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Gallstones-dissolving capacity of lemon (*Citrus limon*) juice, *Herniaria hirsuta* L. extract and lemon juice-based natural vinaigrette *in vitro*

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This paper presents the investigation of the gallstones-dissolving capacity (GDC) of lemon (*Citrus limon*) juice (LJ), *Herniaria hirsuta* L. aqueous extract and olive oil/LJ natural vinaigrette (NV) *in vitro*, for the first time. To start with, the gallstones from an Algerian female patient were characterized in terms of both their chemical composition by UV-VIS spectroscopy and thermal analysis. Then, the GDC was evaluated throughout the reduction of weight of different gallstones which were immersed in different investigated liquid media over the time. It was found that the investigated gallstones are composed of bilirubin and cholesterol. Primarily, the results revealed that the GDC of NV reached 100 % after 7 days immersion, while it reached 72, 26, 6 % after 13 days immersion for HHE, LJ and LJ/HHE mixture, respectively. These results shed light on interesting perspectives for NV applications: 1) as functional food to replace the commercial vinegar-based dressings, knowing that the alternate consumption of olive oil and lemon juice to promote the gallbladder flush is widely popularized, and 2) as a promising alternative to conventional solvents used in the treatment of gallstones by contact litholysis. However, additional studies are needed to improve the stability and bioavailability of the NV.

Keywords: DSC analysis, Extract, Gallstones-dissolving capacity, *Herniaria hirsuta* L., Olive oil/lemon juice-natural vinaigrette, UV-Visible spectrum.

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Diseases of the gallbladder are, recently, a common occurrence in many regions of the world[1], including Algeria[2]. They are also known to be costly[3,4] but new preventive measures, including a healthy diet and medicinal plants, have not been investigated thoroughly yet[3,5], taking into account their safety, efficacy, cultural acceptability and lesser side effects as compared to synthetic drugs[6]. Surgical intervention is often applied for the removal of gallbladder and stones[7]. An alternative approach to the development of a non-surgical therapy of gallstones has been the subject of several studies[8]. In fact, the contact dissolution (also known as direct solvent dissolution or litholysis) via the trans-hepatic route is applied in certain circumstances[9]. Various molecules are employed for this purpose: methyl tert-butyl ether (MTBE)[8], monooctanoin[10], etc. Nevertheless, MTBE as the most commonly used solvent presents a high volatility and flammability along with potential toxicologic concerns[11]. Therefore, the substitution of chemical solvents by extracts of medicinal plants is probably a promoting perspective. Unfortunately, few studies have been devoted to the treatment of gallstones by medicinal plants. In this context, it was shown that the extracts of *Berberis vulgaris* Q, *Dioscorea* Q and lemon juice have a dissolving effect on gallbladder stones[12]. Based on case-control study in southern Italy, it was demonstrated that physical activity, dietary monounsaturated fats, dietary cholesterol, and dietary fibers such as cellulose were inversely associated with risk of gallstone formation[13]. Based on cross-sectional analyses, ascorbic acid (abundant in citrus fruits) was found to be a useful therapeutic agent for the treatment of gallbladder disease[14]. More recently, the prevention of experimentally induced formation of cholesterol gallstones by dietary tender cluster beans and garlic was demonstrated in laboratory animals[1]. The antiurolithiasic activity of *Herniaria hirsuta* L. and lemon juice is widely cited in the literature[15,16,17]. However, only one study was found about the dissolution of gallstone by lemon juice[12].

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In the present paper, the gallstones-dissolving capacity (GDC) of lemon (*Citrus limon*) juice (LJ), *Herniaria hirsuta* L. aqueous extract and olive oil/LJ natural vinaigrette (NV) was investigated *in vitro*, for the first time. Through this work, we try to highlight the prospects of substitution of conventional solvents used in the contact litholysis by food and non-food plant products.

Methodology

Gallstone samples and their characterization for UV/visible spectrum and thermal properties

Gallbladder stones (Fig. 1) were collected from a 45 yrs old female patient at private clinic of Bouira city (70km eastern Algiers) in January 2013. UV-visible spectrum of gallstones was studied by means of UV-1800 Shimadzu Corporation spectrophotometer, according to the methodology described earlier[12]. So, 20 mg of powdered stones were mixed with 10ml of ethanol. The filtrate was then studied for its UV-visible properties. The thermal properties of stones were studied using a calorimeter STA-409 / PC-LUXX NETZSCH. For this, the sample (14.470 mg) was heated up to 1000 °C, at a rate of 5 °C /min in air. UV-visible spectrum, DSC and TGA allowed to highlight the composition of stones. Thus, it was reported that DSC associated with thermogravimetry analysis (TGA) can be used to determine the anhydrous or monohydrate state of cholesterol in gallstones[7], knowing that this molecule is the predominant component (80 %)[7,12]. Other authors have already analyzed gallstones of Algerian patients, in order to find out if it is possible or not to avoid invasive surgery in the treatment of stones and eventually to propose an alternative treatment[2].

Fig. 1—Analyzed gallbladder stones of 45 yrs old female.

Herniaria hirsuta L. aqueous extract (HHE)

Dried sample of *Herniaria hirsuta* L. (Caryophyllaceae) was provided by an herborist from Boumerdes city (30 km Eastern of Algiers). The plant (Fig. 2) is identified by the common name “hairy rupturewort”. It is native to Eurasia and North Africa, and it is known on other continents, including North America[18]. The aqueous extract of *Herniaria hirsuta* in the prevention and cure of urolithiasis was already demonstrated *in vitro* and *in vivo* [17]. Presently, 20 gm of whole plant were infused for 15 min in 1 L boiled distilled water. The plant is left soaked for 15 min and the mixture was then filtered. The obtained filtrate was analyzed for its GDC.

Lemon juice

Lemon (*Citrus limon*) fruits were bought at Boumerdes city (30 km Eastern of Algiers). The fruits were cleaned, washed and then cut in half crosswise. The lemon juice (LJ) was then extracted by juice-press. The LJ was filtrated using Whatman paper filter No 1 and then stored at 4 °C until use. Citrus fruits include one of the most important tree crops in the Mediterranean region[19] and they are known to have long history of use for their medicinal properties, among others[20]. Before all, lemon juice is widely used for its urolithiasis virtues[21]. It is a precious source of ascorbic acid with a concentration above 50 mg/100 mL[22]. It was reported that this molecule may reduce the risk of gallbladder diseases[14].

Lemon juice/HH mixture

The mixture of LJ/ (20 gm/l)/HH aqueous extract (50 %, V/ V) was prepared and then tested for its GDC.

Fig. 2—The medicinal plant (*Herniaria hirsuta L.*) used.

Olive oil-in-lemon juice emulsion

The GDC of a natural olive oil-in- LJ vinaigrette (45, 55 %, v/v) was investigated. This emulsion was prepared according to the procedure described elsewhere[23], the lemon albedo powder-in-LJ suspension being used as aqueous phase and as stabilizing agent. As *citrus* fruits, olive oil is very popular for its nutritive and health-promoting potential[24]. The alternate consumption of olive oil and lemon juice to promote the gallbladder flush is widely popularized[25]. But, this therapy is the subject of some controversy. Such the so called "gall-bladder flushing" or "liver cleansing" regimes that were judged to be misleading[26] since "green stones" can be produced by mixing equal volumes of oleic acid and lemon juice after the addition of a small volume of a potassium hydroxide solution[26]. Nevertheless, the body does not produce the caustic chemical potassium hydroxide needed to cause the soap formation[27]. In all cases, it is always stated that the lemon juice/olive oil mixture may help to dissolve gallstones[28]. However, no scientific work has been devoted to GDC of olive oil-in-LJ emulsion.

GDC evaluation

The GDC of investigated liquid media was evaluated using the procedure adapted from that described by Meiouet *et al.*[15] for cystine urinary stones. Five Gallstones were washed with distilled water several times, and then dried at 40 °C for about 5hrs. The stones of different weights were placed in investigated liquid media (130 mL each), at 20 °C: 374.7 (LJ), 299.1 (HH extract), 250.3 (mixture of LJ/HH extract, 50 %, v/v) and 259.1 mg (olive oil/LJ emulsion, 45,55 %, v/v). The samples were subjected to stirring at 400 rpm.

The weight reduction (WR) of stones, as well as *p*H of solutions was measured every 24 hrs.

The WR (%, w/w) was calculated as follows:

$$WR = 100 \times (W_0 - W) / W_0$$

Where, W_0 and W represent the initial and at any time weight of stone, respectively.

The GDC was evaluated throughout the maximal WR value, reached at a given time.

Results and discussion

Characterization of stones

As shown in UV-VIS spectrum of Fig. 3, a characteristic peak appears at 445 nm. The same peak was found by Das *et al.*[12] regarding gallstone samples from a 55-yrs-old female patient. According to these authors, the peak reflects the presence of bilirubin which is an orange-yellow substance made during the normal breakdown of red blood cells[6]. In fact, it is well established that gallstones are mainly composed of cholesterol, bilirubin, calcium carbonate, calcium phosphate, and other salts[29]. Furthermore, they are generally composed of two types of cholesterol: cholesterol monohydrate, and Cholesterol anhydrous[7]. The DSC thermogram showed various peaks at different temperatures (Fig. 4). Kaloustian *et al.*[7] have obtained the same peak at 40 °C concerning gallstones from thirty 15-yrs-old patients. According to Kaloustian *et al.*[7] who analyzed gallsstones from thirty 15-yrs-old patients, the revealed peaks may be attributed to specific chemical compounds: cholesterol anhydrous (35 °C), cholesterol monohydrate (151.1 °C) and decomposition of CaCO3 into its basic components, namely CaO and CO2 (the phase beginning at about 600 °C. The last peak is also found by Das *et al.*[12] at 148.1°C during DSC analysis of gallstones from a female patient. In our case, the peak at 151.1 °C showed a very low mass loss (about 0 %) which may correspond to any phase change. We recall that Zong-chan *et al.*[2] which have already revealed that Algerian gallstone patients have cholesterol gallstones and suggested, among others: 1) to popularize scientific habits of eat and drinking, and 2) to use magnetic treatment instead of surgery operation when diseases mainly are cholelithiasis of bilirubin type.

Gallstones-dissolving capacity of different liquid media

As it is shown in Fig. 5, the GDC increases with immersing time whatever the plant extract. However, the NV caused the highest GDC rate since the latter reached 100 % value after seven days of immersion,

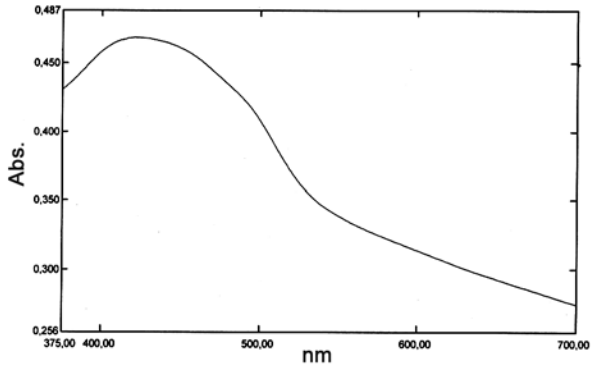

Fig. 3—UV-Visible spectrum of the ethanolic extract of gallbladder stones.

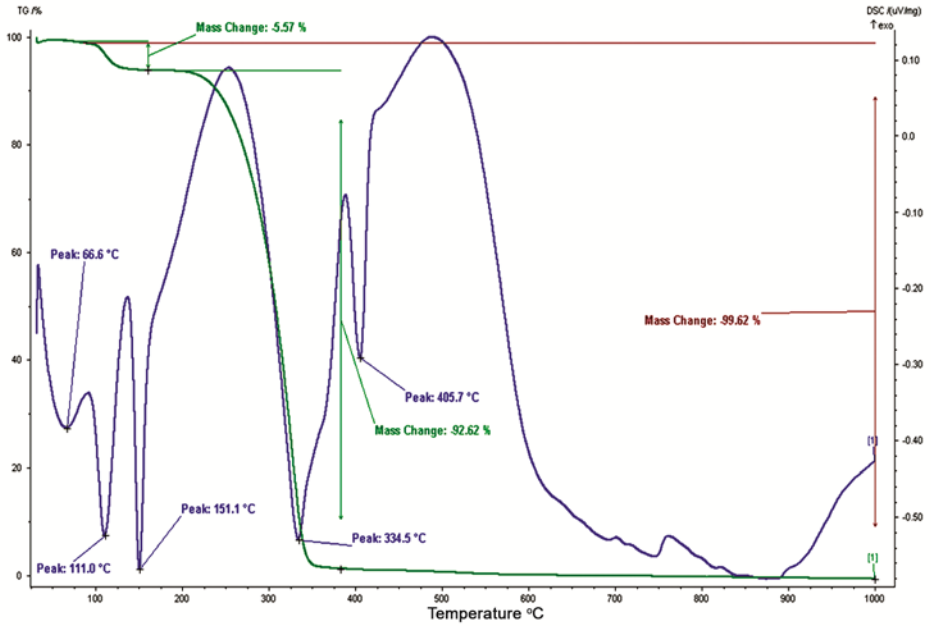

Fig. 4—DSC thermogram of gallbladder stones.

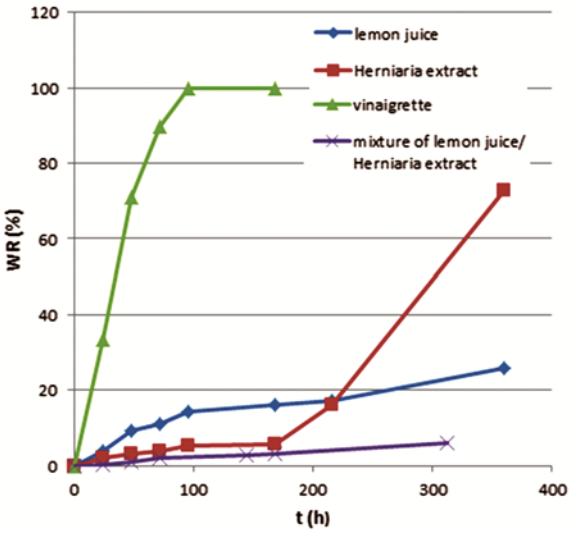

Fig. 5—Weight reduction (WR) of stones versus time, allowing to evaluation the gallstone dissolving capacity of different investigated extracts.

corresponding to dissolution kinetic of exponential type (models not presented here). This efficiency is higher than that (30-80 %) induced by chenodeoxycholic acid[31]. The HHE, LJ and their mixture presented a GDC value of about 72 %, 26 %, 6%, respectively, after thirteen days. There are currently few studies about molecular mechanisms that destroy gallstones once they are formed[32]. However, we think that the observed dissolution of gallstones may be due to different mechanisms, according to the nature of investigated immersion media. Thus, the high GDC of NV, as an outstanding result, may be attributed to the dissolving synergistic effect between oil and aqueous phases of NV. For instance, the in vivo dissolving effect of d-limonen[32] and pectin[33] (two molecules naturally occurring in the lemon peel and to a lesser extent in the lemon juice) on gallstones was reported. In addition, the NV, as emulsion, may favour the *in vitro* dissolution of gallstone bilirubin which can be of hydrophilic or lipophobic character, according to its chemical structure. We recall that the aqueous phase of NV consists of an extract of lemon albedo in lemon juice (submitted)[23], the both components being susceptible to contain naturally limonene and pectin. On the other hand, the cholesterol, other gallstone component, presents certain solubility in olive oil[34] which represents the oil phase of NV[23]. Regarding the synergistic effect between components, Raghavendra & Srinivasan[1] have demonstrated the higher anti-lithogenic effect in animals fed with mixture of cluster beans and garlic, compared with animals fed with theses individual components. Solutions *p*H remained stable throughout the experiment (Fig. 6), reflecting the buffer capacity of studied liquid media. The same phenomenon has been observed by Meiouet *et al.*[15] concerning the dissolution of cystine urinary stones. The stability of *p*H value may explain the

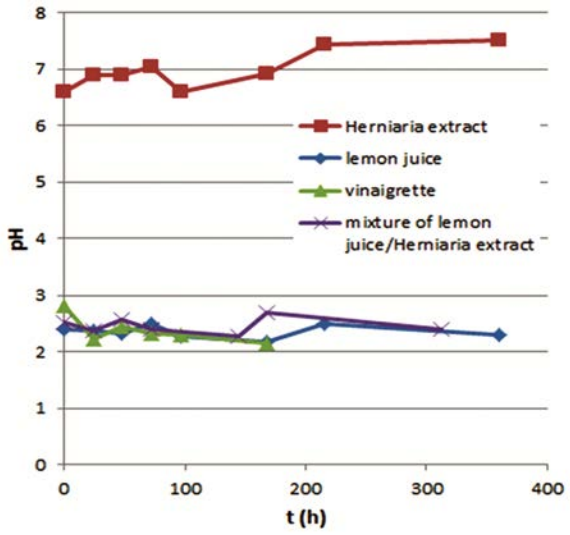

Fig. 6—*p*H of immersion liquid extracts versus time.

uniformity of curves of Fig. 5 since it is well established that the dissolution of organic molecules is generally influenced by the medium *p*H. So, the effectiveness of *in vitro* GDC of HHE, LJ, LJ/HHE mixture and olive oil/LJ natural vinaigrette depends to a large extent on both liquid media in which gallstones were immersed and immersion duration. Thus, the NV showed the outstanding highest GDC (100 % after seven days of treatment), compared to other liquid media investigated. Knowing that *in vitro* assays provide important tools to enhance the extrapolation from in vitro to *in vivo* in humans[35], these findings open interesting perspectives for NV applications: 1) as functional food to replace the commercial vinegar-based dressings, knowing that the alternate consumption of olive oil and lemon juice to promote the gallbladder flush is widely popularized, and 2) as a promising alternative to conventional solvents used in the treatment of gallstones by contact litholysis. However, additional studies are needed to needed to improve the stability and bioavailability of the NV.

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