STUDIES REGARDING THE ANALYSIS OF DIFFERENT EXTRACTION CONDITIONS FOR THE SEPARATION OF *CHELIDONIUM MAJUS* L. ALKALOIDS

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Abstract: This paper focuses on the alkaloid content of greater celandine and the analysis of different alkaloids extraction techniques. The aim of the study is to obtain a reproducible optimum technique for the separation of a limited number of alkaloids for future *in vitro* anti cancer determinations. The thin layer chromatography final analysis shows that different concentrations (70 and 96%) ethanol extracts obtained from the entire plant contain a big number of compounds. We observed an important TLC - profile after four extraction steps (using ethylic ether, ethanol, chloroform and methylene chloride). We can conclude that a big number of extraction steps are needed in order to obtain a concentrated mixture with a limited number of compounds.

INTRODUCTION

Chelidonium majus L. or the greater celandine is well known since ancient times due to its medicinal properties. Because of the popularity of the pharmaceutical industry and the synthetic drugs, in the last century a big number of valuables medicinal plants were forgotten (Grigorescu *et al.*, 2001). Today, the plant is often considered toxic, some studies indicating a high risk of neural and digestive diseases, hemolytic anemia, acute hepatitis, contact dermatitis etc. at the administration of the greater celandine (Benninger *et al.*, 1999; Farina *et al.*, 1999; Etxenagusia *et al.*, 2000; Duke *et al.*, 2002). This toxic effect is attributed to the high diversity of alkaloids from its composition. The scientific data shows the existence of 20 to 30 alkaloids depending on the extraction and isolation methods that are used. The main components are isoquinoline alkaloids, belonging to different classes such as benzophenanthridine (sanguinarine, chelidonine, and chelerithrine), protoberberine (berberine, coptisine) and protopine (protopine, allocryptopine) (Kursinszki *et al.*, 2006). At the same time, the diversity of biologically active components offers a very large spectrum of terapeutical actions such as antioxidant (Then *et al.*, 2003), antibacterial (Kokoska *et al.*, 2002), antiviral (Mlinaric *et al.*, 2000), anti-inflammatory (Jang *et al.*, 2004) and antitumoral (Chan *et al.*, 2003, Vogt *et al.*, 2005).

The aim of this study is to analyze different alkaloids extraction techniques in order to obtain a reproducible protocol for future anti cancer investigations.

MATERIAL AND METHODS

The plant material was collected from the Botanical Garden of Iasi in June 2010. The roots and the aerial parts were separated from the rest of the plant and grounded.

All the extraction methods represent adaptations of previously described approaches. For the first experiment, 5,5 grams of roots were treated with 75 ml ethylic ether using a Soxhlet installation in order to eliminate the containing lipids. The ether phase was collected for chromatographic determinations and, after the complete evaporation, the alkaloids were extracted for 3 hours from the plant material using the same system and 70% ethanol. At the end of the protocol, the sample was dried and extracted with a mixture of acetone and water 9:1 (*v/v*). The ethanol phase was separated in three samples: one sample for chromatographic determinations, 30 ml was mixed with 1M hydrochloric acid until the pH reached the value of 1 (with the alkaloids as quaternary salts) and 30 ml were mixed with 1M sodium hydroxide until the pH became 8.5 (with free base alkaloids). In the last sample, a precipitate has appeared. In order to determine its composition, the sample was centrifuged for 15 minutes at 5000 rpm and the sediment was mixed with 2,5 ml of 70% ethanol and chromatographically analyzed. The supernatant and the sample with acid pH were treated in a separatory funnel with 20 ml chloroform. The chloroform phase was collected and the water phase was treated again with chloroform. At the end, we obtained two chloroform phases and two water phases. The chloroform phases were treated during the night with anhydrous sodium sulphate in order to eliminate the water. All four phases were chromatographically analyzed. The water phase with pH 8.5 was mixed with methylene chloride in a separatory funnel. The methylene chloride phase was mixed in a separatory funnel with 25 ml of water containing 0,75 grams of citric acid. The superior water phase was collected for future investigations and the inferior phase was kept for chromatographic determinations.

For the second experiment, 5 grams of root was mixed with 25 ml of distilled water, 5 ml of methanol and 0,125 g sodium hydroxide and sonicated for one hour. The resulting extract was then centrifuged for 30 minutes at 5000 rpm. The supernatant was mixed in a separatory funnel with 30 ml of methylene chloride. The water phase was kept for future investigations and the methylenic phase was chromatographically analyzed. The sediment was mixed with 30 ml of 70% ethanol, sonicated for one hour, centrifuged and the supernatant was chromatographically analyzed. The experiment was repeated using the roots and the aerial parts. The methylenic and water phase obtained after the treatment with methylene chloride were analyzed. The second one was heated at 60^0C for 30 minutes and 25 ml 30 ‰ citric acid was added. Then, the extract was treated during the night with 10% sodium chloride. Due to the apparition of a precipitate, the sample was centrifuged for 30 minutes at 5000 rpm. The sediment was treated with concentrated acetic acid and chromatographically tested (Boulware and Schlowsky, 1989).

The third experiment consists in treating 5 grams of roots with 75 ml etylic ether using a Soxhlet installation followed by drying and ultrasonication for 30 minutes in the presence of 70% ethanol. The result was centrifuged for 30 minutes at 5000 rpm and the supernatant was treated with 30 ml of methylene chloride in a separatory funnel. The methylenic phase was chromatographically analyzed (Artamonova and Kurkin, 2008).

For the last experiment, 3 grams of plant including the aerial parts were treated with 70% ethanol using a Soxhlet installation. The extract was concentrated under vacuum and centrifuged. The sediment was mixed with 3 ml of 70% ethanol and chromatographically analyzed. A part of the supernatant was kept for future investigations and the other part was chromatographically tested. The experiment was repeated in the same conditions, but using 96% ethanol as extraction solvent (Bugatti *et al.*, 1991).

The chromatographic material was represented by 20 x 20 cm Aluminium sheets with TLC Silica gel 60 provided by *Merck Germany*. All the solvents used for the preparation of the mobile phases have analytical purity. The method presents several steps. The first step consists in pre-saturate the chromatographic chamber with the solvent vapor during the night. The next steps are represented by spotting 20 µl of *C. majus* extract on a small area of the starting line, perfectly drying the plate, the ascending migration, air-drying and UV-visualization. Because of the natural UV-fluorescence of *C. majus* alkaloids, no fluorescent indicators are needed. The photos were taken with a Canon A510 photo camera and using a MacroVue UV-VIS 20 transluminator. The retention factor (Rf) for each obtained spot was calculated.

RESULTS AND DISCUSSION

The isoquinolines are one of the largest groups of alkaloids. The isoquinoline skeleton is a basic building block of various types of alkaloids including benzyl-isoquinolines, protopines, benzophenanthridines, protoberberines and many others (Grycová *et al.*, 2007). To this category belong the alkaloids from the composition of greater celandine. These types of compounds are generally extracted into alcoholic mixtures (ethanol or methanol) and quaternary and nonquaternary alkaloids are subsequently isolated by acidification and basification of the alcoholic residue (Bugatti *et al.*, 1991).

This study focuses on the investigation of different extraction conditions in order to obtain a reproducible optimum technique for the separation of a limited number of alkaloids for future *in vitro* anti cancer determinations.

For the first chromatographic determination, we used as mobile phase a mixture of chloroform, methanol and water (26:14:3 *v/v/v*). Figure 1 shows the chromatographic profile of the ether (A), ethanol (B) and acetone (C) phase obtained in the first experiment. We observe that the lipid extraction using ethylic ether not only eliminates lipids and flavones, but small quantity of at least five alkaloids to. The obtained spots are weak green (Rf = 0,42), brown (Rf = 0,67), green (Rf = 0,75), orange-brown (Rf = 0,88) and yellow-orange (Rf = 0,96). The ethanol extract contains at least eight alkaloids. The spots appear as follows: light blue (Rf = 0,06), grey (Rf = 0,21), dark blue (Rf = 0,28), weak green (Rf = 0,48), brown (Rf = 0,67), bright green (Rf = 0,74), dark green (Rf = 0,86) and yellow-orange (Rf = 0,96). For the same vegetal material finally mixed with acetone and water (9:1 *v/v*), we have obtained two spots: brown (Rf = 0,67) and yellow-orange (Rf = 0,96). Analyzing the colors of the spots and the Rf values, we can conclude that at least two alkaloids are present in all three extraction steps. The appearance of the orange-

brown spot only in the ether phase may suggest the elimination of one alkaloid in the first step of extraction. The bright green compound from the ethanol phase was partially eliminated in the ether phase and entirely in the ethanol phase. Because of the extraction of a limited number of compounds in the acetone phase, we consider this extract an important object of future investigations.

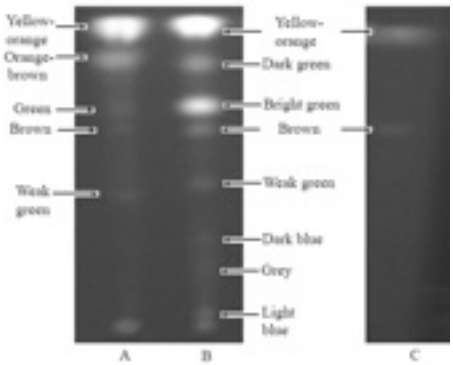

Fig. 1. TLC - profile of the ether (A), ethanol (B) and acetone extracts

For the second determination we used as mobile phase also a mixture of chloroform, methanol and water (26:14:3 *v/v/v*). The analyzed samples consist in the water and chloroform phases obtained in the first extraction experiment (fig. 2). The TLC-profile for the first chloroform phase (pH 1) presents eight spots: blue (Rf = 0,11), blue (Rf = 0,21), dark blue (Rf = 0,43), blue (0,56), orange-brown (Rf = 0,67), bright green (f = 0,73), dark green (Rf = 0,83) and yellow-orange (Rf = 0,93). The TLC-profile for the second chloroform phase (pH 1) shows a total number of five spots: grey (Rf = 0,46), orange-brown (Rf = 0,64), bright green (Rf = 0,72), dark green (0,83) and yellow-orange (Rf = 0,93). The TLC-profile for the water phase (pH 1) contains three spots: bright green (Rf = 0,73), yellow-green (Rf = 0,83) and yellow-orange (0,93). The TLC-profile for the first chloroform phase (pH 8,5) presents seven spots: blue (Rf = 0,08), dark blue (Rf = 0,33), grey (Rf = 0,48), orange-brown (Rf = 0,64), bright green (Rf = 0,72), weak green (Rf = 0,83) and yellow-orange (Rf = 0,93). The TLC-profile for the second chloroform phase (pH 8,5) reveals three spots: grey (Rf = 0,48), orange-brown (Rf = 0,64) and bright green (Rf = 0,72). The TLC-profile for the water phase (pH 8,5) contains three spots: bright green (Rf = 0,72), dark green (Rf = 0,83) and yellow-orange (Rf = 0,93). We observe the presence of the last two compounds or mixture of compounds in all the samples except in the second chloroform phase corresponding to pH 8,5. The bright green compound is extracted using all the techniques in bigger or smaller quantities. The grey compound is extracted only in the second chloroform phase with pH 1 and in both chloroform phases with pH 8,5. We consider this compound a trace of the blue substance previously separated. The blue spots can be observed only in the first chloroform phases (both pH values), which may suggest a complete elimination after the first step of extraction. Because of the extraction of a limited number of compounds in the biggest quantity in the water phases, we consider these extracts an important object of future investigations.

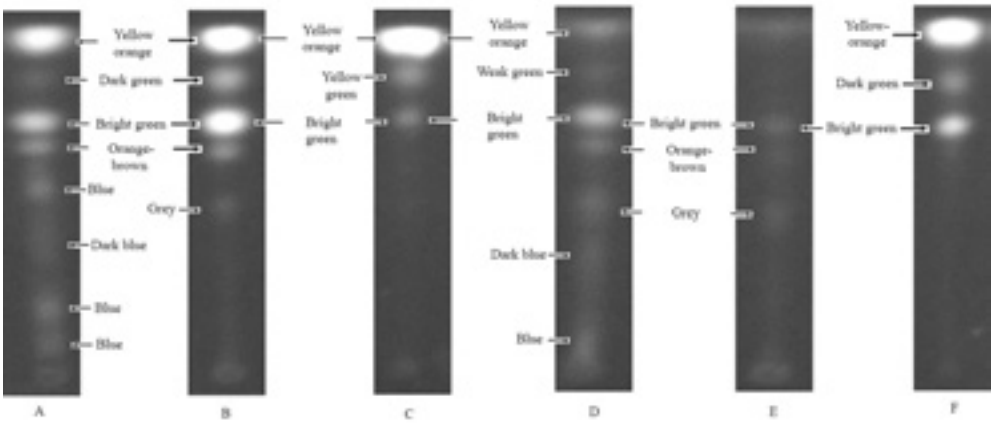

Fig. 2. TLC – profile for the chloroform and water phases: A. 1st chloroform phase pH 1, B. 2nd chloroform phase pH 1, C. water phase pH 1, D. 1st chloroform phase pH 8,5, E. 2nd chloroform phase pH 8,5, F. water phase pH 8,5

Using the same mobile phase, we analyzed the precipitate that appear in the pH 8,5 sample (fig. 3). We observe the apparition of three spots as follows: bright green (Rf = 0,75), dark green (Rf = 0,85) and orange-brown (Rf = 0,98). The retention factor values and the colors of the spots may suggest the presence of the same compounds as in the water phases previously analyzed.

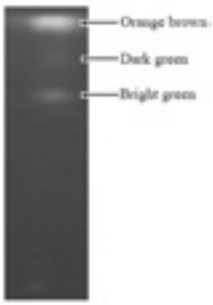

Fig. 3. TLC - profile of the precipitate (pH 8,5 sample) mixed with 2,5 ml of 70% ethanol

Using the same mobile phase, we analyzed the result of treating the water phase (pH 8,5) with methylene chloride. The TLC-profile of obtained methylene chloride and water phases is illustrated in figure 4. The profile of the methylene chloride phase contain three spots: bright green (Rf = 0,77), dark green (Rf = 0,88) and yellow-orange (Rf = 0,96). The water phase contains the same compounds (the same colors and Rf values) but also a brown compound with 0,67 as Rf value. This may indicate a better separation of the alkaloids in the water phase after the methylene chloride treatment.

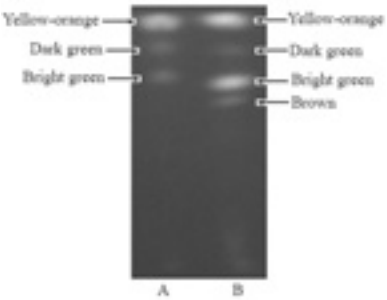

Fig. 4. Water phase (pH 8,5) treated with methylene chloride
A. TLC – profile for the methylene chloride phase; B. TLC – profile for the water phase

Treating the methylene chloride phase previously obtained with an aqueous solution of citric acid, we obtained a very similar TLC-profile, but with the compounds in less important concentration (fig. 5).

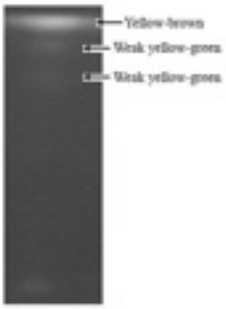

Fig. 5. TLC – profile of the citric acid phase obtained from the methylene chloride phase

For the second experiment the sediment prelevated in ethanol was analyzed, using the same chromatographic conditions. The result may be observed in figure 6. The apparition of a single spot (Rf = 0,95) may suggest the separation of a unique alkaloid or a family of alkaloids with similar structure.

Fig. 6. TLC – profile of the ethanol sediment

The next step was the analysis of the methylenic and water phase supernatant from the same primary extract but using both the aerial and underground parts of the plant. The mobile phase was represented by a mixture of 1-propanol, water and formic acid (90:9:1 *v/v/v*) (fig. 7). In both cases, we observe the apparition of four spots: blue (Rf = 0,064), yellow-orange (Rf = 0,13), green (Rf = 0,18) and blue (Rf = 0,3, respectively 0,26).

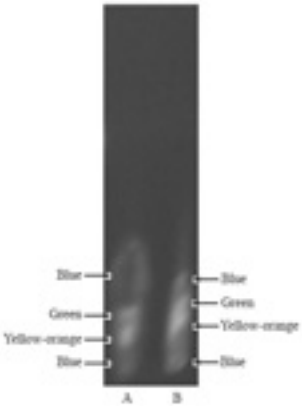

Fig. 7. TLC-profile for the methylene chloride phase (A) and water phase supernatant (B) from the aerial and underground parts

Using a mobile phase composed of n-propanol, water and formic acid (90:9:1 *v/v/v*), we analyzed a mixture of concentrated acetic acid and the water phase sediment obtained from the entire plant extraction and after the citric acid and sodium chloride treatment. We observe the appearance of five spots: two orange spots (Rf = 0,11, respectively 0,15), a yellow-green spot (Rf = 0,21), a blue spot (Rf = 0,6) and a yellow spot (Rf = 0,69). No traces of chlorophyll were observed (fig. 8).

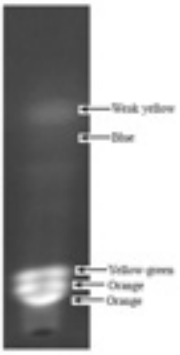

Fig. 8. TLC-profile of the water phase sediment (obtained after the citric acid and sodium chloride treatment)
mixed with concentrated acetic acid

Analyzing the methylene chloride phase obtained in the third experiment, using a mobile phase composed of chloroform, methanol and water (26:14:3 *v/v/v*), we observe the

apparition of four spots: yellow-orange (Rf = 0,72), bright green (Rf = 0,8), dark green (Rf = 0,88) and yellow-orange (Rf = 0,95) (fig. 9). This extract may represent a good starting point for future investigations because of the limited number of compounds and also theirs concentration which is proportional with their color intensity.

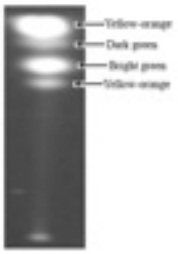

Fig. 9. TLC-profile of the methylenic phase obtained in the third experiment

Finally, the ethanol extracts from the last experiments were analyzed. The mobile phase is composed of n-propanol, water and formic acid (90:9:1 *v/v/v*). Figure 10 shows the profile of supernatant obtained in 70% ethanol, respectively 96% ethanol. The first profile contains six spots and the second one contains seven spots. The first six spots are identical: blue-green (Rf = 0,064), yellow (Rf = 0,14), orange (Rf = 0,29), green (Rf = 0, 45), blue (Rf = 0,58) and yellow-orange (Rf = 0,68). The only difference consists in a larger amount of compounds extracted in 96% ethanol. The seventh spot appeared in figure 10B is represented by chlorophyll (brown-red).

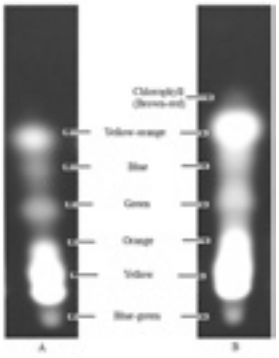

Fig. 10. TLC-profile of the supernatant obtained in the last experiment:
A. in 70% ethanol, B. in 96% ethanol

Figure 11 shows the TLC-profile of the sediment obtained in the last experiment. We obtained eight identical spots: bright green (Rf = 0,11), orange (Rf = 0,15), yellow (Rf = 0,21), green (Rf = 0,43), orange-yellow (0,49), green (Rf = 0,56), orange (Rf = 0,69) and brown-red (Rf = 0,76) represented by chlorophyll. Unlike the supernatant, the biggest concentrations of compounds appear in the sediment derived from the extraction in 70% ethanol. The largest amount of chlorophyll appears in the sediment derived from the extraction in 96% ethanol.

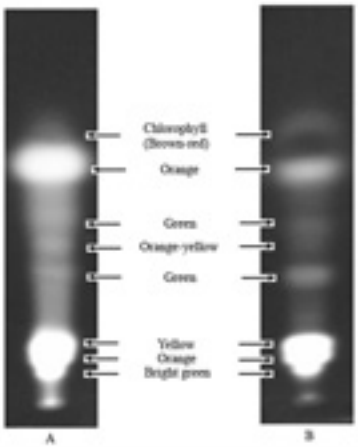

Fig. 11. TLC-profile of the sediment obtained in the last experiment:
A. in 70% ethanol, B. in 96% ethanol

The separation of a big number of compounds using 70 and 96% ethanol suggests that other treatments are needed in order to obtain a more valuable extract for future investigations.

CONCLUSIONS

The ethanol extraction of *Chelidonium majus* L. alkaloids leads to a very complex compounds mixture. Future treatments are needed in order to obtain an extract with a limited and more quantifiable number of alkaloids, suitable to develop *in vitro* anti cancer investigation techniques.

More appropriate extracts were obtained after a big number of extraction steps including ethylic ether, ethanol, chloroform and methylene chloride treatments. The number of spots is limited to three or four that may represent single compounds or closely related compounds families.

The previously specified extracts will represent the object of future investigations using alkaloids standards for an appropriate identification.

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