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In-Vitro Anti-Oxidant, Anti-Amylase, Anti-Arthritic and Cytotoxic Activity of Important Commonly Used Green Leafy Vegetables

Vivek Kumar R[1]*, Satish kumar[2], Shashidhara S[1], Anitha S[1]

[1]Dept of pharmacognosy and [2]Dept of pharmacology Government College of pharmacy, Bangalore, Karnataka, India

***Corres.author: vivek.jsspharma@gmail.com**
Mobile no: +91 9480770847

Abstract: Over the past two decades, there has been an increased recognition of the importance of wild or locally cultivated food plants as sources of micronutrients and plant secondary metabolites. Green leafy vegetables (GLVS) have proved over time that they provide more protective energy to human body than any other vegetable.The present study concern about Anti-Oxidant, Anti-Amylase, Anti-Arthritic and Cytotoxic properties of Commonly Used Green Leafy Vegetables i.e. *Amaranthus viridis (*AV), *Amaranthus gangeticus (*AG), *Anethum sowa* (AS). The phytochemical analysis of aqueous extract of *three GLVS* has indicated the presence of steroid, flavonoid, and saponins types of compounds.EC_{50} values of DPPH free radical and Nitric Oxide scavenging activity of AV, AG, AS and gallic acid standard were found to be 104.64 µg/ml, 110.34 µg/ml, 90.24 µg/ml, 88.42 µg/ml and 105.68 µg/ml, 109.46 µg/ml, 91.28 µg/ml, 87.58 µg/ml respectively. The AV, AG, AS exhibited appreciable α-amylase inhibitory activity with an IC_{50} values 32.24µg/ml, 47.86µg/ml, and 72.34µg/ml respectively, when compared with acarbose (IC_{50} value 82.72µg/ml).The maximum membrane stabilization and protein denaturation inhibition of AV extract was found to be at 90.42% and 70.48% respectively. Among the three extract tested AS (LC_{50}-62.44µg/ml) showed more and AV(LC_{50}-64.16µg/ml) AG(LC_{50}-98.67µg/ml) showed moderate cyto toxicity activity compared with standard vincristine sulfate(LC_{50} of 0.52 µg/ ml) assayed by brine shrimp lethality bioassay. The results demonstrate that GLVs studied contained highly pharmacological active moiety. More work should carry out to isolate and characterize the chemical constituents to ascertain its pharmacological properties.

Key words: *Amaranthus viridis, Amaranthus gangeticus, Anethum sowa,* Anti-Oxidant, Anti-Amylase, Anti-Arthritic, Cytotoxic activity**.**

INTRODUCTION

Providing modern healthcare to rural people in India is still a far-reaching goal due to economic constraints. Hence, people mainly depend on the locally available plant materials to cure various health disorders[1]. The scientific data, from epidemiological studies, indicate that diets rich in fruit, vegetables and grains are associated with a lower risk of several degenerative diseases, such as cancers[2] and cardiovascular diseases[3]. Hence, currently attention is being drawn towards exploring plant sources for substances that provide nutritional and pharmaceutical advantages to humans. Green leafy vegetables (GLVs) are a good source of minerals and vitamins. The ethno-botanical reports offer information on medicinal properties of GLVs like anti-diabetic[4], anti-histaminic[5], and anti-Carcinogenic[6].

Table 1 Botanical, family, common, local names and medicinal properties of three leafy vegetables.

SI NO	Botanical name	Family	Common name	Local name	Medical application
1	*Amaranthus vividis*	Amaranthaceae	Slender amaranth	Harive	Anti-oxidant
2	*Amaranthus gangeticus*	Amaranthaceae	Amaranth	Dantu	Anti-oxidant
3	*Anethum sowa*	Apiaceae	Anethum	Sabsige	carminative

Dietary antioxidants have been hypothesized to have a protective effect against the development of diabetes by inhibiting peroxidation chain reactions[7]. Inhibitory activity against amylase by flavonoid and anthocyanins has been reported[8]. New and highly effective disease-modifying anti rheumatic drugs have continued to emerge until the most recent years. With the growing interest in herbal therapies among the persons with rheumatoid arthritis, there exists a need for investigation into their safety and efficacy of newer drugs[9].It is significant that over 60% of currently used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms[10]. The brine shrimp lethality bioassay has a good correlation with cytotoxic activity in some human solid tumors and with pesticidal activity[11].Botanical, family, common, local names and medicinal properties of three leafy vegetables (Table 1).The present study concern about Anti-Oxidant, Anti-Amylase, Anti-Arthritic and Cytotoxic properties of Commonly Used Green Leafy Vegetables.

MATERIAL AND METHODS:

PLANT MATERIAL

Whole plants of *Amaranthus viridis, Amaranthus gangeticus, and Anethum sowa* were collected from local market, Bangalore. Immediately after collection, the plants were thoroughly washed with water and dried under shade at room temperature.

EXTRACTION

The dried plant materials were coarsely powdered. 20 gm powder plants were subjected to maceration process for 24hrs with water. The solvent was completely removed. This crude extracts was used for investigation.

ANTIOXIDANT ACTIVITY

DPPH RADICAL SCAVENGING ASSAY[12]

DPPH scavenging activity was measured by the slightly modified spectrophotometrically method. A solution of DPPH in methanol (6 x 10^{-5} M) was prepared freshly. A 3 ml aliquot of this solution was mixed with 100 µl of the samples at varying concentrations (50–250 µg/ml). The solutions in the test tubes were shaken well and incubated in the dark for 15 min at room temperature. The decrease in absorbance was measured at 516 nm. The percentage inhibition of the radicals due to the antioxidant property of the isolated fractions was calculated using the formula:

[(A0–A1)/A0] x100, where A0 is the absorbance of the control, and A1 is the absorbance of the extract/ standard. A blank is the absorbance of the control reaction (containing all reagents except the test compound). The %scavenging activity of different concentration of isolated fraction was tabulated in Table.2 & Fig: 1.

Table 2 EC_{50} values (µg/ml) of three GLVS and positive control in DDPH assay method.

SI no.	SAMPLE	EC_{50} µg/ml
1	AV	104.64
2	AG	110.34
3	AS	90.24
4	Standard(gallic acid)	88.42

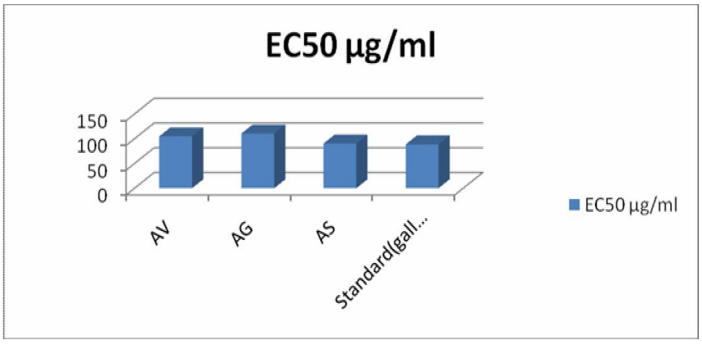

Fig. 1 EC50 values (µg/ml) of three GLVS and positive control in DDPH assay method

NITRIC OXIDE SCAVENGING METHOD[13]

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the Griess reagent. SNP in aqueous solution at physiological pH spontaneously generates NO which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess Reagent. Scavengers of NO compete with oxygen leading to reduced production of NO (17, 16). SNP
(10mM) in phosphate buffer saline (PBS) was mixed with different concentration of isolated fraction (100-500µg/ml) of the drug dissolved in ethanol and water and incubated at 25°C for 180 minutes. The samples from the above were reacted with Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 3% phosphoric acid). The absorbance of the chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine dichloride was read at 546 nm and referred to the absorbance of ascorbic Acid, used as a positive control treated in the same way with Griess reagent.

$$\text{Nitric Oxide scavenged (\%)} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

Where, $A_{control}$ = Absorbance of control reaction,
A_{test} = Absorbance in the presence of the samples of isolated fraction.

The % Nitric Oxide scavenged activity of different concentration of isolated fraction was tabulated in Table.3 & Fig: 2.

Table 3 EC_{50} values (µg/ml) of three GLVS and positive control in Nitric oxide scavenging assay method.

SI no	SAMPLE	EC_{50} µg/ml
1	AD-03	105.68
2	AD-04	109.46
3	AD-05	91.28
4	Standard(gallic acid)	87.58

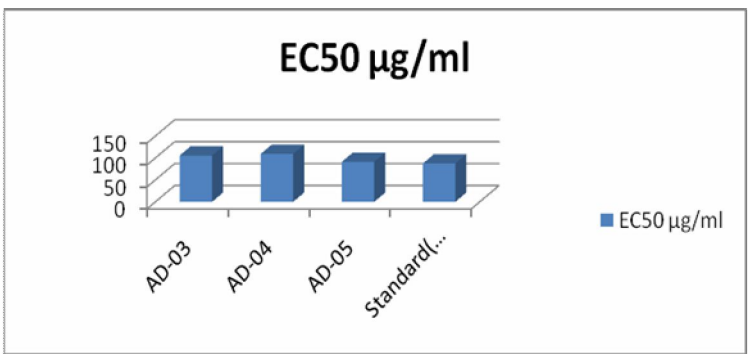

Fig. 2 EC50 values (µg/ml) of three GLVS and positive control in Nitric oxide scavenging assay method.

***IN VITRO* ALPHA AMYLASE INHIBITORY ASSAY**[14]

Starch azure (2 mg) was suspended in a tube containing 0.2ml of 0.5 M Tris-Hcl buffer (pH 6.9) containing 0.01 M calcium chloride (substrate). The tube was boiled for 5 min and then Pre incubated at 37°C for 5 min. 1ml of 0.1% of dimethyl sulfoxide was used to dissolve 1 mg of isolated fraction in order to obtain concentrations of 20, 40, 60, 80 and 100 µg/ml. Then 0.2 ml of isolated fraction of a particular concentration was put in the tube containing the substrate solution. 0.1 ml of porcine pancreatic amylase in Tris-Hcl buffer (2 units/ml) was added to the tube containing the isolated fraction and substrate solution. The process was carried out at 37°C for 10 min. The reaction was stopped by adding 0.5 ml of 50% acetic acid in each tube. The reaction mixture was then centrifuged at 3000 rpm for 5 min at 4°C. The absorbance of resulting supernatant was measured at 595 nm using spectrophotometer.

The α-amylase inhibitory activity = (Ac+) - (Ac-) - (As-Ab) / (Ac+) - (Ac-) X 100

Where, Ac+, Ac-, As, Ab are defined as the absorbance of 100% enzyme activity (only solvent with enzyme), 0% enzyme activity (only solvent without enzyme), a test sample (with enzyme) and a blank (a test sample without enzyme) respectively.

The % α-amylase inhibitory activity and IC_{50} value of different concentration of isolated fraction was tabulated in Table.4 & Fig: 3.

INHIBITION OF PROTEIN DENATURATION [15]

The reaction mixture (0.5 ml) consisted of 0.45 ml bovine serum albumin (5% aqueous solution) and 0.05 ml isolated fraction (250 µg/ml of final volume), pH was adjusted to 6.3 using small amount of 1N hydrochloric acid. The samples were incubated at 37 ºC for 20 min and then heated at 57º C for 3min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. The absorbance's were measured using spectrophotometer at 416 nm. For control tests 0.05 ml distilled water was used instead of extracts while product control tests lacked bovine serum albumin. The percentage inhibition of protein denaturation was calculated-

$$\frac{100-(\text{Absorbance of test solution} - \text{Absorbance of product control})}{(\text{Absorbance of test control})} \times 100$$

The control represents 100% protein denaturation .The results were compared with Diclofenac sodium (250µg/ml. The percentage inhibition of protein denaturation of different concentration was tabulated in Table.5& Fig: 4.

Table 4 IC_{50} values (µg/ml) of three GLVS and positive control in Alpha Amylase Inhibitory Assay

SI no	SAMPLE	IC_{50} µg/ml
1	AD-03	32.24
2	AD-04	47.86
3	AD-05	72.34
4	Standard(acarbose)	82.72

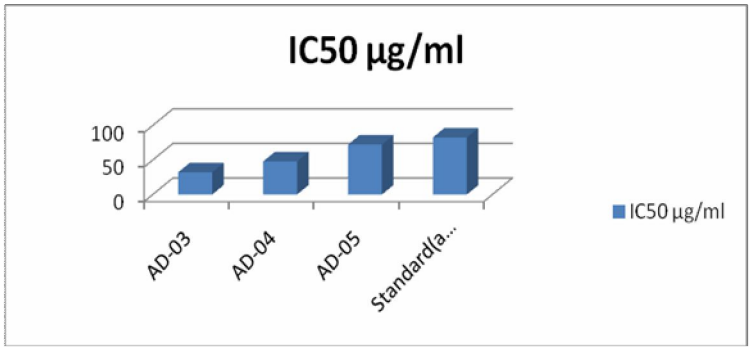

Fig. 3 IC_{50} values (µg/ml) of three GLVS and positive control in Alpha Amylase Inhibitory Assay.

Table 5: Effect of three GLVS on inhibition of protein denaturation

Concentration (µg/0.5ml)	AV	AG	AS	Diclofenac Sodium
250	70.48%	32.24%	36.34%	94%

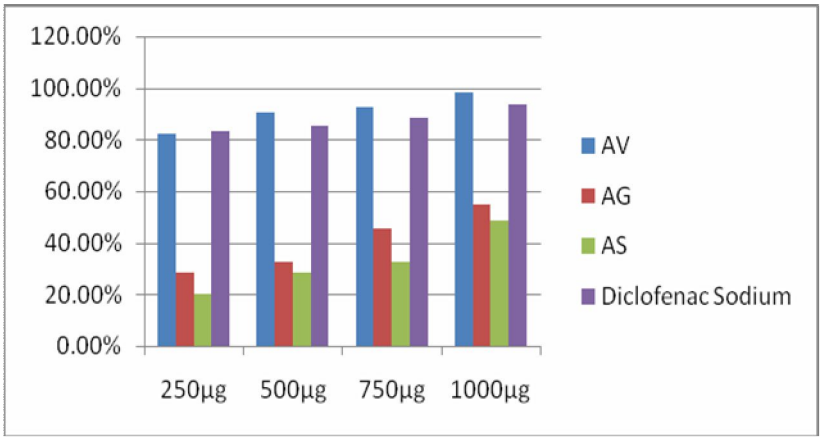

Fig. 4 Percentage inhibition of protein denaturation

EFFECT ON MEMBRANE STABILIZATION[16,17]

The principle involved membrane stabilization is stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis. The assay mixture contains 1ml phosphate buffer [pH 7.4, 0.15 M], 2ml hypo saline [0.36 %], 0.5 ml HRBC suspension[10 % v/v]) with 0.5 ml of plant extracts of various concentrations (250, 500, 750,1000µg/0.5ml), standard drug diclofenac sodium (250, 500, 750,1000µg/0.5ml) and control [distilled water instead of hypo saline to produce 100 % hemolysis were incubated at 37º C for 30 min and centrifuged respectively. The hemoglobin content in the suspension was estimated using spectrophotometer at 560 nm. The percentage hemolysis produced in the presence of distilled water was taken as 100 %. The results were tabulated in Table-6 & Fig-5.

Percentage of HRBC membrane stabilization or protection was calculated using the formula,

$$\text{Percentage Stabilization} = \frac{\text{Absorbance of control- Absorbance of test}}{\text{Optical density of Control}} \times 100$$

BRINE SHRIMP LETHALITY BIOASSAY[18]

The toxic potentiality of the plant crude extract and fractions were evaluated using Brine Shrimp lethality bioassay method where 4 graded doses (25µg/ml, 50µg/ml, 100µg/ml, and 200µg/ml) for isolated fraction and for methanolic extract 4 graded doses (200µg/ml, 400µg/ml, 800µg/ml, and 1000µg/ml) were used. Brine shrimps (Artemia salina) nauplii obtained from natural remedies as a gift sample were used as test organisms. For hatching, eggs were kept in brine with a constant oxygen supply for 48 hours. The nature nauplii were then used in the experiment. DMSO was used as a solvent and also as a negative control. The median lethal concentration LC50 of the test sample after 24 hours was calculated and compared with reference standard Vincristine sulfate. The results were tabulated in Table-7 & Fig-6.

Table 6: Effect of three GLVS on HRBC membrane stabilization

Concentration (µg/0.5ml)	AV	AG	AS	Diclofenac Sodium
250	82.42%	28.50%	20.19%	83.41%
500	90.32%	32.56%	28.68%	85.42%
750	92.42%	45.69%	32.46%	88.69%
1000	98.52%	54.68%	48.78%	93.71%

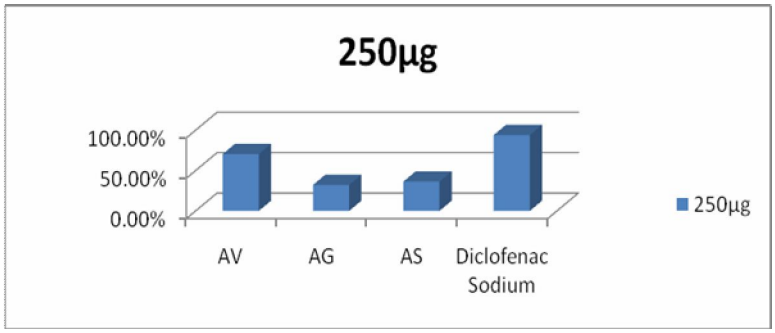

Fig. 5 Percentage protection or stabilization of HRBC membrane

Table 7 the result of cytotoxic activity three GLVS and positive control vincristine sulphate (VS) on brine shrimp

SI NO	SAMPLE	$LC_{50\ \mu g/ml}$
1	AV	62.44
2	AG	64.16
3	AS	98.67
4	Vincristine sulfate	0.52

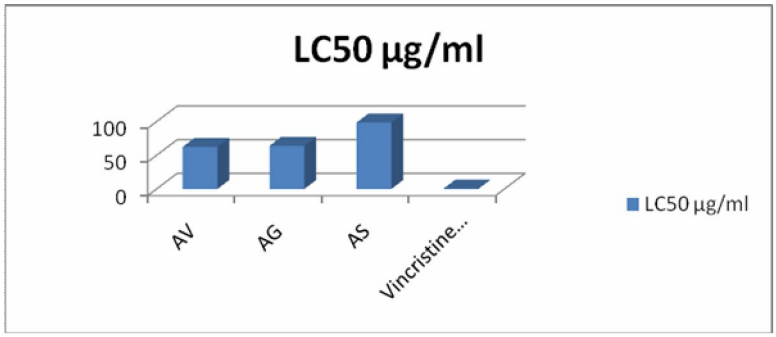

Fig. 6 LC50 µg/ml of three GLVS and positive control vincristine sulphate.

RESULTS AND DISCUSSION

More recently, the role of these biologically diverse species in maintaining human and environmental health has been highlighted, particularly in relation to global food security, sustainable development. The study concern about Anti-Oxidant, Anti-Amylase, Anti-Arthritic and Cytotoxic properties of Commonly Used Green Leafy Vegetables i.e. *Amaranthus viridis (*AV), *Amaranthus gangeticus (*AG), *Anethum sowa* (AS). The phytochemical analysis of aqueous extract of *three GLVS* has indicated the presence of steroid, flavonoid, and saponins types of compounds. Free radical scavenging activity of those three GLVS aqueous extract was confirmed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, Nitric Oxide Scavenging methods. EC50 values of DPPH free radical and Nitric Oxide scavenging activity of AV,AG,AS and gallic acid standard were found to be 104.64 µg/ml,110.34 µg/ml,90.24 µg/ml,88.42 µg/ml and 105.68 µg/ml, 109.46 µg/ml, 91.28 µg/ml,87.58 µg/ml respectively. The AV, AG, AS exhibited

appreciable α-amylase inhibitory activity with an IC50 values 32.24µg/ml, 47.86µg/ml, and 72.34µg/ml respectively, when compared with acarbose (IC50 value 82.72µg/ml). The phytochemical analysis of aqueous extract of three GLVS has indicated the presence of steroid, flavonoid, and saponins types of compounds. Since these compounds are of pharmacological interest, coupled with the use of this plant in traditional medicine, prompted us to evaluate GLVS for its possible anti- arthritic activity by HRBC membrane stabilization and inhibition of protein denaturation method. Among the three extracts tested, AV shown good anti-arthritic activity when compared with standard Diclofenac sodium. The maximum membrane stabilization and protein denaturation inhibition of AV extract was found to be at 90.42% and 70.48% respectively. The cyto toxicity activity of three GLVS aqueous extract was assayed by brine shrimp lethality bioassay. Among the three extract tested AS (LC50-62.44µg/ml) showed more and AV(LC50-64.16µg/ml) AG(LC50-98.67µg/ml) showed moderate cyto toxicity activity compared with standard vincristine sulfate(LC50 of 0.52 µg/ ml). The results demonstrate that GLVs studied contained highly pharmacological active moiety. Further attempt made to isolate and characterized the active moiety which serve as leads for the development of new pharmaceuticals that address hither to unmet needs.

CONCLUSION

Although GLVs used in this study are medicinally important, these are less commonly used for nutritional purpose, which is due to lack of awareness on their nutritional importance. *Amaranthus viridis (*AV), *Amaranthus gangeticus (*AG), *Anethum sowa* (AS) on phytochemical analysis indicated the presence of steroid, flavonoid, and saponins types of compounds and it confirmed anti-oxidant, anti-amylase, anti-arthritic and cytotoxic properties. The results demonstrate that GLVs studied contained highly pharmacological active moiety. It has potential for development into a phytomedicine. More work should carry out to isolate and characterize the chemical constituents to ascertain its pharmacological properties. Moreover it helps to create nutritional awareness among various communities on the importance of these GLVs.

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