Phytotoxicity of clove oil and its primary constituent eugenol and the role of leaf epicuticular wax in the susceptibility to these essential oils

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Abstract and figures

Herbicidal activities of clove oil and its primary constituent eugenol on broccoli, common lambsquarters, and redroot pigweed and the role of crystalline leaf epicuticular wax (LEW) in susceptibility and retention of these essential oils were studied. Clove oil (2.5%) and eugenol (1.5%) were applied to leaves of greenhouse-grown broccoli, common lambsquarters, and redroot pigweed seedlings and effects on seedling growth and leaf cell membrane integrity were studied. Compared with eugenol, clove oil caused greater inhibition of seedling growth in all species. Both eugenol and clove oil caused greater loss of membrane integrity and inhibition of seedling growth in redroot pigweed, which has no crystalline LEW, compared with common lambsquarters, which has a thick layer of crystalline LEW. In broccoli seedlings with LEW, clove oil caused greater inhibition of growth than eugenol. Both clove oil and eugenol caused greater electrolyte leakage from broccoli leaves without LEW than in the leaves with LEW. Removal of LEW increased electrolyte leakage, an indicator of cell membrane damage, by 97% in eugenol-treated and 26% in clove oil–treated broccoli leaves. Susceptibility of broccoli seedlings and possibly some weed species may, therefore, be affected by factors (e.g., genetic, environmental) that influence the amount of LEW. Although the presence of LEW greatly reduced the retention of the essential oil solutions, there was no significant difference between the retention of clove oil and eugenol solutions, indicating that differences in their phytotoxicity to broccoli leaves was not due to differential foliar retention. Nomenclature: Common lambsquarters, Chenopodium album L. CHEAL;

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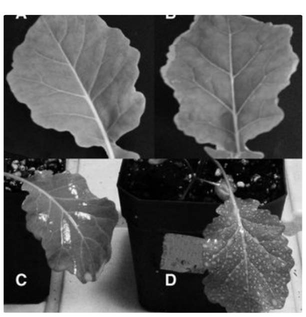

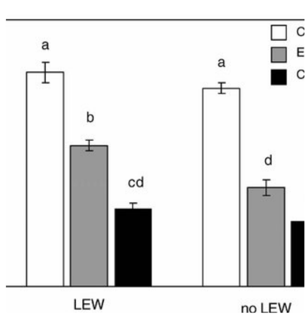

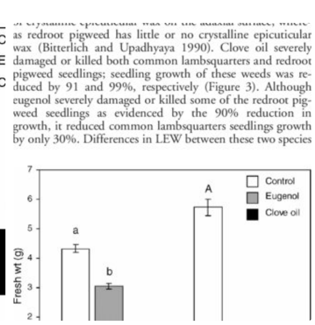

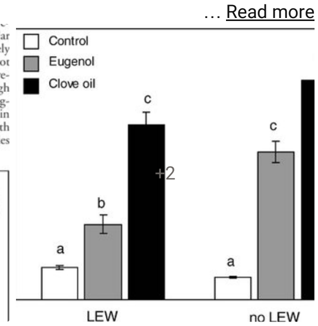

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Phytotoxicity of clove oil and its primary constituent eugenol and the role of leaf epicuticular wax in the susceptibility to these essential oils

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Herbicidal activities of clove oil and its primary constituent eugenol on broccoli, common lambsquarters, and redroot pigweed and the role of crystalline leaf epicuticular wax (LEW) in susceptibility and retention of these essential oils were studied. Clove oil (2.5%) and eugenol (1.5%) were applied to leaves of greenhouse-grown broccoli, common lambsquarters, and redroot pigweed seedlings and effects on seedling growth and leaf cell membrane integrity were studied. Compared with eugenol, clove oil caused greater inhibition of seedling growth in all species. Both eugenol and clove oil caused greater loss of membrane integrity and inhibition of seedling growth in redroot pigweed, which has no crystalline LEW, compared with common lambsquarters, which has a thick layer of crystalline LEW. In broccoli seedlings with LEW, clove oil caused greater inhibition of growth than eugenol. Both clove oil and eugenol caused greater electrolyte leakage from broccoli leaves without LEW than in the leaves with LEW. Removal of LEW increased electrolyte leakage, an indicator of cell membrane damage, by 97% in eugenol-treated and 26% in clove oil–treated broccoli leaves. Susceptibility of broccoli seedlings and possibly some weed species may, therefore, be affected by factors (e.g., genetic, environmental) that influence the amount of LEW. Although the presence of LEW greatly reduced the retention of the essential oil solutions, there was no significant difference between the retention of clove oil and eugenol solutions, indicating that differences in their phytotoxicity to broccoli leaves was not due to differential foliar retention.

Nomenclature: Common lambsquarters, *Chenopodium album* L. CHEAL; redroot pigweed, *Amaranthus retroflexus* L. AMARE; clove, *Syzygium aromaticum* (L.) Merr. & Perr.; purple sprouting broccoli, *Brassica oleracea* var. *italica.*

Key words: Cole crop.

Natural compounds, including essential oils, have been investigated for pest management because of concern for the effects of synthetic pesticides on human and environmental

Weber in Wiggers)leaf disk assay demonstrated that clove oil and eugenol damaged leaves by increasing cell membrane permeability, which caused membrane damage resulting in health and the development of resistance to synthetic pesticides (Duke et al. 2003). Essential oils are particularly attractive in this regard because they generally have low mammalian toxicity and do not persist in the environment (Duke et al. 2003; Isman 2000; Tworkoski 2002). Pesticides containing certain essential oils and their constituents may require less rigid regulatory scrutiny compared with synthetic compounds for registration, which may reduce the cost of their commercialization (Duke 2002; Isman 2000).

Clove oil is obtained by steam distillation from the buds, leaves, and stems of the clove tree, which is native to tropical regions of Asia and South America (Burdock 2002). The primary constituents of clove oil include eugenol, β-caryophyllene, α-humulene, eugenyl acetate, and humulene epoxide (Bauer et al. 1997; Raina et al. 2001). Although the number and concentration of compounds in clove oil can vary depending on geographical location and the plant tissue used to obtain the oil, eugenol remains the primary constituent of clove oil (Raina et al. 2001; Srivastava et al. 2005).

Many essential oils have been shown to exhibit herbicidal activity including clove oil and its primary constituent eugenol (Tworkoski 2002). A dandelion (*Taraxacum officinale* significant leaf injury (Tworkoski 2002). An herbicide containing clove oil (Matran 2) has recently been introduced as a natural product herbicide for organic producers. Matran 2 is a nonselective foliar herbicide for grasses and broadleaf weeds.

The retention capability of foliar herbicides is important, and LEW plays a significant role in the susceptibility of plants to herbicides. LEW offers protection against foliar herbicides by reducing their retention because spray droplets roll off the leaf surface. In general, species with crystalline epicuticular wax on their adaxial leaf surface (e.g., common lambsquarters) are less susceptible to foliar herbicides compared with species with little or no crystalline epicuticular wax (e.g., redroot pigweed) (Bitterlich et al. 1996; Bitterlich and Upadhyaya 1990; Hess 1985).

Essential oils could potentially be used for weed control in cole crops, such as broccoli. The effects of clove oil and its primary constituent eugenol on broccoli and its weeds have not been studied. The objectives of this study were to investigate (1) the phytotoxicity of clove oil and eugenol on broccoli and two major weeds, common lambsquarters and redroot pigweed, and (2) the role of LEW in the susceptibility to and retention of clove oil and eugenol in broccoli.

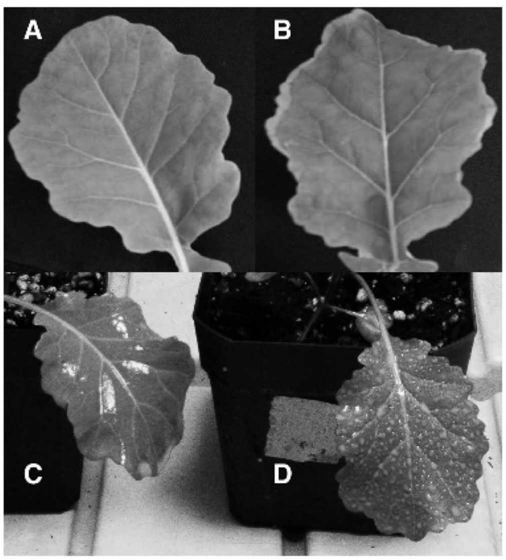

Figure 1. Broccoli leaves (A) with epicuticular wax and (B) after removal of epicuticular wax using the cellulose acetate stripping method and after application of 1.5% eugenol solution containing methyl orange to leaves (C) with and (D) without epicuticular wax.

Broccoli was used in this study because it has a heavy, crystalline epicuticular wax layer on its adaxial leaf surface. Removal of the epicuticular wax allowed comparison of the effect of clove oil and eugenol on a single species with or without LEW. Common lambsquarters and redroot pigweed were chosen for this study because of the presence and absence, respectively, of crystalline epicuticular wax in these species and their significance in broccoli production.

Materials and Methods

Plant Preparation

Purple sprouting broccoli[1] seeds were obtained from West Coast Seeds; common lambsquarters and redroot pigweed seeds were collected from Totem Field at the University of British Columbia campus. Seeds were sown 1 cm deep in soil (75% peat and 25% perlite mix) in 300 ml square

Seedling Growth

Distilled water (control), eugenol[2] (1.5% v/v), and clove leaf oil[3] (2.5% v/v) solutions containing 2% Tween 20[4] were sprayed (50 ml m^{-2}) on broccoli (with or without LEW) seedlings at the three-leaf stage and on common lambsquarters and redroot pigweed seedlings at the ~ eight-leaf stage using a handheld sprayer. After 14 d, the aboveground seedling fresh weight was measured. A completely randomized design with six replicates per treatment was used, and the experiment was repeated.

Membrane Integrity

The effects of clove oil and eugenol on membrane integrity were studied by applying control, eugenol, and clove oil solutions to seedlings of broccoli (three-leaf stage), with and without LEW, and to common lambsquarters and redroot pigweed (eight-leaf stage) as described above. Five leaf discs (10 mm in diameter) were excised from each plant 2 h after application of the solutions, which were transferred into 10 ml of distilled water and placed on a rotary shaker for 3 h at 90 rpm. The solution was then transferred into a glass test tube, and the electrolyte concentration of the bathing medium was measured using a conductivity meter.[5] There were six replicates for each treatment, and the experiment was repeated.

Spray Solution Retention

The role of LEW in retention of clove oil and eugenol was studied by applying control, eugenol, and clove oil solutions containing 0.01% methyl orange[6] to broccoli seedlings (three-leaf stage), with and without LEW. After allowing the excess solution to drip off the leaves (~ 10 s), the second fully expanded leaf was washed in 15 ml of distilled water, and the absorbance (465 nm) of the leaf wash was measured with a spectrophotometer.[7] Each treatment was replicated eight times, and the experiment repeated. A variety of dye solutions have been used to assess retention of foliar-applied solutions (Harbour et al. 2003; Ramsdale and Messersmith 2001; Zawierucha 2000).

Data Analysis

Data were subjected to ANOVA (SYSTAT 9 software[8]), and means compared using the Bonferroni test at $P = 0.05$. Data from two experiments were pooled because there was no significant experiment-by-treatment interaction.

soil (75% peat and 25% perlite mix) in 500-ml square, plastic pots. Plants were grown in a greenhouse under natural light between July and September 2005 at temperatures ranging between 20 and 34 C. Shortly after emergence, seedlings were thinned to one plant per pot.

Epicuticular Wax Removal

Epicuticular wax was removed from broccoli leaves using the cellulose acetate stripping method (Silcox and Holloway, 1986). Cellulose acetate solution (5% cellulose acetate in acetone wt/v) was applied to the adaxial leaf surface with a small paint brush and allowed to dry, and the resulting film was gently peeled to remove the surface wax (Figure 1).

Results and Discussion

Seedling Growth

Compared with controls, clove oil application reduced broccoli seedling fresh weight to a greater extent than eugenol (Figure 2). Removal of LEW from the leaves significantly ($P \leq 0.05$) increased susceptibility of broccoli seedlings to eugenol (Figure 2). Eugenol reduced broccoli seedling growth by only 34% in the presence of LEW, compared with 50% when the LEW was removed. In contrast, growth inhibition by clove oil was 64% with, and 67% without, LEW. These results show that both clove oil and eugenol (1.5 and 2.5%, respectively) are injurious to broccoli seed-

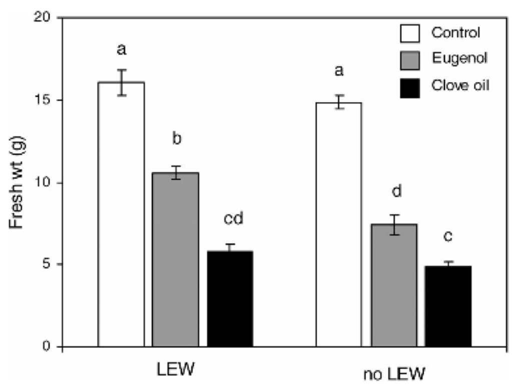

FIGURE 2. Effect of clove oil (2.5%) and eugenol (1.5%) on the fresh weight of broccoli seedlings 14 d after treatment with and without leaf epicuticular wax (LEW). Values are means ± SE of two experiments with six replicates per experiment. Bars with different letters indicate significant differences at $P = 0.05$.

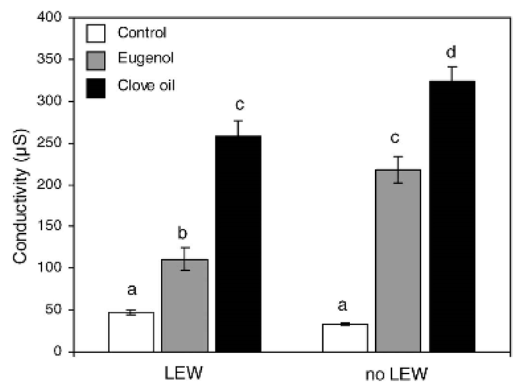

FIGURE 4. Effect of clove oil (2.5%) and eugenol (1.5%) on leakage of electrolytes from leaf discs of broccoli with and without leaf epicuticular wax (LEW). Values are means ± SE of two experiments with six replicates per experiment. Bars with different letters indicate significant differences at $P = 0.05$.

lings, and the phytotoxicity of eugenol is influenced by the presence of LEW.

Common lambsquarters and redroot pigweed, two common weeds in cole crops, differ in their leaf-surface morphology. Common lambsquarters leaves have a heavy layer of crystalline epicuticular wax on the adaxial surface, whereas redroot pigweed has little or no crystalline epicuticular wax (Bitterlich and Upadhyaya 1990). Clove oil severely damaged or killed both common lambsquarters and redroot pigweed seedlings; seedling growth of these weeds was reduced by 91 and 99%, respectively (Figure 3). Although eugenol severely damaged or killed some of the redroot pigweed seedlings as evidenced by the 90% reduction in growth, it reduced common lambsquarters seedlings growth by only 30%. Differences in LEW between these two species could be responsible for this difference, but that remains to be confirmed.

Membrane Integrity

The application of clove oil and eugenol significantly increased electrolyte leakage in broccoli leaf discs compared with the control (Figure 4). Removal of LEW from broccoli leaves significantly increased the electrolyte leakage in response to these essential oils. The removal of LEW increased the amount of electrolyte leakage in eugenol-treated leaves by 97% but only by 26% in clove oil–treated leaves.

Clove oil caused greater electrolyte leakage than eugenol in common lambsquarters and redroot pigweed leaf disks (Figure 5). Clove oil increased the electrolyte leakage over

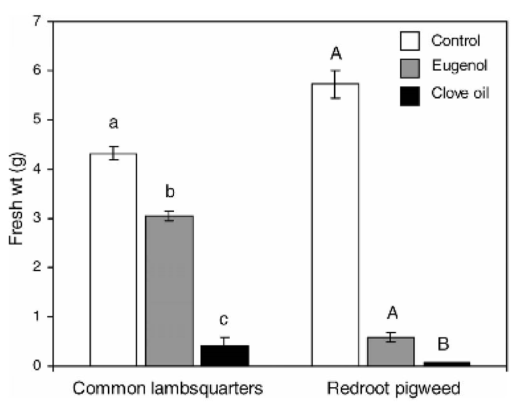

FIGURE 3. Effect of clove oil (2.5%) and eugenol (1.5%) on the fresh weight of common lambsquarters and redroot pigweed seedlings 14 d after treat-

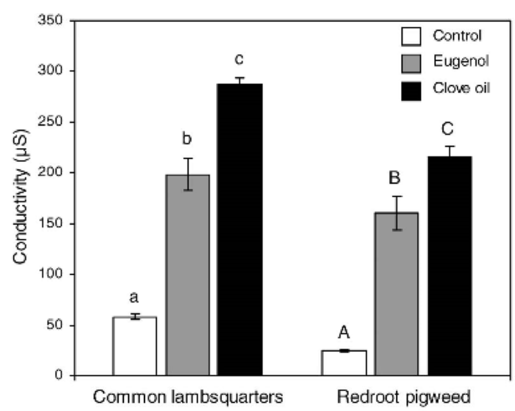

FIGURE 5. Effect of clove oil (2.5%) and eugenol (1.5%) on leakage of

ment. Values are means ± SE of two experiments with six replicates per experiment. Bars with different letters for common lambsquarters (lowercase) and for redroot pigweed (uppercase) indicate significant differences at P = 0.05.

electrolytes from leaf discs of common lambsquarters and redroot pigweed. Values are means ± SE of two experiments with six replicates per experiment. Bars with different letters for common lambsquarters (lowercase) and for redroot pigweed (uppercase) indicate significant differences at P = 0.05.

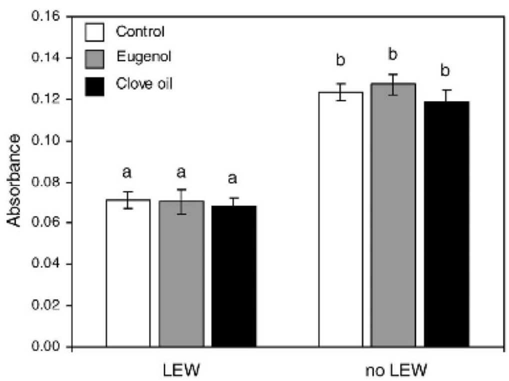

FIGURE 6. Retention of clove oil (2.5%) and eugenol (1.5%) solutions with methyl orange (0.01%) on broccoli leaves with and without leaf epicuticular wax (LEW). Values are means ± SE of two experiments with eight replicates per experiment. Bars with different letters indicate significant differences at P = 0.05.

eugenol-treated leaf disks by 45% in common lambsquarters and 34% in redroot pigweed. These results show that these two essential oils cause significant loss of membrane integrity and that the presence of LEW in broccoli and possibly in common lambsquarters protects leaves from this damage.

Because the clove oil used in this study had a eugenol concentration (63.96%, measured by gas chromatography/mass spectroscopy, R. Bradbury, Ecosafe Natural Products Inc., unpublished data) similar to that of the eugenol solution used (60%), the observed higher phytotoxicity of clove oil compared with eugenol may be because of the other constituents (e.g., β-caryophyllene and α-humulene) in clove oil; the clove oil used in this study contained 14.0% β-caryophyllene and 6.1% α-humulene. β-Caryophyllene has been shown to inhibit seedling growth of radish (*Raphanus sativus* L.), mung bean [*Vigna radiata* (L.) Wilczek], and tomato (*Lycopersicon esculentum* Mill.) plants (Kong et al. 1999). In contrast, α-humulene was not phytotoxic in a dandelion leaf disk assay (Tworkoski, 2002).

Essential Oil Retention

The removal of LEW from the leaves of broccoli seedlings resulted in a higher spray retention of clove oil and eugenol spray solutions (Figure 6). Plants with LEW are generally difficult to wet as the epicuticular wax repels foliar-applied solutions (Bitterlich and Upadhyaya 1990; Bitterlich et al. 1996; Holloway 1993). The results from this study indicate that LEW may play an important role in reducing the susceptibility of broccoli to clove oil and eugenol by lowering the retention of the essential oil solutions. Similarly, when an ammonium nitrate solution was applied to cabbage (*Brassica oleracea* L.) leaves with their LEW stripped, the solution wetted the surface unevenly and was unable to completely kill the leaves (Bitterlich and Upadhyaya 1990). It was suggested that amorphous cuticular wax may offer some protection to the leaves, which may also be an important factor in the susceptibility of broccoli leaves to clove oil and eugenol.

Although the presence of LEW greatly reduced the retention of the essential oil solutions, there was no significant difference between the retention of clove oil and eugenol solutions. The results indicate that differences in the susceptibility of broccoli leaves to these essential oils were not due to their differential foliar retention.

In conclusion, the results of this study indicate that clove oil (2.5%) caused greater inhibition of growth and greater electrolyte leakage compared with eugenol (1.5%) in plants with LEW. The presence of LEW reduced the susceptibility of seedlings to eugenol. Removal of LEW increased the retention of both clove oil and eugenol. The similar spray retention of clove oil and eugenol solutions indicated that differences in their phytotoxicity to broccoli leaves were not due to differential foliar retention.

Sources of Materials

[1] West Coast Seeds, 3925 64th Street, Delta, BC V4K 3N2, Canada.

[2] EcoSMART Technologies Inc., 318 Seaboard Lane, Suite 208, Franklin, TN 37067.

[3] EcoSMART Technologies Inc., 318 Seaboard Lane, Suite 208, Franklin, TN 37067.

[4] Tween-20. Fisher Scientific Company, 1 Reagent Lane, Fair Lawn, NJ 07410.

[5] HI 8733. Hanna Instruments, 3156 Boulevard Industriel, Laval, PQ H7L 4P7, Canada.

[6] Methyl orange. Sigma-Aldrich Canada Ltd., 2149 Winston Park Drive, Oakville, ON L6H 6JB, Canada.

[7] Hach DR/2400. Hach, 1313 Border Street, Unit 34, Winnipeg, MB R3H OX4, Canada.

[8] SYSTAT 9 software. SYSTAT Software Inc., 501 Canal Boulevard, Suite E, Point Richmond, CA 94804-2028.

Acknowledgments

M.K.U. and M.B.I. thank the Natural Sciences and Engineering Research Council of Canada for financial support, and Rod Bradbury (Ecosafe Natural Products Inc.) for analyzing the clove oil by gas chromatography/mass spectroscopy.

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Figure S1

Data

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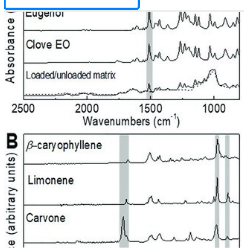
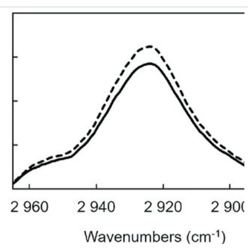
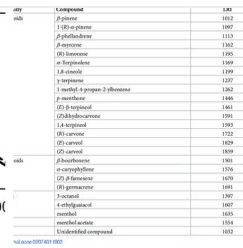
Acetyleugenol infrared spectrum compared to clove oil infrared spectrum (eugenol). Note: Major component of clove oil is eugenol (78%). Abbreviation: AcE, acetyleugenol. ...







Chemical function
Aromatic C = C
Carbonyl C = O
Disubstituted C-H
Trisubstituted C-H

+2

03

Rapid quantification of clove (Syzygium aromaticum) and spearmint (Mentha spicata) essential oils encapsulated in a complex organic matrix using an ATR-FTIR spectroscopic method

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Essential oils (EOs) are often encapsulated in various and complex matrices to protect them against potential degradation or to control their release. To achieve an optimum use in food products, their rapid and precise quantification after encapsulation and storage is required. Hence, a rapid ATR-FTIR method was developed and tested with two encapsulated essential oils (EOs): clove (Syzygium aromaticum) and spearmint ...

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Article

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Ana Lucía Solarte Portilla · Rafael Jesús Astorga Márquez · Fabiana Carolina de Aguiar · [...] · Belen Huerta

To determine the distribution of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oils (EOs) of cinnamon (Cinnamomum zeylanicum), clove (Eugenia caryophyllata), oregano (Origanum vulgare), common thyme (Thymus vulgaris), and red thyme (Thymus zygis) against Salmonella enterica, double serial dilutions of each EO were challenged with 85 Salmonella strains belonging to 23 ...

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Article

Feb 2018 · Industrial Crops and Products

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The selection of suitable technique and apposite operating conditions are the key attributes for efficient extraction of phytochemicals from plant materials. Microwave assisted extraction (MAE) was incorporated to isolate essential oil from clove buds and the parameters were optimized using the grey based Taguchi method to maximize the yield of clove oil (Y, % w/w), eugenol content (y, % w/w) and bacterial inhibition. The results ...

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Jul 2018 · Colloids and Surfaces A Physicochemical and Engineering Aspects

Natalia Sánchez-Arribas · Eduardo Guzmán · Alejandro Lucia · [...] · Ramón G Rubio

Essential oils are highly volatile and non-water soluble natural products which have shown high efficiency against several insect pests. However, their low solubility in water is an important drawback for their practical applications. This work explores the design of new nanocarriers of eugenol based on silica nanoparticles capped with Pluronic F-127, a triblock copolymer of poly(ethylene oxide) and poly(propylene oxide). Dynamic Light ...

FIGURE 2. Effect of clove oil (2.5%) and eugenol (1.5%) on the fresh weight of broccoli seedlings 14 d after treatment with and without leaf epicuticular wax (LEW). Values are means SE of two experiments with six replicates per experiment. Bars with different letters indicate significant differences at P 0.05.

lings, and the phytotoxicity of eugenol is influenced by the presence of LEW.

Common lambsquarters and redroot pigweed, two common weeds in cole crops, differ in their leaf-surface morphology. Common lambsquarters leaves have a heavy layer of crystalline epicuticular wax on the adaxial surface, whereas redroot pigweed has little or no crystalline epicuticular wax (Bitterlich and Upadhyaya 1990). Clove oil severely damaged or killed both common lambsquarters and redroot pigweed seedlings; seedling growth of these weeds was reduced by 91 and 99%, respectively (Figure 3). Although eugenol severely damaged or killed some of the redroot pig-

FIGURE 4. Effect of clove oil (2.5%) and eugenol (1.5%) on leakage of electrolytes from leaf discs of broccoli with and without leaf epicuticular wax (LEW). Values are means SE of two experiments with six replicates per experiment. Bars with different letters indicate significant differences at P 0.05.

could be responsible for this difference, but that remains to be confirmed.

Membrane Integrity

The application of clove oil and eugenol significantly increased electrolyte leakage in broccoli leaf discs compared with the control (Figure 4). Removal of LEW from broccoli leaves significantly increased the electrolyte leakage in response to these essential oils. The removal of LEW increased the amount of electrolyte leakage in eugenol-treated leaves