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EVALUATION OF ANTIOXIDANT AND ANTIGLYCATION ACTIVITIES OF VARIOUS SOLVENT FRACTIONS OF *CISSUS QUADRANGULARIS* STEM

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ABSTRACT

The present study evaluated the free radical scavenging capacity and antioxidant potential of different solvent fractions (Petroleum ether, Ethyl acetate, Butanol and Water) of methanolic extract of *Cissus quadrangularis* stem. Ethyl acetate fraction of *Cissus quadrangularis* stem possessed a high amount of total phenolic and flavonoid contents as compared to other solvent fractions. Also Ethyl acetate fraction exhibited maximum scavenging activity against DPPH (IC_{50} 62.53±5.71µg/ml), superoxide (IC_{50} 50.7±4.86µg/ml), hydroxyl (IC_{50} 24.38±2.34µg/ml) and nitric oxide radicals (IC_{50} 33.83± 3.09µg/ml). These fractions were also screened for their antidiabetic activity via antiglycation assay. Results showed that the ethyl acetate fraction exhibited higher antiglycation activity. Significant antiglycation activity also confirms the therapeutic potential of these fractions against diabetes.

KEY WORDS: **Free radicals, Ethyl acetate fraction, Antioxidant, Antiglycation, Phenolics**

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INTRODUCTION

Free radicals, such as the superoxide anion, nitric oxide and hydroxyl radical, are natural products of metabolism and are toxic to cells[1]. Cellular antioxidant enzymes and the free-radical scavengers normally protect a cell from the toxic effects of free radicals. When the balance between free radical production and antioxidant defenses is lost, 'oxidative stress' results which through a series of events deregulates the cellular functions leading to various pathological conditions including diabetes mellitus, atherosclerosis, ageing and inflammatory diseases[2]. Antioxidant based drugs and formulations for the prevention and treatment of complex diseases like Alzheimer's disease and cancer have appeared during last three decades[3]. Increased consumption of whole grains, fruits and vegetables reduce the risk of chronic diseases like cancer and heart diseases[4]. These protective effects are generally attributed to the presence of various functional components, such as phenolics, vitamin C, vitamin E, provitamins, minerals and fibers. Diabetes is a metabolic syndrome characterized by increased blood glucose levels. Increased oxidative stress which co-exists with reduction of the antioxidant status has been postulated in diabetes[5]. Free radicals have been shown to participate in the glycation reaction resulting in the formation of advanced glycated end products (AGES). Additionally AGEs have a propensity to generate reactive oxygen species (ROS)[6]. Contribution of AGES to diabetes, aging and Alzheimer's disease has received a great deal of attention in recent years[7]. So compounds with combined antiglycation and antioxidant properties may offer therapeutic potential and currently the focus is to explore new herbal drugs.

Cissus quadrangularis Linn. is a perennial tendril climber, belonging to the family *Vitaceae.* It is used by common folk in India to hasten the fracture healing process[8]. It has been prescribed in Ayurveda for skin infections, constipation, piles, anaemia, asthma, irregular menstruation, burns and wounds[9]. The plant is also used as a common food supplement in Southern India[10]. Phytochemical review of *Cissus quadrangularis* (CQ) shows the presence of triterpenes such as δ-amyrin and δ-amyron, β-sitosterol, isopentacosanoic acid, flavonoid such as quercetin and kaempferol, steroidal principles, β-carotene and vitamin C; all these components have potentially different metabolic and physiologic effects[11]. Toxicological evaluation of CQ revealed that the drug is safe and energetic even at higher dose for a prolonged duration of treatment[12]. Previous studies show that CQ stem (CQS) extract effectively reduces the body weight by inhibiting the oxidation of LDL cholesterol and by lowering the blood glucose in obese patients[13]. The present study was designed to evaluate the antioxidant and antidiabetic potential of methanol extract and its different solvent fractions of *Cissus quadrangularis* stem by different *in vitro* models.

MATERIALS AND METHODS

2, 2-Diphenyl-2-picrylhydrazyl (DPPH) and aminoguanidine were purchased from Sigma chemicals Co. (St.Louis, MO, USA). Aluminium chloride was obtained from Merck (Germany). Nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), gallic acid, trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethylene diamine tetra acetic acid (EDTA), deoxyribose, ascorbic acid and ferric chloride were purchased from Sisco Research Laboratories (India). All other chemicals including the solvents used were of standard analytical grade.

(1) Plant material and extraction

Cissus quadrangularis stems were collected from the local areas of Thiruvananthapuram, Kerala, India. The plant was identified and authenticated by Dr. Valsala Devi, Department of Botany, University of Kerala (Voucher no: KUBH 5805). The stems of CQ were shade dried, powdered and extracted with methanol using soxhlet extractor. The crude extract was filtered through Whatman no.1 filter paper and concentrated in vacuum at 40ºC in a rotary evaporator (Heidolph, Germany). The crude methanolic extract (ME) was successively

fractionated with various solvents *viz*, petroleum ether (PE), ethyl acetate (EA), butanol (BU) and water (WA)[14]. These fractions were collected and screened for the antioxidant and antiglycation activities.

(2) Preliminary phytochemical analysis

The qualitative phytochemical analysis of methanolic extract and different solvent fractions of CQS were carried out in order to ascertain the presence of its constituents employing standard conventional protocols[15,16]. The percentage yield of each fraction was also calculated.

(3) Total Phenolic Content

The total phenolic content (TPC) of the CQS fractions was determined according to the method of Singleton and Rossi[17]. Suitable concentrations of CQS fractions were mixed with distilled water (final volume of 3.5 ml) and added 0.5 ml of Folin-Ciocalteu reagent. After 5 min, 1 ml of 20% sodium carbonate solution was added and incubated at ambient temperature (25-27°C) for 90 min. The colour developed was measured at 760 nm using UV-visible spectrophotometer (Shimadzu UV–Vis Spectrophotometer, Model 1240). Gallic acid was used as the reference standard. The content of phenolic compounds was expressed as milligram percentage on dry weight basis.

(4) Total Flavonoid Content

The total flavonoid content (TFC) was determined by aluminium chloride colorimetric assay[18]. Aliquots of plant extract were added to 0.3 ml of 5% (w/v) sodium nitrite. After 5 min, 0.3 ml of 10% (w/v) aluminum chloride and 2 ml 1M sodium hydroxide was added. The absorbance was measured against a blank at 510 nm. Quercetin was used as the reference standard. The TFC was expressed as milligram percentage on dry weight basis.

(5) DPPH radical scavenging activity

The free radical scavenging effect of the CQS fractions were assessed by the decoloration of a methanolic solution of DPPH, as described by the method of Hollman Peter[19]. 2.8ml of 0.1mM DPPH solution was added to different concentrations of CQS fractions. In control, methanol was used in place of the sample. When DPPH reacts with an antioxidant compound that can donate hydrogen, it gets reduced and the resulting decrease in absorbance was recorded at 517nm after 30 min. Ascorbic acid (ASC) was used as a reference free radical scavenger.

(6) Superoxide anion scavenging activity

Superoxide radical scavenging activity was measured by the method of Robak & Gryglewski[20]. Superoxide anions were generated in a non-enzymatic PMS-NADH system through the reaction of PMS, NADH and oxygen and it was assayed by the reduction of NBT. The reaction mixture contained 1ml NBT (156µM), 1ml NADH (468µM), 100µl PMS (60µM) and different concentrations of CQS fractions. Incubated the mixture for 5 min and read the absorbance at 560nm against a blank. Ascorbic acid was used as a positive control.

(7) Nitric oxide radical scavenging activity

Nitric oxide radical scavenging was determined by the method of Garatt[21]. 2ml of 10mM sodium nitroprusside was mixed with 0.5ml of CQS fractions at various concentrations and incubated for 150 min. Then 0.5ml of Griess reagent was added to 0.5ml incubation mixture and absorbance was read at 540nm after 30 min. Curcumin, a potent free radical scavenger, was used as the reference.

(8) Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured by the deoxyribose method[22] and compared with catechin. The reaction mixture, which contained CQS extracts, deoxyribose (3.75 mM), H2O2 (1 mM), potassium phosphate buffer (20 mM, pH 7.4), FeCl3 (0.1 mM), EDTA (0.1 mM) and ascorbic acid (0.1 mM), was incubated in a water bath at 37^0C for 1 hr. 1 ml of thiobarbituric acid (1% w/v) and 1 ml of trichloroacetic acid (2.8% w/v) were added to the mixture and heated in a water bath at 100ºC for 20 min. The absorbance of the resulting mixture was measured at 532 nm.

(9) Total reducing power

The reductive potential of the extract was determined by the method of Oyaizu[23]. The different concentrations of extracts and

standard in 1ml of distilled water were mixed with phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1% w/v). The mixture was incubated at 50ºC for 20 min and then 10% of trichloroacetic acid (TCA) was added to the mixture, subjected to centrifugation for 10 min. The upper layer of solution was taken, mixed with distilled water and 0.1% FeCl3. Read the absorbance at 700 nm. Ascorbic acid was the reference standard. An increased absorbance of the reaction mixture indicated increased reducing power.

(10) Total antioxidant activity

Total antioxidant activity (TAA) of each fraction was determined by phosphomolybdenum method [24]. Briefly 0.3 ml of sample was mixed with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95ºC for 90 min under water bath. Reading was taken at 695 nm after cooling to room temperature. TAA is expressed as the number of equivalents of ascorbic acid.

(11) Antiglycation activity

Antiglycation assay was done by the method of Ulrich and Cerami [25]. Sample mixture contains 20 µl bovine serum albumin (BSA-1mg/ml final concentration), 20 µl of glucose anhydrous (500mM final concentration) and 20 µl test sample (25, 50,75, 100,125 & 150 µg/ml), glycated control contains 20 µl BSA, 20 µl glucose and 20 µl sodium phosphate buffer, while blank control contains 20 µl BSA and 40 µl sodium phosphate buffer. After incubation at 37°C for 7 days, samples are taken out and cooled at room temperature. Then 60µl 100% TCA was added and centrifuged (15000 rpm) for 4 min at 4°C. The supernatant containing glucose, inhibitor and interfering substance was removed and pellet containing AGE-BSA were dissolved in phosphate buffered saline (PBS). The comparison of fluorescence intensity based on AGEs at 370 nm Excitation and Emission at 440 nm is obtained by using Spectrofluorimeter. Aminoguanidine, a hydrazine-like compound which blocks the formation of AGEs by interacting with amadori-derived products was used as the standard inhibitor [26].

(12) Statistical analysis

All assays were carried out in triplicates and results are expressed as mean ± SD (standard deviation). All data were analyzed using the statistical package SPSS (version 17). The sources of variation for multiple comparisons were assessed by the analysis of variance (ANOVA).

RESULTS & DISCUSSION

The phytochemical studies on CQS revealed the presence of phenolics, alkaloids, flavonoids, steroids, terpenoids and tannins. These phytochemical compounds support bioactive processes in higher plants and are known to be excellent antioxidants[27]. The percentage yields obtained were ME-9.6%, PE-34%, EA-19%, BU-8.4% and WA-22%.

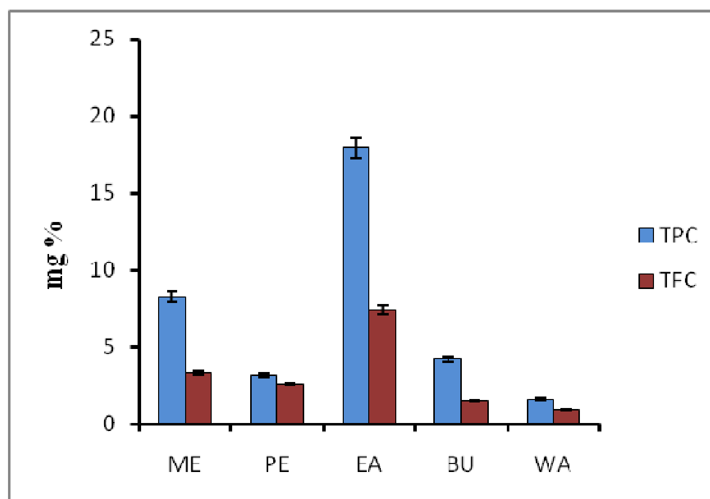
1. Total phenolic content

The amount of TPC determined in different solvent extracts of CQS was given in Fig.1. From a linear calibration curve of gallic acid, in the range 20–100µg/ml, total phenolic content was obtained. The ethyl acetate fraction exhibited the highest total phenolic content (17.94 ± 1.65mg%), whereas the contents obtained with aqueous fraction was much smaller (1.66 ± 0.15 mg%). Phenolic compounds are the principle antioxidant constituents of natural products and are composed of phenolic acids and flavonoids, which are potent radical terminators[28,29]. They donate hydrogen to radicals and break the reaction of lipid oxidation at the initiation step[30]. The high potential of polyphenols to scavenge free radicals may be due to the presence of multiple hydroxyl groups [31].

2. Total flavonoid content

Total flavonoid content (TFC) of CQS was evaluated by aluminium trichloride- sodium nitrite colorimetric assay. Total flavonoid content in ME, PE, EA, BU and WA were 3.36±0.29 mg %, 2.59±0.24 mg %, 7.43±0.67 mg %, 1.55±0.15 mg % and 0.95±0.09 mg % respectively (Fig.1). Among the ME and its fractions, EA fraction contained highest amount of flavonoids. The health benefits associated with the consumption of fruits and vegetables have been partly attributed to the flavonoid content[32].

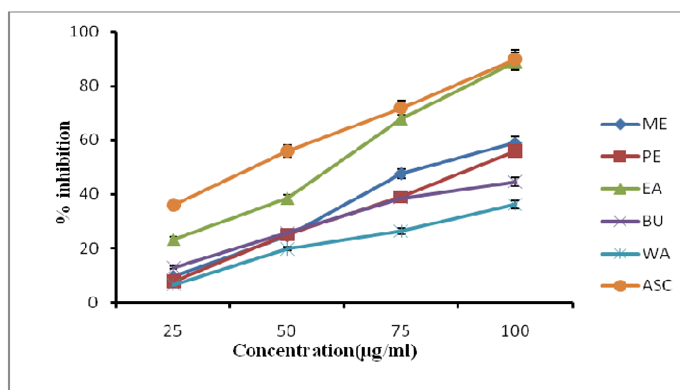
Figure 1
Total Phenolic & Total Flavonoid Content



3. DPPH radical scavenging activity

DPPH is one of the powerful free radical which is used to evaluate the electron donating capacity of antioxidants[33]. All the extracts were able to reduce the stable pink colored free radical DPPH to yellow colored biphenyl picrylhydrazine. Fig.2 shows that the DPPH radical scavenging ability of samples and can be ranked as ethyl acetate (IC_{50} 62.53±5.71µg/ml) > methanol (IC_{50} 87.13±7.95µg/ml) > petroleum ether (IC_{50} 93.28±8.51µg/ml) > butanol (IC_{50} 115.22±10.51µg/ml) >aqueous fractions (IC_{50} 178.59±19.28µg/ml). Phenolic acids and flavonoids have been reported to be the main phytochemicals responsible for the antioxidant capacity of fruits and vegetables. The effect of phenolic compounds, the powerful antioxidants in plants, on DPPH are due to their hydrogen donating ability. These antioxidants could be developed into a drug for prevention and treatment of diseases caused by oxidative stress[34]. There was a positive linear correlation between antioxidant activity and total phenolic content. The results suggested that the phenolic compounds contributed significantly to the antioxidant capacity of CQS.

Figure 2
DPPH Radical Scavenging Activity



4. Superoxide radical scavenging activity

Superoxide radicals are known to be very harmful to cellular components as a precursor of more reactive oxygen species. It was, therefore, proposed to measure the comparative interceptive ability of the antioxidant extracts to scavenge the superoxide radical[35]. Comparison of the

antioxidant activity of the CQS fractions by scavenging superoxide radicals is shown in Fig.3. All of the fractions had a scavenging activity on the superoxide radicals in a dose dependent manner. EA fraction shows highest superoxide radical scavenging activity compared to ME and other fractions.

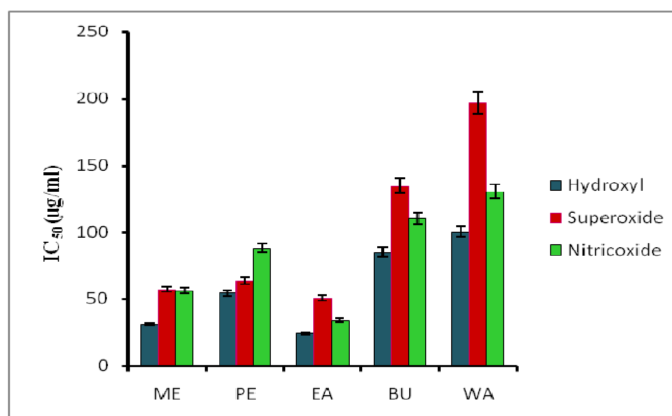
5. Nitric oxide radical scavenging activity

Nitric oxide has an important role in various carcinomas and inflammatory processes. The toxicity of nitric oxide increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion. The plant products have the property to counteract the effect of NO formation and prevent the adverse effects of excessive NO generation in the human body. CQS fractions caused a dose-dependent inhibition of nitric oxide radicals (Fig.3). Among the extracts EA fraction showed superior NO radical scavenging activity (IC_{50} 33.83± 3.09μg/ml).

6. Hydroxyl radical scavenging activity

Hydroxyl radicals are highly potent oxidants that react with almost all biomolecules found in living cells. The effect of extracts in scavenging these and to prevent oxidative degradation of deoxyribose substrate was determined. EA fraction of CQS extract exhibited the highest hydroxyl radical scavenging activity with an IC_{50} value of 24.38±2.34μg/ml (Fig.3). The hydroxyl radical scavenging activity of EA fraction may be due to the active hydrogen donating ability of hydroxyl substitutions of phenolic compounds present in CQS. Phenolic compounds are good electron donors and they may accelerate the conversion of H_2O_2 to H_2O. Generally molecules that inhibit deoxyribose degradation are those that can chelate the iron ions and there by prevent them from complexing with the deoxyribose and render them inactive in a Fenton reaction[36].

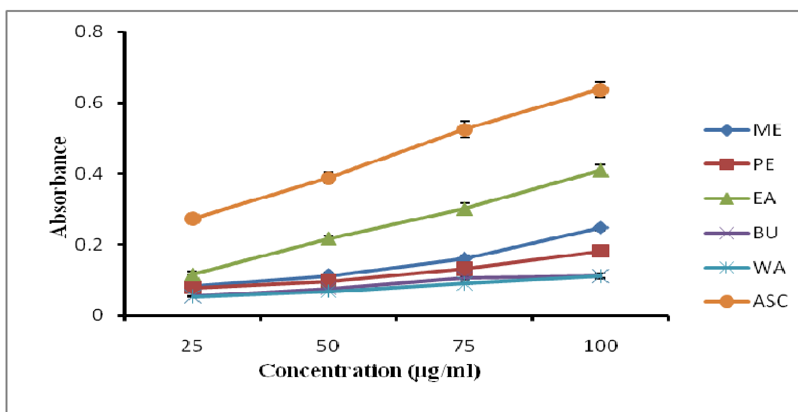
Figure 3
Superoxide, Hydroxyl & Nitric oxide Radical Scavenging Activity



7. Total reducing power

The reducing power of a compound is a supporting feature for its antioxidant capacity. In the reducing power assay, the presence of reductants (antioxidants) in the fractions would result in the reduction of Fe+3/ferriccyanide complex to the ferrous form by donating an electron. The reducing power of the CQS extracts and the reference compound, ascorbic acid increased linearly with increasing concentrations (Fig.4). The greater reducing power was shown by EA fraction and this may be due to the presence of flavonoids which possess potent hydrogen donating abilities. The reducing properties are generally associated with the presence of reductones, which have been shown to exhibit antioxidant action by breaking the chain reactions by donating a hydrogen atom[37]. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation[38].

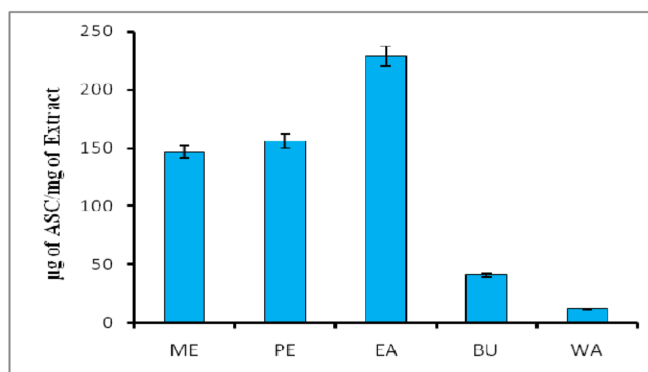
Figure 4
Total Reducing Power



8. Total Antioxidant Activity

The phosphomolybdenum method has been routinely used to evaluate the antioxidant capacity of extracts, mainly by detecting ascorbic acid, some phenolics, α-tocopherol and carotenoids. In the presence of extracts, Mo (VI) is reduced to Mo (V) and forms a green coloured phosphomolybdenum V complex, which shows a maximum absorbance at 700 nm. TAA of different solvent fractions CQS was shown in Fig.5. Ethyl acetate fraction of CQS showed maximum activity with the value of 228.93±20.89μg of ascorbic acid/mg of extract. The results obtained imply that the ethyl acetate fraction has a strong ability to act as antioxidant as compared to other fractions.

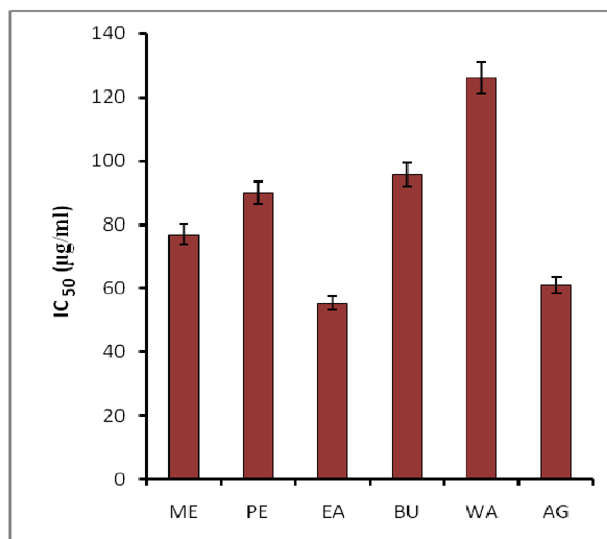
Figure 5
Total Antioxidant Activity



9. Antiglycation activity

Non-enzymatic reaction between reducing sugar and free amino group of protein leads to the formation of glycated protein. Oxidation and reduction of this protein results in the formation of AGES, this is believed to play an important role in the pathogenesis of diabetes. The agents that inhibit the formation of AGES have therapeutic potentials in patients with diabetes and age-related diseases. It was reported that, the naturally occurring compounds with antioxidative and radical scavenging activity posse's antiglycation activity also[39]. The protective effect of CQS on AGE formation was studied. The results were shown in Fig.6. Various solvent fractions of CQS showed good inhibitory potential to glycation reaction at *in vitro* conditions. EA fraction of CQS got a higher activity than other fractions and its activity was comparatively higher than the standard aminoguanidine.

Figure 6
Antiglycation Activity



CONCLUSION

Results of the study revealed that CQS fractions exhibit good antioxidant and free radical scavenging activity. They also exhibited significant antiglycation activity. The study also indicates that EA fraction of CQS have highest antioxidant and antiglycation effects. The activity of this fraction is attributed to the phenolic and flavonoid contents. It is therefore concluded that *Cissus quadrangularis* stem can be used as an accessible source of natural antioxidants with consequent health benefits. However, there is a need for further studies regarding the active compounds present in *Cissus quadrangularis* and the use of its extracts as the natural antioxidants and nutraceutical or pharmaceutical ingredients.

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