New Method for Isolation and Determination of Oleocanthal as carboxylic ester INTRODUCTION

Article *in* International Journal of Scientific and Engineering Research · July 2017

CITATIONS
0

READS
132

3 authors:

H. N. K. Al-Salman
University of Basrah
29 PUBLICATIONS **32** CITATIONS
SEE PROFILE

Usama Ramadhan
University of Basrah
21 PUBLICATIONS **11** CITATIONS
SEE PROFILE

Shaker A.N. Al-Jadaan
University of Basrah
70 PUBLICATIONS **65** CITATIONS
SEE PROFILE

New Method for Isolation and Determination of Oleocanthal as carboxylic ester

H. N. K. Al-Salman, Usama H. Ramadhan, Shaker A. N. Al-Jadaan*

Pharmaceutical Chemistry Division, College of pharmacy,
University of Basrah/ Iraq

*Corresponding author E-mail:shakeraljadaan@yahoo.com
Mobile: +9647811111180

ABSTRACT: A very newly method was described for the Identification and determinationof[2-(4-hydroxyphenyl) ethyl (3*S*, 4E)-4-formyl-3-(2-oxoethyl) hex-4-enoate]. Oleocanthal (OLC) as Water Soluble carboxylic ester. The method extracted it from virgin olive fruits by two main steps with separator funnel, the first step by mix solvent *Ethanol: Chloroform: Water (40:40:10)* and the second step by mix solvent *Ethyl acetate: Water (50:50)*. The Specific estimates ofOleocanthalwith the mixture solvent Acetonitrile: Toluene: Formic acid: Water (4:2:0.5:0.5) was determined by TLC chromatography.The method isolated and purifiedOleocanthal by extracted from olive fruits with methanol/water (80/20, v/v) using a modification of an existing procedure. To obtain one pure material Oleocanthalwas used spectrophotometric IC-UV method in max. wavelength at 278 nm with C18 solid phase column (250mm×4.6mm ID , 5µm) is used for Quantification of Oleocanthal , One peak refers to only one compound in extraction ; Also to Identification of only one extracted compound Via Gas Chromatography-mass spectrometry (MSDCHEM\1\METHODS\MUAFAQ.M) , Oleocanthal is further identified . All methods in procedure have been successfully applied to the determination and identification of Oleocanthal.

Keywords: Oleocanthal (OLC), carboxylic ester,virgin olive fruits,non-steroidal anti-inflammatory drugs

INTRODUCTION:

Oleocanthal (OLC) is a carboxylic ester (phenylethanoid) that is the IUPAC name [2-(4-hydroxyphenyl) ethyl (3*S*, 4E)-4-formyl-3-(2-oxoethyl) hex-4-enoate]. Chemicalformula $C_{17}H_{20}O_5$ and theMolar mass 304.34 g/mol. [1, 2].

Oleocanthal is found in olive fruits but it is not clear whether the natural product is a mixture of E(+)/Z(-) isomers or a single as the two isomers readily interconvert in solution, most pharmacological studies will have been performed using a mixture [3,4,5] .

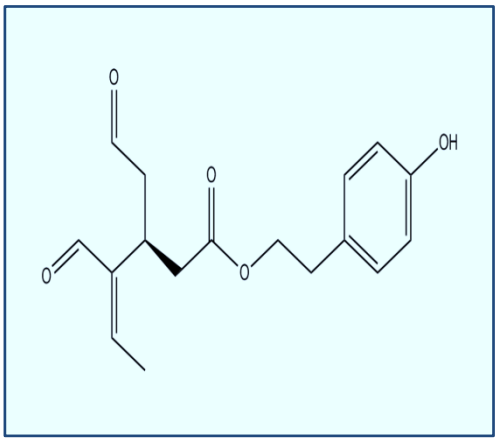


Figure 1: Chemical structure of Oleocanthal

Routine extract able testing has been performed using reflux extraction Soxhlet [6]. These techniques have disadvantages associated with the handling and disposal of significant volumes of potentially flammable and hazardous organic solvents. Extractions usually proceed for 24 h and therefore must be left an attended [7, 8].

Phenolic compounds extracted from the virgin olive fruits have attracted the attention of many researchers in the medical and industrial fields [9]. One of the most important components of this extract is Oleocanthal (+)(-) , which acts as an inhibitor of the enzymes COX1 and COX2 , which have similar efficacy to that of Ibuprofen , the non-steroidal anti-inflammatory drug[10,11] .

Oleocanthal is found in the virgin olive fruits and is working to tear up part of the cancer cells and the

dumping of toxic waste inside and destroy the proteins that cause death within 45 minutes in healthy cells resumes their life cycle after about one day according to the results of the study [12, 13]. In addition to killing cancer cells, Oleocanthal reduces the size of tumor in living tissues [14].

Oleocanthala type of natural phenolic compound found invirgin olive fruits. It appears to be responsible for the burning sensation that occurs in the back of the throat when consuming juices of olive fruits. Oleocanthalis a tyrosol ester and its chemical structure is related to Oleuropein, also found in olive fruits [15, 16].

For the provision of Oleocanthal a phenolic compound with very promising pharmacological properties, isolation from olive fruits is a very important option [17]. Due to the compound's sensitivity to decomposition upon exposure to oxygen and light, a very gentle isolation method has been developed under use of Liquid-Liquid extract and high performance Liquid chromatography (HPLC). By extract method and partition of olive fruits. This method, the isolation of tyrosol ester (hydroxyl tyrosol) and the mixture of (3S,4E)- and (3S,4Z)-OLC was achieved in approx. 50 min for each step[18,19].

A high-performance liquid chromatography (HPLC) method was developed to quantitatively analyze Oleocanthal in virgin olive fruits [20]. Natural phenols spectral

data show a typical UV absorbance characteristic of benzene aromatically at 270 nm. As molecules with higher conjugation levels undergo this bathochromic shift phenomenon, a part of the visible spectrum is absorbed, so; the wavelengths left in the process (generally in red section of the

spectrum) recompose the color of the particular substance. The solvent extract was analyzed by reversed-phase HPLC with UV detection at 278 nm. [21, 22].

Goal of study:

A newdevelopment method in the Isolation, diagnosis and evaluation of the phenolic compound (Oleocanthal) from virgin olive fruits.

Materials and method:

All solvents and reagents were of analytical grade unless indicated otherwise, and all experiments were performed with deionized water (18.2 Ω-cm) resistivity at 25 ^{o}C [**23**].

Equipment:

Chromatography experiments were carried out by HPLC-UV chromatography consisting of:

- LKB Bump 2150 –HPLC, Bromma

Ion Pac Ercus C18 RP-Column; 5µm, (250×4.6 mm id) (P/N 11051194 L) from European was chosen for some Organic compounds separation.

- Metrohme Electric injection valve with 100 µL loop fitted in.
- A PD 303 UV detector single beam (Japan) equipped with an 18 µl flow cell (Helma. UK.) Data logger Lab JackU12 acquisitions (Ocean control/ Australia).
- Personal computer supplied with modifies software programs / cvi programs UV.
- Printer (EPSON-L210 / Japan).
- pH meter (Hana- Italy).
- Gas Chromatography-mass System (MSDCHEM\1\METHODS\MUAFAQ.M) to determination of M/Z Negative Ions.

Reagents and standards:

- Acetonitrile for HPLC grade, BDH Chem. LTD
- Ethyl acetate, BDH Chem. LTD
- Ethanol and Methanol, BDH Chem. LTD
- Chloroform for HPLC grad. LTD
- Toluene and Formic acid, BDH Chem. LTD
- Oleocanthal and analarOleocanthal as standard Sigma-Aldrich German.
- Water was obtained by following purification in a deionized water system.

Working methods:

The fruits of the studied virgin olive were washed well with water to remove the dust and plankton and then dried with a stream of dry air [24, 25]. 1 kg of virgin olive fruit was

taken and cut into small parts and placed in the soxhlet [26]. The extraction process was performed using mixture solvent *Ethanol*: *water* (3:1) as solvent for all the compounds in the fruits such as unsaturated fatty acids, alkaloids, phenols and others. To best an extract phenolic was prepared and subjected by two-steps extract separation under use in the first step of *Ethanol :Chloroform : Water (40:40:10)* mixtures solvent in normal-phase and reverse phase mode, respectively but in the second step the mixture solvent *Ethylacetate : Water (50:50)* . The separation process was then performed for all ingredients in the extraction mix using a mixture of solvents in the separation funnel where total phenols were isolated from the rest of the extracts [27, 28].

Theextract result obtained by the filtration apparatus under vacum to remove plankton and sediment. After drying the solvent using rotary evaporator (20 rpm) at a temperature 80 °C, where a high-density phenolic extract was obtained and dried to calculate the weight of total phenols [29].

Specific estimates of phenols in the studied samples:

The quality of the phenolic extract with the mixture solvent Acetonitrile :Toluene :Formic acid :Water (4:2:0.5:0.5) was determined by TLC chromatography in the research laboratory of the College of Pharmacy / University of Basrah, where three clear and non-trace spots showed three phenolic compounds in the dry mixture [30,32].

Isolation method of Oleocanthal from mixture phenolic compounds:

To isolate and purify Oleocanthal, The irritant was extracted olive fruits with Ethanol/water (80/20, v/v)

using a modification of an existing procedure [33]. The phenolic extract was precipitate by using Methanol : water (3:1), Only one compound is Oleocanthal phenolic extract compound was identified as irritating from the majority of the other co-extracted phenolic compounds using methanol : water as solvent mixtures at three different ratios of eluting solvents . Analysis of the throat-irritating fraction revealed the presence of several unresolved compounds [34, 35].

Quantification of Oleocanthal in olive fruits:

Toobtain pure material Oleocanthal byReversed-phase HPLC with UV detection at 278 nm. A flow rate of 1 ml/min was used and the injection volume is 100 µl, pre-fractionated the olive fruits phenolic extract on a C18 solid phase column was used for Quantification of Oleocanthal in olive fruits at a constant temperature (25°C)using an elution gradient with acetonitrile: water (2:1) V/V, retention information about the throat-irritating principal HPLC method allowed to determination it. A new HPLC gradient was thus developed and only one well-resolved peak was throat-irritating, view in figure 2.

Chromatographic separation of Oleocanthal from other extracted compounds and of the two geometric isomers of Oleocanthal (+) and (-). Both the external standard calibration curve and the internal standard calibration curve were established, and quantitation using both calibration curves gave essentially the same result [36, 37].

Sample Analysis:

Chromatograms of a Oleocanthal (OLC) sample as well as comparison of Peak and retention time allows the identification of (OLC) , recoveries for standard sample ranged from 98-100 % suggesting that the analysis

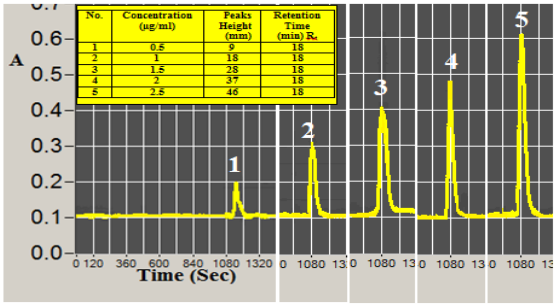
method is accurate [38]. The results were shown in Table 2.

Analyze method by HPLC-UV system:

From a stock solution containing 25.0 µg/ml Oleocanthalin mixture of acetonitrile /water (2:1), a standard curve to analyze by HPLC-UV system was prepared at the concentration of 2.5, 5.0, 7.5, 10.0 and 12.5 µg/ml in mixture of acetonitrile /water (2:1). For standardization, 100 mL of the standard solutions of Oleocanthalwere transferred to glass tubes at room temperature, the concentration range of standard curve was diluted five times in mobile phase and the corresponding solution was submitted to chromatographic analysis at 0.5, 1.0, 1.5, 2.0 and 2.5 µg/ml of Oleocanthal[39].

Table:1:Optimum condition for separation Oleocanthal in HPLC System :

Parameters	*Conditions*
Description Column	Ion Pac Ercus C18 RP-Column; 5µm, (250×4.6 mm id) (P/N 11051194 L)
System Suitability Requirement	USP Tailing Factor @ 5 % From average Peaks Height 1.12 Plates/Column ≥1920.88
Isocratic Mobil phase	acetonitrile and water (2:1) V/V
Test sample	Oleocanthal diluted in the mobile phase
Detection System	UV detection
Maximum Wavelength	278 nm



igure 2: HPLC Peak's for

leocanthal

esults and discussion:

1-Effect of concentration eluent on the separation and retentiontime:-

A Series of experiments were established to find the Optimum eluent concentration. Figure 2 shows the standardchromatogram which obtained by inject 1.5 µg ml^{-1} [1]Oleocanthal (OLC) on a C18 column into the eluent with mixture Eluent from acetonitrile: water (2:1) v/v. The (OLC) peak is well resolved in less than 18 min from the void volume. One peak appearance in chart, the mean cause of separation wasthe properties of column and the type of eluent, the peak refers to (OLC) Extraction which is mean studies, after extract and purity material that is other compound extraction within mean peak [40].

2- Effected Column Temperature on the separation at Oleocanthal (OLC) Active components:-

The effect column temperature in the range 25-45°C on separation of Oleocanthal was evaluated. As expected increasing the column temperature decrease the Retention time and led to good baseline for the separation chromatogram due to difficulty of maintain temperature stability in the IC system.So; 25°C was chosen in the present work. Under the condition established a calibration curve for Oleocanthal was obtained .It is linear in the range (0.5-2.5) µg / ml Typical calibration results are shown in figure 3.The linear graph has a regression coefficient of (0.9997) for five points . Table 3 reports data from the calibration graph [41, 42].

Representative samples (µg mL^{-1})	Peaks Height (mm)	* ±RSD%	Retention Time (t_R) minutes	±*RSD%
1.0	18	±0.517	18	±0.5273
1.5	28	±0.522	18	±0.5270
2.0	37	±0.498	18	±0.4982

3- Method performance (linearity Reproducibility, and Detection Limits):-

Table 2 listed the results to obtain the reproducibility of three consecutive injection of 1.5 µg ml^{-1} of (OLC) sample. Excellent RSD for retention time and peak height were obtained in Figures 2 and 3.

Table 3: Regression statistics of the proposed method with LLOD, LLOQ, Intercept and Slope for Oleocanthal material as standard and Oleocanthal Extraction from Olive fruits.

Table 2: The reproducibility of peaks height and t_R of Oleocanthal

Claimed Conc. (µg mL^{-1})	Found conc. (µg mL^{-1})	Recovery ± RSD
1.0	**0.99**	**99** ±0.5273
1.5	**1.50**	**100** ± 0.5270
2.0	**2.0**	**100** ±0.4982
2.5 µg mL^{-1} for Oleocanthal Extracted	**2.45**	98 ± 0.4333

Figure 3: Standard Calibration curve of Oleocanthal

To evaluate the accuracy of a new method in IC system with recovery experiments were performed for accurate and precision determination of $Oleocanthal_S$ and $Oleocanthal_{Ext}$ by using a standard additions method for all these determinations to avoid the interferences effect Figure 4 and 5 and Table 4. The average recoveries were in acceptable range (98-100 %) which clearly indicated that method could be used successfully for determination Oleocanthal and the matrix of extracted Olive fruits does not effect this determination. [43].

Table: 3: Regression statistics of the proposed Slope, Intercept and method with LLOD, LLOQ.

R^2	**0.9997**
Standard Error	**0.0179**
Standard Error of Estimate	**0.0169**
Intercept	**-0.3**
Slope	**18.6**
LLOD µg mL^{-1}	**0.1402**
LLOQ µg mL^{-1}	**0.4250**
MDL(standard) µg mL^{-1} (SD×$t_{95\%}$) at n= 5-1	**0.0219**

Concentration (µg/ml)

No.	Concentration (µg/ml)	Peaks Height (mm)	Retention Time (min) R_t
1	0.75	14	18
2	1	18	18
3	1.25	22	18

When add 0.25 µg/ml from Oleocanthal $_{Ext}$ to (0.5 ,0.75 and 1.0) µg/ml as Standard Solution from Oleocanthal Compound

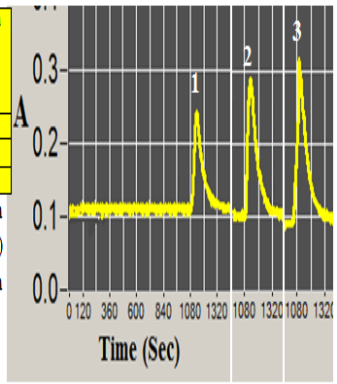


Figure 5: peaks of Standard addition Method

Claimed conc. (µg mL^{-1})	Intra-day		Inter-day	
	Found (µg mL^{-1})	±Recovery % RSD	Found (µg /ml)	± Recovery % RSD
0.5	0.5	100 ± 0.5521	0.5	100± 0.4776
1.0	**0.99**	**99** ±0.5273	0.99	99 ± 0.5870
1.5	**1.50**	**100**± 0.5270	1.5	100 ± 0.4199
2.0	**2.0**	**100** ±0.4982	2.0	100 ± 0.6300
2.5	**2.48**	99.2 ± 0.4698	2.42	96.8 ± 0.5455

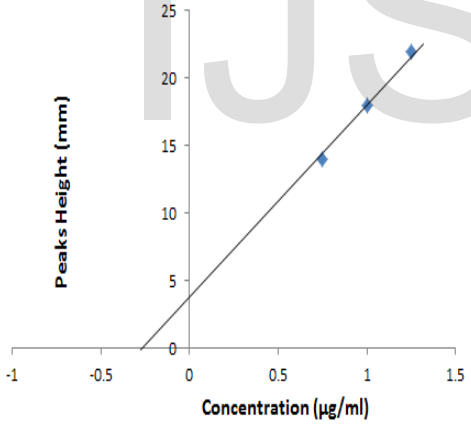


Figure 4: Standard additions for Oleocanthal determination

Table 4: Oleocanthal recoveries obtained by HPLC-UV system

Precision:

Precision of method, reported as % RSD, was estimated by measuring repeatability (intra-day assay) for five replicate injections for all concentrations of Oleocanthal. The intermediate precision (inter-day variation) was also studied for two days using an intermediate concentration solution of Oleocanthal. The Intra-day average recoveries were in the range (98-100) and Inter-day average recoveries (96.8 -100) which thought to be an acceptable result [44]. The obtained results are summarized in Table 5.

Table 5: Intra and inter-day precision and accuracy of standard analysis (n = 5).

Analyze method by Gas Chromatography-mass Spectrum :

The Compound was study through GC-MS to create the molecular ion for the compound and it was found equal to 303 as shown in figure 4 that is confirmed the Oleocanthal molecular weight gave a good indication for isolation and identification of (OLC) [45].

The study demonstrates that with the proper use of Oleocanthal can be analyzed by GC-MS. The reaction conditions may have to response of the Oleocanthal. The fragments, allowing for easy identification by Mass Spectrum. To reduce the overall GC analysis time of these compound, a short, narrow bore column such as the 30 m x 0.250µm I.D. x 0.25µm SS Inlet He is recommended[46].Figure 4 that shown the separation chart by GC-Mass spectrum.

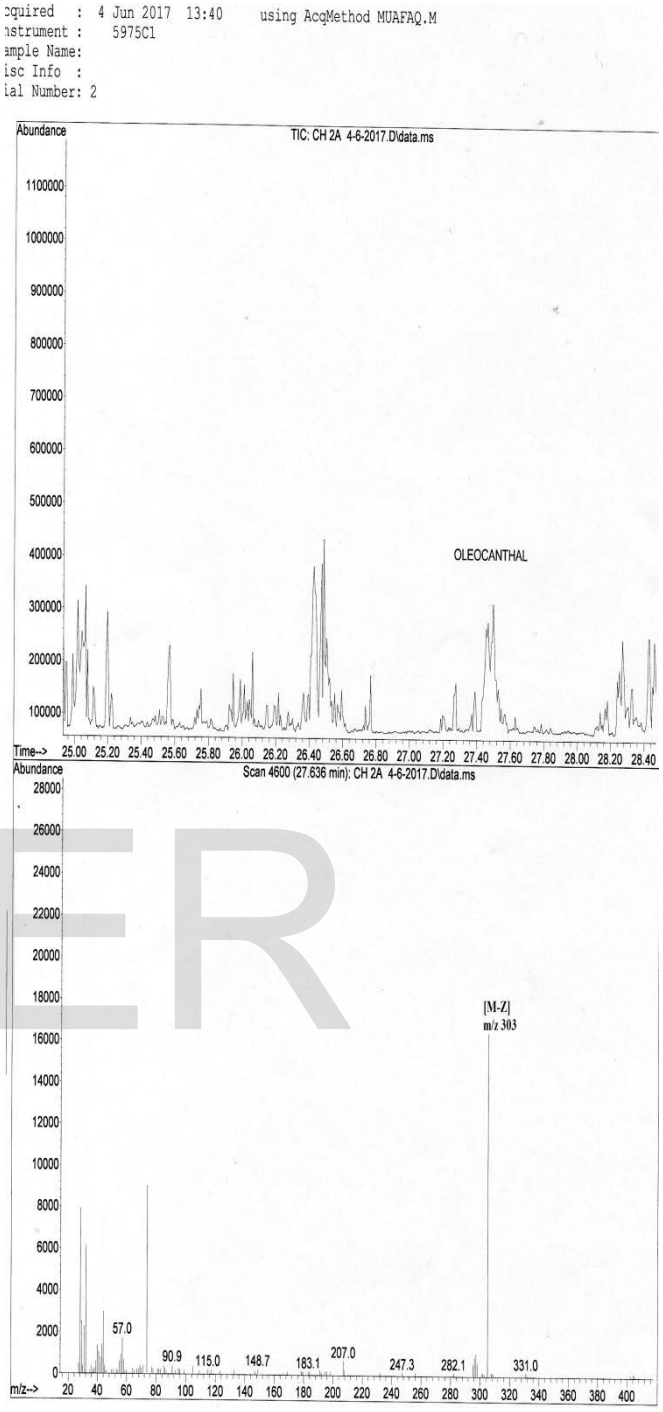


Figure: 4 GC-Mass spectrum for Oleocanthal structure

DATA ANALYSIS PARAMETERS:

Method Name : C: \ MSDCHEM \ 1 \ METHODS \ MUAFAQ.M ,

drying gas : Helium (He) (0.9 ml min^{-1} ,Initialtemperature 90° C) , nebulizer gas , He (8.689 psi), EM voltage 1306 , vaporizer temperature 250 ° C. Figure 4 shows the negative ion mass spectrum of Oleocanthal with the characteristic (M-H) ion at $m/z \approx 303$ highlighted at27.4 minute.

Storage of extraction virgin olive fruits:

Once the oil extraction process out of olive fruit is over, the phenolic quality is possible to fade away due to oxidation catalyzed by oxygen (O_2) and light. The effect of O_2, light and storage time has been investigated by a recent study which covered Oleocanthal concentration. The study showed that Oleocanthal concentration got reduced around (15-37%) over 10-month storage period, depending on the storage conditions. The largest decrease was seen in extraction virgin olive fruits stored under exposure to O_2 and light limiting conditions (15%). It is found that a similar rate of Oleocanthal degradation is caused when oils are stored under sole exposure to O_2 or light for more than 10 months (28%and 25% respectively) [47].

Conclusion:

From the above study we can conclude that Oleocanthal undergoes to different extent under different stress conditions as mentioned above. From the peak purity profile studies, it was confirmed that the peak in HPLC-UV method of the product was not interfering with the peak of Oleocanthal. It confirms that product of Oleocanthal can be separated from the olive fruits by number of methods. Sample extraction with Ethanol: water (3:1) as solvent extraction by Soxhlet, with mixture organic solvent by separator funnel, with HPLC-UV-Refers phase column and with GC-Mas method.

All these methods were successfully applied for the Identification and determination of the Oleocanthal (OLC) in

Virgin olive fruits. Furthermore, the developed methods may be applied for the routine analysis of the more extracts.All standard official methods can be used as analysis methods for routine quality control.

References:

1. H. M. Torkey, H. M. Abou-Yousef, A. Z. Abdel Azeiz, and E. A. HodaFarid. Australian Journal of Basic and Applied Sciences, 2009; 4: 4060-4066 .
2. T. Tannin-Spitz, M. Bergman and S. Grossman, Journal of Biochemical and Biophysical Research Communications, 2007;364 : 181–186.
3. X. Chen, J. Bao, J. Guo, Q. Ding and J. Lu,. Journal of Anti-Cancer Drugs. 2012;23 : 777–787.
4. AH. Abuznait, H. Qosa, BA. Busnena and KA. El Sayed Kaddoumi, Journal of Chemical Neuroscience. 2013; 6: 973–82.
5. A. Khanfar Mohammad K. Bardaweel Sanaa, R. Akl Mohamed and A. El Sayed Khalid , Journal of Chem. Science, 2015;11,101-108.
6. WHO. World Health Statistics and World Health Organization; Geneva, Switzerland: 2010.
7. R. Estruch , E. Ros and J. Salas-Salvador, Journal of Med., 2013;368:1279–1290.
8. K . H . AL - Sowdani and H . N . K . AL – Salman , International Journal of Advanced Research, 2015; 3: 723-730.
9. P. Sexton , P. Black , P. Metcalf and C.R. Wall , Journal of Asthma., 2013;50:75–81.
10. M. A. Martinez-Gonzalez , M. Garcia-Lopez and M. Bes-Rastrollo Toledo. Journal of Nutr. Metab. Cardiovasc., 2011;21:237–244.
11. H. N. K. Al-Salman, Shaker A. N. Al-Jadaan, Maan Al-Nuaim and Hussein H. Hussein,American

Journal of Pharm.Tech. Research, 2017;7:1-10.

12. S. Cicerale , L. Lucas and R. S. Keast, Journal of Biotechnol, 2012;23:129–135.
13. A.Y. Elnagar, P. W. Sylvester , K. A. el Sayed, Journal of Planta Med., 2011;77:1013–1019.
14. E. Karkoula , A. Skantzari , E. Melliou and P. MagiatisJournal of Agric. Food Chem., 2014;62:600–607.
15. X. A. Conlan, N. W. Barnett, and R. S. Keast, Journal of Natural Product Research, 2011; 25, 542-549.
16. S. Cicerale, X. A. Conlan, N. W. Barnett, and R. S. J. Keast, Journal of Food Research International, 2011;26,14-22.
17. L. Lucas and R. Keast, International Journal of Molecular Sciences, 2010;11, 458-79.
18. D. B. Panagiotakos, C. Pitsavos and M. Tampourlou, Journal of Nutritional Biochemistry, 2010; 21, 285-289.
19. K. H. AL - Sowdani and H. N. K. AL – Salman, Journal of Chemical, Biological and Physical Sciences, 2016; 6: 31-38
20. P. Oh, W. K. Yun, H. J. Namgoong, G. M. Ahn and S. G. Kwon, Journal of Carcinogenesis,2010;32, 545.
21. A. Russell, and R. Keast, Journal of Current pharmaceutical Design, 2011; 17, 754-768.
22. P. N. Mitrou, V. Kipnis , A.C. Thiébaut and J. Reedy , Journal of Intern. Med., 2007;167:2461–2468.
23. G. Corona , J. Spencer and M. Dessi , Journal of GI. tract. Toxicol. Ind. Health., 2009;25:285–293.
24. S. Cicerale , X. A. Conlan, A. J. Sinclair and R.S.J. KeastJournal of Food Sci. Nutr., 2009;49:218–236.
25. J. Pitt , W. Roth, P. Lacor, M. Blankenship and P. Velasco, Journal of Toxicol. Appl. Pharmacol., 2009;240:189–197.

26. W. Li , J. B. Sperry and A. Crowe, Journal ofeurochem., 2009;110:1339–1351.

27.G. Grosso, A. Pajak, A. Mistretta and S. Marventano, Journal of Nutr. Metab. Cardiovasc. Dis., 2014; 24, 370–377.

28. R. Estruch, E. Ros, J. Salas-Salvador and M. Covas, Journal of N. Engl. J. Med., 2013; 368, 1279–1290.

29. P. Sexton, P. Black, P. Metcalf and C. R. Wall, Journal of Asthma., 2013; 50, 75–81.

30. M.A. Martínez-González, M. Garcia-Lopez, and M. Bes-Rastrollo, Journal ofNutr. Metab. Cardiovasc. Dis., 2011; 21,237–244.

31. S. J. Carter, M. B. Roberts, J. Salter, and C. B. Eaton, Journal of Atherosclerosis, 2010; 210, 630–636.

32. A. Bach-Faig, E. M. Berry, D. Lairon, and J. Reguant, Journal of Public Health Nutr., 2011; 14, 2274–2284.

33. G. Caramia, A. Gori, and E. Valli, Eur. Journal of Lipid Sci. Technol., 2012; 114, 375–388.

34. S. Cicerale, X. A. Conlan, A.J. Sinclair, R. S. J. Keast, Journal of Food Sci. Nutr., 2009; 49, 218–236.

35. S. Cicerale, L. Lucas and R. S. Keast, Journal of Int. Mol. Sci., 2010; 11, 458–479.

36. E. Karkoula, A. Skantzari, E. Melliou and P. Magiatis, Journal of Agric. Food Chem., 2014; 62, 600–607.

37. E. Karkoula, A. Skantzari, E. Melliou and Journal of Agric. Food Chem., 2012, 60; 11696–11703.

38. S. Cicerale, L. Lucas and R. Keast, Journal of Tech. Rijeka Croatia, 2012; 357–374.

39. G. K. Beauchamp, R. S. J. Keast and D. Morel, Journal of Breslin,

P. A. S. Nature, 2005; 437, 45-46 .

40. B. L. Dixon, A.F. Subar, U. Peters and J. L. Weissfeld, Journal of Nutr., 2007; 137, 2443–2450.

41. Indian Pharmacopoeia, Controller of Publication, Delhi, 2010; 3, 921 - 923.

42. M.V. Dhoka ,V.T.Gawande and P.P. Joshi, J. Pharm. Sci. and Res., 2010; 2 , 477 – 483.

43. The United States Pharmacopeia, Asian Edition, United States Pharmacopeia Convention, Inc, Rockville, MD., 2007.

44. M. Murakami and N. Nishimoto, Journal of Curr. Opin. Rheumatol, 2011, 23, 273–277.

45. J. Scher, M. Pillinger, S. Abramson, Journal of Curr. Rheumatol, Rep., 2007; 9, 9–15.

46. M. Prochazkova, P. Zanvit, T. Dolezal, L. Prokesova and M. Krsiak, Journal of experimental osteoarthritis pain. Physiol. Res., 2009; 58, 419–425.

47. H. Kokkonen, I. Soderstrom, J. Rocklov and G. Hallmans, Journal of Arthritis Rheum. 2010; 62, 383–391.