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QUALITY CONTROL PARAMETERS OF *RIBES NIGRUM* L. BUDS FOR ESTABLISHING THE OPTIMAL HARVESTING PERIOD

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ABSTRACT. The main extract used by the youngest branch of phytotherapy, named meristemotherapy, is obtained from the freshly processed Blackcurrant buds. Due by the lack of information regarding the optimal harvesting time of the buds and buds chemical composition it was begun a larger study, the results presented in this paper being the preliminary screening of the possible active compounds that can be contained by this vegetal material. The apical and axillar buds from *Ribes nigrum* L shrubs were collected in three different development phases. The bioactive compounds composition screening was performed using monodimensional double development TLC on extracts obtained in ethanol, glycerol and their mixture, according to EPh. The analysis results show that the lipophilic compounds are increased in the maximum development stage and are decreasing when the buds begin to open. In the opened stage are increasing the hydrophilic compounds, like the polyphenols that are secondary metabolites of the plants. These changing in chemical composition indicate also the tissues differentiation. In conclusion of this study can decide that the optimal harvesting time of the buds is before the opening and tissues differentiation, period that is according also with the traditional harvesting time of these type of plant materials.

Keywords: *Ribes nigrum L. buds, meristemotherapy, monodimensional double development TLC, optimal chemical composition.*

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INTRODUCTION

The meristemotherapy, or its more used denomination gemmotherapy, was founded as a distinct branch of the phytoptherapy at the begin of the 20th century when a belgian physician, Pol Henry, put the question if the animal stem cells have regenerating power, what are doing the vegetal stem cells. Following this idea begun to use vegetal stem cells extracts, that are the plants meristematic cells, more concentrated in the buds, sprouts, young parts of the plants. Step by step Pol Henry succeeded to put the basis of this new phytotherapy named phytoembriotherapy, than gemmotherapy, but more correctly is meristemotherapy [1,2].

During the time the meristemotherapy was developed at the border of homeopathy and phytotherapy. The benefits upon the people quality of life of the meristematic tissues extracts were observed by the doctors using the observation method of the homeopathy. Many trees and shrubs buds' were used to obtain specific extracts and experimentaly observed their benefit on the people health. From these observation one extract became more popular, mostly for its major beneficial effect. This was the Blackcurrant buds extract used for its cortizon-like antiinflamatory, antiallergenic effects [1-3].

The Blackcurrant, *Ribes nigrum* L., is a commonly cultivated species in Romania and all around world. There are extended cultures in Russia, Europe and America. Its buds are harvested at the begin of the springtime, processed in fresh state, without drying, using a mixture of ethanol and glycerol according to the method described in French Pharmacopoeia from 1965 and now also in European Pharmacopoeia [4,5].

Recent studies identified in the Blackcurrant buds a lot of polyphenols: proanthocyanidines, like prodelphinidines dimers and trimers [6]; flavonols, like: quercetine, kaempferol and their derivatives [7,8]; phenolic acids, like p-coumaric acid, chlorogenic acid and neo-chlorogenic acid [7,8]. Beside these in the buds were identified the vitamin C and also some important aminoacids: arginine, proline, glycine and alanine [9]. The essential oil obtained from Blackcurrant buds had as main compounds sabinene and delta-3-carene [10].

The clinical observations shown that the Blackcurrant buds extract stimulates the adrenal glands, having a cortison-like antiinflammatory effect, but without the cortisons side effects. The proanthocyanidines contained by buds decrease the pro-inflammatory chemokines [11,12]. Some *in vitro* studies show these proanthocyanidines selectivity for COX-2, enzyme implicated in the inflammatory response and they have no negative effect on the colagen synthesis [13]. The essential oil of the buds has antimicrobial and also antifungal effect [10,14].

As can be seen, both the hydrophilic and lipophilic compounds of Blackcurrant buds, have important therapeutical effects, being important to be in highest possible concentration in the harvested buds and then in the obtained extracts. Because the classical phytotherapy does not use the buds, as usual plant material, there are no special indications regarding the optimal harvesting period. The aim of this paper is to establish the optimal harvesting period of the Blackcurrant buds through a TLC screening of both, hydrophilic and lipohilic compounds profile respectively the biometric determinations.

RESULTS AND DISCUSSION

There were established three development periods for harvesting the Blackcurrant buds: sleeping buds, developed buds and opened buds. Through this three development periods the meristematic tissues are changing and begin the differentiation, more intensively in the opened buds. This process will be reflected also in the change of chemical composition.

This study compare not just the chemical composition of the buds from different development phases, but also the freshly respectively dry processed vegetal material, respectively the influence of used solvent on the bioactive compounds profile.

In the figures 1-3 are presented the chromatograms obtained for different buds extracts.

To can compare the bioactive compounds profile all extracts were concentrated by solid phase extraction. This sample preparation was used also to can eliminate the glycerol, solvent that can prevent the proper TLC separation.

The used TLC mobile phase system separate in the lower part of the chromatogram the hydrophilic compounds, mostly the polyphenols like flavonoids and phenolic acids, eventually coumarins and in upper part the lipophilic compounds, like the terpenic compounds.

It can be observed a similar compound profile, but the concentrations are different. The hydrophilic compounds are better extracted in 70 % vol. ethanol or glycerol. The more lipophilic compounds are better extracted in the mixture of ethanol-glycerol, solvent used also by the standardized pharmacopoeial method.

The extraction process depends from the solvents polarity and viscosity, from the drying stage of the cell membranes, all these influencing the mass transfer trough the lipophylic cell membranes. It can be seen on the TLC chromatograms that the glycerol, a more viscous and more lipophylic

solvent, will extract less compounds from dry buds due by the lack of moisture at the cell membranes level and for this reason will penetrate less the cell membranes. The ethanol, a less viscous and more hydrophylic solvent, will extract more compounds due by its higher penetration potential trough cell membranes. The ethanol-glycerol mixture will benefit both by the ethanol less viscosity and hydrophilicity, but also by the lipophilicity of the glycerol, that will more easier penetrate the cell membranes due by the mixture's less viscosity.

Generally, the freshly processed buds will lead to an extract more concentrated in active compounds in comparison with the dry buds extracts. This is a prove of negative effect of the drying process upon the bioactive compounds profile of the plants.

There can be observed also a difference between the compounds profile from a development stage to other. If the polyphenols content appear to be similar, being present the almost in all cases the same bands, in case of the lipophilic compounds are more differences. The lipophilic compound number increases from 3 in sleeping stage to 6 in developed stage and decreases at the tissues differentiation to 2-3 compounds in opened stage.

The polyphenols are more concentrated, showing more intensive bands, in the extracts obtained from opened buds, these compounds being specifically to the mature plant parts.

In table 1 are presented the biometrical data recorded for 25 buds at each stages. These data will help to give also a numerical parameter for quality control of harvesting period of the Blackcurrant buds.

Table 1. Biometrical determinations

Samples	Minimal size, mm	Maximal size, mm
Sleeping buds	18.4	67.0
Developed buds	53.5	138.6
Opened buds	364.1	462.7

If we correlate the TLC screening and the biometrical data we can conclude that the optimal harvesting time is at developed stage when the buds size not exceed 140 mm in hight and the minimal size is 50 mm.

Due by the fact that we can not find studies with similar aims, our result can not be compared with literature data. But these preliminary results encourage us to continue the study for a complex profiling of the bioactive compounds from Blackcurrant buds extract, results that later can offer us the possibility to made correlations with clinical observations, therapeutic effects or pharmacological studies results.

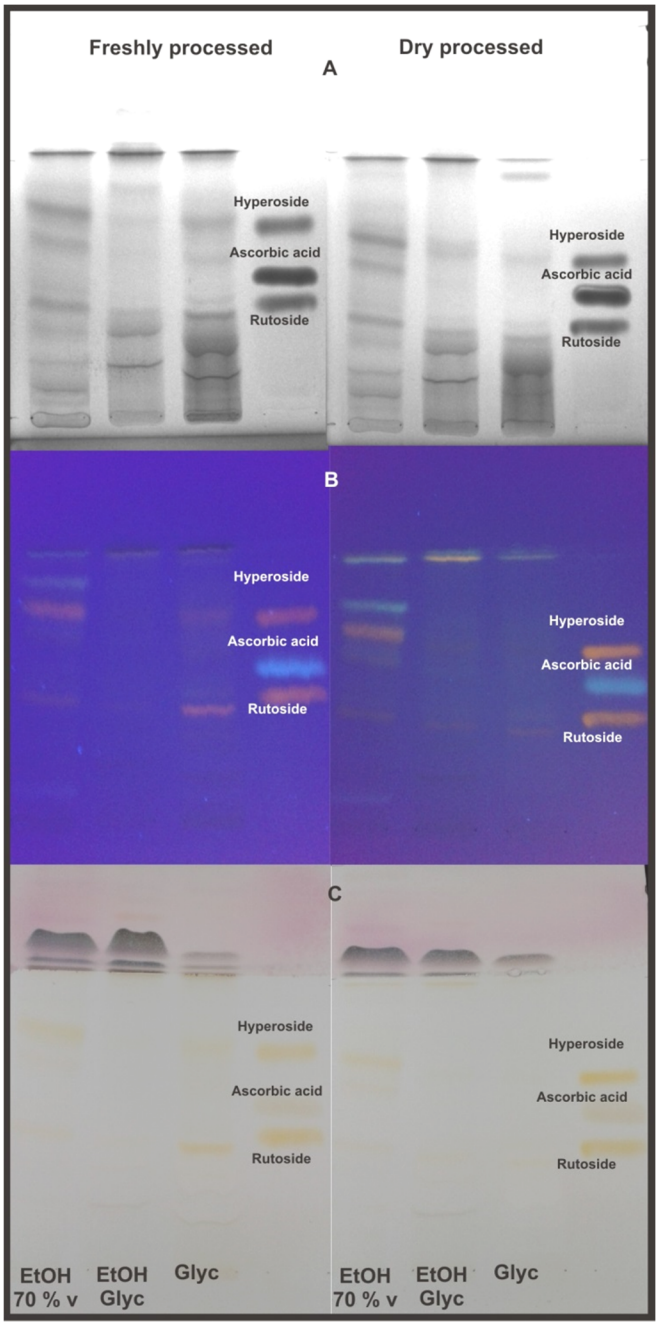

Figure 1. TLC chromatogram of the extracts obtianed from sleeping buds (A = in UV light at 254 nm, B = fluorescence at 365 nm, C = Visible light)

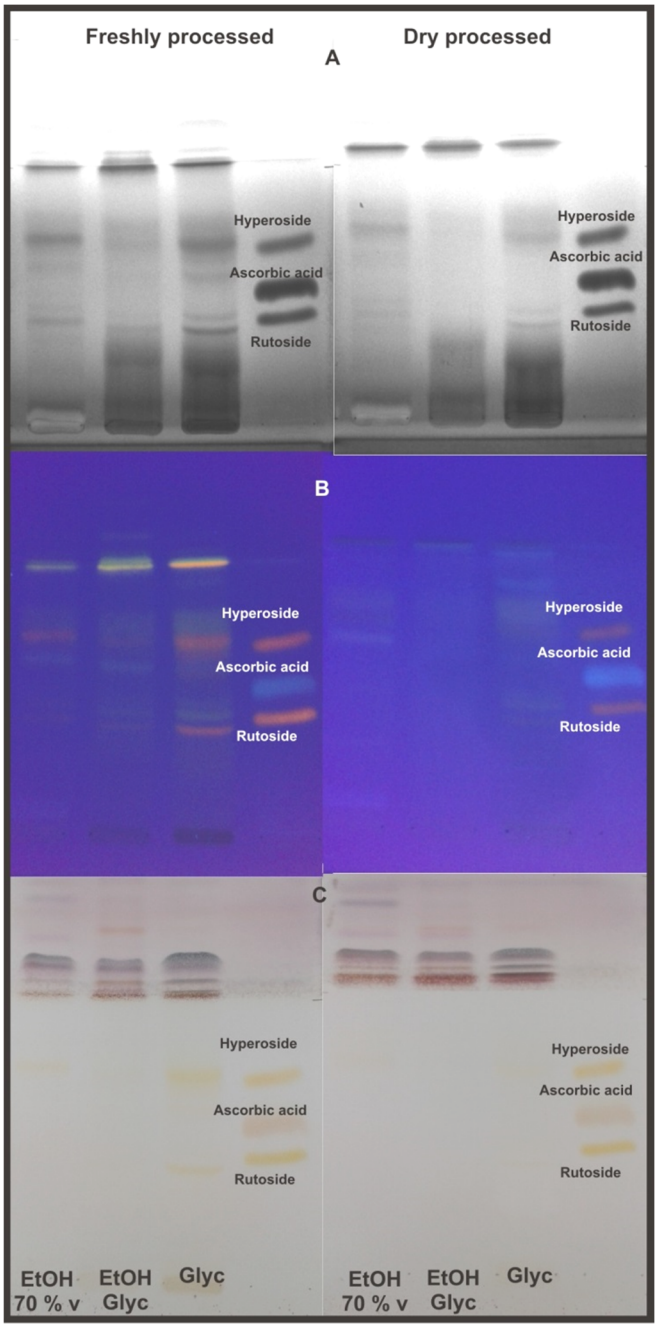

Figure 2. TLC chromatogram of the extracts obtianed from developed buds (A = in UV light at 254 nm, B = fluorescence at 365 nm, C = Visible light)

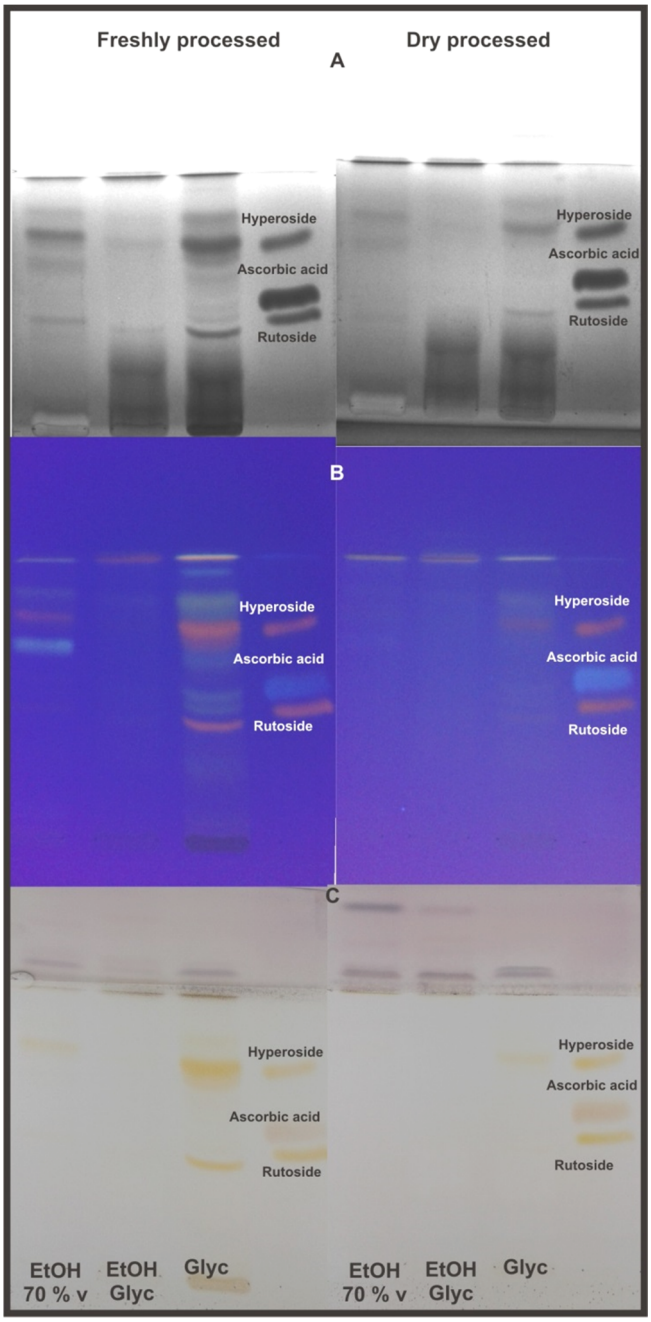

Figure 3. TLC chromatogram of the extracts obtianed from opened buds (A = in UV light at 254 nm, B = fluorescence at 365 nm, C = Visible light)

CONCLUSIONS

Due by the fact that the meristemotherapy and phytochemical study of the buds and its extracts are appeared recently in the researchers interes, this study is the first that wish to establish parameters for a better control of the plant material harvesting in idea to obtain qualitatively the best possible product.

To have a complex mixture of bioactive compounds the optimal harvesting time of the Blackcurrant buds is at developed stage, at the begin of springtime, before the opening and intensive differentiation of the tissues, at a hight of the buds from 50 to 140 mm.

The fresh processing and the using of ethanol-glycerol mixture for extraction will assure the obtaining of Blackcurrant buds extract with established high quality, efficacy and safety.

EXPERIMENTAL SECTION

Harvesting of plant materials

The Blackcurrant buds were harvested from an organic culture established near Cluj, Romania. The organic state of the culture is certified by EcoInspect Ro-008.

The sleeping buds were collected in January 2016, the developed buds in February 2016 and the opened buds at the begin of March 2016. The harvesting was performed at dry weather, after 10 a.m.

Preparation of the extracts

The ethanolic respectively the glycerolic extracts were obtained from fresh buds respectively dry buds using an extraction ratio of dry part of plant – solvent = 1 :10. As solvents were used the 70 % vol. ethanol respectively 100 % glycerol. The extraction ratio used for fresh buds extraction was computed taking in account the humidity of the fresh vegetal material.

The extracts with solvent mixture were obtained from fresh buds respectively dry buds using an extraction ratio of dry part of plant – solvent = 1 :20. As solvent is used a mixture of ethanol – glycerol 1 :1. The vegetal material – solvent ratio respectively the solvent mixture are according to the European Pharmacopoeia monograph from the special chapter of homeopathic preparations.

All extracts were obtained by cold extraction, being maintained at maceration for 20 days, with daily mixing 2 x 10 minutes. The extracts were decanted and the plant materials with the remained solvent were pressed. The extracts were maintained 5 days for stabilization.

Reagents and materials

HPLC grade methanol, ethyl acetate, ethyl-methyl ketone, formic acid, toluene and analytical grade chlorhydric acid were purchased from Merck (Darmstadt, Germany).

The ethanol and glycerol were of pharmaceutical grade, purchased from Coman Prod (Ilfov, Romania) respectively Glacon Chemie (Germany).

Rutoside and hyperoside were purchased from Roth (Karlsruhe, Germany), ascorbic acid from Merck (Darmstadt, Germany). Standard solution (1 mg/ml) of these compounds was prepared in methanol.

The reagent used for preparing the visualising reagents: Neu-PEG and anisaldehyde, respectively the TLC Sil G F254 (20x20 cm) plates and SPE Sil-C18 cartriges were purchased from Merck (Darmstadt, Germany).

The Neu-PEG reagent is composed from two different solutions, used concomitant: 1 % methanolic solution of diphenyl-borate aminoethanol respectively 5 % ethanolic solution of 4000 polyethyleneglycol.

The anisaldehyde reagent was prepared by mixing 0.5 ml of anisaldehyde with 4.5 ml of concentrated sulphuric acid, 10 ml of glacial acetic acid and 85 ml of methanol.

SPE sample preparation

Each sample was extracted on SPE cartridges with silicagel-C18, using methanol, acidulated at pH = 3,5, for elution. Before each extraction the cartridges were activated by flushing with 5 ml methanol and 5 ml water. The SPE 3 ml from each sample was concentrated to 1 ml by this solid phase extraction [15]. By this extraction was eliminated the glycerol that can negatively influence the TLC separation. The sample preparation was applied to all extracts to can compare the compounds profile.

The TLC analysis

TLC analysis was performed using 13 x 20 cm silica TLC plates with fluorescence indicator at 254 nm. The samples were applied as bands (20 mm) at 15 mm from lower edge of the plate. From ethanolic and glycerolic extracts were applied 20 μl, from solvent mixture extracts were applied 40 μl, to can compare also the bands intensity as a visual semi-quantitative evaluation. Hyperoside, ascorbic acid and rutoside bands were also applied as referenced compounds. A double development was performed to achieve a better separation between hydrophilic and lipophilic compounds. First development was performed on 10 cm with ethyl acetate – ethyl-methyl ketone – formic acid – purified water (50:30:10:10, v/v) as mobile phase. After plate was dry a second development was performed in the same

direction with a less polar mobile phase: toluene – ethyl acetate (95:5, v/v) on 15 cm. Hydrophilic compounds were visualized by spraying the first part of the plate (10 cm) with Neu-PEG reagent. The second part (10-15 cm), the zone associated with lipophilic compounds, was sprayed with anisaldehyde reagent and then heated at 105-110°C for 5-10 minutes. The plates were observed in visible light to have a general view (hydrophilic and lipophilic compounds) and under UV light (254 and 365 nm) for emphasize the similarities and differences between the extracts, especially for polyphenolic compounds. For documentation the plate image was captured using a Camag Reprostar cabinet equipped with a HP digital camera.

The biometrical analysis

At each harvesting were sampled 25 statistically representative buds and the longitudinal sizes were measured using a micrometer. Each bud was measured individually.

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