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Determination of Phenolic Content and DPPH Radical Scavenging Activity of Functional Fruit Juices Fortified with *Thymus serpyllum* L. and *Salvia officinalis* L. Extracts

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Abstract: The objective of this study was to spectrophotometric determinate the total phenolic, flavonoid, hydroxycinnamic acid, and flavonol content of orange, pineapple, and apple juices fortified with wild thyme (*Thymus serpyllum* L.), Dalmatian sage (*Salvia officinalis* L.), and wild thyme-Dalmatian sage (3:1, *v*/*v*) extracts, and to evaluate their DPPH radical scavenging activity as a contribution to the development of a new functional beverage. The plant extracts addition increased the amount of phenolic compounds in fruit juices and improved their antioxidant properties. The highest concentrations of bioactive compounds and the greatest DPPH radical activity were obtained by adding Dalmatian sage extract to orange juice. Our study provides the novelty of fortifying fruit juices with wild thyme and Dalmatian sage extracts and offers significant potential for the creation of functional beverages.

Keywords: *Thymus serpyllum* L., *Salvia officinalis* L., phenolic compounds, DPPH, functional beverages, spectrophotometry.

INTRODUCTION

THE increasing popularity of functional foods and beverages with health benefits is largely due to the new century trend of easy access to information and the ability of consumers to strive for a high quality of life.[1] Production of functional beverages has increased, especially non-alcoholic beverages to which natural ingredients from fruit or vegetable, plants, vitamins, minerals, or amino acids are added.[2] Because of their flavor, aroma, composition of chemical molecules, and health-promoting properties, medicinal and aromatic plants are rich sources of bioactive molecules that can be used to produce functional beverages.[3,4] Many medicinal and aromatic plants from the Lamiaceae family are valuable in the field of the functional beverages and provide a variety of health benefits.[5]

Mediterranean plants, including wild thyme (*Thymus serpyllum* L.) and Dalmatian sage (sage in the text below) (*Salvia officinalis* L.), have a particular variety of bioactive molecules that may potentially contribute to the products' functional and sensory qualities. These plants contain a broad spectrum of polyphenols, including various phenolic acids and flavonoids[6,7] with a high water solubility. Plant phenolic compounds exhibit significant molecular diversity, are widely distributed throughout the plant kingdom, and perform an array of structural and protective activities.[8] In addition, phenolic compounds can significantly impact food products as they can be used as natural colorants or as antioxidants to protect food ingredients that are sensitive to

oxidative changes.[9,10] Consuming foods filled with antioxidants may reduce the possibility of oxidative stress, chronic diseases, and related hazards.[11] Therefore, more research has been conducted on the nutritional value of fruit juices, as people worldwide seek to lead better lifestyles.[12]

Plant extracts can be added to fruit juices to enhance the therapeutic potential of the beverage while improving its organoleptic properties.[13] Since plant extracts are very acidic and bitter, they combine well with fruit juices, which have a pleasant flavor and aroma. Another advantage of blended beverages is that the resulting formulations can be modified to make them more palatable to customers.[14] Orange (*Citrus sinensis* (L.) Osbeck), apple (*Malus domestica* (Suckow) Borkh.) and pineapple (*Ananas comosus* (L.) Merr.) are one of the most often consumed fruits within the functional beverage sector and are recognized for their rich sensory characteristics, nutritional value and potential prevention of a number of degenerative diseases.[15–17] In our previous study on the wild thyme, sage and laurel (*Laurus nobilis* L.) extracts, it was determined that wild thyme, sage, and their two-component extract mixture had the greatest total phenolic content among all samples.[18] These extracts were thus selected for the fortification of orange, pineapple and apple juices.

The aim of this study was to provide novelty with the respect to our previous research regarding spectrophotometrically determination (first time report) of the total content of phenols, flavonoids, hydroxycinnamic acids and flavonols in the fruit juices fortified with sage, wild thyme and wild thyme-sage (3:1, *v*/*v*) extracts as well as regarding evaluation of their DPPH radical scavenging activity for the possible development of a new functional beverage. Individual compounds were not investigated from the extracts in present study, but ultra-performance liquid chromatography tandem mass spectrometry (UPLC/MS-MS) was already used in our previous study[18] for identification the present major polyphenols.

EXPERIMENTAL

Chemicals

The Folin-Ciocalteu's reagent was purchased from Fisher Scientific (UK). Sodium carbonate and ethanol were provided by Gram-mol Company (Zagreb, Croatia). Potassium acetate was obtained from VWR Chemicals (Radnor, PA, USA) and hydrochloric acid was obtained from Carlo Erba Reagents S.r.l. (Val-de-Reuil, France). Aluminium chloride and 2,2-diphenyl-1-picrylhydrazyl were supplied from Sigma-Aldrich (Steinheim, Germany).

Plant and Juice Material

Samples of wild thyme (*Thymus serpyllum* L.) and sage (*Salvia officinalis* L.) were obtained from Suban Ltd. (Strmec Samoborski, Croatia). The plants were collected in 2020 and stored in a dark and dry place. The dry weight of sage and wild thyme was 92.76 % and 92.49 %, respectively. Before extraction, the plants were pulverized using an electric grinder (WSG30, Waring Commercial, Torrington, CT, USA). Juice producer Stanić Beverages Ltd. (Zagreb, Croatia) supplied the concentrated orange, pineapple and apple juices.

Preparation of Plant Extracts

In our previous study,[18] the preparation of the plant extracts was described in detail where different ratios of two- and three-component plant extract mixtures containing wild thyme (WT), sage (S), and/or laurel were studied. The highest total phenolic content was found in a two-component extract mixture of wild thyme and sage (3:1, *v*/*v*) (WTS) and pure WT and S extract, so the same ratio and pure extracts were selected for the present study. The samples were stored at 4 °C (less than 7 days).

Preparation of Functional Beverages

Concentrated orange, pineapple, and apple juices were mixed with the S, WT, and WTS plant extracts after being diluted with water to approximately 11 % of soluble dry matter. The amount of each extract added to each juice was 10 %, since we have shown in our previous research that the addition of this percentage leads to favorable sensory properties of fortified fruit juices.[19] Table 1. shows various beverage formulations from fruits fortified with plant extracts.

Total Phenol Content Determination

To determine the total phenol content (TPC) of the extracts, a spectrophotometric method based on the color response of phenols with Folin-Ciocalteu's reagent was utilized.[20] 200 µL of undiluted Folin-Ciocalteu's reagent, 100 µL of the

Table 1. Beverage formulations made with fruit juices fortified with plant extracts.

Juice	Extract	Label
orange	wild thyme	OJWT
	sage	OJS
	wild thyme-sage (3:1, *v*/*v*)	OJWTS
pineapple	wild thyme	PJWT
	sage	PJS
	wild thyme-sage (3:1, *v*/*v*)	PJWTS
apple	wild thyme	AJWT
	sage	AJS
	wild thyme-sage (3:1, *v*/*v*)	AJWTS

plant extract, 2 mL of distilled water and 1 mL of a 20 % sodium carbonate solution, added after three minutes were combined to form a reaction mixture. The mixtures were stirred in a vortex and hold in a water bath at 50 °C for 25 minutes. The solution's optical density (absorbance) was determined using a UV–VIS spectrophotometer (Shimadzu, UV-1900i, Kyoto, Japan) set to 765 nm. The experiments were performed in triplicate, and the reaction was carried out using distilled water as a blank. TPC was determined using gallic acid standard calibration curve ($y = 0.0029x$, $R^2 = 0.9995$), and the results are presented as mean values ± standard error of g L^{-1} of the sample ($n = 3$).

Determination of Total Flavonoid Content

The color reaction of flavonoids with potassium acetate and aluminum chloride forms the basis of the spectrophotometric method for the determination of total flavonoid content (TFC) in plant extracts.[21] The reaction mixture was created by combining 0.5 mL of the plant extract, 2.8 mL of distilled water, 1.5 mL of 96 % ethanol, 0.1 mL of 1 M potassium acetate and 0.1 mL of 10 % aluminum chloride. Measurements were performed in triplicate, and a blank sample was made following the same protocols but with distilled water rather than the plant extract and 10 % aluminum chloride. After the prepared combinations were stored at room temperature for 30 minutes, absorbance was measured at 415 nm. TFC was determined by quercetin calibration curve ($y = 0.0071\ x + 0.0009$, $R^2 = 0.9989$), and results are presented as mean values ± standard error of g L^{-1} of the sample ($n = 3$).

Determination of Total Flavonol and Hydroxycinnamic Acid Content

The content of total flavonols (TFLC) and hydroxycinnamic acids (THCA) was measured following the method developed by Howard *et al.*[22] The reaction mixture was created by adding 250 µL of the extracts, 250 µL of 1 g L^{-1} hydrochloric acid (combined with 96 % ethanol), and 4.55 mL of 2 g L^{-1} hydrochloric acid (combined with distilled water). Absorbance was determined in triplicate at 360 and 320 nm. The blank sample was prepared according to the same protocol, using distilled water rather than the plant extract. TFLC was quantified using quercetin calibration curve ($y = 0.0036\ x + 0.015$, $R^2 = 0.9911$), while THCA was quantified using caffeic acid calibration curve ($y = 0.0047\ x + 0.0231$, $R^2 = 0.9998$). Results are presented as mean values ± standard error in g L^{-1} of the sample ($n = 3$).

DPPH Radical Scavenging Activity

The ability of fruit juices fortified with plant extracts to scavenge free radicals was investigated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay described by Von Gadow *et al.*[23] with slight modifications. Both the fruit juices and the fruit juices fortified with the plant extracts were prepared in distilled water at different concentrations (3.44–55 mg mL^{-1} of soluble dry matter). A volume of 50 µL of the sample was mixed with 2 mL of 6×10^{-5} ethanolic solution of the DPPH radical. The mixtures were stirred in a vortex and kept in a dark place for 30 minutes. Then, the absorbance at 517 nm was measured in triplicate. The control solution was prepared by mixing 50 µL of ethanol and 2 mL of a 6×10^{-5} ethanolic DPPH solution. DPPH radical percentage inhibition was calculated according to the formula of Yen and Duh[24]

$$\% \text{ inhibition} = \left[(A_{C(0)} - A_{A(t)} / A_{C(0)} \right] \times 100$$

where $A_{C(0)}$ is the absorbance of the control solution and $A_{A(t)}$ is the absorbance of the sample. The sample concentration that exhibited 50 % inhibition (IC_{50}) was determined from linear regression analysis interpolation.

Statistical Analysis

Statistical analysis was conducted using STATISTICA v. 8 software (StatSoft Inc., Tulsa, OK, USA). After ensuring that the data set was normally distributed using the Shapiro-Wilks test and that the residuals were homoscedastic using the Levene's test, the results were examined using analysis of variance (ANOVA) for parametric data or the Kruskal-Wallis test for nonparametric data. Marginal means were compared using the Tukey's HSD test or Kruskal-Wallis test, if appropriate, and a statistically significant difference was assumed at $p \leq 0.05$ (95 % confidence interval).

RESULTS AND DISCUSSION

In this study, orange, pineapple and apple juices were fortified with S, WT and WTS and analysed for TPC, TFC, THCA, TFLC and DPPH radical scavenging activity. The results are presented in Table 2, Table 3 (supplementary material) and Figure 1. The values of TPC, TFC, THCA and TFLC differed significantly ($p \leq 0.001$) among the samples studied. The highest levels of TPC (2.05 g GAE L^{-1}), THCA (0.21 g CAE L^{-1}) and TFLC (0.27 g QE L^{-1}) were observed when the plant extracts were mixed with orange juice, especially in the formulation OJS (Table 2.). As for TFC, the highest concentrations were found in pineapple formulations, mainly in PJS (0.19 ± 0.01 g QE L^{-1}). Even without the addition of extracts, pure orange juice contained a significant amount of phenolic compounds. Interestingly, the addition of WTS extract to OJ did not increase THCA and TFLC. AJ had the lowest content of phenolic compounds among all juices studied. However, fortification with plant extracts had a greater effect on TPC, TFC, THCA, and TFLC compared with

the other juices. For example, TFC for AJ increased from about 60 % to 80 % when fortified with plant extracts, while TFC for fortified OJ increased from approximately 15 % to 35 %. The phenolic compounds in S, WT and WTS were described in our previous study. The major polyphenols detected by the UPLC/MS-MS were kaempferol-3-rutinoside (10.80–48,87 g L^{-1} of sample), kaempferol-3-O-hexoside (6.43–137.04 g L^{-1} of sample), apigenin (2.21–6.28 g L^{-1} of sample), catechin (0.36–1.40 g L^{-1} of sample), epicatechin (0.31–1.44 g L^{-1} of sample), caffeic acid (7.11–17.72 g L^{-1} of sample), *p*-hydroxybenzoic acid (1.43–3.80 g L^{-1} of sample) and protocatechuic acid (13.55–40.14 g L^{-1} of sample).[18] These compounds greatly contribute to antioxidant activity, and were previously recognized as the main constituents of sage and wild thyme extract.[7,25–32]

The DPPH assay, one of the most common antioxidant methods, can be used to evaluate the ability of fruit juices and plant extracts to scavenge free radicals. Antioxidants may neutralize DPPH, a persistent free organic radical, by hydrogen atoms or electrons, respectively. With a spectrophotometer, neutralization causes a color change which is simple to observe. Neutralization caused a color change that is easily observed with a spectrophotometer. In the current study, the highest DPPH radical scavenging activity (%) was observed for the OJWTS formulation (91.92 ± 0.02), followed by the OJS formulation (91.61 ± 0.01) (Table 3, supplementary material). To compare the antioxidant properties of fruit juices and fruit juices fortified with plant extracts, the sample amounts required for reducing the original radical concentration by 50 % (IC_{50}) have been calculated (Figure 1). A lower IC_{50} value indicates that the sample has a greater ability to act as an antioxidant. AJ and PJ did not reach an IC_{50} value at the tested concentrations. As expected, better effectiveness was evident in fortified fruit juices with IC_{50} values ranged from 19.69 mg mL^{-1} to 40.98 mg mL^{-1} of soluble dry matter, according to the following order: OJS > AJS > OJWTS > PJS > OJWT > AJWT > PJWT > AJWTS > PJWTS.

To the authors' knowledge, there are no studies comparing TPC, TFC, TFLC, and THCA in formulations containing orange, pineapple or apple juice fortified with wild thyme, sage, and/or wild thyme-sage extract. Therefore, current study provides new insight into the fortification of various fruit juices with several plant extracts. Ivanišová *et al.*,[33] however, fortified apple juice with sage and wild thyme extracts and obtained TPC and TFC that were lower

Table 2. Content of total phenols, flavonoids, flavonols and hydroxycinnamic acids of orange, pineapple and apple juice fortified with wild thyme and sage extracts.

	TPC / g GAE L^{-1}	TFC / g QE L^{-1}	TFLC / g QE L^{-1}	THCA / g CAE L^{-1}
	$p = 0.0002$*	$p = 0.0012$*	$p = 0.0002$*	$p = 0.0012$*
OJ	$1.33 \pm 0.01^{a,b,c}$	$0.05 \pm 0.00^{a,b}$	$0.21 \pm 0.01^{a,b,c}$	$0.17 \pm 0.01^{a,b,c}$
PJ	$0.70 \pm 0.01^{a,b}$	$0.08 \pm 0.00^{a,b,c}$	$0.05 \pm 0.02^{a,b,c}$	$0.04 \pm 0.02^{a,b,c}$
AJ	0.47 ± 0.03^{a}	0.03 ± 0.01^{a}	0.01 ± 0.00^{a}	0.00 ± 0.00^{a}
OJWT	$1.87 \pm 0.02^{b,c}$	$0.06 \pm 0.00^{a,b,c}$	$0.4 \pm 0.00^{b,c}$	$0.19 \pm 0.00^{b,c}$
OJS	2.05 ± 0.05^{c}	$0.08 \pm 0.00^{a,b,c}$	0.27 ± 0.01^{c}	0.21 ± 0.00^{c}
OJWTS	$1.77 \pm 0.08^{a,b,c}$	$0.05 \pm 0.00^{a,b,c}$	$0.19 \pm 0.00^{a,b,c}$	$0.15 \pm 0.00^{a,b,c}$
PJWT	$1.17 \pm 0.01^{a,b,c}$	$0.12 \pm 0.00^{a,b,c}$	$0.09 \pm 0.01^{a,b,c}$	$0.07 \pm 0.01^{a,b,c}$
PJS	$1.05 \pm 0.03^{a,b,c}$	0.19 ± 0.01^{c}	$0.11 \pm 0.01^{a,b,c}$	$0.08 \pm 0.00^{a,b,c}$
PJWTS	$0.83 \pm 0.03^{a,b,c}$	$0.13 \pm 0.00^{a,b,c}$	$0.10 \pm 0.00^{a,b,c}$	$0.08 \pm 0.00^{a,b,c}$
AJWT	$0.96 \pm 0.01^{a,b,c}$	$0.08 \pm 0.00^{a,b,c}$	$0.02 \pm 0.00^{a,b,c}$	$0.01 \pm 0.00^{a,b,c}$
AJS	$1.23 \pm 0.02^{a,b,c}$	$0.16 \pm 0.01^{b,c}$	$0.03 \pm 0.00^{a,b,c}$	$0.02 \pm 0.00^{a,b,c}$
AJWTS	$0.83 \pm 0.00^{a,b,c}$	$0.10 \pm 0.00^{a,b,c}$	$0.02 \pm 0.00^{a,b}$	$0.01 \pm 0.00^{a,b}$

TPC – total phenol content, TFC – total flavonoid content, TFLC – total flavonol content, THCA – total hydroxycinnamic acid content. OJ – orange juice; PJ – pineapple juice; AJ – apple juice; OJWT – orange juice + wild thyme extract; OJS – orange juice + sage extract; OJWTS – orange juice + wild thyme-sage extract (3:1, *v*/*v*); PJWT – pineapple juice + wild thyme extract; PJS – pineapple juice + sage extract; PJWTS – pineapple juice + wild thyme-sage extract (3:1, *v*/*v*); AJWT – apple juice + wild thyme extract; AJS – apple juice + sage extract; AJWTS – apple juice + wild thyme-sage extract (3:1, *v*/*v*); GAE – gallic acid equivalent, QE – quercetin equivalent; CAE – caffeic acid equivalent. Results are expressed as mean ± standard error. *Statistically significant variable at $p \leq 0.05$. Values with different letters within a column are statistically different at $p \leq 0.05$.

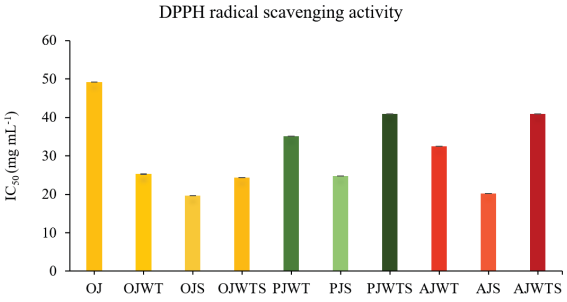

Figure 1. IC_{50} (mg mL^{-1} of soluble dry matter) values of orange, pineapple and apple juice fortified with wild thyme and sage extracts. OJ – orange juice; OJWT – orange juice + wild thyme extract; OJS – orange juice + sage extract; OJWTS – orange juice + wild thyme-sage extract (3:1, *v*/*v*); PJWT – pineapple juice + wild thyme extract; PJS - pineapple juice + sage extract; PJWTS - pineapple juice + wild thyme-sage extract (3:1, *v*/*v*); AJWT – apple juice + wild thyme extract; AJS – apple juice + sage extract; AJWTS – apple juice + wild thyme-sage extract (3:1, *v*/*v*); The data are presented as mean values of three independent experiments ± standard error. *Statistically significant variable ($p \leq 0.05$). The values with different letters are statistically different ($p \leq 0.05$).

compared with our results for apple juice (125.52 mg GAE L^{-1} for TPC and 63.07 μg QE L^{-1} for TFC) and for both sage and wild thyme formulations (342 and 315.89 mg GAE L^{-1} for TPC; 185.50 and 108.27 μg QE L^{-1} for TFC). Our results for TPC in pineapple juice were also higher than 33.1 mg GAE 100 mL^{-1} reported by Piechowiak *et al.*,[34] where the juice was fortified with *Pinus maritima* Miller bark extract (Pycnogenol ®). Similarly, Sarvarian *et al.*[35] and Hashemi *et al.*[36] reported that the addition of extracts of oleaster (*Elaeagnus angustifolia* L.) and *Moringa oleifera* Lam. to orange juice increased its TPC. In addition, Tamer *et al.*[37] fortified lemonade (*Citrus limon* (L.) Osbeck) with the extracts of lemon verbena (*Lippia citrodora* (Paláu) Kunth), mate (*Ilex paraguariensis* A. St.-Hil), clove (*Eugenia caryophyllata* Thunb.), green tea (*Camellia sinensis* (L.) Kuntze), linden (*Tilia argentea* DC.), peppermint (*Mentha x piperita* L.), and ginger (*Zingiber officinale* Roscoe), and heather (*Erica arborea* L.). All formulations had higher TPC compared with lemonade. Saad *et al.*[38] fortified cucumber (*Cucumis sativus* L.) juice with ginger, clove, mint (*Mentha spicata* L.) and cinnamon (*Cinnamomum verum* J. Presl) extracts. The results of their study indicated that TPC was higher in all fortified formulations, as was TFC, except in the formulation with cinnamon, where the results were lower than in unfortified juice. Orange juice had the highest TPC compared to apple or pineapple juice. Our results are in accordance with the paper of Soural *et al.*[39] in which the orange juice concentrate had higher content of total polyphenols compared to apple or grapefruit (*Citrus paradisi* Macfad.) juices. It was expected that formulations containing orange juice would have higher TPC and antioxidant properties. According to the literature, there is a lack of information on the study of TFLC and THCA in fortified fruit juices, so our study was the first to evaluate these aspects in detail and therefore is a contribution to the general knowledge about fortified fruit juices.

All tested fruit juices in our study showed better antioxidant properties when fortified with WT, S or WTS extract. These results are in agreement with our previous study, which showed that fortified fruit juices have higher antioxidant activity by oxygen radical absorbance capacity (ORAC) assay.[19] This is most likely due to the different structures of the phenolic components, which are present in higher concentrations in the plant extracts than in the pure fruit juices. Our findings are also consistent with the results of a previous study by Ogundele *et al.*,[14] who indicated that the ability of fruit juice formulations to scavenge free radicals increased with increasing plant extract amounts. Similar results were reported by Sarvarian *et al.*,[35] who found that orange juice fortified with aqueous oleaster extract significantly increased DPPH free radical activity. Interestingly, the addition of alcoholic and hydroalcoholic oleaster extract resulted in significantly lower DPPH free radical activity. Hashemi *et al.*[36] also confirmed that the amount of phenolic components and antioxidant properties in orange juice increased when *Moringa oleifera* leaf extract was added. Ivanišová *et al.*[33] fortified apple juice with various plant extracts and also confirmed that the antioxidant activity of the juice increased with the addition of plant extracts. Antioxidant activity was also higher in lemonade fortified with the extracts of lemon verbena, mate, clove, green tea, linden, ginger, peppermint, and heather.[37] Moreover, the addition of apple pomace extract to apple juice was found to be more effective against DPPH radicals than without the addition of apple pomace extract.[40] Plant extracts can be used not only to enhance antioxidant activity, but also to improve the sensory properties of functional beverages and to develop new formulations acceptable to consumers.[19]

CONCLUSIONS

The present study was the first to investigate the total phenolic, flavonoid, hydroxycinnamic acid and flavonol content, and antioxidant activity of apple, pineapple and orange juices fortified with wild thyme, sage and wild thyme-sage (3:1, *v/v*) extracts. The best results were obtained by fortifying orange juice with sage, although pure orange juice had a high content of phenolic compounds. Apple juice had the lowest content of phenolic compounds, but showed a greater increase than orange juice after fortification with plant extracts. Based on the profile of phenolic compounds and the associated DPPH radical scavenging activity, it can be concluded that wild thyme and sage extracts can be successfully used to improve the biopotential of fruit juices. The use of wild thyme and sage extracts and their combination to fortify fruit juices showed great potential for the future development of functional beverages.

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Supplementary Information. Supporting information to the paper is attached to the electronic version of the article at: https://doi.org/10.5562/cca4003.

PDF files with attached documents are best viewed with Adobe Acrobat Reader which is free and can be downloaded from Adobe's web site.

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