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Phytochemical Screening, Total Phenolic and Antioxidant Activity of Crude and Fractionated Extracts of *Cynomorium coccineum* Growing in Saudi Arabia

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Aim: This study was carried out to determine the Total phenolic and antioxidant activity of crude and fractionated extracts of *Cynomorium coccineum* growing wild in Saudi Arabia.
Methodology: Phytochemical analysis of the different extracts obtained from *C. coccineum* was carried out by Siddiqui *et al* procedure and the Total phenolic content of the extracts were assessed by the Folin-Ciocalteau's method while the antioxidant activities were determined by two different assays, namely scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) cation radicals methods.
Results: Phytochemical screening of *C. coccineum* revealed the presence of alkaloids, anthraquinones, glycosides, flavonoids, saponins, tannins and terpenoids in all different extracts. In the present study, the butanol extract exhibited the highest total phenolic content (201.36±7.44 mg of GAEs/g of extract) and showed the strongest antioxidant activity in both, the DPPH radicals and ABTS methods. The antioxidant activity of the butanol extract for DPPH scavenging assay was

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95.20%±3.48% (IC_{50} 0.0056 mg/mL) while for ABTS radical scavenging assay was 99.07±0.37 (IC_{50} 0.006 mg/mL) at concentration 0.4 mg/mL.
Conclusion: The high phenolic content in the polar extract accounts for the high antioxidant potential in comparison to non-polar extract. The results of this study substantiates the role of these plant as natural sources of antioxidants.

Keywords: Medicinal plant; C. coccineum; antioxidant activity; DPPH; ABTS; phytochemical; total phenolics.

1. INTRODUCTION

Cynomorium coccineum L. belongs to the balanophora family (family Balanophoraceae). *C. coccineum* is a non-photosynthetic plant, mainly distributed in dry rocky or sandy soils in Mediterranean countries [1]. The Common names for this plant include Maltese fungus, Maltese mushroom, desert thumb, red thumb, tarthuth (Saudi Arabia) and suo yang (Chinese) [2].

C. coccineum is root parasite producing erect dark brownish red fleshy stalks up to 30 cm tall and c. 20 mm in diameter, bearing numerous triangular-lanceolate scale leaves. Inflorescence terminal, club-shaped spike of 1-4 cymosely arranged flower clusters subtended by triangular scaly bracts. Male flowers c. 4 mm long, filaments reddish brown, tepals 1.5-4 mm long, 0.4-0.5 mm broad; anther versatile. Female flowers c. 3 mm long; style c. 2 mm long. Fruit globose, seeded [3].

C. coccineum which is medicinal parasitic plant have been widely applied in folk medicine in Europe, North Africa and Eastern and Arabian Peninsula [4]. It is usually used as a tonic and aphrodisiac and is reported to increase the production of semen [5,6]. It is also reported to have a hypotensive effect [7]. The aqueous extract of *C. coccineum* significantly increases the sperm count, the percentage of live sperms and their motility in addition to decreasing the number of abnormal sperms [8]. The dried stems of this plant showed antitumor activity on melanoma and colon cancer cells [9].

Many studies have been carried out to investigate the bioactive constituents and pharmacological propert of *C. coccineum*. These investigations revealed that the plant contained condensed tannins, steroids, triterpenes, acidic heteropolysaccharides butyl fructosides, flavanoids, lignan glycosides, alkaloids and other compounds [10,11]. Several investigations indicated attempted to evaluate the antioxidant activities of both, the aqueous and methanolic extracts obtained from *C. coccineum* [12,13]. Rached *et al* have investigated the antioxidant potential of *C. coccineum* growoing wild in Algeria by using two complementary assays, namely the DPPH radical and β-carotene bleaching [13].

The present work aimed at evaluating the antioxidant properties of the methanolic, aq. methanolic, butanol and hexane extracts of *C. coccineum* growing wild in Saudi Arabia by the DPPH radicals and ABTS methods.

2. MATERIALS AND METHODS

2.1 Sample Collection

Fresh *C. coccineum* was collected from Sand dunes, near Al-asfar lake that is located on Al-Hassa, Eastern region of Saudi Arabia. The plant was identified by Dr. Jacob Thomas, Department of Botany, King Saud Unversity, Saudi Arabia. The plant material was pulverized into fine powder using a mechanical grinding machine.

2.2 Extraction

Freshly collected plant material was dried under shade and then made into finely powder. A sample weighing 100 g of plant material was extracted with 500 mL of petroleum ether in a Soxhlet type apparatus to remove fatty materials. The residual plant material was extracted with 500 mL of methanol. The solvent was evaporated under vacuum to yield crude residue (9.80 g). The methanolic residue was then partitioned between $CHCl_3$ and H_2O (1:1) solvent system yielding the chloroform (5.30 g) and water (3.88 g) fractions. The dried $CHCl_3$ fraction was partitioned between 10% aqueous methanol and hexane. The polar organic compounds were extracted from water by n-butanol.

2.3 Phytochemical Screening

Phytochemical analysis of the different extracts obtained from *C. coccineum* was carried out using the standard protocols for the presence of flavonoids, alkaloids, Terpenes, saponins, Anthraquinones, glycosides and tannins and according to the procedures described in the literature [14]. Detection of these phytochemicals was based on visual observation following color change or formation of a precipitate after the addition of specific reagents.

2.3.1 Test for anthraquinones

0.1 g of the extract was boiled with 1.0 ml of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1.0 ml of diluteammonia was added. The resulting solution was observed for colour changes.

2.3.2 Test for terpenoids

To 0.1 g each of the extract was added 2 ml of chloroform. Concentrated H_2SO_4 (1.0 ml) was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

2.3.3 Test for flavonoids

A portion of the extract was heated with 3.0 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 1.0 ml of the filtrate was shaken with 1.0 ml of dilute ammonia solution. A yellow colouration indicates the presence of flavonoids.

2.3.4 Test for saponins

To 0.1 g of extract was added 2.0 ml of distilled water in a test tube. The solution was shaken vigourously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigourously after which it was observed for the formation of an emulsion.

2.3.5 Test for tannins

About 0.1 g of the extract was boiled in 2.0 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.

2.3.6 Test for glycosides

To 0.1 g of extract diluted to 3.0 ml in water was added 2.0 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1.0 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

2.3.7 Test for alkaloids

0.1 g of extract was diluted to 3.0 ml with acid alcohol, boiled and filtered. To 2.0 ml of the filtrate was added 1.0 ml of dilute ammonia. 2.0 ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 3.0 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids.

2.4 Determination of Total Phenolic Content

Total phenolic content of the crude plant extract and and the main extract (aq. methanol, butanol, water and hexane) of *C. coccineum* were determined using the Folin-Ciocalteu method as described by Singleton et al. [15] with slight modifications. A volume of 0.5 mL of the extract was introduced into test tubes followed by 2.5 mL Folin-Ciocalteu's reagent (diluted 10 times with water) and then 2 mL sodium carbonate (7.5% w/v) was added. The mixture was allowed to stand at room temperature for 30 min and the absorbance was recorded at 765 nm using UV- visible spectrophotometer. Gallic acid was used as standard for calibration curve, by measuring its absorbance at different concentrations (4-20 µg/mL). All determinations were performed in triplicate. Total phenolic content was obtained from a regression equation, and expressed as mg/g of gallic acid equivalent.

2.4.1 DPPH$^{\bullet}$ free radical scavenging activity

The total radical scavenging capacity of the crude and the main extracts (aq. methanol,

butanol, water and hexane) of *C. coccineum* were determined and compared to those of Ascorbic acid and α- tocopherol, based on the reaction with 2,2-diphenyl-2-picrylhydrazyl radical (**DPPH$^{\bullet}$**). The spectrophotometric method of Hatano et al. was used to estimate the DPPH• free radical scavenging capacity of extract with little modifications [16]. A solution of DPPH• (0.1 mM) was prepared in methanol. A 1.0 mL sample of various concentrations (0.005-0.40 mg/mL) of each extract in methanol were added to 2 mL of 0.1 mM methanol solution of DPPH•. The solutions were allowed to stand at room temperature in the dark for 30 min. The absorbances of the solutions were then measured at 517 nm against blank samples using UV- visible spectrophotometer. All determinations were performed in triplicate. The ability to scavenge the DPPH• radical was calculated using the following equation:

DPPH• scavenging effect (%) $=A_c-A_s/A_c$ *100%

Where A_C is the absorbance of the blank and A_S is the absorbance in the presence of extract. [DPPH•] decreases significantly upon exposure to radical scavengers.

The antioxidant activity of all samples was expressed as IC_{50} which was defined as the concentration (in mg/mL) of extract required to inhibit the formation of DPPH radicals by 50%.

2.4.2 ABTS radical scavenging assay

The total antioxidant activity was determined by radical cation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\bullet+}$) decolorization assay according to the method of Re et al. [17] with some modifications. The $ABTS^{\bullet+}$ cation radical solution was prepared by reaction of similar quantities of 7 mM of ABTS and 2.4 mM of potassium persulfate ($K_2S_2O_8$) solution and allowed to react for 16 hour at room temperature in the dark. Before use, this solution was diluted with methanol to get an absorbance of 0.75±0.02 at 734 nm. The reaction mixture comprised 3 mL of $ABTS^{\bullet+}$ solution and 1 mL of the extracts at various concentration (0.005-0.40 mg/mL). The absorbance of the mixture was measured at 734 nm by using UV- visible spectrophotometer. The blank was run in each assay and all measurements were done after at least 5 min. The ABTS scavenging capacity of the extract was compared with that of Ascorbic acid and α- tocopherol as positive controls. The percentage inhibition was calculated according to the equation:

ABTS radical scavenging activity (%) = (A_{blank} – A_{sample} /A_{blank}) x 100%.

Where A_{blank} is the absorbance of the blank solution and A_{sample} is the absorbance of the remaining $ABTS^{\bullet+}$ solutions in the presence of scavenger. ABTS·+ scavenging ability was expressed as IC_{50} (mg mL^{-1}).

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

The Phytochemical constituents of the butanol, water, aqueous methanol and hexane extracts of *C. coccineum* are shown in Table 1. Terpenes were detected in all extracts except the water fraction. Butanol, water and aq. methanol fractions contained glycosides, tannins and anthraquinones. Flavonoids were detected in butanol, hexane and aqueous methanol fractions. Alkaloids were detected in butanol and water fractions. All fractions contained saponins.

The presence of flavonoids and tannins in most frctions is likely to be responsible for the free radical scavenging effects observed. Flavonoids and tannins are phenolic compounds that are known to act as primary as antioxidants or free radical scavengers [18].

Table 1. Major phytochemical groups detected in crude extract fractions of *C. coccineum*

Test	Butanol	Water	Aq. Methanol	Hexane
Tannins	+	+	+	-
glycosides	+	+	+	-
Flavonoids	+	-	+	+
Anthraquinones	+	+	+	-
Saponins	+	+	+	+
Alkaloids	+	+	-	-
Terpenes	+	-	+	+

Key: (+) detected; (-) not detected

3.2 Determination of Total Phenolic Content

The total phenolic content of the crude and main extract fractions of *C. coccineum* were determined by Folin – Ciocalteu method, and calculated as gallic acid equivalents (Table 2). The total phenolic content was expressed as mg/g of gallic acid equivalent determined from the following regression equation based on the calibration curve: Y=0.019X+0.03, R^2 = 0.969, Where Y is absorbance at 765 nm and X is total phenolic content in the extracts.

Table 2. Total phenolic content of all extracts of *C. coccineum*

C. coccineum extracts	Total Phenol (mg/g of gallic acid)
Original crude	259.3±6.82
Aqueous methanol	89.73±4.95
Butanol	201.36±7.44
Water	39.31±0.84
Hexane	14.11±0.40

Plant phenols are considered amont the most important groups of natural antioxidants and some of them are potent antimicrobial compounds. Therefore, it is necessary to determine the total amount of phenols in the plant extracts for the this study. As shown in Table 2, total phenolic content in the different extracts of the plant varied significantly. The total phenolic content in polar extract of *C. coccineum* was higher than that of the non-polar hexane extract. The phenolic content in butanol, aqueous methanol, water and hexane extracts were 201.36±7.44, 89.73±4.95, 39.31±0.84 14.11±0.40 and mg/g gallic acid equivalent; respectively.

3.3 Scavenging Activity of DPPH Radicals

Many plants extract exhibit efficient antioxidant properties due to their phyto constituents, including phenolic acids and flavonoids [15-21]. In the present study, reduction of DPPH· radicals was significantly observed at 517 nm by the extracts of *C. coccineum.* Measured by **DPPH·** method (Table 3), the order of free radical scavenging activity of the extracts of the plant is butanol > aqueous methanol > water >hexane and increase with increasing the concentration (Fig. 3).

The free radical scavenging activity of the butanol fraction of the plant was the best showing 95.20%±3.48 DPPH inhibitions at 0.4 mg/ mL. By comparison, standard antioxidants Ascorbic acid and α- tocopherol at the same concentration was 95.97%±0.20, 93.92%±1.21, respectively. Percentage DPPH· inhibition by the aq. methanol at the same concentration was 91.90%±0.21. Values obtained for the hexane fraction was 7.99%±0.51 at the same concentration (Table 3). This radical scavenging activity of extract could be related to the nature of phenolics [13], thus contributing to their electron transfer or hydrogen donating ability.

The estimation of antiradical capacity of the different extracts was performed by the value of IC_{50}; as shown in Fig. 1. The order of activity was as the following: butanol (IC_{50} = 0.0056 mg/mL) > aqueous methanol (0.0531 mg/mL) > water (0.017 mg/mL) whereas the hexane fraction could not be determined.

Table 3. Antioxidant activity of the of the crude and main extract fractions of *C. coccineum* and positive controls (ascorbic acid and α-Tocopherol) on DPPH· assay

C (mg/mL)	I% Crude (original)	I% aq. Methanol	I% Butanol	I% Water	I% Hexane	I % Ascorbic acid	I% α- tocopherol
0.005	2.20±1.07	7.58±1.96	42.46±8.88	2.61±1.58	1.41±1.38	49.36±0.52	7.09±4.86
0.01	11.19±1.81	13.33±4.82	68.89±3.65	6.72±2.90	3.08±3.06	68.88±0.68	16.81±2.05
0.02	23.50±2.69	25.41±3.66	87.31±3.43	10.63±4.40	5.39±2.56	94.40±0.15	31.85±6.99
0.04	49.17±3.58	41.40±4.12	90.70±1.54	16.00±2.33	5.50±2.98	94.59±0.24	81.75±1.59
0.05	52.49±1.53	45.84±4.96	92.22±1.13	17.49±1.53	5.53±2.11	94.60±0.75	93.20±1.51
0.06	69.70±5.73	61.99±6.26	92.34±0.72	18.50±3.79	6.49±2.86	95.14±0.40	93.26±1.26
0.08	79.18±2.83	73.60±0.49	92.46±0.82	25.35±1.84	6.94±1.89	95.28±0.33	93.33±0.70
0.1	83.19±2.96	86.15±4.49	92.51±0.91	36.69±5.01	7.05±3.00	95.74±0.13	93.49±0.90
0.2	88.42±4.06	91.17±0.74	94.68±1.70	53.58±1.35	7.12±2.62	95.85±0.26	93.59±1.02
0.4	93.50±0.95	91.90±0.21	95.20±3.48	72.03±10.19	7.99±0.51	95.97±0.20	93.92±1.21

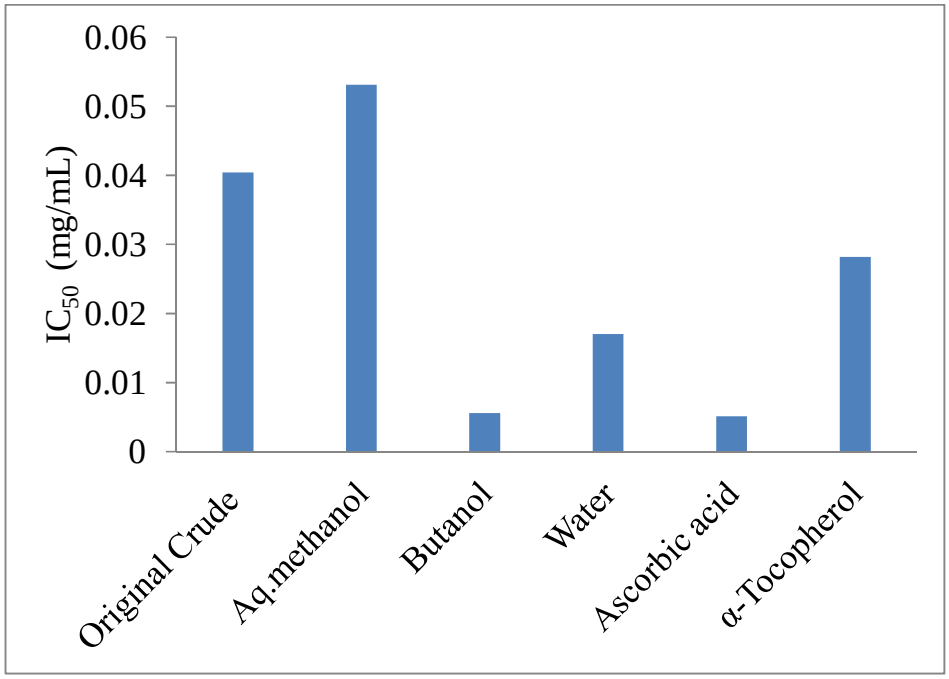

Fig. 1. Free radical-scavenging capacities of the crude and main extract fractions of *C. coccineum* and positive controls (Ascorbic acid and α-Tocopherol) on DPPH· assay

3.4 ABTS Radical Scavenging Assay

ABTS assay is considered as an important method for estimating antioxidant activity. This method is based on the spectrophotometric measurement of ABTS cation radical ($ABTS^{\bullet+}$) concentration changes resulting from the ABTS•+ reaction with antioxidants [17]. In the present study, the ABTS radical scavenging activities were determined for all extracts of *C. coccineum* (Table 4), the order of ABTS radical scavenging activity of the extract of *C. coccineum* is butanol > aqueous methanol > water > hexane. The highest ABTS radical scavenging activity was found in Butanol extract that had a percent inhibition reaching 99.07±0.37 at 0.4 mg/ mL. at this concentration the percentage of inhibition of the standard antioxidant Ascorbic acid and α- Tocopherol was (99.19±0.14) and (98.94±0.44), respectively. From this information, the activity of butanol extract is similar to the activity of Ascorbic acid and α- Tocopherol. At the same concentration, the values obtained for aqueous methanol 91.90±0.21 is higher than the activity of water extract (73.42±8.60). Whereas the Hexane extract has the lowest ABTS inhibition (11.04±3.45) at the same concentration.

The IC_{50} (Fig. 2) estimate of the scavenging activities of the main extracts. From these values the order of ABTS radical scavenging activity of the extract of *C. coccineum* is butanol (0.006 mg/ mL) > aqueous methanol (0.053 mg/ mL) > water (0.11 mg/ mL). Whereas the value of IC_{50} of hexane extract could not be detected.

Two techniques have been used to determine the antioxidant activity *in vitro* in order to allow rapid screening of extracts. Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect us from various diseases [22].

The electron donation ability of natural products can be measured by 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH·) purple-coloured solution bleaching [23]. The method is based on scavenging of DPPH· through the addition of a radical species or antioxidant that decolourizes the DPPH· solution. The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the extract under test [23].

ABTS radical scavenging assay involves a method that generates a blue/green ABTS+ chromophore via the reaction of $ABTS^{\bullet+}$ and potassium persulfate. The $ABTS^{\bullet+}$ radical cation is generated by the oxidation of $ABTS^{\bullet+}$ with potassium persulfate, its reduction in the presence of hydrogen-donating antioxidants is measured spectrophotometrically at 745 nm [24,25]. In the present study all the fractions of *C. coccineum* possessed strong ABTS scavenging activity except thehexane fraction.

Table 4. Antioxidant activity of the crude and main extract fractions of *C. coccineum* and positive controls (ascorbic acid and α-Tocopherol) on ABTS assay

C (mg/mL)	I% Original Crude	I% aq Methanol	I% Butanol	I% Water	I% n-Hexane	% Ascorbic acid	I% α- ocopherol
0.005	12.93±9.50	7.58±1.96	43.93±1.00	6.38±1.23	1.35±1.58	10.96±0.41	12.21±0.50
0.01	20.18±6.75	13.33±4.82	69.09±0.53	10.51±1.62	2. 71±1.68	17.81±0.49	15.86±1.46
0.02	38.72±3.41	25.41±3.66	86.49±0.77	13.10±0.76	5. 24±3.81	66.91±1.92	22.47±1.24
0.04	59.75±9.88	41.40±4.12	90.81±0.63	19.54±2.36	6. 71±2.72	97.43±0.60	23.96±2.19
0.05	74.89±7.57	45.84±4.96	96.01±1.25	28.29±5.75	8. 41±4.01	97.47±0.65	39.04±1.95
0.06	81.92±10.61	62.00±6.26	98.63±0.08	28.56±7.89	9 .94±2.26	97.11±0.73	44.45±2.12
0.08	93.71±4.53	73.60±0.49	98.74±0.12	37.26±0.34	10 .06±7.81	98.11±0.35	56.11±6.28
0.1	98.13±0.44	86.15±4.49	98.96±0.19	48.16±6.21	10. 76±4.97	98.65±0.13	79.79±4.86
0.2	98.75±0.58	90.17±0.74	99.02±0.41	59.11±4.54	10. 94±1.27	99.01±0.15	98.15±0.65
0.4	98.81±0.39	91.90±0.21	99.07±0.37	73.42±8.60	11. 04±3.45	99.19±0.14	98.94±0.44

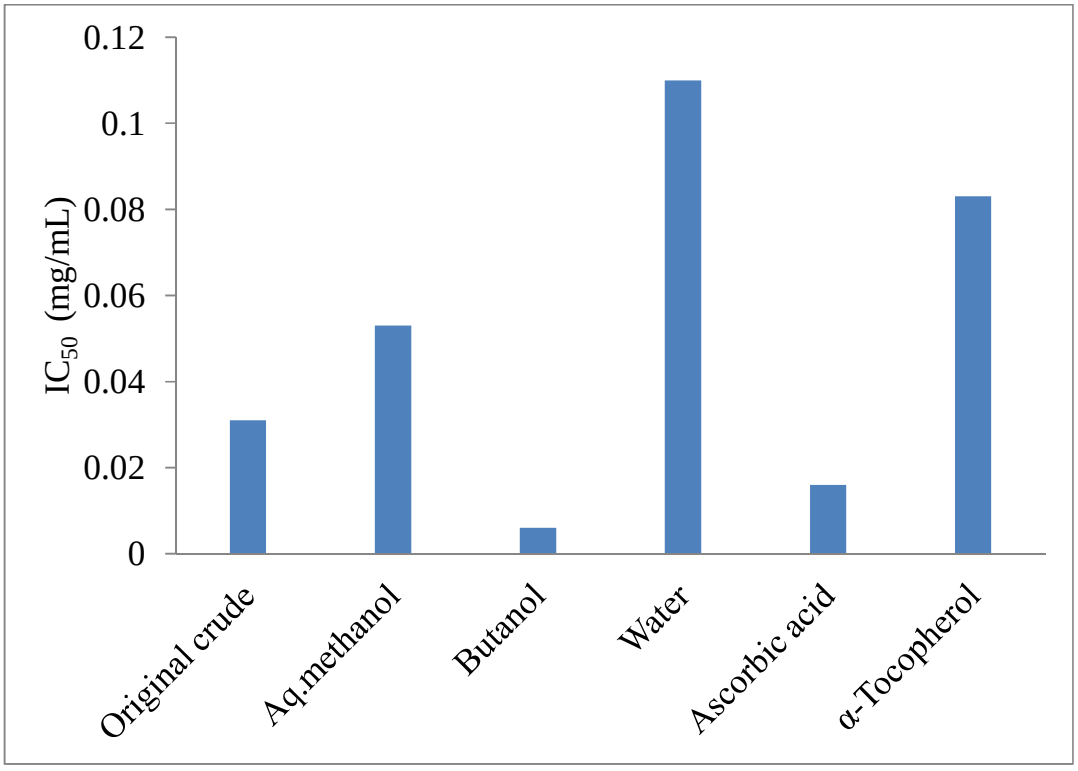

Fig. 2. Free radical-scavenging capacities of the crude and main extract fractions of *C. coccineum* and positive controls (Ascorbic acid and α-Tocopherol) on $ABTS^{\bullet+}$ assay

4. CONCLUSIONS

C. coccineum has been revealed to contain condensed tannins, triterpenes, flavanoids, glycosides, alkaloids, Saponins, Anthraquinones and other compounds. Pharmacological studies have shown that the medicinal herbs have antioxidation, anti-hypoxia/anti-anoxia, *α*-glucosidase inhibition, anti-diabetes, HIV-PR and HCV-PR inhibition, immunity improvement, anti-fatigue, physical endurance enhancement, neuroprotection, anti-dementia, anti-aging, anti-epilepsy, and anti-stress effects. However, further research needs to be carried out to evaluate Pharmacological studies of this plant.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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