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Evaluation of Antioxidant Activity as a Function of the Genetic Diversity of *Canna indica* Complex

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Abstract*—Canna indica* is a prominent species complex in tropical and subtropical areas. They become indigenous in Southeast Asia where they have been introduced. At present, *C. indica* complex comprises over hundred hybrids, are cultivated as commercial horticulture. The species complex contains starchy rhizome having economic value in terms of food and herbal medicine. In addition, bright color of the flowers makes it a valuable ornamental plant and potential source for natural colorant. This study aims to assess genetic diversity of four varieties of *C. indica* complex based on SRAP (sequence-related amplified polymorphism) and iPBS (inter primer binding site) markers. We also examined phytochemical characteristics and antioxidant properties of the flower extracts from four different color varieties. Results showed that despite of the genetic variation, there were no significant differences in phytochemical characteristics and antioxidant properties of flowers. The SRAP and iPBS results agree with the more primitive traits showed by morphological information and phytochemical and antioxidant characteristics from the flowers. Since *Canna* flowers has long been used as natural colorants together with the antioxidant activities from the ethanol extracts in this study, there are likely to be good source for cosmetics additives.

Keywords*—Canna indica*, antioxidant activity, genetic diversity, SRAP, iPBS

I. Introduction

ANTIOXIDANTS are compounds that can interrupt or reduce the oxidation of lipids or other molecules by inhibiting the beginning or propagation of oxidizing chain reactions. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [1]. Commonly, there are two basic categories of antioxidants, natural and synthetic. Nowadays, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity. Therefore, natural antioxidants have become a major area of scientific research. The significance of searching for and exploiting natural antioxidants, especially those of herbal plant, has increased greatly in current years. There is a growing interest in natural additives as potential antioxidants [2], [3]. Although a diversity of herbs is known to be sources of phenolic compounds, their compositional data are inadequate. Moreover, various herbs along with ornamental plant contain numerous phytochemicals in addition to phenolic compounds. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower incidence and lower mortality rates of cancer in several human associates [4], [5].

Canna indica L. originating from tropical and subtropical regions belongs to the family of Cannaceae [6]. It is a perennial rhizomatous herb and widely cultivated as ornamental plants. Although the species was previously considered as simple foliage plants, during the last two centuries of cultivation and improvement they changed them into attractive ornamental flowering plants with decrease in plant height, variability in flower colors and such other positive attributes, currently experiencing significant growth with over hundred cultivars available today [7]. Hundreds of hybrids have evolved from complex crosses between various species of *C. indica*. As the distinction between these hybrid groups have been blurred by further inter-breeding involving parents from one or more than one species, varieties and hybrids. Moreover, all parts of the species like rhizomes, leaves, flowers and seeds are rich source of antioxidants which in turn present evidences of biological activities and medicinal properties [8], [9].

Currently, the rapid progress of molecular biology techniques has provided new approaches to organism identification. One of the most promising is the DNA marker technique as it offers great potential to the analysis of plant genetic structure, diversity, and functionality that are required for marker assisted distinguish schemes. The available DNA marker techniques include random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), and simple sequence repeat (SSR), SRAP, and iPBS [10], [11]. Of these, the SRAP and iPBS techniques are recognized as a simple, efficient, and cost-effective marker system that could be used in multiple molecular biology studies, including genetic diversity analysis [12]. For successful application of SRAP and iPBS authorize an effective polymorphism characterization of target organisms particularly in plant species. In this study, genetic characteristics, agronomic traits and correlation of phytochemical characteristics and antioxidative properties of four *Canna indica* varieties were investigated. Different parameters like presence of total phenols, flavonoids, ferrous reducing power, hydrogen peroxide scavenging assay and ß-carotene bleaching assay of the four color flowers was to

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evaluate the antioxidant properties and to correlate their antioxidant potential to the composition of polyphenols.

II. Materials and Methods

A. Plant Material Collection and Preparation

Four different selected cultivars of *Canna indica* complex were collected (Table I & Fig. 1). All plants were cultivated in a greenhouse at the Faculty of Science and technology of Phranakhon Rajabhat University. Voucher specimens of all accessions were deposited in the Phranakhon Rajabhat University Herbarium.

TABLE I
Morphological Information of Different Cultivars

Code	Plant height/Flower color	Vouchers
C1	100-150 cm/Red	OVC25/5/10
C2	120-250 cm/Yellow, sometimes with orange spots towards the base of the petals.	OVC12/4/10
C3	60-90 cm/Pink, deep pink towards the throat of corolla.	OVC15/2/11
C4	65-95 cm Orange, yellow towards the tip of the petals.	OVC13/7/10

Fig. 1 Four different selected cultivars of *Canna indica* complex: C1 (Red), C2 (Yellow), C3 (Pink), C4 (Orange)

B. DNA Extraction

Genomic DNA was extracted from the leaves of four cultivars using the CTAB method with minor modification. The leaves (500 mg) were ground in a mortar with a pestle. Extraction buffer [(1% (w/v) CTAB, 50 mM Tris–HCl (pH 8), 0.7 M NaCl, 0.1% β-mercaptoethanol)] 500 µl was added and the solution was incubated at 60 °C for 30 min. The homogenate was mixed with 25:24:1 phenol: chloroform: isoamyl alcohol (v/v/v) by gentle inversion. After centrifugation at 13,000 rpm for 15 min, the superior aqueous layer was transferred to a fresh tube. RNA was removed by treating with 2.5 µl of the RNase (10 µg/µl) for 30 min at 37 °C. The removal of DNA with phenol/chloroform/isoamyl alcohol was repeated one or two time until free of color. DNA in the solution was precipitated with 0.6 volume of ice-cold isopropanol and washed with two time 70% ethanol. The final pellet was dissolved in sterile deionized water. The value of DNA was evaluated by reading the absorbance at 260 and 280 nm. DNA quality and quantity were also determined on 1% agarose gel. The DNA was stored at -20 °C, for further utilize as templates for PCR amplification.

C. SRAP-PCR Amplification

Thirty different SRAP primers combinations were employed using five forward primers and six reverse primers (Table II). Each 25 µl PCR reaction mixture consisted of 10x Reaction Buffer, 100 ng template DNA, 0.6 mM dNTP mixture, 5 mM $MgCl_2$, 1 unit of Taq polymerase and 0.6 µM of each primer. The PCR was performed using a Thermohybaid Px2 (Roche Molecular Systems, Inc., USA). The SRAP amplification conditions were 5 min initial denaturation at 94 °C and 5 cycles consisting of 1 min denaturation at 94 °C, 1 min primer annealing at 35 °C, and 2 min extension at 72 °C. In the following 30 cycles, the annealing temperature was increased to 50 °C and a final 8 min extension at 72 °C.

TABLE II
SRAP Primers Used in This Study

Forward primer	Sequence (5'-3')
Me1	TGAGTCCAAACCGGATA
Me2	TGAGTCCAAACCGGAGC
Me3	TGAGTCCAAACCGGAAT
Me4	TGAGTCCAAACCGGACC
Me5	TGAGTCCAAACCGGAAG
Reverse primer	**Sequence(5'-3')**
Em1	GACTGCGTACGAATTAAT
Em2	GACTGCGTACGAATTTGC
Em3	GACTGCGTACGAATTGAC
Em4	GACTGCGTACGAATTTGA
Em5	GACTGCGTACGAATTAAC
Em6	GACTGCGTACGAATTGCA

D. iPBS-PCR Amplification

Twenty iPBS primers were initially screened for analysis (Table III). PCR was performed using a Thermohybaid Px2 (Roche Molecular Systems, Inc., USA). iPBS-PCR reactions were conducted in 25 µl volume containing 10x reaction buffer (100 mM Tris–HCl pH 9, 500 mM KCl, 1% Triton X-100), 100 ng template DNA, 0.6 mM dNTP mixture, 5 mM $MgCl_2$, 1 unit of Taq polymerase and 5 µM primers. The iPBS amplification conditions were: 5 min initial denaturation at 94 °C; 42 cycles consisting of 1 min denaturation at 94 °C, 1 min primer annealing at 47-55 °C, and 2 min extension at 72 °C and a final 7 min extension at 72 °C.

TABLE III
IPBS PRIMERS USED IN THIS STUDY

primer	Sequence (5'-3')
2081	GCAACGGCGCCA
2272	GGCTCAGATGCCA
2076	GCTCCGATGCCA
2077	CTCACGATGCCA
2079	AGGTGGGCGCCA
2080	CAGACGGCGCCA
2083	CTTCTAGCGCCA
2085	ATGCCGATACCA
2374	CCCAGCAAACCA
2378	GGTCCTCATCCA
2380	CAACCTGATCCA
2392	TAGATGGTGCCA
2393	TACGGTACGCCA
2394	GAGCCTAGGCCA
2273	GCTCATCATGCCA
2277	GGCGATGATACCA
2279	AATGAAAGCACCA
2382	TGTTGGCTTCCA
2389	ACATCCTTCCCA
2391	ATCTGTCAGCCA

E. Bands Profile

The SRAP and iPBS products were all analysed by agarose (1.8% w/v) gel electrophoresis at 150 volts for 30 minutes in 0.04 M TAE (Tris–acetate 0.001 M-EDTA) buffer pH 8. The gels were stained with ethidium bromide (10 mg/ml). The gels were viewed and photographed by Bio-Imaging System (Syngene, Genegenuis). To determine iPBS and SRAP profiles, the size of each DNA band was inferred by comparison with a 100 bp DNA ladder (Promega), used as a molecular weight marker (M). Polymorphisms at all loci were confirmed by three repeating tests for each primer at different times.

F. Gel Scoring and Data Analysis

Only strong and reproducible SRAP and iPBS bands were scored. Different patterns observed were scored as distinct variables, using 1 to indicate the presence and 0 to indicate the absence of a unique pattern. A principal component analysis (PCA) was also conducted using a genetic distance matrix obtained from the binary data set. It was negated and rescaled (0–1), using the Euclidean distance between pair-wise comparison of individuals.

G. Flower Extracts

Different colors of fresh Canna flowers (C1-C4) as shown in Fig. 1 were subjected to a hot air oven at 60°C until the weights were not change. The dried flowers were grounded into powder and extracted 3 times with 95% ethanol (flower powder: ethanol = 1:10, w/v). The flower extracts were filtered with cheesecloth and evaporated to dryness in a rotary evaporator and stored in sealed containers until used.

H. Total Phenolic Content Determination

Total phenolic content of the flower extracts was examined by modified colorimetric methods described by [13] The reaction mixture of 0.1ml flower extract (50 mg/ml in 10% ethanol), 2.3 ml Folin-Ciocalteau reagent and 1.6 ml of 7.5% Na_2CO_3 were incubated in the dark at 30°C for 60 minutes. The absorbance at 765nm were measured and compared with gallic acid standard curve. The total phenolic contents were expressed as mg gallic acid equivalent (GAE)/g extract.

I. Total Flavoniod Content Determination

Method according to [14] was modified to determine total flavoniod content of Canna flower extracts. 0.5ml of flower extracts (10 mg/ml) were mixed with 3.5 ml reaction mixture (0.15 ml of 5% $NaNO_3$ and 0.15 ml of 10% $AlCl_3$ and 3.2 ml of 50% ethanol,) and stand at room temperature for 5 min. 1 ml of 1 mM NaOH was then add to the solutions and the absorbance at 510 nm were measured compared with rutin standard curve. The total flavonoid contents were reported as mg rutin equivalent (RE)/g extract.

J. DPPH Free Radical Scavenging Assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging effect of Canna flower extracts were investigated according to the method described previously by [15]. 0.5 ml of flower extract at different concentration was mixed with 0.5 ml of 60 µM DPPH in 95% ethanol. The absorbance at 517 nm was measured after incubated in the dark at room temperature for 15, 30, 45 and 60 min. The DPPH scavenging activity was achieved from (1).

$$DPPH\ scavenging\ activity\ (\%) = [A_{blank} - A_{sample}] \times 100 \quad (1)$$

K. Ferric Reducing Antioxidant Power (FRAP) Assay

Canna flower extracts were subjected to FRAP assay modified from the study of [16]. FRAP reagent [Mixture of 300 mM acetate buffer, pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl and 20 mM $FeCl_3$ at the ratio of 10:1:1] was prepared prior to use. 0.4 ml Canna flower extracts (0.5 mg/ml) were mixed with 3 ml FRAP reagent and incubated in 37°C water bath for 30 min. The absorbance at 595 mm was measured compare to the $FeSO_4$ standard curve and reported as µmol Fe(II)/mg extract.

L. ß-Carotene Bleaching Assay

This assay is a modified procedure according to [17]. 1 ml of ß-carotene (0.2 mg/ml) in chloroform was added to a mixture of 0.02 ml linoleic acid and 0.2 ml Tween40, mix well and subjected to rotary evaporator (40°C) until dry, 100 ml distilled water was then added to the remaining, vigorously shake until emulsion was formed. The emulsion was diluted with distilled water in a ratio of 1:30 (emulsion: H_2O). 40 µl of Canna flower extracts was mixed with 60 µl diluted emulsion in each well of flat-bottom microtiter plate and incubated at 50°C. The absorbance at 470 nm was observed with a microtiter plate reader at every 20 min time interval up to 120 min. 10% ethanol was used instead of flower extract for control. Degradation rate (dr) and antioxidant activity (AA) were calculated from (2) and (3), where A_0 is initial absorbance at time 0; A_t is absorbance at each 20 min time interval; t is 120 min

$$dr_{sample} = \left[\ln\left(\frac{A_0}{A_t}\right)\right]/t \quad (2)$$

$$AA\% = \left[\frac{dr_{control} - dr_{sample}}{dr_{control}}\right]/100 \quad (3)$$

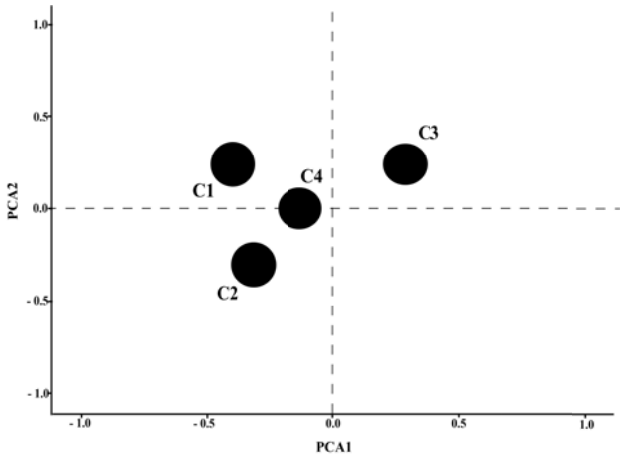

Fig. 2 Plot of PCA analysis of Four different selected cultivars of *Canna indica* complex: C1 (Red), C2 (Yellow), C3 (Pink), C4 (Orange) obtained from iPBS and SRAP marker

III. Results and Discussion

A. DNA Isolation

DNA extracted from leaf using a modified method of [18] gave a good and sufficient quality DNA for PCR reaction, and the amount of DNA extracted from the accessions ranged from 130 to 278 µg/g fresh weight leaf material. The ratios of A260/A280 varied from 1.58 to 1.96. The quality of DNA was also checked by PCR, which confirmed that the DNAs were suitable for PCR reaction.

B. SRAP Analysis

In order to obtain primers that can be used effectively in SRAP analysis, twenty- five random primers were screened and those primers that generated clear bands were identified. A total of 212 reproducible fragments were amplified by 30 combination primers with the number of amplified fragments ranging from three (Me3/Em2) and to sixteen (Me4/Em1). The band size ranged from 100 to 3,000 bp.

C. iPBS Analysis

For iPBS-PCR, a total of 20 primers were screened, and 15 of them that gave clear reproducible fragments were selected for further analysis. A total of 128 reproducible fragments were amplified with the number of amplified fragments ranging from five (2374) to thirteen (2279). The band size ranged from 150 to 2,000 bp.

D. Comparison of SRAP and iPBS Markers

SRAP and iPBS makers are a novel marker system that preferentially detects polymorphisms in coding sequences, which are more conserved among cultivars and have a relatively low mutation rate, making it a more efficient technique due to its capacity to reveal relatively more informative bands. Compared the iPBS primers with the SRAP primers, the SRAP primers generated greater mean number of bands per primer (mean = 11.36) than the iPBS primers (mean = 8.25).

E. Principal Component Analysis

Based on SRAP and iPBS pattern, genetic distances among four samples were calculated and Cluster analysis by principal component analysis (PCA) was constructed. The PCA consisted of two major clusters (Fig. 2). The first cluster contained a group of the eight samples of C1, C2, C4. The second group is made up of C4.

F. Total Phenolic and Total Flavonoid Contents of Canna Flower Extracts

The yield of ethanol extract from dried Canna flowers was 25.77%±2.55%. The phenolic contents of flower extracts shown in Fig. 3. Among four different colors, Red flower extract (C1) contained slightly higher phenolic content (12.24±0.07 mgGAE/g extract) than the others, followed by pink flower extract (C3) with 10.29±0.29 mgGAE/g extract. Yellow (C2) and orange-yellow flower extract (C4) gave not significantly different phenolic content, which are 9.41±0.04 and 9.49 ±0.12 mgGAE/g extract, respectively. As shown in Fig. 4, The C1 flower extract also had highest total flavonoid content (1.17±0.02 mgRE/g extract) followed by C4 (0.94±0.04 mgRE/g extract), C3 (0.83±0.03 mgRE/g extract) and C2 (0.72±0.01 mgRE/g extract), respectively.

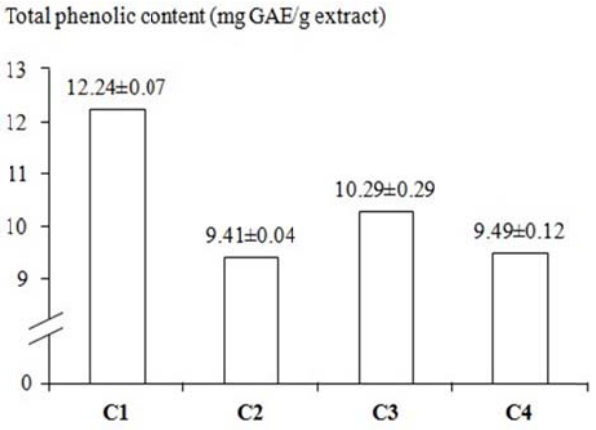

Fig. 3 Total phenolic content of Canna flower extracts

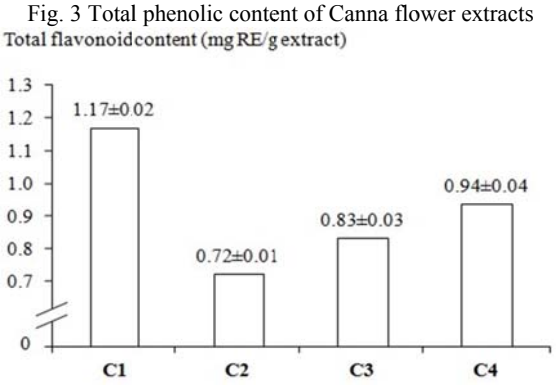

Fig. 4 Total flavonoid content of Canna flower extracts

G.Free Radical Scavenging Activity of Canna Flower Extracts

The radical scavenging ability of Canna flower extracts in terms of donation of hydrogen to a stable radical DPPH were analyzed and report as % inhibition and IC_{50}. The % inhibition of Canna flower extracts in Fig. 5 indicated that they did not show significantly different between different colors and also did not significantly. Elevate when the incubation time increased, at 60 min incubation (Table IV), the lowest IC_{50} was from C3 flower extract (517.80 µg/ml), followed by C1 (625.89 µg/ml), C4 (648.24 µg/ml) and C2 (661.53 µg/ml), respectively.

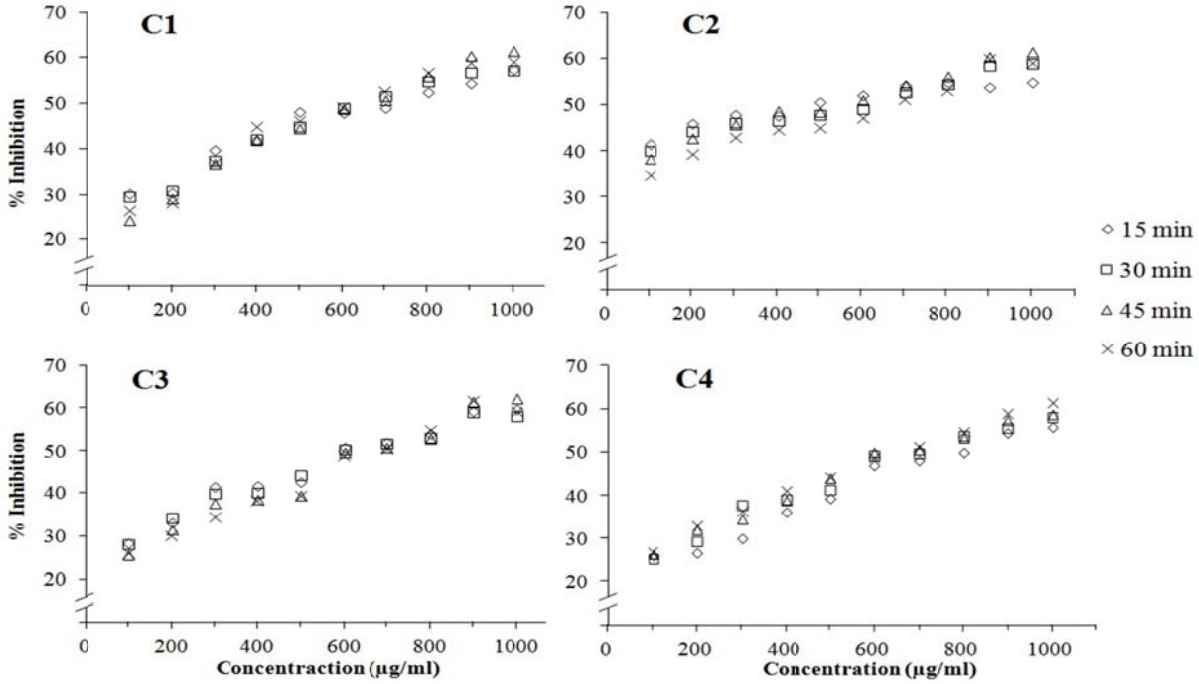

Fig. 5 DPPH scavenging activity of *Canna* flower extracts

TABLE IV
IC_{50} OF *CANNA* FLOWER EXTRACTS AT DIFFERENT INCUBATION TIMES

Time (min)	C1	C2	C3	C4
15	715.62	668.45	654.27	767.69
30	662.88	678.13	562.14	714.19
45	648.71	665.96	519.93	694.17
60	625.89	661.53	517.80	648.24

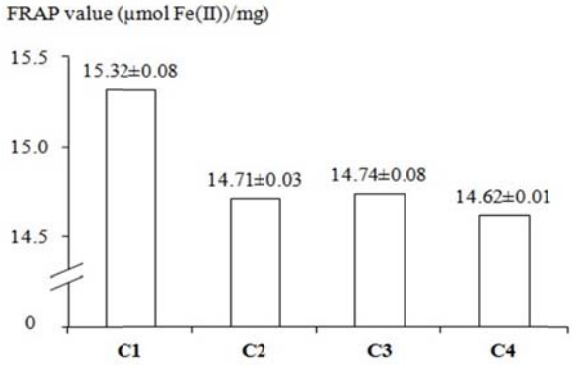

Fig. 6 FRAP value of *Canna* flower extracts

H. Ferric Reducing Antioxidant Power (FRAP) of Canna Flower Extracts

Antioxidant components can be analyzed by FRAP assay. The greater amount of antioxidant content, the higher ability to reduce ferric tripyridyltriazine [Fe(III)-TPTZ] complex to ferrous [Fe(II)] form, which corresponds to an increase in dark blue color. The highest FRAP value [15.32±0.08 mol Fe(II)/µ mg] was obtained from C1 flower extract. The C2 and C3 flower extract showed no significant different which are 14.71±0.03 mol Fe(II)/µ mg and 14.74±0.08 mol Fe(II)/µ mg respectively. The C4 flower extract expressed slightly lower FRAP value (14.62±0.03 mol Fe(II)/µ mg) than C2 and C3 as shown in Fig. 6.

I. ß-Carotene Bleaching Assay of Canna Flower Extracts

ß-carotene bleaching assay is another frequently used method to determine antioxidant components. Hydroperoxide free radical produced from linoleic acid during 50°C incubation attacks ß-carotene molecule cause the color bleaching and reduction in absorbance at 470 nm. The presence of antioxidants can slow down the bleaching reaction by neutralizing the hydroperoxide free radicals. The sample with the highest antioxidant activity shows the lowest ß-carotene degradation rate. Table V shows antioxidant activity from different *Canna* flower extracts. C2 flower extract exhibited the highest antioxidant activity with 98.14%, followed by C4, C1 and C3 with 78.37%, 76.74% and 76.45%, respectively.

TABLE V
ANTIOXIDANT ACTIVITY OF *CANNA* FLOWER EXTRACTS FROM Β-CAROTENE BLEACHING ASSAY

Canna flower extract	Antioxidant activity (%)
C1	76.78
C2	98.14
C3	76.45
C4	78.37

IV. CONCLUSION

The iPBS and SRAP techniques have proven to be successful in supporting the taxonomical identification and providing the genetic structure of *Canna indica*. The present study showed that due to the diversity and complexity of the natural mixtures of phenolic compounds in the different four morphotype of *C. indica* extracts, it is rather difficult to characterize every compound and assess or compare their antioxidant activities. Each flower color generally contained different phenolic compounds, and each of these compounds possessed differing amounts of antioxidant activity. Nevertheless, the results of the present study revealed that the four different colors of *C. indica* do not have equivalent antioxidant powers. The relative high value of antioxidant activity correlated with genetic analysis which high antioxidant activity comprise yellow (C2), red (C1) and orange (C4) flower are cluster in the same grouping while lowest antioxidant activity contains only pink morphotype (C3) is separated.

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