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Research Article

Screened Phytochemicals of *A. esculentus* Leaves and their Therapeutic Role as an Antioxidant

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ABSTRACT

Medicinal plants are valuable natural source for the development of potentially safe drugs. The biological activities associated with these plants are due to the presence of certain phytochemicals which act either individually or synergistically. Hence, the present study deals with phytochemical screening and assessment of antioxidant activity of aqueous extract of *Abelmoschus esculentus* leaves using different assays viz. total phenolics, total flavonoids, total flavonols, reducing power and free radical scavenging capacity for 1,1-diphenyl-2-picrylhydrazyl (DPPH), NO and superoxide anion radicals. The phytochemical analysis of the extract revealed the presence of alkaloids, coumarins, flavonoids, glycosides, saponins, steroids, tannins and terpenoids. The total phenolics, flavonoids and flavonols present in the *Abelmoschus esculentus* leaf extract were 9.61 mg of Gallic Acid Equivalent (GAE)/g, 9.25 mg of Quercetin Equivalent (QE) /g and 6.12 mg of QE/g of dry extract, respectively. Reducing power of the extract was found to be concentration-dependent as it increase with increase in concentration and it was maximum at the highest evaluated concentration of 80 µg/ml. Significant antioxidant efficacy of *A.esculentus* leaves was further confirmed by IC_{50} values of DPPH˚, NO˚ and superoxide anion radical scavenging assays as it was found to be 53.96, 59.15 and 52.50 µg/ml respectively. Since, the results were almost at par with, Ascorbic acid taken as reference therefore, the aqueous extract of *A. esculentus* leaves, having therapeutically important phytochemicals, could be developed not only as an antiaging agent but also as an agent for managing oxidative stress due to diabetic compilcations.

Keywords: *Abelmoschus esculentus*, Phytochemical, antioxidant, radical scavenging activity.

INTRODUCTION

Reactive oxygen species (ROS) are continuously produced in the system being essential for various biological processes but their high levels, due to an imbalance between formation and neutralization of these ROS, causes oxidative stress leading to cell damage[1,2]. Antioxidant agents protect the cell against such damages caused by ROS/free radicals. These antioxidants may be either natural or synthetic[3]. Medicinal plants play vital role in the discovery and development of natural antioxidants with improved efficacy and lower toxicity over synthetic agents. Thus, the Natural antioxidant are always found cost effective and safe. Various medicinal plants as Natural antioxidant have already been reported in ethnomedical literature[4]. Phytochemicals which are secondary metabolites of plants viz tannins, alkaloids, carbohydrates, terpenoids, steroids, flavonoids and phenols are responsible for their bioactivities such as antimicrobial[5], antidiabetic[6] and antioxidant[7] etc. *Abelmoschus esculentus* is a flowering plant commonly known as 'Bhindi' in Hindi, belongs to the family Malvaceae, valued for its edible fruits and therapeutic importance[8]. Hence, the present study deals with the phytochemical screening of *Abelmoschus esculentus* leaves and assessment of their antioxidant potential since its seeds have already been reported for its antioxidant effect[9,10]. Whereas, its leaves are yet to be explored for assessment of their antioxidant efficacy and therefore it is of our choice for the present study.

MATERIALS AND METHODS

Plant material

Fresh leaves of *Abelmoschus esculentus*(500g) were collected from the local area of Allahabad-U.P. (India) and authenticated by Professor Satya Narayan, Taxonomist Department of Botany, University of Allahabad, India. Collected leaves of *A. esculentus* were washed with distilled water and dried completely under shade. The shade dried leaves (150g) were mechanically crushed, powdered and preserved for further use.

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Trichloroacetic acid (TCA), Nitro blue Tetrazolium (NBT), and Quercetin were purchased from Alfa Aesar Pvt. Ltd.. Nicotinamide adenine dinucleotide (NADH), Phenazonium Methosulphate (PMS), Folin-Ciocalteau reagent were purchased from Merck India Pvt Ltd. Sodium phosphate dibasic (Na_2HPO_4), Sodium phosphate monobasic (NaH_2PO_4) were purchased from Hi Media Laboratories and Potassium ferricyanide, Ascorbic acid, Sodium acetate, $AlCl_3$, $FeCl_3$ were purchased from Sisco Research Laboratories Pvt. Ltd,

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India. All other chemicals used including the solvents were of analytical grade.

Preparation of Extract

The dried leaves (150g) were extracted with distilled water (500ml) repeatedly till there was no coloration. All fractions were collected, filtered and concentrated on rotary evaporator at 40̊ C - 50̊ C under reduced pressure to get a semisolid material which was then lyophilized the yield dark brown powder (~13.5% w/w).

Chemical test for Screening of Phytoconstituents

Screening for the phytoconstituents of aqueous extract of *A. esculentus* leaves was carried out using standard methods[11-15] as given below:

Antioxidant Assays - in vitro

Antioxidant study of *aqueous extract of Abelmoschus esculentus* leaves was carried out using various in vitro assays viz estimation of total phenolics, flavonoids and flavanols in addition to reducing power, and free radical scavenging capacity for DPPH, NO and superoxide anion radicals. All the assays were carried out in triplicate and their average values were taken into consideration.

Estimation of Total Phenolics

Total phenolic content was estimated spectrophotometrically using Folin–Ciocalteu reagent[16]. Different concentrations of the Extract and Gallic acid, taken as standard, ranging from 25 to 400µg/mL, were prepared in 60:40 acidified methanol/water (0.3% HCl). 2.0 mL of Na_2CO_3 (2%) was added to100 µl of each test solution. After 2 minutes of incubation, 2.5 ml of Folin-Ciocalteau reagent (diluted with water 1:1 v/v) was added and allowed to stand at room temperature for 30 minutes. Absorbance was measured at 750 nm. Gallic acid was used for standard curve. The total phenolic content of the sample was estimated by comparing with the standard calibration curve and was expressed as mg/g of Gallic Acid Equivalent (GAE).

Estimation of Total Flavonoids

Total flavonoid content was estimated using the method of Ordon.et.al[17]. Different concentration of the Extract and Quercetin as standard ranging from 5 to 25µg/mL were prepared. 0.5 µl of ethanolic $AlCl_3$ (2%) was added to 0.5 µL of each test solution. Appearance of yellow colour indicated the presence of flavonoids after 1 hour of incubation at room temperature. The absorbance was measured at 420 nm. Quercetin was used for standard curve. The total flavonoid content of the sample was estimated by comparing with the standard calibration curve and was expressed as mg/g Quercetin Equivalent (QE).

Estimation of Total Flavonols

Total flavonol content was estimated by the method of Oyaizu[18]. Different concentration of the Extract and Quercetin as standard ranging from 5µg/mL to 25µg/mL, were prepared. 0.5 µl of 2% $AlCl_3$ in 95% ethanol was added to 0.5 µl of each test solution. After 2.5 hours of incubation at 20˚C the absorbance was measured at 440 nm. Quercetin was used for standard curve. The total flavonol content of the sample was estimated by comparing with standard calibration curve and was expressed as mg/g Quercetin Equivalent (QE).

Determination of Reducing Power

Reducing power was estimated by the method of Oyaizu[19]. An aliquot of 1.0 ml extract at various concentrations, ranging from 10 to1000 µg/ml, was mixed with 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated at 50 ℃ for 20 min followed by addition of 2.5 ml of trichloroacetic acid (10%) and then centrifuged at 3000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 0.5 ml of $FeCl_3$ and then absorbance was measured at 700 nm. Ascorbic Acid was used as standard.

Free radical Scavenging Assays

These assays were carried out for DPPH, Nitric oxide and Superoxide anion radicals and the ability to scavenge these radicals was calculated in terms of % inhibition using the following formula:

Inhibition (%) = $[(A_0 – A_1)/A_0] \times 100$

where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample. The lower the value of absorbance of the sample, the higher the radical scavenging ability will be.

DPPH Radicals

Ability of the Extract to scavenge the stable DPPH radical was evaluated by using the method of Mensor[20]. For this assay 0.3 mM methanolic solution of DPPH was prepared, and 1 mL of this solution was added to 1ml of different concentrations ranging from 20-1000 µg/ml of the Extract as well as Ascorbic acid as standard. The mixture was then shaken vigorously and allowed to stand for 30 min at room temperature in dark and finally absorbance of each sample was measured at 517 nm.

Nitric Oxide Radicals

Scavenging of Nitrosyl radical was determined by incubating 0.5ml of 5 mM SNP in10mM PBS (Phosphate Buffered Saline), with different concentrations ranging from 20-1000 µg/ml of the Extract as well as Ascorbic acid as standard. After 150 min, the incubation solution was mixed with 500µl of Griess reagent[21] and then the absorbance was measured at 540 nm, once, pink colour appeared.

Superoxide Anion Radicals

Superoxide anion radicals were generated by the PMS/NADH system according to the method of Kakkar et al.[22]. The reaction mixture was composed of 1 ml of NBT (156 µM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH (468µM in 100 mM phosphate buffer, pH 7.4) and 100 µl of varied concentrations ranging from 20-1000 µg/ml of the Extract as well as ascorbic acid as standard. The reaction was started by addition of 100 µl of PMS (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. After 5 min incubation at 25ºC, absorbance was measured at 560 nm against an appropriate blank to determine the quantity of formazan generated.

RESULTS AND DISCUSSION

Screened Phytoconstituents and their Bioactivities

Table 1, shows the correlation between phytochemicals and bioactivity of *aqueous extract of Abelmoschus esculentus* leaves. The data reveals the significant

Phytoconstituents	Colour Tests	Observation
Alkaloids *(Hager's Test)*	2ml extract + few drops of Hager's reagent	Yellow precipitate
(Wagner's test)	2 ml extract+ 2 drops HCl (1.5%) + 3 drops Wagner's reagent	Brown precipitate
(Mayer's test)	2 ml extract (EtOH) + few drops Mayer's reagent	Yellow precipitate
Anthraquinone (Borntrager's Test)	3ml extract + 3ml Benzene + 5ml NH_3 (10%)	Pink, Violet or Red colour
Anthocyanins	2ml extract + 2ml HCl (2N) + NH_3	Pinkish Red to Bluish Violet colour
Carbohydrate (Molisch's Test)	2ml extract (EtOH) + 10ml H_2O + 2 drops ethanolic α-naphthol (20%) + 2 ml conc.H_2SO_4	Reddish Violet ring at the junction
(Fehling's test)	2 ml extract + 1 ml of Fehling's solution A and B + heat	Red precipitate
Coumarins	2ml extract + 3ml NaOH (10%)	Yellow colour
Emodins	2ml extract + 2ml NH_4OH + 3ml Benzene	Red colour
Flavonoids	1ml extract + 1ml $Pb(OAc)_4$ (10%)	Yellow precipitate
Glycosides (Liebermann's Test)	2ml extract + 2ml $CHCl_3$ + 2ml CH_3COOH	Violet to Blue to Green colour
(Salkowski's Test)	2ml extract + 2ml $CHCl_3$ + 2ml conc. H_2SO_4	Reddish Brown ring at the Junction
Leucoanthocyanins	5ml extract + 5ml Isoamyl alcohol	Organic layer turns into Red colour
Phlobatannins (Precipitate Test)	2ml extract + 2ml HCl (1%) + boil	Red precipitate
Proteins (Xanthoproteic test)	1ml extract + 1ml conc. H_2SO_4	White precipitate to Yellow on heating
(Biuret's test)	1 ml extract + 5-6 drops w/v NaOH + 2 drops $CuSO_4$(30%w/v)	Violet Red colour
Saponins (Foam Test)	5ml extract + 5ml H_2O + heat	Froth appearance
(Emulsion test)	5ml extract + Olive oil (few drops)	Emulsion formation
Steroids (Salkowski's Test)	2ml extract + 2ml $CHCl_3$ + 2ml conc. H_2SO_4	Reddish Brown colour at interface
Tannins (Braymer's Test)	2ml extract + 2ml H_2O + few drops of $FeCl_3$ (5%)	Green colour
Terpenoids	2ml extract + EtOH+2ml $CHCl_3$+ Δ (2 mint.) 3 drops conc. H_2SO_4	Deep red colour

presence of alkaloids, carbohydrate, coumarins, flavonoids, terpenoid and tannins in comparison to saponin, steroid and glycosides which were present in lesser extent. Whereas anthocyanins, anthraquinones, emodins, leucoanthocyanins, phlobatannins and proteins were totally absent. Since all these secondary metabolites are associated with various biological activities[23] therefore, the antimicrobial activity of *A. esculentus* leaves[24] must be due to the presence of alkaloids as they are ranked the most efficient therapeutically significant plant substance used as a basic medicinal agent for its bactericidal effect[25]. The presence of coumarins can be accounted for antiproliferative activity[26] of *A. esculentus* leaves on the basis of its anti-inflammatory and antimicrobial activities, whereas the presence of carbohydrate, coumarins and glycosides are beneficial for the action of immune system by increasing the strength of body and hence these are valuable as dietary supplements[27]. The other important bioactivities of *A. esculentus* leaves viz. antioxidant[28,29] and anticancer[30] are solely due to the presence of flavanoids, as a potent water soluble antioxidant and free radical scavenger, which prevent oxidative cell damage and also have strong anticancer activity. It manages diabetes induced oxidative stress as well. Since, terpenoids are well known for their number of bioactivities[31,32] therefore its presence in *A. esculentus* leaves must be responsible for their antioxidant[33,34], hypoglycemic[35], antimicrobial[36] and anticancer[37] activities. Tannins are polyphenolic compounds and their derivatives are also considered as primary antioxidants or free radical scavengers[38].The presence of Saponins due to their general characteristics of cholesterol binding property[39] and presence of steroids due to their cholesterol reducing property[40] collectively enhance the therapeutic efficacy of the aqueous extract of *Abelmoschus esculentus* leaves.

Antioxidant Assays - in vitro

Table 2, shows the results of quantitative estimation of total phenolics, flavanoids and flavanols. Standard curve equation of Gallic acid was calculated with y = 0.0032x + 0.111, R^2= 0.941 for total phenolics. Whereas, standard curve equation of Quercetin was calculated with y =

Table 1: Screened Phytoconstituents and their Bioactivities.

Phytoconstituents	A. esculentus leaf	Bioactivities
Alkaloids	++	Antimicrobial[25]
Carbohydrate	++	Dietary supplement[27]
Coumarins	++	Antiproliferative activity[26]
Flavonoids	++	Anticancer[30], Antioxidant[28,29]
Glycosides	+	Dietary supplement[27]
Saponins	+	Cholesterol binding property[39]
Steroids	+	Cholesterol reducing property[40]
Tannins	++	Free radical scavenger[38]
Terpenoids	++	Hypoglycemic[31,32]
Anthocyanins	-	
Anthraquinones	-	
Emodins	-	
Leucoanthocyanins	-	
Phlobatannins	-	
Protein	-	

0.049x-0.325, R^2 = 0. 896 for total flavanoids and with y = 0.032x+ 0.002, R^2 = 0.998 for total flavanols. Since, Antioxidant efficacy is directly proportional to the quantity of total phenolic content due to the ability of their hydroxyl groups to scavenge free radicals, therefore the total estimated phenolic content of 9.65mg/g GAE must be contributing towards the significant antioxidant potential of the Extract[41]. Flavonoids are important secondary metabolite of plants and play vital role in modulating lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis. Since, pharmacological effect of flavonoids is associated with their antioxidant activities[42] therefore the total estimated flavonoid content of 9.25 mg/g QE must be responsible for the significant antioxidant efficacy of the Extract. Flavanol is another important phytoconstituent which helps in treating cardiovascular diseases caused by oxidative stress. Hence, estimated flavanol content of 6.12mg/g QE must be attributing to the antioxidant profile of *A. esculentus* leaves[43]. Thus, the extent of significant presence of total phenolics, flavanoids and flavanols may attribute to the antioxidant and free radical scavenging potential of the aqueous extract *A. esculentus* leaves.

Assesment of Reducing Power

Fig.1, depicts the results of Reducing Power of varied concentrations of aqueous extract of *Abelmoschus esculentus* leaves and of Ascorbic acid taken as standard. The reducing power of both, the sample and the standard were found to be concentration-dependent as the absorbance recorded has increased with increase in concentration. Moreover, the reducing power of the *A. esculentus* was found to be significantly greater than that of the standard, at the highest evaluated concentration of 800 µg/ml, confirming thereby the antioxidant potential of *A. esculentus* leaves. The highly significant reducing power of *A. esculentus* leaves have which is even greater than that of the standard, Ascorbic acid must be phenolic contents mediated. Thus, the reducing capacity of the extract is related to the presence of electron-donating reductants which react with free radicals and convert them to stable products resulting into terminate radical chain reaction.

Assessment of Free radical Scavenging Activity

DPPH radical scavenging activity

Fig.2, exhibits the results of DPPH radical scavenging activity of both, the Extract and the Standard, Ascorbic acid, at varied range of concentration. Results depict that the scavenging ability in both the cases was concentration dependent and was found to be maximum at a concentration of 80µg/ml with inhibition of 81.73% in case of Extract and 96.47% in case of Standard, validating thereby the significant antioxidant potential of *A. esculentus* leaf extract. The IC_{50} values of the Extract and the Standard were found to be 53.96 and 32.25 µg/ml respectively, which further confirmed that *A. esculentus* leaves could be developed as an effective antioxidant agent.

NO radical Scavenging Activity

Fig.3, exhibits the results of NO radical scavenging activity of both, the Extract and the Standard, Ascorbic acid, at varied range of concentration. Results depict that the scavenging ability in both the cases was concentration dependent and was found to be maximum at a concentration of 80µg/ml with inhibition of 77.59% in case of Extract and 85.99% in case of Standard, validating thereby the significant antioxidant potential of *A. esculentus* leaves extract. The IC_{50} values of the Extract and the Standard were found to be 59.15 and 50.19 µg/ml which further confirmed that *A. esculentus* leaves could be developed as an effective antioxidant agent. With the help of this data, selected medicinal plant could be further explored for its therapeutic efficacy as an antioxidant.

Superoxide anion radical scavenging activity

Fig.4, exhibits the results of Superoxide anion radical scavenging activity of both, the Extract and the Standard at varied range of concentration. Results depicts that the scavenging ability in both the cases was concentration

Table 2: Quantitative Estimation of Total Phenolics, Flavonoids and Favonols of aqueous extract of *Abelmoschus esculentus* leaves.

Dry exrtract	Total Phenolic (mg/g GAE)	Total Flavonoid (mg/g QE)	Total Flavonol (mg/g QE)
Abelmoschus esculentus	9.65±0.08	9.25±0.05	6.12± 0.14

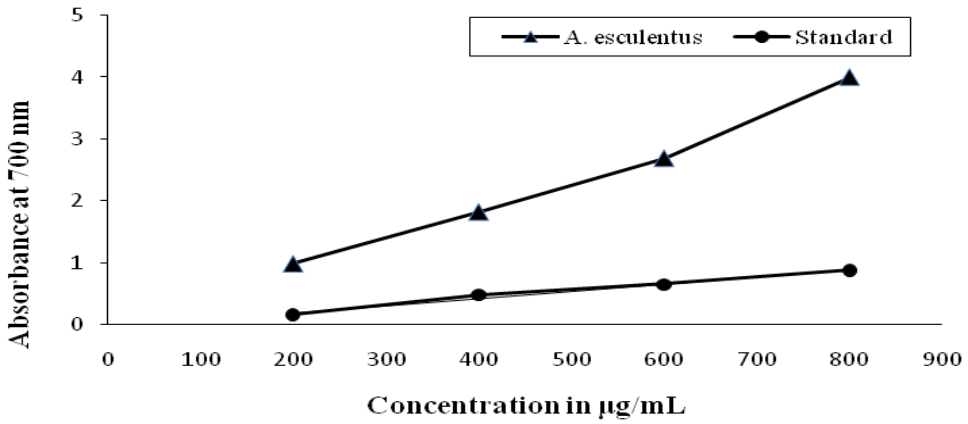

Figure 1: Reducing Power of *A. esculentus* leaves and Ascorbic acid, the standard.

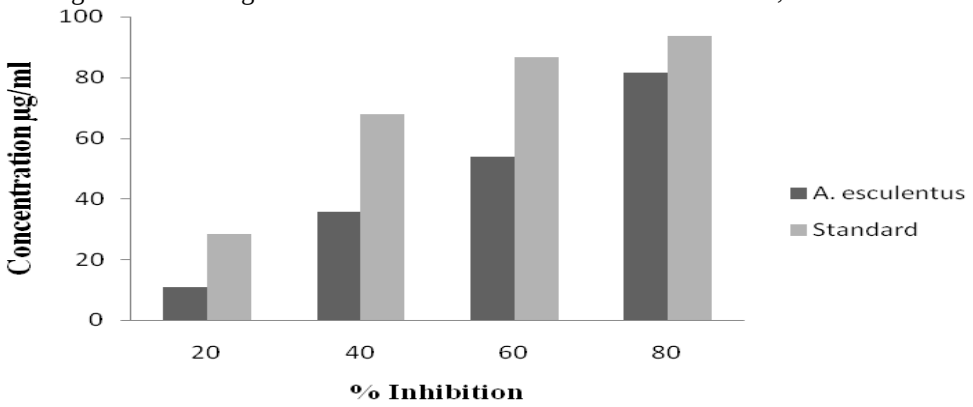

Figure 2: DPPH radical Scavenging Activity of *A. esculentus* leaves and Ascorbic acid.

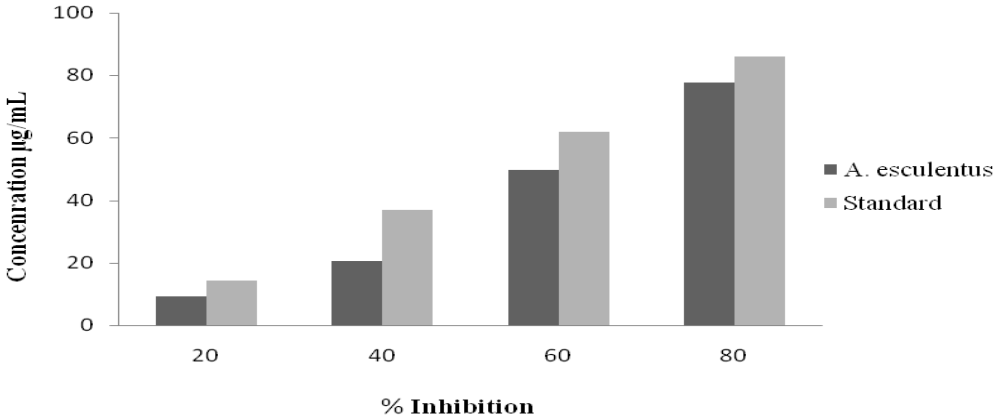

Figure 3: NO radical Scavenging Activity of *A. esculentus* leaves and Ascorbic acid.

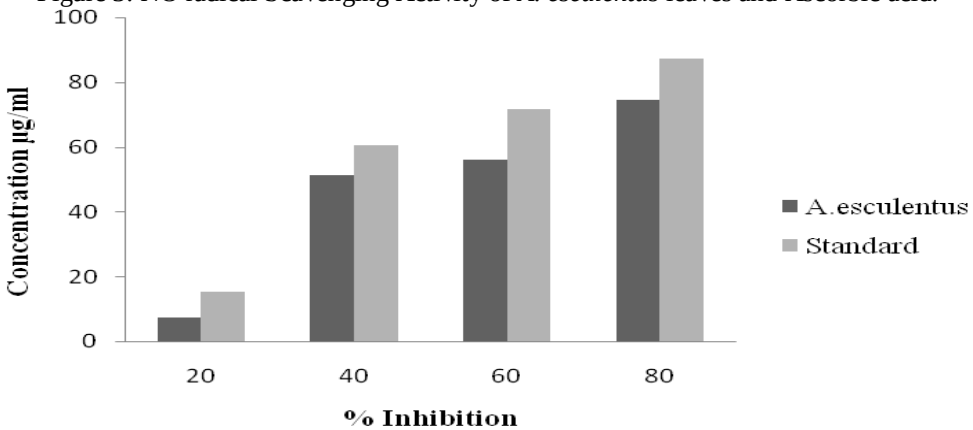

Figure 4: Superoxide anion radical Scavenging Activity of *A. esculentus* leaves and Ascorbic acid.

dependent and was found to be maximum at a concentration of 80µg/ml with inhibition of 74.69% in case of Extract and 87.4% in case of Standard, validating thereby the significant antioxidant potential of *A. esculentus* leaves extract. The IC_{50} values of the Extract and the Standard were found to be 52.50 µg/ml and 42.26µg/ml respectively, which further confirmed that *A. esculentus* leaves could be developed as an effective antioxidant agent.

CONCLUSION

Conclusively it could be stated that *A. esculentus* leaves can be explored further in order to develop a novel antioxidant agent with high therapeutic efficacy due to high content of their polyphenolics including terpenoids in addition to flavanoids. The highly significant antioxidant efficacy of *A. esculentus* leaves was also evident from its high free radical scavenging potential and low IC_{50} values in addition to high reducing power in comparison to the standard drug, ascorbic acid, taken as reference.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

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