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Original Article

Profound Assessment of Phytochemical, Botanical and Antioxidant Characteristics Including Determination of Total Phenolic and Flavonoid Contents of Stem Bark of *Cordia obliqua* L.

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ABSTRACT

Cordia obliqua is known as Bumber. Its fruits and leaves are popularly used because of antioxidant and anti-diabetic activities. The purpose of this study is to evaluate the phytochemicals and antioxidant activity as well as botanical features of stem bark of *Cordia obliqua*. Chemical components were preliminary screened in various fractions based on the polarities including; n-hexane, chloroform, ethyl acetate, butanol and aqueous fractions of using standard procedures. Total phenolic (TPC) and flavonoid (TFC) contents were assessed by Folin-Ciocalteau and aluminium chloride methods respectively. The antioxidant activity was evaluated by ABTS antioxidant procedure, using ascorbic acid as standard. Results confirmed that stem bark of *Cordia obliqua* chemically is characterized by the presences of flavonoids, alkaloids, steroids, saponins, tannins and carbohydrates at different levels in various fractions and the absence of cardiac glycosides and anthraquinones. Microscopically, the plant is characterized by presence of big Ca oxalate clusters, various types of xylem vessels and big amount of cork cells. TPC was ranged from 13.6±1.4 and 220.5±3.4 mg GAE/g dry plant extract and TFC was ranged from 0.029±0.12and 15.46±0.33 mg QE/g dry plant extract. Due to the high phenolic and flavonoid content in butanol and ethyl acetate fractions; results of antioxidant using ABTS assay showed high antioxidant activity with IC_{50} valued 11.84±1.2 µg/ml for butanol fraction and 14.81±1.1 µg/ml for ethyl acetate fraction. Taken together, the research work demonstrated the potential natural antioxidant value of the waste product stem bark of *Cordia obliqua*. The study endorses forthcoming work to isolate and identify the chemical constituents in stem bark of *Cordia obliqua*.

Key words: *Cordia obliqua*, Boraginaceae, Folin-Ciocalteau, DPPH, Total phenolic and flavonoid, ABTS.

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INTRODUCTION

Nature provides the humans with untapped sources including plants which are gifted arsenal of potential therapeutic constituents because of their potency and/or diversity in chemical structure. The flora of Saudi Arabia is considered as one of the richest area of Arabian Gulf countries and contains diversity of families and species of wild and cultivated plants. Several of these plants are well reputed for their therapeutic benefits and traditional uses.[1-4] Boraginaceae family includes more than 148 genera found as 2700 species. The genus *Cordia* is one of the most distributed members of this family. *Cordia* is a genus found as trees or shrubs which is widely distributed in warmer regions.[5] It comprises around 300 species.[6] Several compounds like flavonoids, triterpenes, tannins, alkaloids and fatty acids possessing wide range of bioactivities were isolated from different plant parts of *Cordia* species.[7] *Cordia obliqua* (Synonym *Cordia myxa*) of the family Boraginaceae is plant that grows worldwide including Saudi Arabia. *Cordia obliqua* is a tree of 3-7 meters in height. It has several common names. Locally, it is known as "Bumber" is popularly used for its efficacy in chest and urinary infections and many other recent records stated its antioxidant, anti- diabetic and hepatoprotective activities.[8-10] A literature survey indicated that, many studies revealed the identification of phytochemicals including; alkaloids, flavonoids, saponins and polysaccharides from leaves and fruits of *Cordia obliqua* L.[5,11,12] No records were found on the botanical and phytochemical profiling of stem bark of *Cordia obliqua*. Therefore, the present work is designed to investigate and highlight the botanical features and phytochemical profiling including determination of total phenolic and flavonoid contents of stem bark of *Cordia obliqua*.

MATERIALS AND METHODS

Materials

Stem bark of *Cordia obliqua* was collected from King Faisal University, Al-Hasa, Saudi Arabia (Figure 1). Stem bark was separated and subjected to air-drying according to standard herbarium procedures. A voucher sample (CO-2022) was kept in Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University. All reagents and chemicals were of the finest grade available .

Extraction

The air dried powdered stem bark (1.1 kg) was thoroughly extracted three times (for 1 week) using 10 liters of 70% methanol (Sigma Aldrich, Germany), applying cold maceration method to avoid destruction of active constituents .[13,14] Methanol extracts were compiled and concentrated using

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Figure 1: Photograph of tree of *Cordia obliqua* in gardens of King Faisal University.

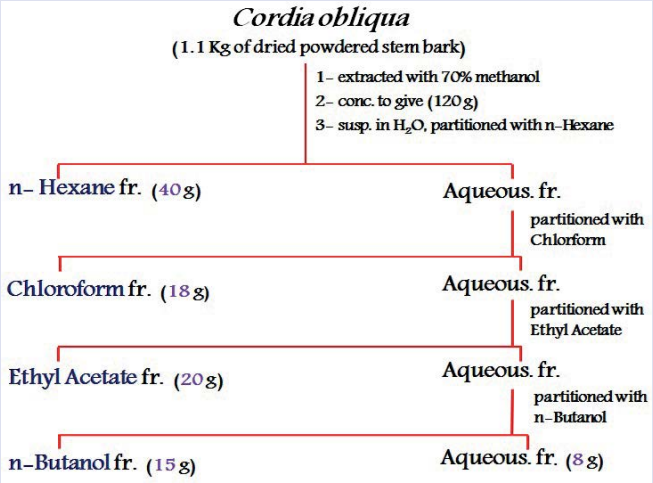

Figure 2: Extraction and fractionation of the stem bark of *Cordia obliqua*.

rotary evaporator and then freeze- dried to yield the total methanol extract of stem bark (120 g), which were kept in freezer for the next steps. The total methanol extract was mixed with 1 liter of distilled water and fractionated with n-hexane, chloroform, ethyl acetate and n-butanol (Sigma Aldrich, Germany), successively according to steps detailed in figure 2 .

Botanical study

Anatomical investigations were carried on cross sections prepared previously using microtome (SLEE, Germany) of stem bark. The photographs were captured for both macro-morphology and micro morphological features using digital camera (Canon IXY, 510-1S, Japan) and Olympus BX41 System Microscope connected to DP-25camera, Japan, respectively.

Phytochemical screening

Qualitative screening of available metabolites was carried out base on the standard protocols available in literature.[15,16]

Flavonoid

Part of corresponding extract (2 ml) was blended with 2 % NaOH (1ml). The production of intense yellow color and turned into colorless on addition of few drops of diluted acid, obviously refer to the presence of flavonoid.

Alkaloid

Part of corresponding extract (0.5.ml) was mixed with dilute HCl (1.5 ml) and followed by filtration. Few drops of Dragendorff's reagent was added to the filtrate and monitored. The presence of alkaloids is confirmed *via* the development of orange or orange-red precipitate.

Saponins

Part of corresponding extract (1 ml) was suspended in distilled H_2O (20 ml) and then the produced mixture was vigorously stirred. The formation of persistence froth for at least 15 min referred to the presence of saponins.

Steroid

Part of corresponding extract (0.5 ml) was dissolved in 5 ml $CHCl_3$ and few drops of acetic anhydride and concentrated H_2SO_4 were added from the side of the test tube. The upper yellow layer with green/blue color indicated the presence of steroids.

Tannins/Phenolics

About few drops of 5% $FeCl_3$ solution was added to 0.5 ml of corresponding extract gives intense blue-greenish indicating the presence of tannins/phenolics.

Glycosides

Part of corresponding extract (2 ml) was mixed Fehling's solution (A) and Fehling's solution (B) and reactants were heated on a water bath for about two minutes. After heating, it gives a brick-red color that indicated the presence of glycosides.

Anthraquinones

Part of corresponding extract (0.5 ml) was boiled with dilute H_2SO_4 then filtered and cooled. The filtrate was extracted with $CHCl_3$ and dilutes NH_3 solution was added to it. The aqueous NH_3 layer became pink to red due to the presence of anthraquinones derivatives.

Cardiac glycosides

Part of corresponding extract (10 ml) was mixed with (4 ml) of solution of glacial acetic acid and 1 drop of 2% $FeCl_3$ followed by 1 ml of concentrated H_2SO_4. A brown ring formed between the layers demonstrated the presence of cardiac glycosides.

Carbohydrates

Part of corresponding extract (2 ml) was mixed with a 10 ml Molisch reagent. Then, 2 ml concentrated H_2SO_4 was added from the side of the test tube. The formation of a violet ring at the intersection of two layers indicated the presence of carbohydrates.

Assessment of total phenolic constituents (ATPC)

ATPC was estimated according to the method described previously applying the protocol of Folin-Ciocalteu reagent.[17] For each of different fractions involved in the study, stock solutions with a concentration

of 1 mg/ml were prepared in methanol. A volume of 0.5 ml of Folin-Ciocalteu reagent in addition to 6 ml of distilled deionized water was successively supplemented to 0.1 ml of stock solution of each fraction. Then, a volume of 1.5 ml of 20% $NaCO_3$ solution was supplemented to complete a final volume of 10 ml of all reactants. The reaction was kept at 25°C for 2 h for completion. Finally, the absorbance of all experiments was measured at 760 nm. Calibration curve of gallic acid (standard) was prepared using serial dilution (0.5, 0.4, 0.3, 0.2 and 0.1 mg/ml in distilled water).

Assessment of total flavonoid constituents (ATFC)

ATFC was achieved in accordance to Khalil *et al* method with minor modifications.[18] A weight of 10 mg from each fraction involved in the study was mixed a volume of 100 ml of previously prepared co-mixture of distilled water and acetone (equal proportion). Serially diluted samples with volume of 0.25 ml were added to 0.75 µl of a $NaNO_2$ (5% w/v) solution, followed by (0.15 ml) of a freshly prepared 10% $AlCl_3$ solution. The previously developed mixture was mixed with 0.5 ml of 1 M NaOH solution. Then, the volume of developed mixture was completed to 10 ml with distilled water. The subsequent developed mixtures were set aside for 5 min. Finally, the absorbance of each developed mixture was observed at 510 nm against the same reactants but without the presence of tested sample. Calibration curve was prepared using quercetin as reference (0.0, 0.1, 0.5, 1, 5 and 10 µg/ml).

Antioxidant properties using ABTS method

The generation of the free radical of ABTS (2, 2'-azino-bis-(3-ethyl-benzothiazoline-6-sulfonic acid) was performed by addition of 7 mM ABTS with 2.45 mM potassium persulfate according to Ezaouine *et al* method with minor modification.[19] The reactant mixture was kept in dark at room temperature for 16 hours before its usage. Ethanol was added to the mixture of generated ABTS•1 free radical solution until an absorbance of 0.70 at 734 nm was achieved. Stock solutions (1 mg/ml) of standard (Sodium ascorbate) or corresponding extracts were made in distilled water and serial dilutions of 1, 5 and 10 µg/mL were prepared. A volume of 0.5 ml of each dilution was added to 2 ml ABTS free radical solution then kept to allow reaction for 30 min. Then, the absorbance was determined at 734 nm. A blank was prepared instead of sample or standard with 0.5 ml methanol. The percentage inhibition of free radical was calculated through the formula: Percentage of inhibition = [Ab.B-Ab.S)/ AbB] x100

Ab.B: absorbance of blank, Ab.S: absorbance of sample or standard. Each concentration of sample or standard was performed in triplicate and mean and standard error of mean were calculated and used in the establishment of calibration curves. Form these curves, linear regression was performed and 50% inhibitory concentration (IC_{50}) was calculated for samples and standard and used in comparison; the lower the IC_{50}, where the greater the antioxidant power.

RESULTS AND DISCUSSIONS

Phytochemical screening of different fractions

The preliminary screening for various chemical constituents in different fractions showed the presence of different chemical constituents such as flavonoids, alkaloids, saponins, steroids, tannins/phenols, glycosides and carbohydrates at different levels in different fractions and the absence of cardiac glycosides, anthraquinones as shown in Table 1.

Micromorphological characteristics of stem bark

The stem bark from *Cordia obliqua* was collected. A transverse cross section throughout the stem bark (Figure 3A) is characterized by presence of some cork cells which are radially arranged in several rows that may reach 5-6 rows and some are ruptured. They are polygonal, tangentially elongated cells with lignified walls, followed by a distinct layer of parenchymatous cells containing big calcium oxalate crystals. On the other hand, the powder is brown in color having a characteristic odor and a bitter taste. Microscopically, it is characterized by the presence of fragments of lignified xylem vessels, which are of spiral and circular thickening (Figure 3B) , calcium oxalate crystals (Figure 3C) and small parts of cork cells which are polygonal cells with lignified walls (Figure 3D).

Constituent/Fraction	nH	CH	EA	BT	AQ
Flavonoids Alkaline solution test	-	+	++	++	++
Alkaloids Dragendorff's reagent	-	+	+	+	+
Saponins Foam test	–	+	+	+	+
Steroids Liebermann-Burchard test	+	+	+	+	+
Tannins/Phenols 10% $FeCl_3$	+	+	++	++	+
Anthraquinones Borntrager's test	-	-	-	-	-
Cardiac glycosides Keller Killiani test	-	-	-	-	-
Glycosides Fehling's test	-	-	+	+	+
Carbohydrates Molisch's test	-	-	+	+	++

nH; n-hexane fraction, CH; chloroform fraction, EA; ethyl acetate fraction, BT; butanol fraction, AQ; remaining aqueous fraction, , Presence in high amount (++), presence in moderate amount (+), absence (-).

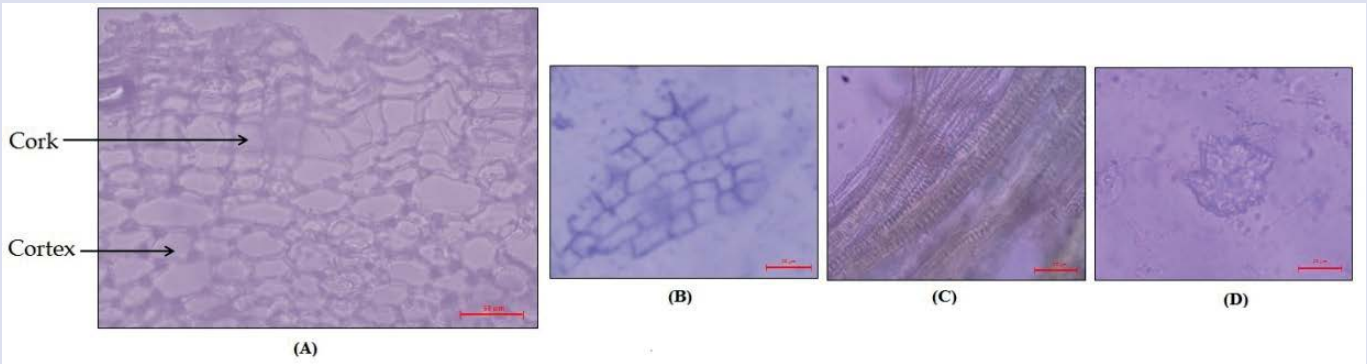

Figure 3: Micromorphology of the stem bark of *Cordia obliqua*. (A) Detailed sector (×200). (B) Cork cells top view (×400). (C) Xylem vessels (×400). (D) calcium oxalate crystals (×400).

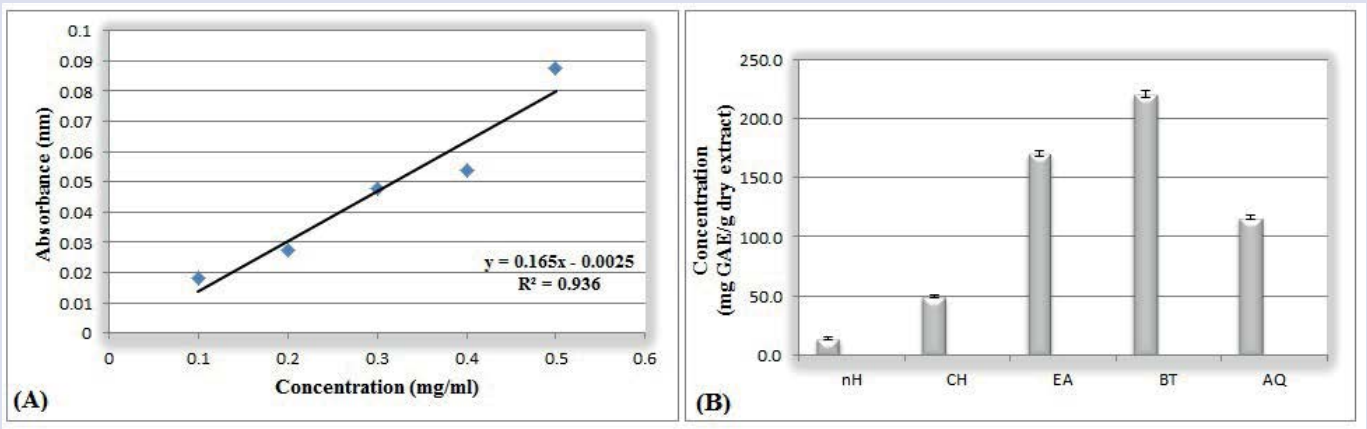

Figure 4: Calibration curve for gallic acid (A) and total phenolic contents (B) of various fractions of stem bark of *Cordia obliqua.* nH; n-hexane fraction, CH; chloroform fraction, EA; ethyl acetate fraction, BT; n-butanol, AQ; remaining aqueous fraction, GAE/g; Gallic acid equivalence per gram of dried plant extract.

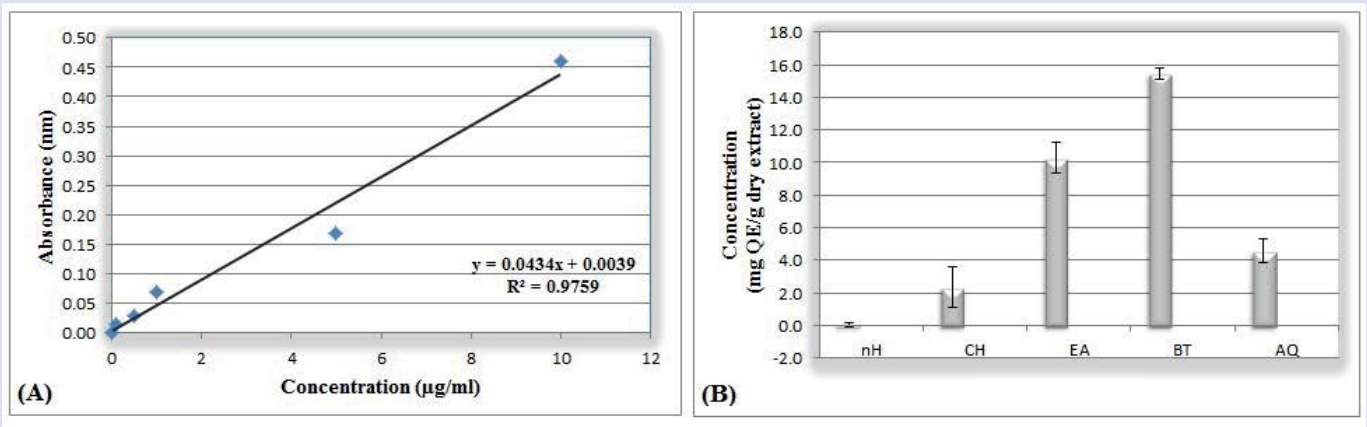

Figure 5: Calibration curve for quercetin (A) and total flavonoid contents (B) of various fractions of stem bark of *Cordia obliqua.* nH; n-hexane fraction, CH; chloroform fraction, EA; ethyl acetate fraction, BT; n-butanol, AQ; remaining aqueous fraction, QE/g; quercetin equivalence per gram of dried plant extract.

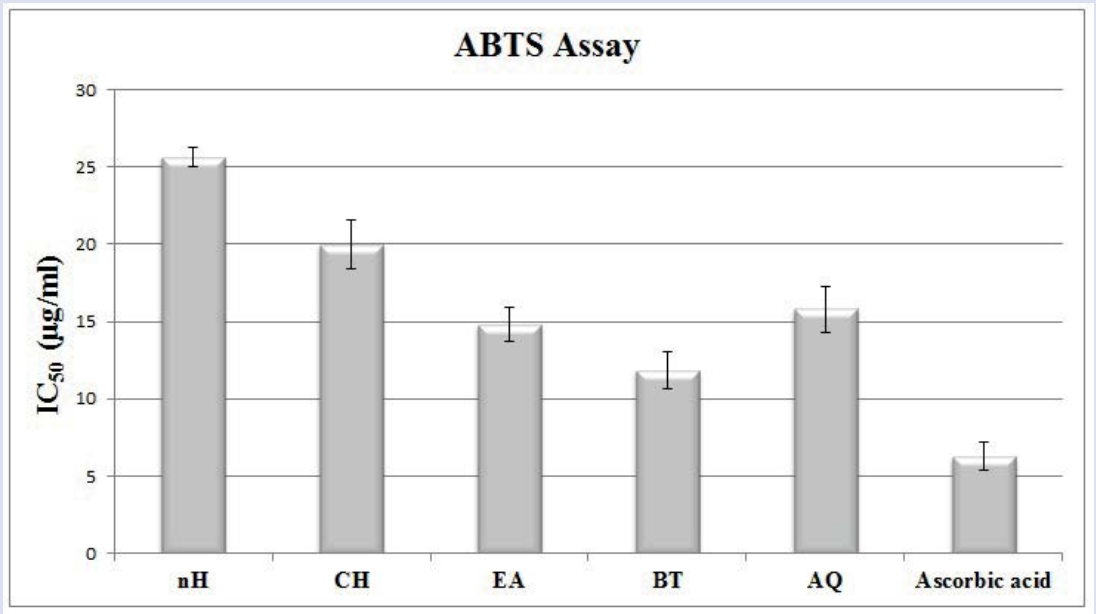

Figure 6: Antioxidant activity (ABTS) of various fractions of stem bark of *Cordia obliqua. nH*; n-hexane fraction, CH; chloroform fraction, EA; ethyl acetate fraction, BT; n-butanol, AQ; remaining aqueous fraction.

ATPC

ATPC in different extracts of different polarities was calculated using the regression equation: y = 0.165x+0.0025, R^2=0.936 . The quantity of phenolic constituents was expressed as the equivalence of milligrams of standard Gallic acid per gram of dried plant extract (mg GAE/g) (Figure 4A). Results demonstrated that the quantity of TPC ranged from (13.6±1.14) to (220.5±3.4) mg GAE/g of dry extract. Butanol fraction demonstrated the highest percentage of TPC followed by Ethyl acetate fraction. While, n-hexane fraction expressed very less phenolic constituents (Figure 4B).

ATFC

ATFC was analyzed using the regression equation: y= 0.0434x + 0.0039, R^2 = 0.9759. The results were expressed as the equivalence of milligrams of quercetin per gram of dried plant extract (mg QE/g) (Figure 5A). Results depicted that the amount of TFC ranged from (0.029±0.12) to (16.46±0.33) mg QE/g of dry extract. Similarly, to those of TPC, Butanol fraction fraction showed to be the richest fraction in flavonoid contents then ethyl acetate fraction. On the other hand, n-hexane fraction was hardly found to contain flavonoid constituents (Figure 5B). The results demonstrated that butanol fraction is worth of for future work to isolate the phenolic and flavonoid constituents.

Antioxidant activity (ABTS)

The ABTS assessment method describes the ability of an antioxidant moiety to scavenge the free radical created by the reagent in reaction media. Interestingly, the decrease in reaction media absorbance reflects the more antioxidant properties of the tested samples.[19] the resultant reduction in values of absorbance is expected due to the interaction between the antioxidant moieties and the produced free radical. The 50% inhibition concentration (IC_{50}) was calculated and used as a mean of assessment and comparison between those of antioxidant material with a well-established standard (Ascorbic acid). The results showed moderate ABTS scavenging activity of all extracts ranged from IC_{50} of 11.8±1.2 µg/ml to 25.6±0.6 µg/ml. Results were compared with IC_{50} of standard (Ascorbic acid) 6.3±0.9 µg/ml (Figure 6). The study demonstrated the efficiency of butanol fraction as free radical scavenger fraction compared to other fractions and its possibility to contain biomolecules that are active against free radicals and could be employed as antioxidant candidates.

CONCLUSION

The present work demonstrated that stem bark of *Cordia obliqua* is characterized chemically by presence of various constituents at different levels in different fractions. Microscopically, the plant is characterized by presence of big Ca oxalate clusters, various types of xylem vessels and big amount of cork cells. Besides, the stem bark is rich in phenolic and flavonoid, which was clear from the higher values of their corresponding mg GAE/g and mg QE/g per dry powdered extracts, hence, the stem bark expressed significant ABTS antioxidant importance. These notable findings provoke the researchers for future investigation to isolate and identify the potential chemical constituents and further biological investigations.

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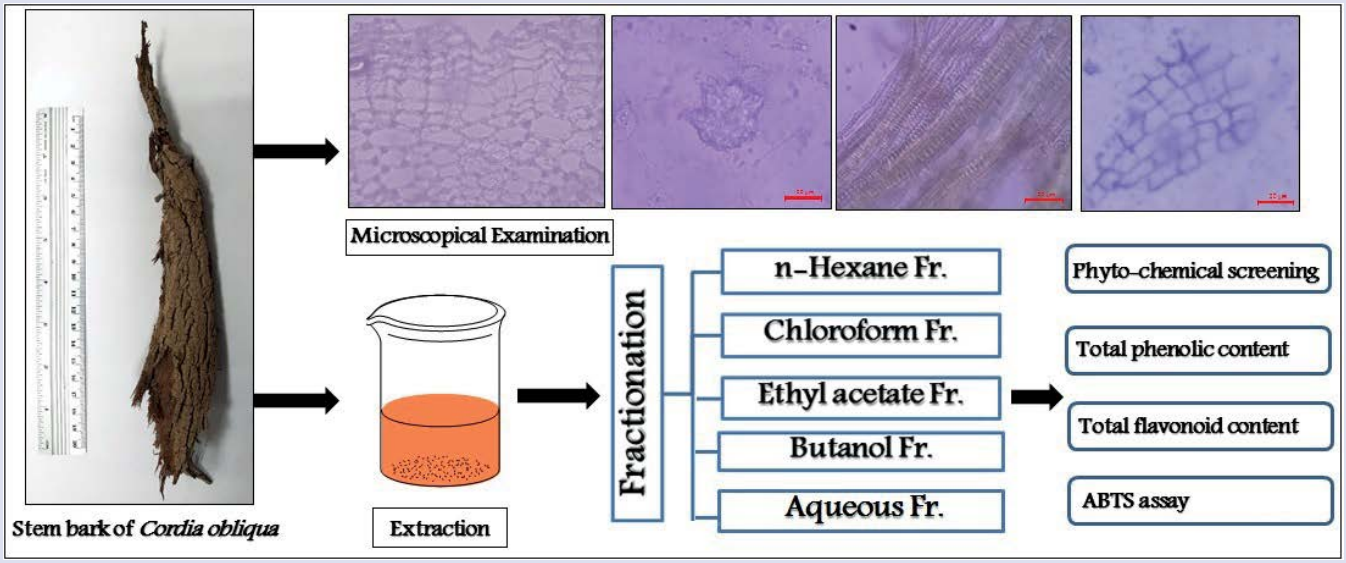
GRAPHICAL ABSTRACT



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