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Novel bioactive flavonoid 5-hydroxy-2-(8-hydroxy 4-oxo-4*H*-chromen-2-yl)-4-oxo-3,4-dihydro-2*H*-1-benzopyran-7-yl-N-[(2-methylpropoxy) carbonyl] carbamate from *Kalanchoe pinnata*

Latha Ophelia George*[a], RadhaH R[b], Pragasam A[c] & Preeti N Tallur[d]

[a] Research and Development Centre, Bharathiar University, Coimbatore 641 046, India

[b] Professor, Department of Chemistry, KSSEM, Bangalore 560 109, India

[c] Associate Professor, Department of Chemistry, Maharani Science College for Women, Mysore 570 005, India

[d] Associate Professor, Department of Chemistry, Maharani Science College for Women, Mysore 570 005, India

E-mail: lathaophelia@gmail.com, radha.sudhakar99@gmail.com, preetiksh2003@gmail.com

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Kalanchoe pinnata is a conspicuous medicinal plant and finds wide applications in pharmaceutical industries. Phytochemical studies have established the mode of action of plant extract as bioactive agent. The foremost constituents accountable for biological activity are polyphenols, which are an important class of naturally occurring antioxidants, having innumerable biological activities such as anticancer, antifungal, antibacterial, antiviral and anti-cholesterol. The present study is focussed on extraction, identification and characterization of flavonoids present in leaves of *Kalanchoe pinnata*. The purified fractions of the methanol extract have been quantised by preparative TLC and RP-HPLC to achieve maximum purity. The isolated purified compounds have been studied for spectral characterisation like IR, ^{1}H and ^{13}C NMR and MS. On the basis of chemical constitution and spectral analysis, the structure has been elucidated as a 5-hydroxy-2-(8-hydroxy 4-oxo-4*H*-chromen-2-yl)-4-oxo-3, 4-dihydro-2*H*-1-benzopyran-7-yl N-[(2-methylpropoxy) carbonyl] carbamate. Combined bioactivities are yet to be evaluated along with other known compounds like gallic acid and quercetin.

Keywords: *Kalanchoe pinnata*, RP-HPLC, Methanol extract, Spectral analysis

Kalanchoe pinnata is one of biologically important widely available medicinal plant having appreciable photochemical like gallic acid, quercetin and polyphenols. Photochemical studies clearly mentioned the mode of action of plant extract as bioactive agent in pharmaceutical industries. Oxidative stress, a result of an overproduction and accumulation of free radicals, is the leading cause of several degenerative diseases such as cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases. Compounds from plant source like terpenoids, alkaloids and flavonoids are utilized as drugs to prevent various diseases[1-6] Flavonoids are known for potent *in vitro* antioxidants for free radical scavenging activity by inducing protective enzymes[7-11] and play vital roles in many of the processes underlying vascular dysfunction and the development of atherosclerosis. Quercetin belongs to set of plant pigments called flavonoids that are mostly responsible for the colours of several fruits, flowers and vegetables. It plays the role as anti-inflammatory, antioxidant, and anticancer agents[12]. Gallic acid is a natural phenolic compound and have also been found in a number of phytomedicines with diverse biological and pharmacological activities, including radical scavenging, interfering with the cell signalling pathways and apoptosis of cancer cells. Among various polyphenols, gallic acid (3, 4, 5-trihydroxybenzoic acid), a naturally occurring low molecular weight triphenolic compound, has emerged as a strong antioxidant and an efficient apoptosis inducing agent[13-15].

Natural products represent a major strategy for discovering and developing new drugs. Moreover, secondary metabolites of the plant display chemical and pharmaceutical properties[1-4]. Flavonoids consists of many subgroups such as flavone, flavanone, flavonol, isoflavonoid, anthocyanidin, and chalcones.

The therapeutic actions of plants are unique to particular plant species or groups and are consistent with this concept as the blend of secondary products in a particular plant is taxonomically distinct. Phenolic compounds embody the largely studied phytochemicals and have been widely explored as model systems in

different areas of plant research. Most flavonoidic compounds exhibit antipyretic, analgesic, anti-inflammatory, anti-arthritic, antioxidant and immuno-modulatory properties. These activities of flavonoidic compounds may be due to the presence of catechin, ellagic acid, gallic acid, vanillin,, quercitin, tannin acid, resorcinol, *etc*.[16-22]

Kalanchoe pinnata (*K. pinnata*) is a crassulescent herb commonly known as air plant which contains numbers of flavonoids. It is a succulent perennial plant that reaches a height of 3-5 feet tall having hollow stems, fleshy dark green leaves and bell-like pendulous flowers. The leaves of the plant have been used by traditional folks for the treatment of numerous diseases as it is considered a sedative, wound-healer, diuretic, anti-inflammatory and cough suppressor. The objective of the research work was to separation and identification of flavonoids. The plant *Kalanchoe pinnata*, especially the leaves, has been reported to possess a wide range of medicinal properties, which has prompted a comprehensive investigation of leaves. This paper was mainly focussed to isolate and characterise the important bioactive flavonoids from leaves with help of spectroscopic techniques like TLC, RP- HPLC, IR, ^{1}H NMR and MS.

The objective of this work was to ascertain the fingerprint profile of *Kalanchoe pinnata* using thin layer chromatography (TLC) and reversed phase high performance thin layer chromatography (RP-HPLC) technique for two prominent phenolic compounds found in medicinal plants using two mobile phases having different elution gradients and run times. These RP-HPLC fingerprints of standard phenolic compounds could be used as benchmarks for the purpose of comparison when doing the qualitative and quantitative analysis of unknown compounds present in any plant sample. The multiple chemical constituents present in any herbal sample could be benchmarked against these standards to assess the quality and quantity of phenolic flavonoids actually present, thereby giving a clear indication of its likely therapeutic efficacy.

Materials and Methods

Chemicals and reagents

All chemicals and reagents used in the study were of analytical grade and purchased from reliable firms and institutes [Sd-fine chemicals, SIGMA, SRL (India), MERCK, RANBAXY, and SUYOG]. Silica gel (G) 60F and 0.25 readymade aluminium sheets (Merck KGaA, Germany). Silica gel 60 F254, HPTLC aluminium sheets 20×20 cm, Merck KGaA, Germany.

Sample collection

The plant, *Kalanchoe pinnata* was collected from Mandya district of South India. The plant parts and leaves were separated. The leaves were then washed and carefully shade dried. The dried material was homogenized in an electric blender to fine powder. The powder was sieved and stored in air tight container for experiments.

Extraction of phytochemicals

The sample powder was first defatted using petroleum ether and then extracted with 500 mL of Chloroform followed by Methanol using Soxhlet apparatus. The extraction was carried out for 8 hours using various solvents such as chloroform, acetone, and ethyl acetate and methanol. The extract was concentrated by evaporation of solvent using Rota-vacuum evaporator. Then extracts were poured in to Petri plates, dried completely and stored in screw cap tubes and kept at 4°C.

100 g of the air dried coarse powdered defatted plant material was percolated with alcohol (95%) for 16 h and repeated three times until exhaustion. The alcoholic extract was evaporated by Roto-evaporator at 50°C. Flavonoid contents and their presence were determined by the method of Harborne (1998), using quercetin as a standard.

Column Chromatography

A column of sized 2 inch diameter and 90 inch length was used. The column was done using silica gel bed and toluene as solvent and allowed to settle for 30minute. 10gm methanol extract was mixed with 5 mL toluene and poured on the top of silica gel bed and the solvent was drained by maintaining 1cm on the silica gel bed in the column. Different fractions were collected for further characterisation using chloroform, methanol and toluene in definite ratios as eluting solvent mixtures. The eluted fractions were further purified by TLC using Hexene: Chloroform: Methanol (6.5:3.5:0.5) over silica gel as solvent system.

Thin layer chromatography (TLC)

Thin-Layer Chromatography was performed on silica gel 60F254 (2 cm× 5 cm; 0.25 mm layer thickness; Merck). KDM extract 25mg/mL prepared and filtered through a 0.45 micron syringe filter,

25 microlitre of these extracts were subjected to spotting on (silica gel 60F254 (Merck, Darmstadt, Germany) TLC plate. The plate was air dried and then developed by using the solvent system hexane: ethyl acetate: methanol (7:2.5:0.5 v/v) as mobile phase20 min. Plate was dried at 65ºC for 2 min and then it was observed under UV chamber 254 and 360nm. The coloured spots were visualized under the UV light between 254 and 360 nm. The dark brown bands that were produced after keeping the plates in iodine chamber were scratched and suspended in the mobile phase separately for 3–4 days. It was vacuum evaporated and the residue left was collected for analysis through RP-HPLC and IR spectrum.

RP-HPLC

Optimized Chromatographic conditions:

Detector	: Shimadzu spd10A uv-vis, Japan
Column	: Phenomenex Gemini-NX-5 µm C18(2) 110 Å, LC Column 250×4. mm, Ea
Elution Type	: Isocratic
Elution A	: Methanol
Elution B	: Methanol: Phosphate buffer *p*H 3.2 (70:30)
Flow Rate	: 1mL/min
Col. Temp	: ambient
Detection	: UV-Vis Abs.-Variable Wave. (UV) @ 280nm

The isolated constituent was further subjected to purification with Hexane chloroform wash then for characterisation by NMR, IR, LC-MS TLC and HPLC.

Results and Discussion

Thin-Layer and column Chromatographic separation of the novel flavonoid

Methanolic extraction of the plant leaves is used to fractionate the different available compounds. The different fractionated compounds are further purified by thin layer chromatographic techniques.

The TLC studies showed that among the four solvents (petroleum ether, chloroform, ethyl acetate and methanol) used for extraction, the high polarity solvent methanol extracted higher quantity of secondary metabolites of medicinal importance *viz*., alkaloids, flavonoids, phenols and tannins from the leaves of K. pinnata. The composition of the mobile phase for TLC was optimized by testing different solvent mixtures of varying polarity. The combination of Hexane: Ethyl acetate: Methanol in the ratio of 6:2:1 was used as

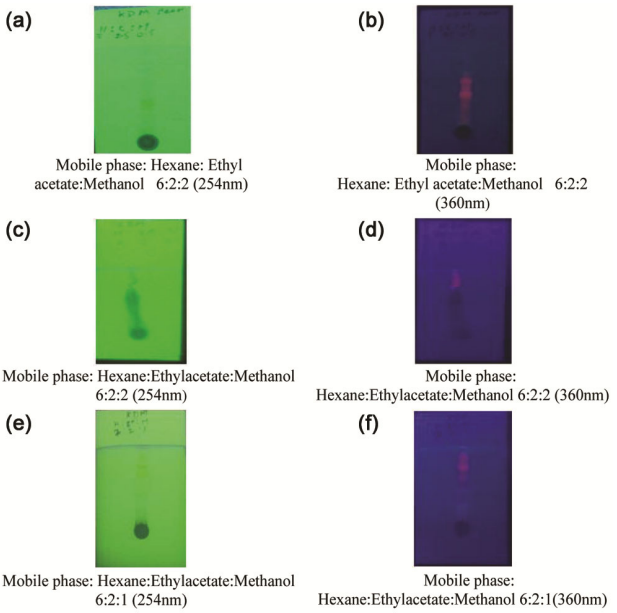
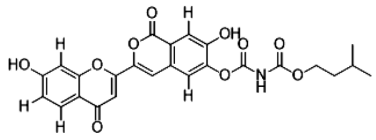


Fig. 1 — Thin-layer and column chromatographic separation of the novel flavonoid



Chemical Formula: $C_{25}H_{21}NO_{10}$
Exact Mass: 495.12
Molecular Weight: 495.43
m/z: 495.12 (100.0%), 496.12 (27.7%), 497.12 (5.8%)
Elemental Analysis: C, 60.61; H, 4.27; N, 2.83; O, 32.29

M (m/z)	M (m/z)ob	Fragment Peaks	The sample mass spectrometry of mass recorded in +ve mode. The sample mass obtained exact mass of sample.
495.12	495.00	178 and 255	

Fig. 2 — Proposed chemical structure of compound isolated from *Kalanchoe pinnata*

suitable mobile phase for the secondary metabolite flavonoid. At UV- 254 and 360 nm the spots were identified. The corresponding TLC is presented in Fig. 1a-f. These phytoconstituents in the methanolic extract had several visible colour spots on the TLC plate. The coloured spots were visualized under the UV light between 254 and 360 nm after the TLC plate was sprayed with specific spraying reagents, thus indicating the presence of phytoconstituents.

The identified spots on the TLC plate were carefully scrapped and dissolved in methanol. All similar methanol fractions are quantitatively collected together and evaporated the solvent to get partially pure compound. These compound fractions were further purified by RP-HPLC and probable structure was assigned by elementary analysis and LC-MS as shown in the Fig. 2.

Structural Elucidation of the of the expected compound

IR- Spectral analysis

IR stretching frequencies for the identified compounds were listed in Table 1.

NMR spectral analysis

^{1}H and ^{13}C NMR shielding and deshielding values were listed in Table 2 and Table 3. The final structure has been confirmed by ^{13}C NMR and elucidated structure is shown the Fig. 2. ^{13}C NMR Spectrum of the novel compound is shown in Fig. 3.

Elucidated structure and Name of the novel compound identified in *Kalanchoe pinnata*

Elucidated structure and name of the novel compound identified in *Kalanchoe pinnata* is shown in Fig. 4.

The fractionated was purified by column chromatography, TLC and the reversed-phase HPLC method was developed for the quantitative estimation. Exactly quantized novel compound was identified and used for the structure elucidation. Elementary analysis and LC-MS spectral data clearly indicates the corresponding molecular mass and elementary composition as 495.12 and $C_{22}H_{21}O_{11}$.The compound separated was characterised under IR spectroscopy to confirm various bond stretching frequencies and functional groups. Relative stretching frequencies for the isolated compound of *K. pinnata* was tabulated in

Table 1 — Stretching frequencies of various type of bonds

S.No.	Frequency (cm^{-1})	Functional group
1	795.29	Ar-mono sub-
2	955.37	=C-H Stretch
3	1125.25	-C-O-C- Stretch
4	1189.25	Ar-O-H Stretch
5	1412.29 -1461.11	-C=C- Stretch
6	1530.54	R-CO-NH-R Stretch
7	1609.43	Ar-CO-O-R Stretch
8	1735.59	C=O Stretch
9	2850.77, 2919.29, 2947.71	-C-H Stretch
10	3331.93	-NH Stretch

Table 2 — ^{1}H NMR spectral data

S.No.	Functional group	Proton NMR (δ)	Signals	Coupling constant (J)	No. of Protons
1	N-H	9.92	Broad singlet (bs)	NA	(1.0H) 1H
2	Ar-H	7.880-7.859	Multiplet (m)	8.4Hz	(1.49H) 2H
3	Ar-H	7.355-7.285	Multiplet (m)	28Hz	(2.97H) 3H
4	Allylic (=C-H)	4.95-4.89	Quartet (q)	24Hz	0.73H (1H)
5	$-CH_2$	2.87	Singlet (s)	NA	(2.27H) 2H
6	$-CH_2$	2.466	Singlet (s)	NA	(2.46H) 2H
7	-CH	1.278	Singlet (s)	NA	0.529H (1H)
8	$-CH_3$	1.83-1.64	Multiplet (m)	76Hz	(5.54H) 6H

Table 3 — ^{13}C NMR spectral data

S.No.	Functional group	Carbon NMR (δ)	Remarks
1	-C=O	161.93-157.14	C^{13} NMR recorded in $CDCl_3$, The $CDCl_3$ signals observed at 77.3028-76.790
2	Ar-C	131.63	
3	Ar-C	131.24	
4	Ar-C	129.81-129.70	
5	Ar-C	127.86-127.35	
6	Ar-C	122.99	
7	Ar-C	120.99	
8	Ar-C	117.12-115.93	
9	Ali-C (CH_3)	40.55	

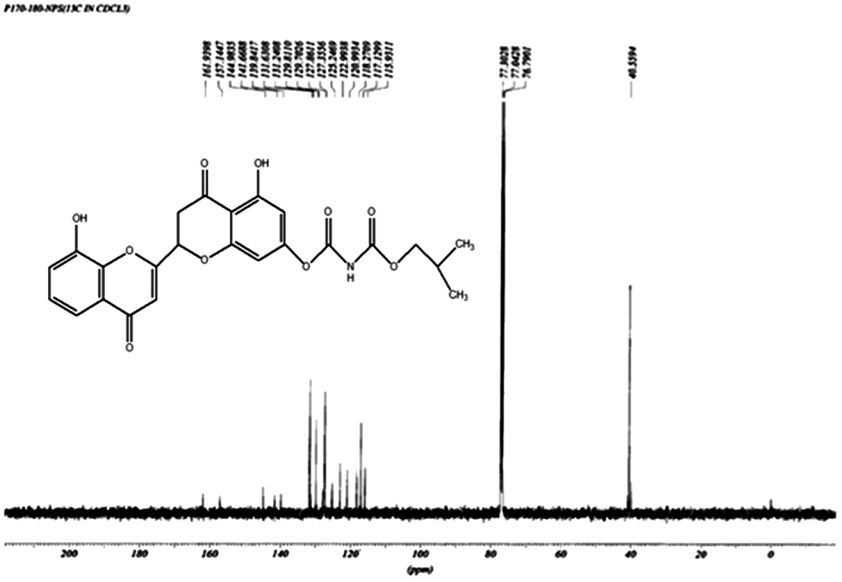
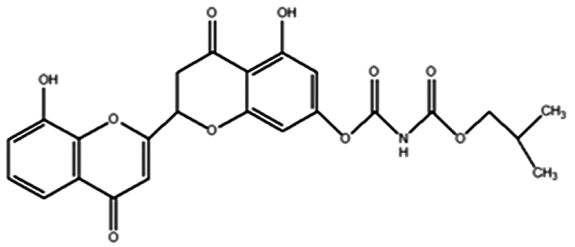


Fig. 3 — ^{13}C NMR spectrum of the novel compound



5-hydroxy-2-(8-hydroxy 4-oxo-4Hchromen-2-yl)-4-oxo-3,4-dihydro-2H-1-benzopyran-7-yl N-[(2-methylpropoxy)carbonyl]carbamate

Fig. 4 — Chemical structure and name of compound isolated from *Kalanchoe pinnata*

Table 1. ^{1}H and ^{13}C NMR characterisation conspicuously indicates and differentiates the vicinity of various protons and carbons attached in the molecule. The spectral data spectrum of the novel compound was recorded in Table 2 and Table 3 and that of spectrum is in the Fig. 1. With reference to spectral data and spectrum, it is clear that the confirmed structure of the novel compound is as shown in the Fig. 2.

Compounds like gallic acid and quercetin are also identified in the methanolic extracts of *K. pinnata* through RP-HPLC whose concentrations were in the order 3.395 µg and 2.685 µg. The *K. pinnata* morphological parts shown combined pharmaceutical effect, the isolation and characterisation finds lot of future scopes.

Conclusion

Compounds like gallic acid and quercetin are also identified in the methanolic extracts of *K. pinnata* through RP-HPLC whose concentrations were in the order 3.395 µg and 2.685 µg. The structure of the novel compound was elucidated on the basis of chemical constitution and spectral analysis as a 5-hydroxy-2-(8-hydroxy 4-oxo-4*H*-chromen-2-yl)-4-oxo-3, 4-dihydro-2*H*-1-benzopyr an-7-yl N-[(2-methylpropoxy) carbonyl] carbamate. Studies done have shown that the most important pharmacological properties of gallic acid are attributed to its antioxidant and anti-inflammatory potentials. In addition, gallic acid is involved in various signaling pathways that regulate the wide range of biological functions including pro- and inflammatory pathways, NO signaling pathway, intrinsic and extrinsic pathways of apoptosis. Gallic acid and its derivatives demonstrated a broad range of beneficial effects in prevention and management of several disorders, also their acceptable safety and stability profiles, make them significant options to be introduced as dietary supplements. Quercetin, a remarkable flavonoid with potent antioxidant and anti-inflammatory properties, is expected to revolutionize the pharmaceutical industry as a multi-faceted therapeutic agent. The extensive research on quercetin has unveiled a multitude of health benefits, offering a promising outlook for various medical conditions. As advancements in science and technology continue, the precise mechanisms underlying quercetin's effects are becoming clearer. Its ability to scavenge free radicals and combat oxidative stress makes it a potential shield against age-related diseases and other conditions influenced by cellular damage. Study must be initiated the role of the novel compound in the presence of quercetin and gallic acid.

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