In vitro biological activities and phytochemical analysis of Pakistani medicinal plant *Caralluma tuberculata*

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Abstract- A Current study was done to evaluate the maximum therapeutic potential of methanol and distilled water stem extracts of *C. tuberculata* on the basis of polarity, on a series of in vitro biological and antioxidant assays (i.e., Phytochemical analysis, total phenol flavonoid and tannin contents, 2,2-Diphenyl-1- picryl hydrazyl (DPPH) assay and alpha-amylase inhibition assay). The Methanolic extract has maximum potential and revealed a higher number of compounds similarly, showed a high % inhibition of alpha-amylase activity 99.12 % rather than distilled water stem extract which is 65.55 % and plant extracts exhibited a higher IC_{50} value of 759.35 µg/ml with minimal inhibitory activity as compared standard 25.60 µg/ml. The comparative efficacy of the anti-diabetic potential of the *C. tuberculata* revealed good results. The activity pattern observed in this study was Methanol > Acarbose > Distilled water. DPPH free radicals can be easily scavenged by *C. tuberculata* stem extracts at concentrations of 50, 100, 125, 250, and 500 µg/mL. It has been concluded that *C. tuberculata* stem extracts are beneficial against diabetes and have possessed anti-oxidant activity as well. The extracts of *C. tuberculata* stem have a broad spectrum of anti-diabetic and anti-oxidant activity and support the traditional use of this plant as medicine.

Index Terms- alpha amylase inhibition assay, *Caralluma tuberculata*, DPPH assay, Phytochemicals, Therapeutic potential

I. Introduction

Medicinal plants are a valuable source of novel medications around the world [1-3]. Medicinal plants are now being recognized as an important source for treating a variety of diseases [4]. Each plant contains numerous essential components that can be utilized in the medical sector and can be incorporated in the development of various types of drugs [5]. Many poor countries, as well as developed countries use herbal medicine to keep human good health, personal medical care and to treat specific diseases including cough [4]. In comparison to modern treatment, medicinal herbs in folk medicine are more cheap and have less side effects [6]. About half of all exports and 45 % of worldwide traditional medicine revenue come from medicinal plants from Asia [7]. They have commercial uses and for home purposes, there are around 38,660 medicinal plant species in Asia [8-13]. China, Bangladesh, Nepal, Pakistan, Indonesia and Myanmar all have medicinal herb cultivation and extraction as one of their culture [14-20].

Caralluma tuberculata is herb and perennial plant [21]. It is belong to the family Asclepiadaceae and genus *Caralluma.* More than 2000 species of this family can be found in these area i.e. North Asian, south American, south eastern and Africans countries [22]. In Pakistan, *C. tuberculata* is both wild and cultivated [23]. *C. tuberculata* is known in Pakistan by a variety of names, including Pamankay in Pushto, Caralluma in English and Chunga in Urdu [24]. *C. tuberculata* has traditional dietary and medicinal importance, the traditional medicinal value of the plant is consistent with various biological activities gastric problem and carminative, diabetes, leprosy, fever and paralysis [25]. Different chemical components contained in the plant may have a role in the *Caralluma* genus possessing anti-inflammatory and anti-tumor properties [26]. In Pakistan *C. tuberculata* stem is referred to as juicy and can be cooked and used as ground meat, while root can be eaten as a vegetable either raw or cooked [25].

Whole plant in Hai valley, district Buner Pakistan used to treat diabetes and also used as vegetable [27]. People of central Kurrum used aerial part to treat stomach problem, diabetes and high blood pressure [28]. A comprehensive review of literature showed that *C. tuberculata* specie is neglected one and is most in danger of going extinct because of improper harvesting and less knowledge of people. In district Haripur, Pakistan study with special focus on *in vitro* diabetic study on alpha amylase level not conducted also review revealed limited studies on antioxidant activity. Therapeutic profiling is not complete and therefore current study will be an attempt to evaluate maximum therapeutic potential of different extracts of *C. tuberculata* plant on the basis of polarity. Figure 1 shown fresh sample of *C. tuberculata* stem.

Fig.1 Fresh sample of *C. tuberculata*

II. MATERIALS AND METHODS

This study was designed and conducted at Department of Biology whereas few of experiments were performed at Food Science and Technology (FST) Department, The University of Haripur, Pakistan.

The healthy and fresh sample of *C. tuberculata* was purchased from Rawalpindi district vegetable market, Pakistan and authenticated by Dr. Aziz Ullah, Department of Biology (Botany), The University of Haripur, Pakistan. The stem part of plant was shade dried, pulverized into powder then sieved with stainless steel sieve before extraction. Voucher specimens were deposited in the herbarium, Department of Biology, The University of Haripur Pakistan.

Extract from stem part of plant was isolated as described previously [29]. Briefly, 75 g and 40 g of fine powder of stem part of *C. tuberculata* plant were soaked in methanol 376 ml, distilled water 500 ml respectively, for five days. The extract was filtered by using Whatman number 1 filter paper in a plastic funnel. After filtration process filtered solutions were evaporated through rotary evaporator at 40°C under low pressure. Plant dried crude extract was stored at 4°C for research [29].

2.1 Qualitative analysis

20 mg plant stem extracts were taken and mixed with 4 ml DMSO (Dimethyl Sulphoxide), shake well and stock solution was prepared. Three concentrations of plant stock 125, 250 and 500 µg/ml were taken respectively, to check the presence and absence of different compounds which is represented by positive or negative sign.

2.1.1 Alkaloids (Wagner's test)

Some Wagner's reagent drops prepared by 2 g iodine and 6 g potassium iodide in 100 ml distilled water were added to 25, 50 and 100 µl of plant stem extracts after 1ml HCl addition. Yellowish brown precipitates or red will indicate presences of alkaloid [30].

2.1.2 Tannins (Ferric chloride test)

In 25, 50 and 100 µl of plant stem extracts, one drop $FeCl_3$ was added. Tannin is indicated by a bluish black tint, which can be seen [30].

2.1.3 Flavonoids (Alkaline reagent test)

Two drops of sodium hydroxide (NaOH) were added in 25, 50 and 100 µl of plant stem extracts. Yellow color turns to colorless the presence of flavonoids can be detected with two or three drops of HCl addition [31].

2.1.4 Saponins (Foam test)

In a test tube 25, 50 and 100 µl of plant stem extracts were added in two ml of distilled water, and then the test tube was allowed to cool and thoroughly mixed. When saponins are present, foam will develop [32].

2.1.5 Sterols (Salkowski's test)

25, 50 and 100 µl of plant stem extracts and 1 ml of Chloroform added in glass test tubes mix well and few drops of sulphuric acid were added shake and after one minute, the lower layer begins to become red, signifying the presence of sterols [30].

2.1.6 Glycosides (Salkowski's test)

1 ml H_2SO_4 was added into 25, 50 and 100 µl of plant stem extracts and shakes well and stands for 2 minutes. The presence of glycosides will be indicated by a reddish brown coloration [33].

2.1.7 Amino acid (Ninhydrin test)

In boiling water bath few drops of ninhydrin solution was added with 25, 50, 100 µl plant stem extracts for 10 minutes. Purple color will indicates the presence of amino acid [30].

2.1.8 Carbohydrates (Benedict's test)

Benedict's reagent was prepared by copper sulfate pentahydrate 17.3 g, Na2CO3 100 g and 173 g sodium citrate added in distilled water. 0.5 ml added in 25, 50 and 100 µl of plant stem extracts and heated for five minutes in water bath. Red precipitate color will indicates presence of carbohydrates [30].

2.1.9 Anthocyanins

25, 50 and 100 µl plant stem extracts combined with 200 µl of NH_3 and HCl. Anthocyanin presence will be indicated by pink red to blue violet [30].

2.1.10 Leucoanthocyanins

400 µl of isoamyl alcohol was mixed with 25, 50 and 100 µl plant stem extracts. Red color in the upper layer will indicates presence of leucoanthocyanins [30].

2.1.11 Anthraquinones

400 µl each of benzene and ammonia (NH_3) were mixed with 25, 50 and 100 µl plant extracts. Anthraquinones will be present if the ammonical layer is red, pink, or violet [30].

2.1.12 Terpenoids (Salkowski test)

Few drops of H_2SO_4 and 2 ml of chloroform were added to 25, 50 and 100 µl plant stem extracts. The presence of terpenoids will be indicated by a reddish brown tint at interface [30].

2.1.13 Quinones

When 25, 50 and 100 µl of plant stem extracts were combined with 500 µl of KOH the mixture turned red to blue showing the presence of quinones [30].

2.1.14 Lactones

5 mg plant stem extracts was added to 500 µl pyridine also NaOH and was added sodium nitroprusside mixture deep red color indicate the presence of lactones [30].

2.1.15 Lignin

- **Labat Test**

Plant stem extracts 25, 50 and 100 µl were dissolved into 25, 50 and 100 µl of gallic acid. The presence of lignin is indicated by the color olive green [30].

- **Dahlmann Test**

A few H_2SO_4 drops and 2 drops aniline were mixed with 25, 50 and 100 µl of plant stem extracts [30].

2.1.16 Phlobatannins (HCl test)

Plant stem extracts 25, 50, 100 µl were added to 2 ml HCl and boiled for 10 minutes. Red precipitate will indicates presence of phlobatannins [30].

2.2 Quantitative analysis

2.2.1 Estimation of total phenolic contents

Total phenolic contents in *C. tuberculata* stem crude extract was checked by Folin- Ciocalteu method with minor modification [34]. Briefly 5 mg/ml stock solution was prepared. From stock solution 25 µl, 50 µl and 100 µl plant extract were taken in 1.5 ml eppendorf tubes. Then 740 µl distilled water and 60 µl Folin-Ciocalteu reagent was added into plant extract. Mixture was incubated for 5 minutes. Following incubation 500 µl (Na2CO3) 7% were added; again, incubate it for 90 minutes. Total solution of 200 µl was added in 96 well plates with three replications to check absorbance. After incubation absorbance were checked through spectrophotometer at 550 nm. Gallic acid was used as a standard.

Total phenolic contents calculation T = C × V/M

2.2.2 Estimation of total flavonoid contents

The total flavonoid contents was determined by Aluminum Chloride Colorimetric method with minor modification [35]. Briefly 5 mg/ml stock solution was prepared. From stock solution 25 µl, 50 µl and 100 µl plant extract were taken in 1.5 ml eppendorf tubes. Then 300 µl methanol, 20 µl aluminum chloride, 20 µl potassium acetate and 660 µl distilled water was added into plant extract and incubate it for 30 minutes. Total solution of 200 µl was added in 96 well plates with three replications to check absorbance. Absorbance was checked through spectrophotometer at 415 nm. Quercetin dehydrate was used standard.

Total flavonoid contents calculation T = C × V/M

2.2.3 Estimation of total tannin contents

Total tannins contents was checked by following procedure with minor modification [36]. Folin- Ciocalteu protocol was used to determine the tannins. Briefly 5 mg/ml stock solution was prepared. From stock solution 25 µl, 50 µl and 100 µl plant extract were taken in 1.5 ml eppendorf tubes. Then 50 µl of Folin- Ciocalteu reagent, 850 µl of distilled water, 100 µl of 35% of Na2Co3 were added. Again 50 µl of distilled water was added into plant extract and incubate it for 30 minutes. Total solution of 200 µl was added in 96 well plates with three replications to check absorbance. Absorbance was taken at 725 nm by using spectrophotometer. Standard Gallic acid was used.

Total tannin contents calculation T = C × V/M

2.3 Anti-diabetic activity

2.3.1 Alpha-amylase inhibitory assay

The alpha amylase inhibitory activity was checked by following method with minor modification [37]. Briefly 5 mg/ml stock solution was prepared. From stock solution 25 µl, 50 µl and 100 µl plant extract were taken in 15 ml falcon centrifuge tubes. Then 160 µl of alpha amylase and 60 µl of phosphate buffer solution were added, incubated for 20 minutes at 37 °C. Then 80 µl of starch solution was added and incubated for 15 minutes in boiling water at 37 °C. 400 µl of DNS (3, 5-dinitroslalicylic acid) was added and dilute it with dilution factor e.g., 1 ml, 2 ml, 3 ml distilled water. Total solution of 200 µl was added into 96 well plates with three replications to check absorbance. Absorbance was taken at 540 nm by using spectrophotometer. Acarbose was used standard.

Alpha amylase inhibitory assay % I = ((A0-A1)/A0) × 100

A0 = Absorbance of negative control and A1 = Absorbance of sample/standard

2.4 In vitro anti-oxidant activity

2.4.1 Free radical scavenging activity through DPPH

The plant stem extracts with different concentrations compared with commercial available standard ascorbic acid 50, 100, 125, 250 and 500 µg/ml respectively to see how effect they will scavenge free radicals (DPPH) with minor modification procedure reported by [38]. A 10 mg/ml stock solution was made from stock solution 12.50 µl, 25 µl and 50 µl plant extract were taken in 1.5 ml eppendorf tubes and incubated for 20 minutes. After incubation plant sample stock solution (All Concentrations) with three replications 100 µl was taken in 96 well plates. 100 µl of DPPH methanol solution of 0.04 % was added. The samples were incubated for 30 minutes and absorbance was taken at 517 nm on spectrophotometer. Methanol was used negative control.

Free radical scavenging activity (%) = ((A0-A1)/A0) × 100

A0 = Absorbance of negative control and A1 = Absorbance of sample/standard

2.5 Statistical analysis

All tests were performed in triplicates. For statistical and graphical representation Microsoft Excel 2010 and software Graph Pad Prism (6.01) (La Jolla CA, USA) were used. One-way analysis of variance (ANOVA) was performed through SPSS 16 version software with Tukey's multiple comparison test to determine the significance of differences and *p*-value < 0.05 was considered statistically significant

The presence or absence of different phytochemicals was examined using qualitative analysis of the stem extracts of *C. tuberculata*. The findings of this research are presented in Table 1, Figure 2 and figure 3 respectively.

III. RESULTS

3.1 Qualitative analysis

Table 1 List of different phytochemicals reported from *C. tuberculata* stem extracts

Sr. No	Phytochemicals	Test name	Observed color	Methanol	Distilled water
1	Alkaloids	Wagner's method	Yellowish brown / Red precipitates	+	+
2	Tannins	Ferric chloride	Bluish black	-	-
3	Flavonoids	Alkaline reagent test	Yellow color turns colorless	+	+
4	Saponins	Foam	Foam appearance	+	-
5	Sterols	H_2SO_4 and $CHCl_4$	Red color in lower layer	+	+
6	Glycosides	H_2SO_4	Reddish brown color	++	+
7	Amino acid	Ninhydrin	Purple color	-	-
8	Carbohydrates	Benedict's reagent	Red precipitates	-	-
9	Anthocyanins	NH_3 and HCl	Pink-red turns blue violet	-	-
10	Leucoanthocyanins	Isoamyl alcohol	Red color in upper layer	-	-
11	Anthraquinones	Benzene and NH_3	Pink red turns to blue violet	-	-
12	Terpenoids	H_2SO_4 and $CHCl_4$	Reddish brown color at interface	+	+
13	Quinones	KOH	Red to blue color	+	-
14	Lactones	C_5H_5N + NaOH + SNP	Deep red color	-	-
15	Lignin	Labat and Dahlmann	Olive green and yellow	+	+
16	Phlobatannins	HCl	Red precipitates	-	-

(+) Presence; (-) Absence; ++: Strong positive; +: Positive; - : Negative

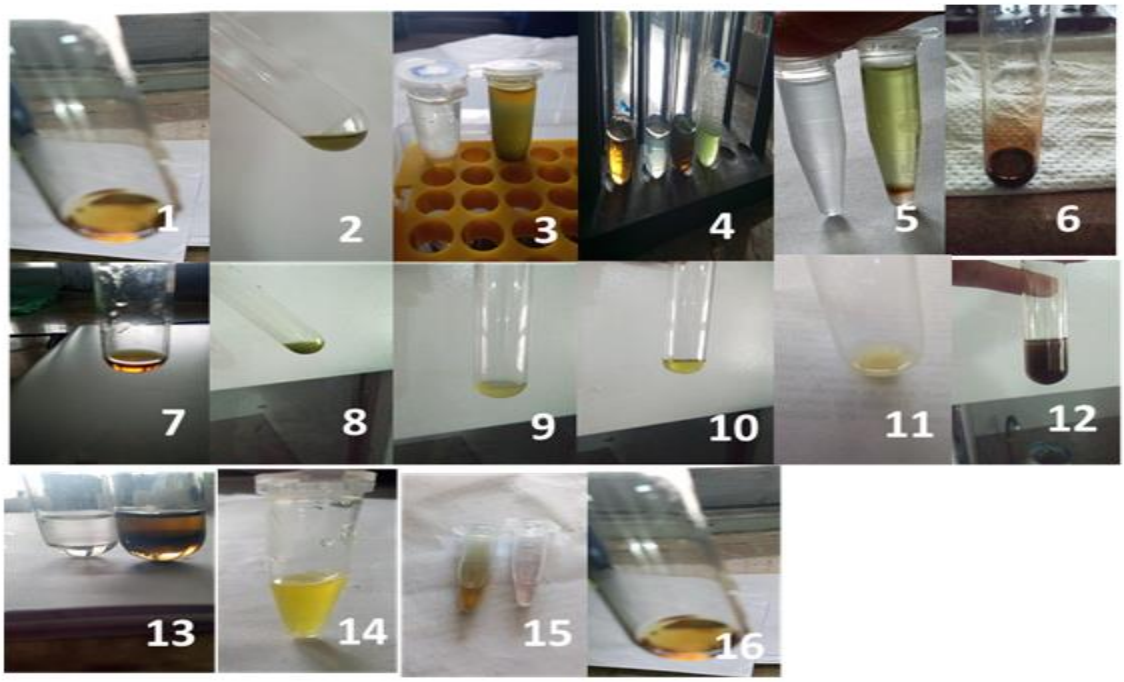

Fig. 2 Qualitative tests in methanolic stem extracts of *C. tuberculata*

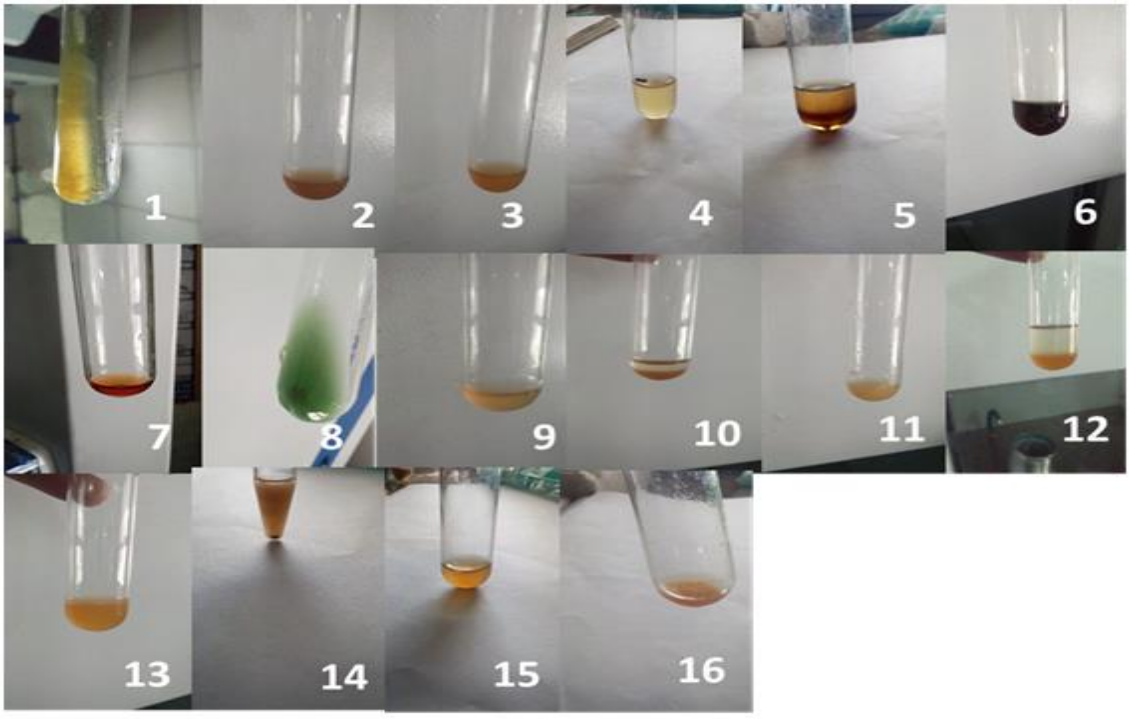

Fig. 3 Qualitative tests in distilled water stem extracts of *C. tuberculata*

3.2 Quantitative analysis

3.2.1 Determination of total phenolic contents

In the body, free radicals are scavenged by phenol in a significant way. Figure 4 shows phenolic amounts present in *C. tuberculata* of different stem extracts i.e., methanol and distilled water at different concentration. Total phenolic amount was calculated with the help of gallic acid standard curve regression equation ($y = 0.0007x + 0.0475$ $R^2 = 0.9697$). Methanol extract exhibited a high amount of phenolic compound 13.57 ± 1.714 at 125 µg/ml concentration and 30.52 ± 1.28 at 500 µg/ml concentration while distilled water extract exhibited low amount 8.33 ± 2.49 mg GAE/g of dry extract at 125 µg/ml concentration and 18.33 ± 2.02 mg GAE/g of dry extract at 500 µg/ml.

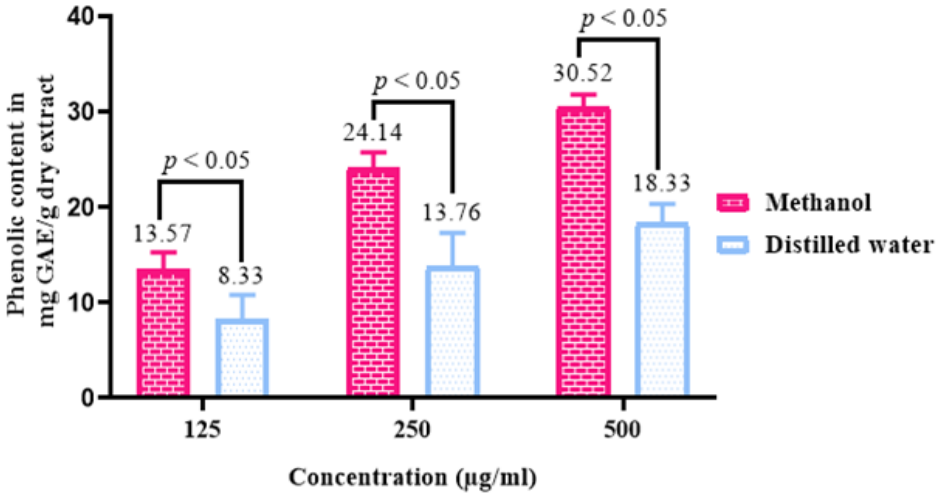

Fig.4 Phenolic contents in methanolic and distilled water stem extracts of *C. tuberculata*. Values are mean of three replicates with the error bars showing standard deviation

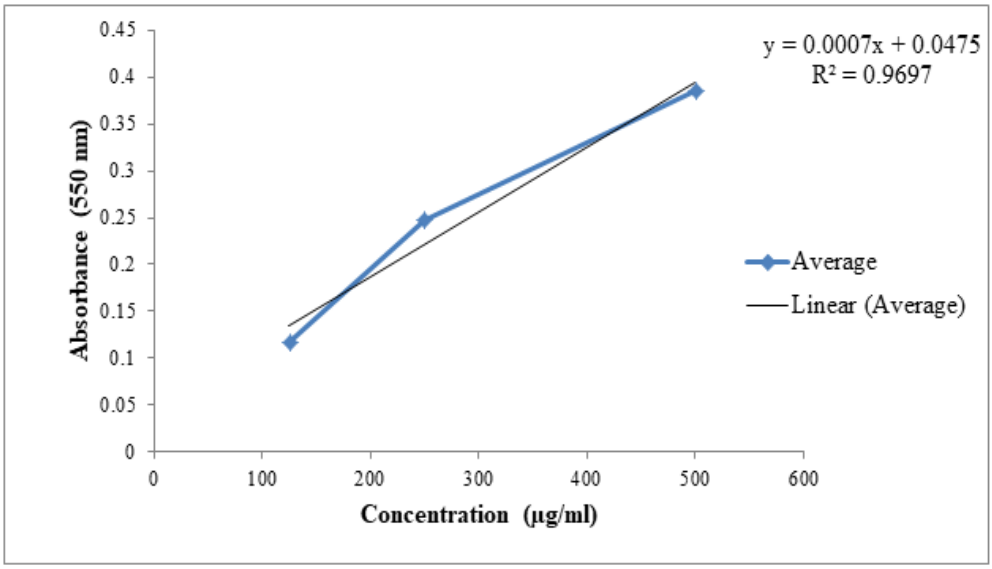

Fig. 5 Gallic acid Standard curve for total phenolic contents determination in *C. tuberculata* stem extracts

3.2.2 Determination of total flavonoid contents

Flavonoids are anti-oxidant and it fights against toxic effect in body. Methanolic and distilled water stem extracts at different concentration in figure 6 are given. Total flavonoids amount was calculated with the help of Quercetin dehydrate standard curve regression equation (y = 0.0034x - 0.0058 R^2 = 0.9988). Methanolic extract showed more flavonoids compounds 12.26 ± 0.86 mg QE/g of dry extract at 125 µg/ml concentration and 30 ± 0.48 mg QE/g of dry extract at 500 µg/ml rather than distilled water, exhibited low flavonoid compounds 7.59 ± 0.56 mg QE/g of dry extract at 125 µg/ml concentration and 22.57 ± 0.41 mg QE/g of dry extract at 500 µg/ml concentration.

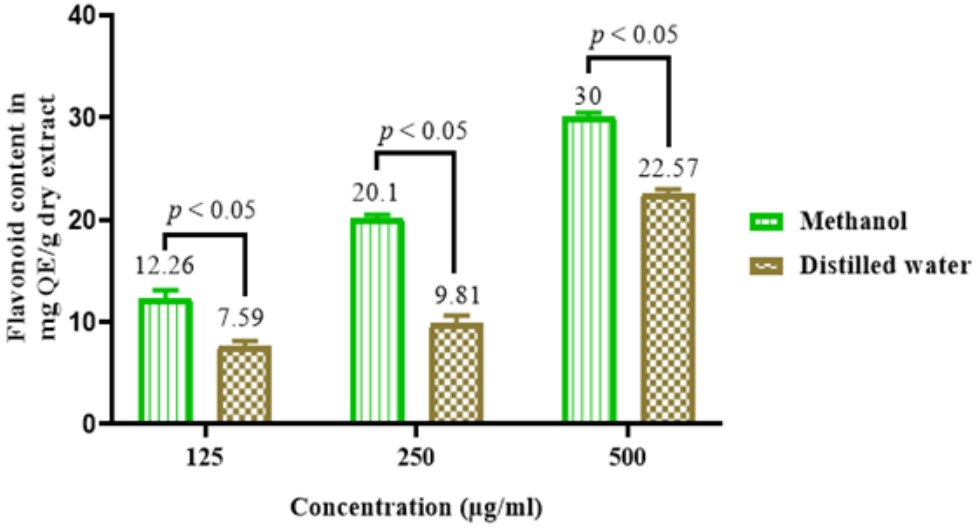

Fig. 6 Flavonoid contents in methanolic and distilled water stem extracts of *C. tuberculata*. Values are mean of three replicates with the error bars showing standard deviation

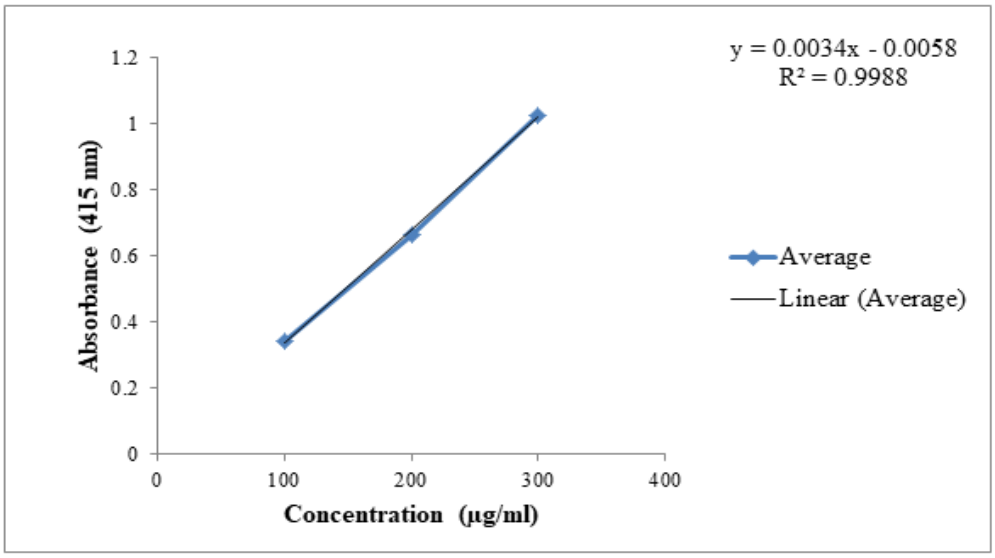

Fig. 7 Quercetin dehydrate standard curve for determination of total flavonoid contents in *C. tuberculata* stem extracts

3.2.3 Determination of total tannin contents

Figure 8 shows tannins content of methanol and distilled water stem extracts at different concentration. Tannin content was calculated with the help of gallic acid standard curve regression equation (y = 0.0014x + 0.0174 R^2 = 0.9853). Methanol extract showed high tannin amount 35.56 ± 1.57 mg GAE/g of dry extract at 125 µg/ml concentration and 51.41 ± 0.71 mg GAE/g of dry extract at 500 µg/ml concentration and distilled water extract exhibited lower tannin amount 26.65 ± 0.85 mg GAE/g of dry extract at 125 µg/ml and 50.37 ± 0.79 mg GAE/g of dry extract at 500 µg/ml concentration respectively.

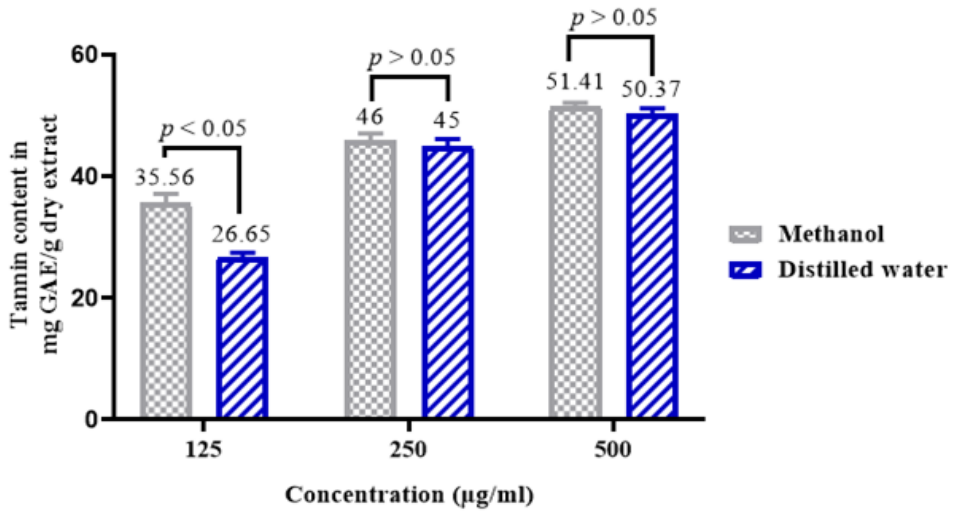

Fig. 8 Tannin contents in methanolic and distilled water stem extracts of *C. tuberculata*. Values are mean of three replicates with the error bars showing standard deviation

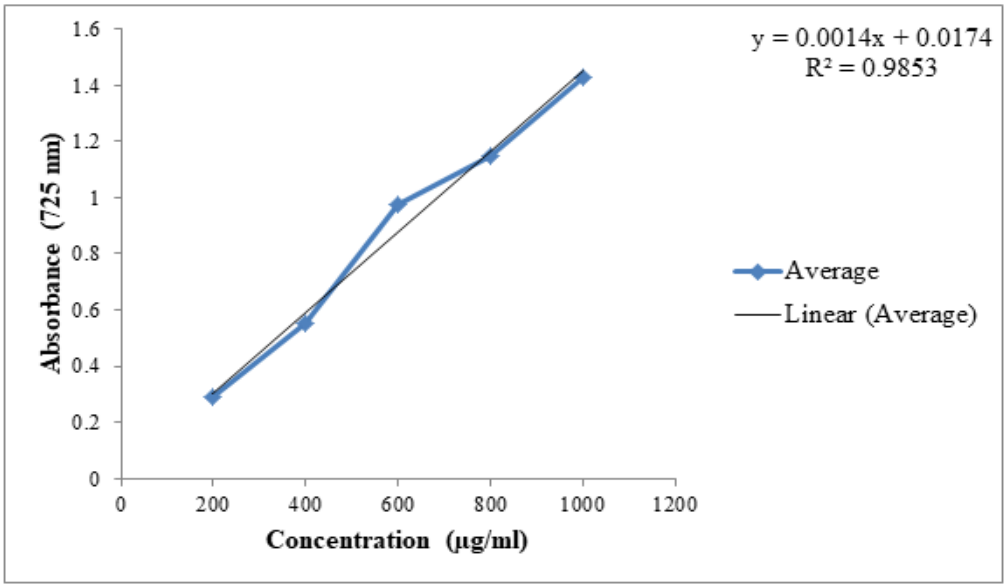

Fig. 9 Gallic acid standard curve for determination of total tannin contents in *C. tuberculata* stem extracts

3.3 Inhibition of α-amylase activity

Alpha amylase is an enzyme that is present in pancreas, small intestine and salivary gland of human body. It converts starch into glucose we check its activity through plant extracts to inhibit alpha amylase enzyme and starch cannot convert into glucose that's why glucose level not rise or high and sugar level maintain and stable. In figure 10 plant extracts were compared with standard acarbose to check alpha amylase inhibition activity. *C. tuberculata* methanolic extract showed 99 % high inhibition activity at 125 µg/ml concentration and 99.12 % inhibition at 500 µg/ml concentration. Distilled water extract of *C. tuberculata* exhibited 29 % inhibition at 125 µg/ml concentration and 65.55 % inhibition activity at 500 µg/ml concentration. Acarbose used as positive control displayed 56.59 % inhibition activity at 125 µg/ml concentration and 88 % inhibition activity at 500 µg/ml concentration. The value of *p* in extracts is statistically different. Based on the statistical results of extracts, it can be concluded that methanolic extract shown the best activity.

3.4 DPPH free radical scavenging activity

Anti-oxidant activity of methanolic stem extract of *C. tuberculata* and standard ascorbic acid are given in figure 11. IC_{50} value (Sample concentration need to reduce or scavenge 50 % free radical) was checked with the help of regression equation created from concentration of sample extract vs % inhibition of free radical formation. Lower IC_{50} value represents greater DPPH radical scavenging activity. Methanolic extract of *C. tuberculata* showed highest IC_{50} value 759.35 µg/ml with minimal inhibitory activity as compared Ascorbic acid 25.60 µg/ml IC_{50} value with best anti -oxidant activity and current DPPH assay ascorbic acid IC_{50} value is much closer to the value mentioned by [38].

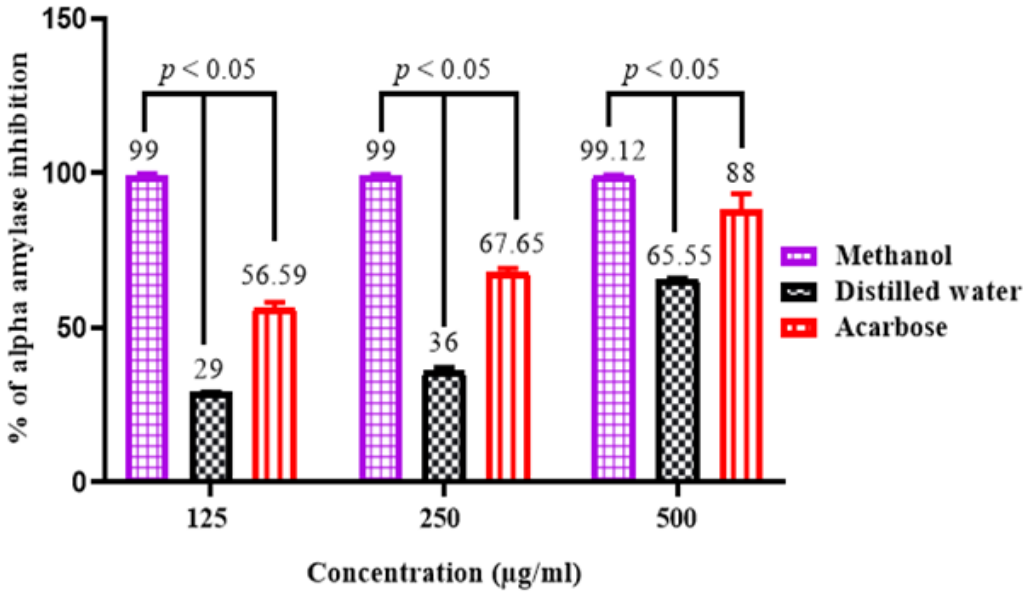

Fig. 10 Inhibition percentage of α-amylase at different concentrations of methanol and distilled water stem extracts of *C. tuberculata* and standard acarbose. Values are mean of three replicates with the error bars showing standard deviation

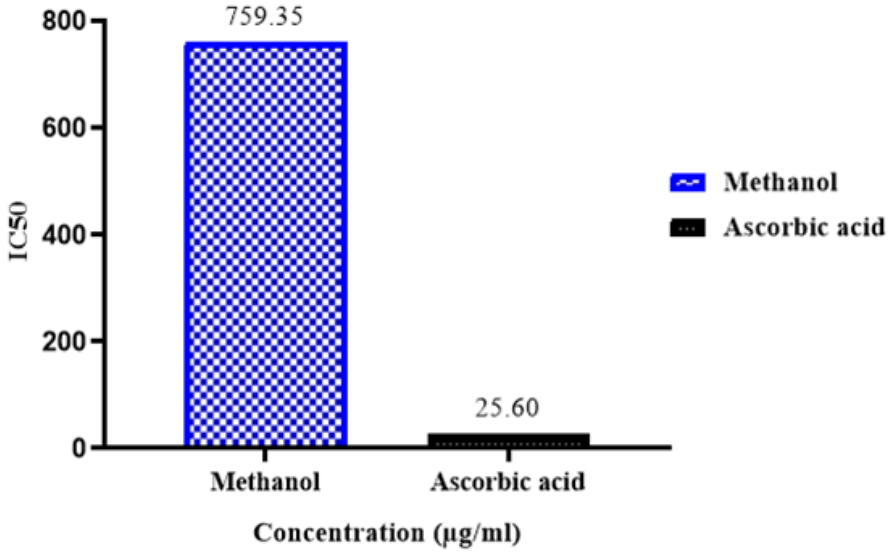

Fig. 11 IC_{50} values of methanolic stem extract of *C. tuberculata* and standard ascorbic acid from DPPH assay

IV. DISCUSSION

The results of qualitative analysis of stem extracts of *C. tuberculata* shows that Flavonoids, glycosides, alkaloids, saponins, quinones and terpenoids were discovered to be rich in methanolic stem extract of *C. tuberculata*. Different

phytochemicals reported in distilled water extract i.e., alkaloids, flavonoids, sterols, glycosides, terpenoids and lignin. These all compounds have therapeutic potential and responsible to cure many diseases. According to our results, the antidiabetic activity of *C. tuberculata* methanolic and distilled water stem extracts was potent against alpha amylase enzyme. The reducing effect of stem extracts rises with concentration. The majority of herbs and their components have the ability to lower blood glucose levels. The reason for this is because of presence of phenols, flavonoids, tannins, and antioxidants [39]. Flavonoids and tannins have the ability to inhibit alpha amylase [40]. Tannins inhibited alpha amylase due to their capacity to bind proteins and carbohydrates. The studies above demonstrated that secondary metabolites such as phenols, flavonoids and tannins successfully inhibited alpha amylase. In the present study, the methanolic and distilled water extracts of *C. tuberculata* effectively inhibits alpha amylase enzyme with different concentrations 125, 250 and 500 µg/ml respectively. In the methanolic and distilled water stem extracts potential number of phenols, flavonoids and tannins were found. The anti-oxidant activity of *C. tuberculata* confirmed its therapeutic value. This action supports the medicinal use of *C. tuberculata* methanolic and distilled water stem extracts. Traditionally, *C. tuberculata* stem was used to cure diseases, which may destroy entire plant and bring the herb in danger. The current research confirmed the anti-oxidant and anti-diabetic properties of methanolic and distilled water stem extracts of *C. tuberculata*.

V. CONCLUSION AND RECOMMENDATION

The results of the current study indicated that different phytochemicals of methanolic and distilled water extracts of *C. tuberculata* stem reported. Methanolic extract showed best activity rather than distilled water at alpha amylase level and also present minimal anti-oxidant inhibitory activity. *C. tuberculata* stem needs to be further evaluated at pharmacological and molecular level.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest

AUTHOR'S CONTRIBUTION

Investigation, research, writing and statistical analysis, Haania Ishaq; Formal analysis and editing, Kainat Nisar. All authors have read and agreed to the published version of the manuscript.

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