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HPTLC FINGER PRINT ANALYSIS FOR FLAVONOIDS AND EVALUATION OF ANTIOXIDANT POTENTIAL OF *CISSUS VITEGENIA*

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ABSTRACT

Methanolic extract of *Cissus vitegenia* (Vitegeniacea) was analysed for the presence of flavonoids by HPTLC and evaluated for antioxidant property by *invitro* models. The extract showed dose dependent free radical scavenging property in all models. Percentage inhibition values were found to be significant in reducing power assay, phosphomolybdenum assay, polyphenol oxidase, catalase and total glutathione activities respectively. The results indicate that the antioxidant property of extract may due to the presence of flavonoidal compounds.

Keywords: *Cissus vitegenia*, Vitegeniacea, free radicals, antioxidant, flavonoids, HPTLC.

INTRODUCTION

The exogenous chemicals and endogenous metabolic process in the human body might produce highly reactive oxygen species, which include free radicals such as superoxide anion radical; hydroxyl radical, non free radical species such as hydrogen peroxide are various forms of activated oxygen. The importance of free radicals and reactive oxygen species has attracted increasing attention over the past decades[1, 2]. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Antioxidants terminate the free radical production and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acids or polyphenols[3]. The free radicals are known to implicate in causations of several diseases such as liver cirrhosis, inflammation, atherosclerosis, diabetes, cancer, neurodegenerative diseases and so forth[4]. Although initial studies suggested that antioxidant supplements might promote health, large clinical trials conducted later did not detect any benefit and suggested excess supplementation may be harmful[5]. So we made an attempt to fulfil the paucity of studies by carrying out preliminary *invitro* antioxidant work in the aerial parts of *Cissus vitegenia*, an initiation for the future drug.

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MATERIALS AND METHODS

Plant collection and authentication

The aerial of *Cissus vitegenia* was collected from Nallamalla forest region, Kurnool, Andhra Pradesh in June. Plant material was thoroughly washed with water to remove adhered particles and debris and shade dried. The air dried plant was powdered using Pulveriser and passed through sieve no.20. The plant specimens were identified and authenticated by Dr. Madhava Chetty, Assistant Professor, Department of Botany, S.V. University Tirupathi, Andhra Pradesh, India.

Extraction

About 1kg of powdered plant material was extracted by Soxhlet extractor using methanol as a solvent. Percentage of extract was determined. The extract was dried under reduced pressure using rotator vacuum evaporator afforded semisolid extract[6].

Preliminary phytochemical analysis

The methanolic extract was subjected to systematic qualitative phytochemical screening to identify the phytoconstituents[7].

Chromatographic Analysis

Chromatography was performed on silica gel F254 HPTLC precoated plates. Samples were applied on the plate as a band of 7mm width using Camag Linomat V sample applicator at the distance of 14mm from the edge of the plates. The mobile phase was constituted of ethyl acetate – acetic acid -formic acid-water 100:11:11:27(v/v/v/v). After development, plates were dried and derivated in NP-PEG reagent. The finger prints were evaluated at 366nm in fluorescence mode with WinCats and video scan software[8]. The chromatogram was given as Figure No.1.

IN VITRO ANTIOXIDANT ASSAYS

Reducing power assay

20-100µg of extracts was taken in 1 mL of phosphate buffer and 5 mL of 0.2 M phosphate buffer (pH 6.6) was added. To this, 5 mL of 1% potassium ferricyanide solution was added and the mixture was incubated at 50°C for 20 min. After the incubation, 5mL of 10% TCA was added. The content was then centrifuged at 1000 rpm for 10 min. The upper layer of the supernatant (5mL) was mixed with 5mL of distilled water and 0.5mL of 0.1% ferric chloride. The absorbance of the reaction mixture was read spectroscopically at 700 nm[9].

Phosphomolybdenum reduction assay

The antioxidant activity of samples was evaluated by the phosphomolybdenum method according to the method of Prieto *et al*[10]. An aliquot of 0.1 mL of sample solution was combined in a 4 mL vial with 1 mL of reagent solution (0.6 M sulphuric acid, 28mM sodium phosphate and 4 mM ammonium molybdate). The vials were capped and incubated in a water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 765 nm against a blank. The results reported are mean values expressed as milligrams of ascorbic acid equivalents per gram sample (AEAC).

Polyphenol oxidase Activity

Assay of Polyphenol oxidase activity was carried out according to the procedure of Sadasivam and Manickam[11]. To 2.0 ml of plant extract and 3.0ml of distilled water added and mixed together. 1.0ml of catechol solution (0.4mg/ml) added to the above solution and the reactants were quickly mixed. The enzyme activity was measured as change in absorbance/min at 490nm.

Catalase Activity

Catalase activity was assayed by the method of Sinha[12]. The enzyme extract (0.5 ml) was added to the reaction mixture containing 1ml of 0.01 M phosphate buffer (pH 7.0), 0.5 ml of 0.2 M H_2O_2, 0.4 ml H_2O and incubated for different time period. The reaction was terminated by the addition of 2 ml of acid reagent (dichromate/acetic acid mixture) which was prepared by mixing 5% potassium dichromate with glacial acetic acid (1:3 by volume). To the control, the enzyme was added after the addition of acid reagent. All the tubes were heated for 10 minutes and the absorbance was read at 610 nm. Catalase activity was expressed in terms of µmoles of H_2O_2 consumed/min/mg protein.

Total Glutathione

The following working solutions were made from stock buffer:(125mM sodium phosphate, 6.3 mM sodium EDTA was adjusted to pH 7.5) 0.3mM NADPH, 6mM dinitrothiobisnitroso benzoic acid (DTNB) and approximately 50 units of glutathione reductase per ml stored at 4ºC .NADPH (700µl), glutathione reductase (10µl), deionized water (165µl) 30 were incubated at 30ºC and absorbance was immediately read at 420nm.[13]

Statistical Analysis

Data analyzed using One Way Analysis of Variance (ANOVA, SPSS Version 16) and expressed as mean ± SEM and comparisons was done using Tukey's test as post-hoc. Difference between means were regarded significant at $P<0.01$.

RESULTS

Phytochemical screening showed the presence of phytosterols, saponins, alkaloids, flavonoids, glycosides and phenolics in the extract. HPTLC analysis of the extract shown 1.35µg/ml of gallic acid, 9.18 µg/ml of ferulic acid, 313.65µg/ml of chlorogenic acid and 10.71 µg/ml of rutin. Reducing power assay and catalase activity were based on the dose dependent manner, the maximum concentration of the extract showed maximum inhibition of the enzymes respectively. Polyphenol oxidase and total glutathione inhibition assays produced maximum significant free radical action which was measured in minutes. Phosphomolybdenum assay shown 239.79±1.72 mg of inhibition compared with the standard drug ascorbic acid.

DISCUSSION

Free radicals are chemical molecules that can exist separately with one or more unpaired electrons. The propagation of free radicals can brings about thousands of reactions and thus makes excessive tissue damage, lipid, protein, DNA are all susceptible to attack free radicals[14, 15]. Antioxidants may offers resistant against oxidative stress by scavenging the free radicals, inhibiting lipid peroxidation etc.

The results obtained from the present studies are clearly indicated that the methanolic extract of *Cissus vitegenia* had powerful antioxidant activity against various antioxidant systems in *invitro*. These radicals are generated inside the body during the normal metabolism or in presence of xenobiotics. The stable free radical was also scavenged by methanolic extract. The reducing property implies that, it is capable of donating hydrogen atom in a dose dependent manner. The content of flavonoidal compounds in the extract may be contributing factor towards antioxidant action. Flavonoids, phenolics, tannins are well known to scavenge the free radicals dose dependently. Thus, these ingredients may contribute the free radical scavenging potential of *Cissus vitegenia* methanolic extract.

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Figure 1: HPTLC Chromatogram of methanolic extract of *Cissus vitegenia*

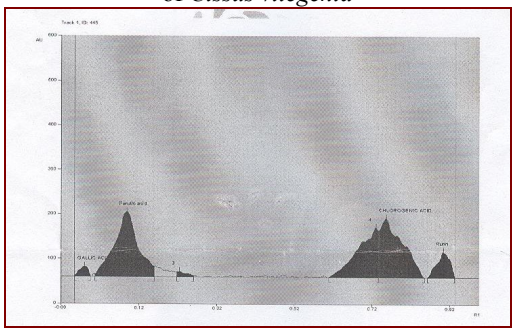

TABLE 1: HPTLC finger print analysis of methanolic extract of *Cissus vitegenia*

Flavonoids	Rf	Peak Height	Peak Area	Concentration µg/ml
Gallic acid	0.05	21.929	374.273	1.35
Ferulic acid	0.09	144.589	6583.06	9.18
Quercetin	Not detected	Not detected	Not detected	Not detected
Chlorogenic acid	0.76	131.433	5677.73	313.65
Rutin	0.91	57.291	1442.965	10.71

TABLE 2: Reducing power assay of methanolic extract of *Cissus vitegenia*

Concentration (µg)	Percentage reduction
350	0.049±0.0026
700	0.087±0.0013
1050	0.124±0.0027
1400	0.168±0.0013
1750	0.200±0.002

TABLE 3: Phosphomolybdenum reduction assay of methanolic extract of *Cissus vitegenia*

Extract	**Phosphomolybdenum** mg ascorbic acid eq/g extract
Cissus vitegenia methanolic extract	239.79±1.72

Table 4: Polyphenol oxidase activity of methanolic extract of *Cissus vitegenia*

Cissus vitegenia extract	Enzyme activity (Unit/g)					
	30 Sec	1 min	2 min	3 min	4 min	5 min
Polyphenol oxidase	0.112 + 0.110	0.165 + 0.121	0.353 + 0.113	0.492 + 0.151	0.523 + 0.171	0.633 + 0.141

Values are mean ± SD; n=3; 1 unit=Activity of catechol oxidase/ lactase that transforms 1 unit of dihydrophenol to quinine/minute

Table 5: Catalase activity of methanolic extract of *Cissus vitegenia*

Sample	Concentration (µg)	Percentage activity (%)	IC_{50} (µg/TRV)
Cissus vitegenia extract	100	14.33 ± 1.24	
	200	29.70 ± 0.83	
	300	39.35 ± 1.32	236.85±1.44
	400	46.33 ± 1.09	
	500	52.75 ± 1.73	
Ascorbic acid	5	8.81	
	10	16.05	
	15	25.15	
	20	40.43	28.21±2.01
	25	49.32	

Table 6: Total glutathione activity of methanolic extract of *Cissus vitegenia*

Cissus vitegenia extract	Enzyme activity (Unit/g)					
	30 Sec	1 min	2 min	3 min	4 min	5 min
Glutathione reductase activity	3.3±0.2	3.0±0.6	3.8±0.4	4.2±0.4	4.5±0.6	6.5±0.92

Values are mean±SD n=3, 1unit=.moles of NADPH oxidized / minute

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