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High-performance thin-layer chromatography/bioautography and and liquid chromatography-mass spectrometry hyphenated with chemometrics for the quality assessment of *Morus alba* samples

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Highlights

- A new HPTLC-based approach for quality assessment of herbal drugs was streamlined.
- The method allows for a fast chemical and biological profiling of complex extracts.
- Best sāng bái pí samples with regard to yield and health benefits were identified.
- Radical scavenging and antimicrobial constituents were disclosed from sāng bái pí.

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ABSTRACT

Quality control is a crucial step in the production of effective and safe herbal remedies. The aim of this study was to develop a new, simple, and high throughput procedure for the quality assessment of herbal drugs using a high-performance thin-layer chromatography (HPTLC)/bioautography and UPLC-MS/MS approach combined with chemometrics. This was exemplarily shown for *Morus alba* L. root bark (sāng bái pí; SBP). Bioautography assays were developed for the identification of constituents with radical scavenging (DPPH assay) and antimicrobial activities (*Bacillus subtilis, Escherichia coli*) of 18 different *M. alba* samples, which was supported by UPLC-MS/MS analysis. Further, the combination of bioautography and chemometrics identified those samples with the most bioactive constituents.

Plant materials collected from Serbia (11 samples) showed higher both radical scavenging and antimicrobial activities compared to samples provided from China (7 samples). Principal component analysis (PCA) confirmed the discrimination of geographically different samples and recognized their main markers responsible for differences between Serbian and Chinese samples. Most importantly for quality assessment, the combined HPTLC/bioautography and UPLC-MS/MS approach not only allowed for a fast chemical profiling of the investigated samples and their unambiguous identification, but also for the disclosure of major and minor bioactive constituents present in SBP.

Key words: HPTLC, bioautography, UPLC-MS/MS, pattern recognition, quality control, sāng bái pí

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1. Introduction

Herbal products have been widely used in traditional Chinese medicine (TCM) for thousands of years [1]. Several factors such as geographical and/or, botanical origin, meteorological conditions, collection seasons, and processing methods can influence the chemical composition of the herbal material, affecting authenticity, quality, and safety of the raw material before it is converted to the final products [2]. In accordance with the above mentioned factors, quality assessment of natural products is still challenging for both analysts and pharmacologists.

Due to different pharmaceutical properties, *Morus alba* L. is one of the most popular medicinal plants in Asian countries. Different parts of this meanwhile widespread species such as fruits, leaves, twigs, and root bark hold a monography in the Chinese Pharmacopoeia. In contrast to the other organs, the root bark (sāng bái pí; SBP) has been widely used in TCM against lung heat, cough, edema, and oliguria [3]. The chemical composition of SBP is highly complex due to a variety of different chemical compound classes, such as Diels–Alder-type adducts, flavonoids, benzofurans, stilbenes, and polyhydroxylated alkaloids [3, 4]. These compounds have shown a wide range of bioactivities including antioxidant, antiviral, anti-inflammatory, antimicrobial, antidiabetic and antitumor etc. [3, 4]. Recently, different flavonoids, among them also Diels-Alder adducts have been identified as potent dual inhibitors of both, influenza viral and pneumococcal neuraminidases, thereby able to disrupt the lethal synergism between these two detrimental microorganisms [5].

Although chromatographic techniques such as HPLC and GC hyphenated with different detectors such as MS allow for a comprehensive chemical profiling of complex mixtures, yet, they do not provide information about the biological activity of the individual compounds [6, 7]. Recently, α-glucosidase inhibitors from SBP were identified using ligand fishing combined with

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high-performance liquid chromatography-mass spectrometry (HPLC-MS) and molecular docking [8]. In contrast to solely LC-MS based approaches, planar chromatography is an ideal technique to combine with different biochemical and biological detection methods, as the open layer enables solvent evaporation, hence allowing the separated compounds (*i*) to directly interact with enzymes, (*ii*) to undergo microchemical reactions, and (*iii*) to stimulate or inhibit cellular/microbial growth [6]. The combination of sophisticated multivariate techniques with planar chromatography further allows the extraction of full information from HPTLC concerning the similarity/dissimilarity between samples, identification of characteristic markers responsible for classification, as well as the prediction of biological activity [9-12]. Recently, a novel HPTLC-partial least squares (PLS) regression based methodology was successfully applied to identify constituents from *Morus alba* fractions responsible for tyrosinase inhibition [4].

To the best of our knowledge, there are no reports related to the quality control of herbal drugs integrating HPTLC/bioautography and UPLC-MS/MS with multivariate data analysis. The combination of HPTLC/bioautography with UPLC-MS/MS becomes very crucial when analyzing complex samples: in addition to the information from the bioautogram the latter method provides MS-based structural insight into the constituents of the mixtures with high selectivity and resolution.

As an application example we selected *Morus alba* root bark (SBP) perceiving its potential as a whole focusing on its radical scavenging and antimicrobial activities, since they both are highly relevant for the reported traditional applications. Accordingly, our aim was to develop a new, effective, and fast high-throughput procedure for quality control of SBP samples based on a combined chemical and biological profiling.

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2. Material and methods

2.1. Chemicals and solvents

All reagents and solvents used in our experiments were analytical grade. Methanol, ethyl acetate, HPTLC glass plates 20 ×10 silica gel 60 F_{254} were purchased from Merck, Germany. *n*-Hexane was bought from VWR International, France. Thiazolylblue tetrazolium bromide (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Fluka. DPPH (2,2-diphenyl-1-picrylhydrazyl), 2-aminoethyl diphenyl borate, vanillin, nalidixic acid, chloramphenicol, ampicillin, triton X-100 Luria-Bertani (LB) broth were purchased from Sigma Aldrich, Austria (purity of Sigma Aldrich chemicals ≥ 98%); sulphuric acid (95-98.6%) from Gatt-Koller GmbH, Absam, Austria. Reference compounds (sanggenons B, C, D, G, sanggenol A, kuwanon L, and moracin M, (MS, NMR; HPLC-MS purity ≥ 95%) were isolated and characterized as described previously [5]. Antibacterial activity was tested using *Bacillus subtilis* (strain 168) and *Escherichia coli* (DH5α) as representatives for the activity against Gram positives and Gram negatives, resp.; bacteria were obtained from the collection of Prof. Sergey Zotchev (University of Vienna, Austria) [13, 14].

2.2. Sample preparation

Eleven *Morus alba* root bark samples (***1-11***) were collected in western and southern parts of Serbia during autumn 2017 and spring 2018, while five *M. alba* root bark (***13-17***) and two *M. alba* twigs samples (***12***, ***18***) were bought in China during August and September 2017 (**Table S1**). Voucher samples are deposited in the Herbarium of the Department of Pharmacognosy, University of Vienna, Austria. The collected samples were stripped and washed with distilled water. After drying overnight at 40°C, the samples were cut into small pieces, and milled for 5

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minutes into a powder (M 20 Universal grinder mill, Thermo Fisher Scientific, USA). List of samples, voucher numbers, geographical origins, and collection seasons are given in **Table S1**.

2.3. Extraction

Five g of *M. alba* samples each were extracted with 50 ml of methanol for 45 min in an ultrasonic bath (BANDELIN electronic GmbH & Co. KG, Berlin, Germany). Any possible decomposition of constituents by using this extraction method could be excluded by analytical comparisons with extracts obtained by maceration; the solution was filtered (Whatman® 595, Schleicher & Schuell GmbH, Dassel, Germany), evaporated to dryness (at 30°C and 120 mbar), and dissolved in 4 mL methanol. The obtained extracts were stored at -20°C prior to analysis. Dry extracts obtained from 5 g of herbal substance was dissolved in methanol to a drug extract ratio (DER) of 1.25:1. The described extraction procedure was applied in order to compare the quality of investigated raw-material samples. Extraction procedures were independently repeated three times for one Serbian (***5***) and one Chinese SBP sample (***18***). The obtained extracts were then analyzed by HPTLC.

2.4. HPTLC analysis

200 µL of the initial methanolic extracts were diluted to 1.8 mL with methanol, of which 2 µL were applied on a 20×10 cm HPTLC plate silica gel 60 F 254, as 8 mm bands using the Automatic TLC Sampler 4 (ATS4; CAMAG, Muttenz, Switzerland). The migration distance was 70 mm from the lower plate edge. The chromatographic separation was performed in the Twin Trough Chamber 20×10 cm (CAMAG, Muttenz, Switzerland) with a mixture of *n*-hexane, ethyl acetate and methanol (6:4:3.5, *V*/*V*/*V*) as a mobile phase. Developed HPTLC chromatograms were visualized under 254 and 366 nm using a CAMAG TLC Visualizer. Afterwards, the

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HPTLC plate was immersed in vanillin-sulphuric reagent (500 mg of vanillin was dissolved in 200 mL of 10% *conc*. sulphuric acid/ethanol) using the Chromatogram Immersion Device (CAMAG) with a speed of 3.5 cm/s for 3 seconds. The derivatized HPTLC plate was heated for 5 min at 103°C. The photo was saved as JPEG file format for further image processing. HPTLC chromatograms without derivatization under 254 nm and 366 nm are presented in Figure S1A.

2.5. DPPH HPTLC assay

200 µL of the initial methanolic extracts were diluted to 1.8 mL with methanol; thereof, 2 µL were applied on the HPTLC plate. 2 µL of reference compounds (1 mg/mL) were applied on the plate (Figure S2). The developed plate was dipped into the 0.1% methanolic solution of the DPPH radical at a speed of 3.5 cm/s for 2 s using the TLC Immersion Device. Afterwards, the plates were dried for 90 s in a dark chamber at ambient temperature. The obtained HPTLC autograms were visualized by the TLC Visualizer 2 for white light [10].

2.6. The bacterial culture

The cell number was monitored during incubation using the optical density (OD) factor at 600 nm (measured with a Thermo Scientific™ Evolution™ 260 Bio UV-Visible Spectrophotometer, Waltham, Massachusetts, USA). Bacterial suspensions (100 µL) were added to 10 mL LB broth each and shaken for 16 h (37°C, 200 rpm; OD_{600}=2.018 for *B. subtilis,* OD_{600}=2.021 for *E. coli*). One mL of the cultures of *E. coli* and *B. subtilis* strains were inoculated to 200 mL of LB broth in 500 mL flasks, resp., and incubated on an incubator shaker at 37^0C for 115 min in case of *E. coli*, and for 90 min in case of *B. subtilis*. OD_{600} were measured and HPTLC plates were dipped into the bacterial suspension. The OD_{600nm} of *B. subtilis* at 37°C was 0.539, while in case of *E. coli* the OD_{600nm} was 0.341.

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2.7. B. subtilis and E. coli bioautography assay

For the *B. subtilis* assay, 60 µL of the initial methanolic extracts were diluted to 1.8 mL; 1 µL thereof was applied on the HPTLC plate. After development, HPTLC plates were dried for 15 min under cold air to remove the mobile phase, and then immersed into cell suspensions manually for 6s. During dipping in *B. subtilis* cells suspension, HPTLC plates were incubated for 90 min in a plastic box lined with wet filter paper at 37°C. The zones with antibacterial effects were visualized by dipping the bioautograms into an aqueous solution of MTT vital dye (1 mg/mL) for 3 s. The bioautogram was additionally incubated for 30 min, and then dried at 60°C for 5 min [15]. After incubation with MTT, metabolically active bacterial cells convert the light yellow MTT to a blue-like formazan, revealing the inhibition zones as whitish spots against a bluish background. Reduction of MTT to formazan is associated with the oxidation of NADPH-dependent cellular oxidoreductase enzymes of living cells [15]. 1, 2, 3, and 4 µL of reference compounds (C = 0.1 mg/mL) were applied as bands to test the antimicrobial activity against *B. subtilis* (Figure S3). For *B. subtilis,* 2µL of chloramphenicol (C = 100 µg/mL) and ampicillin (C = 30 µg/mL) were applied as positive controls (Figure 1C).

For the *E. coli* assay, we used the method developed by Grzelak and co-workers [16] with some modifications. In case of *E. coli* 100 µL of the initial methanolic extracts were diluted to 1.8 mL, and 3 µL were applied on the HPTLC plate. The developed plate was dried for 15 min under cold air flow and dipped in an *E. coli* suspension for 6s manually. Afterwards, the bioautogram was incubated at 37°C for 100 min. For visualization, the plate was immersed manually into a MTT dye (1 mg/mL in water). One drop of Triton X-100/10 mL aqueous MTT solution was found to enhance the intensity of the color. After staining with MTT, the HPTLC plates were incubated again for 1 h at 37°C. In case of *E. coli,* 2 µL of nalidixic acid (C = 30 µg/Ml, Figure

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1D) were applied as positive control. 1, 2, and 3 μL of reference compounds (C = 0.1 mg/mL) were applied as bands in order to test the bioactivity against *E. coli* (Figure S4).

2.8. HPLC-LTQ-MS/MS analysis

200 μL of the initial methanolic extracts were diluted to 1.8 mL with methanol. Obtained extracts were pipetted into 250 μL glass inserts. Mobile phase A (0.2% aqueous formic acid) and mobile phase B (0.2% formic acid in acetonitrile:methanol (80:20, *V/V*) were degassed prior to their usage. Using Dionex Ultimate 3000 ultrahigh-performance liquid chromatograph (UHPLC), a 45 min gradient flow method was applied using a Kinetex 2.1 mm × 15 cm, 2.6 μm, C18, 100 Å reversed-phase column (Phenomenex); the flow rate was set to 250 μL/min, and a gradient was applied (0−2 min, 5% mobile phase B; 2−28 min, 5−85% mobile phase B; 28−35 min, 95% mobile phase B; re-equilibration with 5% mobile phase B). 5 μL of each sample were injected followed by a blank injection to ensure proper column washing and equilibration.

MS detection was performed with a LTQ-XL linear ion trap mass spectrometer (Thermo Fisher Scientific) using a ESI source (300°C heater temperature, 40/10/1 arb. units for the sheath, aux and sweep gases respectively and 3.5 kV spray voltage at 275°C capillary temperature) to achieve a negative ion mode ionization. MS scans were performed with an m/z range from 150 to 1500 and a resolution of 5000 with 0.3 (Da, fwhm) (at m/z = 300). MS/MS scans of the 3 most abundant ions were achieved through higher-energy collisional dissociation (HCD) fragmentation at 30% normalized collision energy. UPLC-MS/MS chromatograms of SBP samples are presented in Figure S5.

2.9. Multivariate analysis

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Principal component analysis was applied on HPTLC chromatograms using rTLC software (http://shinyapps.ernaehrung.uni-giessen.de/rtlc/) [9]. The preprocessing techniques such as filtering, warping, centering, normalization, baseline correction, were applied to improve PCA models [17]. In the case of UPLC-MS/MS, PCA was performed via PLS Tool Box (version 6.2.1) for MATLAB (version 7.12.0, R2011a; Eigenvector Research, Wenatchee, WA).

3. Results and discussion

3.1. HPTLC analysis

A new HPTLC method was developed using a mixture of *n*-hexane, ethyl acetate and methanol (6:4:3.5, *V*/*V*/*V*) as mobile phase to investigate the chemical profile of the crude SBP extracts and to enable a comparison of 16 different SBP samples derived from China (***13-17***) and Serbia (***1-11***). Additionally, two twig samples (***12***, ***18***) from *M. alba* were included to check whether the HPTLC method is able to distinguish these closely related organs. After HPTLC plate development and derivatization with vanillin-sulphuric acid, the chromatograms showed a colorful profile and revealed significant differences between *M. alba* samples: By visual inspection, SBP samples collected from Serbia (11) showed a richer metabolite profile and higher intensity bands in the region between hR_F 50 and 80 compared to Chinese SBP samples (5) (Figures 1A). In order to identify some unknown constituents, seven previously isolated compounds [18], namely sanggenon B (hR_F=70), sanggenon C (hR_F=65), sanggenon D (hR_F=57), sanggenon G (hR_F=55), sanggenol A (hR_F=77), moracin P (hR_F=63), and kuwanon L (hR_F=58) were analyzed together with *M. alba* samples on the HPTLC plate (Figure S1B; chemical structures of isolated compounds are depicted in Figure 2B). In comparison to the SBP

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extracts, the two twigs samples (no ***12*** and ***18***) exerted only one slightly visible red band supposed to be sanggenon G (Figures 1A and 2Aa).

3.2. HPTLC-DPPH

A chemical assay was performed with DPPH to identify HPTLC spots with radical scavenging activity [10, 19]. These were detectable as light yellow bands against a dark purple background in almost all investigated samples: SBP extracts collected from Serbia showed yellow bands with higher intensity compared to samples from China. *M. alba* twigs samples did not show any radical scavenging activity (Figures 1B and 2A).

The seven references compounds were additionally applied on the HPTLC plate (Figure S2). Out of these reference compounds moracin P, kuwanon L, sanggenons C, D, and G showed moderate radical scavenging activities (Figure S2), which is in agreement with literature [20, 21]. In case of almost all SBP samples, the main constituents, *i.e.*, sanggenon D (hR_F 55) and sanggenon G (hR_F 57), showed intensive yellow bands on the HPTLC plate (Figures 1B and 2Ab).

3.3. HPTLC-bioautography

After incubation with *B. subtilis* and staining with MTT solution, compounds with antimicrobial activity against *B. subtilis* were observed as whitish bands against dark bluish background. Major inhibiting bands were observed for those at hR_F corresponding to sanggenons G and D (Figure 1C). Although these compounds displayed moderate activity against *B. subtilis* compared with other reference compounds (Figure S3), clear and intense inhibition bands might be due to their high amount in SBP samples. Further, sanggenol A and an unknown compound with hR_F 79 were observed as minor antimicrobial constituents for all SBP samples (Figures 1C and 2Ac).

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In analogy to the *B. subtilis* bioautogram, inhibitors of *E. coli* growth were visible on the HPTLC plate by whitish bands. Dominant antimicrobial spots were caused by sanggenons D, G, and two hardly visible bands identified as sanggenon C and sanggenol A (Figures 1D and 2Ad). As shown in Figure S4, the bioautogram obtained by incubation with *E. coli* confirmed that sanggenon B, kuwanon L and moracin P showed weak activities, while sanggenons C, D, G, and in particular sanggenol A exhibited a distinct antimicrobial activity against *E. coli*. These results are in accord with previous findings [22]. In general, extracts from *M. alba* twigs exerted neither an activity against *B. subtilis* nor *E. coli*. Further, Serbian SBP samples showed more active bands than Chinese ones against both investigated strains. *3.4. Principal component analysis based on chemical screening*

PCA was applied to obtain a basic insight into the specific grouping patterns among the *Morus* samples, thus to evaluate the ability to distinguish samples derived from Serbia and China as well as to differentiate between twigs and root bark. The loading plot was used to identify the most influential compounds able to discriminate between groups of *Morus* samples. PCA was applied on HPTLC plates without derivatization (under 254 nm and 366 nm), and after derivatization with vanillin-sulphuric acid.

PCA applied on HPTLC plates under 254 nm showed a good separation between samples collected in Serbia and China according to PC1. *M. alba* twigs samples (no ***12*** and ***18***) were clearly positioned outside (Figure 3A). The two PCs describe 73% variance, in which the first one (PC1) refers to 51.59%, while PC2 accounts for 21.43% of the total variance. Sanggenons G, D, sanggenol A, and the constituent with hR_F 3 were recognized as markers for separating between SBP samples (Figures 3B and 3C).

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PCA based on 366 nm resulted in a five-component model that explained 72.62% of the total variance. There are two groups of *M. alba* samples: SBP collected from Serbia were positioned in the right upper side of the PCs score, while the twigs samples were positioned between the Serbian and Chinese samples (Figure 3A; no ***12*** and ***18***). PC1 and PC2 were highly affected by compounds with hR_F at 3, 10, 15, 37, moracin P, and kuwanon L (Figures 3B and 3C), which revealed as markers for geographical origin of SBP samples.

Colorful HPTLC data (after derivatization with vanillin-sulphuric acid) are highly suitable for multivariate analysis: selectivity is enhanced by splitting a photo through red (R), green (G), and blue (B) channels. In the case of the red channel, the total variance explained by the first three PCs was 78.80% (PC1:47.01%, PC2:25.27%, PC3:6.52%) (Figure 4A1). The discrimination between samples is mainly driven by PC2. Based on loading plots, highest influences on separation were found for sanggenol A, sanggenon B, kuwanon L, and two compounds with hR_F at 3 and 80 (Figure 4A2). Also, in the case of green and blue channels there was a clear discrimination between Serbian and Chinese samples (Figures 4B1 and 4C1). Applying the green channel, the first two PCs referred to 61.57%, while for the blue channel they accounted for 58.28% of the total variance. Loading plots recognized compounds such as sanggenons B, C, D, G, sanggenol A, kuwanon L and a compound with hR_F 13 as discriminating markers (Figures 4B2 and 4C2). In all three channels, *M. alba* twigs samples were positioned between Serbian and Chinese SBP samples.

3.5. Multivariate analysis based on biological screening

PCA was additionally performed on HPTLC chemo/bioautograms to classify the *Morus* samples according to their radical scavenging and antimicrobial activities. PCA applied on the HPTLC-DPPH chemical assay showed a good discrimination according to their provenience. PC1

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accounted for 45.91%, PC2 for 22.01% of the total variance. *M. alba* twigs samples were positioned close to those SBP samples which showed no or moderate radical scavenging activities (Figure 5A1). PC1 and PC2 were highly influenced by the compound with hR_F 5, sanggenons D and G, kuwanon L, as well as sanggenol A (Figure 5A2).

In the *B. subtilis* assay, the first three PCs described 81.14%, while for *E. coli* the first three PCs accounted for 83.79% of total variance. Based on Figures 5B1 and 5C1, there is good discrimination between Serbian and Chinese samples according to PC2. Sanggenons C, D and G, sanggenol A and kuwanon L were recognized as the most important compounds for the classification of SBP samples based on their antimicrobial activity (Figures 5B2 and 5C2). *M. alba* twigs samples formed a subgroup close to SBP samples collected from China, which showed distinctly less bands with antimicrobial activities.

3.6. Multivariate analysis based on UPLC -MS/MS fingerprint

The concurrent use of HPTLC and UPLC-MS/MS provides an increased confidence level for the developed approach, because these complementary methods deliver full information regarding bioactivity and identification of minor and major bioactive compounds in SBP extracts (S1.1). Incorporating UPLC-MS/MS in an offline fashion as demonstrated in this study, allowed the dereplication of 18 compounds (**I-XVIII**), which were presumably identified in the *Morus* samples according to their retention time, molecular mass $[M-H]^-$ and fragmentation pattern as reported in literature (Table 1) [21, 23]. Fragmentation pathways are described in the Supplementary information (1.1 S). As a future prospect, a bioactive band could be identified by using a combination of HPTLC and online coupling with mass spectrometry [10]. PCA was applied on the data matrix (18 samples×2712 variables (t_R)) obtained from UPLC-MS/MS chromatograms. The first four PCs described 68.80 % of total variance. From the mutual

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projections of PC factor scores (Figure 6A), all investigated samples were clustered into two groups; samples collected from Serbia formed one group (right hand side), while samples provided from China formed one subgroup (middle left hand side): the two twigs samples were clearly positioned outside the SBP samples (Figure 6A). Based on Figure 6B and 6C, most influential compounds discriminating between samples are kuwanon G, sanggenon B, sanggenol B, mulberroside A, and three unknown compounds with t_r 1.48 and 20.32 min (Figures 6B and 6C).

4. Conclusion

The HPTLC method allowed for a simple, low-cost, and high throughput metabolite screening of the investigated herbal material (requiring a few minutes per sample). Additionally, HPTLC plates immersed in DPPH solution, *B. subtilis* and *E. coli* bacterial suspension provided the simultaneous identification of compounds with potential biological activities from 18 *Morus* samples (only requiring a few minutes per sample). Multipotent components with radical scavenging and antimicrobial (Gram positive and Gram negative) effects were detected directly from the investigated crude extracts without any need for pretreatment. UPLC-MS/MS analysis confirmed the presence of potential bioactive compounds identified by the HPTLC method and allowed for a concrete assignment of major and minor compounds from the complex SBP samples. As exemplified in our investigation on SBP and its potential antioxidant and antimicrobial activity, the applied autographic assays (using DPPH, *E. coli*, and *B. subtilis*) revealed in particular SBP rich in sanggenons C, D, and G with both, high radical scavenging and antimicrobial activity.

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Further, HPTLC bioautography combined with multivariate analysis allowed for the identification of markers responsible for a classification between our samples. Five PCA models obtained from the data set of derivatized and underivatized HPTLC plates showed disparity between samples collected from Serbia and China. To summarize, the best PCA models were obtained at 254 nm, vanillin-sulphuric acid (green channel) for the DPPH assay and the *E. coli* assay. Beside some unidentified metabolites, the PCA models mainly highlighted moracin P, sanggenons B, C, D, G, sanggenol A, and kuwanon L as markers for the geographical classification.

The introduced HPTLC-bioautography/UPLC-MS/MS clustering approach turned out as a highly suitable tool for the identification of those herbal drugs which have the highest potential to contribute to health-related activities in terms of quality and quantity, and thus, to select starting material for herbal preparations with the most beneficial profile.

Conflict of interest

The authors declare no conflict of interest.

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Figure captions

Fig.1. HPTLC profiles of *M. alba* samples: A) after derivatization with vanillin-sulphuric acid, B) after DPPH assay, C) after *B. subtilis* bioassay, D) after *E. coli* bioassay; CL, chloramphenicol; AMP, ampicillin; NAL, nalidixic acid.

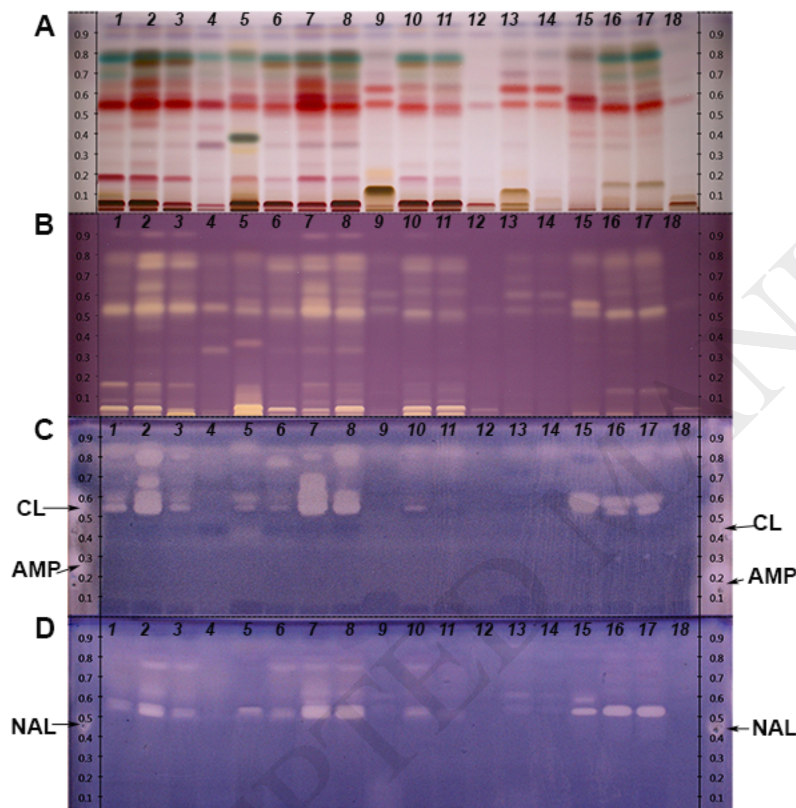

Fig.2. A) HPTLC fingerprints of *M. alba* sample no ***8*** after a) derivatization with vanillin-sulphuric acid, b) DPPH˙ assay, c) *B. subtilis* bioassay, d) *E. coli* bioassay; B) chemical structures of isolated *M. alba* constituents.

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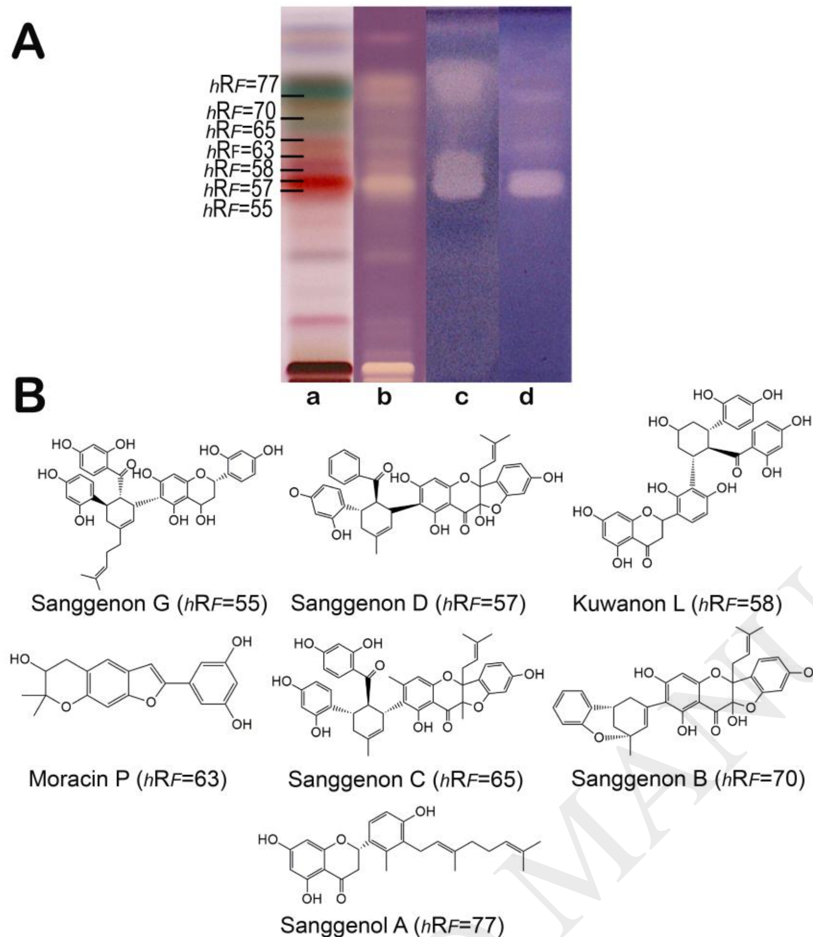

Fig.3. PCA of *M. alba* samples based on 254 nm and 366 nm: A) PCs score plots, B) loading plot based on PC1, C) loading plot based on PC2.

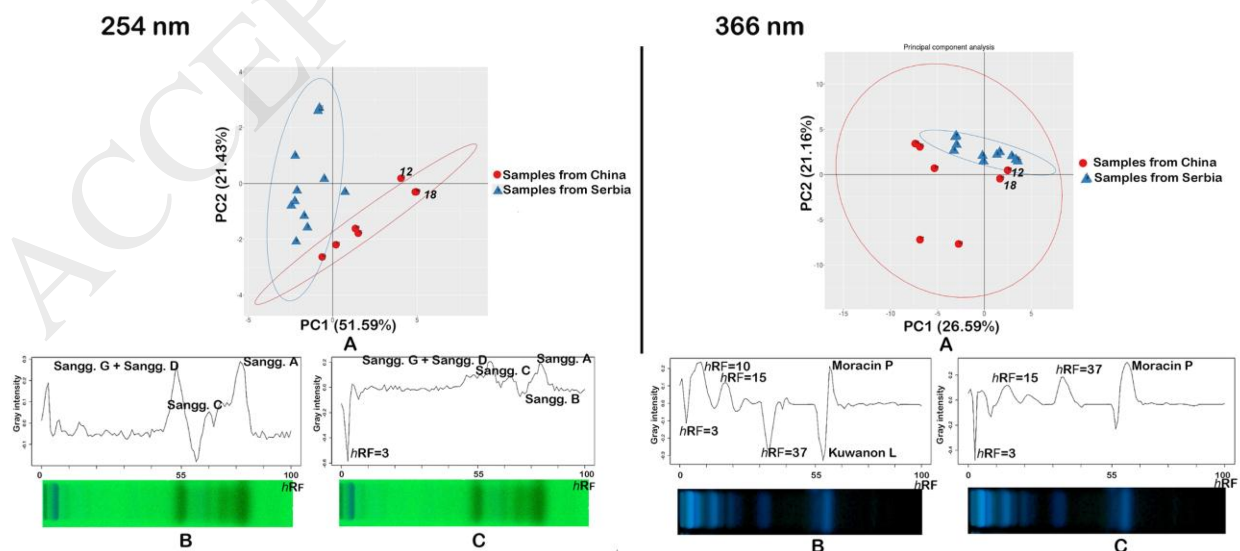

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Fig.4. PCA of *M. alba* samples after derivatization with vanillin-sulphuric acid based on red channel: A1) PCs score plot, A2) loading plots; green channel: B1) PCs score plot, B2) loading plots; blue channel: C1) PCs score plot, C2) loading plots.

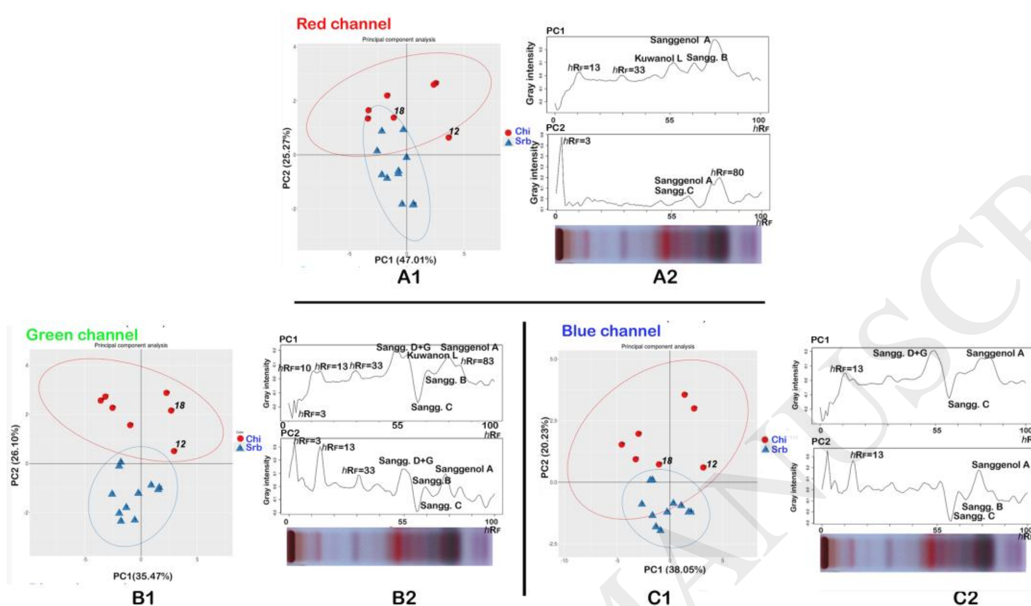

Fig.5. PCA of *M. alba* samples based on DPPH assay: A1) PCs score plot, A2) loading plots; B. subtilis assay: B1) PCs score plot, B2) loading plots; E. coli assay: C1) PCs score plot, C2) loading plots.

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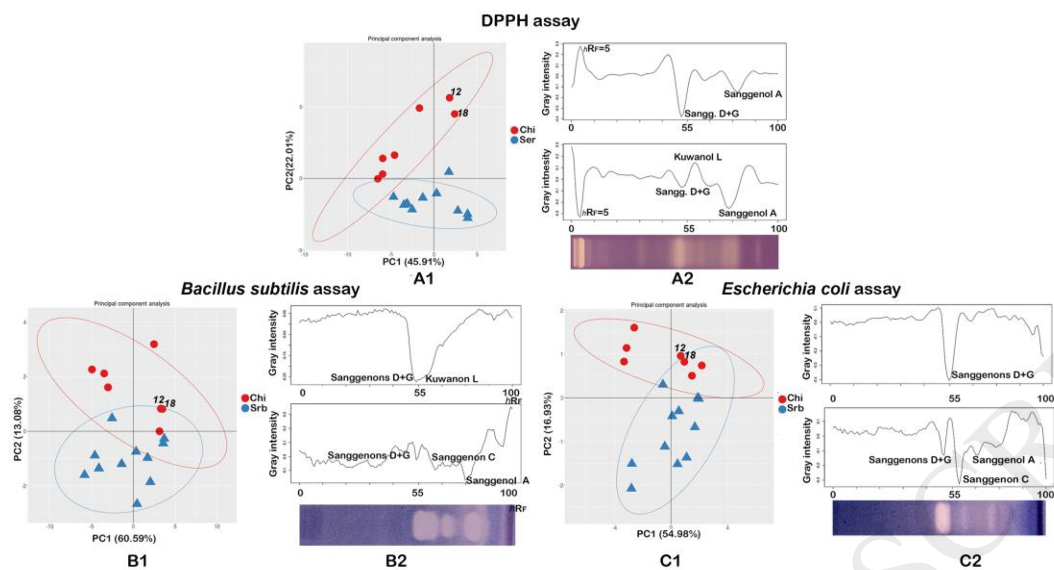

Fig.6. PCA of *M. alba* samples based on UPLC-MS/MS profiling: A) PCs score plots, B) loading plot based on PC1, C) loading plot based on PC2.

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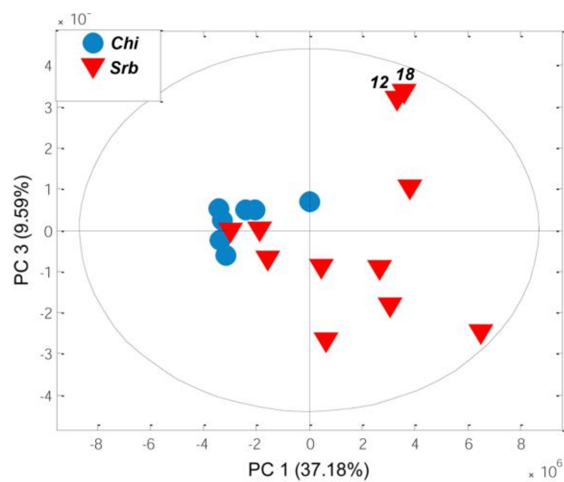
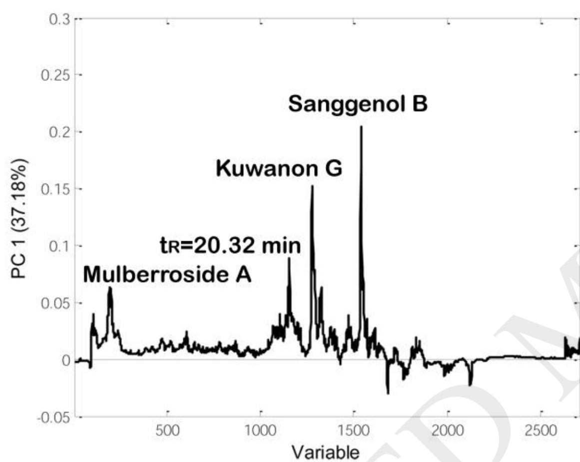
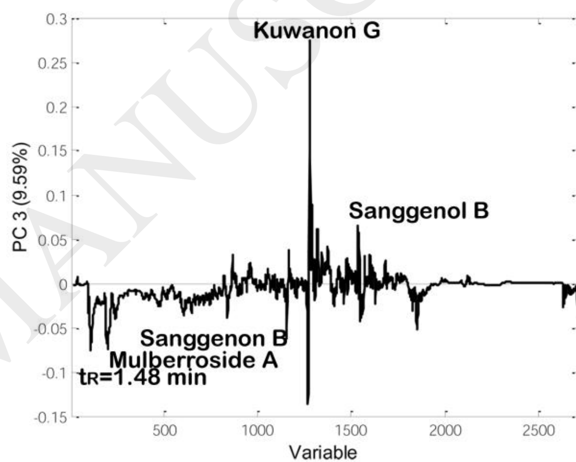


A





B C

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Table 1. Proposed metabolites in SBP samples using UPLC-MS/MS

No.	**Proposed metabolites**	t_R ***(min)***	***m/z* [M−H]$^-$**	**Fragmentation**
I	Mulberroside A	3.00	567	405, 243, 225, 199
II	Moracin M	9.99	241	198, 197
III	Moracin P	15.40	325	283, 253
IV	Sanggenon T (or isomer)	17.83	711	601, 549
V	Sanggenon T (or isomer)	18.34	711	601, 600, 549, 548, 490
VI	Kuwanon L	19.11	625	499, 471, 389
VII	Sanggenon D	21.14	707	489, 369, 325, 300, 259, 227
VIII	Sanggenon B	21.75	569	551, 525, 500, 459, 325, 243, 215
IX	Kuwanon G	22.03	691	581, 471, 459, 353
X	Sanggenon G	22.99	693	583, 531
XI	Sanggenon C	24.02	707	689, 597, 528, 489, 463
XII	Sanggenol A	24.12	423	298, 245, 151, 126
XIII	Kuwanon C	24.29	421	352, 309, 231
XIV	Morusin	24.53	419	375, 350, 309, 297
XV	Morunigrol	25.51	437	419, 379, 315, 312
XVI	Sanggenol B	27.49	489	445, 364, 351, 309, 257, 243,231
XVII	Andalasin A	30.30	487	349, 231
XVIII	Kuwanon G (or isomer)	30.43	692	581, 419, 353