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Comparative phytochemical analysis & free radical scavenging potentials of tubers, shoots, of wild & *in vitro* extracts of *Ruellia tuberosa* L. [Acanthaceae]

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ABSTRACT

Ruellia tuberosa L. (Acanthaceae) is an important medicinal plant, traditionally used to cure diseases. People make a tea with the roots to treat kidney stones and urinary tract infections. *Hence the present study was carried to promote the synthesis of shoot production from different explants such as axillary and terminal meristems. For shoots, MS medium supplemented with different combination of hormones such as Kinetin and BAP were used and for roots NAA and IBA were used. A maximum shoot length was obtained in kinetin and BAP with 2 mg/l concentration and maximum root length was obtained in NAA and IBA with 1mg/l concentration. The aim of this study was to estimate and compare the concentration of bioactive compounds between wild and Invitro studies of R.tuberosa plants. A marked decrease in the total carbohydrate, flavonoids, quinones, and terpenoids were observed between Invitro propagated and wild type plants. This study clearly indicates that the environmental factors play a crucial role in the plant metabolic activities.*

Key words: Traditional, *Invitro* propagation, Wild, Phytochemical.

INTRODUCTION

Herbal medicine is still the mainstay of about 75–80% of the world population, for primary health care because of better compatibility, better cultural acceptability with the human body and lesser side effects, mainly in the developing countries. Medicinal herbs are moving from fringe to mainstream use with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals. Recently, considerable attention has been paid to utilize bio-friendly and eco-friendly plant-based products for the cure and prevention of different human diseases [26]. It is documented that the world's population of 80% has faith in traditional medicine, particularly plant drugs for their primary healthcare [27]. The medicinal importances of secondary metabolites of plants are reported to have pharmacological benefits to mankind. Their extraction and formulations form the basis of modern day herbal alternatives to synthetic medicines. Natural antioxidants, particularly in fruits and vegetables have gained increasing interest among consumers and the scientific community, because epidemiological studies have indicated that frequent consumption of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer [28 and 34]. Reactive oxygen species (ROS) such as singlet oxygen (1O2),superoxide anion (O2–), hydroxyl (.OH) radical and hydrogen peroxide (H2O2) are often generated as by –products of biological reactions or from exogenous factors [20]. These reactive species exert oxidative damaging effects by reacting with nearly every molecule found in living cells [29]. Such species are considered to be important causative factors in the development of diseases such as cancer, stroke, diabetes, arteriosclerosis, cardiovascular diseases and the aging process [36]. The diseases of Human body have multiple mechanisms especially enzymatic and non-enzymatic anti-oxidant systems to protect the cellular molecules against reactive oxygen species (ROS) induced damage [2]. However, the innate defense may not be enough for severe or continued oxidative stress. Hence, certain amounts of exogenous anti-oxidants are constantly required to maintain an adequate level of anti-oxidants in order to balance the ROS in human body. Many synthetic anti-oxidants such as butylated hydroxyl anisole (BHA) and butylated

hydroxyl toluene (BHT) are very effective and are used for industrial processing but they possess potential health risk like carcinogenicity and toxic properties to human health and should be replaced with natural anti-oxidants [1]. Among the various natural anti-oxidants, phenolic compounds in herbs act as anti-oxidants due to their redox properties, allowing them to act as reducing agents, hydrogen donors, free radical quenchers and metal chelators [17]. Several natural anti-oxidants have already been isolated from plant materials such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs. *Ruellia tuberosa* L. (Acanthaceae), known as cracker plant is traditionally used as diuretic, anti-pyretic, analgesic, anti-hypertensive, anthelmentic, abortifacient, emetic, in bladder disease, kidney disorder, bronchitis, gonorrhoea and syphilis [7]. Many phytoconstituents have been identified. It has been experimentally proved to possess anti-oxidant, anti-microbial, anti-cancer and gastroprotective activity and antinociceptive and anti-inflammatory activity [10]. Previous bioactivity studies on this plant revealed its antioxidant [8] and antimicrobial [38] properties; phytochemical analysis led to the isolation of a variety of plant secondary metabolites, including long-chain alkane derivatives [24], flavonoids [25 and 37].

The objective of the current study is to focus comparative phytochemical content and free radical scavenging potentials of the tuber, stem of wild and *Invitro* grown parts of R.tuberosa.

EXPERIMENTAL SECTION

2.1. PLANT COLLECTION

The young axillary and terminal buds and meristams of healthy branches of R.tuberosa were used as explants. They are collected from disease resistant plants .Nodal explants with a single axillary bud is also used as explants. For surface sterilization the collected buds and nodes were washed with running tap water for 5 to 10 minutes and then treated with 5% teepol solution for 5 minutes followed by rinsing with double distilled water. To eliminate the fungal contamination, explants were further treated with 0.1% mercuric chloride for 3 to 5 minutes followed by 4-5 rinses in sterile double distilled water. MS medium [23] containing 3% sucrose solidified with 1% agar (Tissue culture grade, Hi-media). The pH of the medium was adjusted to 5.6 - 5.8 by adding sodium hydroxide and hydrochloric acid [11] and agar was added before autoclaving at 121°C for 15 minutes under 15 lb pressure was used. The cultures were incubated under 16 hours of the photoperiod (2000 lux) provided by cool white fluorescent tubes at 25±2°C.

2.3. SHOOT INDUCTION

MS medium containing different combinations and concentrations of kinetin and BAP (0.5, 1.0, 1.5, 2.0 mg/l) were used for shooting attributes. BAP and kinetin both were checked in combinations with 0.1mg/l of IBA and IAA to induce multiple shooting [3 and 9]. The explants with bud proliferation cultures were transferred to fresh MS media for shoot multiplication. The cultures were maintained by regular subculture on fresh medium with the same composition. After proper shoot induction, the plantlets were carefully removed from the medium and washed with sterile double distilled water properly to avoid any trace of the roots.

2.4. INDUCTION OF ROOTING

The excised shoots with 9 to 10 cm long with 6-8 compound leaves were transferred to half strengthened MS medium containing 3% (w/v) sucrose supplemented with NAA and IBA concentrations (0.5, 1.0, 1.5, and 2.0 mg /l) respectively. After proper root formation the rooted plantlets were transferred to hardening. The well developed rooted plantlets were removed from the culture medium and then washed with sterile double distilled water. Then the plantlets were transferred to the tea cups containing a mixture of autoclaved vermicompost in the ratio 1:1:1 covered with a plastic bag and maintained in the tissue culture lab at 22±2°c for 2 to 3 days, minute holes have been put on the plastic bag. The acclimatized plants were then transferred to the normal room temperature for the next 4 days and finally they were maintained in green house condition to know the survivability rate (fig C). The pots under natural conditions and survivability in nature were recorded. From these *Invitro* hardened plants, the entire plant has been taken for comparative work for further studies.

2.4. SAMPLE PREPARATION

The powdered samples were subjected to sequential extraction using Hexane, Chloroform and ethanol solvents. The extracts were filtered using Whatman filter paper No. 1 and concentrated with rotary evaporator.

2.5. PHYTOCHEMICAL ANALYSIS

The phytochemical tests were done to detect the presence of carbohydrates, tannins, saponins, flavonoids, alkaloids, quinines, glycosides, cardiac glycosides, terpenoids, triterpenoids, phenols, coumarins, proteins, steroids, phytosteroids, phlobatannins and anthraquinones. The tests were based on the visual observation of a change in colour or formation of precipitate after the addition of specific reagents by following the standard phytochemical methods, [35 and 14].

2.6. DPPH ASSAY

The ability of the extracts to annihilate the DPPH radical (1, 1-diphenil-2-picrylhydrazyl) was investigated by the method described by Blois [6]. Stock solution of leaf extracts was prepared to the concentration of 1mg/ml. 100µg of each extracts were added, at an equal volume, to methanolic solution of DPPH (0.1mM). The reaction mixture was incubated for 30min at room temperature; the absorbance was recorded at 517 nm. The experiment was repeated for three times. Ascorbic Acid was used as standard on controls. The annihilation activity of free radicals was calculated in % inhibition according to the following formula: % of Inhibition = (A of control – A of Test)/A of control * 100.

RESULTS AND DISCUSSION

3.1. *INVITRO* PROPAGATION

The data on effective *Invitro* regeneration of nodal explants in terms of shoot regeneration, multiple shoot formation and rooting of R. tuberosa in standardized MS medium given in Table.1 and the response for these attributes was shown in figures A and B. The shoots by formation was effective (90%) in the medium containing kinetin and BAP at 2.0 and 2.0 mg/l respectively. Multiple shooting by sub culturing of the secondary explants, the shoots was highly appreciable in the MS medium containing Kn and BAP 2.0 mg /l respectively (fig A). The further sub culturing for rooting was better in (100%) in the medium containing NAA and IBA at 1.0 mg/l respectively i.e., shown in (fig B) and table 1. The survival of plants was well established i.e. 90% in the hardening medium containing red soil, coco peat, vermicompost in the ratio 1:1:1 in (fig C). The plantlets were developed within 45 days from nodal explants and they were maintained further in the green house for 45 days.

After hardening, the growth rate of the plantlets was slow initially and increased gradually. New leaves emerged from the hardened plantlets after three weeks. Most of the plantlets survived after hardening. Nearly 90% of the regenerated plantlets survived under green house conditions and it is shown in (fig D). Loss of regenerants was already observed in *Eucalyptus tereticornis* [12], *Solanum nigrum* [4], *Rauvolfia serpentina* [16].

3.2. Phytochemical analysis

The results of the preliminary phytochemical investigation on chloroform extract of selected parts of R.tuberosa revealed the presence of various phytoconstituents. The tubers of R.tuberosa showed the presence of cardiac glycosides, carbohydrates, terpenoids, flavonoids, quinones, and steroids. Phenols were the dominant group present in all the extracts of shoot (wild type). The tissue cultured samples showed the presence of tannins, flavonoids, alkaloids, phenols and coumarins.

This presence of a variety of secondary metabolites hinted the potential application of these extracts for pharmacological purposes. This is in validation of the fact that this plant has been used across the globe as a medicinal herb for varied reasons.

3.3. DPPH assay

DPPH (1, 1-diphenyl-2-picrylhydrazyl) is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule yellow-colored diphenylpicrylhydrazyl acid was used as standards. The DPPH radical of reduction capability is determined by the decrease in absorbance at 516 nm induced by anti-oxidants. It has been found that ascorbic acid, cysteine, glutathione, tocopherol, flavonoids, tannins, and aromatic amines (p-phenylenediamine,p-aminophenol, etc.), reduce and decolorize DPPH by their hydrogen donating ability. This DPPH activity was expressed as decrease in absorbance of the samples data different concentration levels.

The free radical scavenging potentials of the R .tuberosa extracts were analysed by DPPH assay. The results revealed that the hexane extracts of the stem concentration of 600ug showed the highest inhibition at 84.44%. The extracts of ethanol of the *Invitro* samples also showed significant inhibition at 1000ug (72.02%).

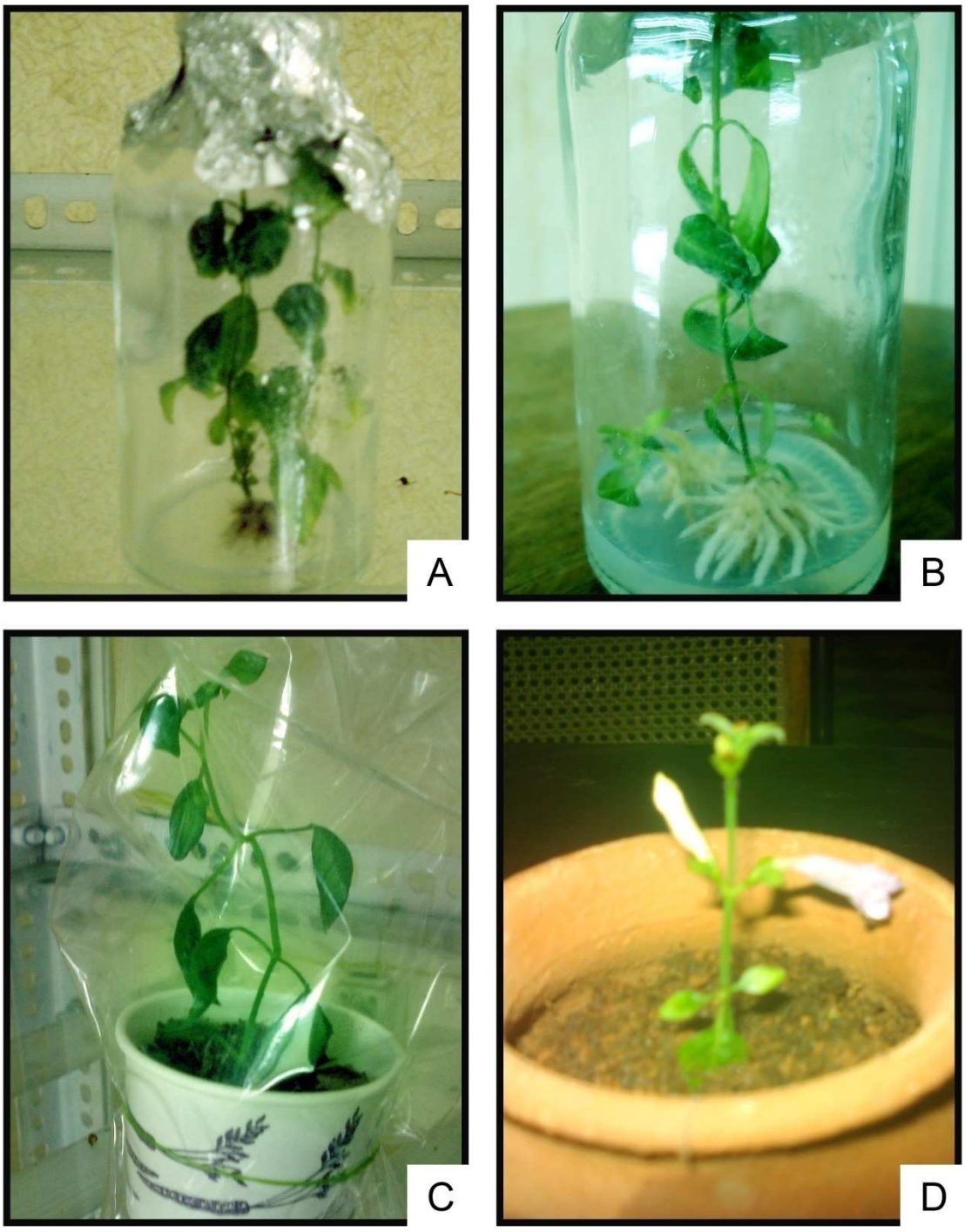

FIGURE : 1. Stages of regeneration of *Ruellia tuberosa* L.
(A) Multiple shoot formation, (B) Root regeneration (C) Hardening and (D) Plants acclimatized in Green house condition.

Table 1: Different growth regulators for shooting and rooting response of *R.Tuberosa* L.

Growth regulators mg/l	Conc mg/l	% of explant showing response	No of shoots (cm)
BAP	2.0	70.0	8.0± 0.5
Kinetin	2.0	95.0	7.0±1.41
	Conc mg /l	% of rooting response	No of roots
NAA	1.0	95.0	2.5±0.31
IBA	1.0	95.0	1.6±0.19

Table. 2: Tuber extracts of *Ruellia tuberosa*

	Phytochemical Tests	Results		
		ETHANOL	HEXANE	CHLOROFORM
1	Carbohydrates test	Weakly+	Weakly+	Weakly +
2	Tannins test	-	-	-
3	Saponins test	-	-	-
4	Flavonoids test	W+	W+	W+
5	Alkaloid test	-	-	-
6	Quinones test	W+	W+	W+
7	Glycosides test	-	-	-
8	Cardiac glycosides test	+	+	+
9	Terpenoids test	-	-	W+
10	Phenols test	-	-	+
11	Coumarins test	-	-	-
12	Steroids and Phytosteroids test	-	-	Steroids +
13	Phlobatannins test	-	-	-
14	Anthraquinones test	-	-	-

Table.3: Shoot extracts of *Ruellia tuberosa*

S. No	Phytochemical Tests	Results		
		ETHANOL	HEXANE	CHOLROFORM
1	Carbohydrates test	W+	W+	W+
2	Tannins test	W+	w+	w+
3	Saponins test	-	W+	-
4	Flavonoids test	-	-	-
5	Alkaloid test	-	-	-
6	Quinones test	-	-	-
7	Glycosides test	-	-	-
8	Cardiac glycosides test	+	+	+
9	Terpenoids test	-	-	-
10	Phenols test	+	+	+
11	Coumarins test	-	-	-
12	Steroids and Phytosteroids test	-	-	-
13	Phlobatannins test	-	-	-
14	Anthraquinones test	-	-	-

Table. 4: In-Vitro extracts of *Ruellia tuberosa*

S.No	Phytochemical Tests	Results		
		HEXANE	CHLOROFORM	ETHANOL
1	Carbohydrates test	**W+**	-	+
2	Tannins test	-	W+	+
3	Saponins test	-	-	-
4	Flavonoids test	-	+	-
5	Alkaloid test	-	+	-
6	Quinones test	W+	-	W+
7	Glycosides test	-	-	-
8	Cardiac glycosides test	-	-	+
9	Terpenoids test	-	-	-
10	Phenols test	+	+	+
11	Coumarins test	-	+	-
12	Steroids and Phytosteroids test	-	-	-
13	Phlobatannins test	-	-	-
14	Anthraquinones test	-	-	-

+ Present
- Absent

Fig: 2 *Invitro* DPPH Scavenging Activity of different extracts of *Ruellia tuberosa*

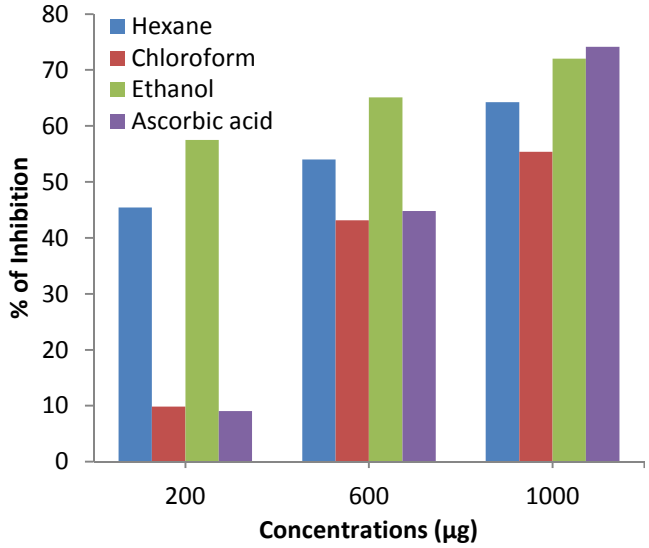

Table: 5 *Invitro* DPPH Scavenging Activity of different extracts of *Ruellia tuberosa*

DPPH Scavenging assay				
Concentration (µg)	Hexane	Chloroform	Ethanol	Ascorbic acid
200	45.4154	9.878419453	57.52279635	9.067882472
600	54.0274	43.13576494	65.12158055	44.78216819
1000	64.2351	55.39513678	72.01114488	74.16413374

Fig: 3 Shoot DPPH Scavenging Activity of Different Extracts of *Ruellia tuberosa*

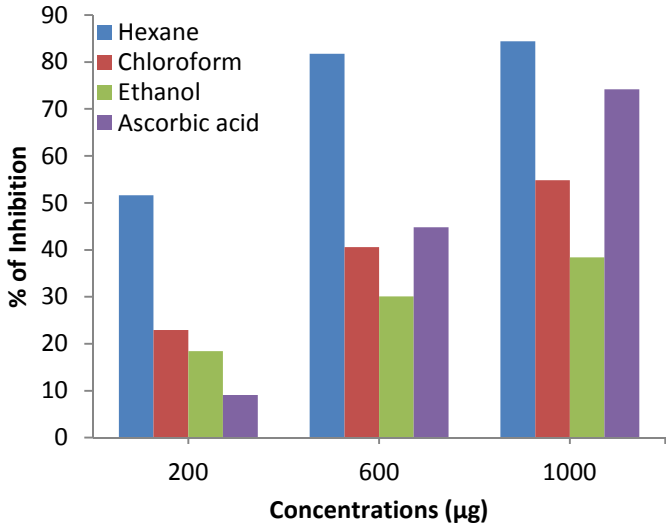

Table:6 Shoot DPPH Scavenging Activity of Different Extracts of *Ruellia tuberosa*

DPPH Scavenging assay				
Concentration (µg)	Hexane	Chloroform	Ethanol	Ascorbic acid
200	51.6464	22.94832827	18.43971631	9.067882472
600	81.7376	40.60283688	30.06585613	44.78216819
1000	84.4478	54.81256332	38.39918946	74.16413374

Fig: 4 Tubers DPPH Scavenging Activity of Different Extracts of *Ruellia tuberosa*

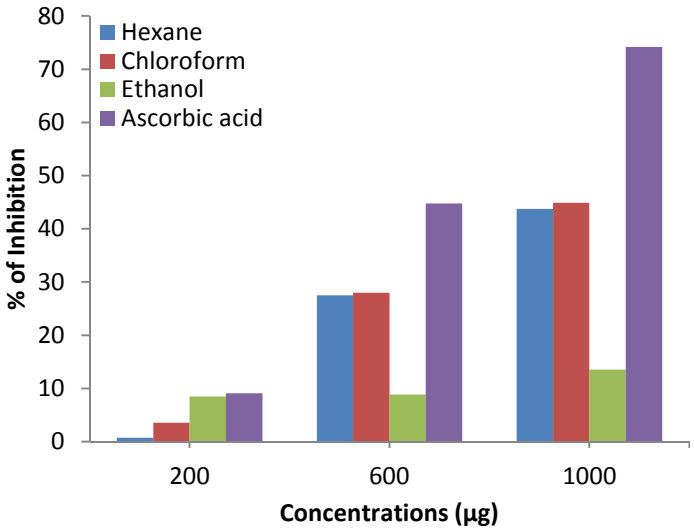

Table: 7 Tubers DPPH Scavenging Activity of Different Extracts of *Ruellia tuberosa*

DPPH Scavenging assay				
Concentration (µg)	Hexane	Chloroform	Ethanol	Ascorbic acid
200	0.73455	3.571428571	8.459979737	9.067882472
600	27.5076	27.96352584	8.865248227	44.78216819
1000	43.769	44.88348531	13.52583587	74.16413374

CONCLUSION

Plants contain a wide variety of free radical scavenging molecules, such as flavonoids, anthocyanins, carotenoids, dietary glutathionine, vitamins and endogenous metabolites and such natural products are rich in antioxidant activities [21]. These plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, peroxide decomposers, enzyme inhibitors and synergists. Electron acceptors, such as molecular oxygen, react easily with free radicals to become radicals themselves, also referred to as reactive oxygen species (ROS). The ROS include superoxide anions, hydrogen peroxide (H2O2) and hydroxyl radicals (+OH) [13]. There are increasing suggestions by considerable evidence that the free radicals induce oxidative damage to bio molecules (lipids, proteins and nucleic acids), the damage which eventually causes atherosclerosis, ageing, cancer, diabetes mellitus, inflammation, AIDS and several degenerative diseases in humans [15].

The present study is an attempt to understanding the differences in the phytochemical content and antioxidant potentials of various parts of the wild plant and their *Invitro* tissue cultured counterparts.

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