**Original Research Article**

**TOTAL PHENOLICS, FLAVONOID’S CONTENT AND *IN VITRO* ANTIOXIDANT ACTIVITIES OF *CUSCUTA REFLEXA***

**Abstract**

**Background:** The aim was to identify functional groups and antioxidant activity of the extract. Soxhlet extraction method was employed to extract phenolic compounds from *Cuscuta reflexa* (*C. reflexa*) stems. The effect of extraction time (1-4 h) and concentration of ethanol (45%, 60%, 75% and 90%) on the percentage of yield, total phenolic (TPC) and flavonoid content (TFC) was investigated. The functional groups of phenolic compounds were characterized by using Fourier Transform Infrared Spectrometry (FTIR). DPPH and ABTS•+ radical scavengers were used to evaluate antioxidant activity.

**Results:** Data showed the highest % of yield (10.22 ± 0.14 w/w), TPC (64.11 ± 0.17, mg GAE/g d.w.) and TFC (41.08 ± 0.34, mg QE/g d.w.) at 3 h with 75% ethanol. FTIR results revealed the presence of functional groups associated phenolic compounds. DPPH and ABTS•+ radical scavengers were showed very potent antioxidant activity with IC50 295.12±1.33 and 245.43±0.78 μg/mL.

**Conclusion:** Phenolic and flavonoids enriched *C. reflexa* extract may play a potential role as a natural nutritional and therapeutic source in Bangladesh.

**Keywords:** *Cuscuta reflexa*, phenolic content, flavonoid content, antioxidant.

**1. Background**

*C. reflexa* is one of the well-known parasitic plants which is frequently utilized as a nutrient ingredient in traditional herbal tonics, functional foods as well as often used in alcoholic beverages[1-2]. *C. reflexa* belonging to Convolvulaceae family, commonly known as amarbel or dodder or algushi or swarnalatha, is an enormously found traditional medicinal herb grows in Bangladesh [3]. This plant has been found most in tropical countries likeMalaysia, Thailand, Afghanistan, and Indian subcontinent such as Bangladesh, India, Pakistan, and Nepal [4]. Different sorts of phenolic constituents were found in *C. reflexa* extracts- glycosides, sterols, terpenes, unsaturated and saturated fatty acids, saturated aliphatic hydrocarbon[5-6]. The stem extract of *C. reflexa* containing phenol (23.49%) [7], kaempherol and quercitin [8]. Furthermore, many biological active compounds were isolated from this plant extract previously like myricetin, quercetin and kaempferol[9], which possess mainly antioxidant and antidiabetic activities. Moreover,some therapeutically active compounds also been reported like coumarin, α-amyrin, astragalin, linoleic acid, palmitic acid, isorhamnetol,oleic acid, luteolin, stearic acid, ß-sitosterol, n-hentriacontane, and sesamin [10]. The 4-vinylphenol isolated from this plant which is using as a flavoring agent[11]. The stem extract of this plant showed potent antibacterial, antioxidant, anti-inflammatory activities [3]. In addition, diuretic, anti-viral and anti-cancer [12], fever, diaphoretic, insanity, demulcent, melancholy, and fits effects also been claimed for traditional use [13]. Besides, several studies were investigated with the extract of *C. reflexa* for antioxidant[14], antitumor [15], anti-epileptic [16], antihypertensive [17], anti-arthritic and nephroprotective [18], antiobesity [19],antispasmodic[20], antibacterialand antifungal [21-22], hypoglycemic, hemodynamic, antiviral, and effects [23-26]. Traditionally, people in Bangladesh and Nepal are consuming this plant or extract using hot water for the therapeutical benefits from jaundice, tumor, skin infections, pain, and edema[27]. The present study was designed to characterize of total phenolic and flavonoids compositions using FTIR fingerprinting and in vitro antioxidant activity.

**2.0. Methods**

**2.1. Sample collection**

Fresh *C. reflexa* stems were obtained from Dhaka district, Bangladesh. The stems were manually separated and clean with tap water. The plant sample was kept in a laboratory dryer chamber at 25°C until fully dried for one week. It was crushed by using normal a blender, separating uniform size of samples using a fine mesh strainer No. 100 (Sigma-Aldrich, St. Louis, Missouri, 63103, USA) in 0.149 mm. The separated fine sample powder was packed in a sealed bag for further usage.

**2.2. Chemicals and reagent**

Ethanol (99.5% purity) was obtained from Thermo Fisher Scientific (81 Wyman Street Waltham MA, 02454, USA), gallic acid, quercetin, Folin–Ciocalteu reagent, 2,20-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate anhydrous, aluminium chloride salt, STZ, and Glibenclamidewere procured from Sigma-Aldrich (Sigma-Aldrich, St. Louis, Missouri, 63103, USA) and all other reagents and chemicals used in the present study were of analytical grade.

**2.2. Extraction of plant samples using Soxhlet technique.**

The*C. reflexa* stem powder sample (20 g) was placed in the Soxhlet extractor. Different concentrations of ethanol (30, 45, 60, 75, 90%) was employed with feed-to-solvent ratio (1:10, 1:15, 1:20, and 1:25 g/mL). The sample mixture was reflux by using heating mantle with different time point as 1, 2, 3 and 4 h [26]. After reaching the pre-determined extraction time, the extraction solution was left at 25°C to cool. The extraction solution was then filtered using filter paper and left in a beaker for some time. The mixture was further concentered to dry using a Buchi R-215 rotary evaporator (BUCHI Malaysia Sdn. Bhd. MY – 47301 Petaling Jaya, Selangor, Malaysia). The percentage of extraction yield was calculated based on the following equation Eq. (1):

$$\% Yield of extract= \frac{Weight of extracts (w1)}{Weight of dried sample (w2)}×100\%…………...(Eq.1)$$

**2.3. Determination of total phenolic content**

The Folin-Ciocalteu reagent procedure was employed to determine the TPC of *C. reflexa* stem extract with partial modification of previous method described by Alara et al. [26]. *C. reflexa* stem extract at 5 g/L (1 mL) and 200 μL of Folin-Ciocalteu reagent were mixed properly at room temperature. After 5 minutes, 0.2mM Na2CO3 (0.6 mL) solution was mixed well with the previously prepared mixture (Extract + Folin-Ciocalteu reagent). The absorbance was taken at 560 nm by UV-vis Spectrophotometer (Shimadzu UV-1800, Kyoto 604-8511, Japan). Subsequently, the mixture was kept at 25°C for two hours. TPC concentration of the extract was then measured using the gallic acid standard calibration curve (ranging from 50 to 500 mg/L). TPC was calculated as equivalent to mg GAE/g d.w. using Eq. (2).

$$TPC=\frac{c\*V}{m}……………………………………………………………..(Eq.2)$$

where c= TPC concentration (mg/L), V= volume (L) of solvent used in the extraction, and m= weight (g) of the dried sample used.

**2.4. Total flavonoid content in the extracts**

The quantitative determination of TFC for the extract of *C. reflexa* was done by using the methods described in the studies of Alara et al. [26]. In brief, an aliquot of 100 μL (1 g/L) plant extract and the same volume of 2% AlCl3 (100 μL) solutions were vigorously mix. Afterwards, mixture solution was permitted to place at 25°C for 60 min. Then supernatant was collected from previous mixture, absorbance measured at 560 nm with UV-vis Spectrophotometer (Shimadzu UV-1800, Kyoto 604-8511, Japan). TFC was calculated using Equation (3)

$$TFC=\frac{c\*V}{m}…………………………………………………………………….(3)$$

where c= TPC concentration (mg/L), V= volume (L) of solvent used in the extraction, and m= weight (g) of the dried sample used.

**2.5. *In vitro* antioxidant activities of the extract**

***2.5.1. DPPH assay***

Verification has been done on the hydrogen atom or donating ability of electron of extract from *C. reflexa* through DPPH assay [3]. Briefly, 0.1 mM DPPH was taken as a volume of 2000 μL, added with 200 μL of extract (or ascorbic acid). The mixture was then serially diluted to different concentrations ranging from 100 to 500 μg/mL. After incubating the mixture in a dark place for 30 minutes, the absorbance of the sample was measured at 560 nm. The IC50 of the sample was calculatedthrough the sample absorbance, where each measurement was done as triplicate. The data was represented as mean ± SD. The DPPH radical scavenging activity was determined according to Eq. (4).

$$\% DPPH inhibition= \frac{A\_{control-}A\_{sample}}{A\_{control}}×100\%…………………………….(4)$$

In the above equation, Acontrol = absorbance of solvent and DPPH solution, Asample = absorbance of *C. reflexa* extract and DPPH solution.

***2.5.2. ABTS assay***

Scavenging ability (ABTS) of *C. reflexa* extract or ascorbic acid was determined by previously described assay [26]. In brief, *C. reflexa* extract (150 μL) was serially diluted to different concentrations ranging from 100 to 500 μg/mL. Then, ABTS solution (285 μL) (2.45mM potassium persulfate solution and 7mM ABTS) was added into the extract solution. After incubating the mixture in a dark place for 120 minutes, the absorbance of the sample was measured at 734 nm. Afterwards, the IC50 of the sample was calculatedthrough the sample absorbance, where each measurement was done as triplicate. The data was represented as mean ± SD. The ABTS radical scavenging activity was determined according to Eq. (5).

$$\% ABTS inhibition= \frac{A\_{control-}A\_{sample}}{A\_{control}}×100\%…………………………………..(5)$$

In the above equation, Acontrol = absorbance of solvent and ABTS solution, Asample = absorbance of *C. reflexa* extract and ABTS solution.

**Statistical analysis**

Each experimental test and analysis were performed in triplicate. A statistical method analysis of variance (ANOVA, IBM, SPSS 20.0, Chicago, Ill., USA) with p< 0.05 considered as significance difference.

**3.0. Results**

**3.1. Determine the recoveries of Soxhlet extraction factorseffects on the yieldsTPC and TFC.**

It isdepending on the concentration of extraction solvent like ethanol and extraction duration. The extraction time and the concentration of ethanol are determined by the recoveries of extracts, TPC, and TFC from *C. reflexa* using the Soxhlet extraction technique.Duration of extraction is authoritative in minimizing cost and energy of the whole extraction process. Among the key factors, extraction time plays an important role in altering the recovery capacity of phenolic contents from herbal crude extract. The reason behind this is the overexposure of the plant sample on heating degrades the phenolic compounds. That is why, it is very important to fix the exact extraction duration to obtained maximum recovery of the targeted compounds. In this study, the Soxhlet extraction process was fixed with different concentration of ethanol at 1:10 g/mL feed-to-solvent ratio. The extraction time was varied as 1, 2, 3, and 4 hours. After completion of extraction time, the maximum yields of extract [Fig. 1. (A)], TPC [Fig. 1. (B)], and TFC [Fig. 1. (C)] were presented.



Fig. 1.Effects of extraction time and concentration of solvent (ethanol) on the recoveries of extracts% yields (A), TPC (B) and TFC (C) from *C. reflexa* Soxhlet extract. Data were expressed as mean±S.D.

**3.2. Identified functional groups in the Soxhlet extract.**

The functional groups of the compounds were selected from obtained peak number in FTIR chromatograph. The representative absorption peaks value is reported in Fig. 2.



Fig.2. FTIR spectra of *C. reflexa*extract using Soxhlet extraction with (a) 45%, (b) 60%, (c) 75% and (d) 90% ethanol.

The extract represents the phenolic component by the presence of a broad peak at 3471 cm− 1 with O-H bending. Moreover, few peaks were found that can be attributed to the existence of lipid-carbohydrate in the sample due to the presence of lipids usually assigned in the peak area at 3000-2000 cm−1 region and 1500-1200 cm−1 represent for carbohydrate. In the extract, the peaks at 2123, 2963, 2960 and 2953 cm−1 specify the existence of lipids and 1449, 1428, and 1300 and 1260 cm−1 for carbohydrate. The band at 1260 cm−1 illustrations the C–O groups of polyols existence which represent the hydroxy flavonoids in the extract. In addition, the sharp peaks were found at 1085, 1010 and 1043 cm−1 that indicate the existence of secondary alcohols or ester groups in tested extract. Aromatic ring vibration could be associated with the peak value at 877 cm−1. Glycosides, flavonoids, and carboxylic acid could be associated due to the existence of C=O stretching and N-H bending that possible peaks area at 1663 and 1607 cm−1 [26]. Patle et al. reported the band obtained at 1520 -1500 cm-1, 1449 –1400 cm-1 and 1260 –1200 cm-1 due to be NO2 bending vibration, C –O, and C – O –C of ester for presence of quercetin in the sample extract [27]. Moreover, CH2 asymmetric and symmetric stretching group was found at 2963 and 2853 cm-1 in the sample indicated ethanol [27].FTIR data revealed the characteristic of the fingerprintsof *C. reflexa*extract using the Soxhlet extraction process thatis reflected the existence of various functional groups related to flavonoids or polyphenols compounds.

**3.4. *In vitro* antioxidant activity of the extracts**

DPPH and ABTS•+ radical scavengers were used to examine the antioxidant properties of *C. reflexa* extract with maximum extraction operating conditions. The results were compared with standard (Ascorbic Acid) shown in the Table 1.

**Table 1.***In vitro* scavenging activities (DPPH and ABTS•+) of *C. reflexa* Soxhlet extract.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Sample | TPC(mg GAE/g d.w.) | TFC(mg GAE/g d.w.) | DPPHIC50 (μg/mL) | ABTS+•IC50 (μg/mL) |
| *C. reflexa* extract | 65.32 ± 1.07 | 40.10 ± 0.42 | 295.12 ± 1.33a | 245.43 ± 0.78a |
| Ascorbic acid | - | - | 110.30 ± 1.10b | 69.34 ± 1.21b |

a,bmeans ± SD (p < 0.05)

It has been found in several studies that the lowest IC50 value indicates the strong antioxidant effect. In this sense, the extract showed potent antioxidant in ABTS•+ assay with IC50 at 245.43±0.78 μg/mL compare to DPPH assay which showed IC50 at 295.12±1.33 μg/mL. The obtained data indicated the potent antioxidant properties of the extract. Dissimilar scavenging activities were observed between DPPH and ABTS•+ assay with the same extract. It might be due to the different assay followed by the different pathways to exhibit the antioxidant activities.

**4.0. Discussion**

There are numerous methods to recover of antioxidants enrichment of bioactive compounds from natural sources, i.e., cold maceration, Soxhlet extraction, supercritical fluid extraction, microwave assisted extraction, and ultrasound assisted extraction. However, percentage of yield extraction and antioxidant capacity not only vary on the extraction technique but also on the solvent used for extraction. The existence of a variety of antioxidant enrichment bioactive constituents with their polarities and diverse of chemical characteristics may or may not be soluble in a specific solvent [28]. Polar solvents are commonly employed for regaining polyphenols from plant materials. Although, methanol,acetone, ethanol, and ethyl acetate containing aqueous mixtures are considering the most suitable solvents for extraction of polyphenols. However, ethanol has been known as a good solvent for polyphenol extraction and is safe for human consumption [29-30]. Do et al. studied with 50%, 75% and 100% ethanol Soxhlet extracts, while 100% extracted sample showed the highest TPC at 40.5 mg gallic acid equivalent/g and TFC at 31.11 mg quercetin equivalent/g from*Limnophila aromatica* crude extract [31]. Baba et al. reported the TPC at 45.17 ± 1.70 gallic acid equivalents/gand TFC at 35 ± 2.20 rutin equivalents/g using100% methanol in Soxhlet extracted up-to 4 h [32]. The current study found that the 75% ethanol extract showed the highest amount of TPC (65.32 ± 1.07 gallic acid equivalents/g) and TFC at 3 h. However, the content recovery was drastically reduced at 4 h due to the maximum recoveries was obtained at 2 h and 3 h. The similar finding was reported by Tanruean et al. with 65.45 mg GAE/g extract of TPC in acetone extract of *C. reflexa* [33]. The possibility of bioactive compounds meeting extracting solvent expanded with increase amount of extraction solvent, leading to higher rates of contents [34]. However, the % yields of antioxidant rich active bioactive compounds will not continue to increase once equilibrium is reached. The solid-to-solvent ratio could considerably affect the equilibrium constant and considered the correlation between yield and solvent use as a steep exponential increase followed by a steady state to give the maximum yield [35]. Moreover, 75% ethanol extract with the highest antioxidant activity was observed by Turkmen et al. [36], which is in line with the current findings.

**5.0. Conclusion**

Studied found that the time of extraction and solvent concentration had major role in recoveries of extract, TPC and TFC in Soxhlet extraction process. *C. reflexa*extract exhibited potent scavenging activities compared to ascorbic acid, suggested its possible potential use as the natural antioxidant.

**Ethics approval and consent to participate.**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and materials**

All data and materials should be available upon request.

**Competing interests**

The authors declare that there is no conflict of interest.

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**Authors’ contributions**

A. K. A, conception and design of the work; A. K. A.; acquisition, analysis, interpretation of data; the creation of new software used in the work; provided funding acquisition, project administration, and resources and A. K. A.; wrote the paper A. K. A.; review, editingA. K. A.; All authors have read and approved the manuscript.

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**Plant authentication**

The plant was identified by a taxonomist in Bangladesh National Herbarium, Dhaka, Bangladesh with a voucher specimen (DACB Accession No. 41879)

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