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PHYTOCHEMICAL INVESTIGATION AND *IN VITRO* CONSERVATION OF *AMARANTHUS VIRIDIS* L. ALEAFY VEGETABLE OF KERALA.

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Abstract

Antioxidant compounds in food play an important role as a health protecting factor. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Highly reactive free radicals and oxygen species can initiate degenerative diseases. *Amaranthus viridis* of Amaranthaceae is a wild species used by tribal people of Kerala for treating various diseases and for culinary purposes in other countries. The plant exhibits sufficient characteristics related to claims of its therapeutic properties. Crude methanolic leaf extract is used for preliminary phytochemical analysis. Phytochemicals detected in the study can have direct or indirect effects on properties attributed to the plant. Quantitative phytochemical analysis showed higher amounts of total carbohydrate, total proteins in addition to a considerable amount of reducing sugar, starch and pigments like chlorophyll-a, chlorophyll-b and Total chlorophyll. Antioxidant activity of the leaf was evaluated for different enzymatic and non- enzymatic antioxidants. Total polyphenol and superoxide dismutase were present in higher amounts, satisfying its use as a potential source of antioxidants. Anticancer analysis in EAC (Ehrlich's Ascites carcinoma) and DLA (Dalton's Lymphoma Ascites) showed Higher cytotoxicity in EAC compared to DLA against standard revealing promising anticancer effects of the leaf extract. *In vitro* conservation of different explants on MS medium supplemented 2 mg/L BAP showed bare survival capacity of nodal explant with axillary bud. Present study evaluated various nutritional, medicinal and regenerative aspects of the plant *Amaranthus viridis*, exhibiting diverse potentialities of the plant and providing supporting information for its use as an ethnomedicinal plant.

Keywords: *Amaranthus viridis*, Amaranthaceae, Ethnomedicinal plant, EAC, DLA and *in vitro* conservation

Introduction

Leafy vegetables played crucial roles in complementing diets for humans and animals (Amadi*et al.,* 2013). *Amaranthus viridis* is sometimes eaten as cooked vegetable, fodder for cattle and green manure; the leaves are diuretic and purgative and are used for treating inflammations, boils and abscesses, gonorrhoea, orchitis and haemorrhoids. (Alegbejo, 2013). *Amaranthus viridis* L., the main type of vegetable amaranth, seems to have originated in south or south-east Asia, and is consequently dispersed throughout the tropical and temperate regions (Martin and Telek, 1979). Boiled leaves and roots of Amaranthus are used as laxative, diuretic, antidiabetic, antipyretic, anti-snake venom, to relieve breathing in acute bronchitis. It also has anti-inflammatory properties, immunomodulatory activity, anti-androgenic activity and anthelmintic properties (Janet, 2013). To address this lacuna, the present study was carried out for preliminary phytochemical analysis, evaluation of nutritional properties, evaluation of antioxidant properties and evaluate anticancer property of the crude methanol extract in DLA and EAC cell line and *in vitr*o conservation of plant. In future *Amaranthusviridis* is important natural source of developing new drugs. So have select the present plant for this study.

Materials and Methods

Collection and Preparation of Sample for Phytochemical Analysis.

The plant *Amaranthus viridis* was collected as fresh from Kalady, Thiruvananthapuram district and Panmana, Kollam district of Kerala. The plant specimen was made into herbarium and deposited in the herbarium repository of Botanical Survey of India (BSI), Southern Regional

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Centre (SRC), Coimbatore-3 and authenticated No.: BSI/SRC/5/23/2023-24/Tech-466. For sample preparation, fresh leaves and stem were separated, shade dried, ground well using a mechanical blender to fine powder and transferred into airtight containers for further analysis.

Phytochemical Screening

Preparation of Plant Extract

The dried plant materials were extracted with methanol for 8 hours by Soxhlet apparatus and extracted as green, black solid respectively. After which, the residues were transferred to a preweighted sample container for storage and later used for phytochemical screening. The phytochemicals like reducing sugar, flavonoids, alkaloids, tannins, terpenoids, steroids, saponins and anthraquinones were tested (Pradeesh and Swapna, 2018).

Preparation of Plant Extract

The dried plant materials were extracted with methanol for 8 hours by Soxhlet apparatus and extracted as green, black solid respectively. After which, the residues were transferred to a preweighted sample container for storage and later used for phytochemical screening.

Phytochemical Screening

Phytochemical analysis of plant extract was done as described by Harborne (Harborne,1977). The different phytochemicals like reducing sugar, flavonoids, alkaloids, tannins, terpenoids, steroids, saponins and anthraquinones were tested.

Biochemical analysis

The fresh leaves of *Amaranthus viridis* were used for the nutritional and antioxidant analysis and experiment was repeated thrice to confirm the result. The analysis was preformed following standard methods for estimation of reducing sugar (Miller, 1972), total carbohydrate (Hedge and Hofreiter, 1962), total protein (Lowry *et al*., 1951), chlorophyll (Witham *et al*., 1971), starch (Thayumanavan and Sadasivam, 1984) and antioxidant like proline (Bates *et al*., 1973), lycopene (Zakaria *et al*.,1979), carotenoids (Bendich and Olson, 1989), total polyphenol (Eom *et al*., 2008), polyphenol oxidase (Esterbauer *et al.*,1991) superoxide dismutase (Gong *et al.*, *2005*) and Lipid peroxide (Ewa, 2006).

Results and Discussion

Fresh leaves and stems of *Amaranthus viridis* were studied for its nutritional and antioxidant properties. Crude methanolic extract was used for the preliminary phytochemical investigation and *in vitro* anticancer analysis. The different explants of the plant were used to carry out *in vitro* conservation.

Phytochemicals screened in the present study include reducing sugar, flavonoids, alkaloids, tannins, terpenoids, steroids, anthraquinones. The concentrations of tannins and saponins were high for both leaves and stem of *Amaranthus viridis*. While the concentrations of reducing sugars, flavonoids, alkaloids, steroids, anthraquinones are very low concentration, terpenoids were not found in *Amaranthus viridis* leaf extract. The concentrations of steroids, terpenoids and anthraquinones were not found in *Amaranthus viridis* stem extract (Table 1).

Table 1. ***Preliminary*** *Phytochemical Evaluation of Amaranthus viridis*

Sl No:	Phytochemicals	*A.viridis* Leaf	*A.viridis* Stem
1	Reducing sugar	+	+
2	Flavonoids	+	+
3	Alkaloids	+	+
4	Tannins	++	++
5	Terpenoids	-	-
6	Steroids	-	--
7	Saponins	++	++
8	Anthraquinones	+	-

Quantitative Analysis

Nutritional Evaluation

Plant extract has a significant effect in reducing blood sugar level and therefore can be used as an alternative and effective insulin enhancer for individuals who have a symptom of diabetes mellitus (Floramyr, 2019). Reducing sugar from the aerial plant parts were extracted and analysed by Dinitro salicylic acid method and the results were found 0.982 mg g^{-1} in leaves of *Amaranthus viridis* and 0.912 mg g^{-1} in the stem of *Amaranthus viridis* (Fig.1). Carbohydrates are macronutrients present in plants that forms major constituents ofhuman diet that can be metabolized to yield energy (Pradeesh and Praveena, 2020). Present study estimated total carbohydrates present in leaf tissue using the anthrone method. The amount of carbohydrates in leaves of *Amaranthus viridis* was found to be higher 35.908 mgg^{-1} and in stem 33.128 mgg^{-1} as shown in (figure 2).

Proteins are complex macromolecules that have structural, functional and catalytic roles in organisms with amino acids as structural units. They form the building block of tissue. (Ghali and Alkoaik, 2010). Estimation of protein from the leaves of *Amaranthus viridis* was done by Lowry's method and the result was found to be high in leaves (32.719 mg g^{-1}) andstem (30.921 mg g^{-1}) as shown in Fig. 2. Dietary chlorophyll derivative has the ability to scavenge long lived free radicals such as DPPH and ABTS (Mishra *et al*., 2011). The estimation of Chlorophyll in *Amaranthus viridis* was carried out using Arnon's method with a result of high content of chlorophyll-a (0.898 mg g^{-1}), chlorophyll-b (0.862 mg g^{-1}) and total chlorophyll (1.436 mg g^{-1}) in leaves and chlorophyll-a (0.628 mg g^{-1}), chlorophyll-b (0.592 mg g^{-1}) and total chlorophyll (1.136 mg g^{-1}) in stem (Fig. 3). Commercial production of starch can be done by degradation of starch. It is used as thickening agents in sauces and as colloidal stabilisers in salad dressings (Manthey and Xu, 2009). The amount of starch in *Amaranthus viridis* was found to be 0.832 mg g^{-1} in leaves and 0.631 mg g^{-1} in stem as shown in Fig. 4. The nutritional analysis of leaves of *Amaranthus viridis* showed the presence of a high amount of reducing sugar, carbohydrates, proteins, pigments and starch.

Evaluation of Antioxidant Properties

Plants are the reservoir of antioxidants that can act as an endogenous antioxidant source through diet which can help in resolving free radical mediated health conditions and its degenerative effects in human beings. Evaluation of enzymatic and non-enzymatic antioxidants in *Amaranthus viridis* can help in understanding the therapeutic potential of the plant in terms of its antioxidant properties. Present study evaluated non-enzymatic antioxidants like proline, lycopene, carotenoids and polyphenols and enzymatic antioxidants such as superoxide dismutase (SOD), polyphenol oxidase (PPO), amylase and lipid peroxidase (LPx) by standard estimation methods. Proline is an amino acid molecule that plays an important role in physiological as well as cellular processes (cell proliferation, cell death and gene expression) in plants (Szabados and Arnould, 2010). The amount of proline in *Amaranthus viridis* was estimated 0.618 mg g^{-1}in leaf and 0.519 mg g^{-1} in stem (Fig. 5). Lycopene comprises the most important class of carotenoids which at physiological concentration has the potential to act against cancerous cells and its proliferation. It interferes with growth factor receptor signalling and cell cycle progression in prostate cancer cells without toxic effects and cell apoptosis (David and Lu, 2002). The estimated amount of proline in methanolic leaf extract of *Amaranthus viridis* (0.152 mg g^{-1}) and in stem extract of *Amaranthus viridis* (0.082 mg g^{-1}) as shown in figure 5.

Carotenoids are widely distributed lipophilic pigment molecules with antioxidant properties. They act as antenna molecules in capturing light and in transfer of energy to chlorophyll molecules during photosynthesis. They also give protection to the photosystem by interacting with free radicals and to light induced tissue damage. Carotenoids can quench highly reactive singlet oxygen and block free radical mediated reactions (Bendich and Olson, 1989). The estimated

amount of carotenoids in leaves of *Amaranthus viridis* is 0.418 mg g^{-1} and in stem 0.396 mg g^{-1} (Fig.5). Flavonoids are a type of secondary metabolites obtained from plants. It is usually found in fruits, vegetables, nuts, seeds, stems and flowers. Flavonoids are also the best sources of natural antioxidants in human diets (Sankhadip*et al*., 2018). The amount of total flavonoids is 0.920 mg g^{-1} in the leaves and 0.892 mg g^{-1} in the stem of *Amaranthus viridis* and found to be high (Fig .5).

Superoxide dismutase (SOD) are metalloenzymes catalysing dismutation or reaction of superoxide radicals. On the basis of the cofactor present, they are classified into Fe-SOD (found in mitochondria) and Cu-SOD or Zn-SOD (found in chloroplast, peroxisomes and cytosol). They are activated under different abiotic stress conditions such as water deficiency, chilling, heat, hypoxia and Heavy metals in toxic concentrations. Intracellular SOD may play a key role in protection of cancer cells against reactive oxygen species generated by anticancer drugs and radiation (Shingo *et al*., 1994). The amount of superoxide dismutase is 1.296 mg g^{-1} in leaves and 1.819 mg g^{-1} in the stem of *Amaranthus viridis* as shown in Fig .6. Polyphenol oxidases (PPO) mediated reactions play an important role in alteration of colour, flavour, texture and nutritional values of fruit and vegetable crops (Yurok and Marshall, 2003). The estimated amount of enzymatic antioxidant polyphenol oxidase is 0.956 mg g^{-1} in leaf and 0.813 mg g^{-1} in stem of *Amaranthus viridis* (Fig 6.). Amylases are one of the most thoroughly researched digestive enzymes catalysing degradation of polysaccharides or reserve carbohydrates (starch in plants) to simple sugars providing energy source in organisms. Amylase play an important role in inducing growth of embryos by the breakdown of starch to sugar in the seeds (Pradeesh and Swapna,2018). The estimated amount of enzymatic antioxidant amylases is 0.948 mg g^{-1} in leaf and 0.892 mg g^{-1} in stem of *Amaranthus viridis* as shown in Fig 6. Free radicals such as H_2O_2 attacks unsaturated fatty acids producing lipid hydro peroxides and further occurrence of chain reactions changes lipid structure and organisation of cell membrane in the process of lipid peroxidation. Peroxidase enzymes (stress enzymes) are hemi-containing enzymes that can oxidise various substrates using H_2O_2 that prevent its accumulation under metabolism during stress conditions thereby preventing lipid peroxidation (Pradeesh and Swapna, 2018). The result revealed that the amount of enzymatic antioxidant lipoperoxide is 0.912 mg g^{-1} in leaves and 0.826 mg g^{-1}in the stem of *Amaranthus viridis* (Fig 6.).

Evaluation of Pharmacological Property

Plants have been known to be effective in treating various diseases since ancient times. Cancer is one of the leading fatal diseases of man. The relevance of active compounds presents in plants in treating malignant tumours and preventing cancer is acquiring more popularity in recent years.Present study evaluated *in vitro* anticancer activity of *Amaranthus viridis* leaf extract and stem extract in methanol. Anticancer effect was analysed using Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cell lines. Viability was determined by Trypan blue exclusion method. The viable cell suspension 1×10^6 cells in 0.1 ml was added in the tubes containing various concentrations (100, 500 and 1000 μg/ml) of test compounds and the volume was made up to 1 ml using phosphate buffer saline (PBS). The mixtures were incubated for 3 hours at 37^{o}C and were added with 2 drops of Trypan blue dye. Dead cells take up the blue colour of the dye while the live cells do not. Reduction in the viable cell count and increased non-viable cancer cell count towards normal in tumour host suggest antitumor effect against EAC and DLA cells in mice. Cyclophosphamide is used as a standard anticancer compound. The result obtained from anticancer study revealed that the leaf methanol extract of *Amaranthus viridis*showed 46.2, 73.9, 86.4 cytotoxicity in EAC compared to 41.9, 66.9, 78.2 cytotoxicity in DLA and the stem methanol extract showed 39.8, 60.3, 79.4 cytotoxicity in EAC compared to 35.6, 49.2, 62.1 cytotoxicity in DLA at concentrations of 100,

500 and 1000 µg/ml (Fig 7.) and (Table 2.). Results obtained in the present study demonstrated that the methanol extract of the *Amaranthus viridis* exhibits *in vitro* anticancer activity against DLA and EAC cell lines. The leaf extract showed concentration dependent cytotoxicity which was found to be effective against solid tumours induced by DLA and ascites tumours induced by EAC. Fijesh (2011), reported that the extracted cells showed membrane blebbing, vacuole formation and nuclear condensation which was absent in untreated cells. Thus, the cytotoxic and antitumor effects of the leaf extract can provide possibilities to novel therapeutic findings for treating cancer cells.

In vitro Conservation of Nodal Explants of *Amaranthus viridis*

In vitro conservation of *Amaranthus viridis* was carried out by using different explants such as stem, node with axillary bud and terminal bud in MS medium supplemented with 2 mg/L BAP (Plate 1 and 2). Results revealed that nodal explant with axillary buds possess comparatively higher survival chance.

Table 2. *In vitro* Anticancer Activity in *Amaranthus viridis*

		LEAF		STEM	
Concentration	**Standard**	**DLA**	**EAC**	**DLA**	**EAC**
100 $\mu g/ml$	61.908	41.9	46.2	35.6	39.8
500 $\mu g/ml$	86.39	66.9	73.9	49.2	60.3
1000 $\mu g/ml$	98.19	78.2	86.4	62.1	79.4

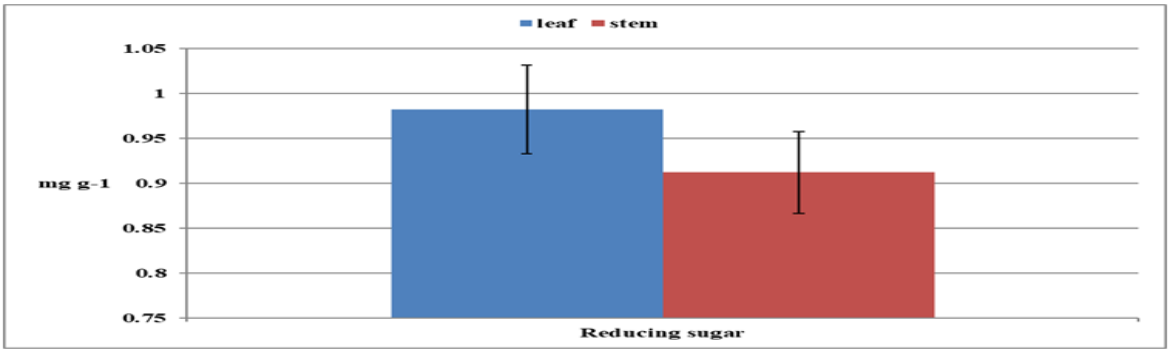

Figure 1. Reducing sugar of *Amaranthus viridis*

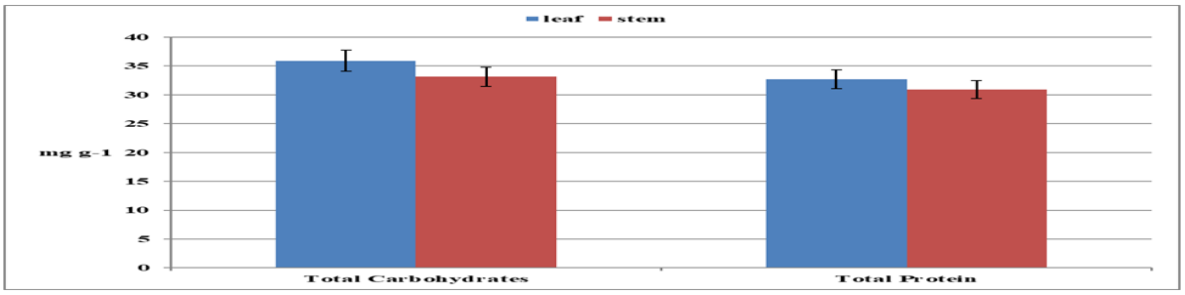

Figure 2. Total carbohydrates and Total protein in *Amaranthus viridis*

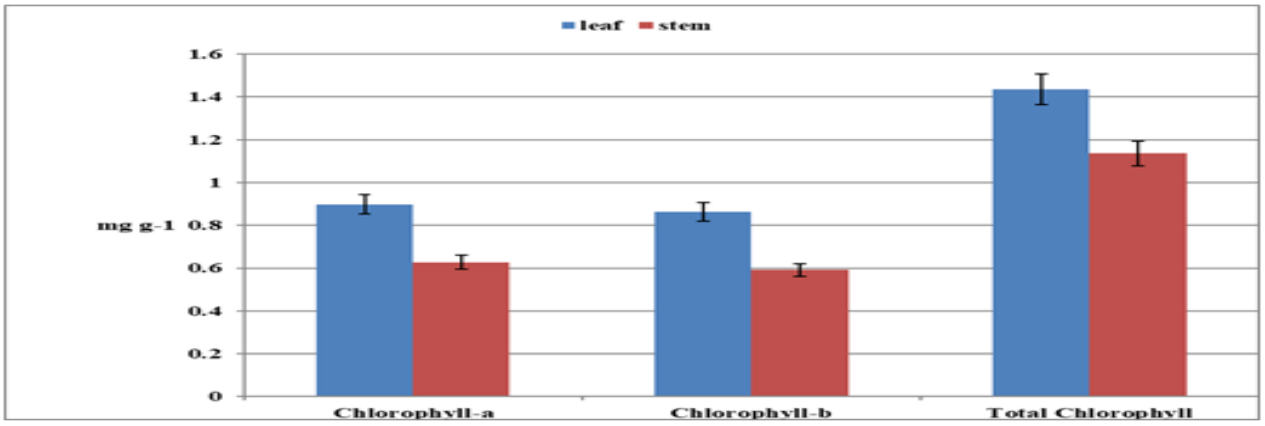

Figure 3. Pigments in *Amaranthus viridis*

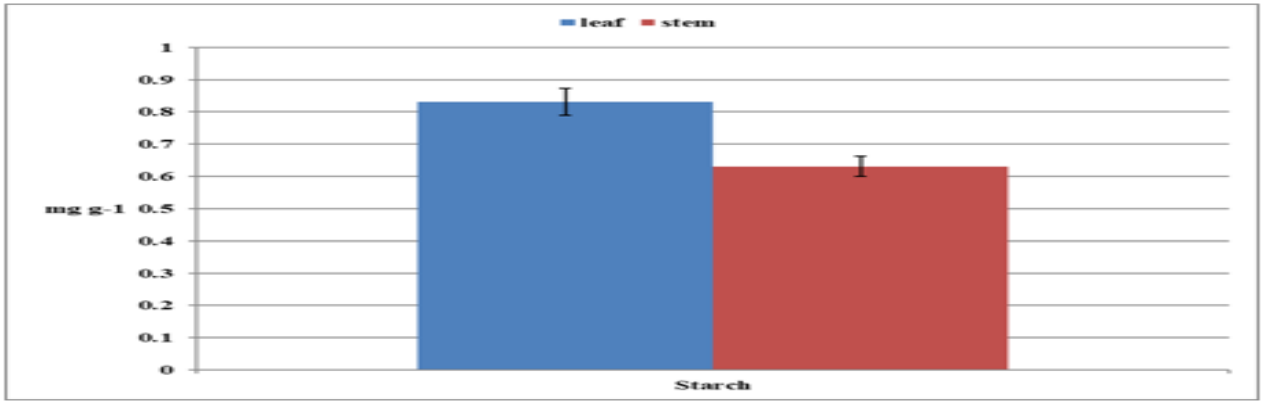

Figure 4. Starch in *Amaranthus viridis*

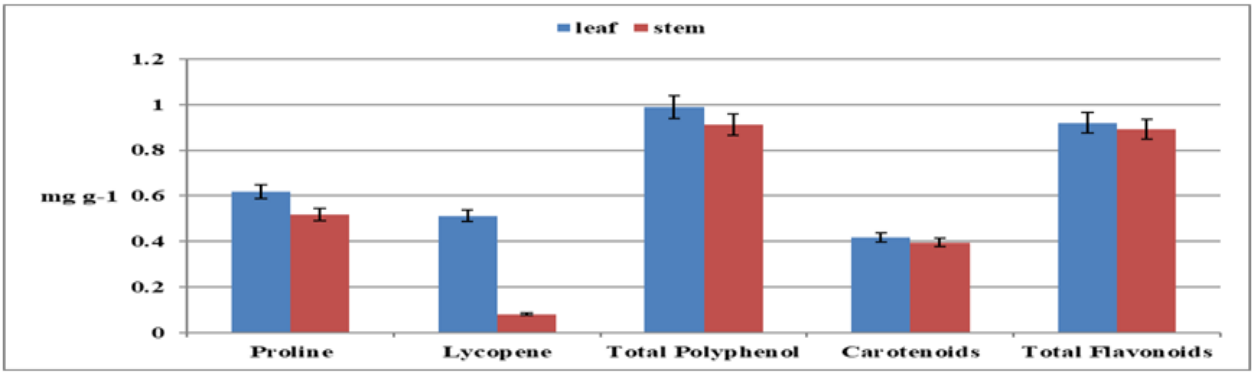

Figure 5. Non-Enzymatic Antioxidants in *Amaranthus viridis*

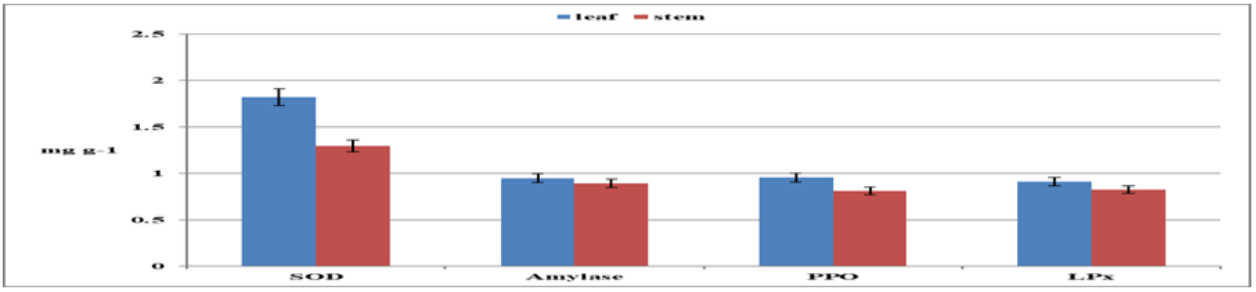

Figure 6. Enzymatic Antioxidants in *Amaranthus viridis*

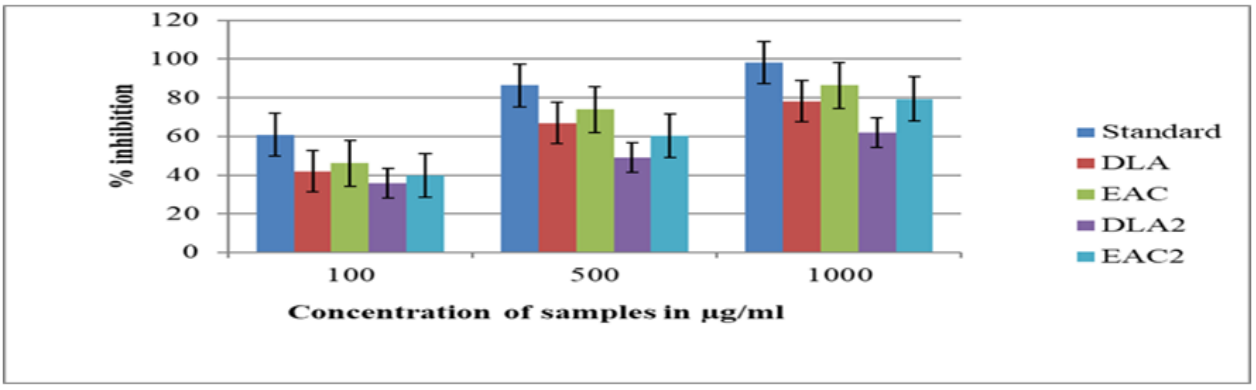

Figure 7. *Invitro* anticancer activity in *Amaranthus viridis*

Plate 1. inoculated explant of *Amaranthus viridis*

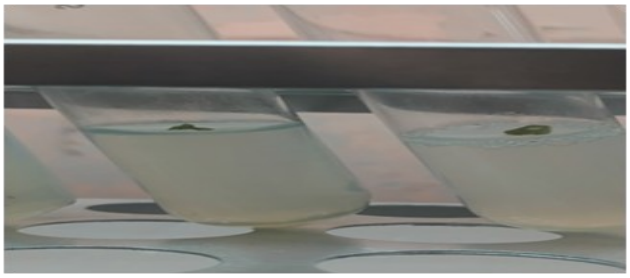

Plate 2. Node with axillary bud

Conclusion

Tribal communities and local people of Kerala use the leaves of *Amaranthus viridis* for treating diseases like asthma, gastrointestinal symptoms, skin disorders, respiratory and urinary problems and hepatic and cardiovascular disease. Present study on *Amaranthus viridis* is concerned with preliminary qualitative analysis of phytochemicals, quantitative analysis of nutritional and antioxidant properties, anticancer analysis on DLA and EAC cell lines and *in vitro* conservation. Anticancer analysis on the crude methanolic extract was carried out. Results obtained in the present study demonstrated the concentration dependent anticancer effect of methanolic extract of *Amaranthus viridis* in DLA and EAC cell lines which was found to be moderate to high. The results from the current study indicated that methanol extract of the *Amaranthus viridis*contained various types of compounds with potential pharmacological activity. The presence of various bioactive compounds justifies the use of *Amaranthus viridis* for various ailments by traditional practitioners. From data, identification of more compounds in their extract and it previously reported that these compounds have antibacterial, antifungal, antioxidant and anticancer activity but further research should be made to isolate and purify natural products in their extract. *In vitro* conservation of the nodal explants of *Amaranthus viridis* was done with different explants (node with axillary bud and terminal bud) explants were inoculated in MS medium supplemented with BAP and the culture was maintained at 16 hours' photoperiod at a temperature of 26^{o}C. This generated information on phytochemical, nutritional, and medicinal characteristics and therapeutic potential of *Amaranthus viridis* provide scientific proof for identifying the plant bio resource and its effective utilisation in the future. The behaviour of plants in culture can provide basis for future conservation strategies and aid in selection of suitable explant of the plant for future studies. In brief the wild species of *Amaranthus viridis* fits its claims of nutritional and medicinal properties which satisfy its use as an ethnomedicinal plant by tribal communities of Kerala.

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