Research article

Isolation and identification of cholestane and dihydropyrene from Calophyllum inophyllum

David Febrilliant Susanto a, Hakun Wirawasista Aparamarta a, Arief Widjaja a, Nurul Jadi b, Setiyo Gunawan a,*

a Department of Chemical Engineering, Institut Teknologi Sepuluh Nopember, Surabaya, 60111, Indonesia
b Department of Biology, Institut Teknologi Sepuluh Nopember, Surabaya, 60111, Indonesia

ARTICLE INFO

Keywords:
Chemical engineering
Biochemical engineering
Pharmaceutical engineering
Transport process
Industrial chemistry
Materials characterization
Calophyllum inophyllum leaves
Extraction
Separation
Solvent

ABSTRACT

The highest population of Calophyllum inophyllum is in Indonesia. One of the bioactive compound contained in C. inophyllum leave is cholestane. The extraction method was employed to obtain crude extract from these leaves. It was followed by identification of the bioactive compounds. The purpose of this research was to develop a process for isolating and identifying cholestane and dihydropyrene from methanolic extract of C. inophyllum leaves in high yield and purity. The effect of crude extract to non-polar solvent mass ratio and non-polar solvent types on the separation of the bioactive compounds were also systematically investigated. New compounds (trans-2-[2-(trifluoromethyl)phenyl]-10b,10c-dimethyl-10b, 10c-dihydropyrene and anti-4-aza-B-homo-5.alpha-cholestane-3-one) were also identified in C. inophyllum leaves. The successful separation was obtained by employing CS2 as the solvent and crude extract to CS2 mass ratio of 1/10 (g/g).

1. Introduction

In recent years, the interest in identifying new sources of bioactive compounds has increased. Bioactive compounds, including flavones, lignans, and phenolic acids from natural products are the main source of herbal drugs, functional foods, and food additives [1]. Mangrove is the most common plant species in Indonesia. One of the mangrove plants that has high economic value is Calophyllum inophyllum. C. inophyllum is widely distributed in tropical areas and has been traditionally consumed as folk medicine. Pharmacological studies have reported that this plant has functioned as anti-bacterial, anti-cancer, anti-neoplastic, anti-inflammatory, anti-platelet agents, and anti-viral [2]. Its oil is usually used for biodiesel production. Meanwhile, its leaves contain bioactive compounds, such as friedelin, xanthones, coumarins, steroids, and dihydropyrene [3].

Friedelin is a kind of triterpenoid compounds. Its molecular formula and molecular weight are C30H50O and 426.72 g/mol, respectively. It has activity as an anti-fungal, anti-nociceptive effect in rodents [4], and hepatoprotective [5]. Xanthone is a polyphenolic compound that has a molecular formula of C13H16O2. Xanthone is known to have a variety of bioactive properties, notably the ability of anti-oxidants. Mangosteen xanthone was isolated from Garcinia mangostana found against free radicals and prevent oxidative damage of low-density lipoprotein [6]. In addition, xanthone isolated from mangosteen can inhibit HL60 leukemia cells [7].

Coumarin is one of the members of benzopyrone components and known as a phenolic substance [8]. In the coumarin structure, there is a benzene ring which is tied with a pyrone ring [9]. It is a component that has high activity as anti-tumor and anti-bacterial. Coumarin and its derivatives act as a barrier against cellular proliferation in various carcinoma cell lines [10]. Besides, coumarin also has anti-coagulant, anti-oxidant, anti-microbial, anti-viral, anti-inflammatory, anti-malarial, and analgesics activity [11].

Campesterols and cholesterols are steroid compounds that have been isolated from C. inophyllum leaves. They have an analgesic activity [12]. Another, dihydropyrene compound was used for photochromic dyes which are very useful in fabricating photoswitchable biomaterials for application in biomedicine, including photocontrol of imaging, on-demand cell attachment, photopharmacological chemotherapy, delivery and biomacromolecular interactions [13].

The previous works are limited to extraction and identification of mixture bioactive compounds in plant extracts. The separation of...
individual bioactive compounds and their recoveries remains unknown. Therefore, the aim of this research was to develop a process for isolating and identifying cholestanol and dihydropyrene from the methanolic extract of \textit{C. inophyllum} leaves in high yield and purity. The effect of crude extract to non-polar solvent mass ratios and non-polar solvent types on the bioactive compounds separation were also systematically investigated.

2. Materials and methods

2.1. Materials

Dried \textit{C. inophyllum} leaves were obtained from the “Koperasi Jarak Lestari”, Cilacap, Central Java, Indonesia. Thin-layer chromatography (TLC) aluminum plates were purchased from Merck (Darmstadt, Germany). CS$_2$, acetic acid, and ethyl acetate analytical reagents were also purchased from Merck (Darmstadt, Germany). Adventec filter papers were obtained from Toyo Roshi Kaisha Ltd. (Tokyo, Japan). Standard of xanthone, coumarin, and friedelin were obtained from Sigma Aldrich (St. Louis, MO). Aquadest, hexane technical grade (98%), and methanol technical grade (99%) were obtained from commercial sources.

2.2. Preparation of \textit{C. inophyllum} leaves methanolic extract

The age of \textit{C. inophyllum} leaves used was about 10 years. The image of \textit{C. inophyllum} is shown in Fig. 1. The leaves of \textit{C. inophyllum} were dried under the sunlight for 3 days. The moisture content of \textit{C. inophyllum} leaves was 11.24%. The dried \textit{C. inophyllum} leaves (1 kg) were chopped to a homogeneous size by a mill and soaked in 3 L methanol for 72 h. Then, the solutions were filtered through filter paper. The residue was soaked again in 3 L methanol for another 72 h. Afterward, the filtrates were collected and combined. Then, the methanol was evaporated by distillation at 80°C to obtain a methanolic crude extract of \textit{C. inophyllum} leaves.
2.3. Separation of dihydropyrene from C. inophyllum leaves methanolic extract

Dihydropyrene was separated from methanolic extract (semi-solid crude extract) of C. inophyllum leaves by washing method with the non-polar solvent. The flowchart of the separation of dihydropyrene and cholestane from C. inophyllum leaves is shown in Fig. 2. The effect of crude extract to non-polar solvent mass ratios (1/10, 1/30, and 1/50 (g/g)) and non-polar solvent types (hexane and CS2) were investigated.

Firstly, the crude extract (about 3 g) was mixed with a non-polar solvent at a certain mass ratio in beaker glass, which was fully covered to avoid solvent loss. The mixture was stirred for 30 min. Then, it was separated into two layers: a lower extract residue and an upper solvent layer. The polar compounds (such as dihydropyrene) were remained in the extract residue (fraction A), whereas the non-polar compounds were dissolved in the non-polar solvent. This step was repeated until there were no compounds dissolve in the non-polar solvent as confirmed by TLC analysis.

The fraction A was analyzed by TLC, gas chromatography and gas chromatography-mass spectrophotometry analyses. Afterward, the non-polar solvent fractions were collected and combined. Then, the solvent was evaporated and designated as fraction B.

The fraction B was fractionated again to identify residual dihydropyrene with 30 mL hexane as the solvent. The mixture was stirred for 30 min and separated into two layers: a lower extract residue and an upper solvent layer. This step was repeated until there were no compounds dissolve in hexane as confirmed by TLC analysis. The fraction C was solid and recovered as a residual dihydropyrene. Afterward, hexane fractions were collected and combined. Then, hexane was evaporated and designated as fraction D for further treatment.

2.4. Isolation and identification of cholestane from C. inophyllum leaves methanolic extract

The fraction D (50 g) was introduced into silica gel (0.05 g) adsorption. The mixture was stirred vigorously for 30 min. Afterward, it was filtrated by filter paper. The filtrate was employed into silica gel adsorption again. This step was repeated until 8 times. The resulting hexane fractions were collected, combined, and named as fraction E. On the other hand, the compounds that adsorbed into silica gel were extracted by methanol. Then, methanol was evaporated and named as...
fraction F (rich in cholestane). All fractions were analyzed by TLC, gas chromatography and gas chromatography-mass spectrophotometry analyses.

2.5. Thin-layer chromatography (TLC) analysis

TLC was employed as described by previous work [14]. TLC plate that has been stained by the sample was immersed in a mobile phase of hexane: ethyl acetate: acetic acid at 80:20:1 (v/v/v).

2.6. Gas chromatography (GC) analysis

Bioactive compounds contents were analyzed by GC analysis [15]. It was performed on a Shimadzu GC-2010 gas chromatograph equipped with a flame ionization detector. The DB-5HT (5%-phenyl)–methylpolysiloxane non-polar column (15 m × 0.32 mm i.d.; Agilent Tech. Palo Alto, California) was used. The injector and detector temperatures were set at 310 °C, respectively. The initial temperature of the column was 80 °C. It was raised to 300 °C with a rate of 15 °C/min, and kept at 300 °C for 8 min. The carrier gas was nitrogen with a linear velocity of 30 cm/s at 80 °C and split ratio of 1:50.

2.7. Gas chromatography-mass spectrophotometry (GC-MS) analysis

The GC-MS analysis was analyzed as described by previous work [16] with some modification. It was performed by an Agilent 6890 GC system with Agilent 6971 inert mass selective detector. The capillary column, HP5 5% phenylmethylsiloxane non-polar column (30 m × 0.32 mm i.d.; Agilent Tech. Palo Alto, California) was used. The initial oven temperature was set at 80 °C and kept for 5 min. Then, it was raised to a temperature of 300 °C with rate of 15 °C/min and kept for 2 min. Helium ultrapure (99.999%) was used as carrier gas. The temperature of the ion source, interface, and injector were 230, 280, and 310 °C, respectively. The sample injection was 1 μL, using a model split inlet with ratio of 1:10, with a flow rate of gas in the column at 1.3 mL/min.

3. Results and discussion

The yield of crude extract of *C. inophyllum* leaves had been investigated with various solvents (methanol, ethanol, and hexane) by soxhlet extraction. It was found that the yield extract of *C. inophyllum* leaves with methanol (24.94%), ethanol (15.21%), and hexane (5.44%) by soxhlet extraction. It was found that methanol can produce a higher yield of crude extract. Therefore, methanol was used to extract the *C. inophyllum* leaves by the percolation method in this research. The yield of the crude extract obtained in this work was 2.88%. It was also found that the crude extract of *C. inophyllum* was brownish semi-solid at room temperature. No other study was found regarding the yield of methanolic extract of *C. inophyllum* leaves. However, this result was comparable with previous work that the methanolic extract yield of *Oroxylum indicum* bark was 3.84% [17].

The chromatogram of *C. inophyllum* leaves crude extract was shown in Fig. 3. It was found that coumarin, xanthone, and friedelin contents were 0.03%, 0.21%, and 1.68%, respectively. They were in a small amount with the retention time of 8.64, 12.21 and 20.72 min. Moreover, 4 major compounds (peak 1, 2, 3 and 4) were detected in the methanolic extract of *C. inophyllum* leaves. They were 18.4, 11.68, 1.36, and 9.69%, respectively. Therefore, identification and elimination of major compounds are important factors for the enrichment of friedelin, xanthone, and coumarin.

The isolation and characterization of bioactive compounds from plants mostly rely on the selection of proper extraction method [18]. Extraction is the first and crucial step for the isolation and purification of bioactive compounds from natural products. The selection of solvents affected the efficiency of extraction [19]. There are many factors for selecting solvent, such as the polarity of targeted compound must be relatively equal with solvent, the molecular affinity between solvent and solute, mass transfer, use of co-solvent, environmental safety, human toxicity, and solvent cost [20]. Many of non-polar compounds from leaves have high pharmacological activity, such as triterpenoid compounds. Hexane and carbon disulfide (CS2) were used for the solvent because of non-polar solvents.

The multi-stage extraction was employed for separating compounds into two fractions: polar and non-polar compounds. The best separation was obtained when dihydropyrene and cholestane were perfectly separated. The separation was conducted with 2 factors at different levels, such as (i) effect of various non-polar solvent types at levels of n-hexane and CS2 and (ii) effect of crude extract to non-polar solvent mass ratios at levels of 1/10, 1/30 and 1/50 (g/g), to obtain dihydropyrene and cholestane from methanolic extract of *C. inophyllum* leaves. A single replicate of the one-factor-at-a-time approach was employed in this study.

3.1. Isolation and identification of dihydropyrene

3.1.1. Effect of the crude extract to non-polar solvent mass ratios

In this study, n-hexane was first used as a solvent to investigate the optimal crude extract to non-polar solvent mass ratio due to the economic and commercial value. The mass ratios of crude extract to hexane of 1/10, 1/30, and 1/50 (g/g) were investigated. The washing method was done by multi-stages extractions until there were no compounds dissolve in the non-polar solvent.

Table 1 shows the effect of the crude extract to n-hexane mass ratio on friedelin, xanthone, coumarin, peak 1, peak 2, peak 3, and peak 4 separation from methanolic extract of *C. inophyllum* leaves. The higher solvent used to extract and the less washing stages were preferred to obtain the best separation. Peak 1, peak 2, peak 3, and peak 4 were concentrated in n-hexane with recoveries of 89%, 81%, 55%, and 80%, respectively. Furthermore, it can be seen that friedelin, xanthone, and coumarin were also dissolved in hexane and concentrated in one fraction (fraction B) with recoveries of 61.8%, 79.6%, and 74.9%, respectively, at a crude extract to n-hexane mass ratio of 1/50.
Table 2
Effect of solvent types on separation of bioactive compounds from C. inophyllum leaves. a

| Solvent type | Hexane | CS2 |
|--------------|--------|-----|
| Fraction     | Fraction A | Fraction B | Fraction A | Fraction B |
| Friedelin    | 2.51% a | 1.39% | 0.167% | 3.61% |
| (38.20%) b   | (61.80%) | (2.90%) | (97.10%) |
| Xanthone     | 0.17% | 0.22% | 0.057% | 0.33% |
| (20.40%)     | (79.60%) | (9.94%) | (90.06%) |
| Coumarin     | 0.03% | 0.03% | 0.036% | 0.01% |
| (25.11%)     | (74.89%) | (63.42%) | (36.58%) |
| Peak 1       | 7.89% | 22.01% | 45.54% | 0.94% |
| (10.96%)     | (89.04%) | (96.89%) | (3.11%) |
| Peak 2       | 8.65% | 12.73% | 0.63% | 18.79% |
| (18.93%)     | (81.07%) | (2.11%) | (97.89%) |
| Peak 3       | 2.41% | 1% | 0.40% | 1.98% |
| (45.18%)     | (54.82%) | (11.48%) | (88.52%) |
| Peak 4       | 7.45% | 10.46% | 0% (0%) | 15.92% |
| (19.65%)     | (80.35%) | (100%) |
| Yield (g/g crude) | 0.26 | 0.74 | 0.39 | 0.61 |

a crude extract to solvent mass ratio = 1/10 (g/g). b %wt.

extract to n-hexane mass ratio of 1/10 (g/g). No other study was found regarding the separation of friedelin, xanthone, and coumarin from the methanolic extract of C. inophyllum leaves. However, this result was comparable with Sabri et al [20] reported that many anticancer potent compounds were detected in the non-polar solvent extraction of Phoenix dactylifera leaves. The yield of fraction A was larger by separation with CS2 (39.17%) than that of hexane (25.57%). It was found that the best separation was obtained by CS2 as the solvent and crude extract to CS2 mass ratio of 1/10 (g/g). The methanolic crude extract was separated into a non-polar fraction (rich in friedelin, xanthone, peak 2, peak 3, and peak 4) and residue extract fraction (rich in coumarin and peak 1).

In summary, it was found that CS2 was a more effective solvent to separate non-polar compounds than that of hexane. It was found that the best separation was obtained by CS2 as the solvent and crude extract to CS2 mass ratio of 1/10 (g/g). The methanolic crude extract was separated into a non-polar fraction (rich in friedelin, xanthone, peak 2, peak 3, and peak 4) and residue extract fraction (rich in coumarin and peak 1).

The next step was a separation of residual peak 1 from the fraction B with n-hexane. The fraction C obtained was 0.28 g. There was only one spot in TLC of fraction C and A as confirmed by GC analysis. The fraction A and C were analyzed by GC-MS. Peak 1 was identified as trans-2-[2-(trifluoromethyl)phenyl]-10b,10c-dimethyl-10b, 10c-dihydropyrene. Its characteristic ions m/z (relative intensity) was 28 (2.74%), 73 (5.48%), 96 (1.37%), 115 (2.74%), 135 (2.74%), 156 (2.05%), 179 (2.05%), 207 (13.7%), 227 (4.11%), 253 (6.85%), 281 (8.22%), 305 (23.29%), 341 (5.48%), 361 (100%), 380 (21.92%), 405 (1.37%), 429 (3.42%).

This result is agree with previous work that the dihydropyrene was identified in C. inophyllum leaves [2]. The structure was shown in Fig. 4a. Another, compound isolated from Juncus effusus which has dihydropyrene skeleton had the anti-microbial and anti-fungal activities [21].

3.2. Isolation and identification of cholestanone from C. inophyllum leaves

Peak 2, peak 3, and peak 4 were still contained in the fraction D. Silica gel adsorption was used to isolate the peak 2. Silica gel was the polar absorbent which has a hydroxyl group, so polar compounds, such as alcohols, phenols, and amines (which can form hydrogen bonds) and unsaturated hydrocarbons (which can form π-complexes) were absorbed [14]. In the silica gel fraction, there was only one compound identified as known as peak 2.

Peak 2 was identified as anti-4-aza-B-homo-5.alpha-cholestane-3-one. It appears at retention time 19.34 min. Its characteristic ions m/z (relative intensity) was 28 (16.33%), 55 (16.33%), 77 (4.08%), 165 (4.08%), 202 (4.08%), 401 (100%), 416 (8.16%). The structure was supported by M+ and a base peak at m/z 401. The structure was shown in Fig. 4b. This compound was aza sterol that is plant hormonal components. It was antiandrogenic effective steroid derivatives that had anti-oxidant activity used for medicinal usage [22]. Sterols have anti-cancer activity and immune regulatory effects [23].

Fig. 4. The structure of trans-2-[2-(trifluoromethyl)phenyl]-10b,10c-dimethyl-10b, 10c-dihydropyrene (a) and anti-4-aza-B-homo-5.alpha-cholestane-3-one (b).
4. Conclusion

A process for isolating dihydropyrene and cholestanone was developed. The trans-2-[2-(trifluoromethyl)phenyl]-10b,10c-dimethyl-10b, 10c-dihydropyrene and anti-4-aza-B-homo-5.alpha-cholestan-3-one were identified and separated in this study. Moreover, the crude extract to CS2 mass ratio of 10 (g/g) gave the best separation condition. CS2 was a more effective solvent to separate non-polar compounds than that of hexane. The yield of the dihydropyrene was 22.2% (79.2% purity). It was found that the C. inophyllum leaves can be used for anti-cancer herbal medicine. The isolation and separation of other bioactive compounds from C. inophyllum leaves (such as flavonoid and terpenoid compounds) will be a trending research topic in the future.

Declarations

Author contribution statement

David Febrilliant Susanto: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Hakim Wirawasista Aparamura, Arief Widjaja, Nurul Jadid & Setiyono Gunawan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, material, and analysis tools or data.

Funding statement

This work was supported by The Directorate General of Resources for Science, Technology, and Higher Education, Ministry of Research, Technology, and Higher Education of the Republic of Indonesia.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

[1] X.K. Huang, M. Bai, L. Zhou, L.L. Lou, Q.B. Liu, Y. Zhang, L.Z. Li, S.J. Song, Food byproducts as a new and cheap source of bioactive compounds: lignans with antioxidant and anti-inflammatory properties from Crateagus pinnatifida seeds, J. Agric. Food Chem. 63 (32) (2015) 7252–7260.

[2] K.H. Ling, C.T. Kian, T.C. Hoon, A Guide to Medicinal Plant, World scientific, Singapore, 2009.

[3] D.F. Susanto, H.W. Aparamura, A. Widjaja, S. Gunawan, Identification of phytochemical compounds in Calophyllum inophyllum leaves, Asian Pac. J. Trop. Biomed. 7 (9) (2017) 773–781.

[4] J.S.S. Quintans, E.V. Costa, J.F. Tavarez, T.T. Soua, S.S. Araujo, C.S. Estevam, A. Barison, A.G.S. Cabral, M.S. Silva, M.R. Serafim, L.J. Quintans-Junior, Phytochemical study and antioxidant potential of the hexanic extract of leaves from Combretum durarenium and friedelin, a triterpene isolated from the hexanic extract, in orofacial nociceptive protocols, Rev. bras. farmacogn. 24 (1) (2014) 60–66.

[5] P. Dzubak, M. Hajduch, D. Vydra, A. Hustova, A. Kvanica, M. David, D. Biedermann, L. Markova, M. Urbanc, J. Sarek, Pharmacological activities of natural triterpenoids and their therapeutic implications, Nat. Prod. Rep. 23 (3) (2006) 394–411.

[6] P. Williams, M. Ongsakul, L.J. Quintans-Junior, J. Proudfoot, K. Croft, L. Beilin, Mangostin inhibits the oxidative modification of human low density lipoprotein, Free Radic. Res. 23 (2) (1995) 175–184.

[7] K. Matsumoto, Y. Akas, E. Kobayashi, K. Oyghuchi, T. Ito, T. Tanaka, M. Linuma, Y. Nozawa, Introduction of apoptosis by xanthones from mangosteen in human leukemia cell lines, J. Nat. Prod. 66 (8) (2003) 1124–1127.

[8] R.O. Kennedy, R.D. Thomes, Coumarin: Biology, Applications and Mode of Action, John Wiley & Sons, Inc., New York, 1997.

[9] R. Bezwada, Chemistry of Coumarins, Indofine Chemical Company, 2008.

[10] A. Lacy, R.O. Kennedy, Studies on coumarins and coumarin related compound to determine their therapeutic role in the treatment of cancer, Curr. Pharmaceut. Des. 10 (30) (2004) 3797–3811.

[11] S.S. Sahoo, S. Shukla, S. Nandy, H.B. Sahoo, Synthesis of novel coumarin derivatives and its biological evaluations, J. Exp. Biol. 2 (4) (2012) 899–908.

[12] S. Silpa, B. Shrivastava, P. Sharma, S.S. Rai, A review article of pharmacological activities and importance of Calophyllum inophyllum, Int. J. Adv. Res. 2 (12) (2014) 599–603.

[13] H. Cheng, J. Yoon, H. Tian, Recent advances in the use of photochromic dyes for photocatalyst in biomedicine, Coord. Chem. Rev. 372 (2018) 66–84.

[14] S. Gunawan, N.S. Kasim, Y.H. Ju, Separation and purification of squalene from soybean oil deodorizer distillate, Separ. Purif. Technol. 60 (2008) 128–135.

[15] N.S. Kasim, S. Gunawan, Y.H. Ju, Separation and identification of steroidal hydrocarbons in soybean oil deodorizer distillate, Food Chem. 117 (1) (2009) 15–19.

[16] D.F. Susanto, S. Humphari, T. Trifluthani, A. Borteb, H.W. Aparamura, A. Widjaja, S. Gunawan, Effect of solvent polarity levels on separation of xanthone and coumarin from Calophyllum inophyllum leaves extract, IOP Conf. Ser. Mater. Sci. Eng. 334 (2018), 012071.

[17] V. Rajkumar, G. Gunjan, R.A. Kumar, Isolation and bioactivity evaluation of two metabolites from the methanolic extract of Oxyrurus indicum stem bark, Asian Pac. J. Trop. Biomed. 2 (1) (2012) 57–511.

[18] S. Sasidharan, Y. Chen, D. Saravanam, K.M. Sundram, Y.L. Latha, Extraction, isolation and characterization of bioactive compounds from plants extracts, Afr. J. Tradit. Complementary Altern. Med. 8 (1) (2011) 1–10.

[19] M.M. Cowan, Plant Products as Antimicrobial Agents, Clinical Microbiology Reviews, American Society for Microbiology, 1999, pp. 564–582.

[20] N.E. Sabri, B. Khalilah, R. Dzulfadli, S. Shamala, S.H. Mohd, H. Roshada, Solvents extraction effects on bioactive compounds of Ajwa date (Phoenix dactylifera L.) flesh using mixture design, Chem. Eng. Trans. 63 (2018) 817–822.

[21] W. Zhao, L.L. Xu, X. Zhang, X.W. Gong, D.L. Zhu, X.H. Xu, F. Wang, X.L. Yang, Three new phenanthrenes with antimicrobial activities from the aerial parts of Junci esculentus, Fitoterapia 130 (2018) 247–250.

[22] A. Sayik, A.S. Yusufoglu, L. Acik, G. Turker, B. Aydin, L. Arslan, DNA-binding, biological activities, and chemical composition of wild growing Epilobium angustifolium L. Extracts from Canakkale, Turkey, J. Turkish chem. soc. 4 (3) (2017) 811–840.

[23] B.J. Grattan, Plant sterols as anticancer nutrients: evidence for their role in breast cancer, Nutrients 5 (2) (2013) 359–387.