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Research Article

HEMOLYTIC ACTIVITY OF CICER ARIETINUM L. EXTRACTS BY TWO EXTRACTION TECHNIQUES

FARAH J. HASHIM

University of Baghdad College of Science Department of Biotechnology, Baghdad Iraq. Email: farah_bio_2020@yahoo.com

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ABSTRACT

This study were designed to test the efficiency of saponin which extracted from *C. arietinum* L. by two different extraction techniques as hemolytic agent, one of them carried out by using ultra sonication-Oven assistance extraction (UOAE)with ethanol as extraction solvent of choice while the other technique deals with more than one solvent with longer extraction time Phytochemical analysis of both crude extracts was performed and revealed positive result for saponins, resins, tannins and alkaloids while resins appeared only in liquid-liquid extraction (LLE) meanwhile results showed that flavonoids, terpenes, steroids, glycosides and polyphenols were absent for both crude extracts. Reversed high performance liquid chromatography carried out to detect presence of saponin in these crude extracts.

The results of hemolytic effect on human erythrocytes showed that both crude extracts significantly potent as lytic agent compared with synthetic purchased saponin at $P \leq 0.05$. The highest hemolytic activity was76.27 ± 0.18% at concentration 0.375mg/ml with UOAE technique while the opposite pattern appeared with LLE and the hemolytic activity increase to 79.40 ± 0.12%with the lowest concentration 0.094mg/ml,statistical analysis proved that is saponins involved both of crude extracts which obtained by UOAE and LLE techniques considered as effective hemolytic agent.

Keywords: Saponin, Chickpea, *Cicerarietinum*, Hemolytic activity, Ultra-sonication

INTRODUCTION

Chickpeas (*Cicer arietinum* L.) are one of the oldest and most widely consumed legumes in the world commonly used as food and provide an important component of the diets of those individuals who cannot afford animal proteins or those who are vegetarian by choice due to its nutritional value. Chickpeas comprise a good source of protein and carbohydrates especially Starch which is the major fraction of the total carbohydrates [2, 11]. Saponins one of the major classes of plant secondary metabolites occurs in a broad range in legumes. It's consisted of main components of Chickpeas and comprises about 3.6% of dry weight of the seeds. A diverse class of glycosidic saponins comprises a steroidal or triterpene aglycon linked to one or more sugar moieties. The most common sugar linked to an aglycon (or sapogenin) are galactose, xylose or glucose. It is generally characterized by their ability to making soap like foam when shaken in aqueous solution and that's belonging to their detergent properties and responsible for imparting a bitter taste [9].Otherwise many biological and pharmacological activities have been reported about saponins such as anti-fungal, anti-virus, anti-oxidant, and haemolytic activity by interactions with cellular and membrane components. Saponins cause hemolysis of red blood cells by non-specific interactions with membrane proteins, and insertion of saponin in to lipid bilayer membrane then binding to cholesterol of erythrocytes, after formation of cholesterol-saponin complex, cell lysis occur. [4,5].Therefore, this property gave saponins an importance by using it in lysis red blood cells during RNA extraction process and DNA micro array technique form infected blood samples [7].

Extraction of secondary metabolites consider as the first most important step to obtain these active ingredients, therefore increasing interest to extract it from plant materials accompanied with needed to develop an extraction method beside high efficiency in extraction, it has to be reduce solvents amount, energy, time and environmental friendliness. The aim of this study, is to detect the hemolysis ability of saponins extracted from Chickpeas using two different extraction methods

Materials and methods

Materials

Dried *C. arietinum* L. seeds were purchased from local market in Baghdad and grinding by electrical grinder until became fine powder which then kept in closed container until use. Otherwise synthetic saponin was purchased from sigma/ USA and lysis buffer purchased from Promega/USA.

Chemicals

Solvents for extraction (methanol, n-butanol , Diethyl ether and ethanol) were purchased from sigma/ USA, lead acetate ferric chloride and potassium iodide (which was used for prepare Mayer and Wagner test) purchased from Merck/Germany, H_2SO_4, chloroform, glacial acetic acid and Potassium hydroxidepurchased from Riedel-deHaën / Germany, mercuric chloride 3, 5 di nitrate benzoic acid (which was used for prepare Ked's test) and iodide (which was used for prepare Wagner's test) purchased from BDH chemicals/England.

Extraction procedure

Ultra sonication- Oven assisted extraction (UOAE):

C. arietinum L. seeds powdered subjected for extraction by following [13] and [8] methods with slight modifications. Powdered plant material were defatted by maceration using hexane for 6 hr., after removing the fats (5 gm) mixed with 25 ml of ethanol as a solvent of choice and the mixture were exposed to ultra sonication with working frequency 60 KHz for 15 min. of sonication time at room temperature then kept constant in oven at 65^{0}C for 10 min., the contents were filtered and evaporated to dryness. The extract was collected and kept in closed vial at 2 ^{0}C until use.

Liquid- liquid extraction (LLE)

C. arietinum L. seeds powdered (300 g) was subjected to extract with 500 ml of methanol by cold extraction for two weeks. After filtration

the crude methanol extract was concentrated by Oven at 50ºC the extract then (20 g) of the crude methanol extract was dissolved in water saturated with n-butanol in a separating funnel. After thatthe n-butanol phase collected and Diethyl ether was added to the n-butanol portion in a flask then crude saponin which was precipitated, collected and kept in closed vial at 2 ^{0}C until use [3].

Phytochemical analysis

The chemical analyses of *C. arietinum* L. seeds extracts by (UOAE) and (LLE) methods were carried out to detect the following compounds using different reagents as in table 1:

Table 1: detection of phytochemical compounds in *C. arietinum* L. seeds extract:

Chemical compounds	Reagents	Indication
Tannins	Lead Acetate 1% [17]	White precipitate
Glycosides	Ked's reagent [17]	Violet ring
Flavonoids	95% ethanol, water bath, KOH, [17]	Yellow precipitate
[Saponins]	1-Shaking	Foam
	2- Mercuric chloride 1% [19]	White precipitate
[Alkaloids]	1Wagner's reagent [19]	Brown precipitate
	2Mayer's reagent [18]	White precipitate
Terpenes	H2SO4, chloroform, Glacial acetic acid [17]	Pink color
Steroids	Similar reagent for terpenes/ leaving sample for 1 min.	Blue color
Phenols	Ferric chloride 1% [17]	Blue-green color
Resins	Ethanol, Boiling D.W. [17]	Turbidity

High performance liquid chromatography (HPLC) method

HPLC analyses were performed using a liquid chromatograph (Shimadzu), UV variable wave length detector (at 203 nm). Saponins was analyzed using a Nucleoil® column C-18, 5µm, 250 mm x 0.6 mm, The mobile phase consisted of acetonitrile: water (70:30,v/v)in isocratic manner. A sample of 50µg/ml was dissolved sample powder in 1 ml of same solution used as mobile phase and filtered by Whatman filter paper No. 2. The flow rate was 1.0 ml/min. and injection volume was 10µl, The HPLC system was operated at room temperature (23 ± 1^{0}C) [10].

Hemolytic activity

The assessments of hemolytic activities of both Chickpea's extracts were carried out according to the procedure reported by [1] with slight modifications. The assay involved pipetting 0, 0.25, 0.5, 0.75 and 1 ml of each extract (1.5 mg/ml) in triplicates in to clean dried test tubes. The volume was adjusted to 1 ml of distilled water. Then 2.5 ml of normal saline was added to each tube followed by the addition of freshly prepared 2% (v/v) red blood cells. The reaction mixture was incubated at 37^{0}C for 1 hr. followed by centrifugation at 3,500 for 15 min. the supernatant were collected and the absorbance was recorded at 630 nm. The red blood cells treated with lysis buffer served as control and represented 100% lysis. Percentage hemolysis was calculated using the expression [6]:

$$\text{Hemolysis \%} = \frac{(\text{OD of sample} - \text{OD of blank})}{(\text{Highest OD of positive control})} \times 100$$

RESULTS AND DISCUSSION

Ultra sonication- Oven assisted extraction (UOAE)

The first extraction method was carried out by using ultrasonic and oven in order to increase the efficiency of extraction from chickpea de-fatted powder in addition to reduce number of solvents using ethanol as solvent of choice. An extract obtained was found 0.8gm/ 4 gm of the chickpea seed dry weight. Period of extraction steps which were scheduled as 15 min. by exposing to ultrasonic and 10 min. by using oven gave efficient extract yield.

Extraction of secondary products from plant materials by UOAE technique significantly decreased the extraction time with enhancing the extraction yield. enlargement in the pores of the cell walls which resulting from Actions such as swelling and hydration can be accelerated by ultrasonic and this probable pores enlargement causing to transfer a good amount of intracellular products in to a solvent. Otherwise the penetration rate of solvent into plant material tissue will be increase as a result of destroying cell wall by frequencies of ultrasonic. For that, ultrasonic technique helps active ingredients dissolve in the solvent faster with improving yield in a short time.

High temperature allowed boosting yield of saponin, since higher temperature caused to dissolve active components in addition to enhancing sample wetting and matrix penetration due to decreasing in surface tension and solvent viscosity decrease with temperature, respectively. Additionally, ultrasonic technique which has the main effect as cavitational bubbles collapse will a raise to two factors play a role when high temperature used in extraction methods with ultrasonic, first one, production of more cavitation bubbles due to decreasing in cavitational threshold and the second one bubbles filled with vapor formed by the vapor pressure relevant high temperature leading to cause cushioning effect by cushioned the implosion of those bubbles., generally heating during extraction procedures reduces significantly both the extraction time and the volume of solvent required therefore,65^{0}C was considered as the optimum temperature for saponin extraction [12,8].

Liquid- liquid extraction (LLE)

Solvents system were used in this method to elicitation more quantity of saponins involving crude extract which extraction yield was found to account for 0.72gm/ 4gm of seed powder dry weight.

LLE technique consumed more than one type of solvents by scheduled manner depending on solubility of saponins which is affected by properties of solvent such as hydrophobicity, solvent composition and characteristic of extracted compound therefore, alcohols (methanol, ethanol) more likely for extraction of saponins, due to presence lipid insoluble sugar chain in their structure [16]. In this study the extraction carried out by methanol for saponins dissolving and elicitation while n-butanol was better than water for the second stage of extraction because of amphiphilic nature of saponins (as glycosides containing glycon water soluble and aglycon water insoluble) [14].Finally, ethyl acetate solvent used to obtain crude extract with high content of saponins based on presence of lipid soluble aglycon moiety so it's allowed reduction of other water soluble compounds that is evolved by methanol in order to collect saponin highly content crude extract as much as possible. Additionally advantage of these solvents were working to transfer as much as can of desired component from matrix tissue to the solvents used which mean more amounts of saponins obtained in crude extract.

Phytochemical analysis

Analyses of (LLE) and (UOAE) of *Cicer arietinum L.* phytochemically which presented by table (2) revealed that a plant seed extract by liquid-liquid method(LLE) gave positive tests for the presence of saponins, resins, tannins and alkaloids, while extraction by ultra sonication- Oven(UOAE) showed the existence of same compounds except resins were absent in the extract. Otherwise the results indicated that other phytochemical compounds such as: flavonoids, terpenes, steroids, glycosides and polyphenols were absent for both of (LLE) and (UOAE). The differences of phytochemical analysis for each extract have shown that using different conditions in extraction process affect on the type of active compounds in crude extract [15].

Table 2: phytochemical analysis results of *Cicer arietinum L.* extract obtained by two different extraction methods:

Name of active compound		Extraction method Ultrasonic- oven associated extraction (UOAE)	Liquid- liquid extraction (LLE)
Tannins		+	+
Glycosides		–	–
Flavonoids		–	–
[Saponins]	1-Mercuric chloride	+	+
	2-shaking	+ (Normal Foam)	+ (Dense foam)
[Alkaloids]	1-Mayer's test	+	+
	2-Wagner's test	+	+
Poly Phenols		–	–
Resins		–	+

High performance liquid chromatography (HPLC) method:

Figure 1a shows the peak of saponin which is commercial product purchased from sigma, at retention time (RT) 1.709 min. in the meantime the peak of saponin appeared at RT 1.625 min. in UOAE and revealed at RT 1.820 min. in LLE as showed in figure 1b and figure 1c, respectively and that indicate both LLE and UOAE contain saponin. An isocratic system was used in chromatographic conditions for the simplicity; maximize precision and also decrease the variation of the baseline. Moreover, low wavelength (203 nm) was chosen considering the absence of chromophores moieties in saponin molecular structure.

As general area of saponin peak indicate that amount of saponins from the total crude extract obtained by LLE is higher than those resulted from UOAE technique.

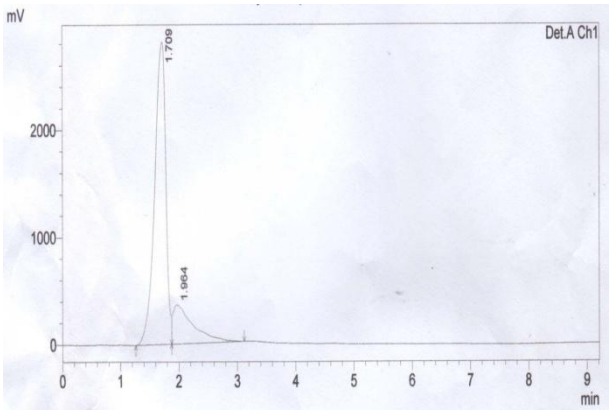

Figure 1 a

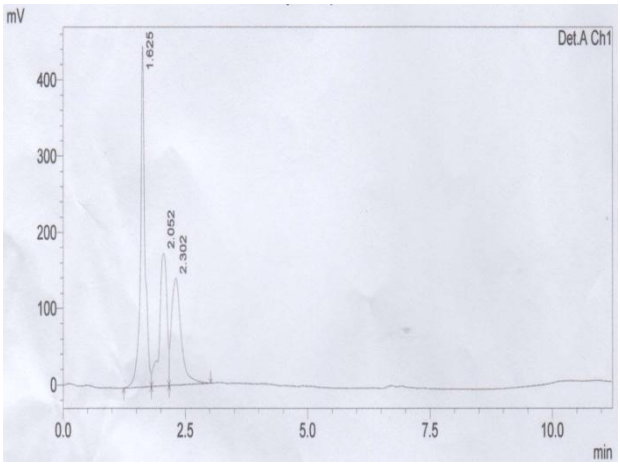

Figure 1 b

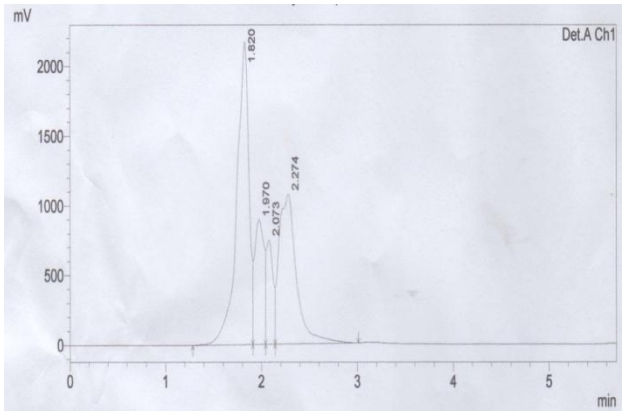

Figure 1 c

Figure (1): isocratic HPLC chromatograms shows analysis of synthetic saponin purchased from sigma (a), UOAE crude extract (b) and LLE crude extract (c) at wavelength 203 nm.

Hemolytic activity

Hemolytic activity considered as one of the main biological effects of saponins thus, it can be used in this study as a tool to determine the efficacy of saponin compounds crude extract extracted by two different methods which is presented in table (3).

Table 3: The percentage of hemolysis activity of human blood for synthetic saponin, Ultrasonication- Oven associated extraction (UOAE) and liquid- liquid extraction (LLE)

Concentration (mg/ml)	Percentage of Hemolysis (Mean ± S.E.) Synthetic Saponins	UOAE	LLE
0.094	$^{b}25.43 \pm 0.07^{C}$	$^{c}55.67 \pm 0.19^{B}$	$^{a}79.40 \pm 0.12^{A}$
0.188	$^{ab}27.00 \pm 0.06^{C}$	$^{b}68.17 \pm 0.18^{B}$	$^{b}75.23 \pm 0.12^{A}$
0.281	$^{a}29.60 \pm 0.07^{B}$	$^{a}73.17 \pm 0.09^{A}$	$^{b}74.83 \pm 0.22^{A}$
0.375	$^{a}31.5 \pm 0.17^{C}$	$^{a}76.27 \pm 0.18^{A}$	$^{c}65.13 \pm 0.03^{B}$

Different Lower Case Letters: Significant difference ($P \leq 0.05$) between means of columns.

Different Upper Case Letters: Significant difference ($P \leq 0.05$) between means of rows.

It is obvious that in synthetic saponin and UOAE, there is a gradual increased hemolytic activity that paralleled the increase in concentration. Hemolytic activity of synthetic saponin starting from 25.43 ± 0.07, 27.00 ± 0.06, 29.60 ± 0.07 and 31.5 ± 0.17 at concentrations 0.094, 0.188, 0.281 and 0.375 mg/ml, respectively while in UOAE extract, the hemolytic activity was 55.67 ± 0.19, 68.17 ± 0.18, 73.17 ± 0.09 and 76.27 ± 0.18 at concentrations 0.094, 0.188, 0.281 and 0.375 mg/ml, respectively. The highest activity was at concentration 0.375mg/ml both of synthetic saponin and UOAE.

Meanwhile in hemolytic activity results of LLE the opposite consequence was observed. Results of hemolytic activity were 79.40 ± 0.12, 75.23 ± 0.12, 73.83 ± 0.22 and 65.13 ± 0.03 at concentrations 0.094, 0.188, 0.281 and 0.375 mg/ml, respectively. The concentration 0.094 mg/ml gave the best effect. An explanation for this finding may be referred to affecting hemolytic by presence of resins as showed in phytochemical analysis results which increase with increasing of extract concentrations so may be its accumulated interfere with hemolytic process. However, both extract recorded a significantly higher percentage of hemolysis for the four assayed concentrations as compared with the corresponding concentrations in synthetic saponin which may be referred either to synergistic effect of the other secondary metabolites that combined with saponins in each crude extract or maybe due to variety of saponins resulted in the extract (differ from each other in glycon and aglycon moieties which seem to play a role in the haemolytic activity of saponins)while a definite type of synthetic saponin was used in commercial product [5]. Hemolytic property of saponins has an advantage at laboratory scale, [7] suggest that uses of saponins as

lytic agent in RNA extraction process increased RNA yield compared with using whole blood.

Saponins have the ability to hemolysis human erythrocytes by form pores in cell membrane based on affinity of aglycon moiety for the membrane sterols particularly cholesterols which leading to form insoluble complexes [9, 14] .

Statistically there is no-significant difference between best hemolytic activity of both extract but as general results of the lytic action of LLE seems to be higher than effect of UOAE and these findings are may be due to many factors such as: composition of the target membrane, the type of side chain, and the nature of the aglycone to which these are attached which all appearing to affect hemolytic action of saponins as reported by. It has been suggested that hemolytic activity increase with increasing number of polar groupsin the aglycon moiety, besides that, Steroid and triterpenoid saponins with a single sugar chain (monodesmosides) were found to have strong haemolytic activity compared with those with two sugar chains (bidesmosides) showed less activity, meanwhile the neutral and acidic triterpenoids, and the acyl glycosides found to be very weakly active, whereas the ester saponins (for example, maesasaponins) shows strong hemolytic activity [9]. Also the hemolysis percentage affected by amount of saponin in the chickpea's crude extract and based on peak area of HPLC result which indicates that saponins of LLE higher than saponins resulted from UOAE technique. That may indicate the extraction by LLE technique gave more potent saponins than those extracted with UOAE.

Comparison between UOAE and LLE

In addition to the nature of desired compound there are many factors playing a role in selection extraction method such as: pollution, energy, extraction period, process cost and extraction yield [8] so a comparison can be arranged between LLA and UOAE according to advantage and disadvantage of each process, LLE consumed large amount of solvents and spend a long extraction time compared with UOAE technique, therefore, this process relatively expensive and unsuitable for commercial purposes. Although HPLC results of saponins peak area for each extract showed that the advantage of LLE by allowed to obtain saponins with more quantity from the total crude extract compared with UOAE technique which seems to be faster and production cost is less due to minimal in amount of solvents used but ultrasonic may be suffer from reduction in low precision which resulting in case the ultrasonic energy is not homogenously distributed so position of extraction vessels have to be carefully chosen and fixed during extraction. Otherwise, the crude extraction yield of UOAE technique (0.8gm) closed to the yield of LLE technique which was (0.72gm) for each 4 gm of chickpea seed powder.

Saponins have long been known to have a lytic action on erythrocyte membranes and this property has been used for their detection and according to statistically analysis for this study, even LLE showed highest activity (79.40 ± 0.12) at lowest concentration 0.094 mg/ml while extract obtained by UOAE technique showed highest activity (76.27 ± 0.18) at highest concentration 0.375 mg/ml, the both extract have hemolysis effect with different concentrations.

From above comparison, the advantage of UOAE in minimal consuming of solvents and time made this technique to be more favorable than LLE based on identity in hemolytic efficiency for both resulted extracts.

CONCLUSION

From these results, it is concluded that ultra sonication– oven assisted extraction (UOAE) technique gave an efficient saponins even with short time extraction and less solvents consumed compared with liquid-liquid extraction (LLE) technique. Therefore, there is no-significant difference in hemolytic activity test between the best two concentrations of both extractions.

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