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Extraction of brown dye from *Eucalyptus* bark and its applications in food storage

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RESEARCH ARTICLE

Abstract

The importance of plant pigments as natural food colourants has been increasing globally in order to avoid from environmental problems and health hazards associated with synthetic colours. The current consumer prefers the naturally derived food colourants due to their health promoting properties. The present investigations were carried out to establish the safety and nutritional value of natural dye produced from the bark of *Eucalyptus globules*. A brown fine powder was obtained by aqueous extraction of finally ground *Eucalyptus* bark. This brown dye contained tannins, reducing sugar, alkaloids, glycosides, saponins, various antioxidants, antimicrobial agents and antiradical compounds. Total phenolic contents were ranging from 66.83 to 534.80 µg of gallic acid equivalents per 100 grams dry weight of the dye. In 2,2-diphenyl-1-picrylhydrazyl assay, IC_{50} value was observed to be 77.20 µg/ml with respect to butylated hydroxytoluene (9.96 µg/ml) as a standard control. The earlier investigations on the coloured candies of various brands suggest that the synthetic dyes used in the production of candies are a very big jeopardy for human health. In the current studies, the natural brown dye obtained from the *Eucalyptus* bark was applied for the production of candies and then the stability of dye in the candies was measured in terms of caffeic acid content. The *Eucalyptus* bark dye has shown an excellent nutritional value with no toxicity against the tested mice looking its possible safe use as a food colorant in future. None of the aflatoxins were detected in the dye.

Keywords: *Eucalyptus* dye; natural food colour; nutritional, antimicrobial, candies

1. Introduction

The demand of safe and nutritious food products with an appropriate shelf life has been increased in the last decade (Junqueira-Gonçalves *et al.*, 2015). Diet plays a most crucial role for human health; improper diet may lead to various chronic diseases and premature deaths (Katz and Meller, 2014). According to International Food Information Council and U.S. Food and Drug Administration (FDA) the appearance, taste, safety, texture and nutritional value of a food depends upon its ingredients (FDA, 2013). Colour is an important quality criterion of a food product for the consumers (Sari, 2016). Earlier investigations especially in late 1800s were mainly concentrated on the synthetic food colours which unluckily, were mostly used to disguise low-quality foods and for decorative purposes. So many synthetic dyes were banned due to their harmful effects on health (Arnold *et al.*, 2012), etc. After getting an increased awareness about the food ingredients in the last 20 years, the consumers showed more preference for the natural foods (Downham and Collins, 2000). In the recent years, the natural pigments have outnumbered the synthetics by 5 to 1 (Shamina *et al.*, 2007). Today, the consumers show more demand for those food supplies which are more colourful, safe, nutritious and flavourful (Piqueras-Fiszman and Spence, 2014; Tesfaye *et al.*, 2015). The natural dyes are advantageous because their sources are biodegradable, renewable and environment friendly. They produce very soft, soothing and uncommon shades which are refreshingly different from the strong bright colours produced by synthetic dyes (Mongkholrattanasit *et al.*, 2011; Zvavamwe *et al.*, 2016).

The naturally derived colourants in foods possess health promoting properties (Chattopadhyay *et al.*, 2008). They are rich in antioxidant nutrients and have the ability to retard the oxidative stress which may lead to various chronic diseases for example diabetes, osteoporosis, cardiovascular disease and cancer, etc. (Giugliano, 2000). The natural pigments e.g. anthocyanins, myoglobins, chlorophylls and carotenoids, etc. can produce various food colourants (Parkinson and Brown, 1981) and are commonly extracted from various plant parts e.g. flowers, stems, roots, leaves, bark, seeds, vegetables, fruits (Sharma *et al.*, 2018). According to the FDA (in Title 21 of the Code of Federal Regulations, Part 73 for food, drug and cosmetics), colour pigments of natural biotic origin are exempt from certification (FDA, 2013; Shamina *et al.*, 2007). The importance of plant pigments as natural food colourants has been increasing globally in order to avoid from numerous environmental problems and health hazards associated with synthetic colours (Shamina *et al.*, 2007; Tesfaye *et al.*, 2015). Colours of plant origin are environment friendly and non-toxic and hence preferred over synthetic food colours (Siva, 2007). By keeping in mind the ingredients of the recipe or formula, light storage, heat and pH, etc., a suitable natural colour can be achieved. For the natural colours, the storage conditions are applied depending upon the specific need of a particular product (Shamina *et al.*, 2007).

Eucalyptus globulus is a commonly found plant all over the world; the growth rate of *Eucalyptus* is 35,000 per year (Albaugh *et al.*, 2013). It is notable in all over the world due to its rapid growth and utilisation in paper and wood industries (Girijashankar, 2011). Its leaves consist of several aromatic compounds; these are eucalyptol, terpinol, terpinen, linalool globule, epiglobule terpinol acetate and geranyl acetate (Ghalem and Mohamed, 2008). The fruits of *Eucalyptus* contains fifteen aromatic components which include beta sitosterol, hydroxybeutilinic acid, methylgallic acid, ellagic acid, etc. (Selvakumar, 2012). The *Eucalyptus* oil finds pharmaceutical applications due to the volatile and aromatic nature of its compounds (Harris *et al.*, 2003). *Eucalyptus* is very beneficial for the treatment of allergiese bronchitis, asthma skin diseases, headache, cough (Maruyama *et al.*, 2006); it possesses antimicrobial, antiseptic, anticancer, inhalant and insect repellent properties (Grattapaglia *et al.*, 2012). Due to its medicinal properties, it has a long history of folk usage (Dixit *et al.*, 2012).

The use of dried *Eucalyptus* leaves to protect the stored grains (e.g. rice) from pests for a long term is common practice in Pakistan. The grains are put in an air tight closed container with the continuous addition of dried *Eucalyptus* leaves. The grain remains protected from the so called pests for many months. The present investigations were carried out to extract the natural brown dye from *Eucalyptus* bark, to apply it in the manufacture of candies and to establish its food preservation value. The investigated dye was subjected to proximate analysis, phytochemical screening and metal analysis. Its antioxidant potential, antimicrobial activities and toxicological studies were also evaluated.

2. Material and methods

For microbiological analysis of the candies containing the dye, standard media and reagents were purchased from Oxoid (Basingstoke, United Kingdom) and LabM (Bury, United Kingdom). All the other reagents and chemicals used were procured from Rapid Labs Ltd (Colchester, UK), Sigma-Aldrich (Gillingham, UK), Scharlab (Barcelona, Spain) and Merck (Darmstadt, Germany). Atomic absorption spectroscopy was performed by AA-7000, Shimadzu (Tokyo, Japan) having voltage range 230 V and frequency 50/60 Hz; air-acetylene flame was used for atomisation of the sample. For antimicrobial activity of the *Eucalyptus* bark dye, the test microorganisms (*Bacillus subtilis*, *Pseudomonas* spp., *Escherichia coli*, *Streptococcus aureus* and *Staphylococcus aureus*) were obtained from the Department of Microbiology and Molecular Genetics, University of Punjab Lahore, Pakistan.

Extraction of brown dye powder

The bark was collected from the trees of *Eucalyptus globolus* in PCSIR Lahore (Pakistan) on June 2016 during hot dry season. The bark was washed with water and the mud particles were removed from the bark. The neat and clean bark was then cut by a knife into small pieces which were spread in thin layers in trays and dried in the hot air oven for 24 hours. The sample was dried and grinded by blender into the fine powder.

The powdered sample (100 g) of *Eucalyptus* bark was added in 1000 ml distilled water and the mixture was stirred vigorously on a hot plate with slight heating (50 °C) for 1 day. The resultant suspension was then filtered through a cloth. The juice was filtered to remove particulates and was freeze dried; then it was kept in a rotary evaporator for complete removal of water. Finally a brown fine powder (dye) was produced, which was subjected to analysis.

Proximate analysis of plant

Proximate analysis was performed by the reported procedure (Nisa *et al.*, 2015; Saeed *et al.*, 2012) to determine the composition and specific characteristics of plants including the moisture, fat, ash, fibre and protein contents.

Phytochemical screening in *Eucalyptus* bark

The dyes were tested for alkaloids, terpenoids, flavonoids, tannins, saponins and glycosides by reported procedure (Ali *et al.*, 2018; Auwal *et al.*, 2014) as given below:

- 2 ml of HCl was added to 2 ml aqueous filtrate of the dye followed by the addition of few drops of Meyer's reagent. Formation of brown precipitates demonstrates the presence of alkaloids in sample.
- 5 ml aqueous filtrate was mixed with 2 ml of chloroform in a test tube and then 3 ml of sulfuric acid was added along the sides of the test tube. Appearance of reddish brown inter phase indicates the existence of terpenoids in sample.
- 5 ml aqueous filtrate was mixed with 2 ml of diluted ammonia and 3 ml of H_2SO_4. Appearance of yellow colour demonstrates the presence of flavonoids in a test sample. The yellow colour disappears after some time.
- 5 ml aqueous filtrate was boiled in 10 ml distilled water followed by the addition of few drops of ferric chloride. Production of bluish black precipitates shows the presence of tannins in a sample.
- The aqueous solution (3 ml) of the dye was taken in a test-tube and diluted with distilled water (10 ml). Then test-tube was vigorously shaken for five minutes and the resulting solution was allowed to stand. The appearance of honeycomb froth after thirty minutes indicates the presence of saponins.
- 2 ml of chloroform and 3 ml of H_2SO_4 were added to 5 ml aqueous solution of the dye. The appearance of red colour then blue and finally green colour indicates the presence of glycosides in the sample.

Metal analyses

The *Eucalyptus* dye was also subjected to the metal analysis by atomic absorption spectroscopy. The aqueous solution of dye (0.5 g dye + 100 ml water) was tested to evaluate the concentrations of potassium, iron, manganese, zinc and copper.

Antioxidant activity

The dye powder was analysed for total phenolic contents spectrophotometrically using gallic acid as calibration standard (Iqbal *et al.*, 2006; Tehseen *et al.*, 2014) and the antioxidant activity was found by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (Brand-Williams *et al.*, 1995; Nisa *et al.*, 2015; Tehseen *et al.*, 2014).

DPPH is a stable free radical which is reduced to 2,2-diphenyl-1-picrylhydrazine after reacting with an antioxidant (Ripa *et al.*, 2009). The reaction of dye with DPPH and lowering of absorbance at 517 nm indicates the scavenging potential of the dye (Algarra *et al.*, 2014). Stock solutions were prepared by mixing specific amount of DPPH in ethanol and shaking the solution very well. Then these solutions were kept at 20 °C for 30 minutes. Similarly stock solution was only mixed with ethanol to obtain the blank reading. The butylated hydroxytoluene (BHT) was applied as a reference standard during the procedure. Results of this activity were determined by the formula:

$$\text{DPPH scavenging activity (\%)} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

Antibacterial activity studies

The methanol extract of *Eucalyptus* bark extract was subjected to antibacterial activity evaluation against five different types of bacteria by disc diffusion method (Hussain *et al.*, 2015a,b). For this purpose the dye solutions of various concentrations were prepared and applied on these bacteria. Dimethyl sulfoxide (DMSO) was used as a negative control and streptomycin as a positive control. The two strains of gram negative bacteria (*Pseudomonas* spp., *E. coli*) and three strains of gram positive bacteria (*Str. aureus*, *Sta. aureus* and *B. subtilis*) were used for the antimicrobial tests.

Toxicological studies

Toxicological tests on mice

Healthy mice were used to assess the toxicological effects of the investigated dye. For this purpose, the solutions of different concentrations of the dye were prepared in water and given orally to the healthy mice for 4 weeks. The results were noted after 4 weeks (Nisa *et al.*, 2015).

Determination of aflatoxin content

50 g of the dye was taken in 500 ml measuring flask and 25 ml distilled water was added into it. Then 200 ml chloroform was also added in it. After covering the flask with cotton and Al foil, the solution was shaken on the shaker for 30 minutes and then filtered with a filter paper. The filtrate (50 ml) was taken in a beaker and was then boiled at 200 °C on a hot plate for half an hour. After boiling, the beaker was removed from the hot plate and kept for cooling. It was tested for aflatoxins by UV spectroscopy (Zahra *et al.*, 2012).

Application of *Eucalyptus* dye for the preparation of candies

450 g of common sugar was mixed with distilled water (500 ml) (Figure 1A). Then the solution was heated slowly to remove moisture from it. Glucose syrup was added to the mixture to make it thick and sticky (Figure 1B). Slow heating resulted in thick treacle which was kept to cool down to 50 °C. Then powdered bark extract was added to the treacle kept at 50 °C; low temperature is compulsory in order to avoid deterioration of colour pigments at high temperature. The mixture was homogenised well to obtain the uniform colour in the treacle. Before complete hardening of the treacle, different shapes of candies were made. Freshly

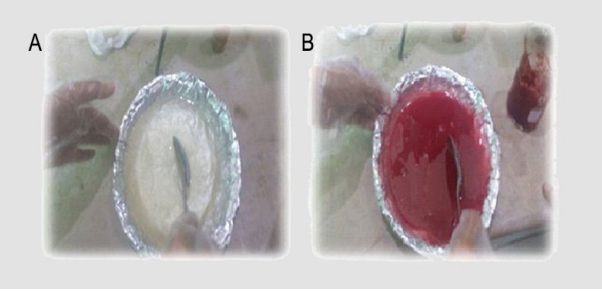

Figure 1. (A) preparation of glucose solution and (B) preparation of candy.

made candies were stored in polythene bags at 4, 25 and 45 °C for further analysis.

Microbiological analysis of candies having dye powder

The candies having *Eucalyptus* bark *dye* were subjected to the microbiological evaluation by a procedure reported in the Manual of Food Quality Control (Andrews, 1992; Nisa *et al.*, 2015). Aseptically weighed (10 gram) brown dye was stored for a period of 03 months. During the storage period, the sample was mixed homogenously on monthly basis with butterfield's phosphate buffer (90 ml) to produce $1:10^{-1}$ dilution from which serial dilutions were prepared up to $1:10^{-6}$. Total aerobic plate counts were measured in the unit of counts per gram. Plate count agar (20-25 ml/plate) was used as the growth medium which was poured into the three plates and then 1 ml from each dilution was added into each plate. These plates were then incubated at 35 °C for 24-48 hours. For the detection of yeasts and moulds, three dilutions from the very first step were added into three plates containing petro dextrose agar. After mixing and solidification, the plates were then incubated at 25 °C for 5 days. The 3 tube most probable number (MPN) method was used to enumerate the faecal coliforms, *Pseudomonas* spp., *E. coli* and the total coliforms, using one ml of serial dilutions. The 0.4 and 0.3 ml of serial dilutions were used on the bared parker agar to enumerate *Sta. aureus*. Then counting of the appeared colonies was performed and an average was taken to express the microbial count in terms of colony-forming units per gram (cfu/g) of the sample. *Salmonella* spp. were evaluated by a pre-enrichment step in lactose broth for 24 hours at 35 °C and then selective enrichment in tetrathionate broth (10 ml) and streaking on bismuth sulphite agar, hektoen enteric agar and xylose desoxycholate agar.

Spectrophotometry of dye powder

0.5 g of each bark extract sample stored at 4, 25 and 45 °C was dissolved in McIlvain's phosphate buffer and then diluted to 100 ml. The solution was centrifuged and measured the absorption with water as reference. The maximum absorption was observed in the range of 0.2 to 0.8. The colour intensity was calculated on the basis of maximum absorption.

Spectrophotometry of candies having dye powder

10 g of each candy stored at 4, 25 and 45 °C was dissolved in McIlvain's phosphate buffer and diluted it to 100 ml. The maximum absorption was observed in the range of 0.2 to 0.8. The solution was centrifuged and its absorption was measured using water as a reference. The colour intensity was calculated on the basis of maximum absorption (at about 530 nm); all red coloured matter being included under betanin with specific extinction. The results obtained through the spectrophotometric analysis were statistically analysed by using a reported analysis technique (Steel *et al.*, 1997).

3. Results and discussion

The neat, clean, dried and small pieces of *Eucalyptus* bark were grinded into fine powder from which reddish brown dye was extracted with distilled water. The brown fine powder was further analysed for its phytochemical constituents, antioxidant potential, antimicrobial activities and toxicity.

The investigations on the coloured candies of various brands suggest that the synthetic dyes used in different candies are a very big jeopardy for human health (Zahra *et al.*, 2016). Hence, the natural food colours are preferred due to their health benefits (Gülçin *et al.*, 2010; Harput *et al.*, 2011) and to decrease the chance of chronic diseases (Saeed *et al.*, 2012). In the current studies, the natural brown dye obtained from the *Eucalyptus* bark was used in the production of candies and then the stability of dye in the candies was measured in terms of caffeic acid content. It was verified that the brown natural dye produced from *Eucalyptus* may be used as a food colorant due to its safety and nutritional value.

Proximate analysis

Proximate analysis of *E. globulus* describes the chemical composition of its bark and nutritional value. This analysis plays a crucial role in evaluating the nutritional importance of the plants (Pandey *et al.*, 2006). According to this analysis, the dye contained 9.3% moisture content, 2.91% total ash content and 18.75% crude fibre. There were significant differences in the protein, fats and carbohydrate contents (1.65, 1.02 and 66.37%, respectively). A very little amount (1.02%) of fat in the *Eucalyptus* bark indicates very low amount of polyunsaturated fatty acids; this low amount may be helpful in the lowering of cholesterol level and esterification's processes occurring in human body. The fibre content was present in moderate amount (18.75%) while significantly higher concentration of carbohydrates

(66.37%) was found in the *Eucalyptus* bark dye. The polymer and derivatives of carbohydrates are found in many biological molecules like coenzymes and nucleic acids (Hassan *et al.*, 2011).

These are the significant quantitative values (2.91%) of ash content determined in the *Eucalyptus* dye. The total material remained after ignition of a plant material is called ash. The presence of ash content demonstrates the presence of some inorganic minerals or salt in the dye. The acid insoluble ash is generally comprised of the silica component of silica.

Metal contents

Suitable amount of metal nutrients is compulsory for proper growth and functioning of the human body. The aqueous solution of dye (0.5 g dye +100 ml water) of *Eucalyptus* bark was also subjected to the metal analysis by atomic absorption spectroscopy. Quantitative tests were performed to evaluate the concentrations of potassium, iron, manganese, zinc and copper. Zinc and copper were not detected in the dye while potassium was present in highest concentration. The concentrations of potassium, iron and manganese were found to be 6.142, 1.177 and 0.1873 mg/kg, respectively.

Phytochemical screening

The various types of constituents present in plant extract are categorised by photochemical screening. The constituents of *Eucalyptus* bark were extracted by using methanol and water as a solvent. The results of phytochemicals screening are given below in the Table 1.

The phytochemicals screening of methanol extract of *Eucalyptus* bark displayed positive results for the presence of tannins, reducing sugar and alkaloids while negative results for flavonoids, terpinoids, glycosides and saponins. The aqueous extract displayed positive results for the presence of tannins, reducing sugar, glycosides and saponins while negative results for flavonoids, terpinoids and alkaloids. It is worth mentioning that both the extracts (methanolic and aqueous) demonstrated the presence of tannins and reducing sugars.

The literature shows that *Eucalyptus* species is a source of tannins for tanning purposes (Bele *et al.*, 2010). It has 10 to 12% of natural tannins and polyphenols which behave as colorants (Ali *et al.*, 2007). The logwood extract of *Eucalyptus* contains mainly two brown substances i.e. tannin and quercetin. This extract can produce purple, blue and red dyes on its exposure to alkali and oxygen (Centeno *et al.*, 2010). Sugars play a very important role in growth and development (Ciereszko, 2018). The alkaloids are common secondary metabolites in plants (Do Carmo *et al.*, 2018). They find the potential therapeutic applications against several neurodegenerative disorders including stroke, schizophrenia, epilepsy, Parkinson's disease, Huntington disease, Alzheimer's disease (Hussain *et al.*, 2018). Saponins are commonly present in most plants; they are steroid or triterpenoid glycosides and find many biological and pharmaceutical applications (Faizal and Geelen, 2013). They possess antioxidant, anti-nociceptive, analgesic, anticarcinogenic, hypocholesterolaemic, immunostimulant and membrane-permeabitising properties. They have the ability to kill molluscs and protozoans and also act as antiviral and antifungal agents. They are important for nutrition of humans and animals (Desai *et al.*, 2009).

Antioxidant studies

The oxidation reactions generate the free radicals which are responsible for the damaging of cells by initiating harmful chain reactions. Antioxidants terminate the free radicals and stop the chain reactions (Naseer *et al.*, 2018). The increased oxidative stress is a source of many life threatening diseases e.g. cardiovascular and neurodegenerative diseases. The antioxidant supplements can be used to overcome the undesirable effects of oxidative stress (Kasote *et al.,* 2013). Plants are considered as an excellent source of dietary nutrients (Naseer *et al.*, 2019; Rehman *et al.*, 2018); about two-thirds of the world's plants which possess medicinal importance, also display an outstanding antioxidant potential (Krishnaiah *et al.,* 2011).

Table 1. Phytochemical screening of bark extract.

Sr. no.	Phytochemical constituents	Test	Methanol extracts	Aqueous extracts
1	Flavonoids	Mg test	–	–
2	Tannins	Gelatin test	+	+
3	Terpinoids	Foam test	–	–
4	Reducing sugar	Fehling solution test	+	+
5	Alkaloids	Mayers test	+	–
6	Glycosides	Iodine test	–	+
7	Saponins	Forthing test	–	+

Antioxidant potential of the dye sample was assessed by total phenolic contents and DPPH methods. Antioxidant tests for total phenolic contents were performed and the results in terms of gallic acid equivalents per 100 grams dry weight are summarised in Table 2. The bark of *E. globulus* is comprised of polyphenolic compounds which can be extracted employing polar solvents (Mota *et al.*, 2012). There were earlier reports regarding the extraction of phenolic compounds from its trimmings using aqueous two-phase systems (salt+polymer+water) based on ammonium sulphate and PEG 2000 (Xavier *et al.*, 2014). There is a direct correlation between the total antioxidant capacity and concentration of phenolic compounds; the greater concentration of phenolics in a sample indicates the greater antioxidant activity (Algarra *et al.*, 2014). Phenolics are secondary plant metabolites of aromatic nature and are associated with the antioxidant, nutritional, sensory and colour properties of food (Tehseen *et al.*, 2014).

The DPPH free radical namely 2,2-diphenyl-1-picryl-hydrazyl was used to evaluate the radical scavenging activity of the dye. The DPPH purple coloured solution always contains stable free radicals which react with antioxidants of sample. The OH free radical scavenging abilities were extrapolated to calculate the IC_{50} (50% inhibitory concentrations) value of the dye (Agu and Okolie, 2017). Figure 2 displays the % scavenging of free radicals caused by various concentrations of *Eucalyptus* brown dye; the dye showed excellent ability to quench the DPPH radicals. The good DPPH scavenging ability of the dye can be attributed to the presence of high contents of phenol and flavonoids (Agu and Okolie, 2017). The scavenging was increased from 41.18 to 82.21% by increasing the sample concentration from 50 to 250 µg/ml, respectively; this is due to increase of antioxidant contents with increasing the concentration of the dye. Table 2 also shows the IC_{50} value observed in DPPH scavenging assay for the *Eucalyptus* bark extract. The IC_{50} value of the *Eucalyptus* dye was observed to be 77.20 µg/ml with respect to BHT (9.96 µg/ml) as the reference standard. The result indicates that the percentage inhibition increases with increasing the sample concentration.

The potential applications of *Eucalyptus* bark extract as antioxidants is also reported in earlier investigations; the use of this extract as phenol substituents in formulation of adhesives and chrome substituents in leather tanning is also reported (Vázquez *et al.*, 2009). There were also earlier investigations on the antioxidant potential of methanolic and petroleum ether extracts and the active ingredients of *E. globulus* separated by the use of 3 different antioxidant assays: 2,2'-azino-bis [ethylbenzthiazoline-6-sulfonic acid] (ABTS), DPPH and β-carotene bleaching assay. The crude methanolic extract demonstrated higher antioxidant potential against both the radicals (ABTS and DPPH) than the petroleum ether extract (El-Moein *et al.*, 2012).

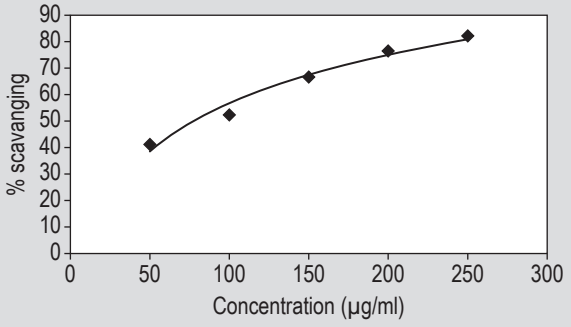

Figure 2. Antioxidant activity by DPPH method of *Eucalyptus* bark extract in terms of % scavenging.

Table 2. Antioxidant activity of *Eucalyptus* bark extract.

Determination of phenol content in terms of gallic acid equivalents per 100 grams dry weight (µg GAE/100 g DW) of the *Eucalyptus* dye

Sr. no.	Concentration (mg/ml)	µg GAE/100 g DW	Sr. no.	Concentration (µg)	Absorbance of gallic acid
1	05	66.83	6	30	391.55
2	10	129.09	7	35	449.73
3	15	166.22	8	40	512.58
4	20	230.92	9	45	534.80
5	25	312.70			

Determination of IC_{50} by DPPH scavenging assay using IC_{50}

Sr. no.	Samples	IC_{50} (µg/ml)
1	Plant sample	77.20
2	Butylated hydroxytoluene	9.96

Antimibacterial potential of *Eucalyptus* bark extract

The antimicrobial studies are very important in order to control the food pathogens which are major causes of illness in the world. The biologically active natural compounds can be used to control the food borne pathogens (Sharma *et al.*, 2013). The methanol extract of *Eucalyptus* bark was subjected to antimicrobial activity by disc diffusion method. The streptomycin was used as positive control and DMSO as a negative control during the procedure (Hussain *et al.*, 2015a,b). The two strains of gram negative bacteria (*E. coli*, *Pseudomonas* spp.) and three strains of gram positive bacteria (*B. subtilis*, *Str. aureus* and *Sta. aureus*) were used for tests. The 0.025 g/ml methanolic extract in DMSO displayed the inhibition zones against all the tested bacteria. Pure DMSO (negative control) was found inactive under the same experimental conditions. The results thus demonstrate that the *Eucalyptus* bark dye possessed antibacterial potential.

Toxicology studies

Healthy mice (4 for each test) were used to check the toxicological effect of prepared dye. The lesser toxicological effects of a compound/sample indicate its possible safe use for future human consumption. The oral dose of dye was mixed in pure water. Various quantities of dose (300, 500, 1000 and 1,500 mg/kg bw) were given to the animals. It was noted that after 4 weeks, all the mice remained alive and no death report was noted after taking the oral doses of bark extract. The results thus indicate the dye of *Eucalyptus* is safe for the tested animals (mice); however, there is need of further investigations before the dye can finally be applied in food used for human consumption.

Aflatoxins are comprised of closely related compounds including aflatoxins B1, B2, G1, G2, M1, and M2. They cause great harm to the food products having improper storage and drying. They can be controlled by stricter quality measures, technical assistance and hygienic precautions (Tahir *et al.*, 2018). In our current investigations, none of the aflatoxins (like G1, G2, B1, B2) was detected in the *Eucalyptus* bark dye as shown by UV spectroscopy.

Antimicrobial analysis of mixture of candies with *Eucalyptus* bark extract

The candies having the dye powder were stored for a period of three months and then subjected to antimicrobial analyses to ensure that the food items having *Eucalyptus* bark dye are safe for human consumption. Total plate count, total coliforms, faecal coliforms, *Sta. aureus*, *E. coli*, *Salmonella*, yeast and mould were determined. The results were compared to the antimicrobial activities of pure *Eucalyptus* bark extracts. The obtained data are summarised in Table 3. According to Table 3 the candy (having *Eucalyptus* dye) stored at 4 °C contains total plate count of 1×10^3 cfu/while that stored at 25 °C showed total plate count of 2×10^3 cfu/g. The pure bark powder stored at 4 °C showed 1.2×10^3 cfu/g of total plate count and 1.8×10^3 cfu/g at 25 °C. Total coliforms, faecal coliforms, *E. coli*, *Sta. aureus* and *Salmonella* were not detected in any sample of candy or bark powder. Yeast was detected in all samples (<10) but the number of yeast colonies were not more than the permitted limit. While mould was detected in candy (having *Eucalyptus* dye) and powder at 25 °C but it was within permissible limit. However, mould was not detected in samples of candy (having *Eucalyptus* dye) and pure bark powder stored at 4 °C. There were reports on the antibacterial and antifungal potential of *Eucalyptus* (*Eucalyptus camaldulensis*) essential oils. A wider use of these essential oils in pharmaceutical and food preparations has been reported (Valizadeh *et al.*, 2015).

Table 3. Microbiological analysis of candies having *Eucalyptus* dye and that of pure dye.

Microorganisms	4 °C candy having *Eucalyptus* dye	25 °C candy having *Eucalyptus* dye	4 °C pure *Eucalyptus* dye	25 °C pure *Eucalyptus* dye
Total plate count (cfu/g)	1×10^3	2×10^3	1.2×10^3	1.8×10^3
Total coliforms (MPN/g)[1]	not detected	not detected	not detected	not detected
Faecal coliforms (MPN/g)	not detected	not detected	not detected	not detected
Escherichia coli (MPN/g)	not detected	not detected	not detected	not detected
Staphylococcus aureus/g	not detected	not detected	not detected	not detected
Salmonella spp./25 g	not detected	not detected	not detected	not detected
Yeast count/g	<10	<10	<10	<10
Mould count/g	not detected	<10	not detected	<10

[1] MPN = most probable number.

Spectrophotometric analysis of dye powder and candies having the dye powder

The pure bark powder and candies (having *Eucalyptus* dye) were stored at various temperatures (45, 25 and 4 °C) and analysed by spectrophotometer for caffeic acid concentration. Caffeic acid content was measured to know the stability of mixture of dye and candies. The reason is that the caffeic acid and its analogues are highly sensitive to decomposition due to their specific chemical nature (such as the carboxyl and ethylene group in the side chain and dihydroxyl in the catechol ring) and the atmospheric conditions (e.g. irradiation, light, heat and so on). Several investigations have described the effects of food processing on the stability of caffeic acid and its analogues (Sun *et al.*, 2013; Vignoli *et al.*, 2011). Caffeic acid is an antioxidant in *vivo* and also in *vitro* (Olthof *et al.*, 2001) and is constituents of all the plants because it plays an important role as the key intermediate in the biosynthesis of lignin which is a principal component of biomass of woody plants (Boerjan *et al.*, 2003). The presence of caffeic acid in *Eucalyptus* species is verified from many earlier reports (Santos *et al.*, 2011; Tian *et al.*, 2012; Vázquez *et al.*, 2008). There are evidences for the measurement of reducing potential of plant extracts in mg of the caffeic acid per g dry weight of plant material (Ferreira *et al.*, 2016).

In our present studies, the spectroscopic results in terms of caffeic acid concentration are presented in Table 4 and Figures 3A and 3B. The highest percentage of caffeic acid content was found in candy (having *Eucalyptus* dye) and the pure bark powder stored at 4 °C while lowest retention of caffeic acid content was observed at 45 °C. It was found that at higher temperatures the colour is deteriorated. The colour retention of the samples stored at 25 °C was lower as compared to those stored at 4 °C and it was higher than those stored at 45 °C. Figure 3A demonstrates gradual decrease of caffeic acid content in

Table 4. Concentration of caffeic acid in pure *Eucalyptus* dye and in the candies at different temperatures.

Weeks	Candy having *Eucalyptus* dye 4 °C	Pure bark powder 4 °C	Candy having *Eucalyptus* dye 25 °C	Pure bark powder 25 °C	Candy having *Eucalyptus* dye 45 °C	Pure bark powder 45 °C
1st	0.00235	0.211	0.00236	0.211	0.00234	0.211
2nd	0.00230	0.210	0.00230	0.201	0.00226	0.201
3rd	0.00229	0.200	0.00229	0.200	0.00222	0.190
4th	0.00222	0.190	0.00223	0.190	0.00204	0.180
5th	0.00220	0.180	0.00220	0.180	0.00198	0.170
6th	0.00214	0.160	0.00214	0.160	0.00193	0.160
7th	0.00204	0.140	0.00207	0.140	0.00185	0.150
8th	0.00201	0.100	0.00198	0.100	0.00180	0.140

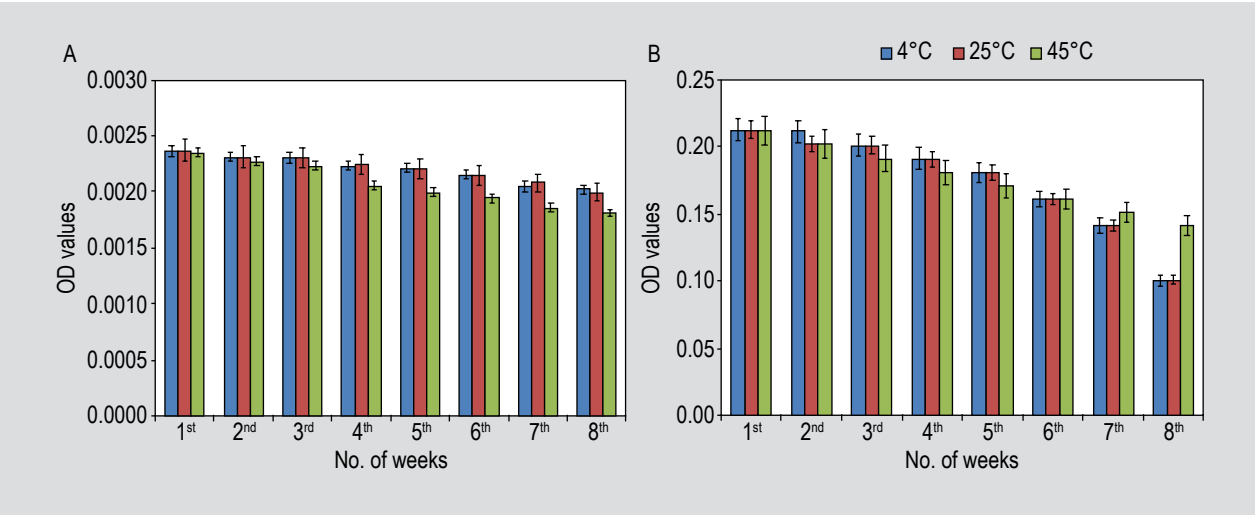

Figure 3. Concentration of caffeic acid in pure *Eucalyptus* dye and those of candies having *Eucalyptus* dye at different temperatures; where the OD represents optical density. (A) represents the behaviour of candies after mixing with bark powder till 8 weeks; (B) displays the behaviour of pure bark powder till 8 weeks.

candies (having *Eucalyptus* dye) with increasing storage temperature. Remarkable degradation of caffeic acid content was observed at 45 °C in the 4th to 8th weeks and caffeic acid content was mainly retained at 4 °C throughout the time period. While at 25 °C very slight change in colour was observed. Figure 3B indicates a gradual decrease in percentage of pure bark powder with increasing storage temperatures. It also shows the same degradation pattern as the candies showed. Statistical data shows the significant results obtained at different storage temperatures of bark dye and candies. Table 5 shows the mean comparisons of bark extracts and candies stored at various temperatures (45, 25 and 4 °C).

Caffeic acid (3,4-dihydroxy-cinnamic acid) is main dietary hydroxycinnamic acid (Clifford, 1999) and widely distributed in plant tissues and many food sources, including apples, blueberries, cider and coffee drinks (Clifford, 2000) and also a part of many important medications, especially those based on propolis (Lustosa *et al.*, 2008). It is a well-known phenolic phytochemical and demonstrates anticarcinogenic effects (Kang *et al.*, 2008), powerful antioxidant activity,

Table 5. Multiple comparison of means of % bark extract and candies at different temperatures.

Dependent variable	(I) Temp. in °C	(J) Temp. in °C	Mean difference (I-J)	Std. error	Sig.	95% confidence interval	
						Lower bound	Upper bound
Candy 1	4	25	-0.00000250	0.00007702	0.974	-0.0001627	0.0001577
		45	0.00014125	0.00007702	0.081	-0.0000189	0.0003014
	25	4	0.00000250	0.00007702	0.974	-0.0001577	0.0001627
		45	0.00014375	0.00007702	0.076	-0.0000164	0.0003039
	45	4	-0.00014125	0.00007702	0.081	-0.0003014	0.0000189
		25	-0.00014375	0.00007702	0.076	-0.0003039	0.0000164
Candy 2	4	25	0.00005000	0.00006195	0.429	-0.0000788	0.0001788
		45	0.00013875*	0.00006195	0.036	0.0000099	0.0002676
	25	4	-0.00005000	0.00006195	0.429	-0.0001788	0.0000788
		45	0.00008875	0.00006195	0.167	-0.0000401	0.0002176
	45	4	-0.00013875*	0.00006195	0.036	-0.0002676	-0.0000099
		25	-0.00008875	0.00006195	0.167	-0.0002176	0.0000401
Candy 3	4	25	-0.00002500	0.00009700	0.799	-0.0002267	0.0001767
		45	-0.00003375	0.00009700	0.731	-0.0002355	0.0001680
	25	4	0.00002500	0.00009700	0.799	-0.0001767	0.0002267
		45	-0.00000875	0.00009700	0.929	-0.0002105	0.0001930
	45	4	0.00003375	0.00009700	0.731	-0.0001680	0.0002355
		25	0.00000875	0.00009700	0.929	-0.0001930	0.0002105
Bark powder 1	4	25	0.001125	0.017135	0.948	-0.03451	0.03676
		45	-0.001375	0.017135	0.937	-0.03701	0.03426
	25	4	-0.001125	0.017135	0.948	-0.03676	0.03451
		45	-0.002500	0.017135	0.885	-0.03813	0.03313
	45	4	0.001375	0.017135	0.937	-0.03426	0.03701
		25	0.002500	0.017135	0.885	-0.03313	0.03813
Bark powder 2	4	25	-0.003875	0.017102	0.823	-0.03944	0.03169
		45	0.001250	0.017102	0.942	-0.03431	0.03681
	25	4	0.003875	0.017102	0.823	-0.03169	0.03944
		45	0.005125	0.017102	0.767	-0.03044	0.04069
	45	4	-0.001250	0.017102	0.942	-0.03681	0.03431
		25	-0.005125	0.017102	0.767	-0.04069	0.03044
Bark powder 3	4	25	0.000000	0.015332	1.000	-0.03189	0.03189
		45	-0.001250	0.015332	0.936	-0.03314	0.03064
	25	4	0.000000	0.015332	1.000	-0.03189	0.03189
		45	-0.001250	0.015332	0.936	-0.03314	0.03064
	45	4	0.001250	0.015332	0.936	-0.03064	0.03314
		25	0.001250	0.015332	0.936	-0.03064	0.03314

antimicrobial activity, prevents premature aging, increases collagen production and may be used to treat the dermal diseases (Magnani *et al.*, 2014), cardiovascular disorders including atherosclerosis (Vinson *et al.*, 2001) and prevents type 2 diabetes mellitus (Higdon and Frei, 2006). Caffeic acid also displays anti-inflammatory and immunomodulatory activities. Caffeic acid outperformed the other antioxidants, reducing aflatoxin production by more than 95% (Wood, 2006). Caffeic acid is susceptible to autoxidation. The thiol compounds (thiocresol, thioglycolic acid, cysteine), glutathione and ascorbic acid have the protective effect on disappearance and browning of caffeic acid (Cilliers and Singleton, 1990).

4. Conclusions

A brown fine powder can be obtained by aqueous extraction of finally ground *Eucalyptus* bark. This powder finds an excellent nutritional value with zero aflatoxin concentration and no toxicity against the tested mice. It contains tannins, reducing sugar, alkaloids, glycosides, saponins, various antioxidants, antimicrobial agents and antiradical compounds. Total phenolic contents in the dye ranged from 66.83 to 534.80 µg of gallic acid equivalents per 100 grams dry weight of the dye. In DPPH assay, the IC_{50} value was observed to be 77.20 µg/ml with respect to BHT (9.96 µg/ml) as a standard control. The *Eucalyptus* dye was applied for the production of candies and assessed microbiologically to check the quality and safety of the products. The mixture of dye and candies showed maximum stability at 4 °C, in terms of caffeic acid content. However, the stability was decreased with the increase of temperature. Total coliforms, faecal coliforms, *E. coli*, *Sta. aureus* and *Salmonella* were not detected in any sample of candy or bark powder even after the storage period of 3 months. The use of dried *Eucalyptus* leaves to protect the stored grains (e.g. rice) from pests is a common practice in Pakistan. The investigated dye may find its use as a food colorant owing to its stability and useful nutritional ingredients, antioxidant activities, antimicrobial potential and no toxicity against the tested animal i.e. mice.

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