 \times 50$  mL), the extracts were filtered and concentrated under reduced pressure. The residue was dissolved in 50 mL water and extracted with ethylacetate ( $3 \times 50$  mL), and then the ethylacetate fractions were dried and con-

centrated under reduced pressure. For the purpose of the analysis, those samples were diluted in 80% methanol to reach concentration of 500  $\mu\text{g}/\text{mL}$ . The extraction of the plant material for individual quantitation of rutin and camelliaside A was performed as reported before (Shkondrov et al. 2019). The extracts were kept at  $-18^\circ\text{C}$ , before the LC-HRESIMS analysis.

### Chromatographic methods

For the individual analysis of rutin and camelliaside a method, previously described (Shkondrov et al. 2019) was applied. The total flavonoid content was determined by a gradient elution: 0 to 3 min 83% (A), 3 to 7 min 80% (A), 7 to 10 min 70% (A), 10 to 20 min 50% (A), 20 to 25 min 40% (A), 25 to 28 min 30% (A), 30 to 35 min 0% (A), 35 to 38 min 90% (A).

UPLC separations for determination of total flavonoid content were performed on a Hypersil Gold C18 column ( $1.9 \mu\text{m}$ ,  $2.1 \times 50$  mm, Thermo Fisher Scientific, USA) at  $30^\circ\text{C}$ . For the quantitation of rutin and camelliaside A, a Kromasyl Eternity C<sub>18</sub> column ( $1.9 \mu\text{m}$ ,  $2.1 \times 50$  mm, Akzo Nobel, Sweden) at  $30^\circ\text{C}$  was used. Analyses were performed with HPLC grade 0.1% formic acid (A) and acetonitrile (B) at flow rate 0.3 mL/min.

HRESIMS spectra were taken with a LC-MS system consisting of a Q Exactive Plus Orbitrap mass spectrometer with a HRESI ion source (Thermo Fisher Scientific, Bremen, Germany) used in ultra-high resolution mode (70 000, at  $m/z$  200). An UHPLC system (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific, Bremen, Germany) was coupled to the mass spectrometer. The operating conditions of the HRESI source ionization device were: 3.5 kV voltage and  $320^\circ\text{C}$  capillary temperature, 25 units of carrier gas flow and 5 units of dry gas flow. All other detector parameters were set in such a way as to obtain the most intense signal from  $[\text{M}-\text{H}]^-$ . Nitrogen was used to atomize the samples.

### Stock solutions

Rutin dihydrate CRS (Sigma-Aldrich, Germany) and camelliaside A (isolated previously, 99.8% purity, Shkondrov et al. 2018) were selected as external standards for the quantitative analysis of individual flavonoids. The calibration for assay of individual flavonoids was based on three concentration levels, ranging from 25 ng/mL to 1014 ng/mL for rutin and from 18.30 ng/mL to 1830 ng/mL for camelliaside A, both in the negative mode.

Determination of total flavonoids was based on calibration curve of rutin: four different concentrations ranging from 0.12  $\mu\text{g}/\text{mL}$  to 15.55  $\mu\text{g}/\text{mL}$  analysed in triplicate in negative mode.

The total flavonoid amount was quantified using the formula:

$$\text{Total flavonoids} = (A_{\text{fl}} * C_{\text{st}}) / (A_{\text{st}} * W_s),$$

$A_{fl}$  – a peak area of deprotonated molecular ions corresponding to flavonoid derivatives based on their MS spectra, Table 1 (Cuyckens and Claeys 2004),  $A_{st}$  – a peak area of a standard,  $C_{st}$  – concentration of the standard,  $W_s$  – weight of the sample.

**Table 1.** Deprotonated molecular ions observed in LC/MS spectra of callus cultures of *A.glycyphyllos* used for determination of total flavonoids.

| Peak | Formula   | $t_R$                      | MW       | $m/z$ [M-H] <sup>-</sup> |
|------|---|----------------------------|----------|--------------------------|
| 1    | C <sub>15</sub> H <sub>18</sub> O <sub>8</sub>  | 2.16                       | 326.1001 | 325.0932                 |
| 2    | C <sub>21</sub> H <sub>28</sub> O <sub>13</sub> | 2.54                       | 488.1529 | 487.1464                 |
| 3    | C <sub>16</sub> H <sub>20</sub> O <sub>9</sub>  | 2.72, 3.04*                | 356.1107 | 355.1040                 |
| 4    | C <sub>16</sub> H <sub>28</sub> O <sub>10</sub> | 3.52, 3.84*                | 380.1682 | 379.1615                 |
| 5    | C <sub>21</sub> H <sub>22</sub> O <sub>11</sub> | 3.14                       | 450.1162 | 449.1094                 |
| 6    | C <sub>22</sub> H <sub>24</sub> O <sub>12</sub> | 3.47                       | 480.1267 | 479.1202                 |
| 7    | C <sub>16</sub> H <sub>16</sub> O <sub>7</sub>  | 4.28                       | 320.0896 | 319.0827                 |
| 8    | C <sub>17</sub> H <sub>30</sub> O <sub>10</sub> | 5.06, 5.33*                | 394.1839 | 393.1773                 |
| 9    | C <sub>22</sub> H <sub>28</sub> O <sub>9</sub>  | 3.86                       | 436.1733 | 435.1665                 |
| 10   | C <sub>24</sub> H <sub>26</sub> O <sub>13</sub> | 4.21, 4.65*, 7.62*, 10.37* | 522.1373 | 521.1308                 |
| 11   | C <sub>19</sub> H <sub>26</sub> O <sub>10</sub> | 4.37                       | 414.1526 | 413.1460                 |
| 12   | C <sub>22</sub> H <sub>24</sub> O <sub>11</sub> | 4.55, 4.78*, 6.14*, 6.40*  | 464.1318 | 463.1254                 |
| 13   | C <sub>22</sub> H <sub>22</sub> O <sub>12</sub> | 4.85, 6.58*, 6.77*, 11.88* | 478.1111 | 477.1046                 |
| 14   | C <sub>23</sub> H <sub>24</sub> O <sub>13</sub> | 5.27, 8.63*                | 508.1217 | 507.1150                 |
| 15   | C <sub>28</sub> H <sub>36</sub> O <sub>13</sub> | 5.7                        | 580.2155 | 579.2088                 |
| 16   | C <sub>22</sub> H <sub>22</sub> O <sub>11</sub> | 8.59                       | 462.1162 | 461.1097                 |
| 17   | C <sub>29</sub> H <sub>38</sub> O <sub>15</sub> | 6.02                       | 626.2210 | 625.2148                 |
| 18   | C <sub>21</sub> H <sub>20</sub> O <sub>10</sub> | 3.25, 5.52*, 6.44*         | 432.1056 | 431.0989                 |
| 19   | C <sub>30</sub> H <sub>38</sub> O <sub>14</sub> | 6.53                       | 622.2261 | 621.2199                 |
| 20   | C <sub>25</sub> H <sub>34</sub> O <sub>14</sub> | 6.63                       | 558.1948 | 557.1885                 |
| 21   | C <sub>16</sub> H <sub>12</sub> O <sub>6</sub>  | 6.96                       | 300.0633 | 299.0564                 |
| 22   | C <sub>23</sub> H <sub>24</sub> O <sub>11</sub> | 7.15                       | 476.1318 | 475.1251                 |
| 23   | C <sub>23</sub> H <sub>38</sub> O <sub>14</sub> | 7.28, 7.46*, 7.74*, 8.07*  | 538.2261 | 537.2197                 |
| 24   | C <sub>26</sub> H <sub>34</sub> O <sub>11</sub> | 4.21, 4.66*                | 522.2101 | 521.2035                 |
| 25   | C <sub>24</sub> H <sub>24</sub> O <sub>13</sub> | 7.85                       | 520.1216 | 519.1153                 |
| 26   | C <sub>24</sub> H <sub>24</sub> O <sub>12</sub> | 9.70, 9.97*                | 504.1267 | 503.1204                 |
| 27   | C <sub>23</sub> H <sub>24</sub> O <sub>12</sub> | 10.14                      | 492.1267 | 491.1201                 |
| 28   | C <sub>23</sub> H <sub>22</sub> O <sub>13</sub> | 10.87                      | 506.1060 | 505.0996                 |
| 29   | C <sub>29</sub> H <sub>36</sub> O <sub>14</sub> | 11.31                      | 608.2105 | 607.2044                 |

\* Isomers

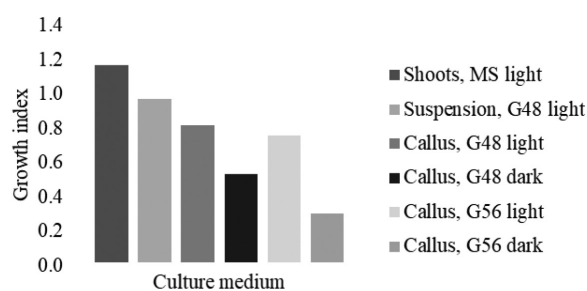
## Statistical processing

Each experiment was done in triplicate. Results were expressed as mean  $\pm$  SD. MedCalc 12.3 (MedCalc Software 2012) was used. The one-way analysis of variance was performed to define the statistical significance of the amount found. Probability values of  $p \leq 0.05$  were accepted as statistically significant.

## Results and discussion

### Determination of growth index, rutin and camelliaside A content

The highest amount of biomass accumulation ( $GI = 1.17 \pm 0.04$ ) of the plant cells of *A. glycyphyllos* was observed on shoot cultures grown on MS medium, while the lowest amount ( $GI = 0.30 \pm 0.03$ ) was detected on calli grown on G56 medium on dark regimen of cultivation. (Fig. 1). Calli cultivated on G48 culture medium reached  $GI = 0.82 \pm 0.03$  under light and  $GI = 0.53 \pm 0.04$



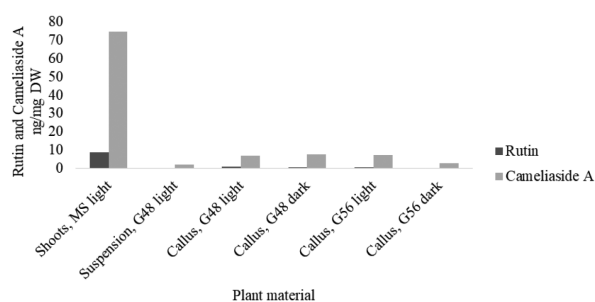
**Figure 1.** Growth index of in vitro cultures of *A. glycyphyllos*.

when cultivated in dark for three weeks. Callus cultures grown on G56 reached  $GI = 0.76 \pm 0.04$  under the light regimen of cultivation and suspension cultures shown  $GI = 0.97 \pm 0.02$  for two weeks of cultivation.

Rutin was observed in the negative ion mode as a deprotonated ion [M-H]<sup>-</sup> with  $m/z$  609.1454 (calc. 609.1456) at  $t_R = 5.71 \pm 0.02$  min and corresponding formula C<sub>27</sub>H<sub>29</sub>O<sub>16</sub>. Camelliaside A was identified as a deprotonated ion [M-H]<sup>-</sup> with  $m/z$  755.2039 (calc. 755.2035) at  $t_R = 4.98 \pm 0.01$  min and corresponding formula of C<sub>33</sub>H<sub>39</sub>O<sub>20</sub>.

A visual evaluation of the linear regression line plots showed that the method was linear for both standards. The determination coefficient for rutin was  $r^2 = 0.9994$  and for camelliaside A  $r^2 = 0.9996$ .

Rutin ( $8.72 \pm 0.09$  ng/mg DW) and camelliaside A content ( $74.65 \pm 0.09$  ng/mg DW) were proved in the highest amount in shoot cultures on MS medium. Camelliaside A was observed in the lowest amount ( $2.19 \pm 0.09$  ng/mg DW) in suspension cultures grown on G48 medium. Rutin was not detected in calli grown on G56 medium in the dark (Fig. 2).



**Figure 2.** Rutin and Camelliaside A content in in vitro cultures of *A. glycyphyllos*.

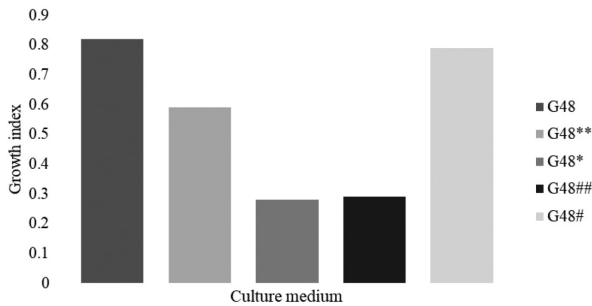
The quantity of rutin reached  $0.39 \pm 0.04$  ng/mg DW in suspension cultures, while callus, cultivated on G48 medium achieved  $0.95 \pm 0.05$  ng/mg DW under light regimen of cultivation and  $0.50 \pm 0.06$  ng/mg DW when cultivated in the dark. Callus cultivated on G56 medium in light achieved  $0.73 \pm 0.05$  ng/mg DW rutin content.

The quantity of camelliaside A reached  $7.30 \pm 0.11$  ng/mg DW in calli, cultivated on G56 medium in the light and  $2.88 \pm 0.08$  ng/mg DW when cultivated on G56 in dark. Callus cultures cultivated on G48 medium achieved

$6.93 \pm 0.12$  ng/mg DW camelliaside A content under light and  $7.60 \pm 0.10$  ng/mg DW when cultivated in dark.

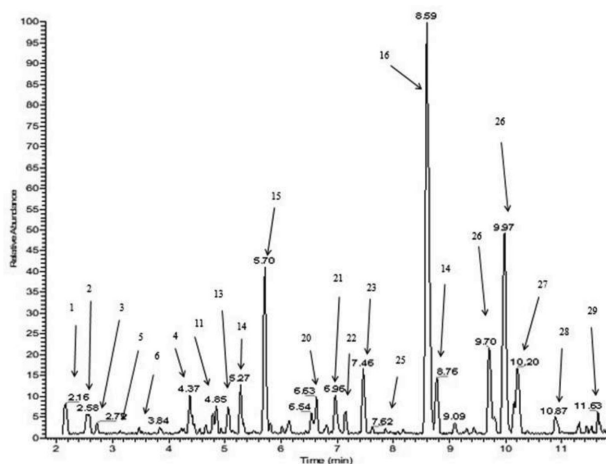
Determination of growth index and total flavonoids content in callus cultures, cultivated on modified MS media supplemented with half and double the amount of  $Mg^{2+}$  and  $Ca^{2+}$

The highest amount of biomass (GI =  $0.82 \pm 0.03$ ) was observed on callus cultures of *A. glycyphyllos* cultivated on G48 medium (control), while the lowest amount (GI =  $0.28 \pm 0.01$ ) was detected on calli established on G48  $\frac{1}{2}Ca^{2+}$  medium (Fig. 3). Calli cultivated on G48 + 2  $Ca^{2+}$  culture medium reached GI =  $0.59 \pm 0.08$ . Callus cultures grown on G48 + 2  $Mg^{2+}$  and G48 +  $\frac{1}{2}Mg^{2+}$  medium reached  $0.29 \pm 0.05$  and  $0.79 \pm 0.05$  amount of biomass, respectively. Therefore, the change in the concentrations of calcium and magnesium salts did not affect callus biomass accumulation.



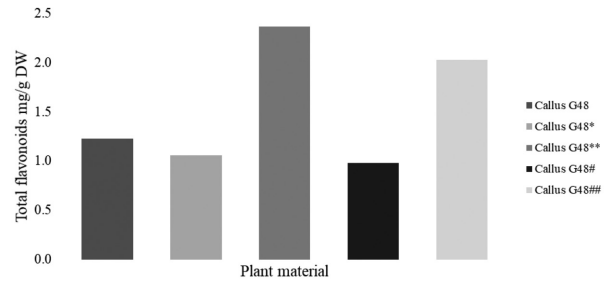
**Figure 3.** Growth index of callus cultures of *A. glycyphyllos*, cultivated on culture mediums with half and double amount of  $Mg^{2+}$  and  $Ca^{2+}$ .

Rutin was observed in the positive ion mode as protonated ion  $[M-H]^+$  with  $m/z$  609.14661 (calc. 609.14560) at  $t_R = 4.40 \pm 0.02$  min and corresponding formula  $C_{27}H_{29}O_{16}$ . A visual evaluation of the linear regression line plots showed that the method was linear. The determination coefficient for rutin was  $r^2 = 0.9989$ . The LC/MS chromatogram of the total flavonoids is shown in Fig. 4.



**Figure 4.** LC-MS chromatogram of flavonoids from callus cultures of *A. glycyphyllos*.

The highest concentration of flavonoids content ( $2.37 \pm 1.08$  mg/g DW) was observed on callus cultures cultivated on G48 + 2  $Ca^{2+}$  medium, while the lowest concentration ( $0.98 \pm 0.85$  mg/g DW) was detected on calli established on G48 +  $\frac{1}{2}Mg^{2+}$  medium (Fig. 5). The quantity of total flavonoids on calli cultivated on G48 (control), G48 +  $\frac{1}{2}Ca^{2+}$  and G48 + 2  $Mg^{2+}$  culture medium are  $1.23 \pm 2.47$  mg/g DW,  $1.06 \pm 0.12$  mg/g DW and  $2.03 \pm 0.69$  mg/g DW, respectively. Therefore, the double increasing the concentration of  $Ca^{2+}$  and  $Mg^{2+}$  increased the total flavonoid contents in callus cultures of *A. glycyphyllos* compared to the control.

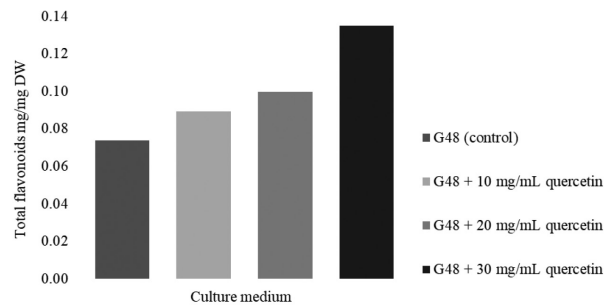


**Figure 5.** Total flavonoid contents (mg/g DW) of callus cultures of *A. glycyphyllos*, cultivated on culture mediums with half and double amount of  $Mg^{2+}$  and  $Ca^{2+}$  under light regimen of cultivation.

### Determination of total flavonoid content in suspension cultures, cultivated on G48 medium, supplemented with 10, 20 and 30 mg/mL quercetin

The addition of quercetin to the G48 medium of suspension cultures of the plant increased the quantity of total flavonoids (Fig. 6). The highest concentration of total flavonoids content ( $0.13$  mg/mg DW) was observed on suspension cultures cultivated on G48 medium, supplemented with 30 mg/mL quercetin, when compared to the untreated control ( $0.07$  mg/mg DW). The total flavonoid contents on suspension cultures, cultivated on G48 medium, supplemented with 10 mg/mL and 20 mg/mL quercetin are  $0.09$  mg/mL DW and  $0.10$  mg/mg DW, respectively.

In addition, biotransformation of quercetin to its monoglycosylated derivative isoquercitrin was obser-



**Figure 6.** Total flavonoid contents (mg/mg DW) in suspension cultures of *A. glycyphyllos*, cultivated on G48 medium, supplemented with 10, 20 and 30 mg/mL quercetin.

ved (Bhatt and Shah 2019). Quercetin *O*-glycoside that is quercetin substituted by an  $\alpha$ -L-rhamnosyl moiety at position 3 *via* a glycosidic linkage was detected as protonated molecular ion  $[M+H]^+$  with  $m/z$  465.10187 (calc. 465.10275) at  $t_R$  5.02 min and corresponding molecular formula  $C_{21}H_{21}O_{12}$ . The highest concentration of isoquercitrin (56.73 ng/mg DW) was observed on suspension cultures cultivated on G48 medium, supplemented with 20 mg/mL quercetin, when compared to the untreated control where the concentration of this metabolite was 19.63 ng/mg DW.

## Conclusion

The medicinal uses of *A. glycyphyllos* and its depletion are the main reasons to apply biotechnological techniques for producing of important flavonoids. The flavonoid

production in cell cultures can be enhanced by varying of the components of the culture mediums. The results from current study demonstrate the potential of biotechnology to induce a flavonoid biosynthesis as a source of pharmaceutically important metabolites such as rutin and camelliaside A. In addition, biotransformation of quercetin to its 3-*O*-glucosyl derivative using *A. glycyphyllos* has been reported. The availability of protocols to establish *in vitro* cultures will allow further studies on secondary metabolism aimed at increasing the production of new compounds of interest.

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