Development of Single-step Protocols for the Separation of Naphtodianthrones from St. John s Wort Extracts

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St. John's Wort (SJW) or Hypericum perforatum L. is a therapeutic plant highly used in pharmacology. Recent in vivo anti-cancer action of naphtodianthrones (NTs) has extended the research related to enrichment methodologies of SJW phyto-extracts. Therefore, the presented study pursuits the optimization of single-step extraction methodologies to obtain NTs-rich extracts from SJW.

Keywords: St. John s Wort, Naphtodianthrone separation, Single-step extraction

The first studies regarding SJW-originated naphtodianthrones (hypericin and its more water-soluble analogue pseudohypericin [1]), appeared in the literature about 20 years ago and depicted the inhibitor action of NTs for a wide spectrum of viruses [2, 3]. Later on, authors reported about naphtodianthrones (NTs) activity *in vivo* against retrovirus infection (Antibacterial action referred to Gram positive and Gram negative bacteria: *Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus aureus* or *Streptococcus* mutans [4, 5]). Other experimental data confirm that NTs inhibit retroviral infections, through unknown mechanisms and thereby appears as a possible explanation of their benefit in AIDS diseases [6]. It seems, however, that involves the prevention of infection or the virus inactivation process, by tying or attaching to the cell membranes. Photodynamic properties of hypericin have also prove to be of help in cancer treatment, inhibiting the growth of cells derived from a variety of neoplastic tissues, including glioma, neuroblastoma, adenoma, mesothelioma, melanoma, carcinoma, sarcoma, and leukemia [7].

NTs extracts used today in therapy are obtained through extraction of biomass with ethylic alcohol/water and standardized in terms of hypericin equivalents [8]. Unfortunately, the low bioavailability of these NTs resulted from SJW (0.06-0.4 wt.% [9]), makes the extraction process to complicated (requiring multiple cycles and quick handling of the plant material as the naphtodianthrones are light sensible substances) and very expensive [10-12]. Most reported procedures for NTs extraction involve the use of water, methanol/ethanol coupled with stirring, ultrasonication, Soxhlet or other modern techniques like high performance liquid chromatography and size exclusion chromatography [12, 13] to obtain various formulas of liquid extracts or capsules.

In this context, this paper evaluates the possibility of applying single-step extraction procedures for SJW phyto-extracts enrichment in NTs [14]. Subsequently, the most convenient extraction method that delivered the highest content of NTs is optimized in terms of plant/solvent ratios, temperature ranges and extraction time. The proposed separation protocols are simple, rapid and delivers NTs-rich extracts in a single step.

Experimental part

Materials and methods

The plant material (SJW aerial part that included the flowers, the leaves and the stem) was collected in spring (P1 batch) and autumn (P2 batch). Extraction solvents: ethylic alcohol 96% (EtOH) (Chimopar), purified water (W). Developer solvents: ethyl acetate p.a, formic acid p.a., acetic acid p.a. (Chimopar). HPTLC Materials: HPTLC silicagel plates (G60F254) from Merck KGaA (200x100 cm; 100x100 cm), cellulose Merck (200x100 cm); reference compounds: rutine, hyperoside, caffeic acid, hypericin, and chlorogenic acid (Fluka Chemie or Sigma-Aldrich); identification reagents (derivatisation): β-ethylamine biphenyl borate (NP), polyethylene glycol 400 (PEG) (Merck KGaA), 2,2–diphenyl-1-picrylhidrazyl (DPPH) (Sigma-Aldrich Co.) and ninhydrine (Merck KGaA).

Instruments and protocols

High Performance Thin Layer Chromatography (HPTLC) profiles were recorded and interpreted accordingly. Apparatus: CAMAG System with an LINOMAT IV band-type applicator, CANON digital camera and a CAMAG data base. Another digital photo camera with 16 mm lens, CAMAG-REPROSTAR III- DIGISTORE II + WinCATS Planar Chromatography Manager Software having Image Comparison Viewer permitted visualization of multiple samples and the retention times of specific compounds compared with adequate references, at a high resolution. Conditions: *Developer* ethyl acetate: formic acid: acetic acid: water = 20 : 2.2 : 2.2 : 5.4 v/v. *Reagents:* (NP) (0.1 g in 10 mL methanol) and PEG 400 (0.5 g in 10 mL methanol) for compounds identification and DPPH (0.02 g in 10 mL methanol) for antioxidant activity. *Reference:* rutine, chlorogenic acid, caffeic acid, as 0.01% in methanol, and hyperoside and hypericin as 0.02% in methanol. *Application parameters*: plate width 100 mm; volume of syringe -100 μL; D2&W lamp. Examination of plates at 254 and at 366 nm is performed before and after spraying the reagents homogenously (process called derivatisation), followed by air drying.

HPTLC Screening or densitometry consists of measuring and quantifying the HPTLC results. All measurements are relative, meaning that the properties of known substance

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are compared with those of unknown substance. Yet, using standards, the system can be calibrated. The essential condition is that both standards and unknown substances to be eluted together on the same plate. It is also used for identification, by comparing the profile curves with individual tracks. UV spectrum measurement is made directly on the chromatographic plate. The characteristic track of hypericin standard by scanning in the appropriate wavelength range is identified. The maximum adsorption of the hypericin standard and its corresponding spot in the samples is further determined. Overlapping absorption curves indicate the presence of the hypericin in the analysed samples and its content is quantified after scanning at 295 nm.

SJW hot extracts obtained by batch reflux extraction procedures

(1)Hydro-alcoholic and Alcoholic Extracts. Studied extraction parameters: extraction solvent ethanol/water 50, 60, 70, 80, 96% v/v; plant/solvent = 1/15; extraction time = 90 min; extraction temperature = 55-60°C. Extraction was carried out at reflux on the heated water bath. The following extracts were obtained: *AE 50%-R (P1, P2), EA 60%-R (P1, P2), EA 70%-R (P1, P2), EA 80%-R (P1, P2), EA 96%-R (P1, P2).*

(2)Hydro-Glycero-alcoholic Extracts. Wetting parameters: humectants = ethanol 96% v/v; plant/solvent = 1/2; wetting time = 24 h; wetting temperature = 20-22°C; stirring periodically in the absence of light. Extraction parameters: extraction solvent water/ethanol (96%)/ glycerine = 1: 2: 1 (vol.%); extraction time = 2 h; extraction temperature = 60°C. Extraction was carried out at reflux on the heated water bath. The following extracts were obtained: *G (P1), G (P2).*

SJW cold extracts obtained by room temperature extraction procedures

(1) Hydro-alcoholic and Alcoholic Extracts. Extraction parameters: The extraction solvent ethanol/water 50%, 60%, 70%, 80%, 96% v/v; plant/solvent = 1/10; extraction time = 10 days; 20-22°C temperature, stirring periodically in the absence of light. The following extracts were obtained: *AE 50%-C (P1, P2), AE 60%-C (P1, P2), AE 70%-C (P1, P2), AE 80%-C (P1, P2) AE 96%-C (P1, P2).*

Optimization of the single-step extraction procedure for naphtodianthrone enrichment

Onwards, particular focus on NT enrichment of primary extract has led to optimizing the main parameters of the single-step extraction protocol. In this respect, the plant/ solvent ratio (DER), extraction temperature and extraction time were varied. Extraction was carried out at reflux on water baths. The obtained solutions were filtered on a fabric filter and the content was analyzed quantitatively for soluble substances.

(1) Optimal plant/solvent ratio (DER). Extraction parameters: extraction solvent ethanol/water 70% v/v; plant/solvent = 1/5, 1/10, 1/15, 1/20, 1/25; extraction time = 2 h; extraction temperature 55-60°C.

(2) Optimal extraction temperature. Extraction parameters: extraction solvent ethanol/water 70% v/v; plant/solvent = 1/15; extraction time = 2 h; extraction temperatures: 20-22, 30, 40, 50, 60, 70°C.

(3) Optimal extraction time. Extraction parameters: extraction solvent ethanol/water 70% v/v; plant / solvent = 1/15; extraction temperature: 55-60°C; extraction time: 60, 90, 120, 150, 180 min.

Quantitative determination of active substances

Determination of extractible substances ES, of flavones (as rutoside), of polyphenolcarboxylic acids (as caffeic acid) and of naphtodianthrones as hypericin were performed according to protocols described in a previous paper [15] and calculated using equations [1-3] and [4], respectively.

$$ES\ (\%) = \frac{m2 - m1}{m2} \cdot 100 \quad (1)$$

where *ES %*= soluble substance percent, *m1* and *m2* (g) the weight of resulted residue and that of the crude plant product, respectively

$$\text{Total flavones (in rutoside equivalents)} = \frac{Ap\ V\ f}{B} \cdot 100 \quad (2)$$

where *Ap*= sample absorbance (a.u.); *f*= calibration factor (0.850x 10^{-3} g/a.u.); *V*=volume of solution A (ml); *B*= volume of solution used for the colorimetric reaction (mL).

$$\text{Total Polyphenolcarboxylic acids (coffeic acid equivalents)} = \frac{Ap \cdot V \cdot f}{B} \cdot 100 \quad (3)$$

where *Ap*= sample absorbance (a.u.); *f*= calibration factor (0.8789x 10^{-3} g/a.u.); *V*=volume of solution A (mL); *B*= volume of solution used for the colorimetric reaction (mL).

$$\text{Total naphtodianthrone content in plant (as hypericin equivalents) \%} = \frac{A \cdot V}{a\ 870\ S} \cdot 100 \quad (4)$$

where *Ap*= sample absorbance (a.u.); 870= absorbance of 1 mg/mL hypericin standard; *V*=total volume of solution (mL); *a*= the content of plant material (g); *S*= percentage of dry content in the plant material (100- U/100); *U*=humidity of plant material.

Results and discussions

High Performance Thin Layer Chromatography (HPTLC) is an effective technique to analyze flavones, but the choice of reagents is extremely important [16]. In this respect, identification was accomplished in two ways: a) with no chemical treatment- in ultraviolet light at 254 nm, and b) with chemical treatment (derivatisation) of plates. Using identification reagents, an intense fluorescence appears at 366 nm, which is specific for each structure. By comparison, the extract constituents were identified at 254 nm and at 366 nm after derivatisation (fig. 1and fig. 2). Intense fluorescence of specific sports in the HPTLC chromatograms proved that all the hydro-alcoholic extracts with ethanol/water 70% v/v from plant batch P2, meaning AE 70%, AE 70%-R and AE 70%-C contained the highest amounts of NTs .

HPTLC screening of antioxidant properties that uses free radical 2,2-diphenyl-1-picrylhidrazyl (DPPH) as an identification reagent . After applying this technique, the characteristic spots identified previously for flavones and polyphenolcarboxylic acids faded after splashing with this DPPH methanol solution, which indicated strong antioxidant activity of the active compounds (Figure 3a and b). It can be mentioned that the most intense scavenging capacity for DPPH free radicals was observed for the hydro-alcoholic extracts 50, 60, 70% v/v, and the weakest for the alcoholic extract 96% v/v. It is also noteworthy the fact that the extracts prepared at room temperature were more active than the ones prepared at 55-60°C.

HPTLC digital scan of chromatoplates allowed further identifying the flavones, the polyphenolcarboxylic acids and the naphtodianthrones. Reference substances were used to underline the specific retention times, *Rf*: 0.45 - 0.46

(rutine), 0.55-0.56 (chlorogenic acid), 0.67-0.68 (hyperoside), 0.96-0.97 (caffeic acid), 0.93-0.94 (hypericin). Figure 4 and figure 5, present the 3D HPTLC diagrams coupled with digital scanning profiles of analyzed samples. Every fraction of a track is represented by a specific peak with a defined *Rf* value, height and area. Comparative analysis of chromatoplates identifying the main classes of active principles - Flv (rutoside, hyperoside), PPC (caffeic acid, chlorogenic acid), and NTs (hypericin) - revealed that the richest are the hot extracts prepared at reflux. If we refer only to NTs, we found that the hydro-alcoholic solvents 50, 60 and 70% v/v ensured a smoother extraction of this bioactive principle and the alcoholic 96% v/v extract had the lowest extraction efficiency.

Regarding the hydro-glycero-alcoholic extracts, it was noticed that the extraction solvent prevented a clear separation of compounds characteristic spots (fig. 2). Therefore, an accurate assessment of chromatograms corresponding to these experimental alternatives was not achievable.

Quantitative analyses of plant product and extracts

SJW plant material evaluation

Determining the loss of moisture and the extractible substances at room temperature from the two plant batches has suggested that batch P2 of SJW was richer in bioactive substances. At the same time, the main classes of active principles relative to extractible substance (ES)

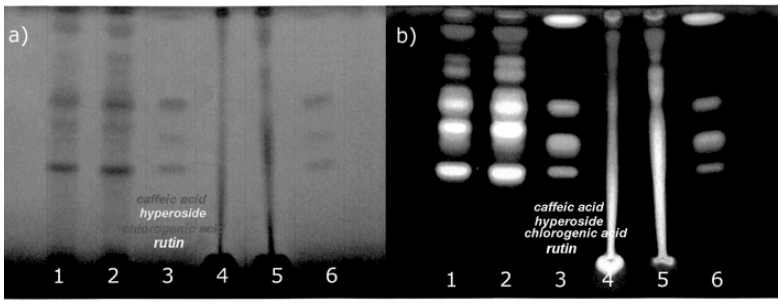

Fig.1. Identification Chromatograms for flavones and polyphenolcarboxylic acids: a) at 254 nm before derivatisation; b) at 366 nm after derivatisation. *Tracks*: 1-AE 70%-R (P1); 2-AE 70%-R (P2); 3- reference substance; 4-AE 50%-R (P1); 5-AE 50%-R (P2).

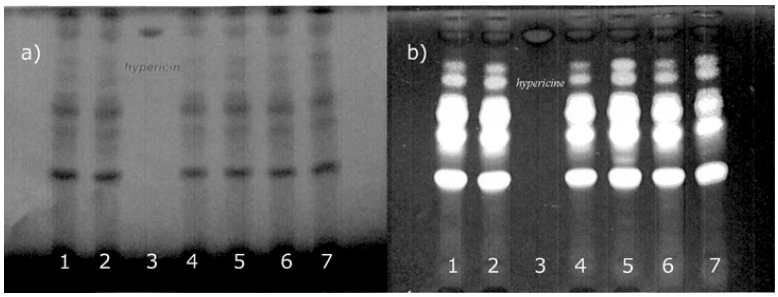

Fig.2. Identification Chromatograms for flavones and polyphenolcarboxylic acids: a) at 254 nm before derivatisation; b) at 366 nm after derivatisation. *Tracks*: 1-AE 70%-C (P1); 2-AE 70%-C (P2); 3- reference substance; 4-G (P1); 5-G (P2); 6-same reference substance as for track 3

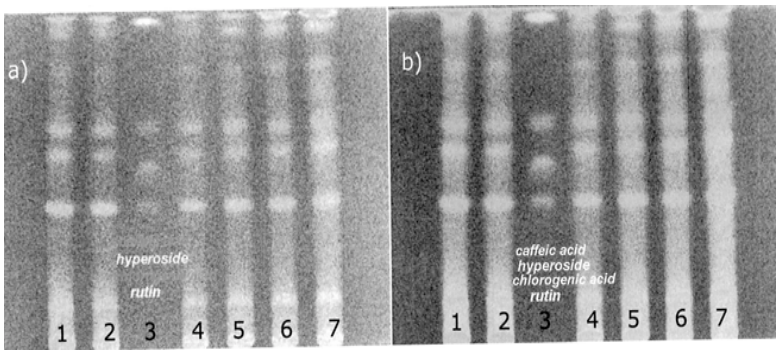

Fig.3 Identification Chromatogram for antioxidant activity: after 72 h with DPPH treatment. *Tracks*: (a) 1-AE 70%-C (P1); 2-AE 70%-C (P2); 3-reference; 4-AE 70%-R (P1); 5-AE 70%-R (P2); 6-AE 50%-R (P1); 7-AE 50%-R (P2); (b) 1-AE 60%-C; 2-AE 80%-C; 3-AE 96%-C; 4- reference; 5-AE 60%-R; 6-AE 80%-R; 7-AE 96%-R

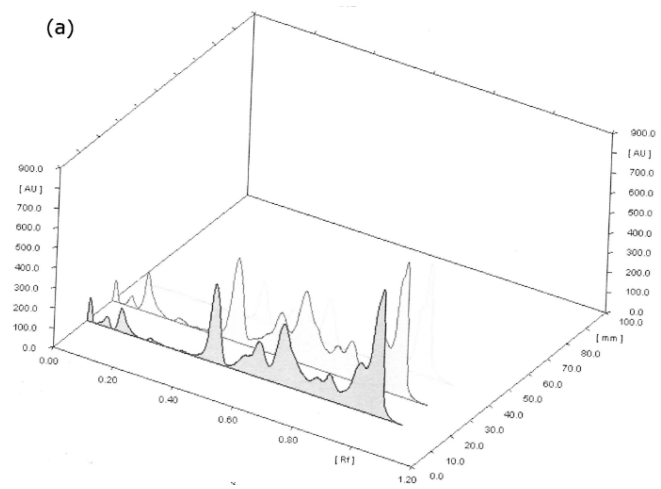

Fig.4. HPTLC 3D profile at 254 nm screening for: (a) rutine/ hyperoside/chlorogenic acid/caffeic acid –plot 3; AE 70%-P1 extract-plot 1 and AE 70%-P2 extracts -plot 2

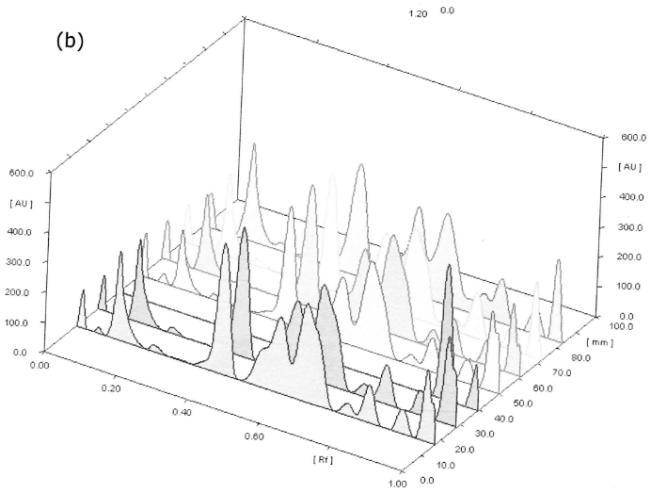

Fig.4. HPTLC 3D profile at 254 nm screening for: (b) hypericin –plot 3; extracts: AE 70% (P1) –plot 1, AE 70% (P2) –plot 2; AE 70% -C (P1) –plot 4, AE 70% -C (P2) –plot 5; AE 70% -R (P1) – plot 6 and AE 70% -R (P2)- plot 7.

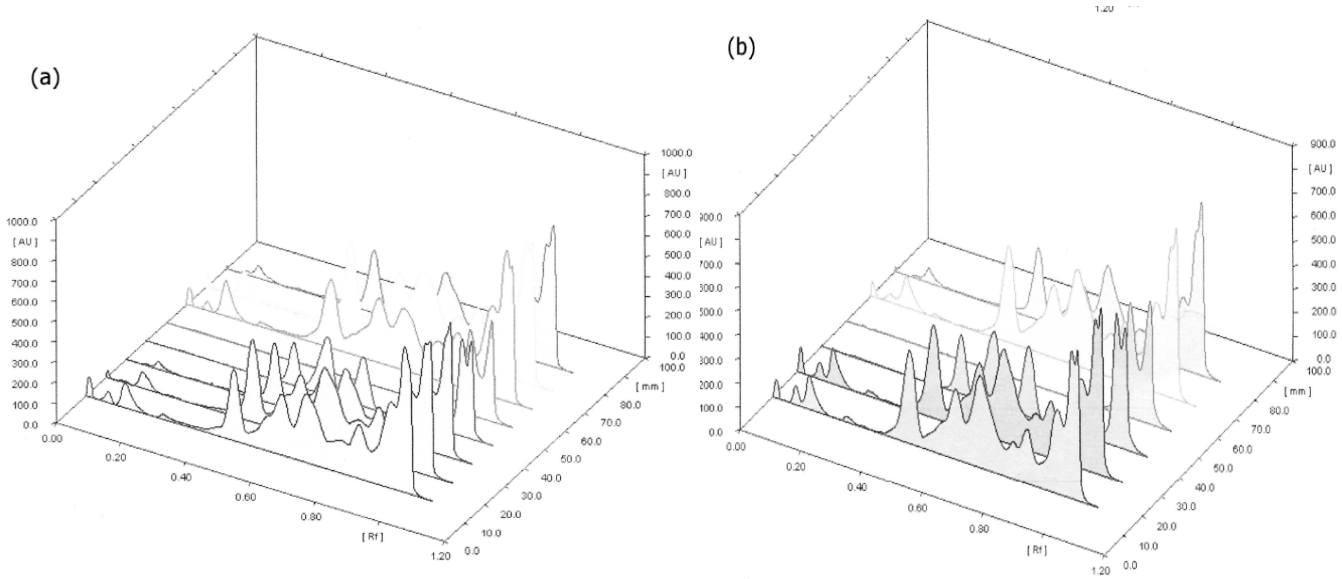

Fig.5 HPTLC 3D profile at 254 nm screening for: (a) rutine/ hyperoside/ caffeic acid / chlorogenic acid –plot 4 and hypericin –plot 5, AE 60%-R (P1)- plot 1, AE 80%-R (P1)- plot 2, AE 96%-R (P1)- plot 3 and AE 60%-R (P2)- plot 6, AE 80% -R (P2)- plot 7, AE 96%-R (P2)- plot 8; (b) rutine/ hyperoside/ caffeic acid/ chlorogenic acid –plot 3 and hypericin – plot 4, AE 50%-C (P2)- plot 1, AE 60% -C (P2)- plot 2, AE 80%-C (P2)- plot 5, AE 96% -C (P2)- plot 6

Table 1
CONTENT OF ACTIVE PRINCIPLES IN SJW PLANT BATCHES DETERMINED ACCORDING TO THE ROMANIAN PHARMACOPOEIA 10TH EDITION [17]

Parameter / Batch	P1	P2
NTs in the ES/ dry plant material (in hypericin) (wt.%)	**0.03/0.006**	**0.04/0.008**
Flv in the ES/ dry plant material (in rutoside) (wt.%)	1.26/0.23	1.62/0.34
PPC in the ES/ dry plant material (in caffeic acid) (wt.%)	1.37/0.25	1.79/0.37

from the dry plant material , i.e. total Flv (expressed in rutoside), PPC (expressed in caffeic acid), and NTs (expressed in hypericin) were found in higher extents in the second batch of plant product, P2; values are listed in table 1.

From this evaluation, we can conclude that harvesting the plants in autumn leads to an increase of bioactive compounds, particularly of NTs with 50%. These values confirm the value of this study for by-passing some successive preparative steps for NTs enrichment/ extraction. Nevertheless, the maximum extent of NTs extracted at room temperature with 70 v/v EtOH/water mixture was limited to 8 mg for 100 g of dry plant material.

Enriched SJW extracts

Since the hydro-alcoholic extract 70% v/v from plant batch P2, meaning AE 70%-R (P2) has proven to be the richest in active compounds, other extraction conditions i.e. DER, extraction temperature and time were further studied only for this particular extract. Figure 6 summarises the results of this study in contents of NTs relative to extractible substances (ES) or dry plant material (S). The optimum extraction efficiency was obtained when using a plant/solvent ratio between 1/10 and 1/15 (fig. 6a). This experimental version delivered a high NTs content (expressed in hypericin) of 0.92 wt.% relative to extractible substances. Comparing to other single-step extraction procedures, like Soxhlet extraction adopted by Anand *et* al. [18] through which they obtained 0.39 wt.% hypericin in the extract, our resulted NTs enrichment protocol reported herein was more successful.

Further on, the temperature range of 50-60°C ensured an elevated content of NTs in the hydro-alcoholic extract 70% v/v of SJW (up to 1.064 wt.% relative to extractible substances) compared with the other experimental trials

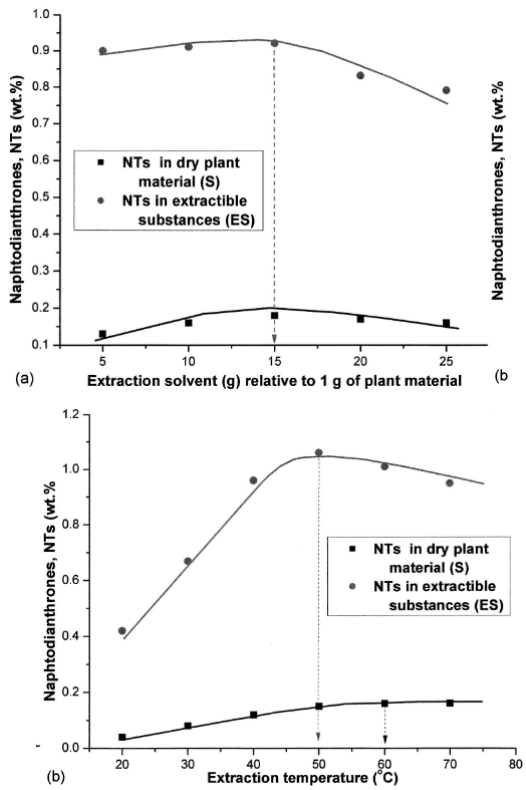

Fig 6. Results of the optimisation study: (a) Variations of NTs content in wt.%, relative to extractible substances (ES) or dry plant material (S), as function of the extraction solvent (EtOH 70% v/v) weight used for 1 g of dry SJW material; (b) Variations of NTs content in wt.%, relative to extractible substances (ES) or dry plant material (S), as function of the extraction temperature

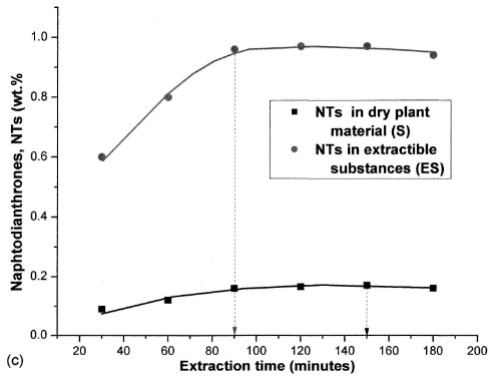

Fig 6. Results of the optimisation study: (c) Variations of NTs content in wt.%, relative to extractible substances (ES) or dry plant material (S), as function of the extraction time.

(fig. 6b). Comparing these results with those obtained previously at room temperature, for the evaluation of the plant content, it can be observed clearly that the content of NTs relative to ES has increased with two orders of magnitude (from 0.04 wt.% to near 1 wt.% in the extractible substance) which corresponded to a range of 0.16-0.18 wt.% of NTs in the dry plant material. Last but not least, the extraction time was also an important factor (fig. 6c), which seemed to favour the extraction of other substances besides NTs. Hence, the cut-off for the extraction time should be 90 min .

Conclusions

This study proposes a single-step reflux extraction method to ease the protocol for the enrichment of NTs extracts, which can stand alone as a straightforward methodology of sample preparation [1]. Hence, some time consuming preparative steps for the SJW extract enrichment in NTs can be by-passed if the plant is collected in autumn. Optimization of the single-step reflux extraction procedure (1/10-1/15 plant/solvent ratio, 55-60°C extraction temperature range and 90 min extraction time), performed with ethanol/water 70% v/v, has led to even richer naphtodianthrone extracts with extraction yields between 0.16-0.18 wt.% of NTs from dry plant materials.

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References

1. FLOREA, A.M., IORDACHE, T.V., BRANGER, C., GHIUREA, M., AVRAMESCU, S., HUBCA, G., SARBU, A., Talanta, **148**, 2016, p.37.
2. LENARD, J., RABSON, A., VANDEROEF, R., Proc. Natl. Acad. Sci. USA, **90**, 1993, p.158.
3. JACOBSON, J. M., FEINMAN, L., LIEBES, L., OSTROW, N., KOSLOWSKI, V., TOBIA, A., CABANA, B.E., LEE, D., SPRITZLER, J., PRINCE, A.M., Antimicrob. Agents Chemother., **45**, no. 2, 2001, p. 517.
4. SCHEMPP, C. M., PELZ, K., WITTMER, A., SCHOPF, E., SIMON, J.C., Lancet, **1**, no. 9170, 1999, p.353.
5. JIANU, C., GOLET, I., MISCA, C., JIANU, A.M., POP, G., GRUIA, A.T., Rev.Chim.(Bucharest), **67**, No. 6 , 2016, p. 1056.
6. MAURY, W., PRICE, J.P., BRINDLEY, M.A., Virol. J., **6**, 2009, p. 101.
7. FOX, F.E., NIU, Z., TOBIA, A., ROOK, A.H., J.Invest.Dermatol., **111**, no. 2, 1998, p. 327.
8. BLOOMFIELD, H.H., NORDFORS, M., MCWILLIAMS, P., Hypericum and Depression, 2nd Ed., Los Angeles, CA: Prelude Press, 1996, p. 49.
9. BARNES, J., ANDERSON, L.A., PHILLIPSON, J.D., J.Pharmacol., **53**, no. 5, 2001, p.583.
10. DRAVES, A.H., WALKER, S.E., J. Chromatogr. B, **749**, 2000, p. 57.
11. SMELCEROVIC, A., LAATSCH, H., LEPOJEVIC, Z., DJORDJEVIC, S., Pharmazie, **57**, no. 3, 2002, p.178.
12. KARIOTI, A., VINCIERI, F.F., BILIA, A.R., J. Sep. Sci., **32**, no. 9, 2009, p.1374.
13. MANNILA, M., WAI, C.M., Green Chem., **5**, no. 4, 2003, p.387.
14. GRACE, M.H., QIANG, Y., SANG, S., LILA, M.A., J. Sep. Sci., **40**, 2017, p.1057.
15. IONESCU, E., IORDACHE, T.V., ÞEBRENCU, C.E. CRETU, R.M., FLOREA, A.M., RADU, A.L., ZAHARIA, A., SARBU, A., Rev.Chim. (Bucharest), **69**, no.7, 2018, p. 1892
16. WILLIAMS, F.B., SANDER, L.C., WISE, S.A., GIRARD, J., J. Chromatogr. A, **1115**, no. 1-2, 2006, p. 93.
17.*** Romanian Pharmacopoeia 10th Edition, 1998, Medical Ed., Bucharest.
18. ANAND, R., VERMA, N., GUPTA, D.K., PURI, S.C., HANDA, G., SHARMA, V.K., QAZI, G.N., J. Chromatogr. Sci., **43**, no. 10, 2005, p.530.

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