

Identification of Apigenin and Luteolin in *Artemisia annua* L. for the Quality Control

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ABSTRACT

Objective: To identify active compounds and establish the chemical fingerprint of *Artemisia annua* L. for the quality control.

Methods: Thin-layer chromatography (TLC) conditions were developed to screen for 2 common flavonoids (apigenin and luteolin). Three mobile phases were used to isolate these flavonoids in 80% ethanolic extract of *A. annua*. Hexane : ethyl acetate : acetic acid (31:14:5, v/v) and toluene : 1,4-dioxane : acetic acid (90:25:4, v/v) were used in normal phase TLC (NP-TLC), and 5.5% formic acid in water : methanol (50:50, v/v) were used in reverse phase TLC (RP-TLC). Chromatograms were visualized under visible light after spraying with Fast Blue B Salt. Apigenin and luteolin bands were checked by comparing their R_f values and UV-Vis absorption spectra with reference markers.

Results: Apigenin and luteolin were simultaneously detected with good specificity in RP-TLC condition, while only apigenin was detected in NP-TLC condition. Apigenin band intensity was higher than luteolin band intensity in both conditions.

Conclusion: This knowledge can be applied to the development of quality control assessments to ensure product efficacy and consistency.

Keywords: *Artemisia annua* L.; TLC fingerprint; apigenin; luteolin (Siriraj Med J 2019;71: 240-245)

INTRODUCTION

Artemisia annua L., commonly known as sweet wormwood, is an annual plant in the Asteraceae (Compositae) family. This herb is native to Asia and throughout much of China. It flourishes in the temperate zone, and also grows wild in central Europe. Aqueous preparations of dried *A. annua* have traditionally been used to treat fever, malaria, skin diseases, jaundice, and hemorrhoids in China. In Thailand, *A. annua* has been used to treat fever, asthma, skin disease, circulatory disorders (e.g., dizziness), and as an expectorant and an anthelmintic.¹ *A. annua* is also used as an herbal ingredient in some

Thai herbal recipes that are published in The National List of Essential Medicines.² Several previous studies reported that *A. annua* possesses various pharmacological activities, including antimalarial, antinociceptive, anti-inflammatory, antioxidant, anticancer, and antimicrobial actions.³⁻¹⁰ Moreover, the essential oil from this plant is postulated to inhibit the growth of Gram-positive bacteria, Gram-negative bacteria, and yeast, and to depress the central nervous system.¹¹⁻¹³ These reported findings support the historical claims and traditional belief that *A. annua* is an effective herbal medicine for treatment of different diseases. The chemical component of *A. annua*

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that makes it effective for treating the parasitic diseases of malaria is artemisinin, a sesquiterpene lactone.⁴⁻⁵ A large number of flavonoides, including apigenin, luteolin, kaempferol, quercetin, casticin, and artemetin, have also been reported to be present in *A. annua*.¹⁴⁻¹⁵ Reported vital pharmacological activities effectuated by flavonoids in *A. annua* include antioxidant, inhibition of immune mediators of angiogenesis, prophylactic effects in malaria and related fevers, and antimalaria parasite.^{8,16-20}

Luteolin and apigenin are flavonoids that are commonly found in different plant species that are associated with a broad spectrum of pharmacological activities, including antimalarial, anti-inflammatory, antioxidant, and anticancer.²⁰⁻²³ However, the efficacy of drugs that contain flavonoids can vary according to environmental conditions and season of harvest or growth stage.²⁴⁻²⁶ Thin-layer chromatography (TLC) is currently the method of choice for identifying a wide variety of substance classes, because it is a simple, rapid, inexpensive, and substances are identified visually. A new method for simultaneously quantifying apigenin and luteolin using reverse-phase TLC in some medicinal plants was recently reported.²⁷ The development of a screening method that could reliably identify the presence of apigenin and luteolin in *A. annua*, and then a chromatogram fingerprint of *A. annua* would facilitate quality control assessments of harvested *A. annua* to ensure the reproducibility of ingredients and the consistent efficacy of the product. Accordingly, the aim of this study was to develop a screening method to identify apigenin and luteolin in *A. annua*, and then establish a chromatogram fingerprint of *A. annua*.

MATERIALS AND METHODS

Chemicals and plant materials

All reagents used were of analytical grade. Methanol, ethanol, and hexane were purchased from Scharlau (Barcelona, Spain), and toluene, formic acid, and acetic acid were purchased from Merck (Darmstadt, Germany). Ethyl acetate was purchased from Riedel-de Haen (Seelze, Germany), and 1, 4 dioxane was purchased from Carlo Erba Reagents, (Val de Reuil, France). Apigenin, luteolin, and Fast Blue B Salt were purchased from Calbiochem (San Diego, California, USA), Extrasynthese, (Genay, France), and Sigma-Aldrich (St. Louis, Missouri, USA), respectively. HPTLC Silica gel 60 F₂₅₄ (NP-TLC) and TLC Silica gel 60 RP-18 F_{254S} (RP-TLC) were purchased from Merck (Darmstadt, Germany). Pulverized aerial plant material of *A. annua*, was obtained from the Manufacturing Unit of Herbal Medicines and Products, Center of Applied

Thai Traditional Medicine (CATTM), Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Instruments

The CAMAG TLC system (CAMAG Chemie-Erzeugnisse & Adsorptionstechnik AG, Muttenz, Switzerland) consisted of a CAMAG Linomat 5 sample applicator, a CAMAG Automatic Developing Chamber (ADC2), and a CAMAG TLC Scanner 3. Densitometry documentation was performed under UV light (254 and 366 nm) and visible light using Reprostar 3 densitometer with CCD camera. CAMAG winCATS software was used to control the system and analyze the data.

Preparation of standard solutions

One milligram of apigenin was dissolved in 2 milliliters of methanol, and 1 mg of luteolin was dissolved in 1 milliliter of methanol. Stock solutions were maintained in a refrigerator at -20°C until use.

Preparation of sample solutions

To obtain the optimal solvent for the extraction of *A. annua*, three common solvents were used, including 100% methanol, 100% ethanol, and 80% ethanol. Ten milliliters of each solvent was added to 1 gram of pulverized aerial plant material of *A. annua* and sonicated for 60 minutes. The 3 mixtures were then centrifuged at 4,000 rpm for 10 minutes at 25°C. The supernatant was separated and evaluated using the TLC systems.

TLC analysis

We employed 3 different mobile phase systems – two normal-phase (NP) and one reverse-phase (RP) TLC. The 2 NP-TLC mobile phase systems consisted of hexane : ethyl acetate : acetic acid (31:14:5, v/v) and toluene : 1,4-dioxane : acetic acid (90:25:4, v/v). The RP-TLC mobile phase system consisted of 5.5% formic acid in water : methanol (50:50, v/v). Sample volumes of *A. annua* extracts applied on HPTLC and RP-TLC plates were varied to obtain the best separation and the most obvious chromatogram fingerprints. One microliter (μL) of luteolin and 2 μL of apigenin were mixed and loaded alongside the sample as markers. Development duration was approximately 20 minutes or the solvent migration distance was at least 7.5 cm. Developed plates were dried in cold air for approximately 5 minutes. Extracted chromatogram fingerprints were visualized under both ultraviolet light (UV) (254 and 366 nm) and visible light after spraying with Fast Blue B Salt (FBS). Chromatograms were further sprayed with 10% NaOH to intensify the result. Presence of apigenin and/

or luteolin was determined by comparing rate of flow (Rf) values and absorption spectra with those of their respective markers.

RESULTS

Chromatogram fingerprints of ethanol, methanol, and 80% ethanol extracts of the aerial part of *A. annua* after FBS staining are shown in Fig 1. Eighty percent ethanol was determined to be the optimal extraction solvent. Chromatogram fingerprints of *A. annua* extract under UV light (254 and 366 nm) and visible light after FBS staining of NP-TLC and RP-TLC are shown in Fig 2. The Rf values in NP-TLC (2 solvent systems) and

RP-TLC (1 solvent system) were 0.21, 0.24, and 0.09 for apigenin marker, and 0.10, 0.13, and 0.15 for luteolin marker, respectively (Fig 3, Table 1). Bands identified in the chromatogram of *A. annua* using RP-TLC expressed migration distance, color, and absorption spectrum similar to that of apigenin and luteolin markers. Using NP-TLC, only bands of apigenin was identified (Fig 4). Accordingly, RP-TLC was found to be the most suitable method for simultaneously identifying both apigenin and luteolin in 80% ethanolic extract of *A. annua*. The band intensity of apigenin was higher than that of luteolin in both NP-TLC and RP-TLC.

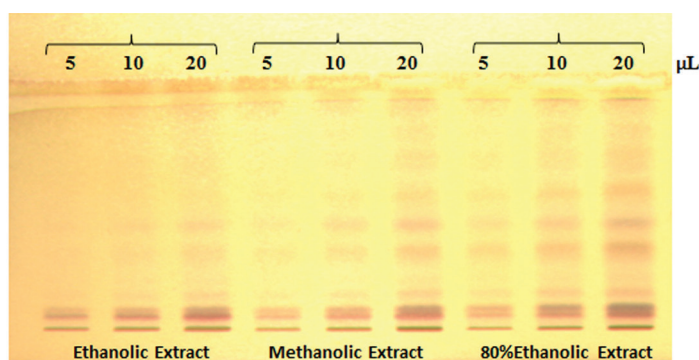


Fig 1. RP-TLC chromatogram of ethanol, methanol, and 80% ethanol extracts of the aerial part of *A. annua* detected under visible light after spraying with FBS and intensification by spraying with 10% NaOH.

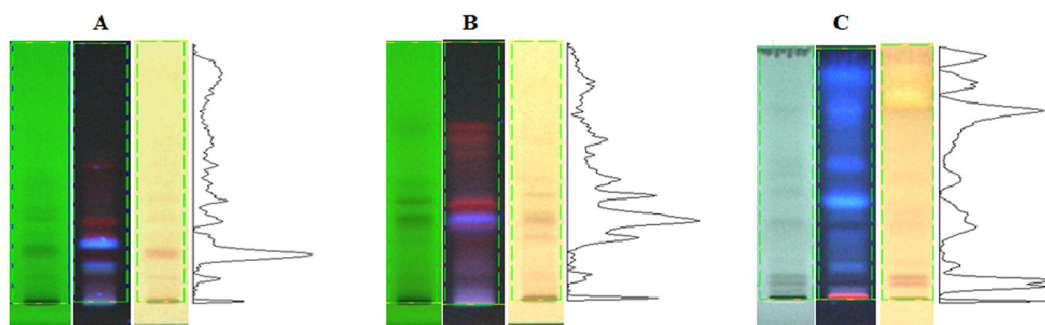


Fig 2. Chromatogram fingerprint of *A. annua* developed in a NP-TLC plate with A) hexane : ethyl acetate : acetic acid (31:14:5 v/v); B) toluene : 1,4-dioxane : acetic acid (90:25:4, v/v); and, C) in an RP-TLC plate with 5.5% formic acid in water : methanol (50:50, v/v). Visualization was performed under UV light (254 and 366 nm) and visible light after spraying with FBS.

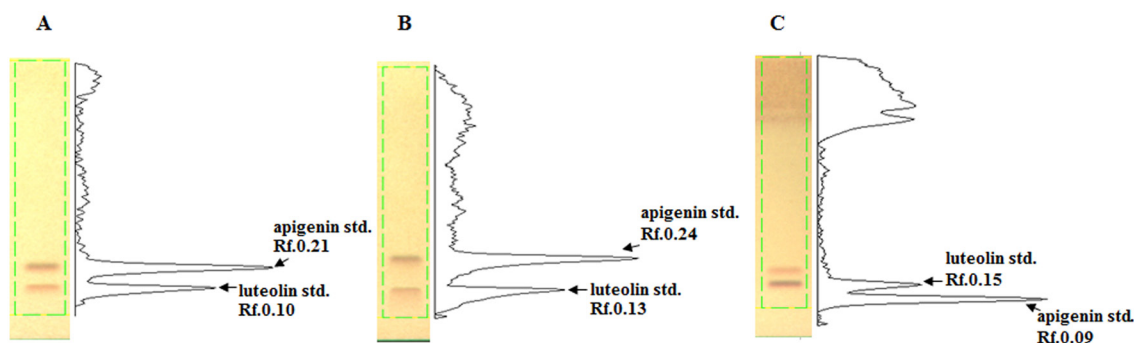


Fig 3. Chromatograms of apigenin and luteolin in a mixed marker solution developed in a NP-TLC plate with A) hexane : ethyl acetate : acetic acid (31:14:5, v/v); B) toluene : 1,4-dioxane : acetic acid (90:25:4, v/v); and, C) in an RP-TLC plate with 5.5% formic acid in water : methanol (50:50, v/v). Bands were detected under visible light after FBS staining.

TABLE 1. Rf values of reference markers by mobile phase

Mobile phase	Marker	Plate type	Rf
Hexane : ethyl acetate : acetic acid (31:14:5, v/v)	Apigenin	NP-TLC	0.21
	Luteolin	NP-TLC	0.10
Toluene : 1,4-dioxane : acetic acid (90:25:4, v/v)	Apigenin	NP-TLC	0.24
	Luteolin	NP-TLC	0.13
5.5% formic acid in water: methanol (50:50, v/v)	Apigenin	RP-TLC	0.09
	Luteolin	RP-TLC	0.15

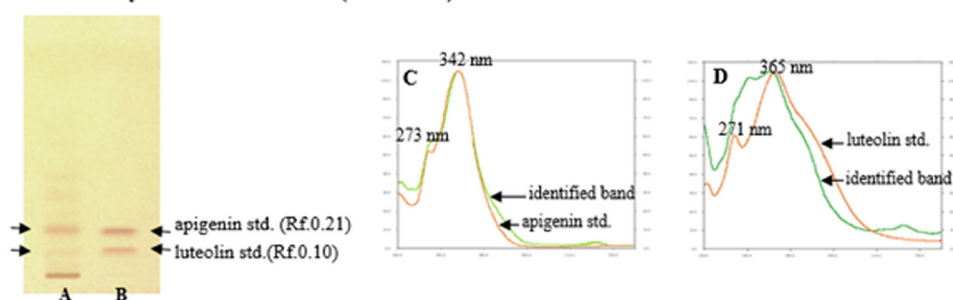
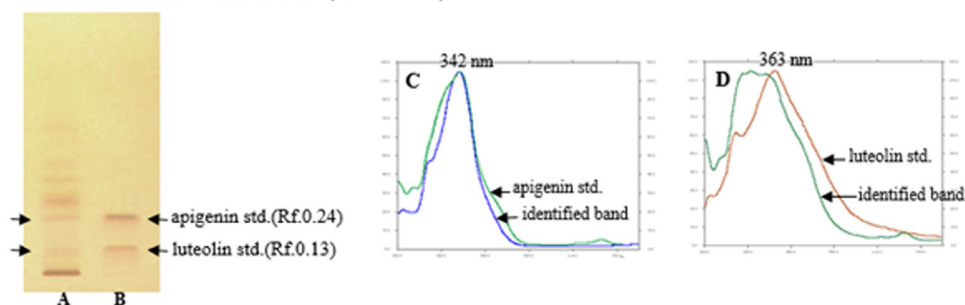
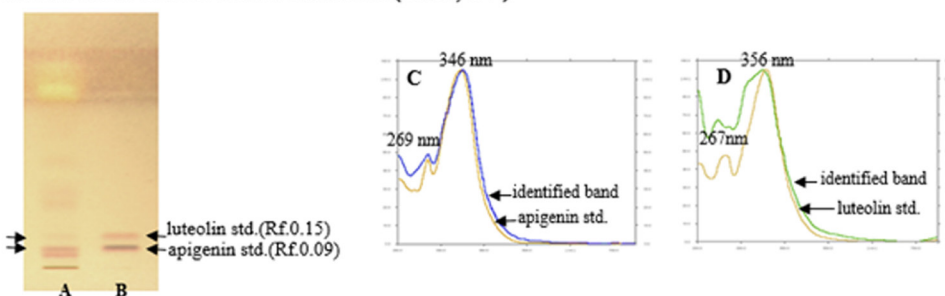
Hexane : Ethyl acetate : Acetic acid (31:14:5 v/v)**Toluene : 1,4 Dioxane : Acetic acid (90: 25:4 v/v)****5.5% Formic acid in Water: Methanol (50:50, v/v)**

Fig 4. Detection of apigenin and luteolin in *A. annua* under visible light after spraying with FBS and intensification with 10% NaOH in 3 condition systems: A) 80% ethanolic extract of *A. annua*; B) mixed marker solution; C) absorbent overlay of apigenin marker along with the sample; and, D) absorbent overlay of luteolin marker along with the sample.

DISCUSSION

Artemisinin is a potent antimalarial drug against *Plasmodium falciparum* that can be found at a range of concentration of 0.01-0.5% of dry weight of *A. annua*.⁴ That study also reported for flavonoid compounds in this plant. Apigenin and luteolin were 2 of several flavonoids identified in the leaves and stems of *A. annua*. These compounds were isolated and characterized by comparing their UV, MS, and 1H NMR spectra with the literature and reference compounds.¹⁴ Luteolin and apigenin are reported to be associated with a broad spectrum of pharmacological activities, including antimalarial, anti-inflammation, antioxidant, and anticancer.²⁰⁻²³ Thin-layer chromatography (TLC) is suitable for initial identification of substances prior to more advanced analysis using more sophisticated analytical instruments. The optimal yield of apigenin and luteolin found in 80% ethanolic extract allowed for further identification of apigenin and luteolin in *A. annua* using the three mobile systems. In this study, RP-TLC enabled simultaneous detection of apigenin and luteolin with specificity while NP-TLC could detect with specificity only for apigenin compound. A previous study reported the use of RP-TLC to identify various flavonoids, including apigenin and luteolin.²⁷ However and based on our review of the literature, no comparison has been made between the efficiency of NP-TLC and RP-TLC. Our finding showed that RP-TLC can identify the two substances of interest and established that RP-TLC is more suitable for detecting the presence of apigenin and luteolin in *A. annua*. The superiority demonstrated by RP-TLC over NP-TLC can be explained by the low selectivity of NP-TLC. The UV-Vis absorption profile of the similar in position band to luteolin reference compound under NP-TLC condition was not similar to that of standard. This indicates that the band might not be free of any interference that was present in the extract. In this study, the fact that the band of apigenin appeared more intensely than the band of luteolin suggests that the aerial parts of the *A. annua* used in this experiment contained more apigenin than luteolin. This result contrasts with the result of a previous study that reported the same amount of luteolin and apigenin isolated from ethyl acetate extract of the leaves and stems of *A. annua* (0.015% on dry weight).¹⁴ In addition to different types of sample preparations and different methods of analysis being the cause of variations in flavonoid content, variation may also be due to geographical area, season of harvest, or phenological growth stages. The previous study on the effects of geographical area and polluted environment on flavonoid contents in *Artemisia vulgaris* and *Veronica chamaedrys* by using

TLC technique reported that apigenin in *V. chamaedrys* was increased at the alpine regions, while the content of quercetin 3,7,3'-trimethyl ether in *A. vulgaris* appeared to be independent of altitude, but it was influenced positively by environmental pollution.²⁴ The study on the seasonal variation in 15 phenolic compounds of 80% ethanolic extract of *Rhododendron tomentosum* leaf found that the quantity of all fifteen phenolic compounds showed significant seasonal variation, but there was no seasonal variation of their total sum.²⁵ The qualitative and quantitative flavonoid aglycones variations in relation to the different phenological stages were also observed in *Artemisia absinthium* and *Artemisia vulgaris* by using HPLC. The detected flavonoids in *A. absinthium* were stable in their qualitatively but different in their contents while the flavonoid profiles of *A. vulgaris* exhibited qualitative and quantitative variability during the life cycle.²⁶

CONCLUSION

This study found RP-TLC to be most suitable for simultaneously identifying both apigenin and luteolin in 80% ethanolic extract of *A. annua*. This knowledge can be applied to the development of quality control assessments of harvested *A. annua* to ensure the reproducibility of ingredients and the consistent efficacy of the product.

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Conflict of interest: The authors hereby declare no personal or professional conflicts of interest regarding any aspect of this study.

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REFERENCES

1. Bunyapraphatsara N, Chokchaicharoenporn O, editors. *Indigenous medicinal herbs* (1). 1st ed. Bangkok: Prachachon Press; 1996.
2. *National Essential Drug List 2009*, National Drug Committee,

- Ministry of Public Health, Thailand, List of Herbal Medicinal Products A.D. 1999, 2006, 2012.
3. Mesa LE, Lutgen P, Velez ID, Segura AM, Robledo SM. *Artemisia annua* L., Potential Source of Molecules with Pharmacological Activity in Human Diseases. *AJPCT* 2015;3:436-50.
 4. Woerdenbag HJ, Lugt CB, Pras N. *Artemisia annua* L.: a source of novel antimalarial drugs. *Pharm Weekbl Sci* 1990;12:169-81.
 5. Elfawal MA, Towler MJ, Reich NG, Golenbock D, Weathers PJ, Rich SM. Dried whole plant *Artemisia annua* as an antimalarial therapy. *PLoS One* 2012;7:e52746.
 6. Favero Fde F, Grandó R, Nonato FR, Sousa IM, Queiroz NC, Longato GB, Zafred RR, Carvalho JE, Spindola HM, Foglio MA. *Artemisia annua* L.: evidence of sesquiterpene lactones' fraction antinociceptive activity. *BMC Complement Altern Med* 2014;14:266.
 7. Kim WS, Choi WJ, Lee S, Kim WJ, Dong Lee C, Sohn UD, et al. Anti-inflammatory, antioxidant and antimicrobial effects of artemisinin extracts from *Artemisia annua* L. *Korean J Physiol Pharmacol* 2015;19:21-27.
 8. Iqbal S, Younas U, Chan KW, Zia-Ul-Haq M, Ismail M. Chemical composition of *Artemisia annua* L. leaves and antioxidant potential of extracts as a function of extraction solvents. *Molecules* 2012;17:6020-32.
 9. Kim MH, Seo JY, Liu KH, Kim JS. Protective effect of *Artemisia annua* L. extract against galactose-induced oxidative stress in mice. *PLoS One* 2014;9:e101486.
 10. Appalasaamy S, Lo KY, Ch'ng SJ, Nornadia K, Othman AS, Chan LK. Antimicrobial activity of artemisinin and precursor derived from in vitro plantlets of *Artemisia annua* L. *Biomed Res Int* 2014;2014:215872.
 11. Marinas IC, Oprea E, Chifiriuc MC, Badea IA, Buleandra M, Lazar V. Chemical composition and antipathogenic activity of *Artemisia annua* essential Oil from Romania. *Chem Biodivers* 2015;12:1554-64.
 12. Bilia AR, Santomauro F, Sacco C, Bergonzi MC, Donato R. Essential oil of *Artemisia annua* L.: An extraordinary component with numerous antimicrobial properties. *Based Complement Alternat Med* 2014;2014:159819.
 13. Perazzo FF, Carvalho JC, Carvalho JE, Rehder VL. Central properties of the essential oil and the crude ethanol extract from aerial parts of *Artemisia annua* L. *Pharmacol Res* 2003;48:497-502.
 14. Yang SL, Roberts MF, O'Neill MJ, Bucar F, Phillipson JD. Flavonoids and chromenes from *Artemisia annua*. *Phytochemistry* 1995;38:255-7.
 15. Bhakuni RS, Jain DC, Sharma RP, Kumar S. Secondary metabolites of *Artemisia annua* and their biological activity. *Curr Sci* 2001; 80:35-48.
 16. Song Y, Desta KT, Kim GS, Lee SJ, Lee WS, Kim YH, et al. Polyphenolic profile and antioxidant effects of various parts of *Artemisia annua* L. *Biomed Chromatogr* 2016; 30:588-95.
 17. Zhu XX, Yang L, Li YJ, Zhang D, Chen Y, Kostecká P, et al. Effects of sesquiterpene, flavonoid and coumarin types of compounds from *Artemisia annua* L. on production of mediators of angiogenesis. *Pharmacol Rep* 2013;65:410-20.
 18. Ogwang PE, Ogwal JO, Kasasa S, Ejubi F, Kabasa D, Obua C. Use of *Artemisia annua* L. infusion for malaria prevention: mode of action and benefits in a Ugandan community. *Br J Pharm Res* 2011;1:124-132.
 19. Ferreira JF, Luthria DL, Sasaki T, Heyerick A. Flavonoids from *Artemisia annua* L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. *Molecules* 2010; 15:3135-70.
 20. Lehane AM, Saliba KJ. Common dietary flavonoids inhibit the growth of the intraerythrocytic malaria parasite. *BMC Res Notes* 2008;1:26.
 21. Funakoshi-Tago M, Nakamura K, Tago K, Mashino T, Kasahara T. Anti-inflammatory activity of structurally related flavonoids, apigenin, luteolin and fisetin. *Int Immunopharmacol* 2011;11: 1150-9.
 22. López-Lázaro M. Distribution and biological activities of the flavonoid luteolin. *Mini Rev Med Chem* 2009;9:31-59.
 23. Patel D, Shukla S, Gupta S. Apigenin and cancer chemoprevention: progress, potential and promise (review). *Int J Oncol* 2007;30: 233-45.
 24. Nikolova MT, Ivancheva SV. Quantitative flavonoid variations of *Artemisia vulgaris* L. and *Veronica chamaedrys* L. in relation to altitude and polluted environment. *Act Biol Szeg* 2005;49: 29-32.
 25. Black P, Saleem A, Dunford A, Guerrero-Analco J, Walshe-Roussel B, Haddad P, et al. Seasonal variation of phenolic constituents and medicinal activities of Northern Labrador tea, *Rhododendron tomentosum* ssp. *subarcticum*, an Inuit and Cree First Nations traditional medicine. *Planta Med* 2011;77: 1655-62.
 26. Nikolova M, Velickovic D. Phenological variations in the surface flavonoids of *Artemisia vulgaris* L. and *Artemisia absinthium* L. *Turk J Bot* 2007;31:459-62.
 27. Bhandari P, Kumar N, Gupta AP, Singh B, Kaul VK. A rapid RP-HPTLC densitometry method for simultaneous determination of major flavonoids in important medicinal plants. *J Sep Sci* 2007;30:2092-6.