Detection and isolation of flavonoids from *Calendula officinalis* (F.Asteraceae) cultivated in Iraq

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Abstract

Calendula officinalis L. (Asteraceae) known as marigold is known to have several pharmacological activities and used for the treatment of several diseases as measles, jaundice, constipation and several inflammations. Marigold flowers contain several chemical constituents mainly flavonoids, triterpenoids and essential oil. In this study marigold flowers cultivated in Iraq had been investigated for its flavonoids content. The study revealed the presence of quercetin and kaempferol glycosides and the absence of myricetin glycosides. The flowers were extracted with ethanol 70% fractionated with different solvent and the flavonoids were isolated by preparative HPLC. The isolated flavonoids were identified by measuring melting points, UV, IR, analytical HPLC and TLC.

Keywords: Marigold, Flavonoids, Kaempferol, Preparative HPLC.

عزل وتشخيص الفلافونويدات من نبات الاقحوان (العائلة : استيراسيا) المستزرع في العراق

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الخلاصة

نبات الأذريون (العائلة أستيراسيا) المعروف باسم القطيفة او الاقحوان الذي يعرف بالعديد من الأنشطة الدوائية ويستخدم لعلاج العديد من الأمراض مثل الحصبة واليرقان، والإمساك، والتهابات عدة. زهور القطيفة تحتوي على العديد من المكونات الكيميائية أساسا الفلافونويدات، التربينات والزيوت. في هذه الدراسة استخدمت زهور نبات القطيفة المستزرع في العراق لدراسة مضمونه من الفلافونويدات. كشفت الدراسة عن وجود جليكوسيدات الكويرستين وجليكوسيدات الكايمبفرول وعدم وجود جليكوسيدات ميريسيتين. تم استخلاص الزهور مع الإيثانول ٧٠% ثم جزأ المستخلص مع المذيبات المختلفة وتم عزل الفلافونويدات باستخدام تقنية الكروماتوغرافيا السائلة ذات الانجاز العالي. وحددت الفلافونويدات المعزولة بقياس درجة الانصهار،وطيف الأشعة فوق البنفسجية والأشعة تحت الحمراء وكروماتوغرافيا السائلة ذات الانجاز العالي وكروماتوغرافيا الطبقة الرقيقة.

الكلمات المفتاحية:الاقحوان ، فلافونويدات، كامبفيرول ، كروماتوغرافيا السائلة ذات الانجاز العالي .

Introduction

Calendula officinalis Linn (Asteraceae) known as marigold is native to central and southern Europe, Western Asia and USA. [(1)] Marigold flowers were used for the treatment of measles, small pox, jaundice, constipation and suppression of menstrual flow [(2)]. It has anti-inflammatory, cytotoxic, antitumor activity [(3,4)], antibacterial and antifungal activity [(5,6)], the flowers contain several chemical constituents as flavonoids and other phenols, triterpenoids, essential oil, polysaccharides [(7,8)], saponins, carotenoids, mucilage and phytosterols [(9,10)]. Various terpenoids have been reported from the petroleum ether extract of *C. officinalis* flowers as β-sitosterol, stigmasterol[(11)] faradiol, calendulodiol, lupeol[(12)], volatile oil [(13)] and also carotenoids as neoxanthin, luteoxanthin, anthroxanthin, and lutein [(14,15)]. *Calendula* flowers also contain coumarins as scopoletin, umbelliferon and esculetin [(16)], flavonoids as quercetin, isorhamnetin, narcissin, calendoflavoside and other flavonoids glycosides [(17,18)] in addition to other constituents as amino acids, bitter constituents and n-paraffin [(19,20)]. Several flavonoids had been isolated from marigold using column chromatography on polyamide sorbent [(21)] or on column using silica gel followed by polyamide column [(17)] or by preparative TLC on silica gel plates [(22)]. Quantitative estimation of total flavonoids in marigold was done using one and two-dimensional TLC or by HPLC on silica gel precoated aluminum plates [(23, 24)]. In our study two flavonoids aglycones were isolated using preparative HPLC and one flavonoid glycoside was detected by analytical HPLC and TLC.

Materials and Methods

Plant material: marigold flowers were collected from the garden of medicinal plants at the College of Pharmacy/University of Baghdad in March and dried in shade at room temperature.

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Extraction: Dried flowers of marigold (5gm) were extracted by maceration with 70% ethanol (150 ml) for 24h, filtered. The procedure was repeated two times; the filtrates were combined together and concentrated under reduced pressure. The obtained extract was fractionated by partitioning with different organic solvents, petroleum ether, chloroform, ethyl acetate, n-butanol (50ml x3 for each solvent) [23].

The first three fractions were dried over anhydrous sodium sulfate, filtered and evaporated to dryness under reduced pressure, while the n-butanol fraction was evaporated to dryness directly. The yields of the fractions were:

Petroleum ether= 0.17gm
Chloroform = 0.23gm
Ethyl acetate = 0.31gm
n-butanol = 0.60gm

Hydrolysis of n-butanol fraction

Part of the n-butanol fraction was hydrolyzed by reflux using 30ml of 10% HCL, cooled and partitioned with ethyl acetate (30ml x3) the ethyl acetate layers were combined together, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness under reduced pressure.

HPLC analysis

ethyl acetate, n-butanol and hydrolyzed n-butanol fractions were analyzed by HPLC using hyperclone ODCC C_{18} column and a mixture of methanol: water (70:30) as a mobile phase with a flow rate of 0.5ml/min and detected at 280 nm.

Thin layer chromatography

Ethyl acetate and hydrolyzed n-butanol fractions using silica gel GF_{254} pre coated aluminum plates developed with following mobile phases:

I- Chloroform: methanol 9:1
II- Chloroform : acetone : formic acid 75:16:8
III- Toluene: chloroform: acetone 40:25:35

The flavonoids contents of these fractions were compared with standards of quercetin (Fluka-Austria), rutin and kaempferol (sigma-Aldrich, USA).

Isolation of flavonoids by preparative HPLC

Two aglycones (I & II) were isolated from the hydrolyzed n-butanol fraction by preparative HPLC using NF 22561/Techno Korma column and methanol: water (70: 30 ratio) as a mobile phase and with a flow rate of 10ml/min, detection at uv 280nm and injection volume of 2 ml the isolated aglycones were recrystallized from boiling ethanol and compared with standard quercetin and kaempferol using the previously mentioned mobile phases.

TLC of n-butanol fraction

n-butanol fraction was analyzed by TLC using the silica gel GF_{254} pre-coated layers developed in the following mobile phases:

I: Methanol: water: formic acid 40: 57: 3 (v/v/v)
II: Ethyl acetate: glacial acetic acid: formic acid: H_2O (100:11:11:25)
III: Ethyl acetate: acetic acid: water 7:1.5: 1.7 (v/v/v)
VI: Toluene: ethyl acetate: methanol: formic acid 32:14: 12:5.

Spiking of n-butanol fraction

part of n-butanol fraction used for analysis in HPLC was mixed with standard rutin and the resultant mixture was re-analyzed by HPLC.

Results

Two flavonoid aglycones were isolated from the hydrolyzed n-butanol fraction and one flavonoid glycoside was detected in the un-hydrolyzed n-butanol fraction.

Flavonoid aglycone I: m.p. 314-317^{0}C, UV λ_{max} (MeOH) 252, 291, 371 nm, IR (KBr) 3418, 3375 (O-H), 1678 (C=O), 1610_s, 1562_s, 1508, 1451 (aromatic).

TLC results of isolated aglycon I are shown in table (1) and HPLC chromatogram of standard quercetin is shown in figure (1).

Flavonoid aglycone II: m.p. 275-278, UV λ_{max} (MeOH) 250, 270 sh, 301, 372sh. IR (KBr) 3321(O-H), 1705 (C=O), 1602, 1612_s, 1558_s, 1508, 1450 (aromatic) TLC results of isolated aglycon II compared with standard kaempferol are shown in table(1) and HPLC chromatogram of standard kaempferol, rutin, and myrecetin are shown in figure(1).

Table (I):- R_f values of standard quercetin and kaempferol compared with isolated aglycons I&II in different mobile phases.

Mobile phase No.	R_f value of standard quercetin	R_f value of isolated aglycon I	R_f value of standard kamepferol	R_f value of isolated aglycon II
I	0.43	0.41	0.62	0.60
II	0.39	0.40	0.5	0.48
III	0.77	0.74	0.86	0.87

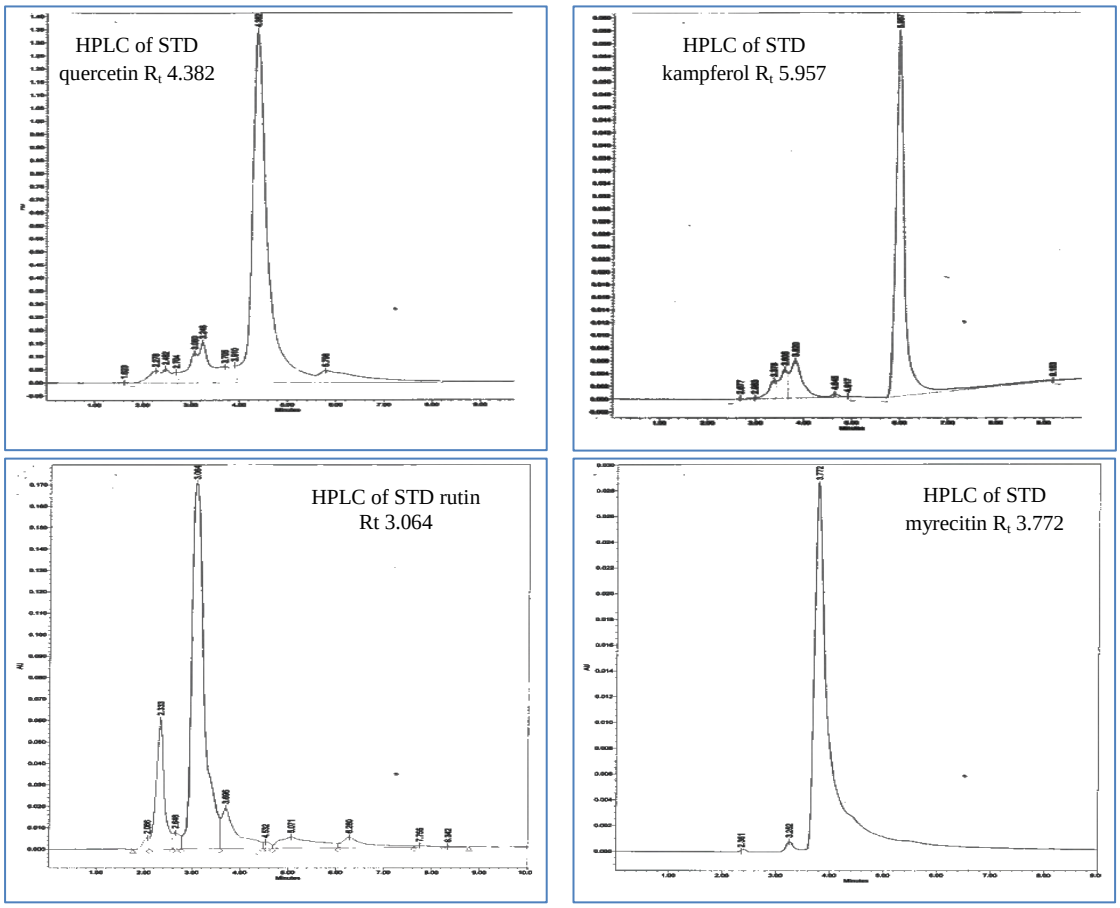

Figure (1):- HPLC of standards quercetin, kampferol, rutin and myrecitin

Detection of rutin

the presence of rutin in n-butanol fraction was detected by HPLC and TLC. HPLC chromatogram of standard rutin is shown in figure 1 TLC of n- butanol fraction compared with standard rutin results are shown in table(2).

Table (2):- R_f values of standard rutin compared with the n-butanol fraction.

Mobile phase No.	R_f value of standard rutin	R_f value of n-butanol fraction
I	0.16	0.14
II	0.42	0.39
III	0.48	0.50
IV	0.11	0.09

HPLC chromatograms of ethyl acetate, n-butanol and hydrolyzed n-butanol fractions are shown in figures 2,3 and 4 respectively.

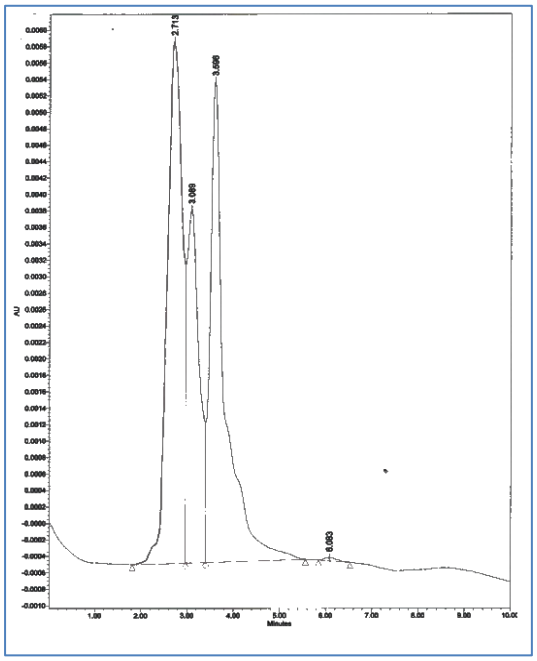

Figure (2): -HPLC of ethyl acetate fraction.

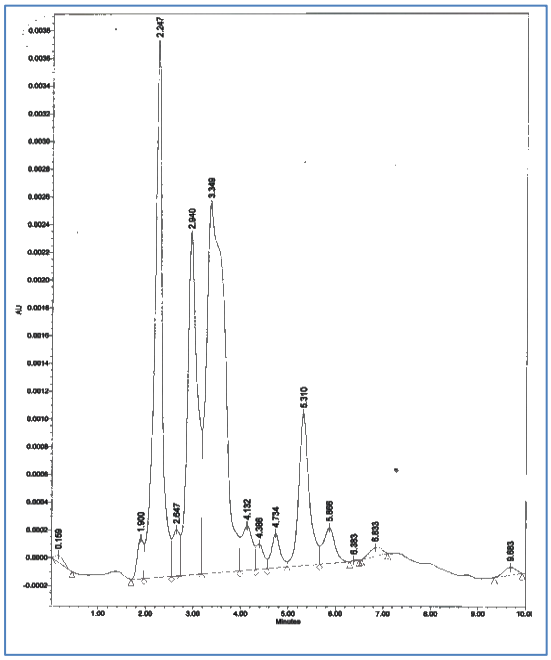

Figure (3):- HPLC of n-butanol fraction.

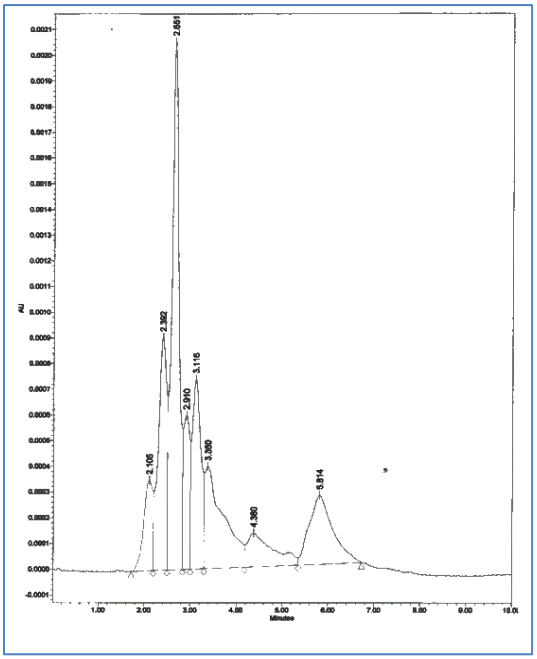

Figure (4):- HPLC of hydrolyzed n-butanol fraction

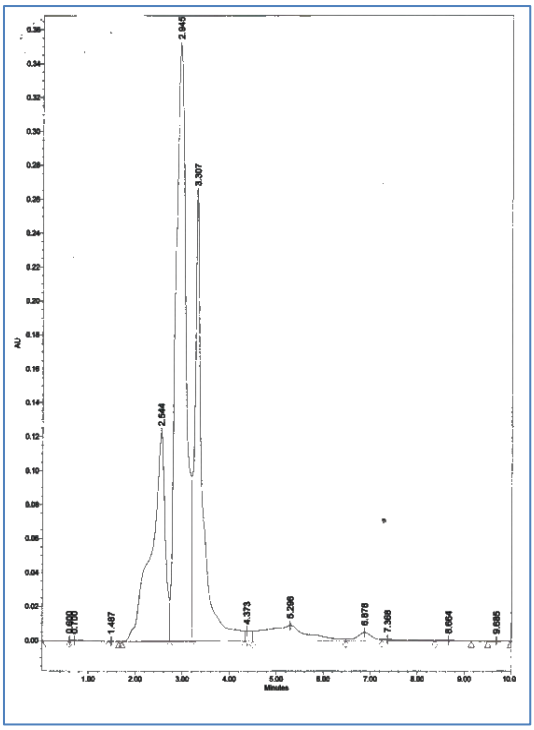

Figure (5):- HPLC of spiking of n-butanol fraction with standard rutin.

The presence of rutin in n-butanol fraction was further confirmed by random spiking with standard rutin & the result is shown in figure (5).

Discussion

Detection of the presence of quercetin and kaempferol glycosides was confirmed after hydrolysis of the n-butanol fraction which

leads to the liberation of these aglycones which could not be detected in the ethyl acetate fraction only traces of kaempferol.

The aglycones are flavonols and their UV absorptions band I appear at 300-350 nm range arise from ring B while band II in the 240-285 nm ranges arise from ring A (24). Melting points, R_f values and IR coincide with that reported for quercetin and kaempferol[25] and the R_f values are identical with the standard. Rutin (flavonol ghycoside) presence in n-butanol fraction was confirmed by analytical HPLC and by spiking with standard rutin and by analytical TLC and co-chromatography with standard rutin in different mobile phases. Rutin is aglycoside of quercetin (quercetin 3-rhamnoglucoside) therefore acid hydrolysis of rutin produces quercetin and this was approved by the isolation of quercetin from the hydrolyzed n-butanol fraction.

Myricetin could not be detected in any fraction of marigold neither as aglycone nor as glycoside.

Conclusion

Quercetin and kampferol are mainly found in *Calendula officinalis* as their glycosides not as aglycones, one of quercetin glycosides detected was rutin (quercetin-3-rhamnoglucoside) while myricetin could not be detected neither as aglycone nor as glycoside.

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