

Article

The Effect of Lactic Acid Fermentation on Extraction of Phenolics and Flavonoids from Sage Leaves

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Featured Application: Improved extraction of polyphenolics and flavonoids from sage leaves.

Abstract: This work analysed the effect of spontaneous fermentation of sage leaves on the release and extraction of flavonoid and phenolic compounds. Chemical profiling based on thin-layer chromatography was used to compare different extracts from two sage species, common sage (*Salvia officinalis*) and white sage (*Salvia apiana*). Non-fermented *Salvia apiana* extracts are richer in antioxidants, phenolics, and terpenoids. Fermentation significantly enhances extraction of total phenolics, flavonoids, and antioxidants only from *Salvia officinalis* leaves, while it does not affect extraction from *Salvia apiana* leaves. In each 20 µL of extract, extraction of polyphenolics increases from 6.55 to 21.01 µg gallic acid equivalents (GAE), anti-oxidants from 0.68 to 2.12 µg GAE, and flavonoid content from 48.01 to 65.33 µg RE (rutin equivalents). Higher antioxidant activity in fermented *Salvia apiana* ethyl acetate extracts is associated with an increased concentration of phenolics and phenolic terpenoids. However, in *Salvia officinalis*, the higher antioxidant activity of fermented extract is a result of the release and improved extraction of flavonoids, as there is no increase in the extraction of phenolics. Lactic acid produced via fermentation and proline from *Salvia officinalis* leaves forms a natural deep eutectic solvent (NADES), which significantly increases the solubility of flavonoids.

Keywords: fermentation; flavonoids; high-performance thin-layer chromatography; phenolics; *Salvia officinalis*; *Salvia apiana*



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1. Introduction

Lamiaceae, or the mint family, is a widespread family of flowering plants known for its aromatic members. These herbs are abundant in phenolic compounds with potent antioxidant activities [1]. In addition to common plant antioxidants, these herbs also contain specific antioxidants, such as carnosic acid, carvacrol, and rosmarinic acid [2]. Many members of this family are used as flavouring and culinary herbs. *Salvia*, known as sage, is the largest genus in the Lamiaceae family, with more than 900 different species worldwide, especially in warm and tropical areas. Many *Salvia* species are native to the Mediterranean region [3]. Many species have been used as medicinal plants for a long time, as suggested by its genus name *Salvia*, which originated from the Latin 'salvare' meaning "to save". Two of the most commonly used sage species worldwide are *Salvia officinalis* and *Salvia apiana*.

This study compares the phytochemical profiles of *Salvia officinalis* leaf extracts with *Salvia apiana* leaf extracts in respect to their polyphenolic, flavonoid, and terpenoid content using high-performance thin-layer chromatography (HPTLC) fingerprint analysis. Different extraction solvent and extraction methods were compared. Most of the phytochemicals that are reported from these two species are isolated from their essential oil, methanol or

ethanol extracts [4], aqueous ethanol extracts [5], ethyl acetate, and hexane extracts [6]. Although closely related, *Salvia apiana* and *Salvia officinalis* are different in both appearance and use. *Salvia officinalis*, or common sage, is the best known and most widely used. The specific name “*officinalis*” in the binomial system of nomenclature, introduced by Linnaeus, embodies the established medicinal use over many centuries [7]. *Salvia apiana*, or white sage, is native to southern California and parts of Mexico. Chumash Native Americans of southern California consider it sacred, burning the leaves for purification ceremonies and using it in Indian native healing practices [8]. As medicinal information was passed on orally, relatively little was known about the therapeutic potential of white sage until recently. HPTLC is an ideal method for chromatographic plant extract fingerprinting, due to its simplicity and speed, low solvent use, and minimal or no sample preparation [9]. Moreover, many samples can be run on the same plate for comparison.

We also wanted to examine whether spontaneous fermentation could enhance the extraction of antioxidants and phenolics. Plant phenolics are present in a soluble or free form, and in an insoluble or bound form. Conventional methods of extraction do not extract significant amounts of bound phenolics, as they need to be released by chemical or enzymatic pre-treatments [10]. These pre-treatments may chemically change the extract and may have negative toxicological effects [11].

Offline hyphenation of HPTLC and attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy allows for chemical characterization of bioactive compound(s). The strong background absorption of the silica gel in the fingerprint region limits direct (in situ) TLC-FTIR measurement from the plate to the region between 3500–1300 cm^{-1} .

2. Materials and Methods

2.1. Chemicals and Materials

Analytical grade reagents were used. Acetic acid, ethanol, methanol, *n*-hexane, and sulfuric acid were bought from Merck (Bayswater, Australia). Ethyl acetate, 2,2-di(4-tert-octylphenyl)-1-picrylhydrazylfree radical (DPPH●), gallic acid (97%), *p*-anisaldehyde were obtained from ACROS organics (New Jersey, USA). Iron(III) chloride (FeCl_3) reagent grade (97%) and aluminum chloride (AlCl_3) reagent grade (98%) were obtained from (Sigma-Aldrich, Macquarie Park, Australia). Deionized (Milli-Q, Millipore) water was used for all aqueous solutions. Glass HPTLC silica gel 60 F254 plates, 20 cm \times 10 cm (Merck, Bayswater, Australia) were used.

2.2. Sample Preparation

Salvia officinalis and *Salvia apiana* plants, purchased from a local nursery, were supplied by Renaissance Herbs Australasia Pty Ltd. (Lilydale Vic, Australia). Aerial parts of two species were collected during the flowering stage. After collection, plant material was freeze-thawed to improve extraction of components. Leaves were washed with distilled water, frozen at $-80\text{ }^\circ\text{C}$ for 6 h, and then thawed for 2 h at $20\text{ }^\circ\text{C}$. This freeze-thaw process was repeated. After a second thawing, samples were air-dried, ground to a fine powder, and stored in a refrigerator at $4\text{ }^\circ\text{C}$.

Ground plant material was extracted using three common solvents (methanol, ethanol, and ethyl acetate). A total of 5 g of powdered leaves was accurately weighed, placed in a conical flask, stoppered, and extracted five times by vigorously shaking for 20 min with 50 mL of solvent and then filtered. Collected extracts of the same solvent were combined and the solvent evaporated to dryness. For HPTLC analysis, a 10 mg/mL solution of dried plant extract was made in the extraction solvent.

2.3. Extractive Fermentation

The initial inoculum of lactic acid bacteria (LAB) for the spontaneous fermentation was from the plant microecosystem. A total of 10 g of powdered leaves was placed in approximately 100 mL of 3% *w/v* sodium chloride solution as fermentation brine. Lactic acid bacteria are salt-loving, so salt creates an environment in which this salt-tolerant

bacteria grow and metabolise lactic acid from sugars. Brined samples were fermented in the dark, at the room temperature (20–25 °C), for five days.

After a week, fermented material was extracted with ethyl acetate. Ethyl acetate was separated from the fermentation broth using fractional freezing. After freezing, the ice and plant material were frozen, while the ethyl acetate, due to its significantly lower freezing point of -83.97 °C [12] was still a liquid. The liquid extract was removed, filtered, and evaporated to dryness. For analysis, a 10 mg/mL solution of extract in ethyl acetate was prepared. Unfortunately, methanol and ethanol could not be used as extraction solvents from fermented material due to their high miscibility with water. When added to brining solution, methanol and ethanol act as anti-solvents for sodium chloride and decrease its solubility. This results in the precipitation of the salt, and further affects solubility and extraction of organic compounds from the plant material.

2.4. Planar Chromatography

Samples were sprayed onto the plate as 8 mm bands, with an 8 mm distance from the bottom and 15 mm from the left edge using a Linomat 5 (CAMAG, Muttenz, Switzerland). Chromatographic separation was carried out in an automated multiple development chamber AMD2 (CAMAG) with a mixture of *n*-hexane-ethyl acetate-acetic acid (60:36:4 *v/v/v*) as a mobile phase, up to a migration distance of 8 cm. Developed plates were documented under UV 254 nm, UV 366 nm, and under white light (in the reflectance mode), with a TLC visualiser with DigiStore 2 documentation system (CAMAG). Digital images of the plates were analysed with VideoScan version 1.02 (CAMAG).

2.5. Post Chromatographic Derivatization

For the chemical profiling study, four chromatograms were prepared with the different derivatization reagents. Antioxidant activity was assessed using a radical scavenging method. Plates were dipped into a 0.2% (*w/v*) DPPH• methanol solution and then placed in the dark for 30 min. Antioxidants appeared as bright yellow zones against a purple background. Total antioxidant activity was expressed in µg of gallic acid equivalents (GAE) per 20 µL of applied extract.

Phenolics were visualized as blue-, green-, or violet-coloured complexes, under white light, after derivatization with neutralized FeCl₃ and then heating the plate at 105 °C for a few minutes. Gallic acid was also used as a model analyte, to quantify the total phenolics content, in the ferric chloride assay.

Flavonoids were seen as bright greenish-blue, fluorescent zones under UV 366 nm, after derivatization with 3% *w/v* AlCl₃ in methanol. Rutin was used as a model analyte to measure total flavonoid content in rutin equivalents (RE).

Natural products in sample extracts were detected after derivatization with anisaldehyde-sulfuric acid (ASA) reagent (0.5 mL *p*-anisaldehyde, 10 mL of refrigerated glacial acetic acid, 85 mL of methanol, and 5 mL concentrated sulfuric acid) and heating the plate at 110 °C for maximum visualization of spots (5–10 min). Plates were evaluated under white light and under UV 366 nm. The amount of total natural products was expressed in sitosterol equivalents (SE) using the calibration curve of a sitosterol standard.

2.6. FTIR-ATR Spectra

All FTIR-ATR spectra were collected with a Cary 630 FTIR (Agilent Technologies Pty Ltd., Mulgrave, Australia) equipped with a diamond attenuated total reflectance (ATR) accessory. They were recorded between 4000 cm⁻¹ to 650 cm⁻¹ with a resolution of 4 cm⁻¹ and 128 scans, against a background scan acquired on a clean crystal. Spectra were analysed with the ResolutionsPro™ software (Ver. 5.3.01964, Agilent Technologies Inc., Santa Clara, CA, USA). A drop of concentrated extract was applied to the ATR crystal, and after solvent evaporation, the spectrum was recorded.

3. Results and Discussion

Salvia officinalis and *Salvia apiana* leaf extracts were analysed via a combination of HPTLC-based chemical profiling and non-targeted screening of bioactive compounds by HPTLC effect-directed detection (HPTLC-EDD). One of the aims was to compare the effect of different extraction solvents (MeOH, EtOH, EtOAc) and spontaneous lacto-fermentation of plant material, on the extraction of antioxidants (Table 1). The selection of a suitable extraction method and selection of suitable extraction solvent are critical for the phytochemical quality of the extract and targeted extraction of bioactive compounds from plant material. Extract yield and bioactivity of the extract prepared using different extraction methods and solvents varies. Bioactive compounds are usually present in low concentration in plants. Therefore, the extraction method used should provide a high yield with minimal degradation of extract components [13]. Hayouni et al. reported that both antioxidant and in vitro antibacterial activity for extracts of *Quercus coccifera* L. and *Juniperus phoenicea* L. fruits is dependent on both the extraction method and the choice of extraction solvent [14]. Previous work reports that organic solvents, such as methanol, are more efficient for extraction of phenolic and flavonoid compounds. Non-toxic ethanol can be used as an environmentally friendly alternative [15] to provide similar or even improved performance [16]. Deep eutectic solvents containing organic acids such as citric and lactic acids are new alternatives to toxic solvents [17].

Table 1. The effect of extraction solvent and fermentation on extraction yield from *Salvia officinalis* and *Salvia apiana*.

Plant Species	Plant Material	Solvent	Yield ¹ /%
<i>Salvia officinalis</i>	Ground dried leaves	Methanol	15.33
	Ground dried leaves	Ethanol	7.33
	Ground dried leaves	Ethyl acetate	6
	Fermented ground dried leaves	Ethyl acetate	3.17
<i>Salvia apiana</i>	Ground dried leaves	Methanol	14.47
	Ground dried leaves	Ethanol	10.32
	Ground dried leaves	Ethyl acetate	9.65
	Fermented ground dried leaves	Ethyl acetate	5.64

¹ Yield (g/100 g) = (W1 × 100)/W2 where W1 is the weight of the crude extract after solvent removal and W2 is the weight of dried and ground plant material.

The ATR-FTIR fingerprints of methanol, ethanol, ethyl acetate extracts, and ethyl acetate extract from fermented *Salvia officinalis* and *Salvia apiana* leaves (Figure 1a–d) were compared to find the variations in their phytochemical compositions that are affected by the polarity of extraction solvent and as a result of fermentation.

The FTIR spectra of both sage species (Figure 1) have a characteristic broad band for the O-H stretching in the range between 3500 and 3100 cm⁻¹, typical for polyphenolic extracts, and symmetric, asymmetric C-H stretches between 3000 and 2800 cm⁻¹. The -CH, -CH₂, and -CH₃ stretching vibrations in carbohydrates and sugars are observed between 2932–2925 cm⁻¹ [18]. The more important peaks appear between 1800 and 1500 cm⁻¹. All recorded FTIR spectra show species specific differences between the peak intensities in this region. The C=O stretching of esters can be seen between 1722–1702 cm⁻¹. Other bands that are easily identified are the C=C-C aromatic and C-O bond stretching. The stretching of the C=C-C aromatic bond is observed between 1611–1444 cm⁻¹, with the C-O bond at 1368–1157 cm⁻¹ and 1031–1023 cm⁻¹ [18].

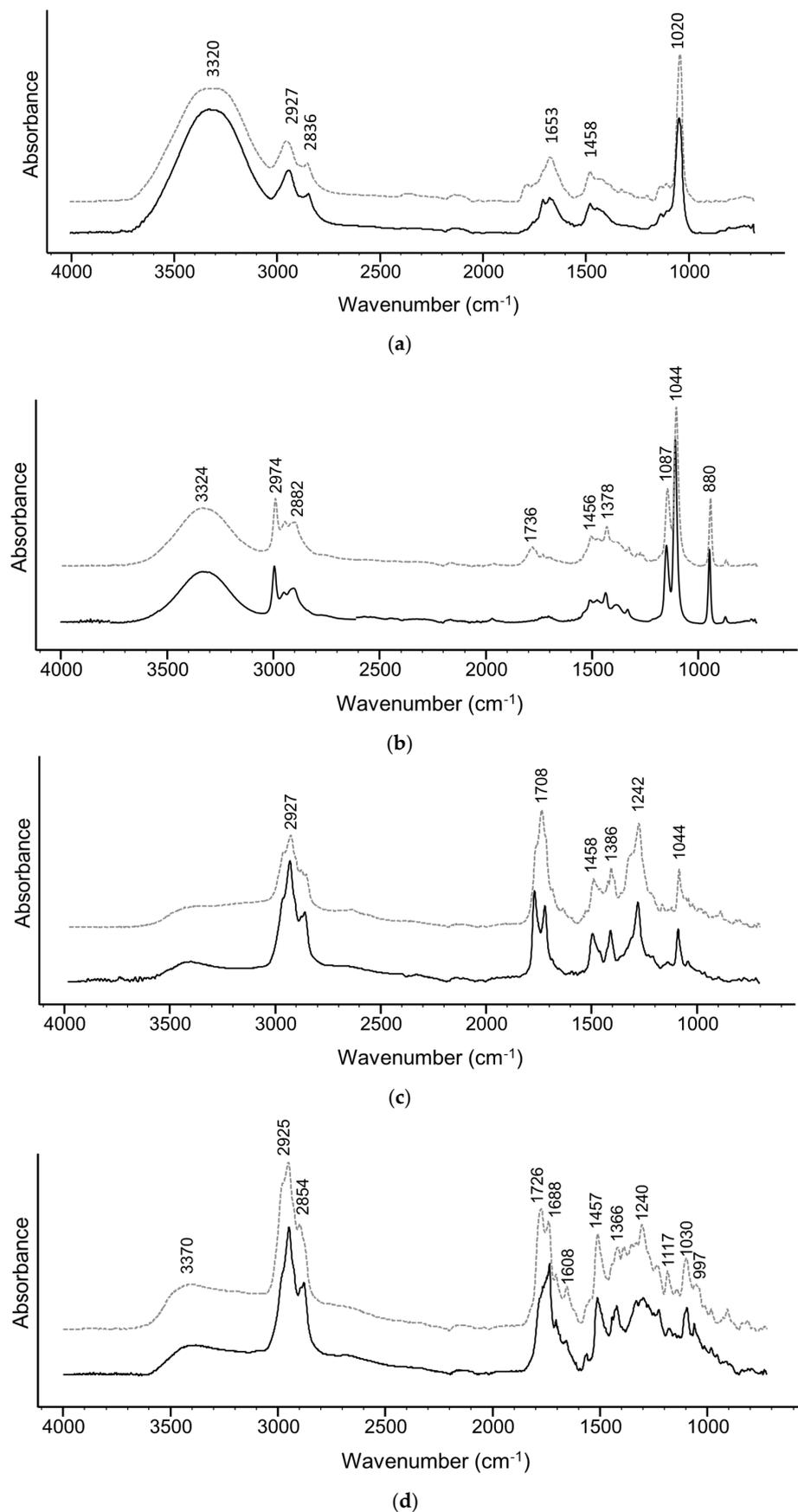


Figure 1. ATR-FTIR superimposed spectra of *Salvia officinalis* (black line) and *Salvia apiana* (dashed line) extracts with methanol (a), ethanol (b), ethyl acetate (c), and ethyl acetate from fermented plant material (d).

FTIR spectra of methanol (Figure 1a) and ethanol extracts (Figure 1b) exhibit vibrations that are characteristic for functional groups seen in flavonoids and phenolic compounds. Carnosol, carnosic acid, methyl carnosate, rosmarinic acids, rosmanol, epirosmanol, rosmadial, and luteolin-7-O- β -glucopyranoside are phenolic compounds with a high antioxidant activity. They are usually extracted from sage with ethanol [19]. The region 3375–3324 cm^{-1} is associated with hydroxyl group (O-H) stretching, and the region of 3026–2832 cm^{-1} corresponds to C-H stretching. The C=C bond of the aromatic ring is located in the range 1660–1466 cm^{-1} . Between 1261–1066 cm^{-1} represents the stretching of the C-O-C bond with an O-glycosidic linkage. Thus, distinct bands at around 1080 cm^{-1} are seen in ethanol extracts, and weaker shoulder bands in methanol extracts, could be due to vibrations of glycosidic bonds and pyranoid rings. The 1071–1022 cm^{-1} region represents C-OH vibration [20].

FTIR spectra of *Salvia Apiana* alcoholic extracts show additional bands for ester carbonyl (C=O) observed at 1765 cm^{-1} in methanol extract and at 1737 cm^{-1} in ethanol extract. In general, this peak falls from 1755 to 1735 cm^{-1} for saturated esters, while the 1765 cm^{-1} peak corresponds to the ester of unsaturated alcohol (vinyl alcohol) having the -CO-O-C=C structure. The C=O stretching frequencies for conjugated systems are generally lower than those of corresponding non-conjugated compounds. Also, vinyl acetate absorbs at 1776 cm^{-1} while the phenyl acetate absorbs at 1770 cm^{-1} . Distortion-induced vibration of aromatic C-H, outside the plane of molecule, depends mainly on the position of substituents, and not on their nature [21]. A distinct peak in the spectra from methanol and ethanol extracts at around ~1100 cm^{-1} can be assigned to cellulose and hemicellulose [22]. A band at 1044 cm^{-1} can be attributed to C-O, C-C stretching, or C-OH bending in xylan in hemicellulose, or at 1053 and at 1030 cm^{-1} , due to C-O stretching at C-3, and C-C and C-O stretching at C-6 in cellulose [23].

Ethyl acetate extracts (Figure 1c) show a significant increase in the intensity of the carbonyl C=O stretching band, suggesting that extracts contain high amounts of triterpenoid acids, ursolic, and oleanolic acids. In *Salvia apiana* extract, there is a single band at 1720 cm^{-1} with a small shoulder at 1660 cm^{-1} , while in *Salvia officinalis* there are two bands, one at 1740 cm^{-1} for carboxylic acid and the band at 1695 cm^{-1} for the conjugated carbonyl. The shoulder peak seen in *Salvia apiana* at approximately 1636 cm^{-1} is from C=C, and this is confirmed by the presence of weak absorption at 970–800 cm^{-1} . Frequencies at 2854 cm^{-1} and 2925 cm^{-1} can be linked to the terminal methylene (=CH₂) and methyl (-CH₃) groups. A weak band at around 1280 cm^{-1} is due to stretching vibrations of C-O and from the wagging of OH. Several bands located in the region 1050–1150 cm^{-1} can be assigned to C-C and C-O bond stretching vibrations. In addition, the sharp and strong band assigned to the C-O-C group of sugar derivatives at around 1044 cm^{-1} in *Salvia apiana* and in *Salvia officinalis* suggests the presence of glycosides. Ursolic and oleanolic acids have been found in almost all *Salvia* species, in addition to many triterpenoids with oleanane and/or ursane skeletons. Among the oleanane and ursane triterpenoids, double bonds are usually found between C-12 and C-13, and sometimes between C-18 and C-19. Oleanolic and ursolic acids exhibit a variety of biological activities, including antimicrobial, anti-inflammatory, antihyperlipidemic, hypoglycaemic, anticarcinogenic, and antiangiogenic activities [24–27].

ATR-FTIR spectra of extracts from fermented plant material exhibits bands characteristic for the FTIR spectrum of lignin (Figure 1d). Lignin is a plant phenolic polymer that occurs in almost all plants, and is one of the structural components of cell walls of woody plants, such as thyme, sage, and olive plants [28]. Both sage species are aromatic and rather woody perennial shrubs in the mint family (Lamiaceae). Lignin is composed of different 4-hydroxycinnamyl alcohols as building monomeric units (or monolignols) that are linked together by several covalent bonds. Caffeic acid is a hydroxycinnamic acid and a secondary metabolite. It is a key intermediate in the lignin biosynthesis. Hydroxycinnamic derivatives, such as caffeic acids, are involved in the phenylpropanoid pathway and cell wall lignification [29]. Caffeic acid, rosmarinic acid (a caffeic acid dimer), and oligomers of caffeic acid are all constituents of *Salvia officinalis* [30].

The ATR-FTIR spectrum exhibits a broad band between 3500–3100 cm^{-1} from stretching vibrations of alcoholic and phenolic OH. Bands at 2925 and 2854 cm^{-1} are assigned to C-H stretching vibrations of the methoxyl group. The absorption bands at 1687 cm^{-1} and 1657 cm^{-1} are from stretching vibrations of C=O conjugated with an aromatic ring band (aromatic carboxyl groups) [31,32]. Stretching vibrations of C=C bonds at 1626–1608 cm^{-1} indicate a small number of double C=C bonds. Absorption bands at 1610 cm^{-1} and 1511 cm^{-1} are linked to vibrations of aromatic rings present in lignin [33]. The band at around 1457 cm^{-1} can be assigned to both -OCH₃ and -OCH₂- groups. The absorption bands at 1367 cm^{-1} can be associated with symmetric deformation vibrations of C-H in methoxyl groups [34]. The increase in the intensity of the absorption band between 1280–1210 cm^{-1} , as seen in the spectra of acetylated and methylated lignin, could be related to asymmetric stretching vibrations of the C-O-C bonds in phenolic ethers (C-O stretch) and esters, or to phenolic hydroxyls [34]. The bands at 1170, 1127, and 1030 cm^{-1} originate from methoxyl groups. The weak absorption bands, located in the region 1250–1000 cm^{-1} , are due to deformation vibrations of C-H bonds in the benzene ring, due to C-H in-plane bending, although these bands are too weak to be detected for most aromatic compounds [35].

The effects of spontaneous fermentation on the extraction of antioxidants, polyphenolics, flavonoids, and natural products from sage leaves were studied by comparing HPTLC fingerprints. Figure 1 are fingerprints of different extracts before (tracks 1–3) and after derivatization (tracks 4–8). Derivatisations with ASA reagent were used to detect natural products, especially terpenoids (Figure 2, tracks 4 and 5) [36]. The use of this reagent is very helpful, when the presence of terpenoids and/or steroids is expected, because it reveals specific colours for monoterpenes, triterpenes, and steroids. Phenolic compounds were visualized under white light as violet–blue bands after derivatisation with FeCl₃ (Figure 2 track 7). Phenolics produce strongly coloured complexes (blue, green, or violet) with ferric chloride [37]. Phenolic acids such as caffeic or rosmarinic acids produce grey–green bands, as seen in the lower part of the chromatograms of the fermented extracts. Flavonoids in track 8 are detected after derivatisation with 3% AlCl₃ in methanol. TLC bioautography assay with DPPH• was used to screen for the free radical scavengers, i.e., components in extracts that can donate an electron and scavenge the stable purple DPPH• free radical to the pale yellow non-radical diphenylpicryl hydrazine (Figure 2 track 6). Zones of strong DPPH• scavenging activity are seen as bright yellow bands against a purple background on the plate.

Due to the presence of hydroxyl and carbonyl groups in their structure, flavonoids have the ability to coordinate metal ions, such as aluminium, and form coloured and often fluorescent chelates. After derivatisation, flavonoids are detected under UV 366 nm as bright greenish-blue fluorescent zones (Figure 2, track 8).

Methanol is a better solvent for the extraction of flavonoids (Figure 2a, track 8) except in the case of fermented *Salvia officinalis* plant material, where fermentation significantly increases the release and extraction of phenolics and flavonoids (Figure 2d, track 8). While *Salvia apiana* is richer in free antioxidants (track 6), phenolics (track 7), and terpenoids (track 4), *Salvia officinalis* has significant amounts of bound phenolics/flavonoids in its leaves.

Method calibration curves and validation data are given in Table 2, while the amounts of antioxidants, polyphenolics, terpenoids, and flavonoids in extracts expressed in equivalents of reference analytes are given in Table 3. The amounts of antioxidants, polyphenolics, flavonoids, and natural products in extracts are expressed in equivalents of reference compounds. Gallic acid is used as a model analyte for antioxidants quantification in the DPPH• assay and for the total phenolics content in the ferric chloride assay; rutin is used as a model analyte to express the total flavonoids in rutin equivalents (RE), and beta sitosterol as a model analyte for natural products (Table 3).

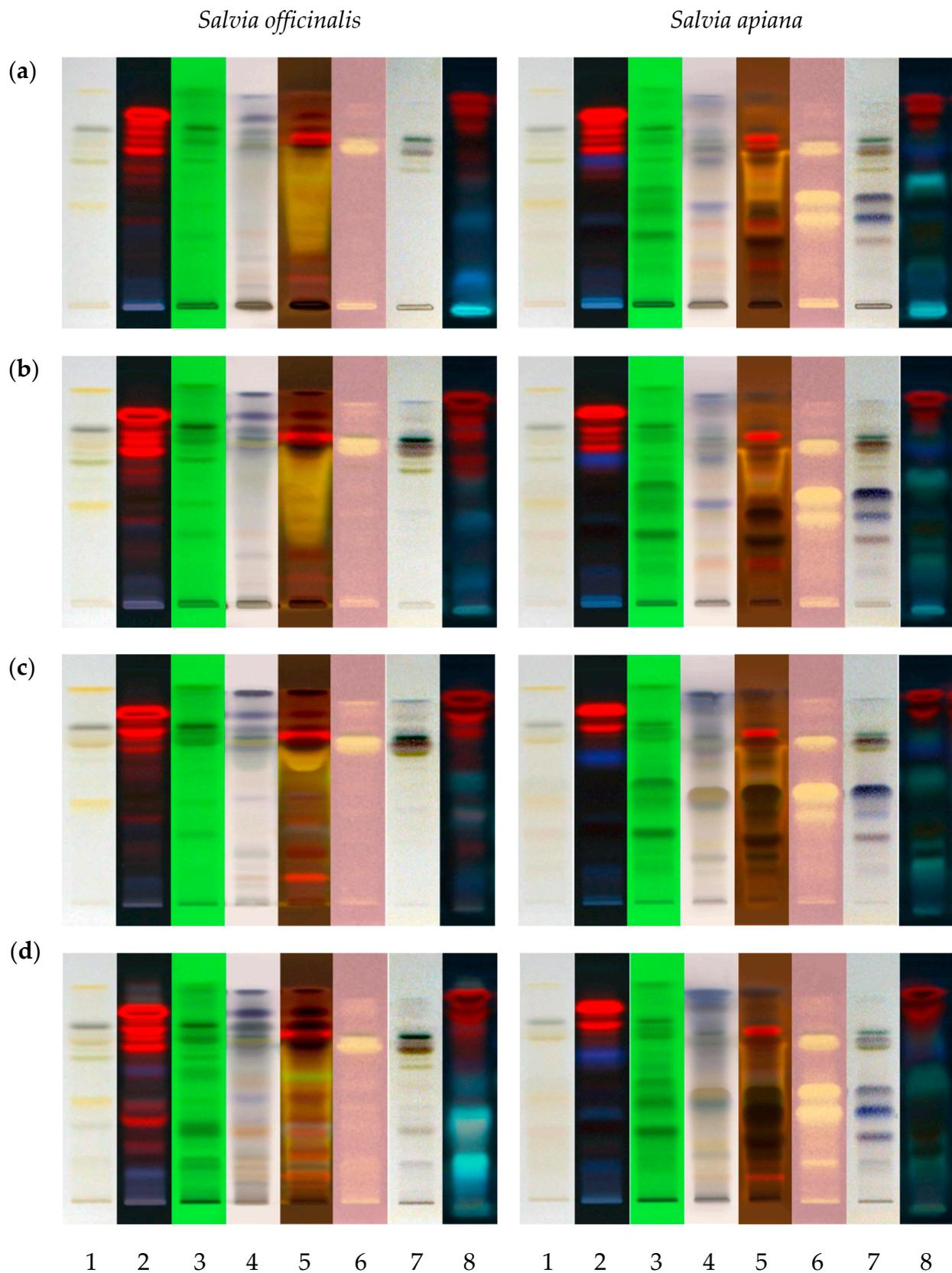


Figure 2. Fingerprints of *Salvia officinalis* and *Salvia apiana* in methanol (a), ethanol (b), ethyl acetate (c), and ethyl acetate fermented plant material (d) extracts. Track 1, white light; track 2, UV 366 nm; track 3, UV 254 nm; track 4, ASA under white light; track 5, ASA under 366 nm; track 6, DPPH• assay; track 7, ferric chloride under white light; track 8, with AlCl₃ under UV 366 nm.

Table 2. Calibration curves and methods validation.

	Linear Regression	RSD (%)	LOD (μg)	LOQ (μg)	Linearity (Analytical Range)
DPPH•	$y = 131,547x + 72,883$ $R^2 = 0.95$	3.20–5.09	0.11	0.35	0.2–6.0
	$y = -16,690x^2 + 217,442x + 27,344$ $R^2 = 0.985$				
FeCl ₃	$y = 21,093x + 15,402$ $R^2 = 0.96$	2.42–5.41	0.22	0.74	0.5–10
	$y = 1252x^2 + 32,564x - 1086.2$ $R^2 = 0.99$				
AlCl ₃	$y = 84,307x + 37,984$ $R^2 = 0.97$	2.06–2.35	0.35	1.19	1.0–7.0
	$y = 713.27x^2 + 13,883x + 30,954$ $R^2 = 0.99$				
ASA	$y = 554.5x + 13,011$ $R^2 = 0.95$	3.28–6.86	0.44	1.48	0.5–6.0
	$y = 766.54x^2 + 10,599x + 7959.9$ $R^2 = 0.98$				

Table 3. Antioxidants, total phenolics, total flavonoids, and natural products contents in extracts.

		Antioxidant Activity (DPPH• Assay)		Phenolics (FeCl ₃)		Total Flavonoids (AlCl ₃)		Natural Products (ASA)	
		Area (Pixels)	GAE /20 μL	Area (Pixels)	GAE /20 μL	Area (Pixels)	RE /20 μL	Area (Pixels)	SE /20 μL
<i>S. officinalis</i>	MeOH	162,764	0.68	153,566	6.55	442,707	48.01	715,681	126.72
	EtOH	213,722	1.07	321,111	14.49	228,069	22.55	878,126	156.01
	EtOAc	230,009	1.19	347,442	15.74	100,748	7.44	856,353	152.08
	EtOAc f.	351,167	2.12	458,563	21.01	588,739	65.33	1,640,941	293.57
<i>S. apiana</i>	MeOH	532,673	3.50	528,201	24.31	347,686	36.74	913,075	162.31
	EtOH	526,481	3.45	580,221	26.78	242,396	24.25	829,756	147.29
	EtOAc	451,561	2.88	503,363	23.13	173,947	16.13	1,373,288	245.31
	EtOAc f.	598,410	3.99	518,137	23.83	154,577	13.83	1,646,589	294.59

As expected, antioxidant activity in the DPPH• assay is highly correlated with total phenolic content ($R = 0.92$), and positively related to terpenoids and steroids ($R = 0.47$). Methanol is a good extraction solvent for flavonoids and ethanol for phenolics from unfermented material. Fermentation increases extraction of phenolics and antioxidants, and significantly increases extraction of flavonoids from *Salvia officinalis*. The highest amounts of terpenoids and steroids are extracted with ethyl acetate and after fermentation (Figure 3). Ethyl acetate is the most efficient solvent for the extraction of the variety of polyphenolics and phytosterols [18].

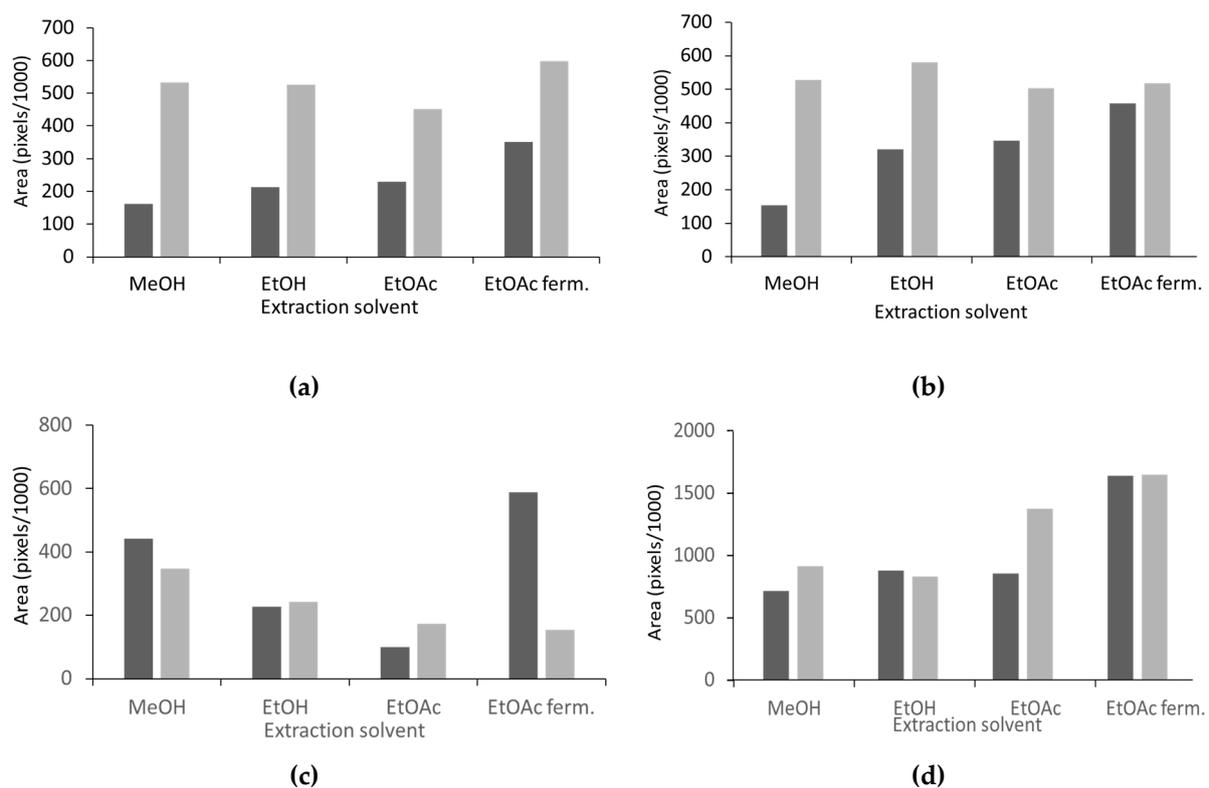


Figure 3. The effect of extraction solvent and method on (a) antioxidant activity (b) phenolic content (c) fla-vonoid extraction, and (d) natural products content; dark grey—*Salvia officinalis*, light grey—*Salvia apiana*.

Fermentation significantly improves extraction of *Salvia officinalis* leaves. Furthermore, common sage is a far woodier plant than *Salvia apiana*. *Salvia apiana* contains higher amounts of antioxidants and non-flavonoid phenolics, and a similar content of terpenoids. According to the number and positioning of carbon atoms, polyphenolics are classified as either flavonoids or non-flavonoids [38]. Ethanol is a better solvent choice than methanol for the extraction of antioxidants and phenolics from non-fermented plant material. Fermentation of plant material increases the extraction of antioxidants and phenolics with ethyl acetate, and significantly increases the extraction of flavonoids from *Salvia officinalis*. Flavonoids in plants are found as free, soluble (covalently linked to sugars), and insoluble or bound phenolics (covalently linked to cell wall structural components). Most flavonoids, except for the dihydroflavonols (catechins subclass), are found in plants, attached to sugars as β -glycosides. Phenolics that are covalently linked to sugars or to structural components of cell walls, must be released by either chemical or enzymatic hydrolysis to be extracted. During fermentation, lactic acid bacteria produces lactic acid from sugars. This production of lactic acid lowers the pH and stops other bacterial cultures from growing. However, LAB also release hydrolytic enzymes, which break down macromolecules and release bioactive compounds from plant material. There is also the possibility that lactic acid produced during fermentation forms a natural deep eutectic solvent (NADES) with one of the amino acids from the plant material. *Sage officinalis* extracts have a high amino acid content, over 1 mg/g [39]. The main amino acids that are found in the leaves of *Sage officinalis* are alanine, glycine, glutamic acid, proline, and valine [39,40]. NADES based on lactic acid are very effective in extraction of polyphenol antioxidants from medicinal plants, with increased solubilizing capacity for flavonoids [41].

Thus, *Salvia officinalis* contains higher amounts of bound flavonoids that must be released by fermentation, while *Salvia apiana* contains significant amounts of free phenolics and antioxidants, accompanied by a variety of terpenoids.

4. Conclusions

This study suggests that the high bioactivity of *Salvia apiana* extracts is attributed to the presence of phenolics and terpenoid antioxidants. Fermentation is shown to be a viable processing technique that increases nutrient bioavailability, and positively alters the levels of health beneficial constituents (particularly antioxidants). The increase in extraction of flavonoids from fermented *Salvia officinalis* leaves is a result of the mobilization of flavonoids from their bound form to a free state, through the activities of hydrolytic enzymes released by LAB during fermentation.

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