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Research Article

Antioxidant Study of Coffea Cruda

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Abstract

Background: Coffea cruda (family Rubiaceae) has great therapeutic significance in Indian system of medicine due to its rich antioxidant activity. Coffea cruda is the major source of antioxidant compounds as it has strong ability to inhibit the process of oxidation in the body which attributes to its high antioxidant activity. The main objective of the present work was to evaluate antioxidant study of Coffea cruda alcoholic extract.

Objectives: The present study was designed to investigate the HPTLC study and anti-oxidant activity of of Coffea cruda alcoholic extract.

Materials and methods: Anti-oxidant activity of alcoholic extract of Coffea cruda was determined by DPPH free radical scavenging activity, Total phenol, Total flavonoid content, ABTS and FRAP assay methods. Whereas, HPTLC study performed on precoated silica gel 60 F254 TLC plate where, mobile phase used was toluene: acetone (7:3, v/v). HPTLC UV detection was performed at 254 nm.

Results: The result revealed that different antioxidant assays of alcoholic extract of Coffea cruda seed shows prominent antioxidant activity and High performance thin layer chromatography study indicates the presence of alkaloid compound caffeine in chloroform extract of Coffea cruda.

Conclusion: The present study justifies Coffea cruda medicinal usage in traditional medicines. High antioxidant potentials are the reason for its cure and healing properties.

Keywords: coffea cruda; rich antioxidant; anti-oxidant

Introduction

Coffea cruda botanical name *Coffea arabica* belongs to the family of flowering plant Rubiaceae [1]. The family contains 13,500 species in about 620 genera. Also, ranks as one of the world most valuable and widely traded commodity crops. *Coffea cruda* has its origin in the highlands of Ethiopia and Boma plateau of Sudan. It is native to tropical and southern Africa and tropical Asia. In India it is widely grown and found in the states like Andhra Pradesh, Karnataka and Tamil Nadu. Its tree looks small and attains a height of five to ten meter. The colors of the leaves are green and flowers are white. Its fruits look like small fleshy drupes which changes color from bright green to yellow to scarlet red [2] (Figure1).

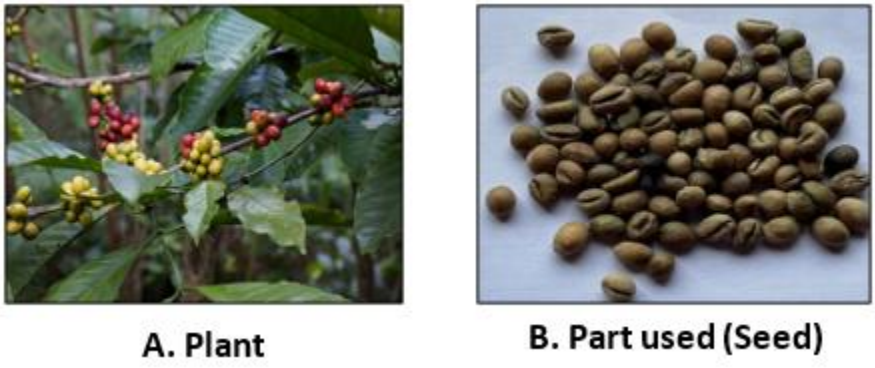

Figure 1: *Image of Coffea cruda plant and its fruit (seed)*

The fruit of the *Coffea cruda* plant i.e. its seed contain a large amount of caffeine [3]. *Coffea cruda* plant is rich in antioxidants [4]. The phytochemical constituents present in the *Coffea cruda* seed are alkaloid, flavonoid, phenol [5], terpenes, tannins and caretenoids which exerts antioxidant activities by quenching free radical production in the body [6-9]. The bitterness of *Coffea cruda* seed is due to the presence of alkaloid caffeine [10]. Caffeine has strong stimulating effect on the central nervous system of the body. The stimulating effect helps in increasing alertness in the body [(11)]. Alkaloid trigonelline is also present in *Coffea cruda* seed but in lower concentration. Green coffee i.e. (*Coffea cruda)* is rich in phytochemical constituents than the robust coffee i.e. (*Coffea tosta*) [12-13]. As in the process of robust, trigonelline starts to decompose and half of the trigonelline is lost in this process. Similarly *Coffea cruda* is also a rich source of acids such as citric acid, malic acid, chlorogenic acid [14] and quinic acid. Since, *Coffea cruda* is the major source of antioxidant compounds so it has strong ability to inhibit the process of oxidation in the body which attributes to its high antioxidant activity [15-17]. In homoeopathy *Coffea cruda* alcoholic extract is used for the treatment of asthma, racing thoughts in children and adults with attention deficit hyperactivity disorder (ADHD), headache, hyperaesthesia, heart, aural neuralgia, hernia, hysteria, labor pains, toothache, metrorrhagia, sciatica and insomnia [18-20]. It has great therapeutic implication in Indian system of medicine and exerts antiviral [21], antibacterial [22] and anti-inflammatory [23,24] effects. Antioxidant components are micro constituents that inhibit lipid oxidation by inhibiting the initiation or propagation of oxidizing chain reactions and involved in scavenging of free radicals [25]. In view of that, we designed the study to evaluate the antioxidant potential of *Coffea cruda* by various assay methods. Present study is helpful for determination and quantity content of antioxidant compounds which is useful for producing safer drugs for the treatment of common various ailments of human beings.

Methodology

Collection of Plant materials

The plant specimen *Coffea cruda* seed was collected authenticated from (hided for blinded review) herbarium for future reference. The seed were shade dried and is made powder mechanically and the fine powder was used for preparation of alcoholic extract. Caffeine ($C_8H_{10}N_4O_2$, M. P. 235°C) with purity by HPLC >99% w/w purchased from Sigma Aldrich, USA. Solvents used were ethanol, methanol, High performance liquid chromatography (HPLC) water and chloroform of analytical grade purity (Merck Ltd., India).

Physicochemical studies for raw drug standardization

Preparation and standardization of alcoholic extract /crude extract

100 g of coarsely dried powdered *Coffea cruda* seeds were taken, in which strong alcohol (95%) was added in sufficient quantity to make one thousand milliliters of the alcoholic extract using the percolation method (as per Homoeopathic Pharmacopoeia of India, Volume 1) [26].

Preparation of standard Caffeine

Dissolved 5 mg of caffeine in 5mL ethanol in volumetric flask and sonicated for 10 minutes to prepare working standard of caffeine with concentration 1mg/mL.

Preparation of chloroform extract

25 mL of alcoholic extract was taken in a 50 mL beaker. To remove the ethanol, solution was evaporated on water bath and extracted three times with 20 mL chloroform. Combined and concentrated chloroform extract upto 2 mL volume. Carried out TLC of chloroform extract and reference standard caffeine on silica gel 60 F_{254} pre-coated plate.

HPTLC fingerprinting profile study

HPLTC fingerprinting study was carried out following the methodology of High-Performance Thin Layer Chromatography for the analysis of Medicinal plant [27]. A densitometric HPTLC Camag Linomat 5 (Switzerland) instrument was used for the study. As a sample applicator Camag Linomat 5 was used for spotting TLC plate. Spots were made on silica gel 60 F_{254} pre-coated plate (Merck) 20 × 10 cm plate with an aid of sampling machine and solvent front was run up to 70mm height. For development of mobile phase, a saturating chamber Camag Twin Trough glass chamber was used. Camag TLC Scanner and software vision CATS were used in for scanning purpose. HPLC grade solvents were used for all the extracts solution. Volume applied for standard 1 to 6 µL and for sample 5-10 µL. Caffeine was used as reference standard. For detection of alkaloid caffeine various mobile phase was used chloroform: methanol (9:1, v/v), toluene: ethylacetate: diethylamine (7:2:1, v/v/v), chloroform: diethylamine (9:1, v/v) and toluene: acetone (7:3, v/v). TLC spots were visualized after illumination at 254 nm.

Study of Antioxidant Potential:

Determination of Total Phenolic Content (TPC):

The total phenolic content of the extracts was determined by Folin-Ciocalteu's reagent [29,30]. procedure reported by Singleton [30]. The total phenol content was estimated in *Coffea cruda* alcoholic extract. Ascorbic acid was used as reference standard. Different concentration (0.0665-8.517 mM) of ascorbic acid were prepared and analysed at 735 nm and calibration curve was plotted as absorbance versus concentration. Total Phenolic content was estimated by using ascorbic acid as standard, approximately 75 µl of the alcoholic extract was mixed with 5 mL of 10 % Folin-Ciocalteu's (phenol reagent) and 4 mL of sodium carbonate. The mixture was allowed to stand for one hour in dark. After one hour the color changed from yellow to blue. The absorbance of the solutions was measured at λmax 735 nm using a UV-VIS spectrophotometer (U. V Spectrophotometer SPECORD 200 plus Analytik Jena, Germany). The total phenolic content was calculated from the calibration curve and in final results total phenolic content of the *Coffea cruda* alcoholic extract was calculated as the ascorbic acid equivalents using standard ascorbic acid ($Y=0.1336x +0.0288$, R^2 0.9984) curve standardised in the lab for the calculation of ascorbic acid equivalent. Total Phenolic content was expressed in mM concentration of ascorbic acid equivalent.

Determination of Total Flavonoid Content (TFC):

Total flavonoid content was determined by aluminium chloride colorimetric assay method [31] using rutin hydrate as standard. An aliquot of 1 mL of the alcoholic extract mixed with 4 mL distilled water add 300 µl (5%) sodium nitrite in it. After five minute add 300 µl (10%) aluminium chloride then after five minute add 2 mL methanol and 2 mL (1M) sodium hydroxide then add 2.4 mL distilled water to make up the volume up to 10 mL. The mixture was shaken vigorously and left to stand in dark at room temperature. The resulting mixture color changed yellow to pink. The absorbance of the resulting mixture was determined using a UV-VIS spectrophotometer (U. V Spectrophotometer SPECORD 200 plus Analytik Jena, Germany) at λ max 510 nm. A standard calibration curve was constructed using rutin hydrate standard solutions of (31.25-500 µg/mL) of each standard was treated in the same manner as the samples above to generate calibration curve. The total flavonoid content of alcoholic extract was reported as rutin hydrate equivalents**,** using the following equation based on the calibration curve ($Y= 0.0148+0.0009*x$, $R^2=0.9960$).

Determination of ABTS assay:

Free radical scavenging activity of Coffea cruda alcoholic extract were determined by ABTS (2, 2-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) radical cation decolourization assay. $ABTS.^+$ cation radical was produced by the reaction between 7mM ABTS in water and 2.45 mM

potassium persulfate (1:1) stored in the dark at room temperature for 16 hours before use. $ABTS^{\cdot+}$ solution was then diluted with methanol to obtain an absorbance of 0.700 at 746 nm. After the addition of 10 µl of alcoholic extract or standard in 2 mL of diluted $ABTS^{\cdot+}$ solution, the absorbance was measured at 5 min after the initial mixing. An appropriate solvent blank (methanol) was run in each assay [32]. Percent inhibition of absorbance at 746 nm was calculated using the formula

$$\text{ABTS ion scavenging effect (\%)} = [(AB - AA) \times 100] \div AB$$

Where,

AB is absorbance of ABTS radical + methanol

AA is absorbance of ABTS radical+ sample/ standard.

Trolox was used as standard substance.

DPPH Radical Scavenging Assay:

The free radical scavenging activity of *Coffea cruda* alcoholic extract was measured by 2,2-diphenyl-1-picryl-ydraxyl (DPPH) radical scavenging assay [33-34]. The standard solution of DPPH was prepared by dissolving 0.025 g in 25 mL methanol and different concentration of standards/ alcoholic extract sample (100µl) was mixed with 4 mL methanol and 1 mL of DPPH solution. The mixture was allowed to stand for one hour in dark, after which the absorbance was measured at 515 nm using a UV-VIS spectrophotometer (U. V Spectrophotometer SPECORD 200 plus Analytik Jena, Germany). The percentage inhibition was determined by comparing the result of the test and the control (methanol used as solvent blank) [32]. Percentage degradation was calculated by the formula:

$$\text{DPPH radical Scavenging (\%)} = [(B - A) \times 100] \div B$$

Where,

A= absorbance of sample

B= absorbance of control

The inhibiting effects of alcoholic extract showed varied levels of DPPH radical scavenging activity, expressed as percentage degradation.

Ferric Reducing Antioxidant Potential (FRAP) Assay:

Ferric reducing power of alcoholic extract was determined by using FRAP assay[35-36]. This method is based on the reduction of colorless ferric complex (Fe^{3+} tripyridyltriazine) to blue colored ferrous complex (Fe^{2+} tripyridyltriazine) by the action of electron donating antioxidants at low pH. The reduction was monitored by measuring the changed of absorbance at 597 nm. The working FRAP reagent was prepared freshly by mixing 300 mM acetate buffer, with 1 volume of 10mM TPTZ (2, 4, 6-tri (2-pyridyl)-s-triazine) in 40mm HCL and with 1 volume of 20 mM ferric chloride in the ratio (10:1:1). 100 µl of alcoholic extract /different concentration standards were added to 3mL of prepared FRAP reagent. Then the absorbance of the samples was measured at 597 nm through UV-VIS spectrophotometer (U. V Spectrophotometer SPECORD 200 plus Analytik Jena, Germany) taking water as the reference after vortexing at 20 minutes. Also, a blank solution was prepared whose absorbance was also noted in the same intervals. The difference between absorbance of sample and the absorbance of blank was calculated and used to calculate FRAP value. FRAP value was expressed in terms of mM Fe^{2+}/g of sample using ferric chloride standard curve ($Y= 0.3358-0.9661^{*}x$), $R^2=0.9916$. All measurements were calculated from the value obtained from assays.

Results and Discussion

Result of HPTLC study:

In HPTLC study chloroform extract was used for extraction. For alkaloid determination various mobile phase were tried for extract. The satisfactory resolution obtained for the phytochemical constituent alkaloid was in the mobile phase toluene: acetone (7:3, v/v). Presence of alkaloid caffeine was confirmed with a very light brown spots observed at R_f 0.25 visible at UV 254 nm (Figure 2).

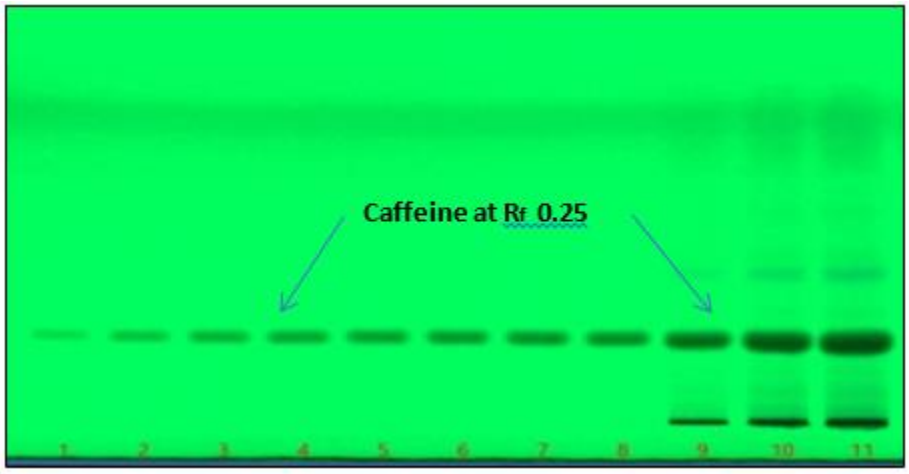

Figure 2: *High-performance thin layer chromatography fingerprints of Coffea cruda at UV 254nm. Standard caffeine Track (1-8), Track (9-11) chloroform extract of alcoholic extract.*

Result of Antioxidant activity:

Total Phenolic Content (TPC)

In the present study the total phenolic content of *Coffea cruda* alcoholic extract was determined by Folin–Ciocalteu method and reported as ascorbic acid equivalents. Study reveals total phenolic content found in *Coffea cruda* alcoholic extract was 2.29 AAE (Ascorbic acid equivalents) (Table 1).

S.No.	Sample	Concentration in (mM) of AAE	Absorbance
1	*Coffea cruda* alcoholic extract	2.29	0.3349
2.	Control	0.04	0.0233

Table 1: *Result of total phenolic content in alcoholic extract of Coffea cruda.*

Total Flavonoid content (TFC)

The total flavonoid content of *Coffea cruda* alcoholic extract was performed by $AlCl_3$ method and reported as rutin hydrate equivalents. Study reveals *Coffea cruda* alcoholic extract (1ml) contains 1062.22µg/ml amount of flavonoid compound (Table 2).

S.No.	Sample	Concentration in (µg/ml)	Absorbance
1	*Coffea cruda* alcoholic extract	1062.22	0.9929
2.	Control	0.41	0.0052

Table 2: *Result of total flavonoid contents in alcoholic extract of Coffea cruda.*

DPPH Radical Scavenging Assay

In the present study the DPPH assay of *Coffea cruda* alcoholic extract was determined by DPPH radical scavenging assay method and reported as ascorbic acid equivalents (AAE). Study reveals *Coffea cruda* alcoholic extract was able to decolorize DPPH free radical, the DPPH scavenging increased with the concentration of the extract. The result shows the DPPH scavenging activity found in *Coffea cruda* alcoholic extract was 46.89% (Table 3).

S.No.	Sample	Concentration in (mM)	Absorbance	% degradation
1.	*Coffea cruda* alcoholic extract	1.74	1.9380	46.89%
2.	Control	0.80	3.6491	-

Table 3: *Result of DPPH scavenging activity against alcoholic extract of Coffea cruda*

In DPPH assay a significant correlation coefficient (R, 0.9944) was found for antioxidant activity of alcoholic extracts of *Coffea cruda* alcoholic extract. The proton radical scavenging action is known to be one of the important mechanisms for measuring antioxidant activity. This assay determines the scavenging of stable radical species DPPH by antioxidants compounds present in the alcoholic extract. The results showed the greater rate of DPPH scavenging activity in alcoholic extract was probably due to the presence of high content of Phenolic compound. Our study clearly indicates that the *Coffea cruda* alcoholic extract exhibited high content of Phenolic compound i.e. 2.29mM which was significantly correlated with DPPH radical scavenging activity % i.e. 46.89%.

ABTS assay

In the present study the ABTS assay of *Coffea cruda* alcoholic extract was determined by ABTS assay method and reported in terms of Trolox equivalents. A significant correlation coefficient (R, 0.9901) was found for the antioxidant activity of alcoholic extracts of *Coffea cruda*. Study reveals *Coffea cruda* alcoholic extract was able to decolorize $ABTS^+$ free radical, the ABTS radical cation scavenging activity increased with the concentration of the extract. The result showed ABTS cation scavenging activity found in 10 µL volume of *Coffea cruda* alcoholic extract was 99.86% (Table 4).

S.No.	Sample	Concentration in (µg/ml)	Absorbance	% degradation
1.	*Coffea cruda* alcoholic extract	269.65	0.0004	99.86
2.	Control	41.76	0.2832	-

Table 4: *Result of ABTS radical cation scavenging activity against alcoholic extract of Coffea cruda.*

Ferric Reducing Antioxidant Potential (FRAP) Assay

FRAP assay measured the reducing potential of an antioxidant reacting with ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and producing a colored ferrous tripyridyltriazine (Fe^{2+}-TPTZ) [37]. The reducing properties associated with the presence of compounds exert their action by breaking the free radical chain through donating a hydrogen atom [38]. FRAP showed positive correlation between reducing power and phenolic content in *Coffea cruda* alcoholic extract. The FRAP value obtained for *Coffea cruda* alcoholic extract was 3.33 equivalent mM of Fe^{2+}/g of sample. The result shows strong correlation between total phenolic content and FRAP assay (Table 5).

S.No.	Sample	Concentration in (mM) of AAE	Absorbance
1	*Coffea cruda* alcoholic extract	3.33	3.5548
2.	Control	0.08	0.4151

Table 5: *Result of FRAP assay against Coffea cruda alcoholic extract.*

Earlier research work [39-40] also reported that phenolic compounds have redox properties which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers. The redox potential of phenolic compounds played an important role in determining the antioxidant potential.

Conclusion:

In this study HPTLC study confirms the presence of alkaloid i.e. caffeine in alcoholic extract of C*offea cruda* seed. Whereas antioxidant activity of alcohol extract of *Coffea cruda* seed was investigated by total phenol, total flavonoid content, DPPH, ABTS and FRAP (ferric reducing antioxidant potential) assay methods. Results shows, *Coffea cruda* seed is a natural source of antioxidant substances of high importance. The high contents of phenolic compounds and significant linear correlation between the values of the concentration of phenolic compounds and antioxidant activity indicates that these compounds contribute to the strong antioxidant activity of *Coffea cruda*. The present study demonstrates that the studied alcoholic extract of *Coffea cruda* seed has significant concentration of polyphenols. The high polyphenol content correlated with the significant antioxidant activity can be the explanation for the beneficial effect of *Coffea cruda* seed in treatments. There is essential need for the detailed physicochemical, phytochemical and HPTLC finger printing study to start work on quality standard of traditional medicines. The present study helped in further research development work which will increase the usefulness of the plant *Coffea cruda* in alternative system of medicine. Quantitative estimation of rest of the compounds present in this plant responsible for its other pharmacological activity will be evaluated in future studies.

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