IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 3, Issue 1 (Jan. – Feb.2017), PP 31-36
www.iosrjournals.org

Separation and Identification Main Polyphenolic Compounds From *Urginea undulata*

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Abstract:*Chromatographic methods revealed the separation of eight biologically active constituents from Urgineaundulata. Bulb and Identification of the chemical composition as well as the physico-chemical properties of the eight active substances was carried out using Ultraviolet and [1]H-NMR spectral data, the separated compounds are three phenolic acids (Ferulic acid, Gallic acid (3, 4, 5-trihydroxy benzoic acid) andCoutoric acid) and five compounds of flavonoids (4', 7-dihydroxyflavone, Quercetin, Rutin,Apigenin and Luteliolin-7, 3\, 4\, tri-O- glucuronide).*

Keywords:*Urgineaundulata, phenolics, bulbs, flavonoids, phenolic acids, U.V. and 1H NMR.*

I. Introduction

Phytochemical constituents are the main source for the establishment of several pharmaceutical industries. The chemical constituents present in the plant play a significant role in the identification of crude drugs **(Akindele&Adeyemi, 2007)**. Probably originated in central Asia and then introduced in Europe by the Phoenicians around 2000 years ago, onion is grown in every part of the world because it is widely adaptable and can occupy a wide range of ecological niches. World onion production has increased by at least 25% over the past 10 years with current world production being about 85 million tons from 4.3 million hectares making it the second most important horticultural crop after tomatoes **(Faostat, 2014)**. Onion is considered one of the most versatile vegetables: from raw to caramelized and from marinated to roasted, it can be found in a wide range of recipes from breakfast to supper and is accepted by almost all traditions and cultures. However, onion is not only a food, but it has also well-known medicinal and functional properties.The recent literature is rich in both in vivo and in vitro studies reporting the anti-thrombotic- **(Lee et al., 2013)**, hypo-lipidemic**(Lee et al., 2012; Srinivasan, 2013)**anti-diabetic- **(Jung *et al.*, 2011)**, anti-obesity- (Yoshinari*et al*., 2012), antioxidant-**(Alpsoy*et al.*,2012; Lee *et al.*, 2012)**, anti-inflammatory **(Vazquez-Prieto*et al.*, 2013)**, cancer chemopreventive- **(Wang *et al.*, 2012)**, and antiparasiticproperties of onion extracts **(Klimpel*et al.*, 2011; Aboelhadid*et al.*,2013)**. Moreover, some epidemiological studies suggest that a diet rich in onions may have a favorable effect on the risk of acute myocardial infarction **(Galeone et al., 2009)** and benign prostatic hyperplasia**(Galeone*et al.*, 2007)**.

II. Materials And Methods

1. Plant Materials :

Fresh bulbs of *Urgineaundulata* were collected from growing habitats in Lakes Borollos (Elwardisland) rulbs ere dry and grinding to final powder for further investigation.

2. Methods:

*** Preparation of Polyphenolic Compounds Extract**

Phenolic compounds 750 gm Dry powder of Urgineaundulata were extraction by 70% ethanol and purified according to standard procedures reported by **Mabry *et al.*, (1970) and Harborne, (1984)**. Combined filtrates were evaporated under reduced pressure using rotavapour apparatus until a minimum amount of solvent remained. The residue (greenish sticky) was stored in a refrigerator at 5 ºC and kept for using in different analysis.

2.1 Chromatographic Investigation

2.1.1Paper Chromatography

Two dimensional paper chromatography (TDPC) was carried out on Whatman (1MM) for comparative studies of aerial part extracts under investigation using BAW for the first dimension, followed by 15% AcOH for the second dimension. For separation and purification of mixture of the phenolic compounds, the elution techniques on Whatman paper (3MM) were used. After the material under investigation was applied to the paper chromatography, the latter was run using solvent system No: 1-8 as outlined in table (1). Glass chromatographic tanks were used applying the ascending or descending techniques. The developed P.C. were air – dried and examined before spraying under both visible and UV light (λmax= 366 and 245nm). The P.C. were exposed to ammonia vapours for about 2-3 seconds and immediately re-examined the P.C. to observe any possible changes that may eventually appear in color or fluorescence under a long wave UV lamp.

2.1.2 ColumnChromatography

The separation and identification of plant extracts were carried out by column chromatographic techniques (CC) **(Markham, 1982).** Silica gel columns were used for the separation and purification of phenolic compounds mixtures. Fractions produced from a large column often yielded simple mixtures of phenolic compounds. Further separation on small column or by paper and thin layer chromatography (T.L.C.) was carried out to obtain a pure flavonoids and phenolic acids.

2.2 Identification Techniques of Flavonoids

The qualitative analysis of flavonoid glycosides involves three distinct methods:

1- Identification of the aglycone.

2-Identification of the sugar moiety.

3- Identification of the point of the sugar attachment.

This is usually carried out through both chemical and physical investigation as indicated below.

2.2.1 ChemicalAnalysis

Analysis to determine the nature of aglycones and sugars was carried out according to standard procedures performed by **Mabry *et al.*, (1970) and Harborne (1984).**

***Complete Acid Hydrolysis**

Complete acid hydrolysis was carried out for 40-60 min. at 1000C using 5ml 2N-hydrochloric acid. The hydrolysis was then extracted with ethyl acetate, and the received extract was subjected to paper chromatographic investigation to detect the aglycones, the mother liquor was carefully neutralized, and then subject to chromatographic investigation by used HPLC to detect the sugars.

• Identification of the Aglycone

Identification of the aglycone moiety of flavonoid glycosides was based on direct co-chromatographic comparison with standard sample or/and UV spectroscopy followed NMR if needed.

• Identification of the Sugar

Sugars obtained by hydrolysis of the flavonoid glycosides were identified by chromatographic investigation using HPLC.

2.2.2 PhysicalAnalysis

Complete elucidation of the flavonoids should include physical methods such as ultraviolet (UV) and nuclear magnetic resonance (1H-NMR and 13C-NMR) measurements.

2.2.2a. Ultraviolet Spectroscopic Analysis

Ultraviolet visible absorption spectroscopy is perhaps the most useful technique available for flavonoid structure analysis using UV- visible spectrophotometer (Shimadzu model UV-240 and 2401 PC). The technique is used to aid both identification of the oxygenation and glycosylation patterns **(Mabry et al., 1970 and Markham,1982).** The flavonoid spectrum is usually determined in methanol using 2ml quartz cells. The spectrum typically consists of two absorption maxima at the ranges 240-285 nm (band II) and 300-500 nm (band I). The precise position and relative intensities of these maxima give valuable information on the nature of the flavonoid and its oxygenation pattern; the effects brought about by these changes are as follows:

1- Changes in the substitution of the A-ring tend to be reflected in the band II absorption while alternations in the substitution of the B-ring and C-ring tend to be more apparent from the band I absorption.

2- Additional oxygenation (especially hydroxylation) generally causes a shift of the appropriate band to longer wavelength, e.g. band I in 3,5,7,-tri OH flavone, 359 nm; 3,5,7,4\-tetra OH flavone, 367 nm; 3,5,7,3\,4\,-penta OH flavone, 370 nm and 3,5,7,3\,4\,5\-hexa OH flavone, 374 nm.

3- Methylation or glycosylation (especially of 3, 5, 7, and 4\ hydroxyls) causes band shifts to shorter wavelength. The nature of the sugar in glycosides is normally of no consequence.

4- Acetylation tends to nullify the effect of a phenolic hydroxyl group on the spectrum.

5- The presence of cinnamic acid as acyl function on a flavonoid can be detected by the presence of an absorption band at 320 nm in flavonoids that themselves lack significant absorption at this region (e.g. anthocyanins).

6- In flavones and flavonols, the presence of 3\, 4\-di-OH system is generally evidenced by a second peak (sometimes a shoulder) in band II.

2.2.2b. 1H-NMR spectroscopy

The NMR spectroscopy is a well - established method for structure elucidation of flavonoids **(Mabry et al., 1970)** using a Jeol Ex-500 spectroscopy; 500MHz (1NMR), 125 MHz (13C-NMR) or Joel JNM-EX 270 spectroscopy; 270 MHz (1H-NMR), 67.5 MHz (13C-NMR).

There are widely applied techniques for obtaining 1H-NMR spectra, using DMSO-d6 (hexadeutro dimethyl sulphoxide) as a solvent for the direct 1H-NMR analysis.

1H-NMR spectra yield three sets of information: the integrals, the coupling pattern and the chemical shifts.

1- The integrals define the number of protons represented by each signal or group of signals.

2- The coupling patterns reflect the mutual arrangement of the coupling protons. In aromatic compounds, the coupling constants are 7-9 Hz between ortho protons, 1-3 Hz between meta protons and less than 1 Hz between para protons. The Para coupling, however, is usually not resolved, but causes broadening of the single only, which may in turn obscure a meta coupling.
3- The chemical shift of a proton indicates its chemical environment i.e. its position relative to any other part of the molecule. Chemical shift values are nowadays almost exclusively given parts per million (ppm) downfield from the TMS signal (Harborne, 1993). I.e. 1H-NMR analysis has been used to determine the aglycone part of the isolated flavone glycosides, to decide the number of sugars in the flavone O-glycosides, and the positions of the linkages of the sugar units in the falvone C-glycosides.
The great number of published 1H-NMR spectra of flavonoids simplified the chemical shift assignments of the protons in the aromatic region of the flavonoid (Table 2). It also helps to decide the number of sugar units in the flavonoid O-glycosides, since the chemical shifts of the anomeric protons are more downfield than the other sugar protons.
For structure elucidation of flavonoid C-glycoside, measurements have been made on the parent flavonoid C-glycoside in DMSO-d6 **(Mabry et al., 1970)**, and the same solvent could be used for both 1H and 13C-NMR spectroscopy. When only small amounts of a sample were available, the C-glycoside was dissolved in DMSO-d6 and submitted to 1H-NMR analysis followed by permethylation according to the mass spectroscopy analysis. By holding a high temperature (800C) when recording the 1H-NMR spectrum, the signal obtained from the absorbed water was minimal.

2.2.2c. 13C-NMR Spectroscopy

13C-NMR provides a very sensitive method for determination of the structure of complex compounds. With the introduction of Fourier-transform methods, 13C-NMR spectroscopy has developed into a powerful tool for the structure elucidation of small amounts of natural products. Over the past thirty years, 13C-NMR spectroscopy has become well established in the structure analysis of flavonoids. Several articles have been published. Most of them have dealt with flavonoid aglycones**(Chari et al., 1977; Markham et al., 1978 and Shen et al., 1993)** and only a few have dealt with C-glycosides **(Chopin et al., 1978 and Agrawal, 1989)**. The position of a signal relative to the TMS reference (0.0 ppm) is a good guide to the type of carbon represented. This position is shifted markedly by nearby substituents. Such effects are predictable in their extent and have led to the formulation of substituent effect data, which defines the expected effect (on aromatic carbon resonance) of introducing a new substituent into an aromatic ring. Using this type of substituent effect data, it is possible to calculate with great accuracy the spectrum of an unknown flavonoid form. To do this it is necessary to have available a wide range of reference spectra and a number of such complications have appeared.
In general terms sugar-related substitution include shifts are as follows:
1- O-glycosylation of a flavonoid hydroxyl produces an upfield shift of up to 2 ppm in the signal of the adjacent flavonoid carbon and downfield shifts in the signal of the ortho and especially para related carbons (1-4 ppm).
2- C-glycosylation of a flavonoid aglycone causes a 10 ppm downfield shift of the signal of the glycosylated carbon, but leaves other signals relatively unaffected.
3- Glycosylation of sugar by glucose causes an 8 ppm downfield shift in the signal of the glycosylated carbon accompanied by a 1-3 ppm upfield shift in the signal of the adjacent carbons. Glycosylation by rhamnose causes smaller shifts (3-6 ppm downfield and 1-2 ppm upfield, respectively).

Table 1. Approximate chemical shifts of various flavonoids proton types

Chemical shift (ppm)	**Proton type**
0 - 0.5	**Trimethylsilyl ether group**
c.1.0	**Rhamnose C-CH_3 (broad doublet)**
c.1.7	**Prenyl (-CH_2-CH=C(CH_3)$_2$ methyl groups**
c.2.0	**Acetate (-OCOCH_2) and aromatic C-CH_2**
2 – 3	**H-3 of flavanones (two proton – multiplet)**
3.5 - 4.0	**Most sugar protons**
4.2 - 6.0	**H-1 of sugars (also H-2 of dihydroflavanolos, 5.0 ppm & H-2 of flavanones 5-5.5 ppm)**
c 6.0	**Methylenedioxy (O-CH_2-O) singlet**
6.0 - 8.0	**A and B- ring protons**
7.5 - 8.0	**H-2 of isoflavones (singlet)**
12 – 14	**5-OH (observed only when using DMSO- d_6**

III. Results And Discussion

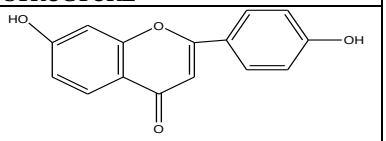
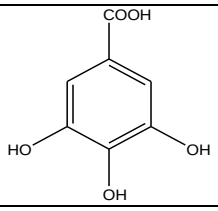
1. Phenolic Compounds

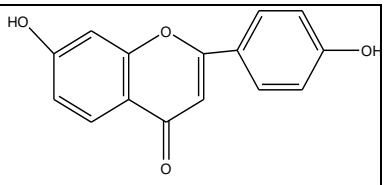
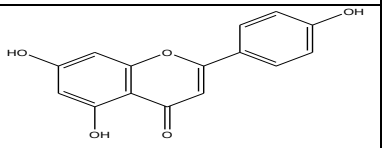
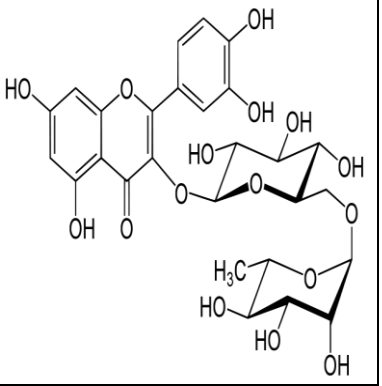
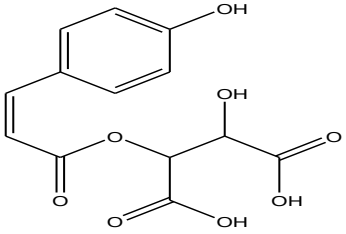
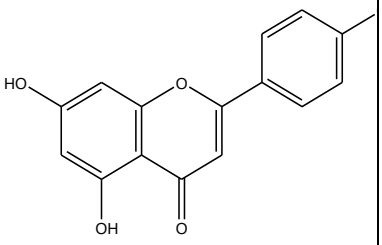
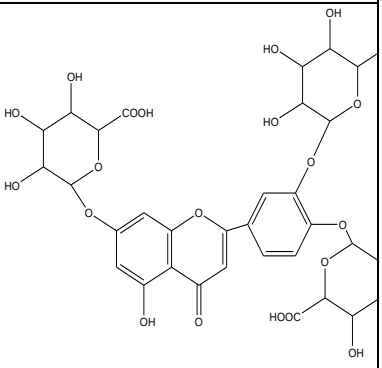
Dried powder of Urgineaundulata total aerial parts (500 g.) in studied habitats, when extracted by ethanol 70% in soxhlet apparatus concentrated to very small volume by used rotavapour under reduce pressure at 40Co to yield 58 gm., fraction of Urgineaundulata under investigation was examined by using AcOH-15% and BAW, dried chromatograms examined under UV light, re-examined after exposure to ammonia then spread AlCl3 to complete evaluated phenolic compounds and chosen the best method for isolation and purification.

1.1. Isolation and Identification of Polyphenolic Compounds of *Urgineaundulata*

70% total ethanol extract after examination by using AcOH-15% and BAW, was applied on the top of silica gel column (12cm X 180cm, 200 gm. silica jell, 20 drops per minutes and 100 ml per fractions) **(Sharaf*et al.*, 2014)** which started from hexane and increased in polarity., in system (hexan : petroleum ether 5:5 revealed the presence of some concentration compounds (fraction 11), which preparative in acetic acid 15% in whattmann paper chromatography 3mm to yield one main blue band, which were subjected to spotting by paper chromatography to using solvent AcOH-15% and BAW revealed the presence of one spot of phenolic acid in nature, compound N1 58 mg. by increased in polarity in system hexan : petroleum ether 1:9 (fraction 17), which preparative in system chloroform :methanol 9:1 in thin layer chromatography TLC to yield one sharp blue band, which were subjected to spotting by paper chromatography to using solvent AcOH-15% and BAW revealed the presence of one spot of phenolic acid in nature, compound N2 35 mg. in system petroleum ether : chloroform 3:7 (fraction 26), which preparative in in thin layer chromatography TLC (hexan : chloroform :methanol 2:4:4) to yield one sharp yellow band, which were subjected to spotting by paper chromatography to using solvent AcOH-15% and BAW revealed the presence of one spot of flavonoids in nature, compound N3 108 mg. while in system chloroform : ethyl acetate 8:2 (fraction 43) which was applied on the top of silica gel column (5cm X 80cm, 80 gm. silica jell, 20 drops per minutes and 50 ml per fractions) which started from hexan and increased in polarity., revealed the presence of one main fractions, in system petroleum ether : chloroform 6:4 , preparative of latter fraction in BAW (butanol :acetic acid : distal water 4:1:5) in whattmann paper chromatography 3mm to yield one main yellow band, which were subjected to spotting by paper chromatography to using solvent AcOH-15% and BAW revealed the presence of one spot of flavonoids in nature, compound N4 44 mg. in ethyl acetate : methanol 9:1 (fraction 52), after examination by using AcOH-15% and BAW was preparative application in paper chromatography 3MM (30 sheet) in BAW as a mobile phase, the presence yellow Band was applied on thin layer chromatography TLC in system TEF (toluene : ethyl acetate : formic acid 5:4:1) which yielded two main band, band 1 was sharp yellow band , elution by methanol to produced N5. 29 mg. while band 2 was phenolic acid in nature N6 57mg. at the end in system ethyl acetate : methanol 4 : 6 were subjected to spotting by paper chromatography to using solvent AcOH-15% and BAW, latter fraction was applied on the top of silica gel column (5cm X 80cm, 85 gm. silica jell, 20 drops per minutes and 50 ml per fractions) which started from ethyl acetate and increased in polarity., in system ethyl acetate : methanol 8:2, revealed the presence of one main fractions, which preparative of latter fraction in BAW (butanol :acetic acid : distal water 4:1:5) in whattmann paper chromatography 3mm to yield two main band, band 1 was sharp yellow band , elution by methanol to produced N7. 48 mg. while band 2 was flavonoids in nature N8 59mg. which were subjected to spotting by paper chromatography to using solvent AcOH-15% and BAW to make purity test for specific compounds.

Table 2. Spectra data for main active constitutes isolated from of *Urgineaundulata* bulbs.

NO.	SPECTRA DATA	STRUCTURE
1	Rf: Acetic acid 15%: 0.87, BAW: 0.5, COLOR :U.V.: Blue, U.V. + NH3: Blue, U.V.: MeOH: 283 sh, 318, NaOMe: 284 sh, 320, IH NMR : (DMSO-D6), δ: 3.8(s, 3H, CH3), 4.9 (s, 1H, Ar-OH), 6.45(d, 1H, J=7.8Hz, Alip-H), 6.5 (m, 2H, J=4.8Hz, Ar-H), 6.95(d, 1H, J=2.7Hz, Ar-H), 7.5 (d, 1H, J=8.5Hz, alip-H), 11(s, 1H, COOH). , C10H10O4: **Ferulic acid**.	
2	Rf: Acetic acid 15%: 0.89, BAW: 0.65, blue salt, U.V. λ_{max}**MeOH**: 273, 334, **NaOMe**: ▲276, ▲344, **^{1}HNMR**. (DMSO-D_6), δ: 3.0 (s, 3H, OH), 6.99 (s, 2H, C_6H_5), 11.0 (s, 1H, COOH). **Gallic acid** (3, 4, 5-trihydroxy benzoic acid),	

3	Rf: Acetic acid 15%: 0.62, BAW: 0.48, COLOR :U.V.: Faint Yellow, U.V. + NH3: Yellow, U.V.: MeOH: 285 sh, 328, NaOMe: 280, ▲397, AlCl3: 278 sh, ▲394, AlCl3+ HCl: 273 sh, ▲401, NaOAc: 291, ▲354, NaOAc+ H3BO3: 290 sh, 328, IH NMR : (DMSO-D6), δ: 5.37(d, 2H, J=5.7Hz, Ar-OH), 6.39(d, H, J=2.7Hz, Ar-H), 6.48(d, 1H, J=3.6Hz, Ar-H), 6.71(m, 2H, J=4.8Hz, Ar-H), 7.31(m, 3H, J=19.2Hz, Ar-H), 7.47(s, 1H, Ar-H) , $C_{15}H_{10}O_4$: **4' 7-dihydroxyflavone**.	
4	**R_f**: Acetic acid 15%: 0.25, BAW: 0.72, **COLOR** :U.V.: Yellow, U.V. + NH_3: Yellow, **U.V**.: MeOH: 261, 302 sh, 375, NaOMe: 290, ▼453, $AlCl_3$: ▲275, ▲450, $AlCl_3$+ HCl: 276, ▼428, NaOAc: 264, ▲385, NaOAc+ H_3BO_3: 265, 302 sh, ▲380, **^{1}H NMR** (DMSO-D6), δ: 5(s, 4H, Ar-OH), 6(s, 2H, Ar-H), 6.5(s, 1H, Ar-H), 7.1(s, 2H, Ar-H), 7.8(s, 1H, Ar-H), $C_{15}H_{10}O_7$: **Quercetin**.	
5	**R_f**: Acetic acid 15%: 0.69, BAW: 0.43, **COLOR** :U.V.: Purple, U.V. + NH_3: Yellow, **U.V**.: MeOH: 250, 273 sh, 292 sh, 359, NaOMe: ▲266, 324, ▲405, $AlCl_3$: ▲268, 298 sh, ▲429, $AlCl_3$+ HCl: ▲268, 300, ▲404, NaOAc: ▲266, 330, ▲394, NaOAc+ H_3BO_3: ▲262, 303, ▲381, **^{1}H NMR** (DMSO-D6), δ: 1.25(d, 3H, J=7.3Hz, CH_3), 2(s, 7H, alip-OH), 3.4(s, 2H, alip-H), 3.52(s, 3H, alip-H), 3.6 (s, 2H, alip-H), 3.7 (s, 2H, alip-H), 3.81(s, 1H, alip-H), 5(s, 4H, Ar-OH), 5.7(d, 1H, J=6.2Hz, Ar-OH), 6(d, 2H, J=8.7Hz, Ar-H), 6.6(d, 1H, J=5.7Hz, Ar-H), 6.64(d, 1H, J=3.5Hz, Ar-H), 7.3 (d, 2H, J=6.9Hz, Ar-H), $C_{27}H_{30}O_{16}$: **Rutin**.	
6	**R_f**: Acetic acid 15%: 0.21, BAW: 0.55, **COLOR** :U.V.: Blue, U.V. + NH_3: Blue, **U.V**.: MeOH: 275 sh, 283 sh, 310s, NaOMe: 276 sh, 284 sh, ▼302, **^{1}H NMR** (DMSO-D6), δ: 2(d, 1H, J=4.9Hz, alip-OH), 5(d, 1H, J=3.8Hz, Ar-OH), 5.3 (d, 2H, J=6.1Hz, alip-H), 5.96(m, 1H, J=9.8Hz, alip-H), 6.68(d, 2H, J=4.8Hz, Ar-H), 7.09(m, 3H, J=12.4Hz, alip-H), 11(d, 2H, J=3.9Hz, COOH), $C_{13}H_{12}O_8$: **Coutoric acid**.	
7	**R_f**: Acetic acid 15%: 0.09, BAW: 0.85, **COLOR** :U.V.: Violet, U.V. + NH_3: Faint Yellow, **U.V**.: MeOH: 271, 340, NaOMe: ▲279, ▲400, $AlCl_3$: 280, 301 sh, 391, $AlCl_3$+ HCl: 275, ▲394, NaOAc: 271, ▼382, NaOAc+ H_3BO_3: 270, 342, **^{1}H NMR** (DMSO-D6), δ: 5(s, 3H, Ar-OH), 6(s, 2H, Ar-H), 6.65(m, 2H, J=14.9Hz, Ar-H), 6.73(m, 1H, J=3.5Hz, Ar-H), 7.16(m, 2H, J=10.7Hz, Ar-H), $C_{15}H_{10}O_5$: **Apigenin.**	
8	**R_f**: Acetic acid 15%: 0.12, BAW: 0.46, **COLOR** :U.V.: Purple, U.V. + NH_3: Yellow **U.V**.: MeOH: 274, 281 sh, 292 sh, 318, NaOMe: 281, 315 sh, ▲375, $AlCl_3$: ▲291, 314 sh, ▲398, $AlCl_3$+ HCl: 276, 305 sh, ▲340, NaOAc: 277, 290 sh, ▲351, NaOAc+ H_3BO_3: 279, ▲342, **^{1}H NMR** (DMSO-D6), δ: 2(s, 9H, J=5.6Hz, alip-H), 3.5(d, 3H, , J=2.7Hz, alip-H), 3.9(m, 6H, J=9.5Hz, alip-H), 4.5(m, 3H, J=5.8Hz, alip-H), 5.9(d, 3H, J=4.6Hz, alip-H), 6.4(m, 3H, J=2.7Hz, Ar-H), 6.7(d, 1H, J=3.8Hz, Ar-H), 6.9(d, 2H, J=10.5Hz, A-H),7.6(d, 1H, J=4.3Hz, Ar-H), 11(s, 3H, COOH), $C_{30}H_{34}O_{24}$: **Luteliolin-7, 3\, 4\, tri-O- glucuronide**	

In the present studies, rutin was separated in pure form ethyl acetate and metanol fraction of ***Urgineaundulata***and identification by use U.V. and ^{1}H- NMR, which clear that the rutin was content multi hydroxyl group attached with heterocyclic ring and four hydroxyl group attached to aliphatic sugar chain, this hydroxyl group refer to polar solvent behavior, "polar solvent dissolved polar solute and non-polar solvent dissolved non-polar solute" this is a rule in the solubility science, but result and rule according to observation was conflict, To explain this contrast is possible to say that the rutin attached with nonpolar compound e.g. lipid by bond and this bond was break by mobile phase with high elute strength according to that the rule may be modification by adding Condition that we should note conjugated compound attached by bonds **(*Sharaf et al., 2014*)**.

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