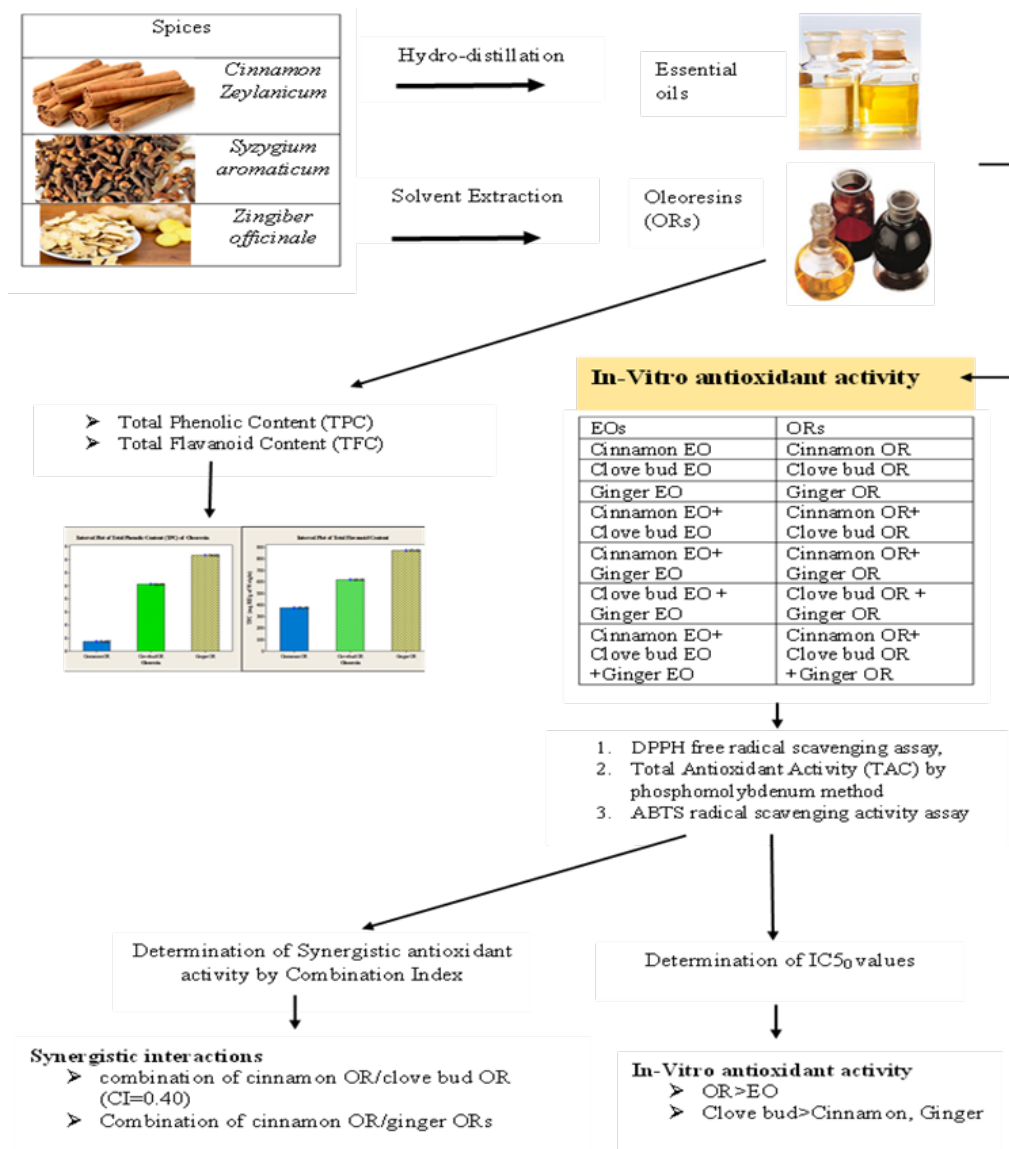


RESEARCH ARTICLE

***In-Vitro* Antioxidant Activity of Essential Oils and Oleoresins of Cinnamon, Clove bud, Ginger and their synergistic interactions**

S.M.M.C. Sethunga, K.K.D.S. Ranaweera, I. Munaweera and K.D.P.P. Gunathilake*

**Highlights**

- Essential oils and oleoresins of *Cinnamomum zeylanicum*, *Syzygium aromaticum* and *Zingiber officinale* showed a wide range of TPC, TFC, and antioxidant activity.
- Clove bud demonstrated higher antioxidant properties compared to cinnamon and ginger, and oleoresins are high in antioxidant properties than essential oils.
- A significant synergistic antioxidant activity is reported by the combinations of cinnamon bark oil with clove bud oil and ginger oil.
- The essential oils, oleoresins and their blends can be used as effective natural antioxidant agents in the food industry.

RESEARCH ARTICLE

***In-Vitro* Antioxidant Activity of Essential Oils and Oleoresins of Cinnamon, Clove bud, Ginger and their synergistic interactions**

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Abstract: Essential oils (EOs) and oleoresins (ORs) are well distinguished for their antioxidant properties and they can be widely used in the food industry as preservatives. The present study was executed for evaluating the synergistic interactions on antioxidant efficacy of EOs and ORs of cinnamon, clove bud and ginger and their combinations. EOs and ORs were obtained by hydro-distillation and solvent extraction. *In-vitro* antioxidant properties were investigated by DPPH free radical scavenging assay, phosphomolybdenum method (Total Antioxidant Capacity, TAC) and ABTS radical scavenging activity assay. EOs and ORs which consisted with different chemical constituents and different Total Phenolic Content (TPC) and Total Flavanoid Content (TFC) exhibited a range of variation in the inhibition percentage of DPPH and IC₅₀ values (0.1645-6.5181 mg/mL). The TAC of EOs and ORs was within the range of 0.27-3.94 mg AAE/mg. The ORs showed higher inhibition percentages and TAC compared to EOs of the same spice. This study revealed that clove buds contains higher antioxidant properties than cinnamon and ginger. Significant reductions in IC₅₀ values were shown in EO blends compared to the ORs blends. The combination of cinnamon EO/ clove bud EO and combination cinnamon EO/ginger EO showed strong synergy in tested samples. Therefore, both EOs and ORs of *Cinnamomum zeylanicum*, *Syzygium aromaticum*, *Zingiber officinale* and their blends are excellent sources for natural and effective natural antioxidant agents in the food industry.

Keywords: Spices; oleoresins; essential oils; antioxidant; extraction

INTRODUCTION

Essential oils (EOs) and oleoresins (ORs) are complex mixtures of secondary metabolites which are derived from aromatic plants. Although they are mainly used for the flavour and fragrance industry, antioxidant activity is a noteworthy functional benefit. EOs and ORs consist of a vast number of different bioactive compounds that contain nitrogen-containing compounds (cyanogenic glycosides, alkaloids, and glucosinolates), phenolic compounds (flavonoids and phenylpropanoids), and terpenes (isoprenoids), hydroxyl groups, conjugated carbon double bonds (War et al., 2019). These bio-actives of EOs and ORs play an antioxidant role in two key ways; as a protector by

maintaining a higher antioxidant level than the formed free radicals in the body after the direct spice consumption and as a preservative for extending the shelf life in processed food and beverages.

Exposure to environmental and chemical pollutants, consumption of processed foods, poor nutrition affects oxidative damage to biomolecules in the cells. The bio-actives of EOs and ORs protect the body by inhibiting Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) which are formed due to redox processes inside the aerobic cells. This leads to induce oxidative damage and helps to protect from degenerative and chronic diseases such as diabetes, various types of cancers, cardiovascular disorders, inflammation, Alzheimer's disease, atherosclerosis, respiratory diseases (Hamidpour, 2017; Abeysekera et al., 2013).

In addition to that, the oil and fat in foods and beverages are more susceptible to oxidation which is caused by external factors such as higher temperature, light, oxygen, metal traces. Because of that, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), tert-butyl hydroquinone (TBHQ) are widely used by food manufactures although many adverse effects such as carcinogenicity are reported. As EOs and ORs are nature-derived substances, they are non-toxic and can be used as a natural antioxidant solution to replace synthetic antioxidants. Therefore, natural antioxidants have gained significant attention from researchers, manufacturers of the food, beverage, cosmetic, pharmaceutical, and nutraceutical industries.

Amidst the vast number of spices, cinnamon (*Cinnamomum zeylanicum*), ginger (*Zingiber officinale*), clove bud (*Syzygium aromaticum*) are highly remarkable spices for their bioactivity other than their uses as flavours, fragrances and culinary ingredients. Cinnamon chemically consists of the class of proanthocyanidins which is made up of dimeric, trimeric and oligomeric proanthocyanidins with doubly linked bis-flavan-3-ol (Varalakshmi et al.,

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2012). Ginger is another significant spice for EOs and ORs which is used for many food formulas and medicinal preparations. The free radical scavenging and its anti-oxidative activity of ginger EO and OR has been studied (Bellik et al., 2013). EOs and ORs of clove bud are highly significant for their therapeutic effects such as antioxidant, antimicrobial, antiviral, anti-inflammatory, antiprotozoal, anticarcinogenic, antimutagenic, anesthetic, repellent effects and clove bud OR was studied for its antioxidant activity (Erukainure et al., 2016). Because of the significance of the above three spices, this study pays attention to the antioxidant activity of the volatile EOs and non-volatile ORs of cinnamon, clove bud and ginger. Although there are some studies which are done for evaluating the antioxidant activity, the previous studies on the antioxidant activity of *Cinnamomum zeylanicum* and the comparisons of EOs and ORs from same spice are limited and have been focused on the antioxidant activity of both EO and OR from the same spice. In addition to that, the combination of spices were used in traditional cuisines not only for the unique flavour but also for enhancing the effectiveness and efficiency of bio-activities of the spices (Benyoucef et al., 2018; Do Nascimento et al., 2020). Therefore, in this study, we exert our effort to identify the synergy of the combination of EOs and ORs of the above three spices as they are highly grown and highly used spices in Sri Lanka.

MATERIALS AND METHODS

Raw Materials

Cinnamon (*Cinnamomum zeylanicum*) quillings (Gemunu Type) were obtained by Cinnamon Research Center, Matara, Sri Lanka. Dried ginger (*Zingiber officinale*) slices (Rangoon variety) and Clove bud (*Syzygium aromaticum*) were purchased from local suppliers in Sri Lanka which were grown in the North Western Province and Central Province, respectively.

Reagents & Chemicals

Ethyl Acetate, methanol, ethanol, sodium nitrite, aluminum chloride, sodium hydroxide, folin-ciocalteu reagent, sodium carbonate, sodium phosphate, gallic acid, rutin, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ammonium molybdate, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS ((2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), potassium persulphate were purchased from Sigma-Aldrich, St. Louis, MO, the USA through the agent of Analytical Instrument Pvt Ltd, Colombo, Sri Lanka. All other standards and chemicals used in these experiments were analytical grades.

Extraction of EOs and ORs

Distillation of EOs

Raw materials (approximately 500 g, 100 g, 35 g of Cinnamon, ginger, clove bud, respectively) were crushed using heavy duty mixer grinder (Panasonic, AC400) and subjected to hydro-distillation (HD) using a Clevenger apparatus following the HD method described in the European Pharmacopoeia (Pharmacopoeia,

1998) with slight modifications. For cinnamon and ginger oil, Clevenger Apparatus for determination of volatile oil lighter than water was used while Clevenger Apparatus for determination of volatile oil heavier than water was used for clove bud oil. Distillation time was 6 hours, 10 hours, 10 hours for cinnamon, ginger and clove bud, respectively. The EOs were collected and were dehydrated over anhydrous sodium sulfate to remove the excess water and then the concentrated oil was weighed and stored in a vial at 4 °C for further analysis.

Soxhlet Extraction

The dried and crushed plant materials (approximately 100 g of cinnamon, 100 g of ginger and 30 g of clove bud) were loaded to the soxhlet apparatus with 750 mL of ethyl acetate and distillation was carried out for 18 hours. The extracts were filtered using Whatman No. 1 filter paper to pre-weighed round bottom flask and the solvent was evaporated using the rotary evaporator at 55°C. The obtained oleoresins were filled into bottles and the weight of the OR were taken. The samples were stored in a refrigerator in the dark at 4°C for analysis. For the EO and OR combinations, extracted crude EO and OR were mixed in same ratios.

Determination of Total Phenolic Content of Oleoresins (Using Folin- Ciocalteu reagent)

The total phenolic content of OR was determined by the Folin- Ciocalteu reagent described by Singleton et al. (1999) and Bellik et al. (2013) with slight modifications. Oleoresin samples were diluted by adding 50 mg of sample to 500 mL of 80% (in distilled water) methanolic solution (100 ppm) and followed by sonication. The volume of 1 mL of diluted sample was added to 0.2 mL of Folin-Ciocalteu reagent (0.5 N) and the mixture was incubated in the dark for 15 min. After incubation, 5 mL of Na₂CO₃ (7.5%) were added to each sample and followed by 2 h incubation in the dark. The absorbance of the reaction mixture was read at 760 nm using a UV/VIS spectrophotometer (Thermo Scientific, Evolution 201 series, USA) against a reagent blank. The concentration of total polyphenols was expressed as mg Gallic Acid Equivalents per mg weight of oleoresin sample (mg GAE per mg W) by using the standard calibration curve of gallic acid which was constructed using different concentrations of gallic acid series. All samples were analyzed in triplicates.

Determination of Total Flavonoid Content of ORs (spectrophotometric method)

The total flavonoid content was determined by the spectrophotometric method as described by Hettiarachchi et al. (2020) with some modifications. Approximately, 1.5 mL from the diluted samples (500 ppm of methanolic extract) were added to the test tubes that were containing 3.5 mL of distilled water. Then, 0.3 mL of 5% NaNO₂ was added to that and the mixture was left to incubate at room temperature for 5 min. Then, 0.3 mL of 10% AlCl₃ was added into each sample and again incubated for 6 minutes at room temperature. After that, 2 mL of 1.0 M NaOH was added for terminating the reaction and distilled water was added for adjusting the total volume to 10 mL.

The absorbance of the samples was measured by a UV-Visible spectrophotometer (Thermo Scientific, Evolution 201 series, USA) at 510 nm. Total flavonoid content was expressed as mg rutin equivalents (RE)/g per mg of oleoresin sample by using the standard calibration curve of rutin which was constructed using different concentrations of rutin series.

Determination of Total antioxidant capacity (phosphomolybdenum method)

As described by Prieto et al. (1999), the samples of EO and OR are analyzed for total antioxidant capacity, using 5 mL of ammonium phosphomolybdate reagent solution (0.6 M sulphuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate) with 0.5 mL of diluted EO and OR samples using methanol. In combined samples of EOs and ORs, same percentages from each EO and OR were added to become 0.5 mL of the total volume. The mixture of reagent and samples were incubated at 95 °C for 90 min and the absorbance was read at 695 nm spectrophotometrically (UV/VIS spectrometer -SP-3000). The antioxidant activity is compared with a reference and expressed as mg Ascorbic Acid Equivalents per mg EO or OR of the sample (mg AAE per g DW).

Determination of radical scavenging activity (DPPH radical scavenging method)

The antioxidant activity of bioactive compounds of EOs and ORs can be simulated by DPPH (1,1-diphenyl-2-picrylhydrazyl) which is a stable free radical as described by Gunathilake & Ranaweera (2016) with slight modifications. EO samples were prepared by adding 50 mg of EO to 50 mL of 80% methanolic solution and 50 mg of oleoresin samples was added to 500 mL of 80% methanolic solution. From each sample, 150 µL (in combined samples of EOs and ORs, same percentages were added to become 150 µL of the total volume of sample combination) was added to the freshly prepared methanolic solution of DPPH solution (1 Mm, 5.85 mL). The mixture of DPPH and sample was vortexed for 15 seconds and followed by incubating at room temperature for 30 min in the dark. The absorbance of the EO and OR were checked spectrophotometrically (UV/VIS spectrometer (SP-3000, OPTIMA INC, Japan)) at 517 nm. Methanol was used as the blank and DPPH in methanol without sample was used as the positive control. The percentage inhibition of the radicals due to the antioxidant activity was calculated using the following equation.

$$\% \text{ inhibited free radicals} = \left\{ \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \right\} \times 100$$

Determination of IC₅₀ values (DPPH Radical Scavenging method)

IC₅₀ values were determined using the DPPH radical scavenging method according to the method described by Purkait et al., (2020). Five different concentrations of 500 ppm to 7000 ppm were tested and the concentration shows 50% inhibitions were calculated as the IC₅₀ value using regression analysis.

Determination of ABTS radical scavenging ability

According to the method, described by Gunathilake et al., (2017) with some modifications. The ABTS reagent (0.768 g of ABTS in 100 mL of distilled water) and 4.9 mM potassium persulphate were mixed at the ratio of 1:1. The mixture was kept in a dark room at 16 h. The prepared ABTS radical solution was diluted with ethanol 100 times and the absorbance was set 0.7 at 734 nm using UV/VIS spectrophotometer (SHIMADZU, UVmini-1240). EOs and ORs diluted with methanol at 250 ppm concentration and eighty µL was added to 3920 µL of ABTS stock solution. The mixture was kept for 10 min at 37 °C and the absorbance at 734 nm was measured. The standard curve was obtained using the Trolox standard. The results were expressed as mg Trolox Equivalent (TE) per 1 mg of EO/OR.

Determination of synergistic activity by calculating Combination Index values

Synergistic antioxidant activity of combined EOs and ORs was determined as described by Bag & Chattopadhyay (2015) using classical isobologram analysis based on IC₅₀ values and Combination Index (CI) equation.

$$CI = (D_1/DX_1) + (D_2/DX_2)$$

Where D1 and D2 were the doses (IC₅₀ values) of combined EOs or ORs; DX₁ and DX₂ were the doses (IC₅₀ values) of individual EOs or ORs. The antioxidant interactions were interpreted based on CI values as follows. CI < 1: synergistic; CI = 1: additive; CI > 1: antagonistic.

Statistical Analysis

All the statistical analyses were done using the analysis of Minitab software (2016). The analyses were carried out in triplicate (n=3) and results were expressed as mean ± standard deviation. The Anderson–Darling test was used for testing the normality assumptions and constant variance using residual versus fits and the independent assumptions were done through randomizations. One-way analysis of variance (ANOVA) was conducted and if p < 0.05, differences were considered to be statistically significant.

RESULTS AND DISCUSSION

Yields and main chemical components of EOs and ORs

The yields of the hydro-distillation and solvent extraction and the chemical components of EOs and ORs are summarized in Table 1. Cinnamon quilling resulted in an average bark oil yield of 1.43±0.01% (w/w) and OR yield of 2.94±0.01% (w/w) based on dried material weight. Moreover, our study resulted 1.42±0.04% of ginger oil yield and 6.91±0.18% of ginger oleoresin yield even though Bellik et al. (2013) reported 0.48±0.19% ginger oil using same method of hydro distillation and the variation of the yield would be due to the different plant materials from different origins. Further, 10.23 ± 1.02% of ginger OR yield was reported by the same authors using methanol as the extraction solvent and the reason for higher yield may be the difference of the extraction conditions such as extraction solvent. In clove buds, 15.69±0.39% of EO yield and 28.40±0.16% of OR yield were resulted. This

clove bud oil yield is higher than the oil yield reported by Purkait et al. (2020) (2.83%) which was obtained by hydro distillation. The variation would be due to the different plant origins. The OR yield of clove buds was lower than the value reported by Hemalatha et al. (2016) which was 37% yield of methanol extract and higher yield than the study of Adaramola & Onigbinde (2016) which was 18.33% yield of 80% acetone extract. Therefore, it can be concluded that the yield depends on many factors such as plant origin, variety of the plant, part of the plant which is used for extraction or distillation, pre-processing or pre-treatment techniques, and methods of the distillation or extraction.

Table 1: Yields of essential oils (EOs) and oleoresins (ORs).

Material	Cinnamon	Ginger	Clove bud
EO Yield (%)	1.43±0.01	1.42±0.04	15.69±0.39
OR Yield (%)	2.94±0.01	6.91±0.18	28.40±0.16

Determination of Total Phenolic Content (TPC) and Total Flavanoid Content (TFC) of ORs

Many studies had revealed that phenolics and flavanoids play a major role in antioxidant activity by directly scavenging the free radicals and inducing various intracellular antioxidant enzymes (Xu et al., 2014). The TPC of samples was determined by Folin-Ciocalteu (FC) assay using gallic acid as the standard which is phenolic acid. Flavanoids is a large family of polyphenolic compounds and they inherited strong free radical scavenging properties due to the capability of hydrogen donation and consisting of many hydroxyl substitutions in skeletal structure (Janarny et al., 2021). TFC was analyzed by following the aluminum chloride method using rutin standard which is a flavanol. The total phenolic and flavanoid content in cinnamon oleoresin, ginger oleoresin and clove bud oleoresin was determined spectrophotometrically and results are displayed in Figure 1. When three ORs were compared,

both TPC and TFC were significantly higher in ginger oleoresin and cinnamon oleoresin gave the lowest value. TPC of cinnamon oleoresin was 75.44 ± 5.85 mg GAE/g which is slightly higher than the study of Abeysekera et al. (2013) (33.43 ± 0.51 mg GAE/g) who extracted using solvent ethanol. The TFC of cinnamon oleoresin was 375.78 ± 10.18 mg RE /g. Clove bud oleoresin had given TPC content of 512.69 ± 15.18 mg GAE/g which is a higher TPC content compared to the study of Adaramola & Onigbinde, 2016 (200.20 ± 0.09 mg GAE/g) and TFC of 620.22 ± 13.88 mg RE /g. The highest TPC and TFC were recorded in ginger oleoresin where TPC content was 734.7 ± 29.8 mg GAE/g and TFC content of 873.56 ± 10.18 mg RE /g. Many studies were done for determining TPC content of ginger extracts which gave TPC content of 67.6 ± 1.08 mg GAE/g of dry methanolic extract (Bellik et al., 2013), 60.34 ± 0.43 mg GAE/g of chloroform: methanol (1:1, v/v) extract (Ali et al., 2018).

Evaluation of antioxidant activity

The spices inherit the antioxidant properties due to their secondary metabolites such as flavanoids, phenolic compounds, and volatiles. As characterized above, the bioactive chemical constituents of the EOs and ORs of cinnamon, clove bud, ginger consists of those phytochemicals which can act as natural antioxidants in the human body and foods itself. The antioxidant activity is evaluated by various types of *in vitro* assays and this study focus on DPPH free radical scavenging assay, total antioxidant activity by phosphomolybdenum method and ABTS radical scavenging assay.

DPPH is one of the prominent and well-proven *in vitro* assay to determine antioxidant activity. DPPH radical is purple-coloured organic nitrogen radical and the absorbance of DPPH is at 517 nm. It is reduced due to the phytochemicals which act as donors of hydrogen atoms to form a stable yellow coloured, non-radical diphenyl-picrylhydrazine (DPPH-H) (Tohma et al., 2017). Therefore, the percentage inhibition of DPPH can be used for determining antioxidant activity (Turgay & Esen, 2015). TAC of the EOs and ORs were determined using the phosphomolybdenum method

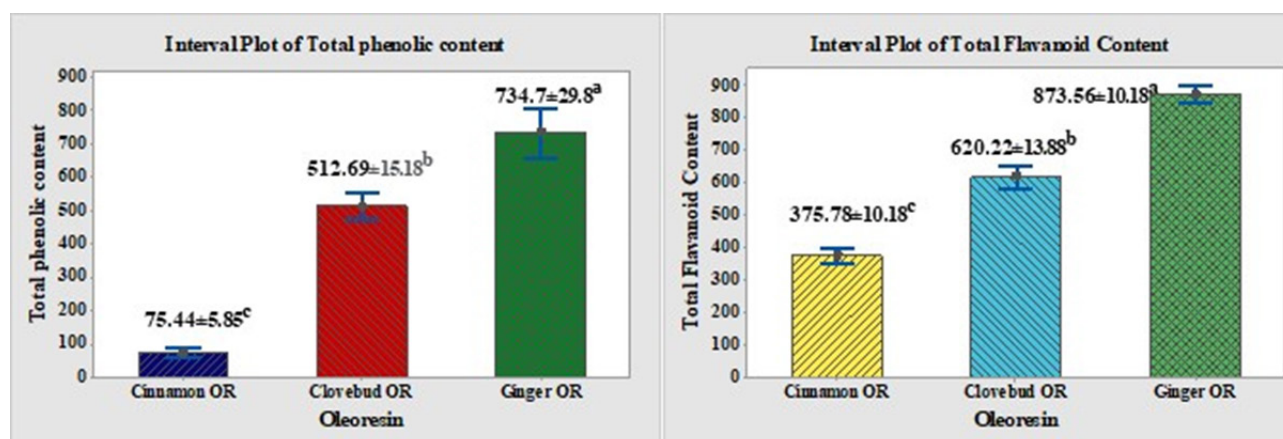


Figure 1: Total phenolic content and total flavanoid content of oleoresins. (95% confidence interval for the mean, The data presented in this graph consist of the Mean \pm SD of three independent samples. Means bearing different superscripts (a–c) differ significantly at a 95% confidence level.

Table 2: DPPH Free Radical Scavenging Assay (% inhibition), Total antioxidant capacity and ABTS radical scavenging activity of selected essential oils (EOs), oleoresins (ORs) and their combinations.

Essential Oil / Oleoresin	DPPH radical scavenging activity (% of inhibition) ^a	Total antioxidant capacity (mg AAE/mg Weight of EO/OR) ^b	ABTS radical scavenging activity (mg TE*/1 mg of EO/OR) ^c
CNO	53.68±0.62	0.27 ±0.00	70.73±9.60
CBO	88.70±0.46	0.48±0.02	2209.41±9.23
GRO	39.48±0.49	0.43±0.00	207.56±58.59
CNO + CBO	96.71±0.51	0.76±0.02	2207.87±9.60
CNO + GRO	68.16±0.68	0.37±0.01	253.69±4.61
CBO + GRO	96.97±0.45	0.70±0.01	960.33±23.06
CNO + CBO + GRO	87.14±0.48	0.52±0.01	2040.28±23.22
CNR	10.91±0.58	0.29±0.01	1019.37±44.00
CBR	34.20±0.40	3.94±0.05	2121.77±9.23
GRR	27.36±1.67	1.78±0.11	1662.05±51.84
CNR + CBR	26.45±0.56	2.00±0.06	1485.24±20.11
CNR + GRR	23.85±0.62	1.07±0.01	2015.68±28.81
CBR + GRR	33.48±0.12	2.33±0.01	2092.56±18.64
CNR + CBR + GRR	26.71±0.43	2.53±0.05	1983.39±9.23

Values are presented as mean ± SD, n = 3 for independent samples

a The inhibition percentage of DPPH is calculated of Essential Oil (1000 ppm concentration)/ Oleoresin (100 ppm concentration)

b mg ascorbic acid equivalent per mg of essential oil/oleoresin weight

c mg Trolox acid equivalent per mg of essential oil/oleoresin weight

AAE – Ascorbic Acid Equivalent, TE – Trolox Equivalent,

Abbreviations; CNO (cinnamon bark oil), CBO (clove bud oil), GRO (ginger oil), CNR (cinnamon oleoresin), CBR (clove bud oleoresin), GRR (ginger oleoresin)

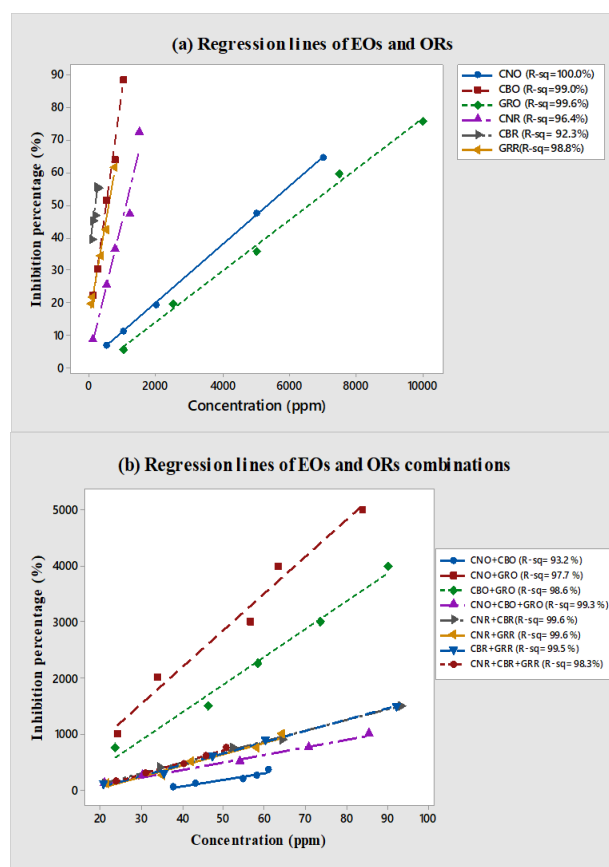


Figure 2: Regression lines of DPPH radical scavenging inhibition percentages (%) with different **essential oil (EO)/oleoresin (OR)** EO/OR concentrations. CNO- cinnamon bark oil, CBO- clove bud oil, GRO- ginger oil, CNR- cinnamon oleoresin, CBR- clove bud oleoresin, GRR- ginger oleoresin).

which is based on the formation of bluish-green coloured phosphate-Mo (V) complex at acidic pH by reducing Phosphate-Mo (VI) by the bio-actives of our samples.

According to the results of our study Table 2, the EOs and ORs exhibited a range of variation in inhibition percentage of DPPH; EOs of cinnamon (53.68±0.62 %), clove bud (88.70±0.46%), ginger (39.48±0.49%) at 1000 mg/L concentration and ORs of cinnamon (10.91±0.58%), clove bud (52.36±0.48%), ginger (27.36±0.48%) at 100 ppm concentration. Therefore, it reveals that, the clove bud gives the highest DPPH free radical scavenging inhibition percentage compared to cinnamon and ginger. According to the previous study done by Lin et al. (2009) using different 42 kinds of EOs, cinnamon bark had given the highest DPPH free-radical scavenging percentage at 5 mg/mL (91.4 ± 0.002%) which is a lower value as we experimented using 1000 ppm concentration of EO. Cinnamaldehyde and cinnamic acetate were reported as the major compounds to contribute to the antioxidant activity and cinnamon bark oil was recorded as the highest antioxidant activity compared to ginger, nutmeg, fennel, laurel and marjoram EOs (Cardoso-Ugarte et al., 2015).

Tohma et al. (2017) had studied ethanolic extract of ginger and 43.8 % of inhibition was given at 30 µg/ mL concentration. In addition to that, ginger EO had shown 19.92±2.80% to 68.31±3.32% at concentration of 1.2 to 19.37 mg/ml and ginger OR had given 25.83±0.30% to 87.65±0.46% at 0.025 to 0.4 mg/ml concentrations. DPPH radical scavenging percentage of clove bud EO at the flowering stage was studied by Alfikri et al. (2020) and 67.65% radical scavenging percentage was shown at 45 µg/mL.

When EOs and ORs were combined with same percentages, a higher radical scavenging percentage and TAC was given in combinations of cinnamon EO with clove bud EO (96.71±0.51 %, 0.76±0.02 mg AAE/mg Weight of EO/OR respectively) and cinnamon EO with ginger EO (96.97±0.45%, 0.70± 0.01 mg AAE/mg Weight of EO/OR respectively).

There are limited previous studies on TAC of cinnamon, ginger and clove bud EOs and ORs. The TAC of EOs

and ORs were within the range of 0.27-3.94 mg AAE/mg weight in EO or OR. Clove bud OR showed the highest TAC (3.94±0.05 mg) followed by ginger (1.78±0.11 mg) and cinnamon (0.29±0.01 mg) per AAE/mg weight of OR. The TAC of EOs was exhibited as 0.48±0.02, 0.43±0.00, 0.27±0.00 AAE/mg weight of OR in clove bud, ginger and cinnamon ORs respectively.

As revealed by DPPH radical scavenging method and TAC, the highest ABTS radical scavenging activity was recorded in clove bud in both clove bud oil (2209.41±9.23 mg TE*/1 mg of EO/OR) and clove bud oleoresin (2121.77±9.23 mg TE*/1 mg of EO/OR). In addition to that, the three tests revealed that ORs are higher in their antioxidant properties compared to essential oil of the same spice in cinnamon, clove bud and ginger. This may be due to the free radical scavenging ability of the non-volatile bioactive compounds of ORs compared to the volatile bio-actives of the EOs.

Table 3: IC₅₀ values of essential oils (EOs) and oleoresins (ORs) and their blends based on DPPH Radical Scavenging Assay.

EOs/ORs	IC ₅₀ Value (ppm)
CNO	5147.3 ± 287.2
CBO	494.3±12.0
CRO	6518.1±219.5
CNR	1063.1±174.3
CBR	164.5±6.1
GRR	557.3±8.8
CNO+CBO	180.9±1.6
CNO+GRO	2846.7±26.5
CBO+GRO	1958.2±41.1
CNO+CBO+GRO	489.9±29.2
CNR+CBR	667.0±31.7
CNR+GRR	632.7±5.2
CBR+GRR	568.4±10.6
CNR+CBR+ GRR	656.8±31.5

CNO- cinnamon bark oil, CBO- clove bud oil, GRO- ginger oil, CNR- cinnamon oleoresin, CBR- clove bud oleoresin, GRR- ginger oleoresin

Table 4: Antioxidant combination indices (CI) values of test EOs and ORs.

Samples	IC 50 Value	CI1 = (D)1 / (DX)1	CI2 = (D)2 / (Dx)2	CI3= (D)3/ (Dx)3	CI = CI1 +CI2+ CI3	Remarks
CNO+CBO	180.90	0.04	0.37		0.40	Synergistic
CNO+GRO	2846.70	0.55	0.44		0.99	Synergistic
CBO+GRO	1958.20	3.96	0.30		4.26	Antagonistic
CNO+CBO+GRO	489.90	0.10	0.99	0.08	1.16	Antagonistic
CNR+CBR	667.00	0.63	4.05		4.68	Antagonistic
CNR+GRR	632.70	0.60	1.14		1.73	Antagonistic
CBR+GRR	568.40	3.46	1.02		4.48	Antagonistic
CNR+CBR+ GRR	656.80	0.62	3.99	1.18	5.79	Antagonistic

(D)1, (D)2 and (D)3 are the doses of combined EOs or ORs ; (DX)1, (DX)2 and (DX)3 are the doses individual EOs or ORs. CI <1: synergistic; CI = 1: additive; CI > 1: antagonistic

CNO- cinnamon bark oil, CBO- clove bud oil, GRO- ginger oil, CNR- cinnamon oleoresin, CBR- clove bud oleoresin, GRR- ginger oleoresin

Determination of IC₅₀ values (DPPH Radical Scavenging method)

The IC₅₀ value (half maximal inhibitory concentration) is the concentration of the antioxidant which is required to decrease the DPPH radical scavenging activity by 50%. Furthermore, the IC₅₀ value is inversely related to the antioxidant activity. It is calculated by linear regression plots (Figure 2) of DPPH radical scavenging activity against the concentration of EO or OR and results are shown in Table 3. The lowest IC₅₀ value was given by clove bud oleoresin and followed by clove bud oil, ginger oleoresin, cinnamon bark oleoresin, cinnamon bark oil and ginger oil. The IC₅₀ value of Cinnamon bark Oil was 5.1473 ± 0.2872 mg/mL which is much higher compared to the study of Nanasombat & Wimuttigosol (2011) (0.29 mg/mL) and cinnamon oleoresin resulted 1.063 ± 0.1743 mg/mL. Ginger oleoresin resulted 0.5573 ± 0.0088 mg/mL which is close to the experiments done by Ezez & Tefera (2021) (0.481 ± 0.015 mg/mL) while ginger oil resulted 6.5181 ± 0.2195 mg/mL which slightly lower compared to the experiments of Bellik et al. (2013) (6.5181 ± 0.2195 mg/mL). Previous studies on the IC₅₀ value of clove bud oil revealed that 15.80–108.85 µg/mL (Alfikri et al., 2020) which is lower than our study (0.4943 ± 0.0120 mg/mL) and clove bud oleoresin resulted in 0.1645 ± 0.0061 mg/mL which is higher than the studies done by Adaramola & Onigbinde (2016) (7.82 ± 0.06 µg/mL) and lower than value (136.6 mg/mL) reported by Turgay & Esen (2015). The difference in the DPPH radical scavenging activity and IC₅₀ values may be due to the variation of extraction method, extraction solvent, plant origin, climate and other environmental factors (Ezez & Tefera, 2021).

IC₅₀ values of combined EO and OR are shown in Table 4. Remarkably, EO blends were very effective over the individual EOs tested. The cinnamon bark oil/ clove bud oil, cinnamon bark oil/ginger oil, clove bud oil/ ginger oil, cinnamon oil/clove bud oil/ ginger oil combinations gave a higher synergistic effect by decreasing the half-maximal inhibitory concentration compared to individual EO. In addition to that, some previous studies on other essential oil blends were reported synergistic effects. The study of (Benyoucef et al., 2018) revealed that strong synergistic effect by combining *T. fontanesii*, *A. herba-alba* and *R. officinalis* EOs. (Crespo et al., 2019) reported highest antioxidant activity in the mixture of 66.7% of *T. vulgaris*, 16.7% of *C. sativum* and 16.7% of *A. graveolens*.

Determination of synergistic activity by calculating CI values

Synergistic antioxidant activity of the EOs and ORs are occurring due to the interactions between the main constituents of the extracts. The synergistic antibacterial and antioxidant activities of EOs of spices and herbs were firstly reported by Bag & Chattopadhyay (2015) using coriander/cumin seed oil combination (CI = 0.79).

According to the Combination Index (CI), the synergistic antioxidant activity of EO and OR was given in Table 5. The combination of cinnamon oleoresin/clove bud oleoresin and combination of cinnamon oleoresin/ginger

oleoresin showed synergy with CI values of 0.40, 0.99 respectively. Furthermore, amidst tested combinations, the combinations of cinnamon oleoresin/clove bud oleoresin and cinnamon oleoresin/ginger oleoresin are higher in proton donating ability at a lower concentration than the individual. Similarly, the study which was done by Purkait et al. (2020) using cinnamon bark oil, clove bud oil and black pepper oil, reported that the synergy in the combination of cinnamon bark oil and clove bud oil (CI=0.82). The absence of observed synergistic or additive effects on antioxidant activity in the ORs of cinnamon, clove bud, and ginger, in contrast to the positive outcomes observed with cinnamon bark oil, clove bud oil, and ginger oil, may be attributed to disparities in chemical composition and potential chemical interactions, including interactions with nanoscale antioxidative compounds. Notably, ORs encompass a broader spectrum of constituents, encompassing both volatile and non-volatile components, whereas EOs are predominantly characterized by volatile compounds. The presence of these non-volatile components within ORs introduces a complex chemical milieu that may exert multifaceted effects on antioxidant activity, possibly negating the synergistic phenomena observed in EOs.

As elucidated by the findings of this research, both ORs and EOs exhibited heightened antioxidant activity. Particularly noteworthy were the observed instances of heightened synergy, notably in combinations of cinnamon bark oil with clove bud oil and ginger oil. These synergistic interactions have the potential to facilitate a reduction in the required dosage of antioxidants, thereby conferring the dual advantages of cost reduction and mitigation of potential adverse effects associated with higher dosages.

CONCLUSION

In conclusion, this study demonstrated that the EOs and ORs of cinnamon (*Cinnamomum zeylanicum*), clove bud (*Syzygium aromaticum*), ginger (*Zingiber officinale*) exhibited wide range of antioxidant properties. Furthermore, the ORs were more antioxidative compared to essential oils amidst the studied three spices. It was remarkable that, clove bud had given highest antioxidant activity in both EOs and ORs compared to cinnamon and ginger. Synergistic antioxidant efficacy of EOs was observed in the combination of cinnamon OR/clove bud OR (CI=0.40) and a combination of cinnamon OR/ginger ORs. Therefore, this will provide a path for developing a novel and natural antioxidant blend at a lower concentration which will reduce the adverse impacts of using synthetic and higher concentration of preservatives.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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