

Compound's Pre-Screening of *Withania somnifera*, *Bacopa monnieri* and *Centella asiatica* Extracts

Steffi Witter^{1,2,3}, Georg Arju², Marina Junusova², Maria Kuhtinskaja³, Ago Samoson¹, Raiker Witter^{4,5}, Raivo Vilu²

¹Department of Health Technologies, Tallinn University of Technology, Tallinn, Estonia

²Competence Center of Food and Fermentation Technology (TFTAK), Tallinn, Estonia

³Department of Chemistry and Biotechnology, Tallinn University of Technology, Tallinn, Estonia

⁴Institute of Nanotechnology, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany

⁵Institute for Quantum Optics, Ulm University, Ulm, Germany

Email: steffi.witter@mail.com, ago.samoson@gmail.com, raivo.vilu@ttu.ee, georg@tftak.eu, marina@tftak.eu, mariab@chemnet.ee, raiker.witter@mail.com

How to cite this paper: Witter, S., Arju, G., Junusova, M., Kuhtinskaja, M., Samoson, A., Witter, R. and Vilu, R. (2020) Compound's Pre-Screening of *Withania somnifera*, *Bacopa monnieri* and *Centella asiatica* Extracts. *Journal of Biosciences and Medicines*, 8, 80-98.

<https://doi.org/10.4236/jbm.2020.89007>

Received: July 21, 2020

Accepted: September 11, 2020

Published: September 14, 2020

Copyright © 2020 by author(s) and Scientific Research Publishing Inc. This work is licensed under the Creative Commons Attribution International License (CC BY 4.0).

<http://creativecommons.org/licenses/by/4.0/>



Open Access

Abstract

Spectral fluorescence signature, Gas Chromatography-Mass Spectrometry and Liquid Chromatography-Mass Spectrometry for identification of chemical and bioactive compounds were applied to study the plant extracts of *Withania somnifera*, *Centella asiatica* and *Bacopa monnieri* which are related to the possible treatment of mental diseases as Alzheimer, Parkinson and Depression. These plants are known for different positive phytotherapeutic effects on the human brain without negative post-, adverse or after effects to the treated individuals, and have been recommended in several medical studies. Therefore, we selected these plants for further analysis, based on the inhibition results of *in vitro* Amyloid Beta fibrillation tests made by previous measurements. With this study a first screening of the complex plant extract mixtures was performed, to get an initial overview about known and unknown ingredients. In all three plants, similar main compounds were identified, however in different quality and quantity. These may provide substantial information on which compound combinations might be mainly responsible for the positive effects and should be further investigated being responsible for reducing the fibrillation process of Amyloid Beta.

Keywords

Gas Chromatography-Mass Spectrometry, Liquid Chromatography-Mass Spectrometry, Principal Component Analysis, Spectra Fluorescence

Signature, Extracts, *Withania somnifera*, *Bacopa monnieri*,
Centella asiatica

1. Introduction

In contrast to plant-derived synthetic medicines which are often associated with adverse effects and mostly target onto a single symptom, phytotherapy is commonly based on traditional knowledge of plants and their expected medical functions on so-called “medicinal wisdom of centuries” [1] [2] [3] and its increasing importance has been discussed [2] [3] [4] [5]. This motivates the usage of natural products which provides promoting medical effects without complications [4]. Such herbal medicines and medical plant extracts are complex bioactive compound mixtures for which an improved insight with specifications and proof-of-effectiveness of the ingredients is recommended [6] [7] [8] [9] raising several questions. What are the compounds within these extracts? What makes the plant mixtures more efficient than a pure synthetic drug? Why the plants’ extracts have in the obtained dosages and mixtures almost none negative side effects?

In this study we performed an ingredients’ screening of promising Ayurvedic plants—*Withania somnifera*, *Centella asiatica*, and *Bacopa monnieri*—to make first steps gaining insight into the prevention or treatment of diverse mental disorders like Alzheimer, Parkinson, Schizophrenia, and Depressions [10]. Extracts were selected from eight traditional Indian medicines—used since more than 2000 years—for a possible Alzheimer treatment and are known for several positive phytotherapeutic effects on the human brain and body without negative post-, adverse or after effects to the treated individuals (positive memory influence, stress reduction, mental health regeneration and reduction of anxiety), and have been recommended in several medical studies [11]-[22] which we previously investigated by *in vitro* Amyloid Beta fibrillation inhibition measurements by luminescence spectroscopy [10] [23]. Here, we applied Gas Chromatography-Mass Spectrometry (GC-MS), Liquid Chromatography-Mass Spectrometry (LC-MS) and Spectral Fluorescence Signature (SFS) on related nutraceuticals and plant extracts.

2. Material and Methods

2.1. Plant Material

Centella asiatica (GK) and *Withania somnifera* (AS) seeds were supplied by Botanik Sämereien (Switzerland).

Bacopa monnieri (BR) juvenile plants were obtained from Hellwig (gardening center, Germany).

Breeding conditions in the green house were kept stable at 20°C - 24°C with humidity of 40% - 60%.

The LED-based illumination system (LED Company, Estonia) provided a light wavelength distribution of 380 - 780 nm. Defined light conditions were achieved for 24 hours: daylight from 8:00 - 19:59 and darkness from 20:00 - 7:59 [10] [23].

2.2. Nutraceuticals

Capsules of BR were filled with a mixture from leaves and stems powder, in case of AS with root powder, which were purchased from the Himalaya Drug company, Bangalore, India.

GK was acquired in form of capsules, filled with leaf powder, from SHAG Psoriasis EX, Berlin, Germany.

For the investigations, the powder material interior of capsules was removed for usage. The nutraceuticals were stored at 4°C, but 30 minutes before experimental application, samples were taken out of the fridge, to equilibrate and handle them at room temperature.

2.3. Extraction Methods

SFS extracts were prepared with maceration with mortar and pestle (3 g plant material with 30 ml solvent) and were directly used after [10] [23].

For Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) measurements two different kinds of plant extractions were utilized:

2.3.1. Soxhlet Extraction

Soxhlet Extraction (SE) was applied to obtain ingredients of the fresh and green plant material. The stock solvent of 96.7% ethanol (EtOH) with MilliQ water was used to prepare 65% and 50% EtOH solution (EtS). The 65% EtS was added to 15 g fresh plant material shortly before the extraction process which was initialized at a temperature of 95°C for 30 minutes and continued at a stable temperature of 90°C for 16 h. The SE of 50% EtS with 15 g green and fresh plant material run at a stabilized temperature of 100°C for 14 h. Hereby, an initial temperature of 120°C for 30 minutes was applied. The SEs were annealed and stored at 4°C and at -80°C in 2 ml tubes till further usage [10] [23]-[28]. All prepared extracts were centrifuged at 15.000 rpm (4°C) to separate solutions from suspended particles and poorly dissolved materials. The upper solvent layer was pipette into marked tubes and stored in an ice box until experimental application [10] [23].

2.3.2. Methanol Extraction

Methanol (MeOH, Sigma Aldrich) extraction for compound analysis was tested with MeOH 95% (5% MilliQ water). 10 ml MeOH was put into a marked falcon tube and 3 g green and freshly harvested plant leaves were added to the solvent. The sample was stored overnight at room temperature (12 hours) to soak for an extract. This solution was sterilized via centrifugation (1 min at 15.000 rpm and room temperature) and further membrane filtrated into the measurement tube.

2.4. Alcohol Evaporation

For GC-MS the SEs were needed to be prepared free from water. Due to the huge sample amount the evaporation was carried out by water bath evaporation which was achieved by placing 50 ml vessel with sample via slow spinning system into an 80°C water bath [10] [23]. Over the duration of two hours, the entire solvent was evaporated and the dried remains were dissolved in methanol for further measurements. (For such sample amounts, vacuum drying can be considered inefficient and would have taken a long time.)

2.5. Liquid Chromatography-Mass Spectrometry (LC-MS)

The Chromatographic separation was performed by use of an Agilent 1290 UPLC (Agilent Technologies, Santa Clara, CA) system equipped with an Agilent RRHD SB-Aq (2.1 × 150 mm, 1.8 µm) column (AGI859700914) and an Agilent SB-Aq (2.1 mm, 1.8 µm) guard column (AGI821725936). The mobile phase A contained an aqueous solution of 0.1% formic acid, and the mobile phase B an acetonitrile solution of 1% MilliQ water and 0.1% formic acid. A solvent gradient of 95% A 0 - 2 min, 95% - 0% A 2 - 22 min, 0% A 22 - 25 min, 0% - 95% A 25 - 26 min, 95% A 26 - 30 min were used. The flow rate was 0.3 ml/min. The analytical column was maintained at 40°C whilst the autosampler was maintained at 4°C. 5 µl of the sample was injected for each run.

Mass spectrometry was performed by using a G6540A QTOF (Agilent Technologies, Santa Clara, CA), a quadrupole and orthogonal acceleration time-of-flight tandem mass spectrometer equipped with an Agilent Jet Stream Ion Source (AJS). The scan range was set between 50 and 1000 *m/z*. Electrospray capillary voltages (negative and positive) were adjusted to 4500 V, nozzle voltage to 1000 V, fragmentor to 175 V, skimmer 1 to 65 V and OctopoleRFPeak to 750 V. Gas temperatures were set to 325°C at 8.5 l/min, sheath gas to 325°C at 8 l/min and the nebulizer to 45 psig. The mass spectrometer was operated in the extended dynamic range mode (1700 *m/z*) with an acquisition rate of 1 Hz. Data acquisition was performed by using the Mass Hunter LC/MS Data Acquisition Workstation Software (Build 6.01.6172 SP1), the data analysis by the Mass Hunter Qualitative Analysis Workstation Software (Build 7.0.7024.0) and the Mass Hunter Mass Profiler (8.0.136), all from Agilent Technologies, Santa Clara, CA. Requested peak picking was carried out with an ion intensity threshold over 2000 counts, an unbiased isotope model and a charge state of 1. Identification was performed using the Mass Hunter ID Browser (Agilent Technologies, Santa Clara, CA) applying the Metlin AM PCD library with a mass tolerance of 5 ppm [29].

All applied chemicals were obtained from Sigma Aldrich and industrial gases were provided by AS Linde Gas, Estonia.

2.6. Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analysis was performed utilizing an Agilent 5973N system with a 6890N MS detector. As a column, a 30 m Phenomenex ZB-5MSi with 0.25 mm

diameter and 0.25 mm film thickness was used. For GC-MS detection, an electron ionization system with ionization energy of 70 eV was applied. Helium gas (99.99%; AS Linde Gas, Estonia) was used as a carrier gas at a constant flow rate of 1.3 ml/min. Injection and mass transfer line temperature were set to 250 and 230°C respectively. The oven temperature was programmed for ramping from 70°C to 150°C with a heating rate of 5°C/min, pursuing to 250°C with a rate of 5°C/min, held there for two minutes and finally increased to 320°C with a heating rate of 10°C/min and then held for 3 min. 1 µL of the sample was inserted with the splitless injector mode by a mass scan of m/z 50 - 700. The relative percentage of each extract constituent was expressed as a percentage with peak area normalization. Interpretation of the mass spectrum of the plant extracts was conducted using the database of the National Institute of Standard and Technology (NIST11) library [30].

The evaporated SE samples for the GC-MS measurements were dissolved in 5 ml methanol before usage. Hereby, 1 ml of this methanol solution was filtrated through a micro-filter.

All applied chemicals were obtained from Sigma Aldrich.

2.7. Spectral Fluorescence Signature (SFS)

To obtain 3D fluorescence matrices of the samples the SFS technique (Laser Diagnostic Instruments AS, Estonia) was used by applying variable excitation and emission wavelengths. Utilizing color patterns, a 2D representation was extracted, where the colors represent the fluorescence intensities to quantify the substances/solvents. A qualitative identification was achieved since various chemical compounds have characteristic fluorescence spectral patterns [6] [31]-[37].

2.8. Principal Component Analysis (PCA)

The PCA were used to preprocess the SFS data with the program named “StandardScaler” (StSc). Hereby the StSc assumes the SFS produced data. Those data are normally distributed within each feature and scaled them in a way, that the distribution is centered around 0, with a standard deviation of 1. Nevertheless, the mean and standard deviation are calculated for the feature and its scale based on: $\frac{x_i - \text{mean}(x)}{\text{stdev}(x)}$ [38].

Furthermore allows the PCA to transform the high dimensional into low dimensional data for the best representation of entire data.

3. Results

3.1. *Withania somnifera* (Ashwagandha, AS)

AS extracts and nutraceuticals affirmed to be complex mixtures consisting of many substances. Different measurements with LC-MS, GC-MS, and SFS were carried out. Hereby, with LC-MS and GC-MS around 10 k possible compounds were denoted, from which around 6 k were identified with 80% - 100% Q Score. The final list of all identified substances without duplicates was found to be

around 2 k (see supporting information), from which a selection of expected compounds, *i.e.* withaferin A, were identified and are listed in **Table 1** [10] [23] [38]-[60]. In addition, approximately 4 k unknown possible ingredients remain unresolved.

The SFS data were analyzed via Principal Component Analysis (PCA), see **Figure 1**. The score SFS plots show differences between samples of spectral regions which were made visible in the loading plots, see **Figure 2**. In the PCA plot of AS, differently prepared samples distribute into separated data clusters in **Figure 1**. The PCA is based on the variation of the AS samples related to two spectral components (**Figure 2**): with maximum variance of the first main component (80.22%) and with a smaller variance for the second main component (-15.47%), all other main components are less presented within percentages (less than 2%; third component have a variance of 1.97% and the fourth main component a variance of -1.24%). In this plot, we observe that AS powder forms very distinct clusters which are related to AS dissolved in 40/50% ethanol, powder and a wider distribution related to the rest of samples. Hereby, the SFS spectra provide intensities, if both—Emission and Excitation—are present. Those Emission and Excitation data of all compared samples were the basis for the PCA measurements.

Table 1. Chemical ingredients of *Withania somnifera*.

Expected components	Possible found Compounds after data analysis
alkaloids, amino acids, anaferine, anahygrine, anthocyanins, anthraquinones, carotenoids, cholesterol, coumarins, cuscohygrine, diosgenin, flavonoids, glycosides, iron, kaempferol, lignins, phenolics, phytosterols, proanthocyanidines, pseudotropine, quercetin, saponins, sitoinodosides VII, sitoinodosides VIII, sitoinodoside IX, sitoinodoside X, somniferine, somniferinine somnisol, somnitol, stigmastadien, stigmasterol, tannins, terpenoids, tropine, vitamin E, withaferin A , withanol, withanolide A, withanolide D, withanolide G, withanone; 6a.chloro-5b, 17a-dihydroxywithaferin A; β -sitosterol	AA861; affinisine; all-trans-pentaprenyl diphosphate; ajugalactone; arabinonic acid; asiatic acid ; asiaticoside ; astemizole; azithromycin; belladonnine; benzeneacetaldehyde; benzoylglutamine; brompheniramine; bruceoside A; butyl citrate; capryloylglycine; citric acid; chlorogenic acid; chlorophyllide b; chlorthiophos; cholesterol sulfate; coniferin; coproporphyrin; cucurbitacin B; cucurbitacin D; cyclofoetoside B; deuteroporphyrin IX; dimethyl phthalate; eicosane; ethyl(dimethyl)methoxysilane; fexaramine; gomisin B; glycobismine A; fludrocortisone; hellicoside; helveticoside; hexadecanamide; indole; hydrocortisone butyrate propionate; kaempferol 3-O- β -D-glucosyl-(1-2)- β -D-glucosyl-(1-2)- β -D-glucoside; KAPA, lappaol B; lappaol D, lauryl hydrogen sulfate, linoelaidic acid; lysoPE(18:4(6Z,9Z,12Z,15Z)/0:0), L-tryptophan; magnesium protoporphyrin, manumycin A; methyl salicylate; N-(3-oxo-octanoyl)-homoserine lactone; nicotinyl; nonactin; oligomycin A; octadecane; PD 123319, pedilstatin; pentadecanoic acid; phosgene; phytol; picein; PIP(16:2(9Z,12Z)/16:0); piperonyl sulfoxide; psychotridine; pyroglutamic acid, quinic acid, roxburghine B; reduced vitamin K; vitamin D2 3-glucuronide; riboflavin; salannin; schisantherin A; schizonepetoside E; silane, (2-methoxyethyl)trimethyl-; spinosin; swainsonine; stearic acid ; stearidonic acid ; sterol 3-beta-D-glucoside ; tetradecanamide; tetradecyl sulfate; tetrahydrocorticosterone; tetranactin; triamcinolone; UDP-2-deoxyglucose; vomicine; withaferin A ; Z-Gly-Pro-Leu-Gly-Pro; 1-Acetylaspidobalidine; 1,2-Di-(9Z,12Z,15Z-octadecatrienoyl)-3-(Galactosyl-alpha-1-6-Galactosyl-beta-1)-glycerol; 1-(3,4-Dihydroxyphenyl)-5-hydroxy-3-decanone; 2-Methylbenzaldehyde; 5-O-Feruloylquinic acid; 6-Deoxoteasterone; 9Z-Octadecenedioic acid; 12alpha-Fluoro-11beta, 17beta-dihydroxyandrost-4-en-3-one; 17beta-Hydroxy-4-mercaptoandrost-4-en-3-one 4-acetate 17-propionate; 6-Gingerol; 9-Octadecenamide, (Z)-

GC-MS and LC-MS data of the chemical compounds found in *Withania somnifera* are selected: expected compounds based on the literature [23] [39]-[60] are provided in the left column and extracted compounds which are selected accordingly the limit of the retention peaks in respect to the highest abundance and on the Q Score of 100% (threshold of 20%). The highlighted compounds are the most probable expected ones listed in the first column.

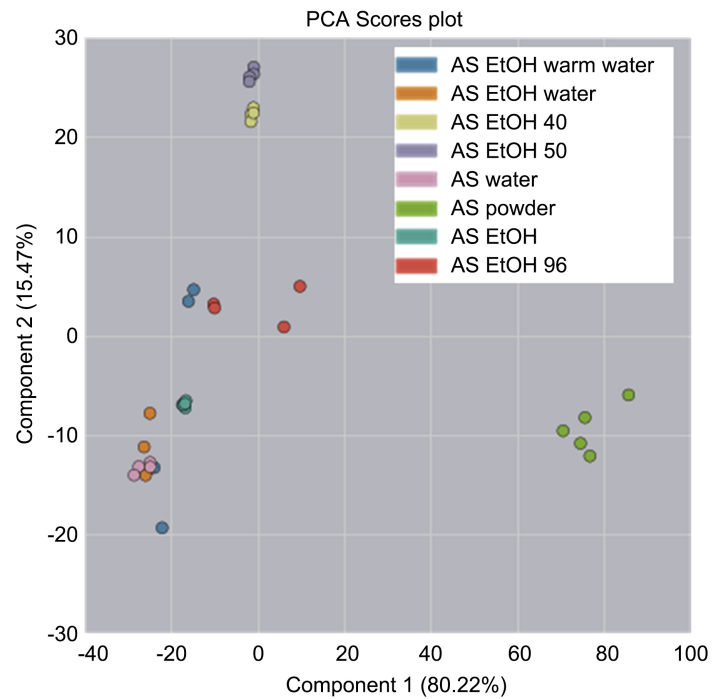


Figure 1. The PCA score plot shows how much the analyzed AS samples differ from each other: spectral component = difference of the sample to the related spectral region. Hereby, component 1 had the highest variance of all data, whilst component 2 represents a lower variance; and hereby, 3 clusters are formed: EtOH 40/50%, powder, and the rest.

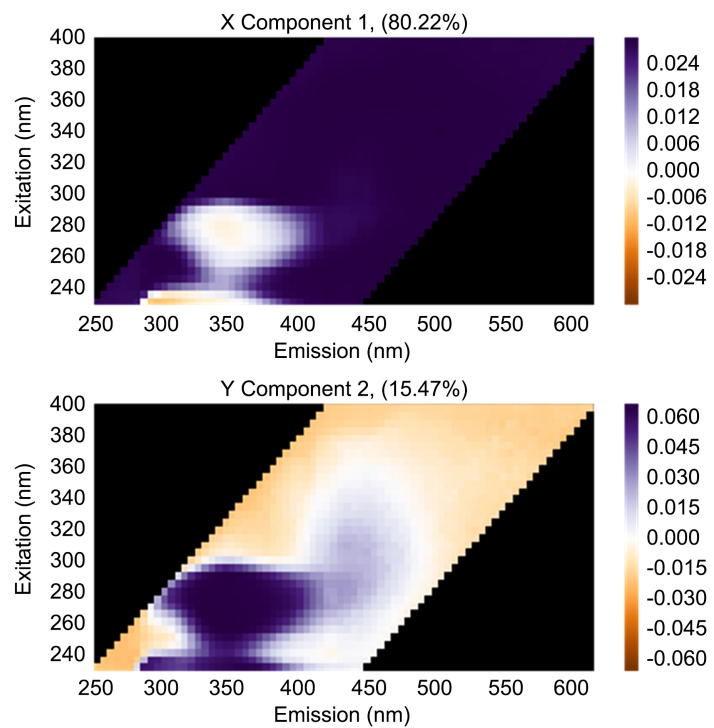


Figure 2. SFS loading plots show which regions cause these differences in the Score plots, hereby the x component 1 shows the first difference in the emission (Em. 280 to 380 nm) and excitation region (Ex. 200 to 300 nm); and the y component 2 show the second difference in the region of Em. 290 to 400 nm and Ex. 200 to 300 nm.

3.2. *Bacopa monnieri* (Brahmi, BR)

BR data were investigated and analyzed as described above (AS data) with LC-MS, GC-MS, and SFS. Hereby, we found several proteins, amino acids, vitamins and most of the expected compounds which are listed in **Table 2** [10] [23] [57] [58] [61] [62] [63], as betulinic acid, alkaloids, and phenols, even brassinolide. However, we could not find the most expected ingredients: bacosides.

The PCA score plots (**Figure 3** and **Figure 4**) show that the most important main component 1 variance of BR samples is 89.80% and the second component variance lay by -9.61%. However, the third and fourth main compounds component variance was less than 1% (component 3: 0.27% and component 4: -0.08%). Hereby, dissolved and macerated BR constitutes a single PCA cluster. Notably, there is a certain difference between ethanol (EtOH) and the other samples (**Figure 3**).

Table 2. Chemical ingredients of *Bacopa monnieri*.

Expected components	Possible found compounds after data analysis
alkaloids; bacosides III; bacosides IV; bacosides V; bacosaponins A; bacosaponins B; bacosaponins C; betulinic acid ; saponin glycosides (jujubogenin bisdesmosides bacosaponins D, E and F); plant sterols ; polyphenols; steroidsaponine (bacoside A, bacoside B); sulfhydryl compounds	A28086B; abequose; abrusoside A; abscisic acid glucose ester; absinthin; acalyphin; acetylglutaloxin; acetyl-L-tyrosine; acetyl-maltose; adiantifoline; agecorynin C; amaranth; amentoflavone; amlodipine; androsterone sulfate; arabinonic acid; asiatic acid ; asiaticoside ; asperuloside; astringin; auriculoside; azithromycin; azukisaponin III; bacampicillin; baccatin III; bacterio-pheophytins; bebeerine; belladonnine; benzoylagmatine; benzylformate; .beta.-Sitosterol ; betulinic acid ; brassinolide ; brevetoxin A, B and C; bruceoside A; cafenstrole; catechin-4beta-ol; chikusetsusaponin IV; chlorophyll b; chlorthiophos; cholesterol sulfate; citric acid; coniferin; cucurbitacin I, A, B, C, D, E and H; curcumin monoglucoside; deltonin; fexaramine; flavonol 3-O-beta-D-glucosyl-(1-2)-beta-D-glucosyl-(1-2)-beta-D-glucoside; forsythiaside; gallopamil; ginkgolide A and C; glyceraldehyde-3-phosphate; hesperetin 7-O-glucoside; KAPA; Lappaol B and D; L-Arginine; L-Cladinose; melampodin; microcystin LR; milbemectin; mupirocin; neocarlinoside; octadecanamide; o-desmethylquinine; oleic acid; oligomycin A, B, C and D; ononin; paeoniflorin; parishin B; p-coumaroylagmatine; pedilstatin; PE(20:2(11Z,14Z)/14:0); PG(18:3(9Z,12Z,15Z)/18:1(11Z)); picrotin; phenol; phloridzin; phenylgalactoside; phosgene; phosphoramidate mustard; phytol; PIP(18:2(9Z,12Z)/16:0); pyrazine, methyl-; pyroglutamic acid; raucaffricine; ruscopine; sakuranin; silanol, ethyldimethyl-; sinapyl aldehyde; stanolone benzoate; stearic acid; stigmasterol; stypanol; verapamil; vitamin E ; zeaxanthin diglucoside; zwittermicin A; 1-acetoxypinoresinol; 1-caffeoyl-beta-D-glucose; 1,2-di-(9Z,12Z,15Z-octadecatrienoyl)-3-(galactosyl-alpha-1-6-galactosyl-beta-1)-glycerol; 1,2-Di-(9Z,12Z,15Z-octadecatrienoyl)-3-(Galactosyl-alpha-1-6-Galactosyl-beta-1)-glycerol; 1-(3,4-Dihydroxyphenyl)-5-hydroxy-3-decanone; 2(3H)-Furanone, dihydro-3,4-dihydroxy; 2-furoic acid; 2-(4-methoxyphenethyl)chromone; 2-keto valeric acid; 2-methoxyestradiol-17 β -3-sulfate; 3-Hydroxyethylchlorophyllide a; 5-(3,4-diacetoxybut-1-ynyl)-2,2'-bithiophene; 6,8a-seco-6,8a-deoxy-5-oxoavermectin "2a" aglycone; 6-gingerol; 12alpha-fluoro-11beta, 17beta-dihydroxyandrost-4-en-3-one; (-)-Apparicine; (+)-Plicamine; (+)-Prosopinine; (+)-Syringaresinol O-beta-D-glucoside; (10S)-Juvenile hormone III acid diol; (1R,6R)-6-Hydroxy-2-succinylcyclohexa-2,4-diene-1-carboxylate; (R)-4'-Deoxyindenestrol; (R)-Benzylsuccinyl-CoA

Selected GC-MS and LC-MS data of the chemical compounds found in *Bacopa monnieri* extracts are listed: expected compounds which are based on literature [23] [57] [58] [61] [62] [63] in the left column and extracted ingredients in the right column. The compounds are limited related to the highest abundance of the retention peak area with a Q Score of 100% and a threshold of 30%, i.e. peptides, proteins, and hormones were discarded (which based on typical plant metabolites and may be found in the most living plants).

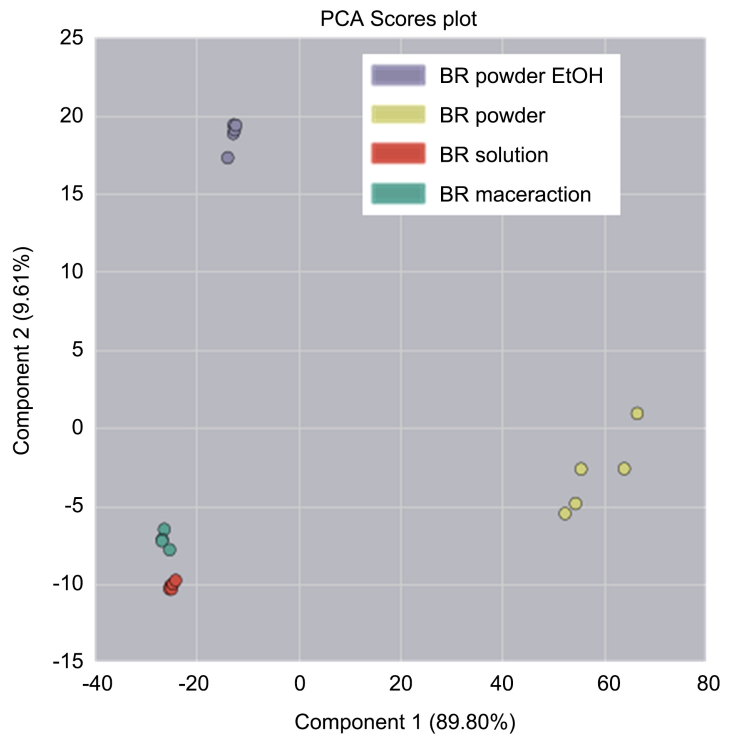


Figure 3. PCA score plots show clusters of the analyzed BR samples. Three clusters are obtained: dissolved/macerated BR, pure powder and powder dissolved in EtOH.

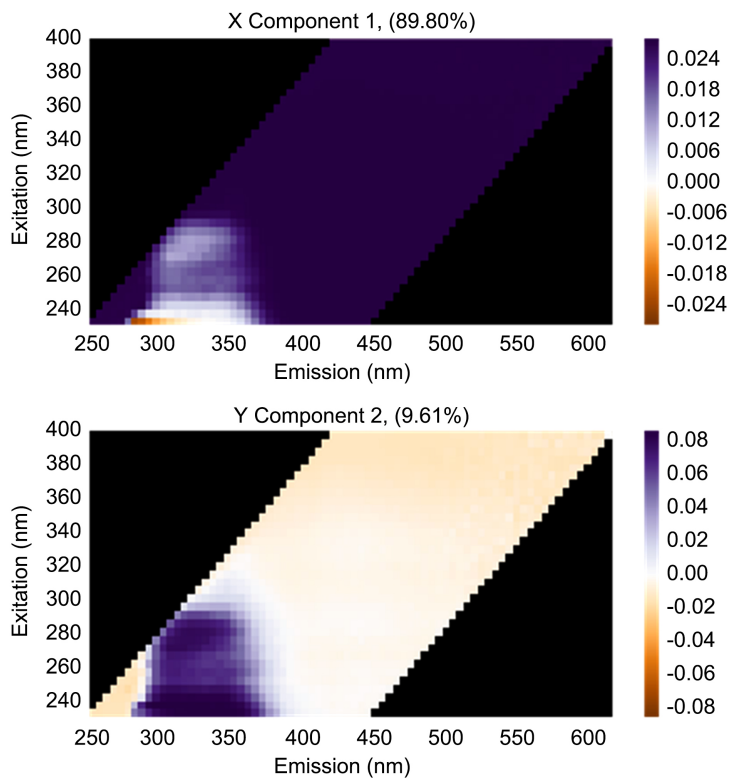


Figure 4. SFS loading plots which show the selected PCA regions: 89.80% (x component 1) in the region of Em. 275 to 360 nm and Ex. 200 to 270 nm; and 9.61% (y component 2) in the area of Em. 270 to 380 nm and Ex. 200 to 280 nm.

3.3. *Centella asiatica* (Gotu kola, GK)

GK LC-MS and GC-MS data are listed in **Table 3** [10] [23] [57] [58] (limited to the main abundance peak areas with a Q Score of 100%; with a threshold around 30%) show more compounds than expected: about 5.000 out of approximately 10.000 components and fragments were identified based on a Q Score of 80% - 100%. Hereby, approximate thousand compounds were specified. Otherwise, several thousand peaks were analyzed but yet not be known. The PCA and score plot of the SFS measurements (**Figure 5** and **Figure 6**) show that between all GK samples (**Figure 5**) exists the highest considerable differences of 64.13% for the first component and only—18.08% of variance for the second component.

Table 3. Chemical ingredients of *Centella asiatica*.

Expected components	Possible found Compounds after data analysis
Asiaticoside derivates, asiatic acid, asiaticoside	asiatic acid; asiaticoside; aurasperone D; avermectin A1a, A1b, A2a, A2b, B1a, B1b, B2a and B2b; bacterio-chlorophyll b; belladonnine; Benzene, 1,3-bis(3-phenoxyphenoxy); benzene, 1-(chloromethyl)-4-nitro-; benzoic acid, 2,4-dichloro-; biflorin; brassinolide; cafenstrole; carvedilol; cephalostatin 1; chikusetsusaponinIa; chlorophyll b; cholesterol sulfate; coniferin; cortisol 21-sulfate; coumermic acid; cyclohexane; cytosine; decoside; dehydrosoyasaponin I; D-Fructofuranose 1,2':2,3'-dianhydride; DG(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/24:1(15Z)/0:0); dianoside A; diginatin; dimethisterone; dodecanoic acid; D-Phenylalanine; eicosane; elatine; emedastine; estradiol benzoate; ethylmalonic acid; euphornin; euscaphic acid; fenclorim; flaccidin B; flavoxanthin; fludrocortisone; 4-fluorobenzyl alcohol; fraxidin; furfural; 2-furanmethanol; 2-furancarboxaldehyde, 5-methyl-; gabapentin; galactosylglycerol; gallic acid; gallopamil; gambirtannine; gamma-Nonalactone; ginkgolide A; glycidyl stearate; glycopeptides; goniiodomin A; hallactone B; hatomasterol; hellicoside; helminthosporol; hematoporphyrin; heptane, 4-ethyl-; heptanoic acid; hildecarpin; huratoxin; hycanthone; hydrocortisone butyrate propionate; 5-hydroxymethylfurfural; inulicin; isoferulic acid; isovalerylglucuronide; istamycin KL1; 3-isoxazolamine, 5-methyl-; kaempferol 3-O- β -D-glucosyl-(1-2)- β -D-glucoside; kanokoside A; karwinskione; ketoconazole; kobusone; lactodifucotetraose; lappaconitine; lappaol C and D; L-Ascorbic acid-2-glucoside; L-cladinoside; lemματοxin; levoglucosenone; limonoate; lithocholic acid sulfate; L-lactic acid; losartan; magnesium protoporphyrinmonomethyl ester; mancinellin; mascaroside; melibiose; methylheptenone; MG(0:0/14:1(9Z)/0:0); neoxanthin; nervonic acid; nocardicin C; n-hexadecanoic acid; O-acetylserine; ochrolifuanine A; octadecanoic acid; ofloxacin; okadaic acid; oleandolide; oligomycin A, B, C and D; ononin; paeonilactone B; papyriferic acid; PD 123177; PE(16:0/22:5(7Z,10Z,13Z,16Z,19Z)); pedilstatin; pentacosanoic acid; pfaffoside A; phthalic acid, di(2-propylpentyl) ester; phosgene; phosphoramidate mustard; phylohydroquinone; piperonylbutoxide; pittoside A; plantagoside; polydine; porphyrin; postin; propofol; protoporphyrin IX; psychotridine; quinic acid; red chlorophyll catabolite; reduced riboflavin; reduced Vitamin K; rehmannioside A; B and C; remikiren; risperidone; rosmarinic acid; ruscopine; saikosaponin A and BK1; sakuranin; salannin; schizonepetoside E; sophoranone; spinoside A; spirodilactone; spironolactone; stearaldehyde; stearic acid; stearidonic acid; stearolic acid; steroid O-sulfate; stevioside; stigmatellin A; succinic acid semialdehyde; teasterone; tetradecanoic acid; tetradecanamide; tetranactin; thymine; tributyrin; triethylene glycol diglycidyl ether; triterpenoid; usambarine; valproic acid; verapamil; vitamin D2; xanthohumol; xylobiose; zearalenone; zeaxanthindigluconide; zingerone; zwittermicin A; 2H-Pyran-3,4,5-triol, tetrahydro-2-methoxy-6-methyl-, 2-Propanol, 1-chloro-, phosphate; 9,12-Octadecadienoic acid (Z,Z)-

GC-MS and LC-MS data of the chemical ingredients of the medical plant *Centella asiatica* are compiled. The expected substances (based on the previous literature [23] [57] [58]) were listed in the 1st column. Most important and unexpected compounds are marked in the 2nd column (unexpected, like stearylaldehyde; stearic acid; stearidonic acid; stearolic acid). Hereby were the compounds limited related to the highest retention peak area with a Q Score of 100% and some components were discarded, *i.e.* peptides, proteins, and hormones, which are only essential for plant survival and may be not relevant for any kind of treatment. Nevertheless the threshold approximately of 20% - 30% was set.

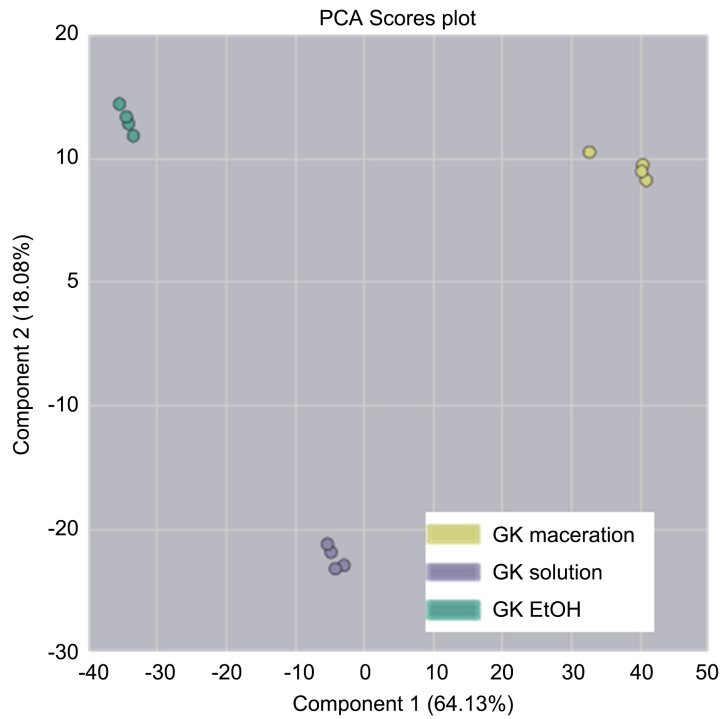


Figure 5. PCA score plots of the GK samples are depicted which show own clusters, *i.e.* there are three separated regions for (1) maceration, (2) EtOH and (3) solution extraction (component = difference).

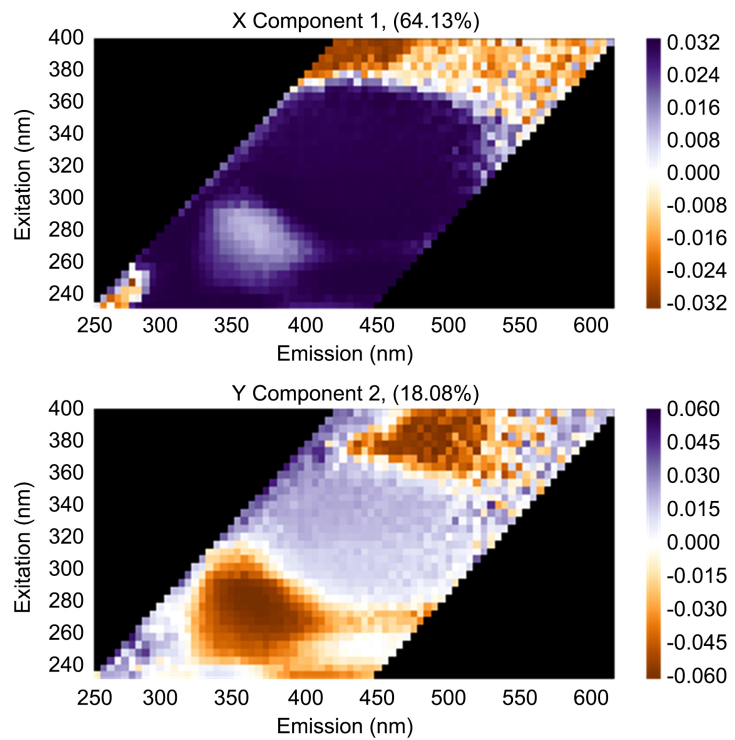


Figure 6. SFS loading plots show for an x component 1 in the region of Em. 330 to 400 nm and Ex. 240 to 300 nm, and a y component 2 in the 1st area of Em. 300 to 470 nm and Ex. 200 to 320 nm; and the 2nd area of Em. 425 to 530 nm and Ex. 350 to 400 nm; which are used for the PCA Score plots of GK.

4. Discussion

Based on LC-MS, GC-MS and SFS investigations AS, GK and BR bioactive compounds were analyzed and statistically significant (by Q score, retention peak threshold, and PCA) substances were indicated according to literature and databases (compound libraries).

The screenings provide evidence of expected compounds, *i.e.* membrane proteins, amino acids, minerals, withaferin A (AS), asiaticosides (GK) and bacoside A (BR), and also some new and unexpected ingredients were identified which were contained in the NIST and Metlin library [29] [30], like reduced vitamin K, vitamin E, coniferin, and cortisol 21-sulfate. Several hundred compounds which have not been listed in the accessible libraries and literature need to be further investigated. These compounds might be fragments due to the influence of Soxhlet extraction and other steps of sample treatments (Table 4) e.g. might have been influenced by certain extracted metabolites that biochemical degradation took place.

For *Withania somnifera* main expected compounds, like withaferin A, some withanolides, and cholesterols, were identified and are listed in Table 1. Typical and expected plant components like amino acids, chlorophyll b, and membrane proteins were found, which not all are provided in detail (approximately 50% - 60% of the entire plant extract content). Please see supplementary material for more information (Table 1 and Table 4). However, from SFS measurements we may come to the conclusion that the maximum difference in PCA plots can be identified between liquid versus powder of AS sample. The largest difference excluding the powder is established between clusters of EtOH 40 and EtOH 50 and all other liquid AS samples, which may indicate the importance of proper selection for optimal medicinal usage.

Bacopa monnieri's expected main components were bacoside A and B which could not be found with LC-MS and GC-MS measurements, which might be due to fragmentation through the Soxhlet extraction or hard ionization: long extraction time with heating and the possible presence of enzymes or high ionization voltages. Nevertheless, we found numerous alkaloids, amino acids, proteins, betulinic acid, phenols and vitamins, from which some of them were expected compounds (Table 2). Furthermore, approximately 50% of the entire extracts and 60% of the dried nutraceuticals' contents were proteins, peptides, vitamins, amino acids, and other substances. Hereby, the SFS measurements (Figure 1 and Figure 3) show that the most important difference of BR samples lays between powdered and liquid samples like AS, whereas dissolved and macerated BR form a cluster.

Centella asiatica plant extracts show the expected main substances, *i.e.* asiaticosides and asiatic acid. Additionally, we found a considerable number of known compounds by NIST and Metlin [29] [30]. Still, a considerable number of approximately 1000 to 5000 unknown compounds remained. Unexpected components like stearaldehyde, stearic acid, stearidonic acid, and stearolic acid seem to

Table 4. List of prepared and investigated samples.

Sample full name	Sample short name	Preparation method	Kind of investigation	Comment	
<i>Withania somnifera</i> sample	sample 2	Soxhlet extraction with ethanol, Methanol extraction	LC-MS		
<i>Withania somnifera</i> methanol	AS MeOH	Soxhlet extraction, evaporation of ethanol and solved in methanol	GC-MS		
<i>Withania somnifera</i> maceration in 50% ethanol/warm MilliQ water mixture	AS EtOH warm water	Maceration	SFS	AS plant extracts and nutraceuticals were tested in previous studies with <i>in vitro</i> Amyloid Beta fibrillation measurements and found as an inhibitor of the fibrillation process dependent on which kind of Amyloid Beta: A β -40 (60% - 90%), A β -42 (80% - 90%) or M-A β -40 (33% - 60%) had been applied [10] [23]. The inhibition efficiency of the extracts was higher compared to the pure nutraceuticals.	
<i>Withania somnifera</i> maceration in 50% ethanol/MilliQ water mixture	AS EtOH water	Maceration	SFS		
<i>Withania somnifera</i> maceration in 40% ethanol	AS EtOH 40	Maceration	SFS		
<i>Withania somnifera</i> maceration in 50% ethanol	AS EtOH 50	Maceration	SFS		
<i>Withania somnifera</i> maceration in MilliQ water	AS water	Maceration	SFS		
<i>Withania somnifera</i> unsolved pure powder	AS powder		SFS		
<i>Withania somnifera</i> maceration in 80% ethanol/20% MilliQ water mixture	AS EtOH	Maceration	SFS		
<i>Withania somnifera</i> maceration in 96% ethanol	AS EtOH 96	Maceration	SFS		
<i>Bacopa monnieri</i>	Sample 3	Soxhlet extraction with ethanol, Methanol extraction	LC-MS		
<i>Bacopa monnieri</i> methanol	BRMeOH	Soxhlet extraction, evaporation of ethanol and solved in methanol	GC-MS		BR plant extracts and nutraceuticals were tested in previous studies with <i>in vitro</i> Amyloid Beta fibrillation tests and found as inhibitors [10] [23]. Also here, the inhibition efficiency of the extracts turned out to be higher than for the nutraceuticals.
<i>Bacopa monnieri</i> powder solved in 80% ethanol	BR powder EtOH	Powder solved in ethanol	SFS		
<i>Bacopa monnieri</i> powder unsolved	BR powder		SFS		
<i>Bacopa monnieri</i> powder in water solved	BR solution	Powder solved in MilliQ water	SFS		
<i>Bacopa monnieri</i> fresh leave maceration extract	BR maceration	Maceration	SFS		
<i>Centella asiatica</i>	Sample 1	Soxhlet extraction with ethanol, Methanol extraction	LC-MS		
<i>Centella asiatica</i> methanol	GK MeOH	Soxhlet extraction, evaporation of ethanol and solved in methanol	GC-MS	GK plant extracts and nutraceuticals were tested in previous studies, see above [10] [23]. Also here, the inhibitions of extracts were favored.	
<i>Centella asiatica</i> fresh leave maceration extract	GK maceration	Maceration	SFS		
<i>Centella asiatica</i> powder solved in water	GK solution		SFS		
<i>Centella asiatica</i> powder solved in EtOH	GK EtOH		SFS		

be of importance. They were mostly considered to be in AS and are suspected to have a relevant medical impact.

Notably, in all three sample classes stearic acid, asiaticosides and asiatic acid were found which might have been caused by fragmentation through Soxhlet extraction, *i.e.* degradation (maybe the sterilization of the extraction filter was not sufficient enough). Still, we may not exclude these compounds which are essential for all three plants and maybe even the reason of their medicinal basic functioning—all three plants were used in the Ayurveda medicine against brain diseases [58]—which has to be verified further.

Also, we noted relevant differences between liquid and pure powder samples which may indicate, that it has to be analyzed, which kind of supply for the treatment might be more efficient: via solid food for dissolving or provided liquid to the stomach, inhalation, rectal, injection, and transdermal.

For future progressing, extended investigations are recommended to clarify unknown compounds and to distinguish between original and metabolized compounds. Quantity and quality of the known ingredients have to be specified. Nevertheless, in this work, we made a step forward in identifying the complexity of ingredients and could provide suggestions in which direction to lead next investigational steps, in order to gain more profound knowledge to develop a natural and simple treatment path for preventing or curing Alzheimer's disease and other brain maladies.

Acknowledgements

Acknowledged are the financial supports from Estonian Research Council for the projects PUT1534 and IUT19-27 for doctoral laboratory studies and usage of technology. This research did not receive any specific grant from commercial funding agencies or not-for-profit sectors.

Grateful thanks to Tallinn University of Technology and Karlsruhe Institute of Technology.

This work has been conducted in motivating memory for doctor Alois Alzheimer, family members and friends: Gudrun and Mario Scholtze, Heinz E. Witter, Ingeburg Thomas, Mr. and Mrs. Samoson.

Availability of Data and Materials

The data that support the findings of this study are available from Steffi Witter but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of Steffi Witter.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

- [1] Goyal, M.R., Suleria, H., Ayeleso, A.O., Joel, T.J. and Panda, S.K. (2020) The Therapeutic Properties of Medicinal Plants: Health-Rejuvenating Bioactive Compounds of Native Flora. Apple Academic Press, Oakville.
<https://doi.org/10.1201/9780429265204>
- [2] Martinez, J.L., Munoz-Acevedo, A. and Rai, M. (2019) Ethnobotany: Local Knowledge and Traditions. CRC Press, Boca Raton.
<https://doi.org/10.1201/9780429424069>
- [3] Patra, J.K., Das, G., Kumar, S. and Thatoi, H.N. (2019) Ethnopharmacology and Biodiversity of Medicinal Plants. Apple Academic Press, Palm Bay, 1.
<https://doi.org/10.1201/9780429398193>
- [4] Goyal, M.R. and Suleria, H.A.R. (2019) Human Health Benefits of Plant Bioactive Compounds: Potentials and Prospects, Innovations in Plant Science for Better Health. Apple Academic Press, Palm Bay, 1. <https://doi.org/10.4324/9780429457913>
- [5] Barba, F.J., et al. (2019) Innovative Thermal and Non-Thermal Processing, Bioaccessibility and Bioavailability of Nutrients and Bioactive Compounds. Elsevier, Washington DC.
- [6] Mazina, J., Vaher, M., Kuhtinskaja, M., Poryvkina, L. and Kaljurand, M. (2015) Fluorescence, Electrophoretic and Chromatographic Fingerprints of Herbal Medicines and Their Comparative Chemometric Analysis. *Talanta*, **139**, 233-246.
<https://doi.org/10.1016/j.talanta.2015.02.050>
- [7] Goyal, M.R. and Chauhan, D.N. (2019) Plant- and Marine-Based Phytochemicals for Human Health: Attributes, Potential, and Use. Apple Academic Press, Oakville.
<https://doi.org/10.1201/9781351251983>
- [8] Goyal, M.R., Joy, P.P. and Suleria, H. (2019) Plant Secondary Metabolites for Human Health: Extraction of Bioactive Compounds, Innovations in Plant Science for Better Health: From Soil to Fork. Apple Academic Press, Palm Bay, 1.
<https://doi.org/10.1201/9780429425325>
- [9] Patra, J.K., Das, G., Kumar, S. and Thatoi, H.N. (2019) Ethnopharmacology and Biodiversity of Medicinal Plants. Apple Academic Press, Palm Bay.
<https://doi.org/10.1201/9780429398193>
- [10] Witter, S., Samoson, A., Vilu, R. and Witter, R. (2019) Screening of Nutraceuticals and Plant Extracts for Inhibition of Amyloid-Beta Fibrillation. *Journal of Alzheimer's Disease*, **73**, 1-10.
- [11] Curry, M., Hazard-Daniel, A. and Daniel, H.J. (1999) Nutraceuticals. *Science*, **286**, 1854-1855. <https://doi.org/10.1126/science.286.5446.1853e>
- [12] Borghi, C. and Cicero, A.F. (2017) Nutraceuticals with a Clinically Detectable Blood Pressure-Lowering Effect: A Review of Available Randomized Clinical Trials and Their Meta-Analyses. *British Journal of Clinical Pharmacology*, **83**, 163-171.
<https://doi.org/10.1111/bcp.12902>
- [13] Carrasco-Gallardo, C., Farias, G.A., Fuentes, P., Crespo, F. and Maccioni, R.B. (2012) Can Nutraceuticals Prevent Alzheimer's Disease? Potential Therapeutic Role of a Formulation Containing Shilajit and Complex B Vitamins. *Archives of Medical Research*, **43**, 699-704. <https://doi.org/10.1016/j.arcmed.2012.10.010>
- [14] Chao, J., Leung, Y., Wang, M. and Chang, R.C. (2012) Nutraceuticals and Their Preventive or Potential Therapeutic Value in Parkinson's Disease. *Nutrition Reviews*, **70**, 373-386. <https://doi.org/10.1111/j.1753-4887.2012.00484.x>
- [15] Chen, Q.M. and Alpert, J.S. (2016) Nutraceuticals: Evidence of Benefit in Clinical

- Practice? *The American Journal of Medicine*, **129**, 897-898.
<https://doi.org/10.1016/j.amjmed.2016.03.036>
- [16] Daliu, P., Santini, A. and Novellino, E. (2019) From Pharmaceuticals to Nutraceuticals: Bridging Disease Prevention and Management. *Expert Review of Clinical Pharmacology*, **12**, 1-7. <https://doi.org/10.1080/17512433.2019.1552135>
- [17] Daniel, O. and Mauskop, A. (2016) Nutraceuticals in Acute and Prophylactic Treatment of Migraine. *Current Treatment Options in Neurology*, **18**, 14. <https://doi.org/10.1007/s11940-016-0398-1>
- [18] Das, L., Bhaumik, E., Raychaudhuri, U. and Chakraborty, R. (2012) Role of Nutraceuticals in Human Health. *Journal of Food Science and Technology*, **49**, 173-183. <https://doi.org/10.1007/s13197-011-0269-4>
- [19] Das, S.K. (2012) Free Radicals, Antioxidants and Nutraceuticals in Health, Disease & Radiation Biology. *Indian Journal of Biochemistry and Biophysics*, **49**, 291-292.
- [20] Davi, G., Santilli, F. and Patrono, C. (2010) Nutraceuticals in Diabetes and Metabolic Syndrome. *Cardiovascular Therapeutics*, **28**, 216-226. <https://doi.org/10.1111/j.1755-5922.2010.00179.x>
- [21] de Silva, A. and Lanerolle, P. (2011) Nutraceuticals: Concepts and Controversies. *Ceylon Medical Journal*, **56**, 171-173. <https://doi.org/10.4038/cmj.v56i4.3901>
- [22] Del Ben, M., Polimeni, L., Baratta, F., Pastori, D. and Angelico, F. (2017) The Role of Nutraceuticals for the Treatment of Non-Alcoholic Fatty Liver Disease. *British Journal of Clinical Pharmacology*, **83**, 88-95. <https://doi.org/10.1111/bcp.12899>
- [23] Witter, S., Witter, R., Vilu, R. and Samoson, A. (2018) Medical Plants and Nutraceuticals for Amyloid Beta Fibrillation Inhibition. *Journal of Alzheimer's Disease Reports*, **2**, 14. <https://doi.org/10.3233/ADR-180066>
- [24] Perez-Serradilla, J.A., Ortiz, M.C., Sarabia, L. and de Castro, M.D. (2007) Focused Microwave-Assisted Soxhlet Extraction of Acorn Oil for Determination of the Fatty Acid Profile by GC-MS. Comparison with Conventional and Standard Methods. *Analytical and Bioanalytical Chemistry*, **388**, 451-462. <https://doi.org/10.1007/s00216-007-1227-x>
- [25] Campanella, B., Pulidori, E., Onor, M., Passaglia, E., Tegli, S., Izquierdo, C.G. and Bramanti, E. (2016) New Polymeric Sorbent for the Solid-Phase Extraction of Indole-3-Acetic Acid from Plants Followed by Liquid Chromatography—Fluorescence Detector. *Microchemical Journal*, **128**, 68-74. <https://doi.org/10.1016/j.microc.2016.04.014>
- [26] Godlewska, K., Michalak, I., Tuhy, L. and Chojnacka, K. (2016) Plant Growth Bio-stimulants Based on Different Methods of Seaweed Extraction with Water. *BioMed Research International*, **2016**, Article ID: 5973760. <https://doi.org/10.1155/2016/5973760>
- [27] Pereira, C., Barros, L. and Ferreira, I.C.F.R. (2016) Extraction, Identification, Fractionation and Isolation of Phenolic Compounds in Plants with Hepatoprotective Effects. *Journal of the Science of Food and Agriculture*, **96**, 1068-1084. <https://doi.org/10.1002/jsfa.7446>
- [28] Sibul, F.S., Orcic, D.Z., Svircev, E. and Mimica-Dukic, N.M. (2016) Optimization of Extraction Conditions for Secondary Biomolecules from Various Plant Species. *Hemijska Industrija*, **70**, 473-483. <https://doi.org/10.2298/HEMIND150531053S>
- [29] Metlin, A.M. (2018) PCD Library. In: Technologies, A., Ed., *Agilent Technologies*, Santa Clara, CA. <https://www.agilent.com/about/companyinfo/index.html>
- [30] Linstrom, P.J. and Mallard, W.G. (2019) NIST Library, NIST Chemistry WebBook.

- National Institute of Standards and Technology, Gaithersburg MD, 20899, Online.
- [31] Bengraïne, K. and Marhaba, T.F. (2003) Comparison of Spectral Fluorescent Signatures-Based Models to Characterize DOM in Treated Water Samples. *Journal of Hazardous Materials*, **100**, 117-130. [https://doi.org/10.1016/S0304-3894\(03\)00071-2](https://doi.org/10.1016/S0304-3894(03)00071-2)
- [32] Poryvkina, L., Tsvetkova, N. and Sobolev, I. (2014) Evaluation of Apple Juice Quality Using Spectral Fluorescence Signatures. *Food Chemistry*, **152**, 573-577. <https://doi.org/10.1016/j.foodchem.2013.11.131>
- [33] Babichenko, S., Leeben, A., Poryvkina, L., van der Wagt, R. and de Vos, F. (2000) Fluorescent Screening of Phytoplankton and Organic Compounds in Sea Water. *Journal of Environmental Monitoring*, **2**, 378-383. <https://doi.org/10.1039/b002780o>
- [34] Dudeizak, A.E., Babichenko, S.M., Poryvkina, L.V. and Saar, K.J. (1991) Total Luminescent Spectroscopy for Remote Laser Diagnostics of Natural Water Conditions. *Applied Optics*, **30**, 453-458. <https://doi.org/10.1364/AO.30.000453>
- [35] He, J.H., Cheng, Y.J., Han, Y.L., Zhang, H. and Yang, T. (2008) Investigation of Quantitative Detection of Water Quality Using Spectral Fluorescence Signature. *Spectroscopy and Spectral Analysis*, **28**, 1870-1874.
- [36] Zhao, N.J., Liu, W.Q., Cui, Z.C., Zhang, Y.J., Liu, J.G., Li, H.B. and Yang, L.S. (2006) Analysis of the Characters of Organic Matter in Water Using Spectral Fluorescence Signature and Fitting Gaussian. *Spectroscopy and Spectral Analysis*, **26**, 922-924.
- [37] Babichenko, L.P.S., et al. (1998) Fluorescent Signatures in Environmental Analysis. In: *The Encyclopedia of Environmental Analysis and Remediation*, John Wiley & Sons Inc., New York, 1787-1791.
- [38] Keen, B.A. (2019) Feature Scaling with Scikit-Learn. <http://benalexkeen.com/feature-scaling-with-scikit-learn>
- [39] Patel, S.B., Rao, N.J. and Hingorani, L.L. (2016) Safety Assessment of *Withania somnifera* Extract Standardized for Withaferin A: Acute and Sub-Acute Toxicity Study. *Journal of Ayurveda and Integrative Medicine*, **7**, 30-37. <https://doi.org/10.1016/j.jaim.2015.08.001>
- [40] Ganguly, B., Kumar, N., Ahmad, A.H. and Rastogi, S.K. (2018) Influence of Phytochemical Composition on *in Vitro* Antioxidant and Reducing Activities of Indian Ginseng [*Withania somnifera* (L.) Dunal] Root Extracts. *Journal of Ginseng Research*, **42**, 463-469. <https://doi.org/10.1016/j.jgr.2017.05.002>
- [41] Tripathi, N., Shrivastava, D., Ahmad Mir, B., Kumar, S., Govil, S., Vahedi, M. and Bisen, P.S. (2018) Metabolomic and Biotechnological Approaches to Determine Therapeutic Potential of *Withania somnifera* (L.) Dunal: A Review. *Phytomedicine*, **50**, 127-136. <https://doi.org/10.1016/j.phymed.2017.08.020>
- [42] Kulkarni, S.K., Akula, K.K. and Dhir, A. (2008) Effect of *Withania somnifera* Dunal Root Extract against Pentylentetrazol Seizure Threshold in Mice: Possible Involvement of GABAergic System. *Indian Journal of Experimental Biology*, **46**, 465-469.
- [43] Kulkarni, S.K. and Dhir, A. (2008) *Withania somnifera*: An Indian Ginseng. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, **32**, 1093-1105. <https://doi.org/10.1016/j.pnpbp.2007.09.011>
- [44] Pandey, A., Bani, S., Dutt, P., Kumar Satti, N., Avtar Suri, K. and Nabi Qazi, G. (2018) Multifunctional Neuroprotective Effect of Withanone, a Compound from *Withania somnifera* Roots in Alleviating Cognitive Dysfunction. *Cytokine*, **102**, 211-221. <https://doi.org/10.1016/j.cyto.2017.10.019>
- [45] Elango, S.R.A.R. (2011) Estimation of Alkaloid Content of Ashwagandha (*Witha-*

- nia somnifera*) with HPLC Methods. *Journal of Experimental Sciences*, **2**, 39-41.
- [46] Trivedi, M.K., Panda, P., Sethi, K.K. and Jana, S. (2017) Metabolite Profiling in *Withania somnifera* Roots Hydroalcoholic Extract Using LC/MS, GC/MS and NMR Spectroscopy. *Chemistry Biodiversity*, **14**, e1600280. <https://doi.org/10.1002/cbdv.201600280>
- [47] Thirugnanasambantham, P.S., Oh, T.-J. and Choi, H.-K. (2015) Comparative Chemometric Profiles between Leaf Tissues of *Withania somnifera* Cultured *in Vitro* and Field. *International Journal of Pharmacy and Pharmaceutical Sciences*, **7**, 66-71.
- [48] Trivedi, D., Nayak, G., Lee, A.C., Hancharuk, A., Sand, C.M., Schnitzer, D.J., Thanasi, R., Meagher, E.M., Pyka, F.A., Gerber, G.R., Stromsnas, J.C., Shapiro, J.M., Streicher, L.N., Hachfeld, L.M., Hornung, M.C., Rowe, P.M., Henderson, S.J., Benson, S.M., Holmlund, S.T., Salters, S.P., Panda, P., Jana, S., et al. (2017) LC-MS, GC-MS, and NMR Spectroscopic Analysis of *Withania somnifera* (Ashwagandha) Root Extract after Treatment with Energy of Consciousness (The Trivedi Effect). *European Journal of Biophysics*, **5**, 10. <https://doi.org/10.11648/j.ejb.20170502.11>
- [49] Saravana Kumar, A., Aslam, A., Shajahan, A., et al. (2011) The Phytochemical Constituents of *Withania somnifera* and *Withania obtusifolia* by GCMS Analysis. *International Journal of Pharmacognosy and Phytochemical Research*, **3**, 31-34.
- [50] Gajbhiye, N.A., Kumar, S., et al. (2017) Effect of Extraction Methods on Yield, Phytochemical Constituents and Antioxidant Activity of *Withania somnifera*. *Arabian Journal of Chemistry*, **10**, 7. <https://doi.org/10.1016/j.arabjc.2013.02.015>
- [51] Sharma, N., Kishor, K., Singh, K., Sharma, N., et al. (2018) Comparative GC-MS Analysis of Leaf and Root Extract of Medicinal Plant *Withania somnifera*. *World Journal of Pharmaceutical Research*, **7**, 17.
- [52] Mirjalili, M.H., Moyano, E., Bonfill, M., Cusido, R.M. and Palazon, J. (2009) Steroidal Lactones from *Withania somnifera*, an Ancient Plant for Novel Medicine. *Molecules*, **14**, 2373-2393. <https://doi.org/10.3390/molecules14072373>
- [53] Mishra, L.C., Singh, B.B. and Dagenais, S. (2000) Scientific Basis for the Therapeutic Use of *Withania somnifera* (Ashwagandha): A Review. *Alternative Medicine Review*, **5**, 334-346.
- [54] Singh, V.K., Jamil, S.S., et al. (2012) Phytochemical and Pharmacological Profile of *Withania somnifera* Dunal: A Review. *Journal of Applied Pharmaceutical Sciences*, **2**, 6.
- [55] Sangwan, N.S., Sabir, F., Mishra, S., Bansal, S. and Sangwan, R.S. (2014) Withanolides from *Withania somnifera* Dunal: Development of Cellular Technology and Their Production. *Recent Patents on Biotechnology*, **8**, 25-35. <https://doi.org/10.2174/1872208307666131218125300>
- [56] Kuboyama, T., Tohda, C. and Komatsu, K. (2005) Neuritic Regeneration and Synaptic Reconstruction Induced by Withanolide A. *British Journal of Pharmacology*, **144**, 961-971. <https://doi.org/10.1038/sj.bjp.0706122>
- [57] Bhutya, R.K. (2011) Ayurvedic Medicinal Plants of India. Scientific Publishers (India), Jodhpur.
- [58] Rao, R.V., Descamps, O., John, V. and Bredesen, D.E. (2012) Ayurvedic Medicinal Plants for Alzheimer's Disease: A Review. *Alzheimer's Research & Therapy*, **4**, Article No. 22. <https://doi.org/10.1186/alzrt125>
- [59] Scartezzini, P. and Speroni, E. (2000) Review on Some Plants of Indian Traditional Medicine with Antioxidant Activity. *Journal of Ethnopharmacology*, **71**, 23-43. [https://doi.org/10.1016/S0378-8741\(00\)00213-0](https://doi.org/10.1016/S0378-8741(00)00213-0)

- [60] Akhil Gupta, A.M., Jha, K.K. and Kumar, A. (2011) Nature's Treasurer: Plants Acting on Colon Cancer. *Journal of Stress Physiology and Biochemistry*, **7**, 217-231.
- [61] Nathan, P.J., Clarke, J., Lloyd, J., Hutchison, C.W., Downey, L. and Stough, C. (2001) The Acute Effects of an Extract of *Bacopa monniera* (Brahmi) on Cognitive Function in Healthy Normal Subjects. *Human Psychopharmacology*, **16**, 345-351. <https://doi.org/10.1002/hup.306>
- [62] Stough, C., Lloyd, J., Clarke, J., Downey, L.A., Hutchison, C.W., Rodgers, T. and Nathan, P.J. (2001) The Chronic Effects of an Extract of *Bacopa monniera* (Brahmi) on Cognitive Function in Healthy Human Subjects. *Psychopharmacology (Berl)*, **156**, 481-484.
- [63] Roodenrys, S., Booth, D., Bulzomi, S., Phipps, A., Micallef, C. and Smoker, J. (2002) Chronic Effects of Brahmi (*Bacopa monnieri*) on Human Memory. *Neuropsychopharmacology*, **27**, 279-281. [https://doi.org/10.1016/S0893-133X\(01\)00419-5](https://doi.org/10.1016/S0893-133X(01)00419-5)