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Increase in Total Phenolic Content and Antioxidant Capacity in Wines with Pre- and Post-Fermentation Addition of *Melissa officinalis*, *Salvia officinalis* and *Cannabis sativa*

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Abstract: Herbs are considered substantial sources of antioxidant compounds, playing an important role in medicines, cosmetics, and distillates. Although they have been used in wine since ancient times, especially in Mediterranean regions, there is limited scientific evidence on how the addition of herbs into wine affects its properties. The aim of the present study was to determine the effects of three herbs, *Salvia officinalis*, *Melissa officinalis* and *Cannabis sativa*, with direct extraction in two different conditions: in must (pre-fermentation addition) and in wine (post-fermentation addition) and investigate potential differences between them. Three Greek indigenous grape varieties of *Vitis vinifera* L. were evaluated (Roditis, Muscat, Fokiano). The extractability of phenolic compounds and the antioxidant capacity of the produced wines were determined by the Folin–Ciocalteu and DPPH methods, respectively. Moreover, HPLC analysis was conducted to identify and quantify rosmarinic acid and caffeic acid, two main components of many Lamiaceae plants. The results indicate that the post-fermentation addition of herbs leads to a significant increase in antioxidant activity and phenolic compounds compared to blank wine. In most cases, the increase is significantly higher in comparison with pre-fermentation addition. Wine, upon the addition of *Melissa officinalis*, was found to extract the highest amount of total phenols compared to the other two herbs.

Keywords: herb extraction; wine total phenols; antioxidant activity; *Salvia officinalis*; *Melissa officinalis*; *Cannabis sativa*; caffeic acid; rosmarinic acid

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1. Introduction

Since ancient times, herbs have played an important role in the traditional medicine of all cultures [1]. Greek culture has a long tradition of using herbs, which is passed down through generations [2,3]. Modern scientific studies on herbs and their constituents confirm many of the healing properties empirically attributed to them in the past. As a result, they are increasingly used in medicines, cosmetics, and distillates. Bibliographical references found in ancient writings relate the preparation of alcoholic beverages with herbal extracts with the aim of treating various diseases [4].

Even though they are found in various forms today, some traditional or ancient flavored wines that were created with specific herbs still exist [5]. Vermouth, Bermet, Retsina and other infused wines containing herbs and spices belong to this category [6]. Such wines have been prepared since antiquity in the Mediterranean basin, with the initial purpose of protecting wines from oxidative or microbiological spoilage [7]. The original goal of macerating herbs and spices in wine has been overcome by technological advancements in winemaking methods, tools and materials, but flavored wines are still produced because of their unique taste, aroma and character [8].

Salvia officinalis, the common Sage, has been an important medicinal plant since ancient times and is still under scientific focus [9]. There is an indication that Romans brought it to Europe from ancient Egypt [10]. It has historically been used to treat pharyngitis, uvulitis, stomatitis, gingivitis, galactorrhea, hyperhidrosis, and flatulent dyspepsia [11], while recent studies detail anti-inflammatory and antinociceptive effects related to pain relief, antioxidant effects, antimicrobial effects related to various infections, anticancer and antimutagenic effects related to various cancers, and significant hypoglycemic and hypolipidemic effects related to metabolic diseases [12]. Also, Sage has proven to be effective on nervous issues like tremors, depression and vertigo [11]. Furthermore, researchers at the MRC's Neurochemical Pathology Unit at Newcastle General Hospital discovered evidence that sage has antidementia effects and can act against Alzheimer's disease. This plant's oil inhibits the action of acetylcholinesterase, which is likely to play a role in memory loss associated with the disease [12].

The medicinal use of *Melissa officinalis*, the common Lemon balm, also dates back to ancient times. Dioscorides used it for dog and scorpion bites but also soaked it in wine to soothe his patients. Greek polymath Theophrastus mentions *Melissa officinalis* in Historia Plantarum, written in c.300 BC, as "bee-leaf" [13]. In recent history, lemon balm was used against fever and flatulence problems. It has also become known in the medical world that the herb's oil can be used as a "surgical dressing" because it kills germs (e.g., *candida albicans*) and seals wounds [14]. Many pharmacological effects have been reported from *Melissa officinalis* extracts, such as antiproliferative, antioxidant, cardioprotective, neuroprotective and many others [15]. Recent research indicates that polyphenols extracted from the leaves of lemon balm have drastic activity towards Gram-positive bacteria and less activity towards Gram-negative bacteria [14]. Both *Melissa officinalis* and *Salvia officinalis* belong to the Lamiaceae family, where rosmarinic acid and caffeic acid are among the most studied constituents [16].

In traditional medicine, leaves, stems and flowers of *Cannabis sativa* are well known for their bitter, intoxicating, tonic, analgesic and aphrodisiac properties, as well as for the production of textiles and rope [17,18]. The National Institute on Drug Abuse (NIDA) of the USA coordinated and sponsored years of research, which resulted in the finding that "Cannabis has proven to lessen the intraocular pressure of glaucoma, which kills the optic nerve and gradually results in blindness" [18]. Clinical observations and statistics from cancer patients show that Δ^9-tetrahydrocannabinol (THC) stimulates appetite, aids in the reversal of chronic weight loss and has some analgesic and antiemetic effects [18]. Drowsiness, dizziness and disorientation were the side effects that were limited due to its use to 25% of patients [19]. Also, the therapeutic value of Cannabis when dealing with chemotherapy side effects is mentioned in the educational manual "Handbook of Cannabis Therapeutics. From Bench to Bedside" [20]. Following those findings, research has discovered that THC and its synthetic analogs (for example, Δ^9-THC) have the ability to control the severe and persistent nausea and vomiting that torment chemotherapy patients [20]. Until now, more than one thousand compounds of Cannabis have been identified, including 278 cannabinoids, 174 terpenes, 221 terpenoids, 19 flavonoids, 63 flavonoid glycosides and 46 polyphenols. Cannabinoids have recently been shown to exhibit anti-inflammatory and immunosuppressing effects against the COVID-19 immune response [19].

On the other hand, wine contains a lot of phenolic compounds and many studies have focused on their antioxidant properties and beneficial health effects [21,22]. The main classes of compounds identified and associated with the beneficial effects are flavanols (3-O-glycosides of myricetin, quercetin, kaempferol, and isorhamnetin), flavonols [(+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate] and, in red wines, anthocyanins [3-O-monoglucosides and the 3-O acylated monoglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin [21]. Several factors, including grape variety, grape ripeness, environmental conditions and winemaking practices can affect the phenolic composition of grapes, pomace and wine, and thus their quality and nutritional properties [23].

Although herbs have been used in wine since ancient times, there is relatively limited scientific research published on infused wines. In the present study, three different herbs (*Salvia officinalis*, *Melissa officinalis* and *Cannabis sativa*) were added both in must (pre-fermentation herb addition) and in stable wine (post-fermentation herb addition) from three indigenous Greek grape varieties (Roditis, Muscat and Fokiano). The main objective of the present work was to evaluate the enrichment of the studied wines in phenolic compounds and to assess if there is a difference between the two methods of herb addition used. Moreover, the presence of caffeic acid and rosmarinic acid in all samples was evaluated.

2. Materials and Methods

2.1. Reagents

All chemicals used in this work were of analytical grade. Folin–Ciocalteu reagent, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 6-Hydroxy-2,3,7,8-tetrameth ylchroman-2-carboxylic acid (Trolox) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) Methanol, acetonitrile, water of HPLC grade and ethanol (analytical grade) were supplied by Merck (Darmstadt, Germany). Rosmarinic acid and caffeic acid, were purchased from DR EHRENSTORFER GmbH (Augsburg, Germany).

2.2. Herbs

Organic dry leaves of Sage—*Salvia officinalis* L. (Lamiaceae), Lemon balm—*Melissa officinalis* L. (Lamiaceae) and flowers of Cannabis—*Cannabis sativa* L. (Cannabaceae) were purchased from the Organic stores Bioplus, Chiron-Kentauros and Arcan (Athens-Greece), respectively, and stored in standard room temperature.

2.3. Wine Preparation with Herbs

The grapes of two white varieties, Muscat of Samos and Roditis from Peloponnese, as well as one red variety, Fokiano from Ikaria were used for the experiments. The following procedure was followed in both vintages 2020 and 2021. At first, grape juice was separated into two parts. The first part was then divided into three small vats, where the three different herbs were added, respectively, and alcoholic fermentation occurred along with the maceration of herbs. The fermenting musts with herbs were stirred twice daily. The second part of the grape juice was fermented with no maceration of any herb. When fermentation finished and the wine was stabilized, a part was kept as blank wine and the rest of it was divided again into three parts, where herbs were added and extracted. In each case, the extraction procedure occurred for fifteen days, under controlled conditions between 20 and 22 °C. Three different ground-dried herb concentrations were used (6 g L^{-1}, 10 g L^{-1} and 15 g L^{-1}) for both pre- and post-fermentation procedures, in order to define the maximum extraction of phenolic compounds in wine. The next step was the filtration of all samples and sealing which took place 30 days post-fermentation. Subsequently, the total phenolic content as well as the antioxidant capacity were determined. No sulfites were used at any step of the winemaking process and the total alcohol % volume of blank wines for Roditis, Muscat and Fokiano were 12.5, 13.0 and 14.5% vol., respectively.

2.4. Total Phenolic Content Determination

The amount of total phenolics (TPC) in each sample was determined using the Folin–Ciocalteu (F-C) method [16]. Briefly, into a 25 mL volumetric flask, the following was introduced strictly in the given order: 0.25 mL of the sample [after 1:10 dilution in the case of white wines (Roditis and Muscat) and 1:20 in the case of red wine (Fokiano)], 12.5 mL of distilled water and 1.25 mL of F–C reagent. After 3 min, 5 mL of sodium carbonate solution (20% w/v) was added. Finally, the volume was made up to 25 mL with distilled water and the content was stirred to homogenize. After 30 min in the dark, the absorbance of all samples was measured at 725 nm using a UV/Vis Shimadzu spectrophotometer. All determinations were performed in triplicate. A calibration curve was prepared using gallic

acid as a standard, in a range 5–50 mg gallic acid per 100 mL. Total phenolic content was expressed as mg of gallic acid equivalents (GAE) L^{-1} wine g^{-1} herb.

2.5. Evaluation of Antioxidant Activity-DPPH Method

The ability of plant extracts to scavenge DPPH free radicals was determined according to the procedure described by Brand-Williams et al. [24]. An aliquot of 0.1 mL of each sample (diluted 1:20 in methanol) was mixed with 3.9 mL of freshly prepared DPPH solution in a concentration of 60 μM in methanol. After 30 min incubation in darkness at ambient temperature, the resultant absorbance was measured at 515 nm. Also, a control of 3000 μL of DPPH/CH_3OH 60 μM solution and 100 μL CH_3OH was used. All determinations were performed in triplicate. The percentages of inhibition of the DPPH radical, as a function of the effect extracted fractions, were calculated using the following equation [25]:

$$\% \text{ of antioxidant activity} = [(A_C - A_S)/A_C] \times 100 \quad (1)$$

where A_C: the absorbance of the control (t = 0), A_S: the absorbance of the samples (t = 30 min). In order to express results as mmol Trolox L^{-1} wine g^{-1} herb, a calibration curve was prepared using 0.1 mL methanolic solutions of Trolox in the range 0–18 nmol Trolox.

2.6. HPLC Analysis of Phenolic Compounds in Plant Extracts

HPLC analysis was carried out using an HPLC system (VWR Hitachi Elite La Chrom system, VWRm Darmstadt, Germany) consisting of an auto-sampler (L-2200), quaternary pump (L-2130), degasser (G 1322 A) and diode array detector (L-2455). Chromatographic separation of compounds was carried out at 30 °C on a RESTEK column C18 (150 × 4.6 mm, 3 μm particle size) with a flow rate of 0.5 mL min^{-1}. For the HPLC analysis of phenolic compounds, a modification method of Kouri et al. was used [26]. The mobile phase consisted of water with 1% formic acid v/v (A), methanol with 1% formic acid (B) and acetonitrile with 1% formic acid (C). The gradient used was 90% A, 6% B, 4% C 0–5 min, 85% A, 9% B, 6% C 5–30 min, 71% A, 17.4% B, 11.6% C 30–60 min, 90% A, 6% B, 4% C 60–65 min.

The injection volume was 20 μL and chromatogram was acquired at 280 nm. All the analyses were made in triplicate. Solutions of available pure known compounds, such as caffeic acid and rosmarinic acid were chromatographed as external standards. All standards were dissolved in methanol before injection in the analytical HPLC system. Individual standard solutions (15 mg) were dissolved in methanol (50 mL) at a concentration of 300 μg mL^{-1} and followed by serial dilutions. A five-point regression curve ($R^2 > 0.98$) was used to quantify each chemical compound separately, ranging from 1 to 100 μg mL^{-1} (LOD = 17.70 μg mL^{-1}, LOQ = 53.63 μg mL^{-1} for caffeic acid and LOD = 15.78 μg mL^{-1}, LOQ = 47.82 μg mL^{-1} for rosmarinic acid). Phenolic compounds of plant extracts were identified by comparing their retention times with those of pure standards. The results were expressed as mg phenolic compound L^{-1}.

2.7. Data Analyses

All data were expressed as Mean ± Standard Deviation (SD) from three independent experiments and differences were analyzed using one-way analysis of variance (ANOVA) followed by a post-hoc test (Tukey's test), using Minitab version 18.0. In most cases, analysis was conducted to compare differences among the blank wine, pre-fermentation addition and post-fermentation addition of herbs for each wine/herb combination. p values ≤ 0.05 were indicative of statistical significance throughout the analyses.

3. Results

3.1. Herbs' Maximum Extraction Level

In order to define the maximum extraction of herb's phenolic compounds and antioxidant compounds in wine, as mentioned, three different herb concentrations were used (6 g L^{-1}, 10 g L^{-1}, and 15 g L^{-1}), following two different manners, pre- and post-

fermentation addition, as described above. According to our results from Folin–Ciocalteu and DPPH method using the three different herb concentrations, in all wine samples, the herbs' maximum extraction level in phenolic and antioxidant compounds was calculated (Table 1).

Table 1. Maximum extraction of herbs in phenolic and antioxidant compounds, after pre- and post-fermentation addition.

	Maximum Her Extraction in Phenolic Compounds (g herb L^{-1} Wine)		**Maximum Herb Extraction in Antioxidant Compounds (g herb L^{-1} Wine)**	
Sample	**Pre-Fermentation**	**Post-Fermentation**	**Pre-Fermentation**	**Post-Fermentation**
Can/Rod 2021	9.31	10.12	7.66	9.70
Can/Mus 2021	8.94	8.38	10.00	10.97
Can/Fok 2020	10.20	10.89	11.30	12.60
Sage/Rod 2021	10.01	10.73	10.86	11.69
Sage/Mus 2021	7.91	8.34	9.10	9.65
Sage/Fok 2020	9.12	9.67	9.65	9.90
Mel/Rod 2021	9.53	11.38	9.70	9.49
Mel/Mus 2021	11.29	11.70	9.38	8.83
Mel/Fok 2020	9.87	9.03	7.83	9.10

Maximum extraction levels of both phenolic and antioxidant compounds were found ~10 g of each herb L^{-1} wine, for pre- and post-fermentation addition and for all herbs.

3.2. Total Phenolic Content

In all wine samples, of 2020 and 2021 vintages, after the pre- and post-fermentation addition of herbs, the total phenolic concentration was determined and expressed in mg gallic acid equivalents (GAE) L^{-1} wine g^{-1} of the selected herb (Table 2). The obtained values refer to the samples containing 10 g of herb, the amount with the maximum extraction level found. According to the results and comparing them with those in blank wine (where no herb was added) the % increase was calculated.

Table 2. Total phenolic content of wine samples expressed in mg gallic acid equivalent L^{-1} g^{-1} and % increase, after pre- and post-fermentation addition of herbs. Reported data are the means of three replications with standard deviation.

	Blank Wine (no Herb)	**Pre-Fermentation Herb Addition (in Must)**		**Post-Fermentation Herb Addition (in Stable Wine)**	
Herb/Wine Harvest Year	**mg GAE L^{-1}**	**mg GAE L^{-1} g^{-1}**	**% Increase**	**mg GAE L^{-1} g^{-1}**	**% Increase**
Can/Rod 2021	140.22 ± 1.07^{a}	151.40 ± 1.33^{b}	7.97	160.51 ± 1.47^{c}	14.47
Can/Mus 2021	192.74 ± 1.98^{a}	213.51 ± 1.71^{b}	10.81	244.33 ± 2.33^{c}	26.78
Can/Fok 2020	337.45 ± 4.79^{a}	387.55 ± 3.52^{b}	14.84	500.35 ± 4.21^{c}	48.27
Sage/Rod 2021	140.22 ± 1.73^{a}	197.10 ± 2.47^{b}	40.56	212.93 ± 3.56^{c}	51.86
Sage/Mus 2021	192.74 ± 1.22^{a}	275.45 ± 2.01^{b}	42.91	356.84 ± 2.72^{c}	85.14
Sage/Fok 2020	337.45 ± 3.56^{a}	443.55 ± 3.89^{b}	31.44	644.75 ± 4.71^{c}	91.06
Mel/Rod 2021	140.22 ± 1.85^{a}	230.93 ± 2.81^{b}	64.71	246.11 ± 2.87^{c}	75.53
Mel/Mus 2021	192.74 ± 1.51^{a}	312.55 ± 2.94^{b}	62.16	374.94 ± 3.75^{c}	94.53
Mel/Fok 2020	337.45 ± 3.44^{a}	461.23 ± 4.38^{b}	36.68	642.35 ± 4.41^{c}	90.35

Can: Cannabis sativa, Sage: Salvia officinalis, Mel: Melissa officinalis. Rod: Roditis-Peloponnese, Mus: Muscat-Samos, Fok: Fokiano-Ikaria. Data are expressed as mean ± standard deviations. Different letters indicate statistical significance at $p < 0.05$ level for each concentration (Tukey test). Statical analysis refers to each herb/wine combination separately.

The amount of total phenols that the must extracted from *Cannabis sativa*, *Salvia officinalis* and *Melissa officinalis* during pre-fermentation addition, increased the total phenolic

content compared to blank wine 7.97–14.84%, 31.44–42.91%, and 36.68–64.71%, respectively. The same herbs extracted in stable wine (post-fermentation addition) increased the total phenolic content compared to the blank wine 14.47–48.27%, 51.86–91.06% and 75.53–94.53%, respectively. In each case, we observed that the total phenolic content of all studied wines was much more enhanced when herbs were added in stable wine, after the end of fermentation and not in the must, where herbs' maceration is taking place during fermentation. Moreover, among the studied herbs, Cannabis enhances the phenolic content of wines the least, as the lowest % increase in TPC was recorded (7.97% in pre-fermentation and 14.47% in post-fermentation addition). On the contrary, Melissa enhances wine's TPC the most (75.53–94.53%). Particularly, the maximum % increase was observed in Muscat (94.53%). Sage also showed a high % TPC increase at post-fermentation addition in Fokiano (91.06%).

According to the TPC obtained values, Figure 1 shows the absolute value increase in total phenolic content in wines after pre- and post-fermentation herb addition compared to blank wine. The percentage difference of the absolute values between pre- and post-fermentation herb addition was also calculated (Table 3). These values represent the % difference in total phenolic content between the higher values of post-fermentation and the lower ones of pre-fermentation addition.

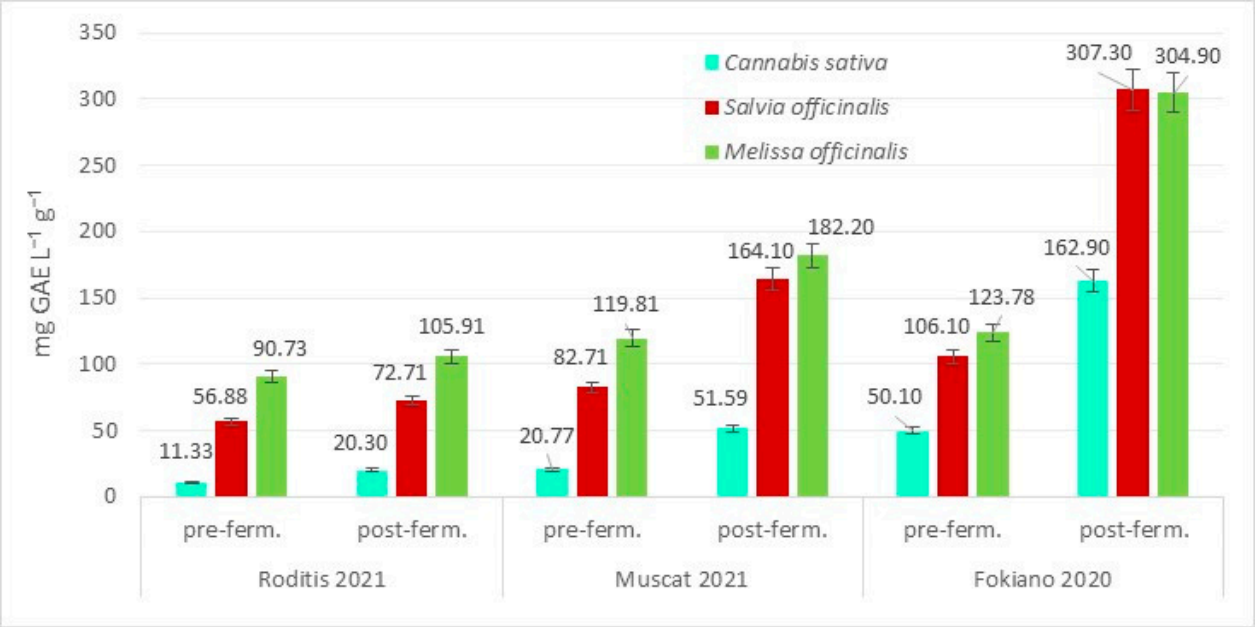

Figure 1. Total phenolic content absolute value increase, after pre- and post-fermentation addition of herbs.

Table 3. Absolute value increase in total phenolic content (mg GAE L^{-1}) in wines after pre- and post-fermentation herb addition, in comparison to blank wine and % difference between pre- and post-fermentation herb addition.

Sample	Pre-Fermentation Herb Addition	Post-Fermentation Herb Addition	% Difference
Can/Rod 2021	11.33	20.30	44.2
Can/Mus 2021	20.77	51.59	59.7
Can/Fok 2020	50.1	162.9	69.2
Sage/Rod 2021	56.88	72.71	22.2
Sage/Mus2021	82.71	164.1	49.6
Sage/Fok 2020	106.1	307.3	65.4
Mel/Rod 2021	90.73	105.91	14.3
Mel/Mus 2021	119.81	182.2	34.2
Mel/Fok 2020	123.78	304.9	59.4

In all cases, it is clear that more phenolic compounds are extracted from herbs, when herbs are added to stable wine compared to the unfermented must (% difference: 14.3 to 69.2%). As expected, in Fokiano, higher values of phenolic content were observed, in comparison to Roditis and Muscat, as Fokiano is a red variety, with plenty of phenolic compounds [27]. On the other hand, Roditis and Muscat as white varieties contain lower amounts of phenolic content [28,29].

3.3. Evaluation of Antioxidant Activity

The antioxidant capacity of all wine samples was determined and expressed in mmole Trolox L^{-1} wine g^{-1} of the selected herb (Table 4). The obtained values refer to the samples containing 10 g of herb, the amount with the maximum extraction level.

Table 4. Antioxidant capacity of wine samples expressed in mmol Trolox L^{-1} g^{-1} (±SD) and % increase, after pre- and post-fermentation addition of herbs. In each case, there was statistical difference between pre- and post-fermentation herb addition (p-value < 0.05). Reported data are the means of three replications with standard deviation.

	Blank Wine (no Herb)	**Pre-Fermentation Herb Addition**		**Post-Fermentation Herb Addition**	
Sample	**mmole Trolox L^{-1}**	**mmole Trolox L^{-1} g^{-1}**	**% Increase**	**mmole Trolox L^{-1} g^{-1}**	**% Increase**
Can/Rod 2021	1.69 ± 0.071^{a}	1.79 ± 0.079^{b}	5.7	1.84 ± 0.083^{b}	9.3
Can/Mus 2021	1.72 ± 0.069^{a}	1.89 ± 0.076^{b}	10.1	2.01 ± 0.084^{b}	16.9
Can/Fok 2020	3.14 ± 0.113^{a}	3.35 ± 0.171^{b}	6.8	3.49 ± 0.182^{b}	11.3
Sage/Rod 2021	1.69 ± 0.077^{a}	1.81 ± 0.084^{b}	7.1	1.96 ± 0.080^{c}	11.1
Sage/Mus 2021	1.72 ± 0.078^{a}	1.99 ± 0.099^{b}	17.1	2.21 ± 0.103^{c}	28.4
Sage/Fok 2020	3.14 ± 0.143^{a}	3.53 ± 0.158^{b}	12.8	3.75 ± 0.146^{c}	19.4
Mel/Rod 2021	1.69 ± 0.064^{a}	2.76 ± 0.092^{b}	63.0	2.91 ± 0.116^{c}	71.6
Mel/Mus 2021	1.72 ± 0.078^{a}	2.67 ± 0.089^{b}	52.7	3.03 ± 0.147^{c}	76.6
Mel/Fok 2020	3.14 ± 0.147^{a}	3.85 ± 0.144^{b}	22.6	4.07 ± 0.153^{c}	29.7

Data are expressed as mean ± standard deviations. Different letters indicate statistical significance at the $p < 0.05$ level for each concentration (Tukey test). Statical analysis refers to each herb/wine combination separately.

As in the case of total phenolic content, the % increase of antioxidant capacity in wines was calculated, after pre- and post-fermentation addition of herbs, compared to blank wine.

The antioxidant capacity resulted by the addition of *Cannabis sativa*, *Salvia officinalis* and *Melissa officinalis* during pre-fermentation, increased the antioxidant capacity of blank wine from 5.7 to 10.1%, 7.1 to 17.1%, and 22.6 to 63%, respectively. The same herbs extracted in stable wine increased the antioxidant capacity of the blank wine from 9.3 to 16.9%, 11.1 to 28.4%, and 29.7 to 76.6%, respectively. The antioxidant capacity of all wine samples increased with the addition of herbs, and a higher increase was observed in the case of post-fermentation herb addition, as well as in total phenolic content. However, the highest % increase in antioxidant activity was observed in Muscat. Between the studied herbs, *Melissa officinalis* induced a higher increase in the antioxidant potential of all wine samples.

The absolute value increase in antioxidant compounds in wines after pre- and post-fermentation herb addition compared to blank wine is shown in Figure 2. The % difference of the absolute values between pre- and post-fermentation herb addition was calculated (Table 5). These values represent the % difference in antioxidant compounds between the higher values of post-fermentation and the lower ones of pre-fermentation addition.

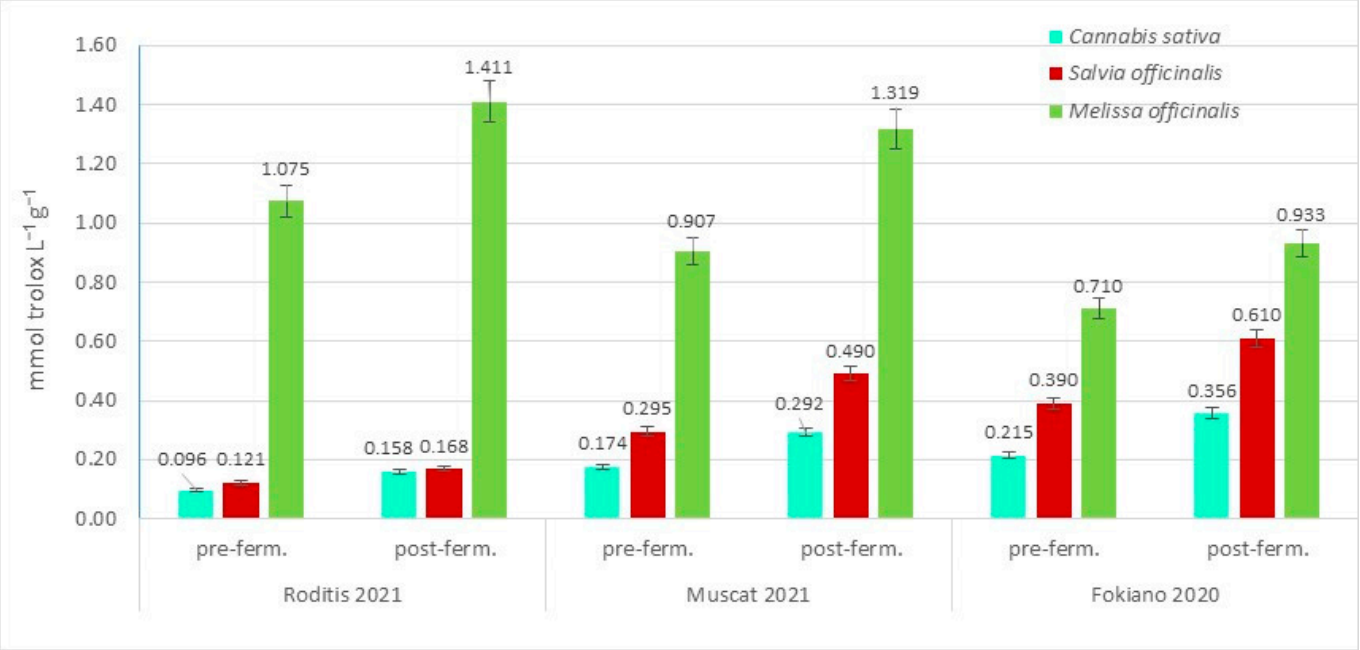

Figure 2. Antioxidant capacity absolute value increase, after pre- and post-fermentation herb addition.

Table 5. Absolute value increase in antioxidant capacity, after pre- and post-fermentation addition of herbs, in comparison to blank wine and % difference between pre- and post-fermentation herb addition.

	mmole Trolox L^{-1} g^{-1}	mmole Trolox L^{-1} g^{-1}	
Sample	**Pre-Fermentation Herb Addition**	**Post-Fermentation Herb Addition**	**% Difference**
Can/Rod 2021	0.096	0.158	39.20
Can/Mus 2021	0.174	0.292	40.40
Can/Fok 2020	0.215	0.356	39.60
Sage/Rod 2021	0.121	0.168	27.90
Sage/Mus 2021	0.295	0.490	39.70
Sage/Fok 2020	0.390	0.610	36.00
Mel/Rod 2021	1.075	1.411	23.80
Mel/Mus 2021	0.907	1.319	31.30
Mel/Fok 2020	0.710	0.933	23.90

In each case, more antioxidant compounds were extracted in all wine samples when herbs were added post-fermentation. As we can conclude from the results shown in Table 5, an average of 34% difference in the antioxidant capacity of wine was observed. The results are consistent with the ones of phenolic compounds, where it was also found that higher extraction occurred in post-fermentation herb addition.

3.4. HPLC-DAD Analysis

HPLC analysis in all wine samples focused on the detection of two main phenolic acids: caffeic acid and rosmarinic acid (Figure 3), both found in the literature to be characteristic compounds of the Lamiaceae family [30]. Caffeic acid is found in cannabis [31], but it is also found in wines, acting as an antioxidant and increasing its percentage as wines age [32]. On the other hand, rosmarinic acid is a compound clearly detected after the extraction of the herbs and it is not naturally found in wine.

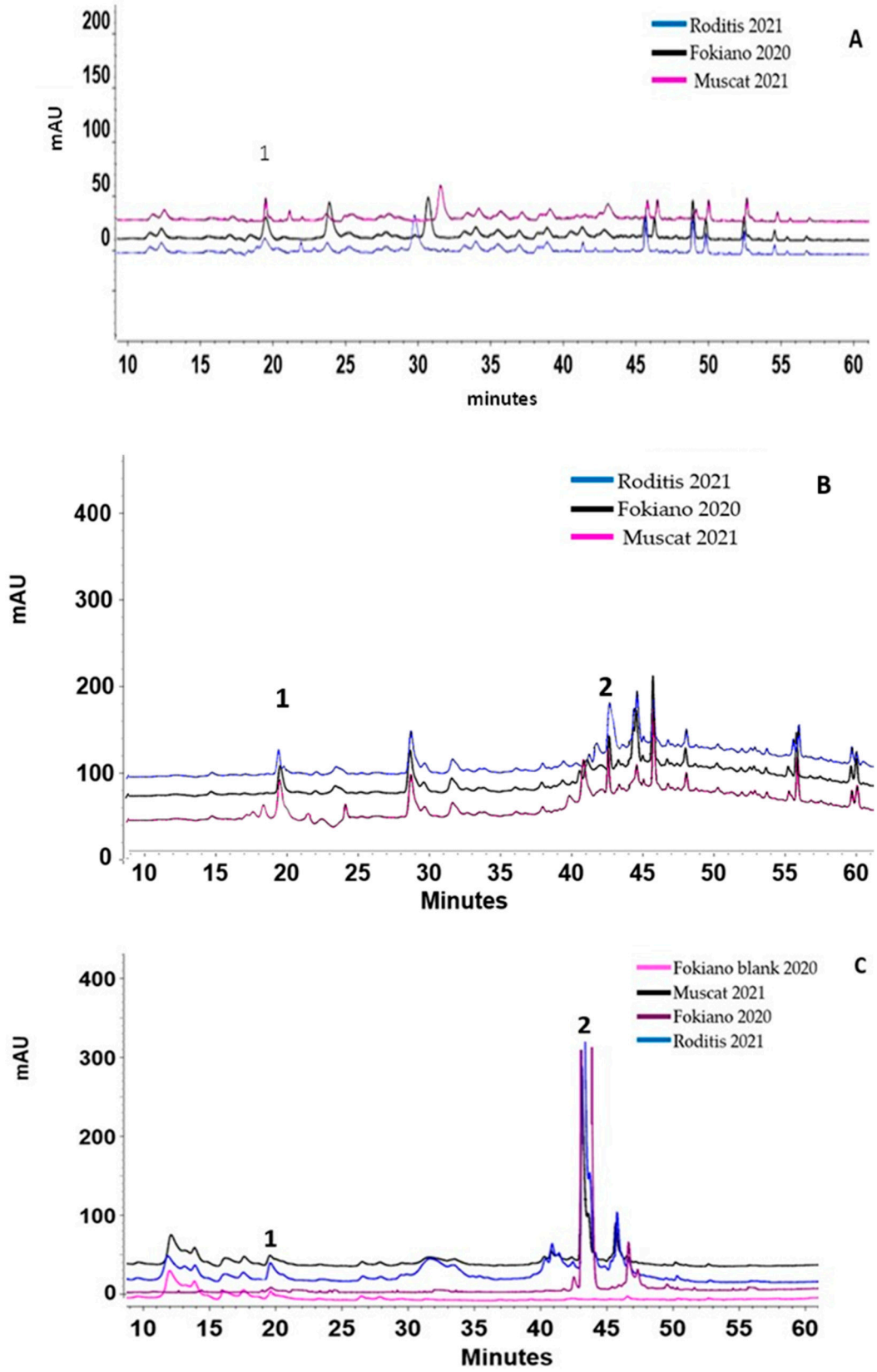

Figure 3. HPLC chromatographic profile of wines after post-fermentation addition of (**A**) Cannabis, (**B**) Sage and (**C**) Melissa. Peaks: 1, caffeic acid (Retention Time = 19.3 min); 2, rosmarinic acid (Retention Time = 42.7 min). Scanning at λ= 280 nm.

According to HPLC results (Table 6), it is worth noting that the amount of both rosmarinic and caffeic acid was undetectable in all samples in which the extraction of the herbs had been pre-fermented, as well as to blank white wines from the varieties of Roditis and Muscat. On the other hand, in the red blank Fokiano, the amount of 0.58 mg L^{-1} of caffeic acid was detected.

Table 6. HPLC analysis of caffeic acid and rosmarinic acid in wine samples, after pre- and post-fermentation herb addition.

	Caffeic Acid (mg L^{-1})		Rosmarinic Acid (mg L^{-1})	
	Pre-Fermentation	**Post-Fermentation**	**Pre-Fermentation**	**Post-Fermentation**
Can/Rod 2021	n.d. *	0.9 ± 0.10	n.d.	nd
Can/Mus 2021	n.d.	0.9 ± 0.18	n.d.	nd
Can/Fok 2020	n.d.	0.9 ± 0.16	n.d.	nd
Sage/Rod 2021	n.d.	17.9 ± 0.53	n.d.	27.0 ± 0.33
Sage/Mus2021	n.d.	19.1 ± 0.38	n.d.	27.8 ± 0.71
Sage/Fok 2020	n.d.	20.0 ± 0.31	n.d.	28.7 ± 0.67
Mel/Rod 2021	n.d.	3.1 ± 0.44	n.d.	39.4 ± 1.06
Mel/Mus 2021	n.d.	3.4 ± 0.46	n.d.	44.8 ± 0.91
Mel/Fok 2020	n.d.	4.8 ± 0.52	n.d.	53.5 ± 1.25

* n.d.: non-detected. Data are expressed as mean ± standard deviations.

4. Discussion

Phenolic content and antioxidant activity in wines depend on many parameters, such as geographical origin, grape variety, aging, climate and vinification techniques [33]. On the other hand, the use of herbs in wines in order to produce the so-called herbal or infused wines (medicinal wines) has a long tradition. Since ancient times, people have been flavoring their wine with various herbs and spices, and current research activities promote the creation of new flavored wines [7].

In the literature, three different methods are used for the aroma extraction of herbs and spices according to the type of botanicals used: (a) direct extraction, (b) concentrate preparation using base wine and (c) concentrate preparation using base wine distillate. Direct extraction is the simplest method, where the calculated amounts of finely ground herbs and spices are infused in the base wine until the desired aromas and flavors are completely absorbed [7]. However, it may also result in the release of undesirable aromas and flavors, so a partial extraction method using cloth-bagged botanicals is usually preferred. In concentrate preparation using base wine, the herbs and spices are placed in a vat outside the extraction tank from which the base wine is circulated through the herbs in the vessel until the extraction of most desired compounds. It is worth mentioning that better extraction can be attained if herbs and spices are softened with hot water in advance [34]. In the present work, herbs Cannabis, Sage, and Lemon balm were added in must and wine with direct extraction. In fact, an amount of approximately 10 g L^{-1} of all herbs studied, seems to extract the highest level of phenolic compounds. This quantity may be sufficient in case of future preparation of medicinal drinks, based on the studied wines, possibly bioactively enhanced. The same amount of herb was also used in the study of Popescu [35], where dried leaves of *Salvia Officinalis* were added in natural red wines for 21 days, and concluded that Sage enriches wines in polyphenolic compounds.

In the present study, for the direct extraction of the studied herbs, two different ways for their addition were followed: both in must (pre-fermentation addition) and in stable wine (post-fermentation addition). For this purpose, two white Greek grape varieties, Roditis and Muscat, and one red, Fokiano, were used. The total phenolic content and the antioxidant capacity of the produced wines were evaluated. According to our results, in each case, the addition of herbs increased both the total phenolic content and antioxidant capacity of the wines. In fact, this increase seems to be higher when herbs are added to stable wine than when they are added to must. So, in all herbs, we observed less increase

in antioxidant capacity by 23.8–40.4% (Table 5), when the extraction of herbs occurred in fermentation must, compared to herb addition in stable wine. To our knowledge, this is the first attempt to produce a herbal wine with herb addition before alcoholic fermentation. The difference occurred, may be due to metabolic processes during fermentation that takes place, where substances extracted from the herbs may be converted into other products with no or less antioxidant activity. The biotransformation of phenolics by different microorganisms during the fermentation of various plant-based foods and beverages has been reported in recent studies [36]. In our study, we also noticed that the amounts of rosmarinic and caffeic acid were not detectable in samples of pre-fermentation herb addition, while they were identified in samples of post-fermentation herb addition. The published research generally demonstrates that after the fermentation of various foods (such as soybean and brown rice) by various microorganisms, including yeasts, the total phenolic content (TPC) and antioxidant activity is increased [36]. However, in some cases, reduction seems to be more common. In the case of wine, further research is needed to investigate the interactions between wine and herb components during alcoholic fermentation.

Among the herbs that were studied in the present work, *Mellissa officinalis* seems to infuse a much higher percentage of phenolic and antioxidant substances into wine, and the highest amount of rosmarinic acid compared to the other studied species of the Lamiaceae family (sage). The total phenolic content extracted in wines was between 230.93 ± 2.81 and 642.35 ± 4.41 mg GAE L^{-1} g^{-1} (Table 2), while antioxidant activity was found between 2.67 ± 0.089 and 4.07 ± 0.153 mmol Trolox L^{-1} g^{-1} (Table 4). In the literature, *Melissa officinalis* has been studied mainly in aqueous, ethanol or methanol extracts, but not in wine. A high percentage of TPC in Melissa soluble extracts was also found by Kennedy et al. [13]. Moreover, Skotti et al. found that aqueous extracts of *Melissa officinalis* L. showed the highest values in total phenolic content (0.985 ± 0.001 mg caffeic acid mL^{-1}) and antioxidant activity (6.61 ± 0.04 μmol Trolox mL^{-1}), independently of the extraction process followed [37]. Also, *Melissa officinalis* was studied by Dastmalchi et al. in aqueous ethanol solutions, where 68.9 ± 21.3 mg gallic acid g^{-1} (dry wt.) was extracted from the herb, a lower amount than the one found in our studies in wine [38]. Wine is probably a better solution in terms of extracting herbs' phenolic compounds [35]. Methanol extracts of Melissa also studied by Jungmin et al. revealed the presence of caffeic acid and rosmarinic acid, as in the case of the wines studied in our work, although a different extraction medium was used [39]. It is noteworthy that HPLC analysis showed that higher amounts of rosmarinic acid are extracted when the addition of *Melissa officinalis* takes place in stable wine, rather than in must (Table 4).

Concerning *Salvia officinalis* (Sage), in the present study, an increase of 11.1–28.4% in the antioxidant capacity was identified when Sage was added to stable wine (Table 4). Our results are in accordance with the increase in the amount of total phenols found in the study of Popescu et al., where Sage was infused in red wine, showing an increase in total phenols from 6931 ± 109 to 10416.7 ± 620 mg GAE L^{-1}, which corresponds to a 28% increase [35]. Also, in the same research, caffeic acid was identified by HPLC in the medicinal wines produced, consistent with our results where caffeic acid was also identified in all wine samples of post-fermentation herb addition. Furthermore, rosmarinic acid has been found to be extracted from Sage in more studies such as the ones of Mouna et al. and Shekarchi et al. using methanol extracts [40,41].

Finally, according to our results, the extraction of *Cannabis sativa* increases the total antioxidant capacity of wines from 1.69 ± 0.071 to 3.49 ± 0.182 mmole Trolox L^{-1} g^{-1} of herb. In fact, Cannabis is infusing the lowest amount of phenolic compounds and displays the least antioxidant activity compared to *Melissa officinalis* and *Salvia officinalis*. Moreover, in our wine samples, a small amount of caffeic acid (0.9 mg L^{-1}) extracted from Cannabis was detected, while rosmarinic acid was not detected. Ahmed et al. also found a small amount of caffeic acid in the non-cannabinoid compounds of the cannabis plant, in methanol extracts, whereas in another research he defines that maximum and minimum phenolic content from *Cannabis sativa* leaves were determined by methanol and distilled

water, respectively. However, minimum phenols were observed in ethyl acetate and ethanol extracts [31]. As far as we are concerned, there is no previous research published on infused wines with Cannabis neither with white nor with red grape varieties.

On the other hand, among studied wines, the highest amount of total phenolic content and antioxidant capacity were observed in samples from the red indigenous variety Fokiano (Tables 2 and 4). This result is in accordance with other studies in Greek wines [27,42], which mention that the red wines produced by grape varieties grown in the Greek islands were richer in phenolic compounds, revealing that there are qualitative and quantitative differences in polyphenolic antioxidants of red and white Greek wines of different geographical origins [42]. Moreover, in the present study, the highest % decrease in TPC between pre- and post-fermentation herb addition was observed in all samples of Fokiano (59.4–69.2%, Table 3). However, the highest % decrease in antioxidant capacity was observed in Muscat samples (Table 5). It is worth mentioning that comparing the % difference between pre- and post-fermentation absolute values, in phenolic content there was a variation between 14.3 and 69.2%, whereas in antioxidant capacity an average of 34% was found.

In general, as resulted from the present study, herbs' addition increases wines' phenolic compounds, which is in agreement with similar studies found in the literature. For example, the addition of *Melissa officinalis* in apple wine has been reported to increase the polyphenol content [43]. In another study conducted by Lakicevic et al., selected aromatic herbs were added to red wines from the Serbian autochthonous variety 'Prokupac' (*Vitis vinifera* L.) and findings indicated that total phenolic and flavonoid contents, along with antioxidant activity, were significantly higher in all examined wine samples [44]. Also, Chamafambria et al. showed that the addition of *Lippia javanica* extracts enhanced the total phenol, color, and sensory properties of a *Uapaca kirkiana* fruit-based wine [45]. Tarapatskyy et al. studied white and red wines of the region of Poland enhanced with cowslip (*Primula veris* L.) and an increase in polyphenol compounds was detected [46]. Recently, Liang et al. enhanced Chardonnay wine in phenolic compounds using green tea and processing pulse electric field [47]. However, in the above-mentioned studies, herbs are added to wines mainly as an extract, while in the present work, herbs were added with direct extraction.

5. Conclusions

This study concludes that *Cannabis sativa*, *Melissa officinalis* and *Salvia officinalis* can enrich the Greek wines Roditis, Muscat and Fokiano with polyphenolic extracts and antioxidant compounds and this enrichment is higher when herbs are added in stable wine (post-fermentation herb addition). Also, *Melissa officinalis* was found to induce a higher increase in phenolic content and antioxidant potential. Further studies are needed to determine the multitude of phenolic compounds extracted from herbs as well as the aromatic compounds that are likely to be extracted, contributing to the organoleptic characteristics of wines. Of particular interest will be the study of biodegradation of wine's phenolic components during alcoholic fermentation, as there are very few studies investigating the changes in the phenolic profile of wine after adding flavor additives. The findings can be applied in the future for the production of wine-based beverages with unique aromas and increased bioactivities.

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