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Original Research Article

Understanding the phytochemical constitution, antioxidant potential and spectral characteristics of aqueous extracts of the chosen leafy vegetables from south India

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ABSTRACT

The phytochemical composition, total phenolic and ascorbic acid contents of the aqueous extracts of six south Indian leafy vegetables viz. *Amaranthus viridis* L, *Hibiscus cannabinus* L, *Spinacea oleracea* L, *Mentha spicata* L, *Murraya koenigii* L and *Coriandrum sativum* L were tested, characterized and the antioxidant potential was evaluated. In addition, aqueous leaf extracts of *A. viridis* and *H. cannabinus* were demonstrated for their ability to form silver nanoparticles. All the aqueous extracts demonstrated the presence of flavonoids, saponins, tannins (except *M. koenigii*) and terpenoids (except *S. oleracea*) while phlobatannins (only in *M. spicata*), steroids, cardiac glycosides, alkaloids and proteins were absent in all the tested leaves. Carbohydrates were found only in the aqueous extract of *H. cannabinus*. The total phenolic content was in the order of 0.9, 0.2, 1.8, 0.8 and 2.1 mgGAE/g of the leaves for *A. viridis*, *H. cannabinus*, *M. spicata*, *M. koenigii* and *C. sativum*, respectively, while *S. oleracea* did not respond to total phenolic content assay by FC method. Ascorbic acid, present in all the leafy vegetables exhibited the lowest value of 0.3 mg/g in *M. spicata* and the highest value of 1.9 mg/g in *C. sativum*. In addition, a highly significant DPPH radical scavenging activity was found in *H. cannabinus* (82.76%) followed by *M. spicata* (78.08%) and *C. sativum* (69.76%) at an extract concentration of 31.25 mg/mL, while *A. viridis*, *S. oleracea* and *M. koenigii* showed 72.95%, 58.27% and 75.38%, respectively at a concentration of 50 mg/mL. FT-IR spectral characterization of the extracts and the synthesized silver nanoparticles (Ag NPs) indicated the presence of N-H amines, O-H stretch, C-H out of plane bending vibrations, C=C stretching of alkenes, C=O stretch of amide and C≡C stretch of alkynes. According to our findings, the leafy vegetables can be used for the synthesis of lead compounds which will cure diseases.

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1. Introduction

Regular consumption of fresh and green vegetables as well as fruits for healthy living has been advocated since ages. However, the mechanism of this action has been understood only for the past few years. Dietary antioxidants are found to reduce the risk of several diseases (Hunter and Fletcher, 2002). Phytochemical constituents which are biologically active but non-nutritious are understood to impart several health benefits (Oomah and Mazza, 2000). It is now well-accepted that food constituents play a vital role as essential nutrients in preventing and delaying the premature onset of chronic disease late in life (Liu, 2003). From the epidemiological studies, it is evident that fruit and vegetable consumption reduces the risk of cancer by 15%, cardiovascular diseases by 30% and mortality by 20% (Rimm et al., 1996; Steimez and Potter, 1996). The higher intake of dietary antioxidants in the form of fruits and vegetables on regular basis is observed to offer protection against chronic diseases like cancers, cardiovascular and cerebro-vascular diseases.

Andhra Pradesh consists of nearly 1577 medicinal plants including both native and exotic species. Traditionally, *Amaranthus viridis* L, *Hibiscus cannabinus* L, *Spinacea oleracea* L are eaten as leafy vegetables and

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Fig. 1. Photographs of the plants: a: *Amaranthus viridis,* b: *Hibiscus cannabinus,* c: *Spinacea oleracea,* d: *Mentha spicata,* e: *Murraya koenigii* and f: *Coriandrum sativum*.

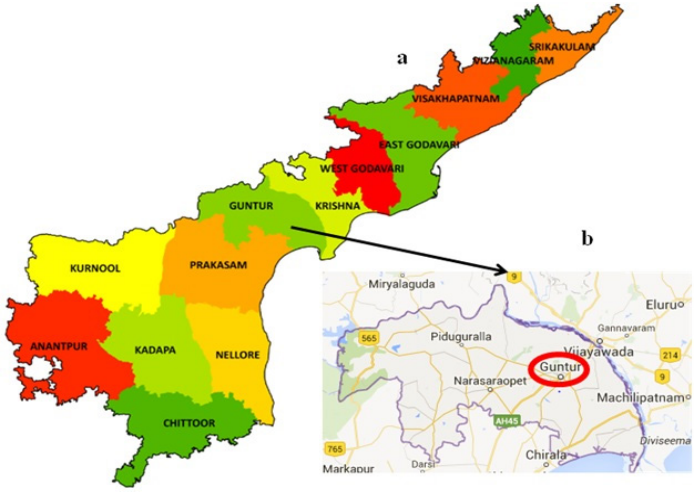

Fig. 2. a: The thirteen districts of Andhra Pradesh b: Sampling area: Guntur town of Guntur Dt.

Mentha spicata L, *Murraya koenigii* L and *Coriandrum sativum* L are used as garnishing vegetables in South India (Fig. 1). *A. viridis* is distributed throughout the world especially in tropical countries, used in treating respiratory problems like asthma, antidote for snake bites and scorpion stings, anti-ulcer, anti-rheumatic, diuretic, laxative and anti-leprotic activities (Sowjanya et al., 2014). *H. cannabinus*, is used as the folk medicine for the treatment of throat and blood disorders, to check excessive secretion of bile leading to jaundice or congestion of liver with acidity, bilious conditions, fever and puerperium, stomach problems, earache and appetizer, pains and bruises (Lee et al., 2007). *S. oleracea* is an annual leafy green vegetable native to central and southwestern Asia and not grown in most parts of the world. It is a rich source of vitamins (A, C, E, K) and minerals like Mg, Mn, Fe, P, Ca, Zn, Cu, K and folic acid, carotenoids (beta-carotene and lutein). This plant has also been shown to be rich in flavonoids and phenolic compounds comprising antioxidant, anti-inflammatory, anti-proliferative, hepato-protective, anti-histaminic, CNS depressant, protection against gamma radiation and used to prevent bone loss associated with osteoporosis (Gaikwad et al., 2010). Mentha, the oil extracted from the *M. spicata* is being used in the treatment of cold, flu, cough, as a strong pain killer and a proper remedy against several diseases like cancer, diabetes, asthma and heart problems. The oil obtained from the plant shows antimicrobial and antiviral activities (Neerj et al., 2013). The pure compounds and crude organic extracts of the leaves of *M. koenigii* possess anti-inflammatory, anti-amnesic, wound healing and phagocytic activity etc (Handral et al., 2012). *C. sativum* is one of the earliest spices used by mankind for analgesic, digestive, carminative, anti-rheumatic and anti-spasmodic etc (Mahendra and Bisht, 2011). Literature survey reveals that intensive research is under way on the plants viz. *Achillea* species (Mohammadhosseini et al., 2017a), *Alternanthera sessilis* (Sobha et al., 2017a), *Ziziphora* species (Mohammadhosseini, 2017a), *Rosa damascene* (Nunes and Miguel, 2017), *Leucas aspera* (Pavunraj et al., 2017), *Borago officinalis* (Aidi Wannes et al., 2017), *Artemisia sieberi* (Mohammadhosseini, 2017b; Mohammadhosseini et al., 2016), *Salvia limbata* (Mohammadhosseini et al., 2017b) accounting for the occurrence of different kinds of phytoconstituents. A few of the leafy vegetables are tested for their ability to form silver nanoparticles viz. *Rumex acetosa* (Sobha et

al., 2017b), *Alternanthera sessilis* (Niraimathi et al., 2013) and *Moringa oleifera* (Prasad and Elumalai, 2011).

Inspired with the importance of traditional leafy vegetables/medicinal plants, the present work is intended to understand the phytochemical ingredients of three major (*Amaranthus viridis* L., *Hibiscus cannabinus* L. and *Spinacea oleracea* L.) and three minor (garnishing) green leafy vegetables including *Mentha spicata* L., *Murraya koenigii* L. and *Coriandrum sativum* L. of Indian cuisine on the basis of their quantity consumed on average per day to evaluate their antioxidant potential and spectral characteristics. The geographical map of the sampling area is depicted in Fig. 2. Further, *A. viridis* and *H. cannabinus* have been tested for their ability to form silver nanoparticles. The main reasons for synthesizing silver nanoparticles from are their safety besides being economical, effective and easy availability (Prakash and Gupta, 2005).

2. Experimental

2.1. Materials and methods

All the chemicals and solvents used were of analytical grade and purchased from either Sigma Aldrich Inc. (Mumbai) or Merck Ltd. (New Delhi). Sterilized borosil glass and tarson grade plastic were used for all the experiments to avoid contaminations. Healthy leafy vegetables containing *A. viridis*, *H. cannabinus*, *S. oleracea*, *M. spicata*, *M. koenigii* and *C. sativum* were purchased from the local vegetable market, Guntur, Andhra Pradesh, India. These herbal materials were thoroughly washed and used for the experimental study.

2.2. Preparation of aqueous extracts

5 g of washed and air dried leaves of the six vegetables were taken from the stem of the respective vegetable and chopped into fine pieces. 100 mL of deionized water was kept in hot water bath and when the water was boiling, the leaves were chopped into pieces and added immediately, allowed to boil for 10 minutes, cooled and filtered. pH of the extract was determined and then stored at 4 ºC till further use.

2.3. Phytochemical investigation

Phytochemical tests were performed using standard procedures (Trease and Evans, 1989; Sobha et al., 2017a).

2.4. Antioxidant activity

2.4.1. Determination of Total Phenolic Content (TPC)

Total phenolic content of aqueous extracts of the leaves was determined by Folin-Ciocalteu (FC) method using gallic acid as a standard phenolic compound (McDonald et al., 2001; Stanly et al., 2011). 100 mg of gallic acid was dissolved in 100 mL of deionized water to give a concentration of 1 mg/mL. Zero to 500 µL of gallic acid stock solution was taken in test tubes labeled S1 to S6 and made up to 1 mL with distilled water. Then, 250 µL of F-C reagent was added, mixed well and allowed to stand for 5 minutes at room temperature for the reaction to complete. In the next step, 2.5 mL of sodium carbonate (Na_2CO_3, 7%) was added to each of the tubes and the final volume was made up to 6 mL with distilled water. Samples were incubated at room temperature for 90 minutes and the absorbance was measured at 760 nm using UV-Vis spectrophotometer (Shimadzu). Standard graph was plotted with the values of absorbance on y-axis and concentration of gallic acid (mg/mL) on x-axis. The same protocol was repeated twice with the aqueous extracts of the green leafy vegetables instead of gallic acid solution and the concentration of phenolic content was calculated using the regression equation, $y=2.2x+1.86$ ($r^2=0.991$) obtained with the standard graph. The concentration of the total phenolic compounds in the extracts was determined using the Eqn. 1 as follows.

$$T=CxV/W \qquad \text{(Eqn. 1)}$$

Where T is the total phenolic content in mgGAE/g of plant extract; C is the concentration of gallic acid calculated from calibration curve (mg/mL); V is the volume of sample in mL; W is the weight of the plant extract in g. Thus, the TPC is expressed as mg of GAE/g of plant extract.

2.4.2. Estimation of ascorbic acid

Ascorbic acid with specific rotation [α^20/D] +20.5º to +21.5º purchased from Merck was used as the standard. 100 mg of ascorbic acid dissolved in 100 mL of 4% oxalic acid (1 mg/mL) was used as stock solution. Bromine liquor was added till the solution turns yellow orange and then excess bromine was removed by blowing in air. Ten times dilution of the stock with 4% oxalic acid was used as working standard. The principle of the colorimetric estimation is that the dehydroascorbic acid formed by bromination of ascorbic acid, reacts with 2,4 dinitrophenyl hydrazine to form osazone which then is dissolved in sulphuric acid to give an orange red color solution and finally its absorbance is measured at 540 nm (Sadasivam and Manickam, 2005). The advantage of the method is that the dehydroascorbic acid alone reacts quantitatively and does not reduce the other substances and hence gives a precise estimation of ascorbic acid present in the sample extract (Al-Ani et al., 2007). Standard dehydroascorbic acid solution containing 25 µg to 50 µg, at intervals of 5 µg, was pipetted out into a series of tubes. Similarly, aliquots of different brominated sample extracts were pipetted out into test series of tubes and finally the volume in

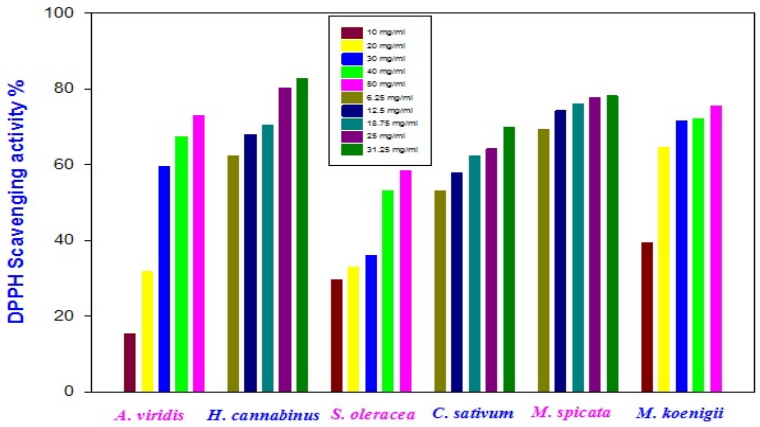

Fig. 3. DPPH radical scavenging activity (%) against indicated concentrations of aqueous leaf extracts in the color coded palette.

all the tubes was made up to 3 mL by adding distilled water. Then, 1 mL of 2,4-dinitrophenylhydrazine reagent which was prepared by heating 2 g of DNPH in 100 mL of 0.5 N H_2SO_4 was added to all the tubes followed by 1-2 drops of thiourea solution (10%). A blank containing all the reagents except the standard/plant extract was set up simultaneously and all the tubes were incubated at 37 ºC for 3 hours. Finally, the orange red osazone crystals formed were dissolved by adding 7 mL of H_2SO_4 (80%) and the absorbance measured at 540 nm using UV-Vis spectrophotometer (Shimadzu). From the linear regression equation obtained with the standard ascorbic acid, y=0.004x+0.08 (r^2=0.997), the concentration of ascorbic acid in the sample extract was calculated.

2.4.3. DPPH radical scavenging assay

The free radical scavenging activity of the aqueous extracts was measured *in vitro* by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Hsu et al., 2007; Alam et al., 2013). A DPPH solution will 0.3 mM in 100% ethanol was prepared and 1 mL of this solution was added to 3 mL of the extract. The mixture was will then shaken and allowed to stand at room temperature for 30 min and the absorbance was finally measured at 517 nm using a UV-Vis spectrophotometer (Shimadzu). Under the experimental conditions, the solution without leaf extract was used as control. Inhibition of DPPH free radical in percentage was calculated by the formula:

Table 1
Total phenolic content, ascorbic acid, and DPPH (%) scavenging activity of six leafy vegetables under investigation.

Leafy vegetables	Total phenolic content (mgGAE/g)	Ascorbic acid (mg/g)	DPPH (%)
A. viridis	0.9	1.3	72.95
H. cannabinus	0.2	1.5	82.76
M. spicata	1.8	0.3	78.08
M. koenigii	0.8	1.5	75.38
C. sativum	2.1	1.9	69.76
S. oleracea	-	1.0	58.27

$$\text{DPPH radical scavenging activity}(\%)=(A_0 - A_s)/A_0 \times 100 \quad \text{(Eqn. 2)}$$

Where A_0 and A_s are respectively due to the absorbance of the blank and the sample. The test was done in duplicate and the average values are plotted in Fig. 3. The results of the antioxidant tests are summarized in Table 1.

2.5. FTIR spectral characterization

All the aqueous leaf extracts were analyzed for the functional groups using FT-IR spectrometer (Bruker, UK) (Fig. 4 and Fig. 5) and the assignment of functional groups to the peaks obtained is presented in Table 2.

2.6. Green synthesis of silver nanoparticles

3 mM Silver nitrate ($AgNO_3$) was prepared by dissolving 0.255 g of $AgNO_3$ in 500 mL of deionized water. Aqueous leaf extracts (*A. viridis* and *H. cannabinus* individually) with pH adjusted to 6.5, 7.5, 8.5 and 9.5 using NaOH (0.1 N) and the silver nitrate solution were mixed in different ratios viz. 1:1, 1:2, 1:4, 1:6, 1:8, and 1:10 by volume respectively, kept at room temperature (28 ± 2 ºC) for 24 hours and monitored for the change in color every 30 minutes initially for 6 hours, and finally

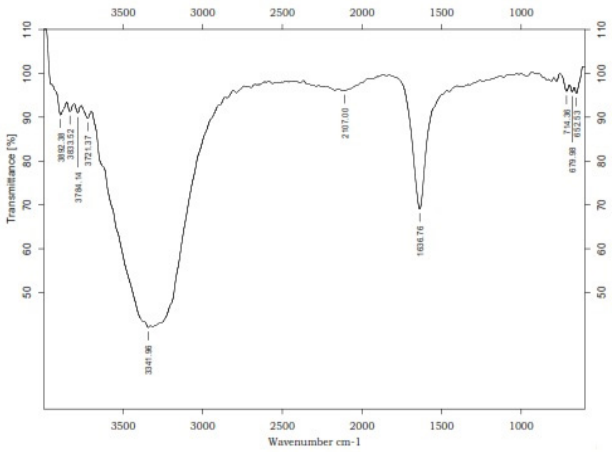

Fig. 4. FT-IR spectrum of the aqueous leaf extract of *A. viridis*.

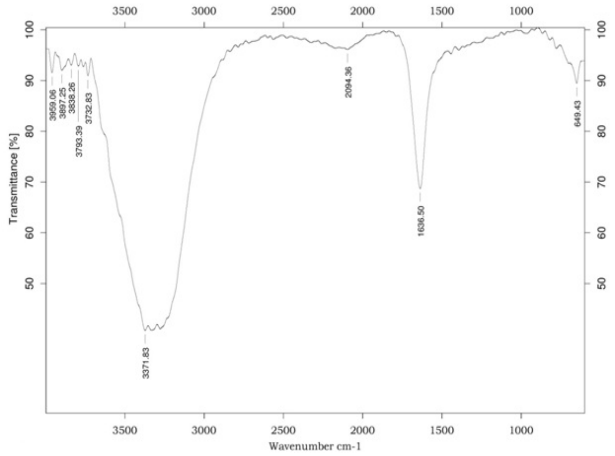

Fig. 5. FT-IR spectrum of the aqueous leaf extract of *H. cannabinus*.

after 24 hours (Sobha et al., 2017b). The appearance of black/brown color was taken as an indication for the formation of silver nanoparticles and the UV-Vis spectra taken from 380 to 600 nm, for confirmation of silver nanoparticles. The solution was then filtered and the obtained nanoparticles were dried in hot air oven at 60 ºC and analyzed by FT-IR spectra (Table 2).

3. Results and Discussion

3.1. Phytochemical composition

All the aqueous extracts of the leaves in the present study demonstrated the presence of flavonoids, saponins, tannins (except *M. koenigii*) and terpenoids (except *S.oleracea*), while phlobatannins (found only in *M. spicata*), steroids, cardiac glycosides, alkaloids and proteins were absent in all the leaves tested. Carbohydrates were found only in the aqueous extract of *H. cannabinus*. These metabolites are similar to those reported in the leafy vegetables studied by earlier researchers in various fruits, vegetables and medicinal herbs across the world (Gacche et al., 2010; Gulcin et al., 2010; Gulcin, 2012; Poojary et al., 2015). Out of the ten phytochemical constituents tested, flavonoids and saponins were found in all the six leafy vegetable aqueous extracts. Tannins were present in rich amounts in *H. cannabinus* and present in considerable amounts in *A. viridis*, *S. oleracea*, *M. spicata* and *C. sativum* but absent in *M. koenigii*. Phlobatannins were absent in all extracts but present in *M. spicata*. Terpenoids were present in all the five leafy vegetables under study except in *S. oleracea*. Steroids, alkaloids, and proteins

Table 2

FT-IR absorption peaks (λ) of leafy and silver nanoparticles their corresponding groups.

Sl.no.	Wave numbers of Absorption peaks (cm^{-1})								Assignment
	A. viridis	Silver nanoparticles of A.viridis	*H. cannabinus*	Silver nanoparticles of H.cannabinus	*S. oleracea*	*M. spicata*	*M. koenigii*	*C. sativum*	
1	652, 679, 714	653	649	643, 702	642	-	-	643, 684	C-H & N-H out of plane bend; C-X (halides) strong strech
2	1636	1637	1636	1636	1637	1638	1636	1637	N-H bend, C=O strech amide, C=C strech alkene
3	2107	2104	2094	2104	2107	2343		2140	C≡C strech alkynes
4	3341, 3721, 3784, 3833, 3892	3347, 3728, 3782, 3839, 3899, 3960	3371, 3732, 3793, 3838, 3897, 3959	3344, 3795, 3836, 3888, 3961	3330, 3725, 3780, 3840, 3895, 3960	3350, 3791, 3838, 3881	3334, 3722, 3781, 3893	3314, 3730, 3837, 3893, 3959	N-H amines, O-H strech, C-H

Table 3

Qualitative analysis of phytochemical constituents of the six leafy vegetables.

Sl. No.	Phytochemical constituent	Plants chosen for the study					
		A. viridis	*H. cannabinus*	*S. oleracea*	*M. spicata*	*M. koenigii*	*C. sativum*
1	Tannins	+	++	+	+	-	+
2	Phlobatannins	-	-	-	+	-	-
3	Saponins	+	+	+	+	+	+
4	Flavonoids	+	+	+	+	+	+
5	Steroids	-	-	-	-	-	-
6	Terpenoids	+	+	-	+	+	+
7	Cardiac glycosides	-	-	-	-	-	-
8	Alkaloids	-	-	-	-	-	-
9	Carbohydrates	-	+	-	-	-	-
10	Proteins	-	-	-	-	-	-

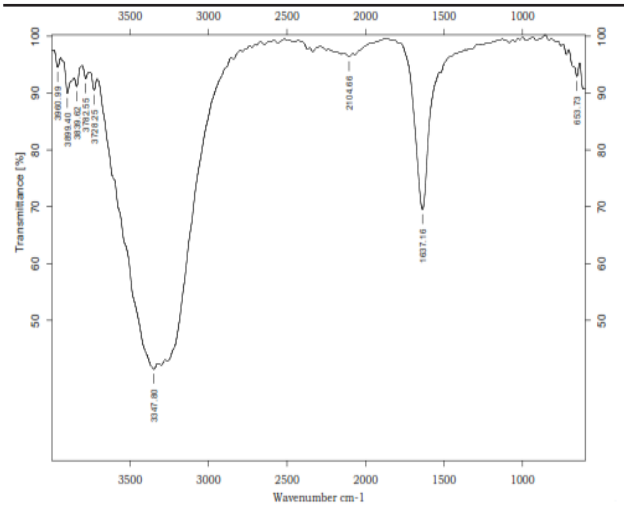

Fig. 6. FT-IR spectral peaks of the silver nanoparticles synthesized with the aqueous leaf extract of *A. viridis*.

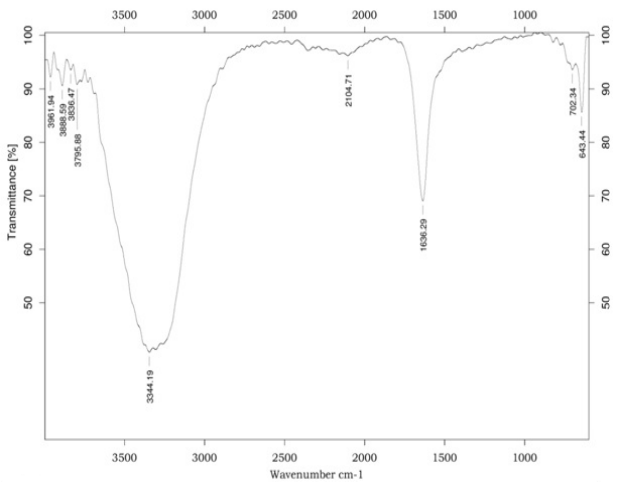

Fig. 7. FT-IR spectral peaks of the silver nanoparticles synthesized with the aqueous leaf extract of *H. cannabinus*.

were absent in all while carbohydrates are identified only in *H. cannabinus* (see Table 3).

3.2. Characterization of aqueous leaf extracts and silver nanoparticles

Formation of silver nanoparticles is confirmed by the change in color from green to brown/black color over a period of time from 2 hours to 24 hours. Maximum absorbance obtained at 448 nm (Fig. S3) confirms the formation of silver nanoparticles, the results are in correlation with those reported by Das et al. (2009). FT-IR spectral peaks with the leaf extracts indicate the presence of N-H amines, O-H stretch, C-H out of plane bending vibrations, C=C stretching of alkenes, C=O stretch of amide and C≡C stretch of alkynes. Similar FT-IR spectral characteristics are shown by the silver nanoparticles synthesized with the aqueous leaf extracts of *A. viridis* and *H. cannabinus* (Fig. 6 and Fig. 7; Table 2).

The FT-IR spectra of leafy vegetables showed characteristic changes at four main regions viz. 640-720 cm^{-1}, 1636-1638 cm^{-1}, 2090-2340 cm^{-1} and 3330-3960 cm^{-1}. In *A. viridis* (Fig. 4), the three peaks at 652 cm^{-1}, 679 cm^{-1} and 714 cm^{-1} combined and formed a single peak at 653 cm^{-1} in silver nanoparticles (Fig. 6). A broad and wide peak at 3341 cm^{-1} and small peaks at 3721 cm^{-1}, 3784 cm^{-1}, 3833 cm^{-1} and 3892 cm^{-1} in *A. viridis* indicate the involvement of N-H and O-H bonds slightly shifted to 3347 cm^{-1}, 3728 cm^{-1}, 3782 cm^{-1}, 3839 cm^{-1}, 3899 cm^{-1} and 3960 cm^{-1}, after the synthesis of silver nanoparticles. A small peak in *H. cannabinus* (Fig. 5) at 649 is split into two 643 cm^{-1} and 702 cm^{-1} peaks after the synthesis of silver nanoparticles (Fig. 7). A peak at 2094 cm^{-1} indicates the aklyne stretch in *H. cannabinus* shifted slightly to 2104 cm^{-1} in silver nanoparticles. In *H. cannabinus,* the peaks at 3371 cm^{-1}, 3732 cm^{-1}, 3793 cm^{-1}, 3838 cm^{-1}, 3897 cm^{-1} and 3959 cm^{-1}, became sharp peaks at 3344 cm^{-1}, 3795 cm^{-1}, 3836 cm^{-1}, 3888 cm^{-1} and 3961 cm^{-1} in its silver nanoparticles which indicate the involvement of N-H amines and O-H bonds. The peak which is common in both the silver nanoparticles is 2104 cm^{-1}.

Some previous investigations on nanoparticle synthesis were focused on green methods, particularly with the leaf extracts of edible plants, to ensure the safety and efficacy of the prepared particles as drug carriers (Kasthuri et al., 2009; Geoprincy et al., 2013). The organic constituents of the phytoextracts used form capping on the surface of silver nanoparticles and provide more functional groups facilitating their binding properties to biological membranes.

3.3. Antioxidant activity

Researchers demonstrated that different antioxidant compounds present such as ascorbic acid, vitamin E, carotenoids, lycopenes, polyphenols and other phytochemicals considerably lower the risk of several diseases (Prior and Cao, 2000; Gupta and Prakash, 2009).

3.3.1. Total phenolic content (TPC)

While the aqueous extract of *S. oleracea* did not show phenolic compounds, *C. sativum* showed highest phenolic content of 2.1 mg/g followed by *M. spicata* (1.8 mg/g), *A. viridis* (0.9 mg/g), *M. koenigii* (0.8 mg/g) and *H. cannabinus* (0.2 mg/g) in the order of decrease (Fig. S1).

3.3.2. Ascorbic acid

Ascorbic acid content was highest in *C. sativum* (1.9 mg/g) followed by *M. koenigii* and *H. cannabinus* (1.5 mg/g), *A. viridis* (1.3 mg/g), *S. oleracea* (1.0 mg/g) and *M. spicata* (0.3 mg/g) in the decreasing order (Fig. S2). Gupta and Prakash (2009) reported that the ascorbic acid content of *Centella asiatica*, *Murayya koenigi*, *Trigonella*

foenum graecum and *Amaranthus* sp. were in the order of 0.1518 mg/g, 0.2931 mg/g, 1.0136 mg/g and 0.64 mg/g, respectively. Meena et al. (2012) reported similar results with *Centella asiatica* (0.0487 mg/g) and for *Baccopa monnieri* the ascorbic acid content was 0.1551 mg/g. In the present study, the ascorbic acid content of *A. viridis* (1.3 mg/g) was found to be higher than that reported by Gupta and Prakash (2009), it may be most probably due to plant species, sampling location and type of extract.

3.3.3. DPPH radical scavenging assay

Out of the six leafy vegetables tested for the ability of DPPH inhibition (Fig. 3), the highest ability was shown by *H. cannabinus* followed by *M. spicata* and *C. sativum* at a concentration of 31.25 mg/mL, while *M. koenigii* showed the highest inhibition followed by *A. viridis* and *S. oleracea* at 50 mg/mL concentration (Table 1). The DPPH activity of *Spinacia oleracea*, *Trigonella foenum graecum*, *Chenopodium album*, *Amaranthus viridis*, *Moringa oleifera* were 34.63, 69.33, 77.88, 40.32, 57.42, respectively (Jaiswal et al., 2017). The present study reports that for the chosen six vegetables, DPPH inhibition was high due to environmental condition and can be used as raw ingredients in the preparation of herbal-based drugs.

4. Concluding remarks

Extensive researches are being carried out worldwide to explore the beneficial effects of dietary and/or medicinal plants, to characterize their constituents and their probable role in the treatment of various ailments. The majority of the researches envisage the preparation of plant extracts using different organic solvents and evaluation of their antioxidant, antibacterial, antiviral, anti-cancerous and other activities. The present study was aimed at evaluating the phytochemical constituents and the antioxidant properties of the chosen leafy vegetables with aqueous extracts as they are consumed in daily life. The results of this study revealed an improvement in the dietary intake through awareness and ward off the ailments. Our ongoing study aimed at isolation of individual phytoconstituents, their characterization and evaluation of their therapeutic potential using animal models and understanding the mechanism of action followed by synthesis of lead molecules from natural resources with good therapeutic activity. Interestingly, the synthesized silver nanoparticles with two of the leafy vegetables tested suggest their potential use in green and ecofriendly synthesis of silver nanoparticles and their further applications in disease treatment.

Conflict of interest

The authors declare that there is no conflict of interest.

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