SHORT REPORT

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Antiproliferative Activities of Chemical Constituents Isolated from *Thymus praecox* subsp. *grossheimii* (Ronniger) Jalas

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Abstract: *Thymus praecox* subsp. *grossheimii* (Ronniger) Jalas is (TPGJ) an aromatic and medicinal plant used as folk medicine and exhibits a variety of biological activities. Aerial part of plant material was boiled in water then extracted with hexane and ethyl acetate sequentially. Flash column chromatography (Sephadex LH-20) and HPLC were used for ethy acetate extract to isolate rosmarinic acid (**1**), apigenin 7-*O*-glucoside (**2**), chrysoeriol (**3**), apigenin (**4**), naringenin (**5**), eriodictiol (**6**), luteolin **(7)** and globoidnan A **(8)**. The structures of isolated compounds were elucidated by spectroscopic techniques basically 1D, 2D-NMR and LC-TOF/MS/MS. Antiproliferative activity, cytotoxicity of compounds and extract were investigated *in vitro* on C6 (rat brain tumor), HeLa (human cervix carcinoma), HT29 (human colon carcinoma) and Vero (African green monkey kidney epithelium) cells lines by using BrdU cell proliferation ELISA and lactate dehydrogenase (LDH) assays. Extract and some compounds exhibited significant antiproliferative effects against various cancerous cell lines.

Keywords: *Thymus praecox* subsp. *grossheimii* (Ronniger) Jalas; antiproliferative activity.

1. Plant Source

The *Thymus* genus including nearly 928 species is mostly found in the Mediterranean region, Asia, northern Europe as well as other parts of the world such as Asia, South America, and Australia [1]. This genus is represented by 41 species in Turkey, 24 of which are endemic [2].

The aerial parts of TPGJ was collected at the flowering stage in June 2014 from Tokat, Turkey and identified by Prof. Dr. Ozgur Eminagaoglu, Artvin University, Faculty of Forest Engineering where a voucher specimen was deposited (ARTH:5361).

2. Previous Studies

Thymus flowers and leaves have been widely used as traditional medicine to alleviate several illnesses such as cough, headaches and stomachache, treatment of antiviral, immune stimulatory effect as well as flavouring agent in many kinds of food products [3,4]. Phytochemical studies on *Thymus* species revealed the isolation of polyphenols [5], flavonoids [6], steroids [6], monoterpene glucosides [7].

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Although a plenty of works were carried out about the essential oils and extracts of other species of *Thymus*, only a work was recently reported about the phytochemical studies and quantitative analysis of this species (TPGJ) [8]. Herein, we isolated and elucidated two flavonones, three flavones, a flavone glucoside, one phenolic acid and a lignan from this species (Figure 1). Antiproliferative activity of isolated compounds and extract were tested *in vitro* on C6, HeLa, HT29, and Vero cells. Moreover, cytotoxicities of these compounds were presented.

3. Present Study

The aerial parts of the plant material (1.0 kg) were boiled in distilled water for 2 h [9]. The aqueous part was sequentially extracted with hexane and ethyl acetate. The ethyl acetate extract was chromatographed on Sephadex LH-20 to yield 100 fractions. The fractions including compounds which have the same R_f value were combined. Fractions 46-50 yielded the rosmarinic acid **(1)** (75 mg) [10] and apigenin 7-*O*-glucoside **(2)** (15 mg) [11], fractions 55-64 were collected then purified by preparative HPLC afforded the chrysoeriol **(3)** (10 mg) [12], apigenin **(4)** (12 mg) [13], naringenin **(5)** (10 mg) [14] and eriodictyol **(6)** (11 mg) [15] respectively, fractions 67-70 yielded the compound of luteolin **(7)** (35 mg) [16-18], fractions 75-81 were collected then purified by preparavite HPLC yielded the globoidnan A **(8)** (10 mg) [19] (Figure 1).

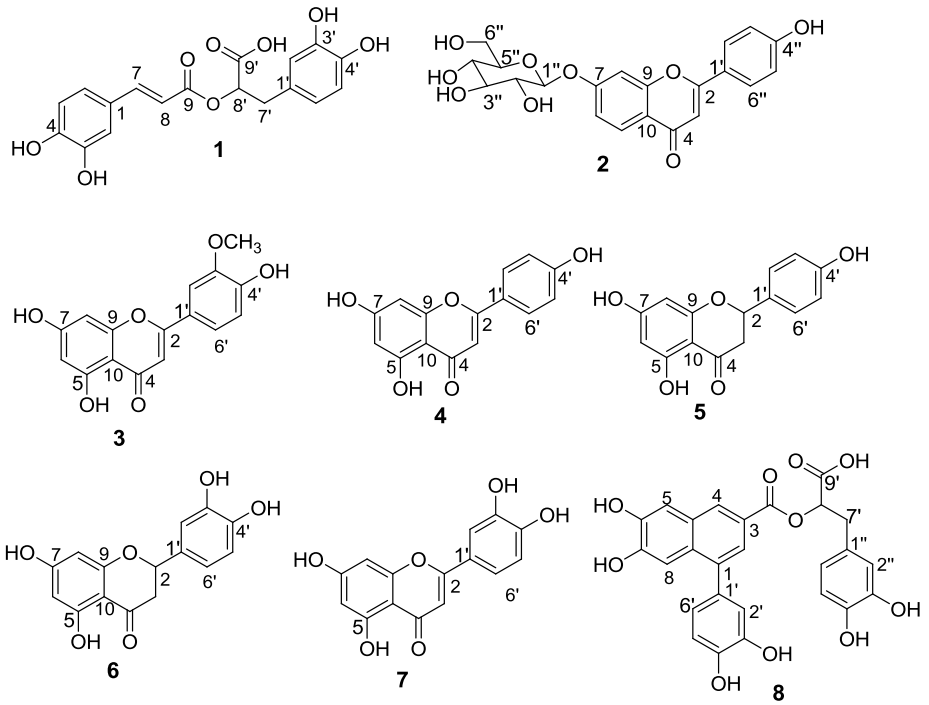

Figure 1. Structure of isolated compounds from *T. praecox* subsp. *grossheimii*

*Rosmarinic acid **(1)**:* LC-TOF/MS: *m/z* 359.0836 $[M-H]^-$ (calcd. 359.0776 for $C_{18}H_{15}O_8$); ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 2.94 (1H, d, *J*= 10 Hz, H-7b',), 2.98 (1H, dd, *J*= 10.1 Hz, *J*= 1.10 Hz, H- 7a'), 5.08 (1H, dd, *J*= 2.8 Hz, *J*= 10 Hz H-8'), 6.24 (1H, d, *J*= 15.8 Hz H-8), 6.54 (1H, d, *J*= 7.92 Hz, H-5'), 6.65 (1H, d, *J*= 7.92 Hz, H-6'), 6.69 (1H, d, *J*= 1.5 Hz, H-2'), 6.79 (1H, d, *J*= 8.12 Hz, H-5), 7.02 (1H, d, *J*= 8.12 Hz, H-6), 7.06 (1H, d, *J*= 1.4 Hz, H-2), 7.46 (1H, d, *J*= 15.8 Hz, H-7); ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 36.6 (C-7'), 73.2 (C-8'), 113.6 (C-8), 115.2 (C- 2), 120.5 (C-5'), 116.2 (C-5), 117.2 (C-2'), 115.8 (C-6'), 122.0 (C-6), 125.9 (C-1), 127.7 (C-1'), 145.4 (C-4'), 144.5 (C-3'), 146.4 (C-7), 149.1 (C-3), 146.0 (C-4), 166.4 (C-9), 171.3 (C-9').

*Apigenin 7-O-glucoside **(2)***: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 3.13 (1H, m, H-6a"), 3.31 (1H, m, H-6b"), 3.29 (1H, m, H-3"), 3.33 (1H, m, H-5"), 3.38 (1H, m, H-2"), 4.02 (1H, d, *J* = 9.2

Hz,H-4"), 5.06 (1H,d, *J* = 7.24 Hz, H-1") 6.46 (1H, d, *J* = 1.5 Hz, H-6), 6.86 (1H, s, H-3), 6.86 (1H, d, *J* = 1.5 Hz, H-8), 6.94 (1H, d, *J* = 8.64 Hz, H-3' and H-5'), 7.96 (1H, d, *J* = 8.64 Hz, H-2' and H-6'); ^{13}C NMR (100 MHz, DMSO-d_6) : δ (ppm) = 61.1 (C-6a"), 70.0 (C-2"), 73.6 (C-3") ,77.0 (C-4"), 77.7 (C-5"), 95.1 (C-8), 99.9 (C-6), 100.4 (C-1"), 103.6 (C-3), 109.9 (C-10), 116.5 (C-3' and C-5'), 121.4 (C-1') 129.1 (C-2' and C-6'), 157.4 (C-9), 161.9 (C-5), 163.7 (C-7), 164.8 (C-2), 182.5 (C-4.

*Chrysoeriol (**3**):* LC-TOF/MS: *m/z* 299.0525 $[M-H]^-$ (calcd. 299.0556 for $C_{16}H_{11}O_6$); ^{1}H NMR (400MHz, DMSO-d_6): δ (ppm) = 6.20 (1H, d, *J*= 2.1Hz, 6-H), 6.52 (1H, d, *J*= 2.1Hz, 8-H), 6.69 (1H, s, 3-H), 6.94 (1H, d, *J*= 8.3 Hz, 5' -H), 7.56 (1H, dd, *J*= 8.3 ,2.3, H-6'), 7.55 (1H, d, *J*=2.3, H-2'), 3.89 (1H, s, 4'-OCH3); ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.2 (C-4), 164.9 (C-7), 164.1 (C-2), 161.9 (C-5), 157.8 (C-9), 150.3 (C-4'), 148.5 (C-3'), 121.9 (C-6'), 120.8 (C-1'), 116.2 (C-5'), 110.6 (C-2'), 104.0 (C-10), 103.62 (C-3), 99.4 (C-6), 94.5 (C-8), 56.4 (3'- OCH3).

*Apigenin (**4**):* LC-TOF/MS: *m/z* 269.0455 $[M-H]^-$ (calcd. 269.0450 for $C_{15}H_9O_5$); ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 7.92 (2H, d, *J* = 8.7 Hz, H-2', H-6'), 6.92 (2H, d, *J* = 8.7 Hz, H-3', H-5'), 6.49 (1H, d, *J* = 2.1 Hz, H-6), 6.20 (1H, d, *J* = 2.1 Hz, H-8), 6.78 (1H, s, H-3); ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.2 (C-4), 161.9 (C-5), 164.3 (C-2), 161.7 (C-4'), 157.7 (C-9), 164.3(C-7), 128.9 (C-2', C-6'), 121.6 (C-1'), 116.46 (C-3', C-5'), 104.1 (C-10), 103.2 (C-3), 99.4 (C-6), 94.47 (C-8).

*Naringenin (**5**):* LC-TOF/MS: *m/z* 271.0612 $[M-H]^-$ (calcd. 271.0606 for $C_{15}H_{11}O_5$); ^{1}H NMR (400 MHz DMSO-d_6): δ (ppm) = 7.32 (2H, d, *J* = 8.36 H-2', H-6'), 6.90 (2H, d, *J* = 8.36 Hz, H-3', H-5'), 5.87 (1H, s, H-8), 5.95 (1H, s, H-6), 5.45 (1H, dd, *J* = 12.74, 2.70 Hz, H-2), 3.25 (1H, dd, *J* = 17.10, 12.74 Hz, H-3b), 2.67 (1H, dd, *J* = 17.10, 2.70 Hz, H-3a); ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.2 (C-4), 161.9 (C-5), 164.3 (C-2), 161.7 (C-4'), 157.7 (C-9), 164.3(C-7), 128.9 (C-2', C-6'), 121.6 (C-1'), 116.46 (C-3', C-5'), 104.1 (C-10), 103.2 (C-3), 99.4 (C-6), 94.47 (C-8).

*Eriodictyol (**6**):* LC-TOF/MS: *m/z* 287.0555 $[M-H]^-$ (calcd. 287.0556 for $C_{15}H_{11}O_6$); ^{1}H NMR (400 MHz DMSO-d_6): δ (ppm) = 5.36 (1H, dd, *J* = 2.46, 12.90 Hz, H-2), 2.65 (2H, dd, *J* = 2.90, 17.12 Hz, H-3a, H-3b), 5.87 (1H, s, *J* = H-6), 5.87 (1H, s, *J* = H-8), 6.87 (1H, s, H-2'), 6.74 (2H, s, H-5', H-6'); ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 78.9 (C-2), 42.5 (C-3), 196.6 (C-4), 163.9 (C-5), 96.3 (C-6), 167.6 (C-7), 95.5 (C-8), 163.3 (s, C-9), 102.1 (C-10), 129.9 (C-1'), 114.8 (C-2'), 145.7 (C-3'), 146.2 (C-4'), 115.8 (C-5'), 118.6 (C-6').

*Luteolin (**7**):* LC-TOF/MS: *m/z* 285.0329 $[M-H]^-$ (calcd. 285.0399 for $C_{15}H_9O_6$); ^{1}H NMR (400 MHz DMSO-d_6): δ (ppm) = 7.43 (1H, d, H-2', *J*= 2.2 Hz), 7.40 (1H, dd, H-6', *J*= 8.1 Hz, *J*= 2.2 Hz), 6.90 (1H, d, H-5', *J*= 8.1 Hz), 6.67 (1H, s, H-3), 6.45 (1H, d, H-8, *J*= 2.0 Hz), 6.20 (1H, d, H-6, *J*= 2.0 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) =182.1 (C-4), 164.6 (C-7), 164.4 (C-2), 161.9 (C-5), 157.8 (C-9), 150.2 (C-4'), 146.2 (C-3'), 122.0 (C-1'), 119.4 (C-6'), 116.5 (C-5'), 113.8 (C-2'), 104.2 (C-10), 103.3 (C-3), 99.3 (C-6), 94.3 (C-8).

*Globoidnan A (**8**):* LC-TOF/MS: *m/z* 491.0913 $[M-H]^-$ (calcd. 491.0978 for $C_{26}H_{19}O_{10}$); ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 3.03-3.10 (2H, m, H-7"), 5.23(1H, dd, *j*=4.44, 7.68 Hz, 8"), 6.64 (1H, brs, H-2"), 6.73 (1H, dd, *J* = 1.70, 8.00 Hz, H-6"), 6.77 (1H, brs, H-3"), 6.85 (1H, s, H-2'), 6.92 (1H, d, *J* = 8.00 Hz, H-5'), 7.27 (1H, s, H-8), 7.36 (1H, s, H-5), 7.51 (1H, s, H-2), 8.23 1H, s, H-4); ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 36.7 (C-7"), 73.9 (C-8"), 108.5 (C-8), 112.0 (C-5), 115.9 (C-6"), 116.3 (C-5'), 117.3 (C-2'), 117.2 (C-3'), 120.7 (C-2"), 120.9 (C-6'), 123.0 (C-2), 123.5 (C-3), 127.9 (C-1"), 128.1 (C-4), 128.8 (C-1), 130.2 (C-4a), 131.7 (C-1'), 138.7 (C-8a), 144.5 (C-4"), 144.6 (C-5"), 145.4 (C-3'), 145.5 (C-4'), 147.9 (C-6), 148.0 (C-7), 166.2 (C-9), 171.3 (C-9").

Rosmarinic acid is commonly found in Lamiaceae family, especially *Mentha* species [20]. It has a variety of biological properties including antioxidant, anti-inflammatory, antiviral, antibacterial effects [21]. Moreover, it is also used as a fragrant additive in cosmetics [22] and a food additive to keep food fresh [23]. The other isolated compounds are mostly existed in plants and are well known secondary metabolites [24].

Antiproliferative assays: Antiproliferative effects of isolated compounds and ethyl acetate extract as well as cytotoxicity tests were carried out. A good antiproliferative material is preferred to have high antiproliferative effect and low cytotoxicity which leading to no harm to normal cells. Therefore,

cytotoxicity tests are generally carried out to determine toxicity of material. In this work, Percent inhibition was reported as mean values ±SEM of three independent assays ($P < 0.05$) for all tests. Chrysoeriol (**3**), globoidnan A (**8**) and ethyl acetate extract revealed better antiproliferative effect than that of the standard (5-FU) on C6 cell lines and rosmarinic acid (**1**) exhibited as good activity as standard at low concentration. However, noticeable effect was not detected for the other compounds on this cell lines. Chrysoeriol (**3**), globoidnan A (**8**) and extract had also excellent effect on HeLa cell lines. In addition, rosmarinic acid (**1**) showed the same activity with 5-FU. Rosmarinic acid (**1**), apigenin 7-*O*-glucoside (**2**), chrysoeriol (**3**), globoidnan A **(8)** and extract displayed outstanding ability to inhibit the proliferation of HT29 cell lines. Still, compounds, **4**, **5** and **6** had not noteworthy activity on HT29 cell lines. Same trends were observed on Vero cells. This results indicated that rosmarinic acid (**1**), chrysoeriol (**3**), globoidnan A (**8**) and extract had selectively effect on cancerous cells (S 40). The activities of extract could be attributed to the synergic effect of the compounds into the extract. Chrysoeriol (**3**) exhibiting the activities bore methoxy group but apigenin (**4**) lack of methoxy did not reveal the activity. Hence, methoxy group was responsible for the activities.

Morphological assessment of the cytotoxic activity of compounds: Morphological assessment of each cell line treated with compounds and extract presented that some compounds have antiproliferative ability. Because treated cells showed characteristic features of apoptotic cell death. Figure 6 showed inverted microscope images of the morphology of untreated or treated C6, HeLa, HT29, and Vero cell lines with 5, 10, 20, 30, 40, 50, 75, and 100 µg/mL of the compounds of TPGJ. The result in figure 6 show the normal structure of numerous control cells growing in the medium, and also shows the same cells line following 24 hours of incubation with compounds and extract of TPGJ. However, according to this, the numbers of cells have importantly decreased and they look different structures from untreated cells. They have lost their astrocyte-like and fibroblast-like structures and they seem to be globular upon treatment. In addition, the treated C6 cells seem to shrink and they developed a round shape and they have lost their neuronal-like structure. They seem to clump together which is typical of apoptosis. Especially, at high concentrations (>50 µg/mL), the numbers of cells seem to lessen and separate from one another and they look smaller. The control groups showed entire confluent growth with healthy proliferating cells. Treatment with **1**, **3**, **8**, and extact showed important degradations on the cells, as the cell growth affected and cellular morphology was similar to that of apoptotic situations. Photomicrograph depicted the strong apoptotic effect of **1**, **3**, **8**, and extract at 30 µg/mL and higher doses for HeLa and HT29 cells. However, the picture revealed that, the treated C6 and Vero cells exhibited apoptotic cellular death at 50 µg/mL and higher doses. It can be seen clearly that the compounds affected the normal morphology of the cells and exhibited apoptotic cellular death, which is evident through the appearance of membrane blabbing and shrinkage, nuclear condensation and apoptotic bodies (S 41).

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

References

[1] S. M. Nabavi, A. Marchese, M. Izadi, V. Curti, M. Daglia and S. F. Nabavi (2015). Plants belonging to the genus *Thymus* as antibacterial agents: From farm to pharmacy, *Food Chem*. **173**, 339-347.

[2] I. Orhan, F. S. Senol, A. R. Gulpinar, M. Kartal, N. Sekeroglu, M. Deveci, Y. Kan and B. Sener (2009). Acetylcholinesterase inhibitory and antioxidant properties of *Cyclotrichium niveum*, *Thymus praecox* subsp *caucasicus* var. *caucasicus*, *Echinacea purpurea* and *E. pallida*, *Food Chem. Toxicol*. **47**, 1304-1310.

[3] M. S. Klaric, I. Kosalec, J. Mastelic, E. Pieckova and S. Pepeljnak (2007). Antifungal activity of thyme (*Thymus vulgaris* L.) essential oil and thymol against moulds from damp dwellings, *Lett. Appl. Microbio.* **44**, 36-42.

[4] C. O. Vandenbroucke and J. A. Lemli (1983). Spasmolytic Activity of the Flavonoids from *Thymus-Vulgaris*, *Pharm. Weekblad*. **5**, 9-14.

[5] S. Aziz, M. Irshad and Habib-ur-Rehman (2014). Isolation of a New Antibacterial Polyphenol from *Thymus serpyllum*, *Chem. Nat. Compd.* **49**, 1023-1027.
[6] S. Kulevanova, T. Stafilov, F. Anastasova, M. Ristic and D. Brkic (1997). Isolation and identification of flavonoid aglycones from some taxa of Sect. Marginati of genus *Thymus*, *Pharmazie* **52**, 886-888.
[7] Z. G. Lu and H. Takeuchi (2006). Synthesis and isolation of a new monoterpene glucoside from *thyme*, *Chinese J. Org. Chem.* **26**, 51-55.
[8] H. G. Sevindik, U. Ozgen, A. Atila, H. O. Er, C. Kazaz and H. Duman (2015). Phtytochemical Studies and Quantitative HPLC Analysis of Rosmarinic Acid and Luteolin 5-*O*-beta-*D*-Glucopyranoside on *Thymus praecox* subsp *grossheimii* var. *grossheimii*, *Chem. Pharm. Bull.* **63**, 720-725.
[9] I. Demirtas, R. Erenler, M. Elmastas and A. Goktasoglu (2013). Studies on the antioxidant potential of flavones of *Allium vineale* isolated from its water-soluble fraction, *Food Chem.* **136**, 34-40.
[10] Y. Lu and L. Y. Foo (1999). Rosmarinic acid derivatives from *Salvia officinalis*, *Phytochemistry* **51**, 91-94.
[11] S. S. Bhujbal, R. K. Nanda, R. S. Deoda, D. Kumar, S. M. Kewatkar, L. S. More and M. J. Patil (2010). Structure elucidation of a flavonoid glycoside from the roots of *Clerodendrum serratum* (L.) Moon, Lamiaceae, *Rev. Bras. De Farmacogn.* **20**, 1001-1002.
[12] D. Rinaldo, C. M. Rodrigues, J. Rodrigues, M. Sannomiya, L. C. Dos Santos and W. Vilegas (2007). New flavone from the leaves of *Neea theifera* (*Nyctaginaceae*), *J. Brazil. Chem. Soc.* **18**, 1132-1135.
[13] T. Ersoz, U. S. Harput, I. Saracoglu, I. Calis and Y. Ogihara (2002). Phenolic compounds from *Scutellaria pontica*, *Turk. J. Chem.* **26**, 581-588.
[14] F. Maltese, C. Erkelens, F. van der Kooy, Y. H. Choi and R. Verpoorte (2009). Identification of natural epimeric flavanone glycosides by NMR spectroscopy, *Food Chem.* **116**, 575-579.
[15] D. R. Encarnacion, C. L. Nogueiras, V. H. A. Salinas, U. Anthoni, P. H. Nielsen and C. Christophersen (1999). Isolation of eriodictyol identical with huazhongilexone from *Solanum hindsianum*, *Acta Chem. Scand.* **53**, 375-377.
[16] E. R. Woo and M. S. Piao (2004). Antioxidative constituents from *Lycopus lucidus*, *Arch. Pharm. Res.* **27**, 173-176.
[17] M. F. Wang, J. G. Li, M. Rangarajan, Y. Shao, E. J. LaVoie, T. C. Huang and C. T. Ho (1998). Antioxidative phenolic compounds from sage (*Salvia officinalis*), *J. Agr. Food Chem.* **46**, 4869-4873.
[18] M. Mizuno, M. Kato, M. Iinuma, T. Tanaka, A. Kimura, H. Ohashi and H. Sakai (1987). Acylated Luteolin Glucosides from Salix-Gilgiana, *Phytochemistry* **26**, 2418-2420.
[19] S. P. B. Ovenden, J. Yu, S. S. Wan, G. Sberna, R. M. Tait, D. Rhodes, S. Cox, J. Coates, N. G. Walsh and B. M. Meurer-Grimes (2004). Globoidnan A: a lignan from *Eucalyptus globoidea* inhibits HIV integrase, *Phytochemistry* **65**, 3255-3259.
[20] H. J. D. Dorman, M. Kosar, K. Kahlos, Y. Holm and R. Hiltunen (2003). Antioxidant properties and composition of aqueous extracts from *Mentha species*, hybrids, varieties, and cultivars, *J. Agr. Food Chem.* **51**, 4563-4569.
[21] F. Ding and W. Peng (2015). Biological activity of natural flavonoids as impacted by protein flexibility: an example of flavanones, *Mol. Biosyst.* **11**, 1119-1133.
[22] M. Martinez-Tome, A. M. Jimenez, S. Ruggieri, N. Frega, R. Strabbioli and M. A. Murcia (2001). Antioxidant properties of Mediterranean spices compared with common food additives, *J. Food Protect.* **64**, 1412-1419.
[23] M. Petersen and M. S. J. Simmonds (2003). Molecules of interest - Rosmarinic acid, *Phytochemistry* **62**, 121-125.
[24] S. W. Jeong, S. Park, J. S. Jin, O. N. Seo, G. S. Kim, Y. H. Kim, H. Bae, G. Lee, S. T. Kim, W. S. Lee and S. C. Shin (2012). Influences of Four Different Light-Emitting Diode Lights on Flowering and Polyphenol Variations in the Leaves of *Chrysanthemum* (*Chrysanthemum morifolium*), *J. Agr. Food Chem.* **60**, 9793-9800.