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In vitro binding capacity of free bilirubin by aqueous extract of *Marrubium vulgare* L.

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ABSTRACT. *Marrubium vulgare* L. *(MV*L.*)* has been traditionally used to treat kernicterus. However, the anti-jaundice effects and mechanisms of *MVS*. on free bilirubin remain poorly understood. The present study assesses the anti-jaundice effect of the aqueous extract of *MV*L, by measuring the percentage of adsorption (binding) of this extract on plasmatic free bilirubin obtained from kernicterus newborns (n = 40). The plant's aerian party and leaf pouder histological and phytochemical properties of *MV*L were examined. Our results show that dry aqueous extract of *MV*L contains total phenols (51.5 ± 0.40 mg EAG g^{-1}), condensed tannins (14.7 ± 0.49 mg ECAT g^{-1}) and flavonoids (25.33 ± 0.41 mg ECAT g^{-1}). Histological examination showed that both the surfaces of leave and stems are covered by a protective hair; glandular and non-glandular trichomes which produced various secondary metabolites. Free bilirubin was significantly bound by the aqueous extract of *MV*L at different concentrations (250-1250 µg ml^{-1}) to the extent of (33.6 - 54.61%). In the light of these preliminary findings and future experimental studies, it indicates that this plant may be used as a source of therapeutic agent for kernicterus and can be an alternative treatment to the currently available treatments such as phototherapy and exsanguino-transfusion, which have serious short and long term complications.

Keywords: histological examination; hypobilirubinemia effect; Kernicterus; phototherapy; phytochemical properties.

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Introduction

Marrubium vulgare L. (*MV*L) is a common plant of the Lamiaceae family, it is native of North Africa, Western Asia, and Southern Europe, locally known as 'Merrioua'. *Marrubium vulgare* L., is a popular traditional medicinal plant and has been extensively used f or the treatment of several diseases, including inflammatory, gastroenterical and respiratory disorders (Stulzer et al., 2006). Recent studies on the hepatoprotective effects of the methanolic extract of *MV*L have revealed the presence of a monoterpene acid: a component that could be responsible for the antihepatotoxic activity (Ahmed et al., 2009). Other medicinal effects are reported for *MV*L, such as antioxidant (VanderJagt et al., 2002), hypotensive (El Bardai et al., 2001), hypoglycemiant (Herrera-Arellano et al., 2004) and insecticidal properties (Pavela, 2004). Although *MV*L is known traditionally for curing Neonatal jaundice, especially the pathological model, widely called Kernicterus, which is evidenced by an increase in the serum concentration of free bilirubin, leading to the death of brain cells because of its neurotoxicity. This entity was associated to lifelong neurological sequelae in newborns, as: coordination, oculomotor disorders and central deafness (Mitra & Rennie, 2017). No previous scientific study has been carried out on the aqueous extract of *MV*L for checking its free bilirubin lowering action.

In the present report, we evaluate *in vitro*, the binding capacity of aqueous extract of *MV*L, by free bilirubin and determine the possible free bilirubin lowering effects of *MV*L (hypobilirubinemia effect), to confirm and justify the use the of *Marrubium vulgare* L. in folk medicine to treat unconjugated bilirubin toxicity (Kernicterus or bilirubin encephalopathy).

Material and methods

Collection and identification of plant material

The plant was harvested from the east of Mascara, region of Algeria, then identified by the botanic laboratory of superior national school in Algiers, Algeria. Voucher specimen of the plant was deposited in the

herbarium of the laboratory of bioconversion, microbiological engineering and sanitary security (voucher reference MV115/20).

Botanical study

Histological examination by double coloration method

A histological study of anatomical sections of the *MV*L (leaf and stem) was performed using the double coloration method described by Bouzabata (2015). Very thin cross-sections performed were immediately collected in water, avoiding dehydration. The sections are then successively placed in bleach at 12° (for 15 min) to empty the cytoplasmic content of the cells, followed by neutralization of the excess bleach with 20% acetic acid (for 3 min). The lignified walls are stained green, blue or violet (depending on the degree of lignification) with Iodine Green (for 3 min). The cellulose walls are stained pink with Alumina Carmine (5 to 10 min). The sections must be rinsed with distilled water between each staining step. Sections are finally mounted between slide and coverslip with a drop of distilled water and observed under a light microscope at G ×10 then G ×40.

Microscopic examination of leaves powder

The purpose of this test is to analyze the specific criteria for this plant's powder. This fast, specialized technique, described by Bouzabata (2015), makes it also possible to quickly identify adulterations and falsifications of medicinal plants. Briefly, on a microscope slide, a drop of lactic acid was placed and mixed with a small amount of the leaf powder until wet. Then, this slide is covered with a coverslip. Microscopic observation was carried out at (G ×10 then G ×40).

Preparation of plant extract

The plant's aerien party was dried in shade and crushed to coarse powder, then 333g of the plant powder was steeped in 2 L of deionized water and extracted by cold maceration method, using a magnetic stirrer for 24 hours with intermittent shaking at room temperature. The macerate was filtered through Whatman filter paper. The extracts were then centrifuged at 8000 rpm for 10 min. The aqueous solution was lyophilized for 72 hours, by using a freeze-drier. Extraction was done following the method of Boubakeur et al. (2017) with slight modifications.

About 76.59 g of aqueous extract with a yield of 23 % was obtained. It was briefly stored at 4°C, until further use. For preparation of different concentrations (concentration range 250-1250 µg mL^{-1}), desired quantity of aqueous extract was dissolved in 0.9% sodium chloride solution (NaCl).

Identification of secondary metabolites

Common phytochemical tests were carried out on the aqueous extract to ascertain the presence of some major natural chemical groups. according to the protocol described by Harborne (1980). The qualitative presence of the following secondary metabolites was analysed: flavonoids, steroids, terpenes, tannins, anthocyanins, saponins, and alkaloids.

Determination of the main chemical groups

Determination of total phenolic compounds

The total phenolic content of *MV*L aqueous extract was assessed using Folin-Ciocalteu method described by Hu et al. (2008). Briefly, 200 µL of extract dissolved in distilled water (40 µg mL^{-1}), were mixed with 1 ml of Folin-Ciocalteu reagent and 800 µl of sodium carbonate (75 mg ml^{-1}). The mixture was agitated and incubated in dark for 45 min. at 25°C. Then, absorbance was measured at 760 nm using a UV-V spectrophotometer (MEDLINE MD 2000, Spain). The total phenolic concentration of *MV*L aqueous extract was expressed in milligram equivalents of gallic acid per gram (g) of dry matter extract (mg EGA g^{-1}).

Determination of total flavonoids

Quantitative analysis of total flavonoids is carried out by a colorimetric method using aluminum trichloride, which forms a yellow complex with flavonoids, and quercitin as reference compound (Akrout et al., 2011). Briefly, 1 mL of extract (10 mg mL^{-1}) was mixed with the same volume of 2% methanolic

solution of aluminum trichloride. After 10 minutes of incubation in dark at 25°C, the absorbance of the mixture was measured at 430 nm. Results were reported in milligram quercitin equivalents per gram (g) of extract (mg QE g^{-1}).

Determination of total tannins

Tannins content was determined using the method described by Julkunen-Titto (1985). A volume of the extract (50 ml) was added to 1500 µL of 4% vanillin solution in methanol. The solution was vigorously stirred. Then, 750 µL of concentrated hydrochloric acid was added. The mixture was incubated for 20 min at 25°C. The absorbance was measured 550 nm. Results were reported in milligram equivalents of tannic acid per gram (g) of dry extract (mg ETA g^{-1} of dry extract).

Isolation of human plasma

Human blood and plasma were obtained from 40 kernicterus newborns usually stay with their mother in a newborn room of the hospital (*Chalabi Abdelkader* Tighennif in Mascara, Algeria). None of the newborn donors had taken any medication, any addictive substances or any antioxidant supplementation. All participants gave informed consent before being inclusion in the study. The study was performed according to the principles given in the Helsinki Declaration (Council for International Organizations of Medical Sciences [CIOMS], 2016). Venous blood was withdrawn between 8 and 10 AM from the antecubital vein using a clean venipuncture technique and immediately centrifuged at 2500 g for 15 min to obtain plasma.

Measurement of plasma direct, indirect, and total bilirubin levels

Plasma bilirubin reacts with diazotized sulfanilic acid to form a color complex, azobilirubin, which can be measured spectrophotometrically using a commercial kit (SPINREACT, Spain). These measures were used as control without extract.

Free bilirubin binding by indirect diazoreaction technic:

The indirect (or free) bilirubin binding assay was executed through modification of the procedure previously by Hu et al. (2008). Briefly, a precise volume of icteric plasma, containing free bilirubin (as substrate), was mixed in the same volume of 0.1M phosphate buffer, pH = 7 and the same volume of each extract concentration. The final mixture was incubated at 37°C for 90 minutes in a shaking water bath. Mixtures were centrifuged at 10°C with 10,000 rpm for 30 min. in an ultracentrifuge (Model J-26XPI, Beckman, USA), to separate the bound from the free unconjugated bilirubin. The supernatant was removed into a second set of tubes and frozen at – 20°C until further use. The free bilirubine concentration was analyzed using SPINREACT kits applied to spectrophotometer at 555 nm. The assessment of the absorption capacity of the aqueous extract on free bilirubin is calculated in relation to the control without extract.

Statistical analyses

The results are presented as mean ± standard errors of mean (SEM) for N = 40, in order to eliminate uncertain data. The mean and statistical significance between the groups was analyzed by means of the Student's t-test or analysis of variance and $p < 0.05$ was considered significant. Statistically significant differences within and between groups were identified by applying the ANOVA test.

Results

Botanical study results

Microscopic features of *Marrubium vulgarea* L. leaf and stem

Description of leaf cross-section

Leaves of *MV*L. are petiolate, roundish, ovate, and arranged in opposite pairs on a long stem. The cross section of the leaf lamina presents an epidermis including epidermal cells and non-glandular and glandular trichomes. The dorsiventral mesophyll is constituted by the palisade parenchyma condensed in chloroplasts and spongy parenchyma. The palisade tissue is abundant than the spongy parenchyma. There is a central vascular bundle with an arc shaped form (xylem and phloem) (Figure 1).

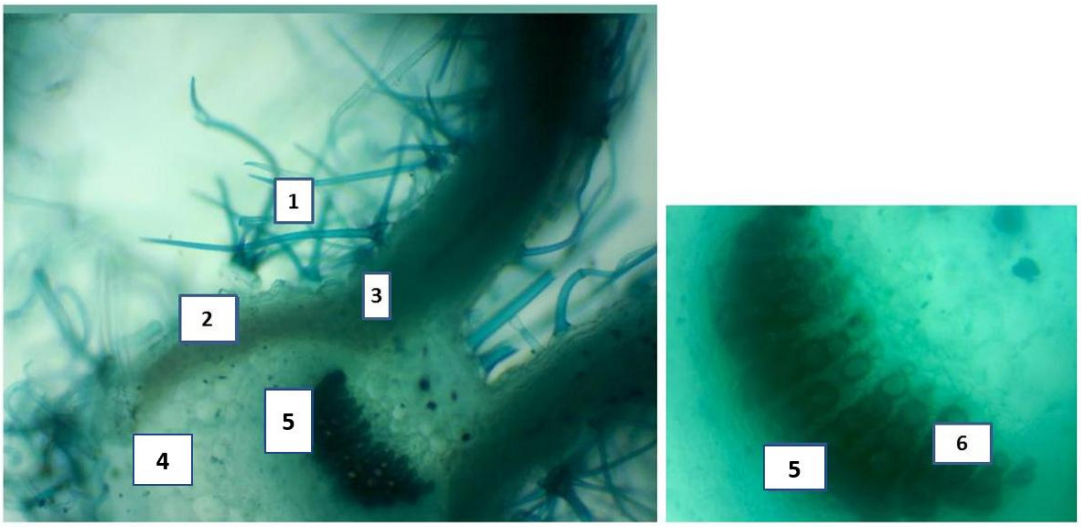

Figure 1. Microscopic characteristics of leaf cross-sections of *Marrubium vulgare* L. (horehound) (G×10) × 10 and (G×40) ×10

Description of stem cross-section

The cross-section of *Marrubium vulgare* L. stem shows a quadrangular form due to the presence of few layers of collenchyma cells situated in the corner, underneath the epidermis. The stem is densely covered with glandular and non-glandular trichomes. The parenchyma cells of the cortex and the pith are clearly distinguishable and the cortex is thickness than the pith. The vascular tissues have a bundled structure (Figure 2).

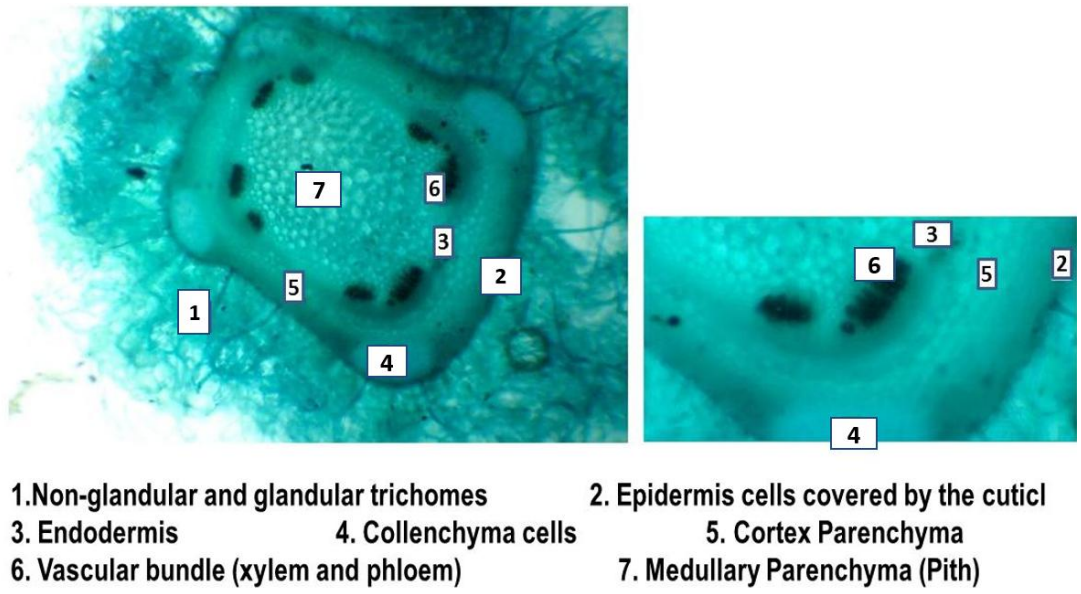

Figure 2. Microscopic characteristics of stem cross section of *Marrubium vulgare* L. (horehound) (G×10) × 10 and (G×40) ×10.

Description of *Marrubium vulgare* L. leaf powder

A yellowish-green powder without characteristic aromatic odour, with some elements observed under the optical microscope:

- Fragment of lower epidermis made up of thin-walled cells with smooth cuticle, numerous and characteristic (Figure 3, legend 8).
- Fragment of glandular and non-glandular trichomes, numerous and characteristic (Figure 3, legend 9).
-Fragment of Palisade parenchyma, few in number and characteristic (Figure 3, legend 10).
- Fragment of xylem, few in number and characteristic (Figure 3, legend 11).
- Fragment of vascular bundle, few in number and characteristic (Figure 3, legend 12).

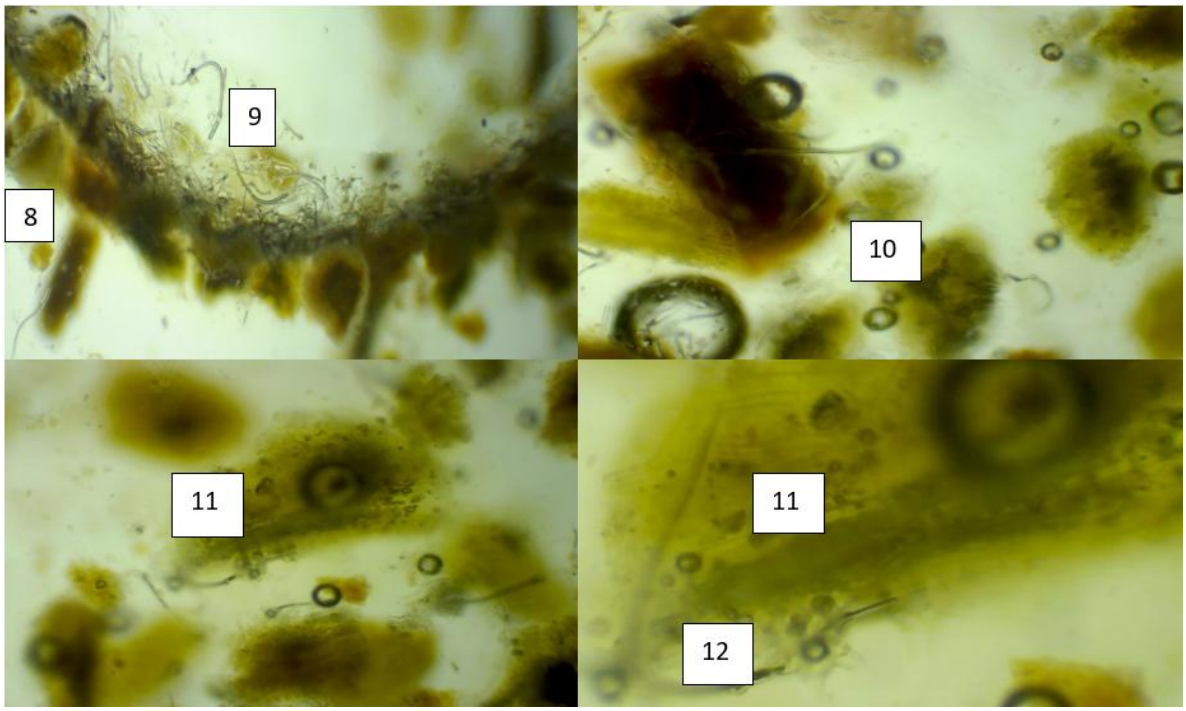

Figure 3. Description of *Marrubium vulgare* L. leaf powder (G x 40) x 10. 8: Fragment of lower epidermis, 9: Fragment of glandular and non-glandular trichomes, 10: Fragment of Palisade parenchyma, 11: Fragment of xylem, 12: Fragment of vascular bundle.

Phytochemical screening of *Marrubium vulgare* L. aqueous extract

Phytochemical analysis revealed the presence of flavonoids, anthocyanin, tannins, alkaloids, saponins, terpenes and steroids (Table 1).

Table 1. Phytochemical screening on the aqueous extract of *Marrubium vulgare* L.

Metabolites	*Marrubium vulgare* L. aqueous extract
flavonoids	**+++**
Anthocyanins	-
tannins	+++
alkaloids	+++
saponins	+++
terpenes	++
steroids	++

Test results are classified as strongly positive (+++), moderately positive (++), weakly positive (+), absence (-).

Total phenol, flavonoid and condensed tannins levels

The quantitative analysis in total phenols, condensed tannins, and flavonoids shows that the aqueous extract contains total phenols, condensed tannins, and flavonoids levels of 51.5 ± 0.40 mg EAG g^{-1} dry extract, 25.33 ± 0.41 mg ECAT g^{-1} and 14.7 ± 0.49 mg ECAT g^{-1} dry extract, respectively (Figure 4).

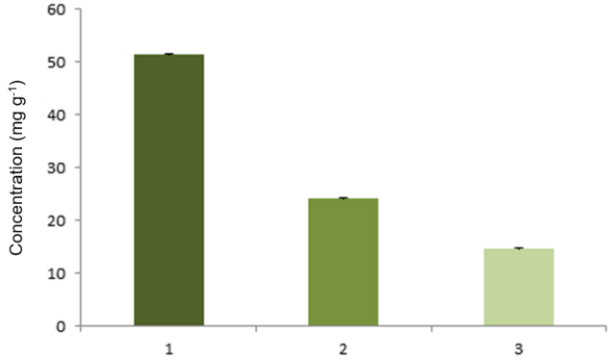

Figure 4. Total phenolic compounds, total tannins, and total flavonoids compounds of *Marrubium vulgare* L. aqueous extract. **1:** total phenolic (mg EGA g^{-1}), **2:** total flavonoids (mg QE g^{-1}), **3:** total tannins (mg ETA g^{-1})

In vitro Marrubium vulgare L. aqueous extract binding capacity of free bilirubin

The percentage of free bilirubin binding by the aqueous extract of *MV*L is shown in figure 5. The results show that the extract had the ability to bind the free bilirubin with different degrees. This difference depends on the extract concentration (250-1250 µg mL^{-1}): the free bilirubin was bound significatively by the extract with 250, 500, 750, 1000, 1250 µg mL^{-1}, to the degree of 33.6, 37.28, 39.56, 52.88 and 54.61% respectively.

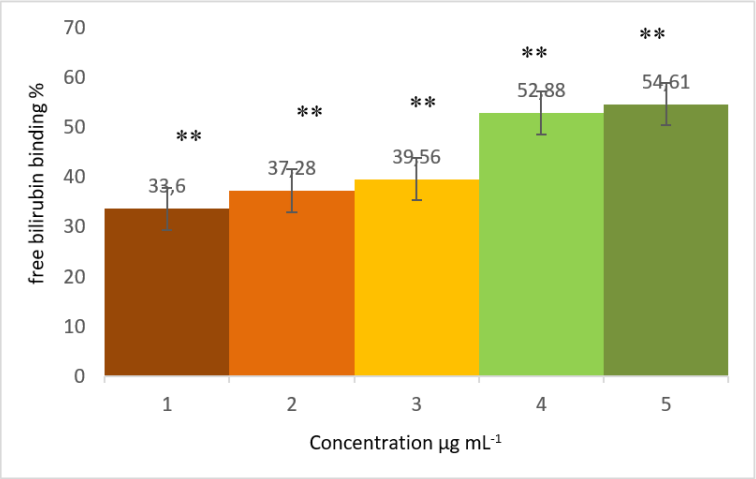

Figure 5. Free bilirubin binding by aqueous extract of *MV*L in different concentration of (250-1250µg ml^{-1}). Each column represents the mean of 40 experiments and the vertical lines indicate the S.E.M.. (**) indicate significant differences by ANOVA test: **p < 0.01.

Discussion

Various medicinal plants are used in many societies. It is used worldwide as a remedy for many diseases processes. Neonatal jaundice has become the most common disease in newborns. In most cases, neonatal hyperbilirubinemia resolves without treatment and is called 'physiologic jaundice'. However, it is important to distinguish this from a more serious condition called pathologic jaundice. It is commonly called kernicterus or bilirubin encephalopathy. The term refers to the long-term clinical consequences of unconjugated bilirubin toxicity. The treatment of this disease involves phototherapy: the most common treatment, and exchange transfusion. Phototherapy imposes the respect of a number of requirements with close medical supervision, because of the serious complications that they can cause: hyperthermia, dehydration, ophthalmological mutagenic and gonadal risks. Exchange transfusion has risks that are difficult to quantify: infectious, hypocalcaemia, thrombocytopenia, convulsions, bradycardia, apnea and mortality (3%) (Mitra & Rennie, 2017). In a program to obtain new hypobilirubinemia effect from medicinal plants, we have selected *Marrubium vulgare* L., based on its using in folk medicine to treat neonatal hyperbilirubinemia, especially kernicterus. The results of the present study demonstrate, for the first time, that *Marrubium vulgare* L. exhibits significant hypobilirubinemia effect. The interpretation of the hypobilirubin binding effect is based on discussions of Woodward and Reed (1989) which indicate that phytochemicals have the ability to form complexes with biomolecules such as carbohydrates and proteins. Barry and Manley (1986) and Wamatu et al. (2006) have shown that tannins preside over the interference with the protein fraction, and they are known to complex with proteins (protein-binding or precipitation capacity).

Phytochemical analysis performed on our aqueous extract of locally *Marrubium vulare* L. revealed that the plant is rich in secondary metabolites such as flavonoids, tannins, anthocyanins, saponins, steroids, and terpenes. These results enhance the previous interpretation, the fact that there is tannins in the aqueous extract reinforces the hypothesis of the role of tannins of *Marrubium vulgare* L. in the hypobilirubinemia effect by binding to the free bilirubin.

Phytochemical screening's results are similar to those obtained by the phytochemical study carried out by Mittal et al. (2016), Djahra et al. (2015) and Kahlouche-Riachi et al. (2015) The high content of flavonoids and phenolic compound justified the use of *Marrubium vulgare* L. in the diseases (hypertension, diabetes, cardioprotective etc) caused by oxidative stress.

Histological examination of the plant's aerien party shows, that both the surfaces of leave and stems are covered by a protective hair; glandular and non-glandular trichomes which produced various substances such

as lipids, polysaccharides, phenolic compounds, terpenes, tannins, and flavonoids with the most accumulated compounds known as marrubiin (Popoola et al., 2013). Indeed, at present, the available therapeutic options for *MV*L in Algerian folk medicine to treat neonatal jaundice are mainly based on the cutaneous administration for a general action by penetration of secondary metabolites through the various cell layers and diffusion through the bloodstream (percutaneous or transdermal administration). Because of these potential benefits, the aqueous extract of *MV*Lmerits a full evaluation in terms of efficacy.

Conclusion

In summary, the results show that the aqueous extract of *MV*L exhibits a hypobilirubinemia effect through the ability of its components to adhere to free bilirubin, but it has not been possible to determine the precise nature and mechanism of complexation. In the light of these results, *MV*L's effective anti-icteric properties were judged to be satisfactory. Finally, the results of this study confirm and justify, at least in part, the use of *MV*L in folk medicine to treat neonatal jaundice and encourage further research on this plant and its synthetic derivatives in order to obtain more powerful anti-icteric agents, which could be phytobiotic alternatives with fewer damaging effects on the health of newborns.

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