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Cholesterol lowering effect in the gall bladder of dogs

by a standardized infusion of *Herniaria hirsuta* L.

Ines van Dooren[a*], My El Abbes Faouzi[b], Kenn Foubert[a], Mart Theunis[a], Luc Pieters[a], Yahya Cherrah[b], Sandra Apers[a]

[a] Natural Products & Food Research and Analysis, Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium

[b] Laboratory of Pharmacology, Faculty of Medicine and Pharmacy, Rabat-Institute, Al Karm street 11, Hay Riad, Rabat 10104, Morocco

[*]Corresponding author. Tel.: +32 3 265 27 20; fax: +32 3 265 27 09

E-mail address: Ines.vanDooren@uantwerpen.be

Abstract

Etnopharmacological relevance. Infusions of *Herniaria hirsuta* L., *Herniaria glabra* L. and *Herniaria fontanesii* J.Gay are well known in Moroccon folk medicine for the treatment of biliary dyskinesia, (uro)lithiasis or as a diuretic. *Herniariae Herba* which can contain *H. glabra* and *H. hirsuta* is known in Europe as an urological drug.

Aim of the study. To investigate the efficacy of a standardized infusion of *Herniaria hirsuta* against choleltihiasis, and evaluation of its genotoxicity.

Methods and materials. An analytical HPLC-UV method to quantify flavonoids and saponins present in the extract of *H. hirsuta* was developed and validated. An *in vivo* experiment to evaluate the cholesterol lowering effect of a infusion of *H. hirsuta* in the gall bladder of dogs was carried out. Dogs were divided into 3 groups i.e. control dogs (CG), dogs treated with ursodeoxycholic acid (UDCA) (2 x 7.35 mg/kg body weight/day) and dogs treated with the standardized infusion (HG) (2 x 48.5 mg/kg body weight/day). Dogs were fed a fatty diet during 120 days after which a diet without additional fat was introduced till day 180. Treatment started 30 days after introduction of the fatty diet and lasted till the end of the experiment. A bile and blood sample of each dog was collected every 30 days, after which the concentration of cholesterol was determined. An Ames test was performed according to the OECD-guidelines.

Results. The validated HPLC-UV method showed a linear calibration model and an acceptable precision for the total flavonoid content (total content 4.51%) as well as the total saponin content (12.74%). The *in vivo* experiments already showed a minor difference for bile cholesterol between CG and HG after 30 days of treatment with the infusion, and the difference was more pronounced after 90 days of treatment. Even 30 days after discontinuation of the cholesterol-rich diet a significant difference remained between CG and HG. There was no statistically significant difference in blood cholesterol. The Ames test showed that the infusion of *H. hirsuta* could be considered as being free from genotoxic risks.

Conclusion. A method for the standardization of a infusion of *Herniaria hirsuta* was developed and validated. Prolonged use of this standardized *H. hirsuta* extract resulted in a cholesterol-lowering effect in the bile of dogs. Since this pharmacological effect prevents the formation of gallstones and can contribute to solving existing gallstones, a standardized infusion of *H. hirsuta* may have a positive effect in the treatment of gallstones in human patients.

Keywords: *Herniaria hirsuta* L., flavonoids, saponins, standardized extract, cholelithiasis, *in vivo,* Ames test, gall stones

1. Introduction

With a prevalence of 10-20% of the adult population in Europe and America, gallstone diseases are a major problem in health care in the Western society (Schafmayer et al., 2006; Stinton et al., 2010). About 80% of gallstones are composed mainly of cholesterol and are caused by saturation of the bile with cholesterol (Das and Verma, 2008; Lambou-Gianoukos and Heller, 2008; Schafmayer et al., 2006). Despite the frequency of the condition, the clinical management of gallstone disease is almost exclusively based on cholecystectomy and endoscopic or medical treatment of complications. Cholecystectomy, although an established procedure, still carries a small but existent complication rate, especially when performed in an acute setting (Schafmayer et al., 2006). Some 20% of patients continue to suffer from pain after cholecystectomy, and symptomatic therapy with analgesia (non-steroidal anti-inflammatory drugs (NSAIDs), narcotic analgesics) is designated. Medical dissolution therapy with ursodeoxycholic acid (UDCA) is an alternative for patients experiencing moderate symptoms due to cholesterol gallstones. The main drawbacks of this treatment are the low efficacy (40%), slowness in action and the possibility of stone recurrence (Konikoff, 2003).

The use of drugs influencing hepatic synthesis and/or secretion of cholesterol like statins, and/or intestinal absorption of cholesterol like ezitimibe might be able to influence the formation of cholesterol gallstones and promote the dissolution of gallstones. These drugs are currently not used against gallstones and to prove the efficacy of the latter, rather expensive drugs in the treatment of cholelithiasis, still long-term well designed experimental studies should be performed (Portincasa et al., 2012).

Because many synthetic chemical drugs show side effects, there is an increasing public interest in natural medicines. On the internet some poorly characterized products and some

homeopathic drugs against cholelithiasis are advertised. However, currently no herbal medicinal products, for which the efficacy against cholelithiasis has been proven, are available.

The genus *Herniaria* (Caryophyllaceae) contains several species which are widely distributed in Europe, Asia and North Africa. Beside the fact that an infusion of either *H. glabra* L., *H. hirsuta* L. (hairy rupturewort) or *H. fontanesii* J.Gay is well known in Moroccon folk medicine for the treatment of biliary dyskinesia, (uro)lithiasis or as a diuretic, *Herniariae Herba* which can contain *H. glabra* and *H. hirsuta* is known in Europe as urological drug (Atmani et al., 2004; Eddouks et al., 2002; Mbark et al., 2000; Settaf et al., 2000). Some phytochemical research on these species revealed the presence of several saponins, flavonoids and coumarins (Charrouf et al., 1996; Freiler et al., 1996; Mbark et al., 2000; Mbark et al., 1995; MBark et al., 1996; Schroder et al., 1993). However, the efficacy and use of a standardized extract against cholelithiasis has never been investigated. In this study an analytical method for the quantification of the main natural compounds of an extract of *H. hirsuta* was developed and validated, while the *in vivo* effect of the standardized extract of the plant on the level of cholesterol in the bile of dogs receiving a cholesterol-rich diet was investigated. In addition to the efficacy of the standardized extract, the genotoxicity was evaluated.

2. Materials and methods

2.1. Chemicals and reagents

Methanol HPLC grade and acetonitril Far UV HPLC grade were purchased by Fisher Scientific (Hampton, NH, USA). Butanol (99%), formic acid (99+%), acetic acid (99.8%), sulfuric acid (85% g/g) and ethylacetate HPLC grade were provided by Acros (Geel, Belgium). Hederacoside C (98.19% purity and 97.70% purity) was obtained by Extrasynthese (Genay, Cedex, France) and Roth (Bonheiden, Belgium), rutin (99.70% purity) was purchased by Extrasynthese (Genay, Cedex, France) and Sigma (96% purity) (Bornem, Belgium). Ursodeoxycholic acid (UDCA) was purchased in the form of Ursofalk® 250 mg (Dr. Falk Pharma Benelux B.V.). Other chemicals included praziquantel (Droncit®, Bayer), oxibendazole and myclozamide solution (Vitaminthe®, BCI), marbofloxacin 2% (Marbocyl®, Vétoquinol), acepromazin (Calmivet®, Vétoquinol), atropin (Promopharm), tolfenamic acid 4% (Tolfidin®, Vétoquinol), sodium thiopental (Nesdonal®, Sanofi-Aventis), xylazine (Rompun®, Bayer), ketamin (Clorketam 1000®, Vétoquinol) and isoflurane (Cooper Maroc). A kit for cholesterol determination was obtained by DiaSYS Diagnostic Systems GmbH (Holzeim, Germany).

2.2. Plant material and infusion

For the preparation of a standardized extract the aerial parts of *Herniaria hirsuta* L. (hairy rupturewort) were collected in d'Oujda, Morocco. A voucher specimen of the plant is kept at the Muséum National d'Histoire Naturelle -Institut scientifique-Université Mohamed V Agdal – (Reference number: 5902). The material was air dried. The extract was prepared by the infusion of 80 g of the dried plant material in 4 l boiled water during 30 min with continuous stirring. After filtration the infusions were combined and lyophilized. Typically, 100 g of plant material yielded about 15 g of lyophilizate.

2.3. Quantification method for the determination of saponin and flavonoïd content

2.3.1. Method optimization

For the optimization of the chromatographic conditions of the method several analytical columns and mobile phases were tested. A Luna C18 column (4.6 x 250 mm, 5 µm), an Econosphere C18 column (4.6 x 250 mm, 5 µm) and an Apollo C18 column (4.6 x 250 mm, 5 µm) were compaired. As mobile phases, water, methanol and acetonitrile (ACN) with 0.05% formic acid (FA) were evaluated. For all solvent systems, the gradient was optimized to get an optimal analysis time with an acceptable resolution.

Different extraction parameters were evaluated in order to fully extract all compounds of interest from the lyophilized infusion. The extraction solvent composition (5%, 20%, 50% and 80% methanol), sonication time (20 and 60 minutes) and number of extractions (1 – 3 times) were tested.

As a final method the following procedure was performed. About 100 mg of the lyophilized infusion was accurately weighed in a measuring flask of 10.0 ml. Methanol 50% was added and the sample was ultrasonicated during 20 min.

For the subsequent HPLC analysis, 50 µl of the extraction solution was injected on an Apollo C18 column (4.6 mm x 250 mm, 5 µm) (Grace, Deerfield, USA). A flow rate of 1 ml/min was chosen and the following gradient was used: mobile phase A = H_2O + 0.05% FA, B = methanol + 0.05% FA; gradient: start at 5% B – stay at 5% B during 5 min - from 5% to 100% B in 55 min – stay at 100% during 2 min – from 100% to 5% B in 1 min – stay at 5% B during 2 min.

2.3.2. Method validation

The method was validated according to the ICH guidelines (ICH, 1994, 1996).

The calibration model of the two selected standards was investigated. Therefore five concentration levels of both rutin (1.28 – 489.50 µg/ml) and α-hederacoside C (19.52 – 780.61 µg/ml) were prepared. All solutions were analyzed in duplicate.

For the repeatability of the injection, one sample was analyzed 6 times. Also the repeatability and the intermediate precision were investigated. Therefore six independently prepared samples (100%; 100 mg) were analyzed according to the above described method. The procedure was repeated on three different days. The intermediate precision on different concentration levels was determined by analyzing six samples weighing 50% of the normal mass (50 mg) and six samples weighing 200% of the normal mass (200 mg). A solution of both standards, used to determine the amount of flavonoids and saponins was freshly prepared each day and analyzed twice, using the same HPLC method.

To investigate the accuracy of the method, a recovery experiment for both rutin and α-hederacoside was performed. To 50% of the infusion of *H. hirsuta* a known amount of rutin or α-hederacoside C was added until a total concentration of 100% of either rutin or the saponins. For both compounds the samples were prepared in triplicate according to the described procedure.

2.4. Experimental protocol for the *in vivo* evaluation

All experiments were approved by the Ethical Committee of the University of Rabat, Morocco. 21 dogs (14.5 ± 4.9 kg) were collected for the experiment; temperature in the animalarium ranged between 16 – 35 °C on a natural light-dark cycle. Dogs used in the experiment were divided into one group of control dogs (CG, seven dogs), one group of dogs treated with UDCA (UG, seven dogs) and one group of dogs treated with a standardized extract of *H. hirsuta* (HG, seven dogs). Each dog was caged individually and was subjected to

an acclimatization period of 15 days in the new environment before starting the experiment. During this period all dogs received an anti-parasitic treatment. Subsequently all dogs were daily fed 200 g horse meat containing 50% sheep fat during 120 days, after which all dogs were daily fed 200 g horse meat without sheep fat till the end of the experiment (day 180). At day 30 of the experiment treatment of the different groups started. While CG dogs received no additional treatment, UG dogs received two times a day a dose of 7.35 mg/kg body weight UDCA (based on the human posology using the extrapolation of animal dose to human dose) and HG dogs received two times a day a dose of 48.5 mg/kg body weight (based on Settaf et al., 2000) of the herbal extract (patent filed, Pieters et al., 2013) till the end of the experiment (day 180). UDCA or the herbal extract were mixed with a small quantity of meat making sure the entire treatment dose was administered to each dog. A bile and blood sample of each dog was collected every 30 days, after which the concentration of cholesterol was determined. For the collection of the bile the following surgical method was used: All dogs received marbofloxacin 2% (1 mg/kg body weight), acepromazin (0.05 mg/kg body weight, IM), atropine (0.04 mg/kg body weight, IM) and tolfenamic acid (4 mg/kg body weight, IM) before surgery. Induction of anesthesia was done depending on the weight of the dogs with sodium thiopental (15 mg/kg body weight, IV) or xylazine (0.5 mg/kg body weight, IV) combined with ketamin (15 mg/kg body weight, IV). For the maintenance of sedation isoflurane (1 l/min) (xylazine, ketamine) was used. During surgery all dogs received artificial breathing through a tracheal tube. Each surgery bile was collected by puncturing the gallbladder. Bile and blood samples were immediately stored at -20 °C after which quantification of the cholesterol in both bile and blood was performed by an enzymatic method. After each surgical procedure each dog received marbofloxacin 2% (1 mg/kg body weight) during 4 days. The body weight of all dogs was monitored during the entire experiment. Data are expressed as mean ± SEM. Data were analyzed using one way analysis of variance (ANOVA), followed by

the Bonferroni test or Kruskal-Wallis analysis and Dunnett T3. $P \leq 0.05$ was considered significant.

2.5. Ames test

An Ames test was performed according to the OECD-guideline ((HMPC), 2007; Abdillahi et al., 2012). The standardized extract was tested on 5 *Salmonella typhimurium* strains (TA 1535, TA 100, TA 98, TA 1537, TA 102), whether or not in the presence of metabolising S9-fraction. Six concentrations of the extract were tested (5 mg/plate, 1.5 mg/plate, 0.5 mg/plate, 0.15 mg/plate, 0.05 mg/plate, 0.015 mg/plate) and depending on the bacterial strain and the absence and presence of the S9-fraction different positive controls were included in the test. The respective positive controls for TA 1535 were sodium azide (50 µg/ml) and 2-aminoanthracene (25 µg/ml), for TA 100 sodium azide (50 µg/ml) and 2-aminoanthracene (10 µg/ml), for TA 98 4-nitroquinoline-1-oxide (2 µg/ml) and 2-aminoanthracene (25 µg/ml), for TA 1537 9-aminoacridine (500 µg/ml) in both situations and for TA 102 4-nitroquinoline-1-oxide (10 µg/ml) and 2-aminoanthracene (25 µg/ml). Each concentration was tested in triplicate, while negative controls were performed in quadruplicate. Results are expressed as mean number of revertants ± SEM.

3. Results

3.1. Method optimization and validation

A tentative identification by UV, NMR and MS (data not shown) of the main compounds present in the chromatogram of the extract of *H. hirsuta*, obtained after HPLC analysis by the final method (Fig. 1), resulted in the assignment of peaks to the class of flavonoids or saponins. Suitable standard compounds were selected for the quantification of the flavonoids as well as for the saponins. Rutin which is present in the infusion was chosen as a standard for the flavonoids. But since the saponins present in the infusion of *H. hirsuta*, are not commercially available and their large scale isolation would be very time consuming and expensive, which would in addition make the method not useful for other research groups, α-hederacoside C was selected. The extraction experiments showed that 50% (v/v) methanol gave the best results and was chosen as extraction solvent. The best resolution was obtained with the Apollo C18 column, using water and ACN with 0.05% formic acid as mobile phases. A gradient, starting with 5% ACN + 0.05% FA going to 100% ACN + 0.05% FA was used.

The calibration model of both compounds was investigated. The regression line was constructed, the equation was generated and the correlation coefficient calculated. The slope and intercept were investigated with a Student t-test. The residuals were graphically evaluated. Also an ANOVA lack of fit test was performed. A visual inspection of the regression line and residuals plot showed that the method was linear and homoscedastic. The correlation coefficient was higher than 0.99. The slope of the regression line was significant and the intercept of the line did include the point (0,0) for rutine but not for α-Hederacoside C (Table 1). The F-value was smaller than the critical value for the ANOVA lack of fit test, meaning that there was no lack of fit for both calibration lines.

For the investigation of the precision the mean, the standard deviation and the RSD% were calculated for each day and each concentration level. The overall mean, standard deviation

and RSD% were calculated for the three days and also for the three different concentration levels. All results are shown in table 2. The repeatability and intermediate precision were evaluated by an ANOVA single factor test. For the repeatability, the within mean squares were used to calculate the standard deviation and RSD%. For the intermediate precision, the standard deviation was calculated using the following formula: $((MS_{between} - MS_{within}) / n + MS_{within}) \wedge 0.5)$. Before performing the ANOVA single factor, a Cochran test was done. The calculated Cochran value was smaller than the critical C-value, as well as for different days as for the different concentration levels, implying that the variances are not significantly different and an ANOVA single factor could be carried out.

For the interday precision, the calculated and theoretical F-values are presented in table 2. Though for most of the flavonoids and for the saponins, there is a significant effect of the factor day and/or factor concentration, the precision of the method is acceptable taking into account the complexity of the analysis. The $RSD_{between}$ for the determination of flavonoïds is smaller than 3%, respectively 1.72%, 2.02%, 1.95%, 2.66%, 256% and 1.81%. $RSD\%_{between}$ values for saponins ranged from 3.13% to 14.54%. Saponins are detected at a wavelength of 210 nm causing higher variation in results, especially when the concentration is low, which is the case for one of them. Therefore only the total content of saponins can be determined with an acceptable $RSD_{between}$ (3.13%).

The accuracy of the method was determined by means of a recovery experiment. For the flavonoids a mean recovery of 99.18% was found with a RSD% of 0.64% while for the saponins 109.20% was recovered with a RSD% of 5.51%. Although for both groups of compounds 100% was not included in the 95% confidence interval of the recovery results, for the flavonoids the method can be considered as accurate since a recovery% of 97% - 103% is generally accepted. For the saponins however, the acceptable recovery limits were not reached, therefore recovery% should be mentioned with every result obtained by this method.

The lyophilysed infusion was analyzed with the validated method and contained 4.51% flavonoïds and 12.74% saponins, taking in account the amount of moisture present in the lyophilizate.

3.2. *In vivo*

The level of cholesterol in the bile was determined for all dogs starting at day 30 of the experiment and with time intervals of 30 days (Fig. 2). It was observed that the control group (CG) and the group that received UDCA (UG) showed an increase in bile cholesterol over time, until day 120 (90 days of treatment) when the cholesterol-rich diet was stopped. However, the bile cholesterol values of the group which received the standardized extract of *H. hirsuta* remained at the starting levels in spite of the continuous administration of a cholesterol-rich diet. Although a minor difference ($p \leq 0.05$) was observed between CG and HG after 30 days of treatment with the extract, this dissimilarity between CG and HG was more profound after 90 days of treatment ($p \leq 0.001$). Even 30 days after discontinuation of the cholesterol-rich diet a significant difference ($p \leq 0.001$) remained between the untreated group and the group which received the standardized extract of *Herniaria hirsuta.* At 150 days of treatment a large standard deviation could be observed in CG, due to the bile cholesterol values of one dog. However, since this value was not marked as an extreme outlier by statistical analysis it was not excluded. Also, elimination of this value did not cause any profound difference in the statistical results after 150 days of treatment, as all three groups remained statistically equal. At any time-point no statistical difference could be observed between CG and UG.

Although the values for cholesterol in blood seem to have a tendency to decrease over time (Fig. 3), no statistically significant difference could be observed for CG and UG between the different time-points. For HG a difference could be observed which started after 90 days of treatment with the standardized extract and remained till the end of the experiment. Despite this difference over time, all three groups (CG, UG and HG) remained statistically equal for cholesterol values in blood at the different time-points. Concerning the body weight of the dogs, all groups were statistically equal and dogs gained or lost no body weight over time.

3.3. Ames test

The results obtained for the Ames test are displayed in table 3. For none of the tested strains a dose-response relationship was observed. In addition, for none of the strains there was a doubling of the amount of revertants in comparison with the negative control. Therefore it can be concluded the extract is not genotoxic.

4. Discussion

Since the treatment and avoidance of recurring problems of gallstone diseases remains a challenging and costly problem in Western countries (Di Ciaula et al., 2010; Schafmayer et al., 2006; Stinton et al., 2010), the availability of a standardized natural product with proven efficacy might provide an alternative solution for patients in the treatment or maintenance therapy of gallstones. Because of the promising activty of *H. hirsuta* on bile cholesterol in normal conditions (Settaf et al., 2000), the extract of this plant was standardized and investigated for its efficacy against gallstones in dogs receiving a cholesterol rich diet. In spite of the continuous administration of a cholesterol rich diet the standardized extract of

Herniaria hirsuta was able to retain the level of cholesterol in the bile nearly constant at the starting level and to induce a difference with the untreated group. In the present study administration of a standardized extract of *H. hirsuta* had no significant effect on blood cholesterol levels.

UDCA is often used in the treatment of gallstones because of the production of unsaturated gallbladder bile resulting from a decreased hepatic secretion of biliary cholesterol and reduced intestinal absorption of cholesterol (Di Ciaula et al., 2010). However, in the current study this drug was unable to reduce the level of cholesterol in the bile or in the blood of the treated dogs. This can possibly be explained by compensatory mechanisms, for example, increased cholesterol synthesis or decreased excretion of fecal bile acids (Pertsemlidis et al., 1973). It is clear that the standardized extract of *H. hirsuta* either has different mechanisms of action than UDCA, and/or eliminates the compensatory mechanisms which may arise in dogs treated with UDCA.

Because of its established traditional use in Morocco and European countries, and the absence of reported toxicity in folk medicine and in the *in vivo* experiments (Atmani et al., 2004; Mbark et al., 2000; ÖAB-Kommission, 2010; Settaf et al., 2000) an extract of *Herniaria hirsuta* can be considered as non-toxic according to the legislation on herbal medicinal products (traditional use). However, since pharmacovigilance and long-standing use cannot be used as evidence for absence of genotoxic risks, the genotoxicity was evaluated according to the guideline on the assessment of genotoxicity of herbal substances/preparations (Vlietinck et al., 2009). The basic requirement is to assess genotoxicity in a bacterial reverse mutation tests using a test battery of different bacterial strains ((HMPC), 2007). Based on this test it was clear that the standardized extract of *H. hirsuta* could be considered as being free from genotoxic risks.

5. Conclusions

A method for the standardization of an extract of *Herniaria hirsuta* was developed and validated. Prolonged use of this standardized *H. hirsuta* extract results in a cholesterol-lowering effect in the bile but not in blood of dogs when maintaining a cholesterol-rich diet. Since this pharmacological effect prevents the formation of gallstones and can contribute to solving existing gallstones, a standardized preparation of *H. hirsuta* may have a positive effect in the treatment of gallstones in human patients.

Conflict of interest

The authors have declared no conflict of interest.

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References

Anonymous (HMPC), C.o.H.M.P., 2007. Guideline on the assessment of genotoxic constituents in herbal substances/preparations. EMEA/HMPC/107079/2007, London, 21 May 2008.

Abdillahi, H.S., Verschaeve, L., Finnie, J.F., Van Staden, J., 2012. Mutagenicity, antimutagenicity and cytotoxicity evaluation of South African Podocarpus species. J. Ethnopharmacol. 139, 728-738.

Atmani, F., Slimani, Y., Mimouni, M., Aziz, M., Hacht, B., Ziyyat, A., 2004. Effect of aqueous extract from *Herniaria hirsuta* L. on experimentally nephrolithiasic rats. J. Ethnopharmacol. 95, 87-93.

Charrouf, Z., NaitMbark, A., Guillaume, D., Leroy, Y., Kol, O., 1996. Herniaria saponin B, a novel triterpenoid saponin from *Herniaria fontanesii*. Sapon. Used in Food and Agric. 405, 241-245.

Das, I., Verma, S., 2008. Human stones: dissolution of calcium phosphate and cholesterol by edible plant extracts and bile acids. J. Sci. Ind. Res. India 67, 291-294.

Di Ciaula, A., Wang, D.Q., Wang, H.H., Bonfrate, L., Portincasa, P., 2010. Targets for current pharmacologic therapy in cholesterol gallstone disease. Gastroenterol. Clin. of N. Am. 39, 245-264, viii−ix.

Eddouks, M., Maghrani, M., Lemhadri, A., Ouahidi, M.L., Jouad, H., 2002. Ethnopharmacological survey of medicinal plants used for the treatment of diabetes mellitus, hypertension and cardiac diseases in the south-east region of Morocco (Tafilalet). J. Ethnopharmacol. 82, 97-103.

Freiler, M., Reznicek, G., Jorenitsch, J., Kubelka, W., Schmidt, W., Schubert Zsilavecz, M., Haslinger, E., Reiner, J., 1996. New triterpene saponins from *Herniaria glabra*. Helv. Chim. Acta 79, 385-390.

ICH, 1994. Text on Validation of Analytical Procedures − ICH Harmonised Tripatite guideline.

ICH, 1996. Validation of Analytical Procedures: methodology − ICH Harmonised Tripartite Guideline.

Konikoff, F.M., 2003. Gallstones − approach to medical management. MedGenMed : Medscape Gen. Med. 5, 8.

Lambou-Gianoukos, S., Heller, S.J., 2008. Lithogenesis and bile metabolism. The Surg. Clin. N. Am. 88, 1175-1194, vii.

Mbark, A.N., Charouf, Z., Wray, V., Nimtz, M., Schopke, T., 2000. Monodesmosidic saponins from *Herniaria hirsuta*. Pharm. 55, 690-692.

Mbark, A.N., Charrouf, Z., Wieruszeski, J.M., Leroy, Y., Kol, O., 1995. Herniaria Saponin-a, a Novel Saponin from *Herniaria fontanesii*. Nat. Prod. Lett. 6, 233-240.

MBark, A.N., Guillaume, D., Kol, O., Charrouf, Z., 1996. Triterpenoid saponins from *Herniaria fontanesii*. Phytochemistry 43, 1075-1077.

ÖAB-Kommission, 2010. *Herba Herniariae*, Österreichisches Arzeneibuch, Vienna, pp. 335-336.

Pertsemlidis, D., Kirchman, E.H., Ahrens, E.H., Jr., 1973. Regulation of cholesterol metabolism in the dog. II. Effects of complete bile diversion and of cholesterol feeding on pool sizes of tissue cholesterol measured at autopsy. J. Clin. Investig. 52, 2368-2378.

Pieters L., Apers, S., Theunis, M., Vaeck, M., El Mazouari, K., Cherrah, Y., 2013. Medicinal Plant Extract. European Patent Application EP13188906.5

Portincasa, P., Ciaula, A.D., Bonfrate, L., Wang, D.Q., 2012. Therapy of gallstone disease: What it was, what it is, what it will be. World journal of gastrointestinal pharmacology and therapeutics 3, 7-20.

Schafmayer, C., Hartleb, J., Tepel, J., Albers, S., Freitag, S., Voelzke, H., Buch, S., Seeger, M., Timm, B., Kremer, B., Foelsch, U.R., Faendrich, F., Krawczak, M., Schreiber, S., Hampe, J., 2006. Predictors of gallstone composition in 1025 symptomatic gallstones from Northern Germany. BMC Gastroenterol. 6, 36.

Schroder, H., Schubertzsilavecz, M., Reznicek, G., Cart, J., Jurenitsch, J., Haslinger, E., 1993. A Triterpene Saponin from Herniaria-Glabra. Phytochemistry 34, 1609-1613.

Settaf, A., El Kabbaj, S., Labhal, A., Cherrah, Y., Slaoui, A., Hassar, M., 2000. *Herniaria hirsuta* reduces biliary cholesterol in dogs. Induced changes in bile composition. Biolog. Santé 1, 44-49.

Stinton, L.M., Myers, R.P., Shaffer, E.A., 2010. Epidemiology of gallstones. Gastroenterol. Clin. N. Am. 39, 157-169

Vlietinck, A., Pieters, L., Apers, S., 2009. Legal requirements for the quality of herbal substances and herbal preparations for the manufacturing of herbal medicinal products in the European union. Planta Med. 75, 683-688.

Fig. 1. Chromatographic profiel (detection wavelength 210 nm) of the *H. hirsuta* extract containing several major peaks identified as a caffeic acid derivative (1), quercetin-3-*O*-(2-**O**-rhamnosyl)-glucuronide (2), a flavonoid (3 and 4), rutin (5), isorhamnetin-3-*O*-rutinoside (6), hydroxymedicagenic acid heptaglucoside (7), a saponin (8), medicagenic acid heptaglucoside (9)

Fig. 2. Cholesterol level in the bile for the three groups. CG: control group; HG: *H. hirsuta* group; UG: UDCA group

Fig. 3. Cholesterol level in blood for the three groups. CG: control group; HG: *H. hirsuta* group; UG: UDCA group

Table 1. Data of the linear regression of rutin and α-hederacoside C in a limited concentration range.

	Rutin	α-hederacoside C
Correlation coefficient	1.0000	0.9994
Slope ± standard error	85458.3 ± 154.4	7389.1 ± 49.7
Intercept ± standard error	33.6 ± 16.5	58.4 ± 17.6
Confidence interval (95%)	-5.4 – 72.7	20.7 – 96.2
Range (μg/mL)	1.28 – 489.50	19.52 – 780.61
Number of standards (in duplicate)	9	8

Table 2. Precision results. $F_{theoretical}$ = 2.76 (different days, n = 18); $F_{theoretical}$ = 3.68 (different concentration levels, n = 30)

Compound		Mean	RSD%	RSD_{within}	$RSD_{between}$	RSD_{max}	Cochran	F_{calc}
2	Conc. 50%	2.0460	2.09	1.16	1.72	2.40	0.4042	1.84
	Conc. 150%	2.0387	2.28					
	Day 1 - 100%	2.0033	1.12	1.06	1.12	2.40	0.5001	1.79
	Day 2 – 100%	2.0155	1.30					
	Day 3 – 100%	2.0261	0.66					
3	Conc. 50%	0.2653	1.71	1.43	2.02	3.26	0.5024	6.88
	Conc. 150%	0.2673	2.23					
	Day 1 - 100%	0.2617	0.67	0.85	1.49	3.27	0.5186	19.58
	Day 2 – 100%	0.2630	1.05					
	Day 3 – 100%	0.2567	0.77					
4	Conc. 50%	0.3713	1.38	1.55	1.95	3.10	0.4592	4.48
	Conc. 150%	0.3705	2.32					
	Day 1 - 100%	0.3630	1.05	1.24	1.18	3.11	0.6706	0.46
	Day 2 – 100%	0.3626	1.76					
	Day 3 – 100%	0.3607	0.65					
5	Conc. 50%	0.9443	2.37	1.81	2.66	2.69	0.4237	7.90
	Conc. 150%	0.9347	2.63					
	Day 1 - 100%	0.9222	1.22	1.12	2.97	2.70	0.5774	37.54
	Day 2 – 100%	0.9085	0.00					
	Day 3 – 100%	0.9587	0.33					
6	Conc. 50%	0.6825	2.88	2.16	2.56	2.83	0.4102	3.45
	Conc. 150%	0.6717	3.07					
	Day 1 - 100%	0.6739	1.60	1.31	1.86	2.84	0.5168	7.21
	Day 2 – 100%	0.6585	1.51					
	Day 3 – 100%	0.6565	0.47					
Total flavonoid	Conc. 50%	4.3094	2.10	1.64	1.81	2.14	0.4878	2.27
	Conc. 150%	4.2829	2.43					
	Day 1 - 100%	4.2241	1.14	1.01	1.14	2.15	0.4425	2.60
	Day 2 – 100%	4.2032	1.23					
	Day 3 – 100%	4.2588	0.51					
7	Conc. 50%	2.8682	4.68	2.84	6.72	2.24	0.4387	28.53
	Conc. 150%	3.2053	2.95					
	Day 1 - 100%	3.2470	1.88	2.07	3.81	2.23	0.6488	15.40
	Day 2 – 100%	3.4114	1.02					
	Day 3 – 100%	3.1951	2.99					
8	Conc. 50%	1.1332	6.93	4.13	14.54	2.53	0.3663	69.34
	Conc. 150%	1.4766	2.04					
	Day 1 - 100%	1.3346	4.50	3.37	11.78	2.51	0.3692	68.32
	Day 2 – 100%	1.6731	3.32					
	Day 3 – 100%	1.4118	3.93					
9	Conc. 50%	8.1356	2.08	2.28	4.70	1.96	0.3289	20.52
	Conc. 150%	7.3644	1.11					
	Day 1 - 100%	7.9517	2.53	2.07	4.27	1.96	0.5371	20.65
	Day 2 – 100%	7.6582	2.19					
	Day 3 – 100%	7.4629	3.02					
Total saponin	Conc. 50%	12.1370	2.18	1.99	3.13	1.83	0.2330	9.86
	Conc. 150%	12.0463	2.78					
	Day 1 - 100%	12.5334	1.77	1.59	3.21	1.82	0.5407	19.53
	Day 2 – 100%	12.7427	1.98					
	Day 3 – 100%	12.0675	0.58					

Table 3: The amount of revertants (mean ± SD) observed for the different bacterial strains in the Ames test. (n=3, negative control n=4, *=high amount of revertants, not counted)

	TA 98		TA 100		TA 1535		TA 1537		TA 102	
S9		X		X		X		X		X
Negative control	38.8 ± 8.5	34.7 ± 2.9	187.0 ± 15.9	191.3 ± 33.7	8.8 ± 4.0	11.3 ± 4.3	8.5 ± 1.3	6.5 ± 1.3	218.5 ± 7.8	270.0 ± 34.0
Positive control	221.0 ± 19.7	1841.3±348.6	904.0 ± 110.5	498.7 ± 48.6	714.7 ± 64.3	50.1 ± 5.5	135.7 ± 52.6	517.3 ± 23.5	*	*
Extract 0.015 mg/plate	47.0 ± 15.5	29.0 ± 7.9	248.7 ± 38.6	202.5 ± 47.4	8.7 ± 4.7	11.0 ± 4.4	8.7 ± 5.5	6.3 ± 1.5	369.0 ± 58.1	259.3 ± 10.0
Extract 0.05 mg/plate	53.0 ± 18.4	31.7 ± 2.1	263.4 ± 10.5	137.0 ± 4.2	7.0 ± 5.0	10.3 ± 2.1	4.3 ± 1.5	6.0 ± 3.5	356.3 ± 10.4	297.7 ± 21.1
Extract 0.15 mg/plate	30.3 ± 3.1	31.3 ± 2.1	257.3 ± 24.0	156.0 ± 50.9	13.3 ± 3.0	13.7 ± 2.5	12.0 ± 1.4	5.3 ± 1.2	295.7 ± 28.6	254.7 ± 12.3
Extract 0.5 mg/plate	44.3 ± 19.1	37.7 ± 9.3	220.0 ± 11.1	196.7 ± 55.6	12.0 ± 2.7	9.0 ± 2.7	8.7 ± 1.5	7.0 ± 2.7	308.7 ± 18.0	375.3 ± 27.6
Extract 1.5 mg/plate	31.0 ± 5.3	35.0 ± 1.7	275.0 ± 46.0	212.0 ± 42.7	9.3 ± 2.5	13.0 ± 4.4	9.0 ± 1.7	11.7 ± 1.5	420.0 ± 9.1	441.0 ± 52.3
Extract 5 mg/plate	28.7 ± 1.5	64.0 ± 6.6	290.3 ± 40.5	261.3 ± 68.6	15.7 ± 4.0	12.0 ± 7.0	11.3 ± 2.9	11.3 ± 2.1	429.0 ± 42.4	513.7 ± 41.3

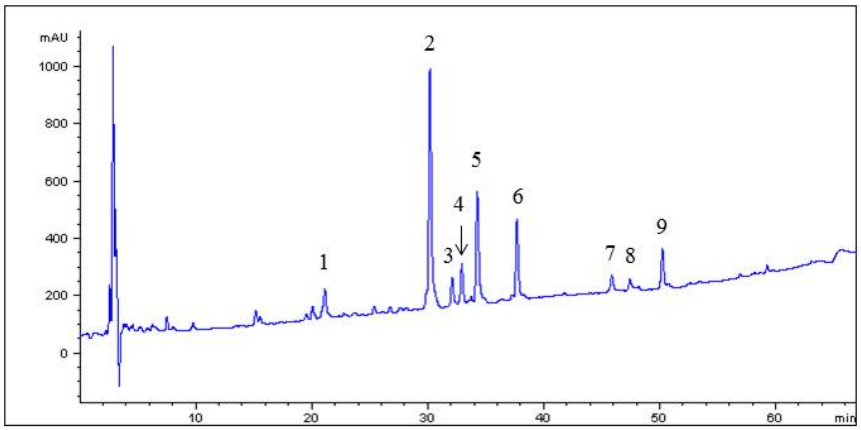

Fig. 1. Chromatographic profile (detection wavelength 210 nm) of the *H. hirsuta* extract containing several major peaks identified as a caffeic acid derivative (1), quercetin-3-*O*-(2-**O**-rhamnosyl)-glucuronide (2), a flavonoid (3 and 4), rutin (5), isorhamnetin-3-*O*-rutinoside (6), hydroxymedicagenic acid heptaglucoside (7), a saponin (8), medicagenic acid heptaglucoside (9)

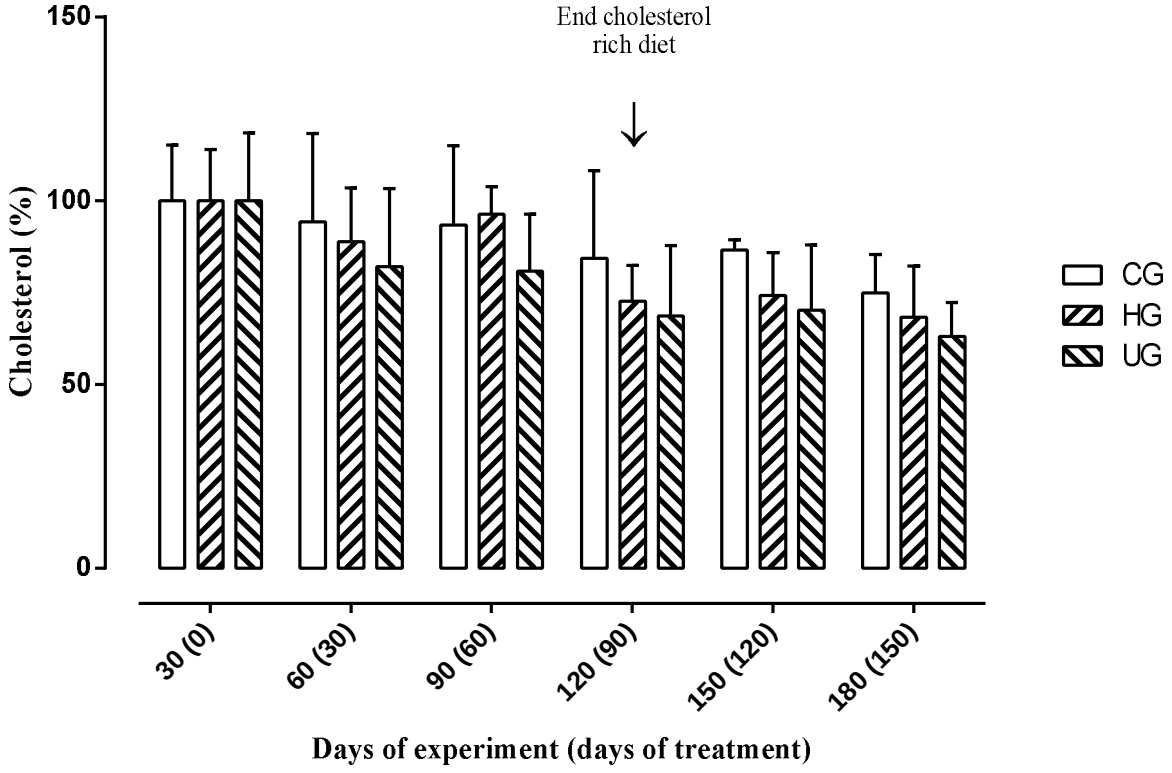

Fig. 2. Cholesterol level in the bile for the three groups. CG: control group; HG: *H. hirsuta* group; UG: UDCA group

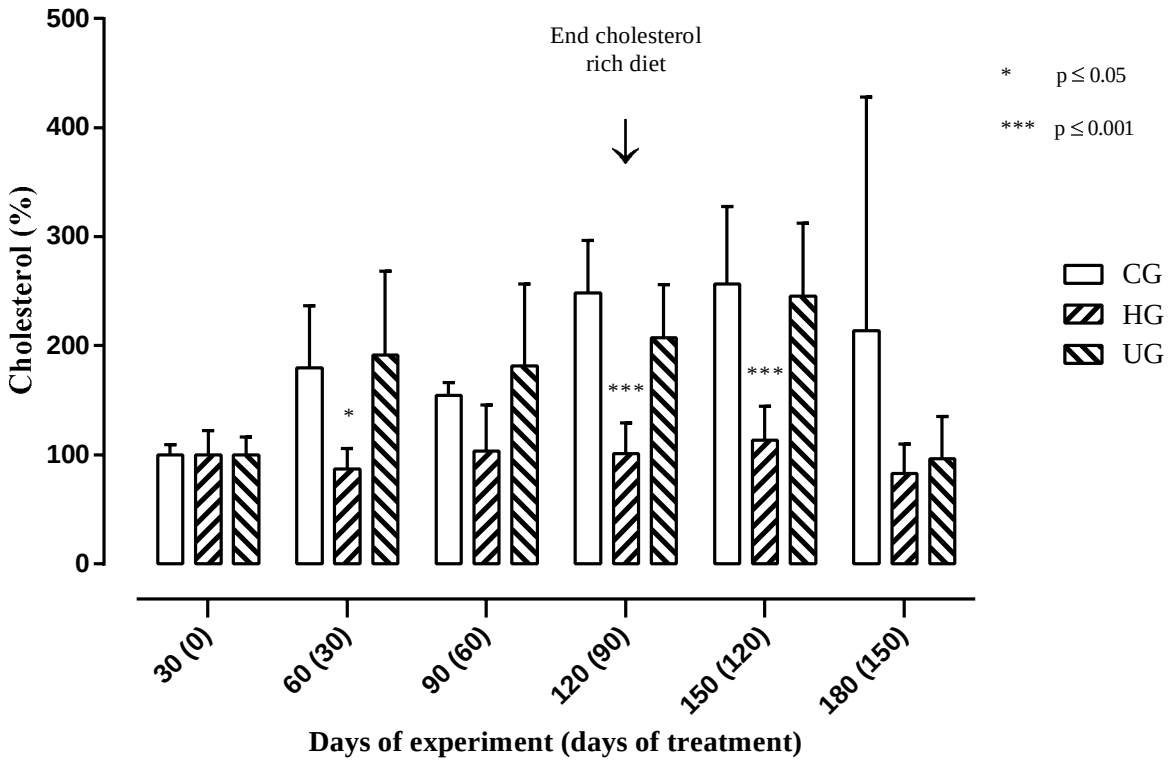

Fig. 3. Cholesterol level in blood for the three groups. CG: control group; HG: *H. hirsuta* group; UG: UDCA group