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Research Article
Content of Vitamin C, Phenols and Carotenoids Extracted from *Capsicum annuum* with Antioxidant, Antimicrobial and Coloring Effects

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Abstract

Background and Objective: *Capsicum annuum* is considered a good source of various natural compounds. The current study aimed to assess the vitamin C and total phenolic and carotenoid contents in *C. annuum* using standard methods. **Materials and Methods:** Microwave and Soxhlet extraction by using water and acetone were used to extract vitamin C and phenols. Saponification extraction was used to extract carotenoids. The antioxidant activities of each extract were assessed using a DPPH assay. The Minimum Inhibitory Concentration and Minimum Bactericidal Concentration (MIC and MBC) against *Escherichia coli* and *Staphylococcus aureus* were determined using the broth microdilution method. The coloring capacity for the acetone extract was evaluated and determined using glass wool fiber at different concentrations and then used in the formulation of multivitamin hard candy. **Results:** The acetone extract showed the highest phenol and vitamin C content (1.03±0.02 and 9.7±1.3 mg mL^{-1}, respectively), antioxidant activity (67.12±3.8 mg mL^{-1}) and MIC and MBC of 0.96 and 1.88 mg mL^{-1} against *E. coli* and 3.75 and 7.5 mg mL^{-1} against *S. aureus*. It also showed an intense orange shade on wool fiber and on the prepared multivitamin candy at concentrations of 6 and 0.5% (w/w), respectively. Saponifications of the acetone extract yield (23.49±0.13 µg g^{-1}) of carotenoids. **Conclusion:** The prepared acetone extract of *C. annuum* stands as a potential pharmaceutical additive, which can be used as coloring and preservative agents in the formulation of kids multi-vitamin candy.

Key words: Vitamin C, phenols, carotenoids, *Capsicum annuum*, antioxidant, antimicrobial, coloring agent

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Internationally, the market for natural products is substantially expanding by annual increases of 10-15% due to the introduction of new categories of natural products, including natural food additives such as; preservatives and coloring agents[1]. Historically, the use of food additives goes back to the 1800 sec, when additives were used to prevent food adulteration. Today, more than 2500 additives are deliberately added to food in order to improve its properties or to prolong its shelf life[2]. Recently, a growing numbers of natural food additives such as natural dyes are being commercially produced due to consumer concerns surrounding the safety of synthetic dyes[3]. Currently, food colorant additives are required to be evaluated for safety. Therefore, the FDA mainly focuses on synthetic colorants[4].

Naturally derived coloring agents are mainly obtained from animals, vegetables, minerals and plant extracts or from synthetic duplicates of naturally existing colors[5]. The composition and properties of these substances as well as the amount that would typically be consumed, the immediate and long-term health effects and various other safety factors must be evaluated[2].

In addition to their use in food products, coloring agents can be used in the pharmaceutical industry for many pharmaceutical dosage forms, especially tablets, caplets, capsules, gels and creams[6]. Globally, many multivitamin formulations used for children contain natural extracts as coloring agents, such as black carrot juice concentrate and purple berry concentrate[7]. In addition, these formulas contain vitamin C and antimicrobial agents that act as preservatives[8]. The most commonly known natural coloring agents are the carotenoids. Carotenoids have been detected in various plants and are found at the highest levels in orange or red plant species. One of these species is *Capsicum annum*, carotenoid pigments give the fruit its appealing red color[9]. The carotenoid content can vary among different cultivars[10]. In addition, these fruits are also a good source of other important phytochemicals such as; phenols, flavonoids and capsaicinoids. *Capsicum* is also considered a good source of vitamin C, though there are significant differences in vitamin C levels among different *Capsicum* species and genotypes[11-13].

Capsicum, commonly known as pepper is a genus of flowering plants of the nightshade family, Solanaceae. It is an annual or perennial flowering shrub plant available in five main species: *C. annuum*, *C. chinense*, *C. baccatum, C. pubescens* and *C. frutescens*[14]. Pepper is an essential in Jordan that is usually cultivated in the lower Jordan valley[15]. The majority of Jordanian people use pepper as food and a spice as well as for other uses[16]. Pepper fruits collected from local sources have been traditionally used as a digestive aid and as an aphrodisiac, according to an ethnopharmacological survey of traditional drugs sold in the Kingdom of Jordan[17].

To our knowledge, this is the first study to evaluate the contents of vitamin C, phenols and carotenoids, the antioxidant and antimicrobial activities and the coloring capacity of red pepper extracts from *C. annuum* fruit available in Jordan. These findings are of special interest for researchers working in the pharmaceutical industry looking for natural compounds and extracts with colorant and preservative properties that can be utilized in the manufacturing of pharmaceutical dosage forms used for children.

MATERIALS AND METHODS

Study area: This study was carried out at the laboratories of Pharmaceutical Sciences and Pharmacognosy Department, Faculty of Pharmacy, Applied Science Private University, Amman, Jordan, from April, 2018-August, 2018.

Plant material: The *C. annuum* fruits were bought from a local market in Amman, Jordan during April. The fresh fruits were cleaned with running tap water and the seeds were removed. The flesh of the fruit was finely ground by using a blender and kept in the freezer at -20°C until use.

Preparation of fruit extract

Soxhlet extraction method: Samples (350 g) of *Capsicum* fruit were used to prepare extracts with acetone or water/acetone (50:50) for duration of 5 h at 50 and 70°C, respectively.

Microwave oven extraction method: Aqueous extracts were prepared using an 800 W microwave oven (Sona, Jordan). Briefly, a sample (50 g) of the plant material was extracted with water for a duration of 2 min at 98°C.

The obtained extracts were then filtered and concentrated using a rotary evaporator and freeze-dried. All extracts were stored in a refrigerator at 5°C until use.

Quantification of the vitamin C content in the *C. annuum* extracts: The quantification of vitamin C in the *C. annuum* extracts was performed by using the direct titration method[18]. Briefly, 1 g of each dry extract was diluted with water (100 mL) and mixed with 1 mL of potassium iodide solution (0.6 mol L^{-1}) and 1 mL HCl (1 M). The titrant used was 0.002 mol L^{-1} potassium iodate solution. The endpoint was detected by the appearance of a blue-black complex using starch as indicator.

Quantification of the total phenol content in the *C. annuum* extracts: The quantification of the total phenol content was carried out by using the Folin-Ciocalteu (FC) colorimetric assay method[19]. Briefly, 1 g of each dry extract was diluted with 3 mL water and mixed with 1 mL of 2% Na_2CO_3. To this mixture, 1 mL of 50% FC reagent was added and the mixture was left in a dark place at room temperature for 2 h. Later, the absorption of the prepared samples was read at 650 nm using UV spectroscopy (Dr. Akid SCO GmbH, Germany). Gallic acid was used as the standard solution and the data were expressed as gallic acid equivalents (mg mL^{-1}).

Determination of the antioxidant activity of the *C. annuum* extracts: This assay was based on the method described previously[20]. Briefly, the dry plant extracts (acetone and water/acetone). Soxhlet extracts and microwave aqueous extracts were dissolved in ethanol at different concentrations ranging from 20-80 mg mL^{-1} and used as reducing agents. An amount of 0.5 mL of DPPH free radical solution was mixed with 3 mL of ethanol plant extract solution. These sample mixtures were then incubated for 100 min in the dark at room temperature. The color of the reaction mixture was monitored as it gradually changed from violet to yellow. The absorption for each sample was measured at 517 nm using a plate reader. Ascorbic acid was used as the standard solution, prepared at various concentrations ranging from 0.2-25 µg mL^{-1}. The effective concentration required to scavenge 50% of the free radicals (EC_{50}) was calculated.

Determination of the antimicrobial activity of the *C. annuum* extracts: According to the method described by Talib and Mahasneh[21], the antimicrobial activities of each *C. annuum* extract against *E. coli* and *S. aureus* were evaluated. Briefly, a stock solution composed of 150 mg mL^{-1} of plant extract in nutritional broth was prepared for each extract. In a microplate, all the wells except the first line were filled with 100 µL nutritional broth. Then, 100 µL of two fold serial dilutions of each plant extract in broth at volumes of 150, 100, 50, 25, 12.5, 6,25, 3.125 and 1.56 µL were incubated with 50 µL of each bacterial suspension.

A stock solution of gentamicin (80 mg mL^{-1}) was used as positive control. The microtiter plates were incubated at 37°C for 24 h. The turbidity of the incubated broth was measured at 600 nm using a spectrophotometer. This broth dilution assay method allows the determination of both the MIC (Minimum Inhibitory Concentration) and the MBC (Minimum Bactericidal Concentration)[22].

Evaluation of the coloring capacity of the *C. annuum* acetone extract: The coloring capacity of the acetone extract was evaluated on glass wool fiber using the method described by Arora *et al.*[23]. The other extracts were excluded from this test as they are completely water-soluble. Briefly, 100 mL samples of acetone extract at different concentrations (1-6%) were prepared and used to dye 1 g of superfine glass wool fiber by maceration and boiling at 85°C for 30 min. Each completely dyed wool piece was spread on a Petri dish for 24 h in the dark at room temperature until completely dried, then washed with an aliquot of water and left to dry completely for 24 h more. Later, these pieces were washed again with water and mild detergent and left to dry completely for 24 h.

A photograph of each wool piece was taken before and after dying with each extract concentration, as well as after each washing step for the comparison of the coloring capacity and the stability of the extracts used.

Saponification and determination of carotenoid content from *C. annum* extracts: The extraction of carotenoids from *C. annum* was performed based on the method described by Giuffrida *et al.*[24] and Baba *et al.*[25]. Briefly, fresh, finely ground *Capsicum* samples were extracted with an excess volume of acetone using a homogenizer. The carotenoid compounds were purified from the concentrated extract using diethyl ether, treated with 10% methanolic potassium hydroxide as a saponification agent, shaken well and kept in the dark for 1 h. For the desaponification, the emulsion was treated with 10% sodium chloride to separate the solution into two layers. The supernatant layer was collected and dried. For the quantitative analysis, the dry residues were dissolved in ethanol and measured spectrophotometrically at 455 nm.

Formulation of multivitamin candy using *C. annum* extract as additive agent: A multivitamin hard candy was prepared according to the method described by Assous *et al.*[26] and Verma and Gupta[27] (Table 1). Briefly, granulated sugar was mixed with water. Citric acid was added and the mixture was heated. Corn syrup was added at the boiling point. The solution was further heated until it turned highly viscous. Then, it was allowed to cool before adding the required vitamins and dry acetone extract. The mass of the dry acetone

Table 1: Kids-multivitamins hard candy formula

Ingredients	Wight
Sugar	48 g
Corn syrup	25 g
Water	25 mL
Citric acid	0.15 g
Vitamin B1	0.5 mg
Vitamin B6	0.4 mg
Vitamin B12	2 µg
C. annum acetone extract	0.5% (g g^{-1})

extract used in the formula was 3.5 g dry weight per 7 g candy, which was calculated based on its vitamin C content equal to 35 mg (POLY-VIT: *MULTIVITAMIN ORAL DROPS (FDA)).

The prepared solution was allowed to cool and then poured into molds to obtain the desired shape. The control sample was made by using 0.10% synthetic red dyes E122 (azorubine) and E110 (sunset yellow FCF)).

Photo pictures for the prepared candy were taken for comparison of the coloring capacity and the stability of the used extracts in relation to the used synthetic dies.

Statistical analysis: All experimental data were expressed as the Means±SD. Statistical analyses were performed by ANOVA with SPSS (IBM SPSS Statistics 22) and Microsoft Excel 2013. Statistical comparisons were carried out by bi-variate correlation. A p-value $\leq$0.05 was considered significant.

RESULTS

Evaluation of chemical composition and biological activities of *C. annum* extracts: Table 2 shows vitamin C, total phenol and carotenoids contents for different *C. annum* extracts. The DPPH antioxidant activity (EC_{50}) compare to ascorbic acid and antimicrobial activities against *E. coli* and *S. aureus* (MIC and MBC) (mg mL^{-1}) compare to gentamicin for all the prepared extracts are also shown. Findings show the highest content of vitamin C was obtained by using acetone/water extraction, with significant differences ($p\leq0.05$) between different extracts. Where phenol content was higher in the acetone extract, with no significant differences between the different extracts were observed. In addition, saponifications of the acetone extract yield (23.490.13 µg g^{-1}) of total carotenoids. The antioxidant activities show significant differences ($p\leq0.05$) between different extracts. However, it revealed that acetone extract to possess the highest antioxidant activity. The antimicrobial test revealed that the MIC and MBC values for *C. annuum* acetone extract to exhibit the highest activity against *E. coli.* The acetone/water extract exhibit the highest activity against *S. aureus*. While, the microwave extract showed to posses very weak antimicrobial activities.

Evaluation of the coloring capacity of the *C. annuum* acetone extract: Figure 1 shows photographs of the intensities of the orange shade developed on glass wool using

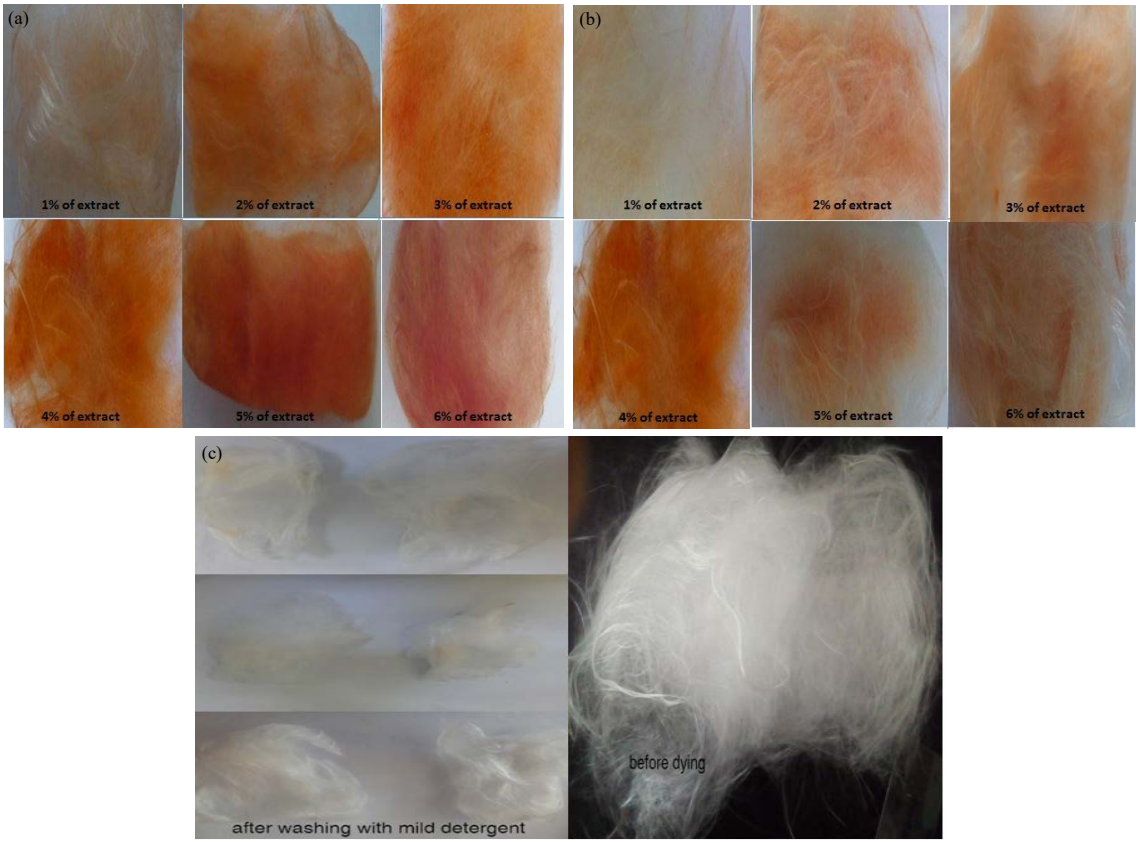

Fig. 1(a-c): Shades of orange color developed on wool fibers, (a) Applying different concentrations of *C. annum* acetone extracts (1-6%), (b) After washing with tap water and (c) After washing with tap water and mild detergent

Table 2: Comparison of chemical composition and biological activities for *C. annum extracts*

Activity	Soxhlet-acetone	Soxhlet-acetone/water	Microwave-water	Ascorbic acid	Gentamicin (80 mg mL^{-1})	p-value
Vitamin C (mg g^{-1})±SD	9.7±1.3	10±2	1.9±0	-	-	0.022
Phenols (mg mL^{-1})±SD	1.03± 0.02	0.81±0.005	0.75±0.015	-	-	0.811
Carotenoids (µg g^{-1})±SD	23.490.13	-	-	-	-	-
EC_{50} (mg mL^{-1})±SD	67.12±3.8	90.86±1.2	99.54±8.27	0.0018±0.0066	-	0.007
E. coli MIC (mg mL^{-1})	0.96	3.75	15	-	0.19	-
S. aureus MIC (mg mL^{-1})	3.75	1.88	7.5	-	0.4	-
E. coli MBC (mg mL^{-1})	1.88	15	15	-	0.4	-
S. aureus MBC (mg mL^{-1})	7.5	7.5	15	-	0.8	-

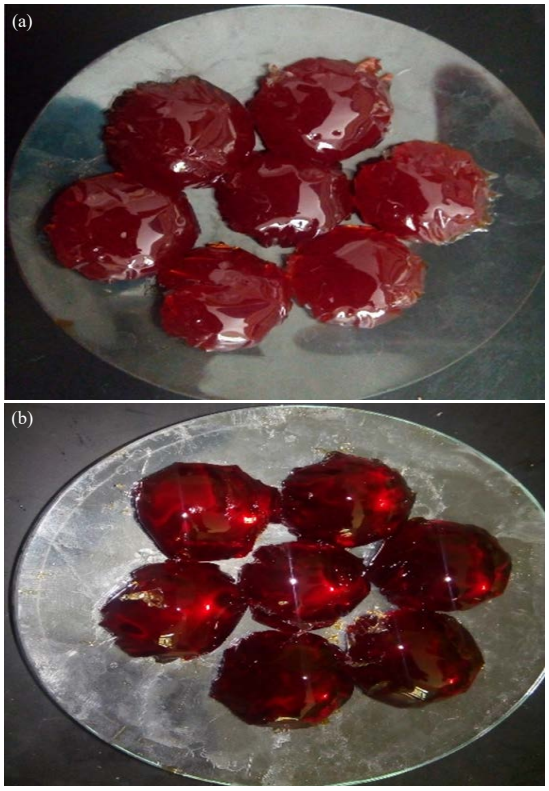

Fig. 2(a-b): Multivitamin hard candy prepared using, (a) *C. annum* acetone extract (0.5%), (b) Synthetic colorant agent E122 and E110 (0.1%)

the *C. annuum* acetone extract at different concentrations (1-6%). Figure 1a shows that the shades orange increase at higher concentrations of the plant extract (Fig. 1a). After washing the wool fibers with tap water only, the orange shade intensity decreased at all applied plant extract concentrations (Fig. 1b). After washing the wool fibers with tap water and mild detergent, the orange color washed out completely (Fig. 1c).

Formulation of multivitamin candy using *C. anuum* extract as additive agent: Figure 2a shows the multivitamin candy prepared using the *C. anuum* acetone extract (0.5%) (Fig. 2a) and the synthetic colorants E122 and E110 (0.1%) (Fig. 2b). This preliminary formula containing the potential coloring extract developed the desired orange shade, like the standard coloring agents. Further stability studies are needed before the extract can be considered a stable and safe additive agent for use in the food and pharmaceutical industries.

DISCUSSION

The findings of this study revealed that the acetone dry extract of *C. annuum* is a rich source of many important natural compounds including vitamin C, phenols and carotenoids has antioxidant and antimicrobial effects as well as an orange coloring capacity that complies with food additive requirements[28]. it can be used as a natural coloring and preservative agent. Supplement formulations such as vitamins and minerals are commonly intended for use by infants and children, these supplements typically contain coloring agents. Many recent studies have shown that the consumption of a mixture of synthetic food colorants may increase the Global Hyperactivity Aggregate (GHA) score and Attention Deficit Hyperactivity Disorder (ADHD) in treated groups' of 3-9 year-old children[29-31].

Therefore, alternative natural colorant compounds such as; anthocyanins, betacyanins, carotenoids and chlorophylls are now being used and extensively investigated in term of their health benefits compared to synthetic colorants[1,32]. Particularly, carotenoids have shown to reduce the risk of many diseases, including cardiovascular disease, cancers, age-related macular degeneration and photosensitivity related to UV exposure[33].

Different varieties of *Capsicum* species showed variations in their vitamin C, phenol and carotenoid content based on their cultivation regions[34-36]. Additionally, different cooking and drying methods can drastically reduce the contents of these compounds[37].

In this study, statistical analysis revealed significant differences ($p \leq 0.05$) between the Soxhlet and microwave extraction methods. Soxhlet extraction preserved five times more vitamin C content than the microwave extraction method. This due to the ability of the Soxhlet method to preserve this heat-sensitive compound, unlike microwave

extraction, which utilized dipolar microwave energy as a heat source. While there were no significant differences ($p>0.05$) between the microwave and Soxhlet extracts in terms of their phenolic compound content, the latter contained fewer phenolic compounds. In addition, the detected carotenoid content extracted from *C. annuum* was lower than the previously reported data obtained from three Mexican varieties of *C. annuum*[38], but was within the range of total carotenoid content for 40 other cultivars [17]. The difference can be attributed to genetic and environmental influences on the synthesis and accumulation of these compounds[34].

Lower antioxidant activity was found in these *C. annum* extracts compared to previous findings using other *Capsicum* species (*C. annuum*, *C. baccatum*, *C. chacoense* and *C. chinense*). These differences could be attributed to the negative effect of the heating processes used during the extraction, which are known to affect the contents of active phytochemicals[39]. Nevertheless, the acetone dry extract had the highest antioxidant activity compared to the other extracts. In addition, the acetone dry extract also had higher contents of vitamin C and phenols, which contributed to its antioxidant activity.

The antimicrobial activity of *C. annuum* extracts was previously reported by Mokhtar *et al.*[40]. Similarly, they found higher activity for the alcoholic extract against *E. coli* than against *S. aureus,* which was explained by the higher content of polyphenolic compounds possessing antimicrobial effects, they also found variations in sensitivity to *C. annuum* extracts between Gram-negative and Gram-positive bacteria[41].

The plant extract showed good coloring capacity as a coloring agent on wool fabrics at relatively low concentrations. The extract showed stability to washing with water, but was removed when washed with detergents. These findings shed light on the possible use of this extract as a safe food coloring agent. Moreover, this extract demonstrated its potent antimicrobial effects on most common pathogens that may cause food contamination in addition to the adulteration in food color caused by its antioxidant activity.

The preliminary formula of *C. annum* extract used in this study at a concentration equivalent to the recommended amount of vitamin C in a multivitamin formula for children showed considerable antimicrobial and antioxidant effects as well as acceptable coloring capacity. The extract acted as a promising preservative and coloring agent and has potential as a substitute for the synthetic alternatives commonly used in pharmaceutical applications. The investigation of *C. annuum* extract as a coloring agent on wool fabrics showed good coloring capacity at relatively low concentrations, but with the ability to leach when washed with detergents. As such, the prepared multivitamin candy utilizing an optimized amount of *C. annum* extract, with the intention of the substitution of the synthetic alternatives was proposed in this study. These findings shed the light on the possible use of this extract as a safe food and pharmaceuticals coloring and preservative agent.

In this study, there were various limitation areas where further research is needed for the complete identification of the chemical compounds and mineral composition extracted from the study plant species. Moreover, determination of other vitamin contents would be valuable.

CONCLUSION

In this study, *C. annum* acetone dry extract is suggested as a potential natural additive with coloring and preservative effects for the formulation of multivitamin candies. These properties of the extract make it a good alternative to synthetic compounds, with the advantages of added health benefits, due to its rich content of vitamin C, phenols and carotenoids. These compounds also contributed to the beneficial antioxidant and antimicrobial activities of the extract. Therefore, it is possible that it could be used in food and pharmaceutical formulations to replace synthetic additives with a natural substitute that provides additional health benefits. Nevertheless, further studies are needed to assess the industrial and economic applicability of this extract.

SIGNIFICANCE STATEMENT

This study discovers the possible synergistic effect between phenols, vitamin C and carotenoids obtained using acetone extract prepared from the Jordanian species of *C. annum*. The prepared extract posses antioxidant and antimicrobial activities against the most common pathogens causing deterioration of food materials. Therefore, it has shown to be applicable as preservative agent. In addition, the prepared extract has shown acceptable coloring capacities that can be used as natural additives in food and pharmaceuticals formulations. Thus, a new source of phytochemicals combination with potential biological activities that can be used for varied pharmaceutical applications may be arrived at. Never the less, further studies are needed to assess their industrial and economic applicability.

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