

Spice plants: Chemical composition and antioxidant properties of *Pimenta Lindl.* essential oils, part 2: *Pimenta racemosa* (Mill.) J.W. Moore leaf oil from Jamaica*

Gewürzpflanzen: Chemische Zusammensetzung und antioxidative Aktivitäten von ätherischen Ölen von *Pimenta Lindl.*, Teil 2: *Pimenta racemosa* (Mill.) J.W. Moore Blattöl aus Jamaika

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Summary

The chemical composition of the essential *Pimenta racemosa* leaf oil ("bay oil") from Jamaica was analyzed by GC and GC-MS and as major compounds eugenol (45.60 %), myrcene (24.97 %) and chavicol (9.31 %) were identified. The antioxidant properties of this *P. racemosa* essential leaf oil were assayed by its ability to scavenge DPPH and hydroxyl (OH•) radicals. The concentrations, which lead to 50 % neutralization (IC₅₀-value), were estimated as 10.0 µg/mL and 0.6 µL/mL for the radicals, respectively. The bay oil prevented the generation of the superoxide radical via inhibiting the xanthine oxidase activity to 49.03 %. High antioxidant activity of the bay oil in a linoleic emulsion system was observed with 54.34 % inhibition of conjugated diene formation and 88.23 % inhibition of the generation of secondary products from lipid peroxidation.

Keywords:

Pimenta racemosa (Mill.) J.W. Moore, essential leaf oil, chemical composition, GC, GC-MS, antioxidant properties

Zusammenfassung

Die chemische Zusammensetzung eines ätherischen Blattöles von *Pimenta racemosa* aus Jamaika wurde mittels GC und GC-MS analysiert und als Hauptkomponenten wurden Eugenol (45,60 %), Myrcen (24,97 %) und Chavicol (9,31 %) identifiziert. Die antioxidativen Eigenschaften dieses ätherischen *P. racemosa* Blattöles wurden in Hinsicht auf die Fähigkeit, DPPH- und Hydroxyl- (OH•) Radikale entgegenzuwirken, untersucht. Die Konzentrationen, welche zu einer 50 %igen Neutralisation (IC₅₀-Wert) führten, wurden mit 10,0 µg/mL respektive 0,6 µL/mL bestimmt. Das Bayöl verhindert die Generierung von Superoxid-Radikalen durch Hemmung der Xanthinoxidase-Aktivität zu 49,03 %. Hohe antioxidative Aktivitäten des Bayöles wurden auch in einem Modellsystem einer Linolensäure-Emulsion beobachtet. Es hemmt 54,34 % der Bildung von konjugierten Dienen und 88,23 % der Ausbildung von sekundären Produkten aus der Lipidperoxidation.

Kennwörter:

Pimenta racemosa (Mill.) J.W. Moore, ätherisches Blattöl, chemische Zusammensetzung, GC, GC-MS, antioxidative Eigenschaften

1. Introduction

Pimenta racemosa (Mill.) J.W. Moore syn. *Pimenta acris* (Sw.) Kostel belongs to the botanical spice-group of *Pimenta* Lindl. ("Allspice"), Myrtaceae family [1]. The tropical, evergreen dried fruits of the *P. racemosa* tree are used around the world as valuable spice, but in lower quantities as the better known species of this family, *Pimenta dioica* (L.) Merr. [1], and also for adulteration of the *P. dioica* spice. The common names of

this spice are known, such as bayrum (tree), West Indian bay (English) and Bayrum(baum) as well as Kronpiment (German) [1].

The bayrum-tree grows or is cultivated especially in Indonesia, West Indies, Venezuela, Mexico, Puerto Rico, Guayana, Jamaica and Africa (Cameroon) [1].

The essential oil is obtained from fruits or leaves with yields of 1.5 – 4.5 %. The major compound of most of the *Pimenta racemosa* oils is eugenol (70 – 80 %) [2, 4, 5]. In bay leaf oils of *P. racemosa* var. *terebinthia* α-terpinyl

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acetate, α -terpineol and methoxyeugenol as well as of *P. racemosa* var. *grisea* 4-methoxy-isoeugenol and 4-methoxyeugenol were found as dominating constituents [6].

The essential *P. racemosa* leaf and fruit oil is used in perfumes, aftershaves, lotions enhancing hair growth and strength or acting against hair loss and commercial food flavoring [1, 3]. The essential bay leaf oil is also important in Caribbean folk medicine and used for the treatment of rheumatism or for toothache as well as for its antiinflammatory and analgesic properties [6].

Oxidation reactions are an essential part of normal metabolism as oxygen is the ultimate electron acceptor in the electron flow system that produces ATP [7]. Free radicals and reactive oxygen species are continuously emerging in living cells as a function of the mitochondrial electron transfer or as bio-products formed by the action of the enzymes xanthin oxidase, lipoxygenase and cycloxygenase [8]. Reactive oxygen species exert a positive impact on living organisms as they participate in energy generation and cell growth, but on the other hand they are fully capable of attacking vital bio-molecules, such as lipids, proteins and deoxyribonucleic acid [9]. The autoxidation of polyunsaturated fatty acids not only reduces the nutritional value of foods [10-12], but also leads to membrane injuries, ageing, cardiovascular and cancerous diseases [13, 14]. When the level of reactive oxygen species surpasses the antioxidant capacity of the human body, the oxidative losses trigger the initiation of various diseases [13, 15]. The damages caused by free radicals and reactive oxygen species are associated with some neuro-degenerative disorders [16], cancer [17], and oxidation of low-density lipoprotein as a major factor in the promotion of coronary heart disease and atherosclerosis [18].

Currently there exists a great worldwide interest in finding new and safe antioxidants from natural sources (e. g. berries, vegetables, corn and seeds, aromatic and medicinal plants) to prevent oxidative deterioration of foods and to minimize oxidative damage of living cells [19-24]. Our study has focused on spice constituents or isolates which are functional antioxidants yet do not have the overpowering or undesirable organoleptic properties of the whole spice. Therefore, essential oils and extracts of *Pimenta* sp. are very interesting samples to investigate on this field, but only a few data are available until now [25-27].

2. Materials and methods

2.1. Essential oil and natural reference compound

The essential leaf oil of *Pimenta racemosa* (Mill.) J.W. Moore from Jamaica and the natural reference com-

pound eugenol are products from Kurt Kitzing Co., Wallerstein, Germany, with number 800116 (charge 12724).

2.2. GC analysis

GC/FID analyses were carried out using a GC-14A with split/splitless-injector, FID and C-R6A-Chromatopac integrator (Shimadzu, Japan), a GC-3700 with FID (Varian, Germany) and C-R1B-Chromatopac integrator (Shimadzu). The carrier gas was hydrogen (flow rate of 1.5 mL/min.); injector temperature, 250 °C; detector temperature, 320 °C; injected volume: 0.1 μ L genuine oil (splitless mode). The temperature programme was: 40 °C/5 min to 280 °C/5 min, with a heating rate of 6 °C/min. The columns were 30 m x 0.32 mm bonded FSOT-RSL-200 fused silica, with a film thickness of 0.25 μ m (Biorad, Germany) and 30 m x 0.32 mm bonded Stabilwax, with a film thickness of 0.50 μ m (Restek, USA). Quantification was achieved using peak area calculations, and compound identification was carried out partly using correlations between retention times [28-32].

2.3. GC-MS analysis

For GC/MS measurements a GC-17A with QP5000 (Shimadzu), split/splitless injector and Compaq-ProLinea data system (class5k-software), a GC-HP5890 with HP5970-MSD (Hewlett-Packard, USA) and ChemStation software on a Pentium PC (Böhm, Austria), a GCQ (Finnigan-Spectronex, Germany-USA) and Gateway-2000-PS75 data system (Siemens-Nixdorf, Germany, GCQ-software) were used. The carrier gas was helium (flow rate: 1.0 mL/min.); injector temperature, 250 °C; injected volume: 0.1 μ L genuine oil (splitless mode); interface-heating at 300 °C, ion-source-heating at 200 °C, EI-mode was 70 eV electron energy, and the scan-range was 41-450 amu. For other parameters, see description of GC/FID, above. Mass spectra correlations were done using Wiley, NBS, NIST and our own library as well as published data [28, 30, 31].

2. Scavenging effect on 2,2-diphenyl-1-picrylhydrazyl radical (DPPH)

The radical scavenging capacity was determined according to the method described by [33]. 1.0 mL from 0.3 mM alcohol solution of DPPH was added to 2.5 mL from the samples with different concentration of bay oil and of eugenol, each. The samples were kept at room temperature in the dark and after 30 min the optic density was measured at 518 nm. The optic density of the samples, the control and the empty samples were measured in comparison with ethanol. Ascorbic acid, rutin, butylhydroxy toluene (BHT) and butylated hydroxyl anisole (BHA) were used as positive control.

2.4. Detection of hydroxyl radicals by deoxyribose assay

The assay was performed as described elsewhere [34] with minor changes. All solutions were freshly prepared. 1.0 mL of the reaction mixture contained 28 mM 2-deoxy-D-ribose (dissolved in $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer 10 mM, pH 7.4), 500 μL solution of various concentrations of the bay oil or eugenol, 200 μM FeCl_3 and 1.04 mM EDTA (1:1 v/v), 10 mM H_2O_2 and 1.0 mM ascorbic acid. After an incubation period of 1 h at 37 °C the extent of deoxyribose degradation was measured by the thiobarbituric acid (TBA) reaction. 1.0 mL of TBA (1 % in 50 mM NaOH) and 1.0 mL of trichloroacetic acid (TCA) were added to the reaction mixture and the tubes were heated at 100 °C for 20 min. After cooling, the absorbance was measured at 532 nm against a blank (containing only buffer and deoxyribose). The percentage inhibition was calculated by the formula:

$$I(\%) = 100 - (\text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}) \times 100$$

The IC_{50} value represented the concentration of the compounds that caused 50 % inhibition of radical formation. Quercetin was used as a positive control.

2.5. Assay of superoxide radical anion

Superoxide anions were generated in an enzymatic system (xanthine-xanthine oxidase) and determined by the reduction of nitroblue tetrazolium. The former comprised a solution of 100 μM xanthine, 60 μM nitroblue tetrazolium in 0.1 M phosphate buffer at pH 7.4 and 0.07 U mL^{-1} xanthine oxidase in a total volume of 1 mL. This mixture was incubated at 25 °C for 10 min and the optical density was recorded at 560 nm against a blank, which did not contain the enzyme [35]. In order to check the inhibitory effect of pimento oil on xanthine oxidase activity, the enzyme was assayed by measuring the formation of uric acid from xanthine [35, 36]. Concentrations of 25 $\mu\text{g}/\text{mL}$ of bay oil, eugenol and BHT were added to the sample before the enzyme. The percentage inhibition of xanthine oxidase was calculated by the formula:

$$I(\%) = 100 - (\text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}) \times 100$$

2.6. Evaluation of antioxidant activity in linoleic acid model system

Linoleic acid emulsions were prepared by mixing 0.285 g of linoleic acid, 0.289 g of Tween 20 as emulsifier and 50 mL phosphate buffer (pH 7.2). The mixture was homogenized for 5 min according to [37]. The antioxidant was added at the final concentrations of 0, 0.01 and 0.02 % wt/vol of bay oil, BHT 0.01 % was used as control. The mixture was incubated in an oven at 37 °C for 10 d. The course of oxidation was monitored by measuring the conjugated diene forma-

tion (CD) and thiobarbituric acid reactive substances (TBARS). The antioxidant activity at the end of the assay time was expressed for each indicator as reduction percent of peroxidation (RP %) with the control containing no antioxidant being 0 %.

$$\text{RP \%} = [(\text{peroxidation indicator value without antioxidant}) - (\text{peroxidation indicator value with antioxidant}) / \text{peroxidation indicator value without antioxidant}] \times 100.$$

A higher percentage indicates a higher antioxidative activity.

2.7. Determination of conjugated diene (CD) formation

Aliquots of 0.02 mL were taken at different intervals during incubation. After incubation, 2 mL of methanol in deionised water (60 %) were added, and the absorbance of the mixture was measured at 233 nm. The conjugated diene concentration was expressed in mL/mg in each sample. The results were calculated as $\text{CD} = B \times \text{vol}/\text{wt}$; where B is the absorbance reading, vol denotes the volume (mL) of the sample and wt is the mass (mg) of emulsion measured [38].

2.8. Determination of thiobarbituric acid reactive substances

A modified thiobarbituric acid reactive substances (TBARS) method was used to measure the antioxidant activity of the essential oil in terms of inhibition on lipid peroxidation. 0.1 mL of sample was taken every day, from the emulsion, the following were sequentially added: the TBA-TCA solution (2 mL TBA in 15 % TCA). The mixture was heated in a 100 °C water bath for 15 min and cooled at room temperature. After 2 mL of chloroform were added, the mixture was mixed and centrifuged at 2000 rpm for 15 min. The chloroform layer was separated and the absorbance of the supernatant was measured at 532 nm against a blank containing TBA-TCA solution. Malonic aldehyde standard curves were prepared by 1,1,3,3-tetramethoxypropane and TBARS were expressed as mg of malonic aldehyde/kg dry matter [39]. The data obtained at each point for all experiments were the average of three measurements.

2.9. Statistical analysis

All experimental data (in triple repetition) were included in an approximation model through polynomial dependences from fourth order. For all cases the plural correlation coefficient R^2 was determined. The level of the concentration, which corresponds to 50 % of inhibition, was calculated according to this approximated dependence for which R^2 is maximum. The statistical processing of the data was carried out with a specialized software MATLAB (5.3/6.0).

3. Results and discussions

Using GC and GC-MS 35 constituents of the essential leaf oil of *Pimenta racemosa* from Jamaica were found with eugenol (45.60 %), myrcene (24.97 %) and chavicol (9.31 %) as the main compounds (see Table 1).

Compound [#]	% ⁺	RI [*]
α-Thujene	0.08	930
α-Pinene	0.45	939
1-Octen-3-ol	0.85	972
β-Pinene	0.07	979
3-Octanone	0.83	984
Myrcene	24.97	991
3-Octanol	0.69	993
α-Phellandrene	0.42	1003
α-Terpinene	0.17	1015
p-Cymene	0.50	1025
Limonene	2.92	1027
β-Phellandrene	0.35	1030
1,8-Cineole	0.69	1032
cis-β-Ocimene	0.11	1037
trans-β-Ocimene	0.88	1048
γ-Terpinene	0.15	1059
Octanol	0.21	1067
cis-Linalool oxide	0.09	1085
Terpinolene	0.42	1088
Linalool	2.25	1093
Terpinen-4-ol	0.45	1177
α-Terpineol	0.07	1184
Methyl chavicol	0.07	1187
Estragol	0.19	1189
Nerol	0.04	1229
Neral	0.09	1237
Chavicol	9.31	1248
Geraniol	0.08	1253
Eugenol	45.60	1357
Geranial	0.06	1265
α-Copaene	0.29	1277
Methyl eugenol	1.02	1402
Isoeugenol	0.72	1405
α-Gurjunene	0.01	1411
β-Caryophyllene	0.47	1418
α-Humulene	0.41	1454
α-(E,E)-Farnesene	0.49	1508
γ-Cadinene	0.18	1513
δ-Cadinene	0.55	1522
α-Cadinene	0.08	1538
trans-Nerolidol	0.51	1561

Tab. 1: Chemical composition of the essential oil of *Pimenta racemosa* leaves from Jamaica.

[#] in order of their retention-times

⁺ relative %-peak area of GC-FID analyses using an apolar column

^{*} retention indices using an apolar OV-5-type column

Further components in concentrations higher than 1.00 % (calculated as relative %-peak area of GC with FID and apolar column, mean-value of three measurements) of the bay leaf oil were identified as limonene (2.92 %), linalool (2.25 %) and methyl eugenol (1.02 %).

Results of the investigations of the DPPH radical-scavenging activity are as follows: DPPH is a stable free radical and accepts an electron or a hydrogen radical to become a stable diamagnetic molecule. It is typically used as a substrate to evaluate the antioxidant activity of various antioxidants of different samples [40-43]. Table 2 shows the concentrations of the essential bay leaf oil, eugenol, rutin, ascorbic acid, BHT and BHA that resulted in a 50 % inhibition of the free radical DPPH (IC₅₀). Following the decrease in antioxidant activities, the components under study were arranged in the order: BHA > eugenol > ascorbic acid > BHT > bay oil > rutin. IC₅₀ values were approximated with statistical significance p ≤ 0.01 and with high regression coefficients.

Test compound	IC ₅₀ *	R ² **
Bay oil	10.00	0.998
Eugenol	1.26	0.999
Rutin	14.65	0.991
Ascorbic acid	4.20	0.994
Butylated hydroxytoluene (BHT)	4.41	0.998
Butylated hydroxyanisole (BHA)	1.12	0.996

Tab. 2: Effect of the test compounds in the DPPH assay

* Concentration (μg/mL) for a 50 % inhibition

** R² – correlation coefficient

Hydroxyl radical-scavenging activity investigations showed following data: Hydroxyl radicals were generated in a reaction mixture containing ascorbate, hydrogen peroxide and iron III-EDTA at pH 7.4 and measured by their ability to degrade the sugar deoxyribose [34, 44]. Bay oil showed a significant OH[•]-scavenging activity that intensified with the increase of concentration, reaching 92.27 % at 6.0 μg/mL, while bay oil's major component – eugenol, caused 91.20 % inhibition of OH[•] at 0.6 μg/mL concentration (Figure 1). The antioxidant activity of quercetin was -77.8 % at 20 μg/mL and therefore substantially weaker. The three studied antioxidants were arranged by their antioxidant effect (expressed as IC₅₀), in descending order, as follows: eugenol -0.06 μg/mL (R² = 0.989), bay oil -0.60 μg/mL (R² = 0.969), quercetin -4.61 μg/mL (R² = 0.834). The same analytical method could also be applied for studying the inhibitory power of essential *P. racemosa* oil against the metal ion-dependant

generation of OH^\bullet , and not only for assaying its ability to capture already formed radicals. When Fe^{3+} -ions are added to the reaction mixture as FeCl_3 instead of EDTA complex, some of the iron ions form complexes with deoxyribose. The Fe^{3+} may be subsequently reduced by ascorbate to Fe^{2+} , which in turn may remain bound to deoxyribose and further react with H_2O_2 . The reaction generates the necessary OH^\bullet , which immediately triggers

the degradation of deoxyribose in a site-specific manner. Only molecules that are able to chelate iron ions and make them inactive may inhibit the degradation of deoxyribose. *Figure 1* (without EDTA) shows bay oil, eugenol and quercetin as scavengers of OH^\bullet and manifests chelative properties, most expressive in the case of eugenol. Like most radicals, OH^\bullet can be neutralized by a hydrogen atom. The capture of OH^\bullet by bay oil is attributed to the hydrogen-donating capacity of eugenol, which is found in the essential oil of *Pimenta racemosa* leaves at a high concentration (45.60 %).

The superoxide anion scavenging activity results can be discussed as follows: Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals [45]. Superoxide anions indirectly initiate lipid oxidation as a result of superoxide and hydrogen peroxide serving as precursors of singlet oxygen and hydroxyl radicals [35]. Xanthine-xanthine oxidase is the system, which is often used as a generator of superoxide radicals. The experimental results from this study indicated that the essential *P. racemosa* leaf oil, eugenol and BHT had inhibitory effect on enzyme activity (*Table 3*). At 25 $\mu\text{g}/\text{mL}$ con-

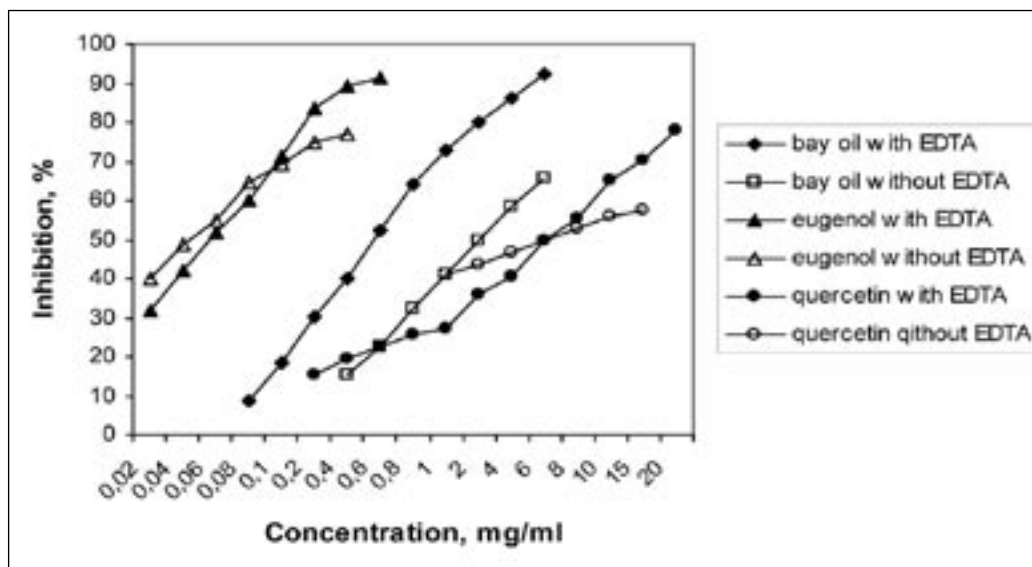


Fig. 1: Metal chelating activity of bay oil, eugenol and quercetin on deoxyribose degradation by OH^\bullet

centration, the xanthine oxidase inhibition power of the studied components arranged them in the following order: BHT > eugenol > bay oil. The xanthine oxidase activity was inhibited by bay oil at 49.03 %. In such manner, by inhibiting xanthine oxidase activity, bay oil prevents the formation of the superoxide radical.

The evaluation of antioxidant activity in a linoleic acid model system was as follows: For the task of evaluating the inhibitory effect of bay oil on lipid peroxidation, a model system of linoleic acid emulsion was applied. The antioxidant capacity was estimated both at the early stages of linoleic acid autoxidation and later, after the emergence of secondary oxidation products, like aldehydes, ketones or hydrocarbons. Two indicators were referred to, corresponding to a different degree of lipid peroxidation – conjugated diene formation and TBARS.

For the determination of conjugated diene (CD) formation the following results were found: It was determined that the peak in conjugated diene formation was reached on the fifth day following the incubation of linoleic acid (*Figure 2A*). At this stage of incubation, with 0.01 % bay oil added, significant inhibition of the process was achieved (-28.35 %), compared to a higher value of 37.31 % inhibition realized with BHT. A maximum inhibitory effect of bay oil was observed on the ninth day of the study, when the essential *P. racemosa* leaf oil acting in an almost analogical manner as the synthetic antioxidant -54.34 % inhibition for bay oil as opposed to 58.69 % for BHT. The implementation of a higher concentration of bay oil -0.02 % brought a significant inhibition of lipid peroxidation -63.04 % on the ninth day of incubation.

Compound tested	A_{295} nm	% inhibition
Control	0.310	-
Bay oil (25 $\mu\text{g}/\text{mL}$)	0.158	49.03
Eugenol (25 $\mu\text{g}/\text{mL}$)	0.143	53.87
BHT (25 $\mu\text{g}/\text{mL}$)	0.137	55.80

Tab. 3: Effect of bay oil, eugenol and BHT on xanthine oxidase activity

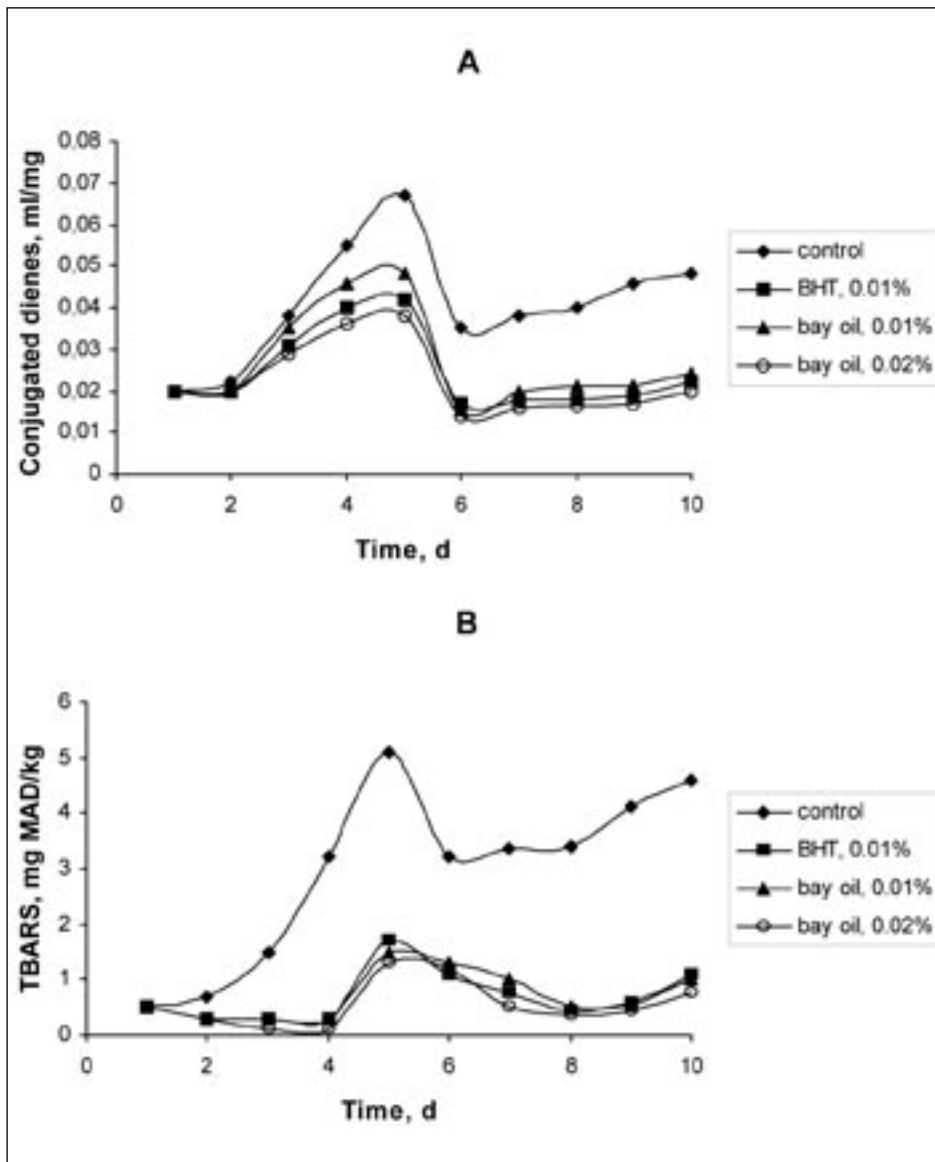


Fig. 2: Effect of bay oil on (A) conjugated dienes and (B) TBARS in a linoleic acid/water emulsion system.

Finally, the determination of thiobarbituric acid reactive substances can be discussed as follows: With the second reference used for estimating the presence of secondary derivatives from linoleic acid oxidation – TBARS – maximum accumulation of malonic aldehyde was found, as the case above, on the fifth day of linoleic acid incubation (Figure 2B), suggesting therefore that the process ran in a manner nearly analogous to the formation of conjugated dienes. The strongest antioxidant action of the compounds under study was exercised on the eighth day of the study, with the inhibitory effect of essential bay leaf oil being almost equal to that of the synthetic standard BHT; moreover, the effect was executed at a concentration twice as lower than that of BHT (0.01 %). The inhibition of lipid peroxidation by bay oil attained

85.58 % compared to 86.76 % inhibition realized by BHT. The application of the higher concentration of the natural antioxidant bay oil, namely 0.02 % resulted in 89.11 % inhibition on the eighth day of the experiment.

Therefore, we can conclude for this investigation of chemical composition and antioxidative properties of the essential leaf oil of *Pimenta racemosa* from Jamaica that eugenol, myrcene and chavicol were found as main compounds with this capacity.

The DPPH and hydroxyl radical scavenging ability of bay oil is attributed to the hydrogen-donating potential of the phenol component eugenol, assayed to be present at a significant concentration in the essential oil. Bay oil manifested weaker antioxidant action on DPPH radicals in comparison with those of eugenol, BHT and BHA, demonstrated by the IC_{50} -values. The inhibitory effect of bay oil on OH^* was assessed as being weaker than that of eugenol, but stronger than that of quercetin. The essential *P. racemosa* leaf oil demonstrated chelative properties with respect to Fe^{3+} , resulting in a prevention of

hydroxyl radicals' initiation. Xanthine oxidase activity was inhibited by bay oil, thus blocking the generation of the superoxide radicals.

Bay oil was capable of an effective inhibition of both the conjugated diene formation and the generation of secondary products from lipid peroxidation, carried out at a concentration twice lower than that of the reference antioxidant BHT. The essential *P. racemosa* leaf oil showed a stronger antioxidant action upon the process of generation of secondary products from lipid autoxidation.

Therefore, our study characterized the essential *Pimenta racemosa* leaf oil from Jamaica as a potent antioxidant, eligible as a natural conservative that would reduce or prevent losses due to oxidative processes.

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