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Antioxidant activity evaluation and phytochemical screening of pigment extract of Indian shot (*Canna indica* L.)

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ABSTRACT

In general, flavonoids, isoflavonoids, polyphenols, anthocyanidins, terpenoids, carotenoids, etc. are considered phytochemicals. Anthocyanins have lately attracted increased interest as natural food colorants due to the detrimental effects of synthetic colors and these possesses antioxidant and radical scavenging properties. Fresh red flowers of Canna indica which is commonly known as Canna or Indian shot were used for extraction of pigment using different solvents and durations by ultrasound-assisted extraction method and subjected for phytochemicals estimation through spectrophotometric analysis. Total phenol content, total flavonoid content and antioxidant activity were estimated by using Folin-Ciocalteu's method, aluminum chloride method and 2,2-diphenyl-1-picryl hydrazyl (DPPH) assay, respectively. Among various treatment combinations, 120 min ultrasound in methanol showed highest amount of total phenol, total flavonoid and antioxidant activity with the values of 236.60 ± 0.21 mg GAE/100 g, 276.77 ± 0.30 mg QE/100 g and 93.73 ± 0.03% RSA respectively. From the results of the study, Canna indica flower can be regarded as a possible source of food color and can be used in pharmaceuticals and nutraceuticals due to presence of phenols, flavonoids, anthocyanin and its antioxidant activity.

Keywords: *Natural pigments, Ultrasound-assisted extraction, Anthocyanin, Flavonoids, Antioxidants*

INTRODUCTION

Canna or Indian shot which is botanically known as *Canna indica* L. comes under the family cannaceae and it is the only genus of cannaceae family. Flowers are red, solitary or in pair, the bract about 1.3 cm long. Flower consists of 1-1.5 cm long sepals, 1 cm long reddish corolla tube, 5 petals and flowers are hermaphrodite [18]. Anthocyanin plays important role in plant physiology, visual attraction and seed dispersal along with its application as a food colorant because it exhibits good antioxidant activity, which in turn suitable for use as a functional food. Nutraceuticals are any safe food extract supplements that have been shown via scientific research to improve health and prevent disease [21].

Anthocyanins have antioxidant and radical scavenging characteristics, which safeguard cells from oxidative damage and lower the risk of various critical disorders such as cardiovascular disease and cancer. As these are soluble in water and is polar so that it can dissolve in polar solvents such as ethanol, methanol, acetone and other solvents [22]. Phytochemicals, the bioactive ingredients of nutraceuticals encourage health and arise from the nexus of the food and pharmaceutical sectors. These phytochemicals can be found in processed foods and drinks, herbal items, dietary supplements, and separated nutrients. These are broadly characterized as isoflavonoids, polyphenols, anthocyanidins, flavonoids, terpenoids, carotenoids etc. Phytochemicals either alone or in combinations provide protection against several illnesses or ailments such as diabetes, high blood pressure, cancers, coronary heart disease, inflammation, microbial, viral and parasitic infections, spasmodic conditions, psychotic diseases, ulcers, osteoporosis and other correlated disorders [16].

Flavonoids are the primary active components of plants that are used as nutraceuticals [21]. The parent compound of flavonoids, 2-phenylchromone, is made up of three phenolic rings, known as A, B, and C rings. These rings display varying levels of hydroxylation and methoxylation [5]. The chemical structure of flavonoids and their metabolites, as well as the relative orientation of their different moieties, dictate their biochemical activities [24]. On a chemical level, flavonoids are made up of a fifteen-carbon skeleton that is connected by a heterocyclic pyrane ring between two benzene rings. They fall into several classes, including flavones, (e.g., flavone, apigenin, and luteolin), flavanols (e.g., quercetin, kaempferol, myricetin, and fisetin), flavanones (e.g., flavanone, hesperetin, and naringenin), and others [19]. The several classes of flavonoids range in the degree of oxidation and C ring substitution pattern, whereas discrete compounds within a class differ in the pattern of A ring substitution [13]. Flavonoids, similar to other polyphenols, are good free radical scavengers (chain-breaking antioxidants), because of their strong hydrogen or electron donor activity. Flavonoids have biological activities that include antiallergenic, anti-inflammatory, antioxidant, antiviral, antithrombotic, and anticarcinogenic properties. Nonetheless, the majority of attention has been focused on their antioxidant activity, which results from their capacity to both scavenge and lessen the production of free radicals [23].

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Antioxidants are compounds that, when they are less concentrated relative to the substrate, substantially slow down or prevent the oxidation of an oxidizable substrate [12]. Currently, the majority of antioxidants in the synthetic antioxidant class are produced artificially. The primary obstacle of synthetic antioxidants is their potential for in vivo side effects [3]. Natural or phytochemical antioxidants are secondary metabolites found in plants [7]. The mechanisms that mediated the antioxidant properties of phenolics and flavonoids are scavenging reactive oxygen species (ROS) and reactive nitrogen species (RNS), hindering certain enzymes or chelating trace metals concerned in the production of free radicals to suppress the formation of ROS/RNS and enhancing or defending antioxidant defense [6].

In chemical terms, element having an aromatic ring with multiple hydroxy substituents, comprising functional derivatives (esters, methyl ethers, glycosides, etc.), is named as phenolic or polyphenol [4]. In recent years, developments in the chemical profile of flavonoids have resulted in the creation of antioxidants that act as protective agents against a number of chronic illnesses. One of the most studied groups of flavonoids is anthocyanin, and efforts by food scientists have aided in advances in the epidemiology of this compound with regard to metabolism and production, phytochemical analysis, and plant occurrence. Main objective of the study is to estimate the total phenol, flavonoid content and antioxidant activity of pigment extracted from red flowers of *Canna indica*.

MATERIALS AND METHODS

Fresh red flowers of canna were selected for extraction of coloring pigments using ultrasound-assisted extraction method with the help of single probe sonicator (BANDELIN SONOPLUS HD 2070). Polar solvents such as methanol, ethanol, ethyl acetate, acetone and a non-polar solvent, aqueous (double distilled water) were used as solvents for pigment extraction at different time durations of 60, 90, 120 and 150 min. 20 g of fresh flowers were incorporated in 50 ml of each solvent to extract pigment and its phytochemical concentration was determined using standard procedures.

Chemicals and reagents used: Gallic acid (98-102%, HiMedia), Folin – Ciocalteu's phenol reagent (2N, Analytical grade, Sigma-Aldrich), Quercetin (98%, HiMedia), DPPH (2,2-Diphenyl-1-Picrylhydrazyl, 95%, SRL).

Standard curve for total phenol estimation: Plotting optical density against gallic acid content (mg/ml) using standard solutions with concentrations of 1, 3, 5, 7, and 9 mg/ml made from 1% stock solution allowed for the creation of a standard curve [8]. After adding 0.5 ml of distilled water and 0.1 ml of Folin-Ciocalteu's reagent (2N), the 0.1 ml standard solution of gallic acid was let to stand for six minutes. The reaction mixture was supplemented with 0.5 ml of distilled water and 1 ml of 7.5% sodium carbonate. This combination was allowed to remain at room temperature for ninety minutes. In a spectrophotometer, the dark blue hue that was created was measured at 725 nm in comparison to the blank.

Determination of total phenolic content (mg/100g): Otto Folin and Vintila Ciocalteu suggested using the Folin-Ciocalteu reagent correctly [9]. With some modifications, the Folin-Ciocalteu's reagent technique [14][20] was used to evaluate the total phenolic content in the various solvent extracts of canna flowers.

In this case, 0.1 ml of fresh extract from flowers was used to create 1 ml of volume. After that, the reaction mixture was given 0.1 ml of 2N Folin-Ciocalteu's reagent (1:10 v/v), and it was let to stand for six minutes. After adding 1 ml of 7.5% sodium carbonate, the mixture was allowed to rest at room temperature for 90 minutes. In a spectrophotometer, the blue color generated was determined at 725 nm in comparison to a blank.

Standard curve for total flavonoid estimation: In order to create a standard curve for calibration, absorbance was plotted against the known concentration of quercetin. Standard solutions were made from a 0.5% stock solution with concentrations of 0.5, 1, 1.5, 2 and 2.5 mg/ml in methanol [14]. After adding 0.5 ml of distilled water and 0.1 ml of 5% sodium nitrate to 0.1 ml of quercetin standard solution, the mixture was left to stand for six minutes. Following the addition of 0.15 ml of a 10% aluminum chloride solution and a 5 min waiting period, 0.2 ml of a 1M sodium hydroxide solution was added one at a time. In a spectrophotometer, the generated golden orange hue was checked at 510 nm in comparison to a blank.

Determination of total flavonoid content (mg/100g): Utilizing the aluminum chloride technique described by [14] along with few modifications, the total flavonoid content of the solvent-based extracts of canna flowers was calculated. After taking 0.1 ml of fresh flower extract, make up the volume to 1 ml. After adding 0.1 ml of 5% sodium nitrate, the mixture was let to stand for 6 minutes. Subsequently, 0.2 ml of 1M sodium hydroxide solution was added in turn after 0.15 ml of 10% aluminum chloride was added and left to stand for 5 minutes. In a spectrophotometer, the generated golden orange color was detected at 510 nm in comparison to a blank. Using the quercetin standard curve, the total flavonoid content was determined and reported as mg of quercetin equivalents (QE)/100g.

Determination of antioxidant activity (%RSA): The decoloration test, which is currently frequently employed for antioxidant quantification, was initially reported on by Brand-Williams and colleagues in 1995 [2]. The protective antioxidant capacity of canna flowers was evaluated in several solvent extracts using the DPPH-based free radical scavenging test, as reported by [14]. One of the few persistent organic nitrogen radicals is the DPPH radical, which has a rich purple hue. Due of its commercial availability, it doesn't need generation [17]. The IC50 values, or the value of concentration at which 50% of the DPPH solution is scavenged, were found by calibrating the percentage inhibition curve using gallic acid.

A 1% stock solution was made by dissolving 1 g of gallic acid in 100 ml of methanol. A standard curve of gallic acid was created for calibration purposes, and several standard solutions with concentrations of 20, 40, 60, 80, and 100 µg/ml were generated from a 1% stock solution [8]. After mixing 50 µl of pure pigment extract with 3 ml of 0.002% DPPH solution, the mixture was left in the dark for fifteen minutes. Following incubation, the reaction mixture's absorbance was measured in a UV spectrophotometer at 517 nm against a blank. We calculated the percentage of radical scavenging activity using the following formula.

$$\%RSA = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad \text{----- Eq. (1)}$$

Where, absorbance of control was pure DPPH solution and %RSA is percentage radical scavenging activity.

Statistical Analysis: The data gathered from the lab trials was examined by factorial completely randomized design. The data obtained was analyzed and appropriately interpreted as per the methods described in "Statistical Method for Agriculture Workers" by Panse and Sukhatme (1967) [15]. All experimental results were performed in two replications and were stated as mean ± SD (Standard Deviation) with the significant difference of $P < 0.01$.

RESULTS AND DISCUSSION

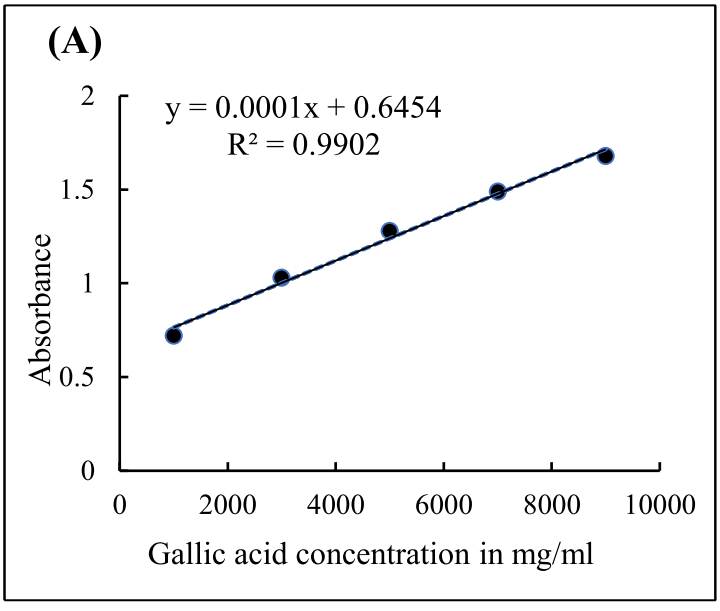
Total phenolic content: The equation for regression of the calibration curve (y = 0.0002x+0.3536) with regression co-efficient R^2 = 0.9902 was used to measure the concentration of phenolic chemicals in solvent extracts (Fig. 1A). It was discovered that phenolic content was high in methanol, ethanol, acetone, ethyl acetate, and aqueous extracts. The values of gallic acid equivalents per 100 g of sample ranged from 9.55 ± 0.43 mg to 236.60 ± 0.21 mg in the samples. The treatment combination, 120 min ultrasound in acetone (224.54 ± 0.41 mg GAE/100 g), recorded the second highest amount of total phenol, whereas the 120 min ultrasound in methanol (236.60 ± 0.21 mg GAE/100 g) recorded the greatest amount. Out of all treatment combinations, the 60 min ultrasound in aqueous extraction produced the lowest total phenol content (9.55 ± 0.43 mg GAE/100 g) (Table 1). This result indicated that total phenolic content of pigment extract is in the order of methanol > acetone > ethanol > ethyl acetate > aqueous extraction.

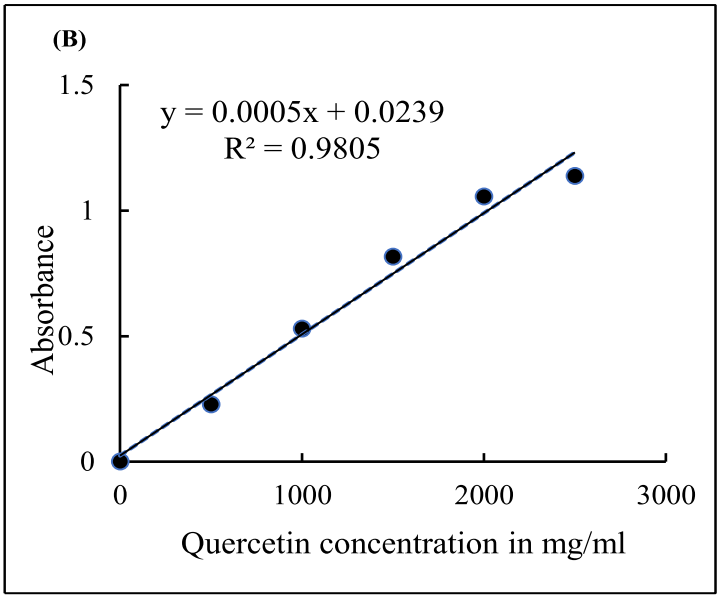
Table 1. Total Phenolic Content (mg/100g) in different pigment extracts

	Total Phenolic Content (mg/100g)			
	60 min Ultrasound	**90 min Ultrasound**	**120 min Ultrasound**	**150 min Ultrasound**
Methanol	188.36 ± 0.50	203.35 ± 0.49	236.60 ± 0.21	221.45 ± 0.42
Ethanol	153.37 ± 0.52	196.62 ± 0.18	214.56 ± 0.43	211.66 ± 0.13
Ethyl acetate	110.48 ± 0.04	142.64 ± 0.16	206.67 ± 0.11	197.38 ± 0.18
Acetone	154.45 ± 0.28	199.53 ± 0.40	224.54 ± 0.41	218.30 ± 0.42
Aqueous	9.55 ± 0.43	39.60 ± 0.49	114.59 ± 0.47	145.46 ± 0.42

Data are to averages of two replications and are represented in the form of mean ± SD (Standard deviation)

Because the solvents have an impact on the extracts' different responses in this assay, a notable variation is seen between the solvents. The kind of solvents (polarity) employed, the extent of phenolic polymerization, and the chemical reactions between phenolics all affect how soluble phenolic compounds are [1] [23]. As seen in Fig. 2A, solvents such as methanol, ethanol, acetone, and ethyl acetate generated the largest phenolic content when compared with aqueous extract. This is because phenolic chemicals are more soluble in organic solvents than in water. Phenols were released and diffused into solvents over a period of 60 to 120 min under ultrasound, with an increase in extraction time corresponding to a larger degree of cell damage [11].





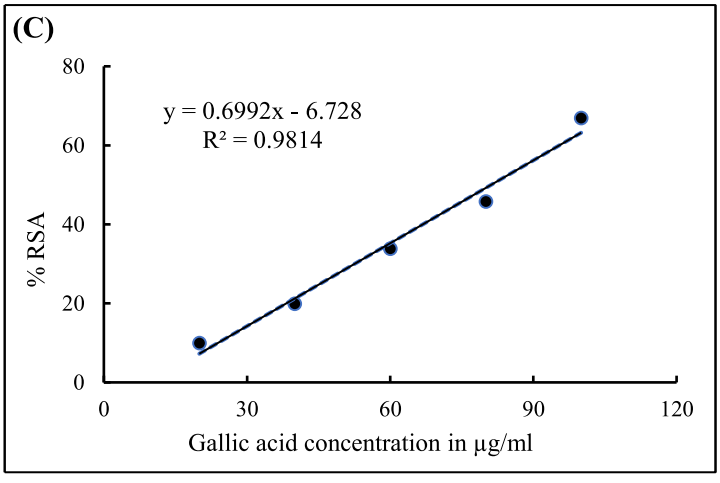


Figure 1. Calibration curves for estimation of (A) Total phenolic content; (B) Total flavonoid content; (C) Antioxidant activity.

Total flavonoid content: The regression calculation of calibration curve (y = 0.0005x+0.0239) with regression co-efficient of R^2 = 0.9805 was used to calculate the flavonoid concentrations in organic solvent (Fig. 1B). 43.13 ± 0.11 mg to 276.77 ± 0.30 mg of quercetin equivalent per 100 g of various solvent extracts were found in the samples. The results indicate that the treatment combination of 120 min ultrasound in methanol and 120 min ultrasound in acetone produced the highest total flavonoid amount 276.77 ± 0.30 mg QE/100 g and 254.17 ± 0.17 mg QE/100 g, respectively and lowest total flavonoid amount in 60 min ultrasound in aqueous (43.13 ± 0.11 mg QE/100 g) (Table 2). According to this data, the pigment extract's total flavonoid concentration was as follows: methanol > acetone > ethanol > ethyl-acetate > aqueous extract (as shown in Fig. 2B).

Table 2. Total Flavonoid Content (mg/100g) in different pigment extracts

	Total Flavonoid Content (mg/100g)			
	60 min Ultrasound	**90 min Ultrasound**	**120 min Ultrasound**	**150 min Ultrasound**
Methanol	97.09 ± 0.06	115.35 ± 0.28	276.77 ± 0.30	226.22 ± 0.23
Ethanol	86.31 ± 0.36	112.29 ± 0.37	144.76 ± 0.30	127.34 ± 0.30
Ethyl acetate	69.14 ± 0.13	74.42 ± 0.18	124.21 ± 0.23	116.45 ± 0.14
Acetone	92.24 ± 0.27	114.15 ± 0.14	254.17 ± 0.17	210.30 ± 0.35
Aqueous	43.13 ± 0.11	44.17 ± 0.17	61.52 ± 0.05	72.68 ± 0.18

Data are to averages of two replications and are represented in the form of mean ± SD (Standard deviation)

Because flavonoids and solvents are polar, methanol, ethanol, acetone, and ethyl acetate are some of the organic solvents that extract flavonoids more effectively than aqueous solvent. The highest flavonoid concentration of floral extracts is found in methanolic extract, which is easily extracted from fresh flowers due to its optimal polarity and suitability for solubilizing flavonoid components [10] [23]. As extraction times increased, flavonoid release and diffusion into solvents accelerated as a result of increased material cell breakdown (Haitang *et al.* 2018).

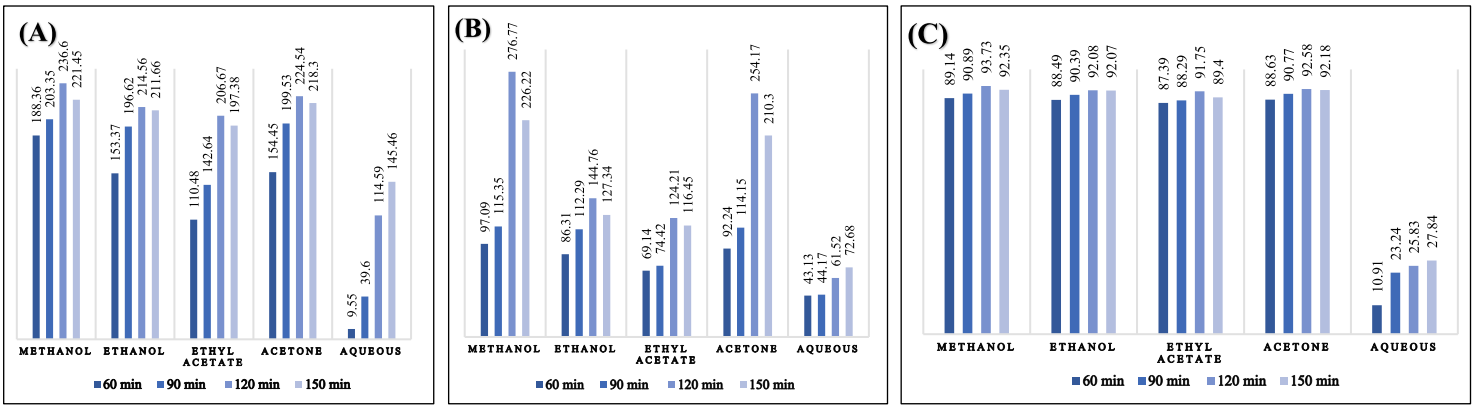


Figure 2. Depiction of (A) Total phenolic content (mg/100g); (B) Total flavonoid content (mg/100g); (C) Antioxidant activity (%RSA) of pigment extracts due to effect of solvents and time durations

Antioxidant activity: The extracts' IC50 values the concentrations at which they scavenge 50% of the DPPH solution were ascertained employing the DPPH free radical scavenging technique. Pigment antioxidant activity represented as a percentage of free radical scavenging activity, or %RSA. The IC50 value was found out using gallic acid as the standard and the calibration curve regression equation (y = 0.699x – 6.728) with a regression co-efficient of R^2 = 0.981 (Fig. 1C). The hydroxyls found in compounds like flavonoids and phenols are what give plants their ability to scavenge radicals. Based on the calibration curve, gallic acid's mean IC50 was found to be 81.13 µg/ml. 120 min ultrasound in methanol recorded highest percentage of free radical scavenging activity with 93.73 ± 0.03% RSA followed by 120 min ultrasound in acetone with 92.58 ± 0.03% RSA (Table 3).

Table 3. Antioxidant activity (%RSA) in different pigment extracts

	Antioxidant activity (% RSA)			
	60 min Ultrasound	**90 min Ultrasound**	**120 min Ultrasound**	**150 min Ultrasound**
Methanol	89.14 ± 0.07	90.89 ± 0.04	93.73 ± 0.03	92.35 ± 0.18
Ethanol	88.49 ± 0.04	90.39 ± 0.01	92.08 ± 0.04	92.07 ± 0.03
Ethyl acetate	87.39 ± 0.03	88.29 ± 0.01	91.75 ± 0.04	89.40 ± 0.01
Acetone	88.63 ± 0.03	90.77 ± 0.02	92.58 ± 0.03	92.18 ± 0.01
Aqueous	10.91 ± 0.02	23.24 ± 0.04	25.83 ± 0.02	27.84 ± 0.04

Data are to averages of two replications and are represented in the form of mean ± SD (Standard deviation)

The treatment combinations of 150 min of ultrasound in acetone, 150 min of ultrasound in methanol, 120 min of ultrasound in ethanol, 150 min of ultrasound in ethanol, 90 min of ultrasound in methanol, and 90 min of ultrasound in acetone demonstrated 92.18 ± 0.01, 92.35 ± 0.18, 92.08 ± 0.04, 92.07 ± 0.03, 90.89 ± 0.04, and 90.77 ± 0.02% RSA, respectively (Fig. 2C). The 60 min ultrasound in aqueous extraction produced the least amount of free radical scavenging activity (10.91±0.02% RSA). The pigment extract's antioxidant activity ranged from 10.91 ± 0.02% RSA to 93.73 ± 0.03% RSA because the amounts of anthocyanin, total phenols, and flavonoid content that were extracted from fresh *Canna indica* flowers differed. Because methanolic extract contains large levels of phenolic and flavonoid compounds, which are responsible for plants' ability to scavenge radicals, it has much stronger antioxidant activity [23].

The longer ultrasound-assisted extraction time resulted in an increase in antioxidant activity, which was likely caused by the presence of more bioactives extracted under the process circumstances. As a result, the difference in time length was determined to be significant [11]. The findings demonstrated a clear relationship between the antioxidant properties of *Canna indica* pigment extract and its phenolic, flavonoid, and anthocyanin content.

Future scope of the study

One of the most important characteristics of food that helps determine its quality is its color. Pigments are fascinating functional ingredients that are now gaining a lot of interest from scientists and consumers because of their biological activity, nutraceutical qualities, and safety. In addition to their significance as natural colorants, they are regarded as components that promote health, have a number of advantageous properties, and show promise as a substitute for artificial colorants. Pigments' limited bioavailability, low water solubility, and susceptibility to process and environmental challenges are only a few of the characteristics that prevent them from being used in food. In future, the study can be done on enhancing the color intensity and stability of natural pigments to strengthen the properties of the desired food product.

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CONCLUSION

On the basis of results obtained from the analysis of total phenol, total flavonoid and antioxidant activity, we conclude that among methanol, ethanol, ethyl-acetate, acetone and aqueous extracts of canna flower, methanolic extract at time duration of 120 min ultrasound possesses significant quantity of phytochemicals indicating its potentiality of anthocyanin in pharmaceutical and nutraceutical industry. The total phenol (236.60 ± 0.21 mg GAE/100 g) and total flavonoid content (276.77 mg ± 0.30 QE/100 g) were found highest in 120 min ultrasound in methanol extract. Antioxidant activity (93.73 ± 0.03% RSA) was found highest in 120 min ultrasound in methanol. These research findings would help for the successful utilization of flowers of canna for pigment extraction and exploiting its antioxidant property for health benefits.

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