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Extraction, separation, and identification of Two Flavonoids from Leaves of Catharranthus roseus (L) G. Don

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Abstract

Catharanthus roseus (L.) G. Don is one of the most important medicinal plants, mainly due to the presence of anticancer alkaloids. For many years, the phenolic composition of this species remained largely unstudied. Recently, detailed phytochemical studies using the latest analytical techniques have helped understand the complex phenolic profile of this species.In this study The investigation of phenolic compounds in Leaves of Catharranthus roseus (L) G. Don by using different chromatographic methods of separation showed the presence of Kampferol-4\-methyl ether and Quercetin-3-rhamnose-4\, 7-dimethyl ether. The structures of these flavonoids were characterized on the basis of their, UV, ^{1}H-NMR spectral data.

Keywords: Catharanthus roseus, ethyl acetate extract, flavonoids, phenolics.
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Introduction

Natural resources including herbal plants, which contain a large variety of phytochemicals promising as traditional medicine to treat chronic and infectious diseases, have been considered as safe and effective alternatives with fewer side effects compared to synthetic agents [1]**.** Amongst the plethora of medicinal plants identified Catharanthus roseus (L.) G. Don (C. roseus) has been widely used to treat various diseases in many countries. The hot water extract of the dried C. roseus leaf has been used for the treatment of diabetes in Jamaica, Kenya and the West Indies or the hot water extract of the dried plant has been taken orally as complementary and alternative therapies for various types of cancers, heart disease and leishmaniasis in Peru [2]**.** More scientific evidence has proved the potential health benefits of individual phytochemicals extracted from this plant. Of note, vinblastine and vincristine from C. roseus, and their synthetic analogues, have been used in combination with other cancer chemotherapeutic drugs for treating advanced testicular cancer, breast and lung cancers [3]**.** The drying and extraction process are crucial steps prior to isolation and purification of bioactive compounds from plant material. Drying aims to remove moisture content and reduce water activity in plant material, thus inactivating the enzymes responsible for degrading many bioactive compounds, decreasing the rate of microbial growth and reducing the costs of transportation and preservation. However, the drying treatment has been found to have a significant effect on the retention of bioactive compounds and antioxidant capacity of plant material. Therefore, selecting a suitable drying method is very important to maintain the high yield of bioactive compounds and antioxidant power within plant material; however, the economic aspect and the accessibility of drying equipment should also be considered. Similarly, the extraction process is also a key step to obtain bioactive compounds from plant materials. Extraction techniques have been reported to significantly affect the extraction efficiency and stability of bioactive compounds. Hence, this paper provides a comprehensive review of different techniques of drying, extracting and isolating bioactive compounds from the target plant material, C. roseus, and discusses its potential health benefits along with its traditional use.C. roseusis native to Madagascar and known as the Madagascar periwinkle. It is now grown in many countries and is a common decorative, easy growing and spreading perennial herb. The C. roseus plant is 30–60 cm tall with young pubescent branches. Its leaves are oblong or oblanceolate, membranous, entire, obtuse or mucronate, and have short petioles. Inflorescence occurs at the axillary with 1–4 flowered cymes, and flowers are from white to red depending on the cultivar. The calyx tube is short, but the corolla tube is long, sparsely pubescent above and the throat is hairy within or below the stamens. The ovary is pubescent, long and the stigma is pentagonal. Its fruits are 15–25 mm long and have two follicles [4]**.** This plant has been used as a traditional medicine in many countries. The dried leaf or entire plant is boiled with water and then the extract is taken orally to treat diabetes in Northeast India, the Cook Islands, Australia, England, Thailand, Natal, Mozambique, Philippines, Dominican Republic, Jamaica, Northern Europe and [5], [6]**.**The aqueous extract of the leaf or the whole plant is also used by Cook Island and Vietnamese people [5], [7]**.** and Kenyans as complementary and alternative therapies for various types of cancer including throat, stomach and

oesophageal [8].The people in the Kancheepuram District of Tamil Nadu, India mix the powder of C. roseus whole plant with cow's milk, which is taken orally to treat diabetes [9]**.** C. roseus root is dried, ground and decocted for curing urogenital infections in the Venda region, South Africa [10]**,** gonorrhoea in Limpopo Province, South Africa [11].and stomach ache in the Mutirikwi area of Zimbabwe. [12].

So, we interest to choose Catharranthus roseus (L) G. Don leaves which belongs to family Apocynaceae.

1. Plant material

The fresh leaves were collected in February 2018 from Elhawamdia South Giza in Egypt. They were washed, air-dried at lab. Temperature then dried in an oven at 50°C till constant weight, and finally ground to fine powder. The taxonomic identification of plant materials was confirmed by cairo univrisity herbarium.

2. Solvent Systems

The solvents used are abbreviated according to the symbols given in table (1). Solvents from 1–5 were used for flavonoids, while solvents 1 and 6 were used for sugars.

Table (1): Solvent's system used for paper chromatography.

No.	Symbol	Composition	Percent by volume
1	BAW	n-Butanol/acetic acid /water	4:1:5(upper phase)
2	H_2O	Water	-
3	15% AcOH	Acetic Acid / Water	15:85
4	50% AcOH	Acetic Acid / Water	50:50
5	PhW	Phenol / Water	80:20 (w/v)
6	BBPW	Benzene/n-butanol/ Pyridine/H_2O	1:5:3:3 (Upper layer)

3. Preparation of Flavonoid Extract.

Two hundred fifty grams Dry powder of Catharanthus roseus (L.) G. Don leaves were extracted by using methyl alcohol 95% . The residue of the methanolic extract was washed with benzene to get rid of chlorophyll, then washed with successive selective organic solvents hexane, petroleum ether, ethyl acetate, chloroform, methanol 70% finally with water, where six main fractions were obtained: hexane, petroleum ether, ethyl acetate, chloroform, methanol 70% and water fraction.

Results of separation techniques clearly indicated that, the ethyl acetate fraction were the most rich by phenolic constituents most of them are of flavonoids nature.

4. Investigation of flavonoids and phenolic acids of Catharanthus roseus (L.) G. Don leaves were done using chromatographic investigation using paper chromatography, column chromatography [13] of polyamide column [14]and SephadexLH-20 (Pharmacia) column chromatography [15].Identification techniques of flavonoids by chemical analysis [16].Complete elucidation of the flavonoids include physical methods: ultraviolet (UV) [13], nuclear magnetic resonance (^{1}H-NMR measurement using a Jeol Ex-500 spectroscopy; 500MHz (^{1}NMR), or Joel JNM-EX 270 spectroscopy; 270 MHz (^{1}H-NMR), [17].

Results and discussion

1. Flavonoids and Phenolic Acids

1.1. Separation of Flavonoids and Phenolic acids

Flavonoids and phenolic compounds more concentrated in the plant leaves than in the plant stem, which was cleared from the phytochemical study so that, these active constituents were separated from the leaves of Catharranthus roseus (L) G. Don. Two compounds were separated from ethyle acetate fraction of the leaves.

1.2. Purification of ethyl acetate fraction

Ethyl acetate fraction was applied on the top of silica gel column chromatography, eluted firstly with hexane followed by hexane/chloroform to increase polarity until pure chloroform. It was followed by chloroform/ethyl acetate until pure ethyl acetate, then it was followed by ethyl acetate/methanol until finally pure methanol, where two main fractions I and II were obtained.

1.2.1 Purification and identification of fraction I

Fraction I when subjected to two-dimension paper chromatography using B: A: W (4: 1:5) and AcOH-15%, two major spots were obtained. Fraction I was subjected to preparative paper chromatography using the solvent system B: A: W (4: 1:5) for 24 h. give one band which was cut carefully and eluted with 70% ethanol.

Identification of compound 1

1.2.3 Purification and Identification of (compound 1)

Fraction I, when subjected to two-dimensional paper chromatography using B: A: W (4: 1:5) and AcOH-15%, one major spots of flavonoid nature was obtained. It was dried under reduced pressure and purified on sephadex LH-20 column using methanol/ water as described by [18]**,** where a pure flavonoid compound was obtained (Compound1). The R_f values of the separated compound (1) (0.72 & 0.46) were illustrated at Table 2.

Table (2): R_f-values and color reaction of the compound 1.

Solvent	R_f-value	Reagent	Color	
			Visible	UV
B:A:W	0.72	Untreated	—	Yellow
AcOH-15%	0.46	NH_3	—	Yellow
		$AlCl_3$	—	Yellow

UV spectral data, λ_{max} nm, in MeOH

The obtained results of UV spectral data (Figs. 1, 2 & 3) were

UV spectral data, λ_{max} nm, in MeOH:

The obtained results of UV spectral data (Figs. 1, 2& 3) were:

MeOH : 265, 305sh, 365.
NaOMe : 275▲, 325(sh.), 415▲.
NaOAc : 273▲, 305, 375▲.
NaOAc + H_3BO_3 : 265, 295(sh.), 315(sh.), 365.
$AlCl_3$: 268,305(sh), 345,425.▲
$AlCl_3$ + HCl : 268,305(sh), 345,425▲.

^{1}H-NMR spectral data:

The ^{1}H-NMR spectrum of compound 1 in CD_3OD (Fig. 4) showed signals at δ(ppm) 8.07 (2H, d, J= 8.4Hz, H-2\ and H-6\), 6.9 (2H, d, J= 8.5Hz, H-3\ and H-5`), 6.4 (1H, d, J= 2.5Hz, H-8), 6.19 (1H, d, J= 2.5Hz, H-6) and 3.9 (3H, s, OCH_3).

It was appeared on paper chromatography under UV light as yellow spot.

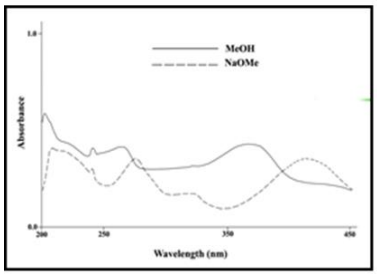


Fig (1)

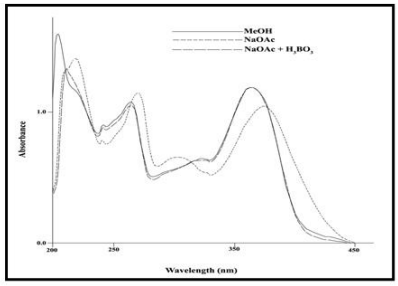


Fig (2)

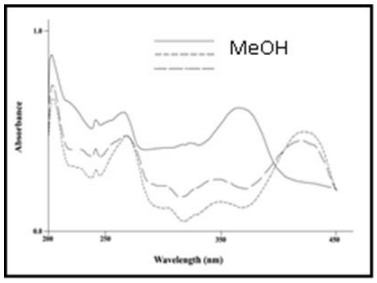


Fig (3)

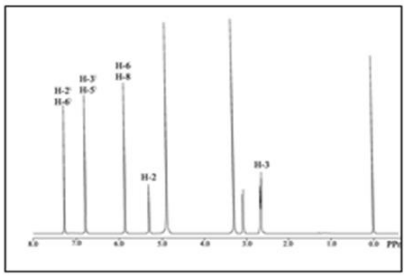
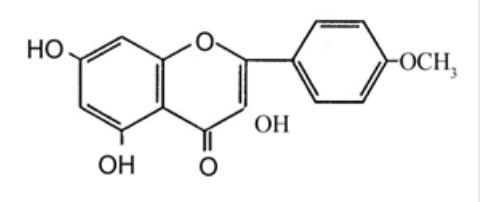


Fig (4)

UV spectral data of the separated compound 1 in methanol showed two bands at 265 and 365 nm, which indicated that 1was a flavonol[15], where addition of NaOMe induced a bathochromic, with decrease in intensity which declared the absences of 4\-OH. Addition of $AlCl_3$ induced a bathochromic which was unaffected on addition of HCl, which proving the absences of catecholic hydroxyl.Additional of NaOAc induced bathochromic shift in band II, which indicated the presence of free OH at position 7, addition of H_3BO_3 induced no shift, to give further confirmation for absences of catecholic hydroxyls.The structure of compound 1 was further confirmed as Kampferol-4\-methylether by ^{1}H-NMR spectrum (Fig. 4), which showed kampferol type protons at δ 8.07 (2H, d, J=8Hz, H-2\ and H-6\), 6.9 (2H, d, J=8.5Hz, H-3\ and 5\), 6.4 (1H, d, J=2.5Hz, H-8) and 6.19 (1H, d, J=2.5Hz, H-6) with addition signals at δ 3.9 (3H, s, OCH_3). Hence, from R_f, color reaction, UV, and ^{1}H-NMR spectral data compound 1 was identified as kampferol-4\-methyl ether.



- **Identification of compound 2**

Fraction II, when subjected to two-dimensional paper chromatography using B: A: W (4: 1:5) and AcOH-15%, one spot of flavonoid nature was obtained. When fraction II was subjected to preparative paper chromatography using the solvent system B: A: W (4: 1:5) for 24 h. give one band, they was cut carefully and eluted with 70% ethanol.

- **Identification of Compound 2**

When band 2 of fraction II, was eluted with 70% ethanol, dried under reduced pressure, purified on sephadex LH-20 column using methanol/ water as described by [18] and subjected to two-dimensional paper chromatography using the solvent system B: A: W and AcOH-15% one major spot of flavonoid nature (compound 2) was detected. R_f values (0.30 & 0.65) were illustrated at Table 4. The purified compound 2 appear on paper chromatography as deep purple spot changed to yellow with ammonia under UV light.

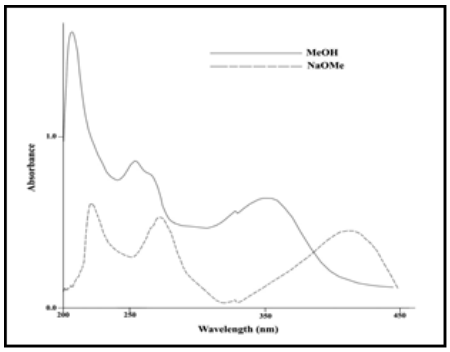
Table (3): R_f-values and color reaction of the compound 2.

Solvent	R_f-value	Reagent	Color	
			Visible	UV
B:A:W	0.30	Untreated	—	Deep purple
AcOH-15%	0.65	NH_3	—	yellow
		$AlCl_3$	—	yellow

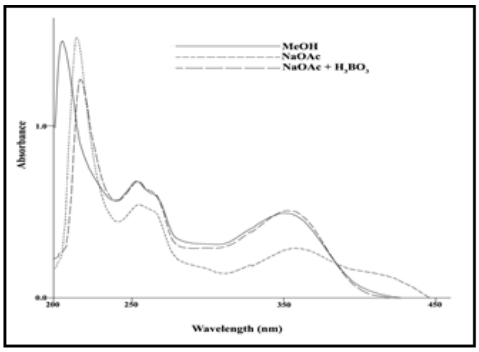
UV spectral data, λ_{max} nm, in MeOH:

The obtained results of UV spectral data (Figs. 5, 6 &7) were:

MeOH : 255, 265(sh.), 350.
NaOMe : 273▲, 415▲.
NaOAc : 255,295(sh.), 360.
NaOAc + H_3BO_3 : 255,295(sh.), 353.
$AlCl_3$:265▲, 302(sh.), 360, 400▲.
$AlCl_3$ + HCl : 265▲, 302(sh.), 345, 395▲.



Fig(5)



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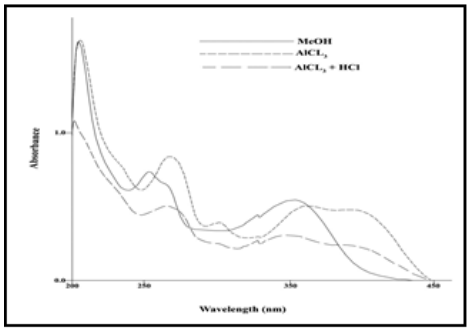
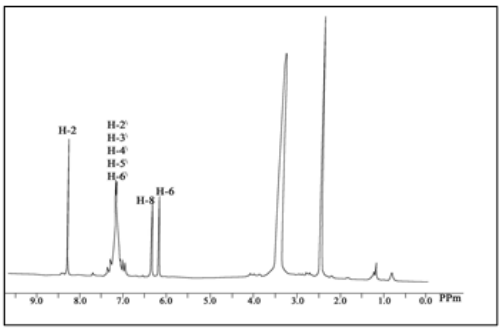


Fig(7)



Fig(8)

^{1}H-NMR spectral data:

The 1H-NMR spectrum of compound 2 in CD3OD (Fig. 8) showed signals at δ(ppm) 7.7(1H, d, J= 8.4Hz, H-2\ ), 7.6(1H, d, J= 8.4Hz, H-6\ ), 6.8 (1H, d, J= 2.3Hz,H-5`), 6.6(1H, d, J= 2.5Hz, H-8), 6.3 (1H, d, J= 2.5Hz, H-6), 5.38(1H, d, J= 6Hz, H-1\\ rhamnose), 3.9(3H,s,oCH3), 3.8(3H,s,oCH3), 3.2-3.8(m, sugar proton) and 1.2 (3H,d, J= 6Hz, CH3 rhamnose).

Compound 2 when subjected to mild acid hydrolysis using 0.1N HCl, refluxed for 15mint, traced every 5minuts and examined by comparative paper chromatography

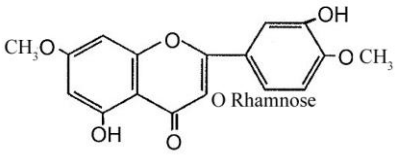
using B: A: Was solvent system with available authentic, gave quercetin-4\,7 dimethyl ether as aglycone and rhamnose as sugar moiety.

UV spectral data of compound 2 in methanol showed maximal absorption bands at 255 and 350 nm, which were typical flavonol with 3-position substitution. Addition of NaOMe induced bathochromic shift for both bands I and II with decrease in intensity which indicated the absence of 4\- OH group and occupation of this position. Addition of $AlCl_3$ induced bathochromic shifts for both bands, which indicated the presence of free OH group at position 5, while addition of HCl induced no change, which indicated the absences of catecholic hydroxyl groups. Meanwhile additional of NaOAc induced no bathochromic shift in band II, which suggested occupation of position 7.

Addition of H_3BO_3 induced no shift, which confirmed the absence of catecholic hydroxyl groups. UV and color reaction of compound 2 showed that it was probably quercetin with 3, 4\, 7 substitutions.

^{1}H-NMR spectrum of (Fig. 8), showed signals characteristic for quercetin with additional signals at δ 5.3 (1H, d, J=6Hz, H-1\\-rhamnose), 3.95 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 3.2-3.8 (m, sugar protons) and 1.28 (3H, d, J= 6Hz, CH_3-rhamnose).

The obtained data showed that, compound 2 can be identified as quercetin-3-rhamnose-4\, 7-dimethyl ether



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ARABIC SUMMARY

استخلاص وفصل وتعريف مركبين من الفلافونيدات من اوراق نبات الونكا

للدكتور
محمد ابراهيم حجاج
قسم النبات والميكروبيولوجى- كلية العلوم- جامعة الازهر- مدينة نصر-القاهرة- مصر

الهدف من الدراسة: اجريت هذه الدراسة معمليا لاستخلاص وفصل وتعريف اثنين من المركبات الفلافونيديه من اوراق نبات الونكا.

نتائج هذه الدراسة: خلصت هذه الدراسه ان اوراق نبات الونكا غنى بنواتج الايض الثانويه وخصوصا الفلافونيدات وان النبات موضوع الدراسه استخلصت منه سابقا ومازالت تستخلص منه مركبات تستخدم كأدويه لمرضى السكري ومرضى السرطان حول العالم ولاهميته حرصت على استخلاص و فصل وتعريف اثنين من ابرز هذه المركبات باستخدام طرق فصل مختلفه واستخدام الاجهزة الطيفية المختلفه للتعريف وهذان المركبين هما (كامفيرول-4- ثنائى ميثيل ايثير – كوارستين-3 رامنوز-7,4 ثنائى ميثيل ايثير).