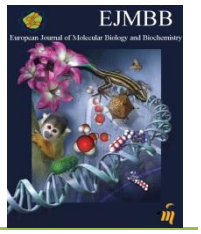
e - ISSN - 2348 - 2206
Print ISSN - 2348 - 2192

European Journal of Molecular Biology and Biochemistry

Journal homepage: www.mcmed.us/journal/ejmbb



ANTIOXIDANT ACTIVITY OF ISOLATED COMPUNDS IN *COCHLOSPERUM VITIFOLIUM*

G. Sandhyarani[1*], K.Swathi[2], Gangarapu.Kiran[3], K. Praveen Kumar[4], Alli Ramesh[4]

[1]Vaageswari College of Pharmacy, Karimnager, Andhra Pradesh, India.
[2]Department of Pharmaceutical Chemistry, University College of Pharmaceutical Sciences, Kakatiya University, Warangal, Andhra Pradesh, India.
[3]Department of pharmaceutical Analysis, Kakatiya Institute of Pharmaceutical sciences, Penbarthy, Warangal, Andhra Pradesh, India.
[4]Vaagdevi College of Pharmacy, Medicinal Chemistry Research Division, Hanamkonda, Warangal, Andhra Pradesh, India.

Article Info

Received 02/01/2014
Revised 16/01/2014
Accepted 02/02/2014

Key words:
Cochlosperum vitifolium; Free radicals.

ABSTRACT

The antioxidant properties of four successive extracts of *Cochlosperum vitifolium* and the successive chloroform extract fraction, CV-11, were tested using standard in vitro model. The amount of the total phenolic and flavonoid content was also determined. The successive chloroform extract, CVC and its fraction CVCF -28 exhibited strong scavenging effect on 2,2-diphenyl-2-picryl hydrazyl (DPPH), Nitric oxide, 2,2 -azino-bis (3ethylbenzo-thiazoline-6-sulphonic acid) diammonium salt (ABTS) radical cation, Reducing power, p-NDA and hydrogen peroxide methods. The free radical scavenging effect of CVC and CV-11 was comparable with that of reference antioxidants. These results clearly indicate the strong antioxidant property of the plant *Cochlosperum vitifolium*. The study provides a proof for the ethnomedical claims and reported biological activities. The plant has, therefore, very good therapeutic potential.

INTRODUCTION

The role of free radicals and active oxygen is becoming increasingly recognized in the pathogenesis of many human diseases, including cancer, aging and atherosclerosis. Human body can be protected from these harmful compounds by enzymatic system, catalase, scavengers and antioxidants. Antioxidants are capable of preventing oxidative processes by inhibiting the initiation and propagation of an oxidative chain reaction. They are important in the prevention of many oxidative-stress related diseases. Several studies have shown that the therapeutic effects of some medicinal plants, fruits and even vegetables which are commonly used in folklore remedies against many diseases can be attributed to the antioxidant properties of their phytoconstituents.

Corresponding Author

G.Sandhyarani
Email:- sandhyaguggilla9@gmail.com

Cochlosperum vitifolium belongs to, Family Cochlo spermaceae. In view of this ethno pharmacological information on the plant, it was proposed screen *Cochlosperum vitifolium* successive extracts and its fraction(s) for the in vitro and in vivo antioxidant activity using standard procedures [1].

MATERIALS AND METHODS

Plant material

The plant *Cochlosperum vitifolium* was purchased from Tirupati Botanicals, Tamilnadu, India, and authenticated by Dr. P.Jayaraman, PARC, India. A voucher specimen has been deposited for further reference.

Chemicals

2,2-Diphenyl-2-picryl hydrazyl (DPPH) and 2,2-azinobis(3-ethylbenzo-thiazoline-6-sulphonicacid) diammonium salt (ABTS) were obtained from Sigma–Aldrich Co., St. Louis, USA. Rutin and p -nitroso

dimethyl aniline (p -NDA) were obtained from Acros Organics, NJ, USA. Naphthyl ethylene diamine dihydrochloride (NEDD) was from Roch-Light Ltd.,Suffolk, UK, ascorbic acid, nitro blue tetrazolium (NBT) and butylated hydroxyanisole (BHA) were from SD Fine Chemicals Ltd., Mumbai, India and 2-deoxy-d -ribose was from Hi-Media Laboratories Pvt. Ltd. Mumbai, India. Sodium nitroprusside and Silymarin were from Ranbaxy Laboratories Ltd., Mohali, India. Sulphanilic acid used was from E-Merck (India) Ltd., Mumbai, India. All chemicals used were of analytical grade.

Animals

Healthy male albino rats of wistar strain (180–220 g) were obtained from the animal house, J.S.S. College of Pharmacy, Ootacamund, India, and were maintained under standard environmental conditions (22–28ºC, 60–70% relative humidity, 12-h dark:12-h light cycle) and were fed with standard rat feed (M/S Hindustan Lever Ltd., Bangalore, India) and water ad libitum . The experiments were conducted as per the guidelines of CPCSEA, Chennai, India.

Extraction procedure

The plant was chopped to small pieces and dried in shade. The dried root was powdered and passed through sieve no.20 and extracted (600g) successively with 4.8 L each of nhexane (60–80ºC), chloroform, ethyl acetate and hydromethanolic (1:1) solvents in a Soxhlet extractor for 18–20 h. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40–50ºC). The n-hexane extract yielded an yellowish green sticky semisolid,(2.37g) The chloroform extract yielded green semisolid (6.07g). The ethyl acetate extract yielded dark blackish green solid (6.73g) and hydro methanolic extract yielded (21.54g), respectively. All the extracts were preserved in a refrigerator till further use.

Isolation and fractionation

The chloroform extract was concentrated and loaded in a column after adsorbing silica gel 60-120 mesh for isolation. About 35 different fractions were collected with different solvent system ranging from low to high polarity by gradient technique. Among all the fractions, the fraction showing good in vitro antioxidant (CV-11) was chosen for further studies. Repeated re-column chromatography of (CV-11) yielded a colourless crystalline compound. This was characterized by melting point, IR, NMR and mass spectrum.

Preparation of test and standard solutions

All the four extracts of *Cochlosperum vitifolium*, fraction (CV-11) and the standard antioxidants (ascorbic acid, rutin and butylated hydroxy anisole) were dissolved in distilled dimethyl sulphoxide (DMSO) separately and used for the in vitro antioxidant assays using five different methods except the hydrogen peroxide method. For the hydrogen peroxide method (where DMSO interferes with the method), the extracts and the standards were dissolved in distilled methanol and used. The stock solutions were serially diluted with the respective solvents to obtain lower dilutions. A suspension of CVC, CV-11 and standard drug silymarin were prepared in sodium CMC (0.5%, w/v) using distilled water and used for in vivo experiments.

Estimation of total phenolic content

The amount of phenolic compounds in all the four extracts was estimated by using Folin–Ciocalteu reagent12.In a test tube, 0.4mL of the extract in methanol was taken, mixed with 2 mL of Folin–Ciocalteu reagent and 1.6mL of sodium carbonate. After shaking, it was kept for 2h and the absorbance was measured at 750nm using a ShimadzuUV-160 spectrophotometer. Using gallic acid monohydrate, a standard curve was prepared. The linearity obtained was in the range of 1–10 g/mL. Using the standard curve, the total phenolic compounds content was calculated and expressed as gallic acid equivalent in mg/g of the extracts.

Estimation of Total flavonoid content

Aluminum chloride colorimetric method was used for the estimation of flavonoids 13.The sample (1mL) was mixed with 3 mL of methanol, 0.2 mL of 10% aluminum chloride, 0.2 mL of 1 M potassium acetate and 5.6 mL of distilled water. The mixture was kept at room temperature for 30 minutes and the absorbance of the reaction mixture was measured at 415 nm a with PerkinElmer UV/Visible spectrophotometer. A calibration curve was prepared by preparing quercetin solutions at 20, 40, 60, 80 and 100 µg/mL concentrations in methanol. The concentration of flavonoid was found using the standard curve and the results were expressed as mg of quercetin equivalents (QE) per g dry weight (mg QE/g DW) of the sample.

***In vitro* antioxidant activity**

The four extracts and the f reaction CV-11 were tested for their in vitro antioxidant activity using standard methods. In all these methods, a particular concentration of the extract or standard solution was used which gave a final concentration of 1000–0.45g/mL after all the reagents were added. Absorbance was measured against a blank solution containing the extract or standard, but without the reagents. A control test was performed without the extracts or standards. Percentage scavenging and IC50 values ±S.E.M. (IC50 value is the concentration of the sample required to inhibit 50% of radical) were calculated.

DPPH radical scavenging method

A 10µL aliquot of the extracts, or the standards was added to 200 µL of DPPH in methanol solution (100 µM) in a 96-well microtitre plate (Tarson Products (P) Ltd., Kolkota, India). After incubation at 37ºC for 20 min, the absorbance of each solution was determined at 490 nm using ELISA reader (Bio-Rad Laboratories Inc., CA, USA, Model 550).

Nitric oxide radical inhibition assay

The reaction mixture (6 mL) containing sodium nitroprusside (10 mM, 4 mL), phosphate buffer saline (1 mL) and the extract or the standard (1 mL) was incubated at 25ºC for 150 min. After incubation, 0.5mL of the reaction mixture was removed and 1mL of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was mixed and allowed to stand for 5 min for completion of diazotization reaction and then 1mL of NEDD was added, mixed and allowed to stand for another 30 min in diffused light. The absorbance was measured at 540 nm against the corresponding blank solution in a 96-well microtitre plate (Tarsons Product (P) Ltd., Kolkata, India) using ELISA reader (Bio-Rad Laboratories Inc., Model 550).

Scavenging of ABTS radical cation

To 0.2 mL of the extract or standard, 1.0 mL of distilled DMSO and 0.16 mL of ABTS solution were added and incubated for 20 min. Absorbance of these solutions was measured spectrophotometrically at 734 nm17.

Reducing power assay

The extract (1mL) or the standard was added to 2.5 mL of 1% potassium ferricyanide and the mixture incubated for 20 minutes at 50ºC. Aliquots of trichloroacetic acid (2.5 mL, 10%) was added to the mixture and centrifuged for 10 minutes. The upper layer (2.5 mL) was mixed with 2.5 Ml of water and 0.5 mL of 0.1% ferric chloride solution. Absorbance was measured at 700 nm against the corresponding blank solutions.

Scavenging of hydroxyl radical by p-NDA method

The extracts or the standard in distilled DMSO (0.5 mL) was added to a solution mixture containing ferric chloride (0.1 mM, 0.5 mL), EDTA (0.1 mM, 0.5 mL), ascorbic acid (0.1 mM, 0.5 mL), hydrogen peroxide (2 mM, 0.5 mL) and p-NDA (0.01 mM, 0.5 mL) in phosphate buffer (pH 7.4, 20 mM), to produce a final volume of 3 mL. Absorbance was measured at 440 nm19.

Scavenging of hydrogen peroxide

A solution of hydrogen peroxide (20 mM) was prepared inphosphate-buffered saline (PBS at pH 7.4). The extract /standard in methanol (1 mL) was added to 2mL of hydrogen peroxide solution in PBS. After 10 min the absorbance was measured at 230 nm.

Statistical analysis

Results were expressed as mean ± S.E.M. Comparisons among the groups were tested by one-way ANOVA using Graph Pad Prism, Version 4.0 (Graph Pad Software, San Diego, CA, USA). When the p-value obtained from ANOVA was significant ($p < 0.05$), the Tukey test was applied to test for differences among groups.

RESULTS

Isolation of stigmasterol

The melting point of isolated compound is 162ºC. IR bands (KBr): 3336, 2936, 2891, 1591, 1459, and 1051cm−1; FAB-MS:m/z 412 (M + 1). In the 1 H-NMR spectrum of the compound, the upfield region exhibited the presence of two tertiary methyls (δ 0.68 and 1.01), three secondary methyls (δ 0.98, 0.85 and 0.83) and a primary methyl (δ 0.85, overlapped with the secondary methyl group). The olefinic protons were observed at δ 5.34 (1H, br.s), 5.16 (1H, dd) & 5.01 (1H,dd), suggesting the presence of two double bonds, whCVE the carbinylic proton centered at δ 3.55 as a multiplet. 13C-NMR spectra showed recognizable signals at δ 14.60 (C-5), 138.26(C-23), 121.65(C-22) and 121.63 (C-6) confirming the presence of two double bonds. Among them the signal at δ 14.60 (C-5) is of weak intensity, indicating that it is a quaternary atom. The signal at δ 71.71 indicates the presence of the carbon atom under the hydroxyl function (C-3). These data confirm the structure of the compound as stigmasterol (Figure 1).

Total phenolic content estimation

The total phenolic content of the four successive extracts were expressed as gallic acid equivalent in mg/g of extracts. Chloroform extract had the highest phenolic content, namely 29.2±0.8 mg/g, followed by ethyl acetate extract 16.5±0.2 mg/g. Ethyl acetate extract had the highest flavonoid content 4.7±0.1 mg/g followed by chloroform extract 19.2±0.15 mg/g.

***In vitro* antioxidant assay**

The four successive extracts and the fraction CV-11 were tested for in vitro antioxidant activity. The results are given in Table I. The data reveal that in the DPPH method potent antioxidant activity was observed for CV-11, CVC and CVE with IC50 value of 19.3 ± 0.91, 25.02 ± 0.05 and 90.69 ± 0.72 µg/ml, respectively. CVE and CVHM showed good to moderate antioxidant activity with IC50 value of 120.20 ± 0.17 and 176.34 ± 0.61 µg/ml, respectively. The successive n-hexane extracts of *Cochlosperum vitifolium* CVH showed poor antioxidant activity.

In the Nitric oxide method, potent antioxidant activity was observed for CV-11 with an IC50 value of 26.72 ± 0.72 µg/ml. CVC also showed potent antioxidant activity (but lower than both the standards) with an IC50

value of 37.97 ± 2.11 µg/ml. CVE showed good to moderate antioxidant activity with IC50 value of 117.12 ± 0.31 and 165.21 ± 0.41 µg/ml, respectively. CVH and CVHM show no or weak antioxidant activity. In ABTS method, CV-11 exhibited potent antioxidant activity with, IC50 value of 54.22 ± 1.12 µg/ml. CVC also showed potent antioxidant activity with IC50 value of 65.19 ± 0.21 µg/ml. CVC, CVE showed moderate antioxidant activity with IC50 value of 287.03 ± 1.22 and 310.57 ± 2.01 µg/ml, respectively. CVP and CVHM showed weak activity even at the highest concentrations tested.In the reducing power method CV-11 exhibited good antioxidant activity with IC50 value of 125.15 ± 1.04 µg/ml. CVC and CVE, showed moderate antioxidant activity with IC50 value of 195.31 ± 2.01 and 362.56 ± 0.02 µg/ml, respectively. CVH showed no antioxidant activity. CVHM showed weak activity even at high concentrations tested.In the p-NDA method CV-11 exhibited moderate activity with IC50 value of 341.01 ± 0.32 µg/ml. CVC and CVE showed weak activity with IC50 value of 402.01 ±0.22 and 589.21 ± 0.55 µg/ml, respectively. CVHM showed weak activity even at high concentrations tested. In H_2O2 method CVC-28 exhibited moderate activity with IC50 value of 480.21 ± 2.01 µg/ml. The remaining extracts and fractions are inactive.

Table 1. In vitro antioxidant activity of different extracts and fractions

Sample	IC 50 values ± SEM*(µg/mL)					
	DPPH	**Nitric oxide**	**ABTS**	**Reducing power**	**p-NDA**	**Hydrogen peroxide**
CVH CVC CV-11 CVE CVHM	225.27±1.20 25.02±0.05 19.31±0.91 90.69±0.72 176.34±0.61	>1000 37.97±2.11 26.72±0.72 117.12±0.31 187.43±1.32	278.36±0.31 65.19±0.21 54.22±1.12 287.03±1.22 353.87±2.10	>1000 195.34±2.01 125.15±1.04 362.56±0.02 416.77±1.22	>1000 402.01±0.22 341.01±0.32 589.21±0.55 >1000	>1000 516.21±3.11 480.21±2.01 >100 790.31±2.11
Ascorbic Acids Rutin BHA	10.41±0.41 0.72±0.31 -	7.32±0.36 19.49±0.22 -	7.22±1.11 1.86±0.15 -	>1000 - -	550 196.32±0.32 -	- - 22.43±1.02

DISCUSSION AND CONCLUSION

Biological systems protect themselves against the damaging effects of activated species by several means. These include free radical scavengers and chain reaction terminators; enzymes such as SOD and CAT system [27]. The SOD converts superoxide radicals (O2 −) into H_2O_2 plus O_2, thus participating with other antioxidant enzymes, in the enzymatic defense against oxygen toxicity. The data obtained in the present study (Table 1) reveals that there is an increase of SOD activity in a dose dependent manner suggesting that the CVC has an efficient protective effect in response to ROS. CAT is a key component of the antioxidant defense system. Inhibition of this protective mechanism results in enhanced sensitivity to free radical induced cellular damage [3-6]. The reduction in the activity of CAT may, therefore, result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide. The data obtained in the present study reveals that administration of CVC increases the CAT level in CCl_4 induced liver damage to rats thus preventing the accumulation of excessive free radicals and protects the liver from CCl_4 intoxication. The phytochemical studies carried out on CVC reveal the presence of carbohydrates, flavonoids, glycosides, steroids and phenols etc. An analysis of the data given in Table 1 reveals that the observed in vitro antioxidant activity of four successive extracts of Ipomoea leari correlates with its phenolic content [5,6]. A number of scientific reports indicate certain terpenoids, steroids and phenolic compounds such as tannins, coumarins and flavonoids have protective effects due to its antioxidant properties 31, 32, 33, 34.In the present study successive chloroform extract, CVC shows the presence of stigmasterol as a major compound which has been shown well to moderate antioxidant activity in different in vitro models by earlier workers35. Stigmasterol thus contributes to antioxidant nature of CVC. In conclusion, the present study clearly reveals that CVC has potent in vitro free radicals scavenging effect in different in vitro models and exhibits a dose dependent antioxidant activity by inhibiting lipid peroxidation. CVC is, therefore, a potential therapeutic, thus making it an excellent candidate for more deta CVEd investigations.

REFERENCES

1. Perry RJ, Watson P, Hodges JR. (2000). The nature and staging of attention dysfunction in early (minimal and mil) Azheimer's disease, relationship to episodic and semantic memory impairment. *Neuropsychol*, 38, 252-271.
2. Gerber M, Boutron-Ruault MC, Hercberg S, Riboli E, Scalbert A, Siess MH. (2002). Food and cancer, state of the art about the protective effect of fruits and vegetables, *Bull. Cancer*, 89, 293–312.

3. Pietta PG. (1998). Flavonoids in medicinal plants. In, CA. Rice Evans and L. Packer, Editors, Flavonoids in health and disease, Dekker, New York, pp. 61–110.
4. Pourmorad F, Hosseinimehr S, Shahabimajd N. (2006). Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants, *Afr. J. Biotechnol*, 5, 1142 -1145.
5. Cao S, Guza RC, Wisse JH, Evans R, van der Werff H, Miller JS, Kingston D. (2005). *G. I. J. Nat. Prod*, 68, 487–492.
6. Anonymous. http,//idl-bnc.idrc.ca/dspace/bitstream/10625/10898/1/95222.pdf.