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EPR and Spectral Investigations on Edible Green Leafy Vegetables.

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ABSTRACT

Edible leafy vegetables are very important dietary components in daily life. Spectral investigations on three different leafy vegetables namely *Hibiscus sabdariffa* (Gongura), *Spinacia oleracea* (Palakura) and *Amaranthus gangeticus* (Thotakura) are carried out by Electron Paramagnetic Resonance (EPR technique). In *Hibiscus sabdariffa* leaf sample, the EPR spectral investigations at different temperatures indicate the presence of Fe³⁺ ions in rhombic symmetry and also the presence of Mn²⁺ ions in divalent state. The EPR spectra of *Amaranthus gangeticus* and *Spinacia oleracea* have also been studied at different temperatures. The EPR spectra exhibited the presence of these Fe³⁺ and Mn²⁺ ions in all the green leafy vegetables which help in oxidation-reaction of many carcinogenic free radicals like superoxide. The infrared spectra are recorded on a FT-IR spectrophotometer and investigations reveal a better understanding of different functional metal complexes present in these edible leafy vegetables. Magnesium and iron present in these leafy green vegetables are quantified using ICP-OES method.

Keywords: EPR, FT-IR, ICP-OES and Green leafy vegetables.

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INTRODUCTION

Vegetables hold an important position in balanced diets. Green Leafy Vegetables (GLV) are believed to occupy a modest place as a source of micronutrients including nutraceuticals of nutritional importance like -carotene, iron, calcium, magnesium, phosphorus, potassium, fiber, folic acid, vitamins K, C and E [1-2]. Green leafy vegetables contribute significantly to overall health and micronutrient security in particular. On one hand, micronutrient (iron) deficiency is rampant in the country on the other hand GLV acts as an economic, affordable, culturally acceptable, sustainable means of mitigating these deficiencies. Greens (GLV) that are dark green are a rich source of vitamin C, K, E, and B, as well as iron, calcium, and fiber, which are essential for good health. The vitamin K plays an important role in preventing osteoporosis, arthritis, diabetes, and atherosclerosis. Vitamin E has been shown to prevent skin cancer. Green leafy vegetables also contain beta carotene (vitamin A), lutein, and zeaxanthin which are powerful antioxidants that destroy free radicals (that damage and weaken our body cells), and help to protect our bodies from cancer. Beta carotene strengthens the immune system, while lutein and zeaxanthin prevent degenerative eye diseases like cataract and they also slow down general age-related cellular degeneration. The greens contain folate, which plays an important role in the repair of damaged cells. Folate is known to reduce the risk of colon, lung, cervix, and breast cancer. The antioxidants in greens contain enzymes that help to fight cancer. These enzymes also help with digestion and keep our digestive tract healthy. Greens are rich source of potassium and magnesium. Potassium helps to regulate blood pressure, and magnesium regulates blood sugar. Greens such as spinach may also help to reduce type 2 diabetes risks due to their high magnesium content.

Electron paramagnetic resonance (EPR) is one of the powerful tools and spectroscopic methods to investigate the biological metal centers and their presence in biological system [3]. So for different trace elements like manganese, iron, cobalt, vanadium, nickel, copper, molybdenum and tungsten have been detected in biological system by EPR technique [4]. The other important spectroscopic method is near infrared spectroscopy for quantitative analysis of vegetables and oils [5]. These trace elements are attached to and transported by the proteins like ceruloplasmin, metallothionin, transferrin, and transmanganin. Ceruloplasmin transports Cu^{2+} – Cu^{+} ; metallothionin transports Zn^{2+} ; transferrin transports Fe^{3+} and transmanganin transports Mn^{2+} ions [6]. There are several standard methods for the analysis of fats, lipids and other essential oils in vegetable plants [5,7]. Almost all GLV contains manganese, and in recent years, the studies on higher valent manganese have attracted many researchers due to their vital role in photosynthetic dioxygen evolution complexes (OECs) in plants [8-10]. These types of complexes have catalase like properties [11-12]. and also contain oxygen donor ligands complexed to manganese in dimeric or polymeric forms of manganese in 2^{+} , 3^{+} and/or 4^{+} oxidation states and higher valent manganese is implicated both in the oxidative and reductive cycles of oxygen in photosynthetic systems. The human body contains about 12 mg of manganese, which is stored mainly in the bones; in the tissue, it is mostly concentrated in the liver and kidneys. In the human brain, the manganese is bound to manganese metalloproteins, most notably glutamine synthetase in astrocytes [13]. Not only manganese, iron is also essential for a large number of biological processes ranging from O_2 transport to DNA synthesis and electron transport. Therefore, iron acquisition is a fundamental requirement in almost all living organisms [14]. In the present study, the interesting features are to study paramagnetic nature of the trace elements that are bound to the protein of greens unintentionally from the soil and also the dietary fibres that are known to bind different minerals in foods [15-17]. In the present work, three different GLV, *Hibiscus sabdariffa* (gongura), *Spinacia oleracea* Linn (Palakura) and *Amaranthus gangeticus* Linn (Thotakura) are taken for the spectral measurements through some systematized experiments. The local names are also included in **Table 1** as subheads.

Table 1: Classification of three varieties of leafy vegetables based on botanical names.

Local name	Gongura	Thotakura	Palakura
Botanical name	<i>Hibiscus sabdariffa</i> Linn.	<i>Amaranthus gangeticus</i> Linn	<i>Spinacia oleracea</i> Linn
Class	Dicotyledons	Dicotyledons	Dicotyledons
Sub-class	Dilleniidae	Caryophyllidae	Caryophyllidae
Order	Malvales	Caryophyllales	Caryophyllales
Family	Malvaceae	Amaranthaceae	Amaranthaceae

The present investigations reveal a better understanding of different functional metal complexes present in these edible leafy vegetables. The spectral results will be very much useful in spectral library information to carry out the future work by other researchers, for the purification of metalloproteins and functional metal centres from these leafy vegetable plants.

2. MATERIALS AND METHODS

All the three leafy vegetables were collected from the local markets of Tirupati (India). These leaves were cleaned with deionized water and dried at room temperature. All the samples were powdered and stored in an air tight zip lock bags. EPR spectra of these powdered samples were recorded at room temperature and also at 100°C using JEOL, FE IX ESR Spectrometer operating at X-band microwave frequencies with a 100 kHz modulation. The samples were heated in an electric furnace at 100⁰ C for 1 hour and again its ESR spectra were recorded.

The Infrared spectra were recorded on an FT-IR spectrophotometer using KBr pellet technique. A Perkin Elmer, Inductively Coupled Plasma system equipped with Optical Emission Spectrophotometer (ICP-OES Optima 8000) and system controlled through Win Lab32 software. A stock solution of leaf extract was prepared 1gm/100ml by dissolving the appropriate amount of the leaf extract powder in distilled water. Standards stocks were prepared as stock preparation depending on validation experiment. The objective of the study is to quantitate magnesium and iron present in leaf green vegetables by ICP-OES method (Table.2).

Table.2: ICP-OEC analysis of leaf extracts

Local name	Gongura	Thotakura	Palakura
Botanical name	<i>Hibiscus sabdariffa</i> Linn.	<i>Amaranthus gangeticus</i> Linn	<i>Spinacia oleracea</i> Linn
Manganese	2.386mg/L	2.383mg/L	2.382mg/L
Iron	2.455mg/L	2.434mg/L	2.433mg/L

RESULTS AND DISCUSSION

Hibiscus sabdariffa Linn. (regional name- Gongura)

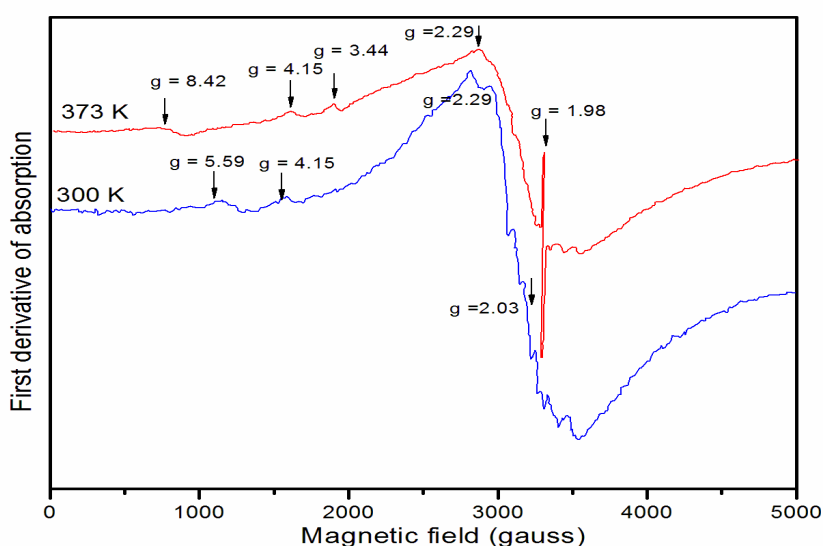
Hibiscus sabdariffa Linn belongs to a family Malvaceae, is an annual dicotyledonous herbaceous shrub plant popularly known as 'Gongura' in Hindi or 'Yerra gogu' in Telugu. *Hibiscus sabdariffa* is known for delicacy and also for medicinal properties. Tender young leaves and stems - raw or cooked use in salads, as a pot-herb and as a seasoning in curries, they have an acid, rhubarb-like flavor. *Hibiscus sabdariffa* is an aromatic, astringent, cooling herb that is much used in the Tropics. It is said to have diuretic effects, help to lower fevers and is antiscorbutic. The leaves are antiscorbutic, emollient; diuretic, refrigerant, and sedative. The plant is also reported to be antiseptic, aphrodisiac, astringent, cholagogue, demulcent, digestive, purgative and resolvent. It is used as a folk remedy in the treatment of abscesses, bilious conditions, cancer, cough, debility, dyspepsia, fever, hangover, heart ailments, hypertension, and neurosis. The plant has several useful applications, such as antidotes to poisonous chemicals (acids, alkali, and pesticides) and venomous mushrooms [18]. The leaves reported to contain proteins, fat, carbohydrates, fiber, ash, calcium, phosphorous, iron, thiamine, β carotene, riboflavin, niacin and ascorbic acid [19-20] also contains flavonoids such as hibiscitrin and hibiscetin [21] and also contains β -sitosterol, anthocyanin, citric acid, pectin, proto catechuic acid, quercetin stearic acid and wax [22]. Anthocyanin and PCA, have been found to have very good antioxidant activity and against atherosclerosis and cancer [23-25]. *Hibiscus sabdariffa* has shown *in vitro* antimicrobial activity against *E. coli* [26]. A recent review stated that specific extracts of *H. sabdariffa* exhibit activities against atherosclerosis, liver disease, cancer, diabetes and other metabolic syndromes [27].

The present study was carried out to identify the presence of important microelements present in three different edible green leafy vegetables such as *Hibiscus sabdariffa*, *Amaranthus gangeticus* and *Spinacia oleracea* by Electron Paramagnetic Resonance studies, and the evaluated spin- Hamiltonian parameters are summarized in Table 3.

Table.3. Spin-Hamiltnian parameters for the dark green leafy vegetables

Sample	Parameters	RT (300K)	373K
1 . <i>Hibiscus sabdariffa</i>	g_1	-	8.42
	g_2	5.59	
	g_3	4.15	4.15
	g_4		3.44
	g_5	2.29	2.29
	g_{sexet}	2.03	2.03
	$g_{\text{free radical}}$	-	1.98
2. <i>Amaranthus gangeticus</i>	$A (\text{Mn}^{2+})$	87 G	88 G
	g_1	-	4.15
	g_2	2.29	2.29
	g_{sexet}	2.03	2.03
	$g_{\text{free radical}}$	1.98	1.98
3 <i>Spinacia oleracea</i>	$A (\text{Mn}^{2+})$	90 G	90 G
	g_1	-	4.15
	g_2	2.40	2.65
	g_{sexet}	2.03	2.03
	$g_{\text{free radical}}$	1.98	1.98
3 <i>Spinacia oleracea</i>	$A (\text{Mn}^{2+})$	90 G	90 G
	g_1	-	4.15
	g_2	2.40	2.65
	g_{sexet}	2.03	2.03
	$g_{\text{free radical}}$	1.98	1.98

The spectra of unheated and heated sample of *Hibiscus sabdariffa* leaves are shown in Fig.1. The EPR spectrum of unheated sample consists of four resonance lines. The first, second and third resonance lines on lower magnetic field side can be explained by the presence of the Fe^{3+} ion. The fourth resonance line with sextet hyperfine structure with g value 2.03 is due to Mn^{2+} ion ($S = 5/2$ and $I = 5/2$). The calculated effective g values for three resonance signals on lower magnetic field side are at $g = 5.59$, $g = 4.15$ and $g = 2.09$. These are due to difference in in-equivalent paramagnetic centers corresponding to Fe^{3+} impurity ion and often observed in many biological systems [28]. The g values of these resonance signals are consistent with those of other reported vegetable leaves and other food materials [29-32]. The number of spins (N_s) participating in the resonance can be calculated by using the formula $N_s = 0.285 I(\Delta H)^2$, where 'I' is the peak to peak height (arbitrary units) and ' ΔH ' is the line width in (in G) [33].


Fig.1. EPR spectra of *Hibiscus sabdariffa* at 300K and 373K

The EPR spectrum recorded at 373K (heated) exhibits an additional resonance signals with effective g values at $g = 8.42$ and $g = 3.44$ corresponding to iron ions and a sharp signal centered at $g = 1.98$ is characteristic of radical species. There is no change in g values at $g = 4.15$, $g = 2.09$ and $g = 2.03$ on heating the sample. The line width and intensity around $g = 2.09$ is considerably increased in heated sample. Number of spins is also

increased from 160×10^4 (at 300K) to 215×10^4 (at 373K). The resonance signal of manganese ions recorded at 373K is poorly resolved which is consistent with results of ICP atomic emission data. The hyperfine splitting constant is calculated from the sextet lines and is found to be 87 G. A sharp signal centered at $g = 1.98$ is attributed to the usual organic radical, probably due to C-O carboxyl radical associated with cellulose [29].

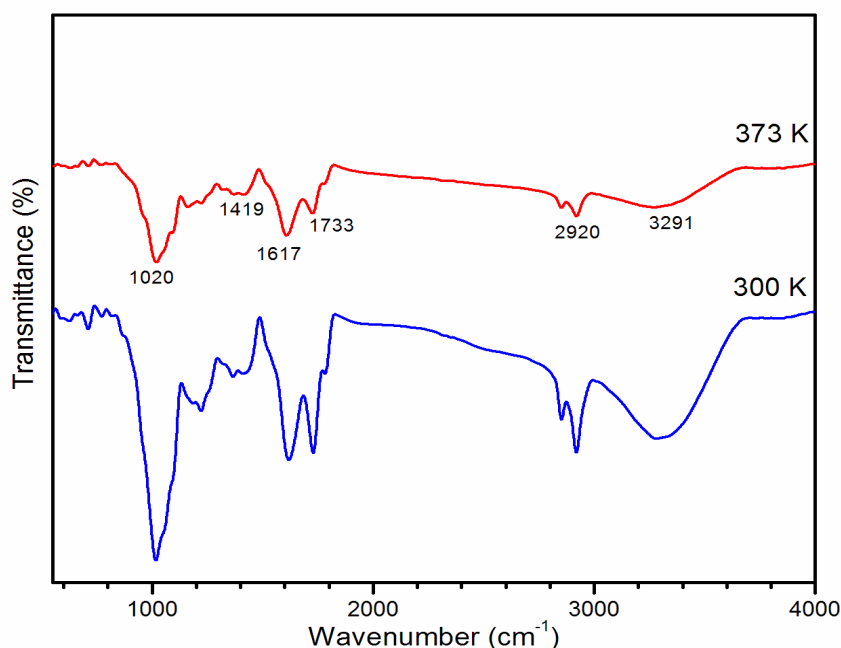


Fig.2. FT- IR spectra of *Hibiscus sabdariffa* leaf extract at 300K and 373K

The FT-IR spectra of the leaf extract at 300 K shown in **Fig.2**, exhibits the absorption peaks at 3854 and 3291 cm^{-1} corresponding to the hydrogen-bonded hydroxyl (OH) and the peak at 2918 cm^{-1} , indicates the presence of C-H mode of vibrations. The absorption peaks situated around 1617, 1362, 1221, 1015 and 709 cm^{-1} are the characteristic peaks for the C=C, mode C-C, C-H, C-O-C and N-H stretching modes respectively (of the aromatics). Where as the IR spectrum of the leaf extract heated at 373 K shows the absorption peaks at 3854 and 3291 cm^{-1} corresponding to the hydrogen-bonded hydroxyl (OH) and the peak at 2920 indicates the presence of C-H. The absorption peaks situated around 1733, 1617, 1419, 1160, 1021 and 709 cm^{-1} are the characteristic peaks for the C=C, C-C, C-H, C-O-C and N-H stretching, respectively (of the aromatics) (Fig.2). The IR data of *Hibiscus sabdariffa* leaf extract at 300 K and 373 K reveals that there is not much difference in functional groups, there is slight stretching in few functional groups which is very negligible, so it is concluded that is the reason why people eat green leafy vegetables after cooking up to 100°C (i.e is 373 K). It is the best example proven experimentally by the above data. The IR bands at 300 K and 373 K correspond to 1362, 1221 and 1015 cm^{-1} indicate the presence of C-O stretching of alcohols, carboxylic acids, and ester and ether groups (Fig.2). The presence of the carboxylic bands in the IR spectra of this *Hibiscus sabdariffa* indicates that the manganese ions are coordinated to carboxylate ligands to form Mn-O-Mn type of complex.

***Amaranthus gangeticus* Linn. (regional name- Thotakura)**

Amaranth is a common leaf vegetable throughout the tropics and in many warm temperate regions. *Amaranthus*, collectively known as amaranth is an annual perennial plants, belongs to family (Amaranthaceae) Amaranth greens, also called as Chau lai (Hindi), and Thotakura (Telugu). Cooked amaranth leaves are a good source of vitamin A, vitamin C, folate, thiamine, niacin and riboflavin [34]. Important dietary minerals including calcium, iron, potassium, zinc, copper and manganese which are comparable to common grains such as wheat germ, oats and others [35]. Amaranth seeds contain lysine, an essential amino acid, limited in other grains or plant sources. According to a 2007 report, amaranth compares well in nutrient content with gluten-free vegetarian options such as buckwheat, corn, millet, wild rice, oats and quinoa [36]. Amaranth is good for those with hypertension and cardiovascular disease; regular consumption reduces blood pressure and cholesterol levels, improve antioxidant status and some immune parameters[37-39].

The EPR spectra of *Amaranthus gangeticus* recorded at 300 K (RT) and 373 K are shown in **Fig.3**. The EPR spectrum at 300K shows resonance signal at $g = 2.03$ and exhibits poorly resolved sextet hyperfine resonance lines with a hyperfine splitting constant $A=90$ G. Also a resonance signal due to the free radical at $g = 1.98$ is observed. In addition to this, there is a resonance signal with effective g value at $g = 2.29$. In the EPR spectrum recorded at 373 K, these lines are seen with increased intensity. The EPR spectrum recorded at 373 K exhibits an additional resonance signal with effective g value at $g = 4.15$. Among these, the resonance signal centred at $g = 4.15$ arise due to paramagnetic ions namely, Fe (III) ions. The sextet hyperfine lines can be attributed to Mn^{2+} ions ($S = 5/2$ and $I = 5/2$) arising from the central sextet $\pm 1/2 \leftrightarrow \mp 1/2$ with $g = 2.03$ [29-32]. A sharp resonance signal centered at $g = 1.98$ corresponds to the usual organic radical, probably due to C-O carboxyl radical associated with cellulose.

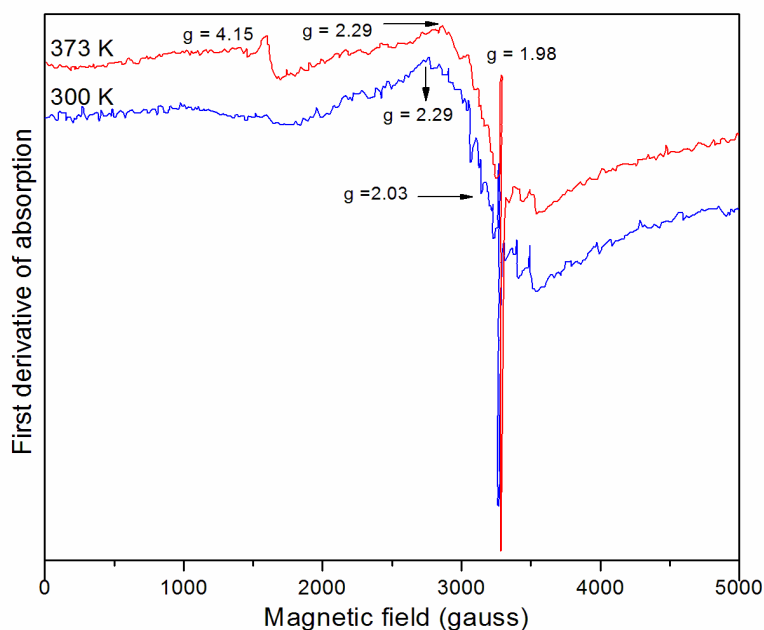


Fig.3. EPR spectra of *Amaranthus gangeticus* leaf extract at 300K and 373K

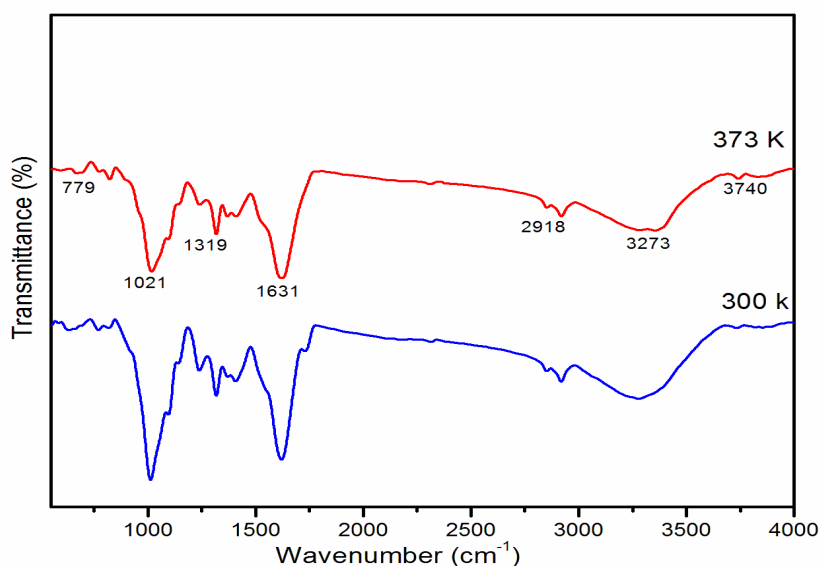


Fig.4. FT- IR spectra of *Amaranthus gangeticus* leaf extract at 300K and 373K

The FT-IR spectra of the *Amaranthus gangeticus* leaf extract at 300 K show in **Fig.4**, the absorption peaks at 3852 and 3275 cm^{-1} corresponding to the hydrogen-bonded hydroxyl (OH) and the peak at 2918 cm^{-1} , indicates the presence of C-H. The absorption peaks situated around 1617, 1417, 1319, 1011, 779 and 666

cm^{-1} are the characteristic peaks for the C=C, C-C, C-H, C-O-C and N-H stretching, respectively, of the aromatics. Where as the IR spectrum of the leaf extract heated at 373 K shows the absorption peaks at 3740 and 3273 cm^{-1} corresponding to the hydrogen-bonded hydroxyl (OH) and the peak at 2918 indicates the presence of C-H. The absorption peaks situated around 1631, 1319, 1021, 779 and 666 cm^{-1} are the characteristic peaks for the C=C, C-C, C-H, C-O-C and N-H stretching, respectively, of the aromatics. The IR bands at 300 K correspond to 1362, 1221 and 1015 cm^{-1} indicate the presence of C-O stretching of alcohols, carboxylic acids, and ester and ether groups (Fig.4). The presence of the carboxylic bands in the IR spectra of this *Amaranthus* indicates that these coupled manganese ions are coordinated to carboxylate ligands to form Mn-O-Mn type of complex.

***Spinacia oleracea* Linn. (regional name- palakura)**

Spinach (*Spinacia oleracea*) is an edible annual flowering plant in the family of Amaranthaceae. It is native to central and south-western Asia. Spinach has a high nutritional value and is extremely rich in antioxidants, especially when fresh, steamed, or quickly boiled. It is a rich source of vitamin A (and especially high in lutein), vitamin C, vitamin E, vitamin K, magnesium, manganese, folate, betaine, iron, vitamin B₂, calcium, potassium, vitamin B₆, folic acid, copper, protein, phosphorus, zinc, niacin, selenium and omega-3 fatty acids. Recently, opioid peptides called rubiscolins have also been found in spinach. Spinach is rich in dietary fiber, which aids in digestion, prevents constipation, maintains low blood sugar, and curbs over eating. Flavonoids are abundant in spinach which has anti-cancer properties and slow down cell division in human stomach and skin cancer cells. Furthermore, spinach has shown significant protection against the occurrence of aggressive prostate cancer. Neoxanthin and violaxanthin are two anti-inflammatory epoxy xanthophylls that play an important role in regulation of inflammation and are present in unusual amounts in spinach. The vitamin C, vitamin E, beta-carotene, manganese, zinc and selenium present in spinach all serve as powerful antioxidants that combat the onset of osteoporosis, atherosclerosis and high blood pressure.

The EPR spectra of *Spinacia oleracea* observed at 300 K (RT) and 373 K are shown in Fig.5. The EPR spectrum at 300 K shows poorly resolved sextet hyperfine lines with a hyperfine splitting constant $A = 90$ G along with a resonance signal due to the free radical at $g = 1.98$. In addition there is a resonance signal at $g = 2.40$. In the EPR spectrum recorded at 373 K, these lines are seen with increased intensity. The EPR spectrum recorded at 373 K exhibits an additional resonance signal at $g = 4.15$. On heating the sample there is a shift in resonance signal ($g = 2.65$) compared to the room temperature resonance signal ($g = 2.40$). The resonance signals at $g = 2.40$ (RT) and $g = 2.65$ (373 K) and $g = 4.15$ (373 K) are due to Fe^{3+} ions. The sextet hyperfine lines can be attributed to Mn^{2+} ions ($S = 5/2$ and $I = 5/2$) arising from the central sextet $\pm 1/2 \leftrightarrow \mp 1/2$ with effective g value 2.03 [29-32]. A sharp signal centered at $g = 1.98$ exhibits an increase in intensity at 373 K. This signal corresponds to the usual organic radical, probably due to C-O carboxyl radical associated with cellulose.

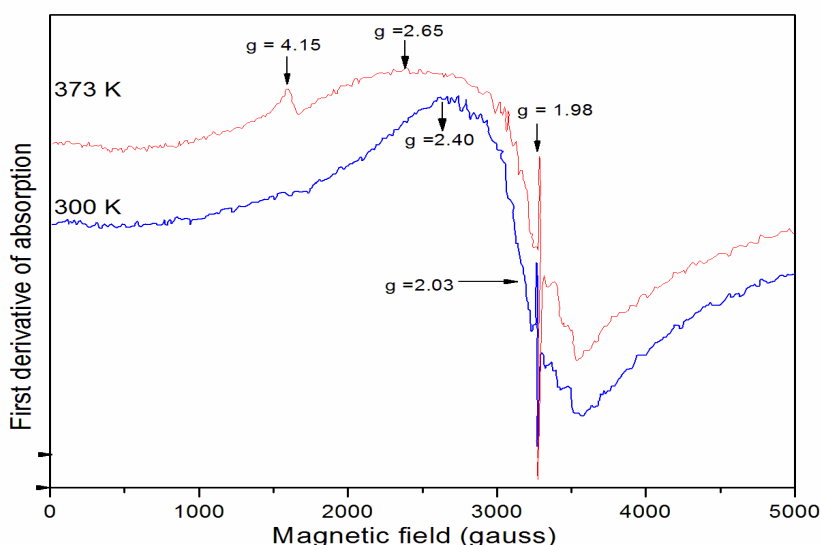


Fig.5. EPR spectra of *Spinacia oleracea* leaf extract at 300 K and 373 K

The FT-IR spectra of the *Spinacia oleracea* leaf extract at 300 K are shown in **Fig.6**. The spectra exhibit the absorption peaks at 3852 and 3291 cm^{-1} corresponding to the hydrogen-bonded hydroxyl (OH) and the peak at 2918 and 2849 cm^{-1} indicates the presence of C-H. The absorption peaks situated around 1635, 1230, 1117, 1017, 830, 774 and 611 cm^{-1} are the characteristic peaks for the C=C, C-C, C-H, C-O-C and N-H stretching, respectively, of the aromatics. Whereas the IR spectrum of the leaf extract heated at 373 K shows the absorption peaks at 3744 and 3354 cm^{-1} corresponding to the hydrogen-bonded hydroxyl (OH) and the peak at 2920 indicates the presence of C-H. The absorption peaks situated around 1633, 1317, 1027, 825, 774 and 615 cm^{-1} are the characteristic peaks of the aromatics for the C=C, C-C, C-H, C-O-C and N-H stretching, respectively. The IR bands at 300K correspond to 1362, 1221 and 1015 cm^{-1} indicate the presence of C-O stretching of alcohols, carboxylic acids, and ester and ether groups. The presence of the carboxylic bands in the IR spectra *Spinacia oleracea* indicates that these coupled manganese ions are coordinated to carboxylate ligands to form Mn-O-Mn type of complex. Iron plays an important role in oxygen transport. In humans, the haemoglobin molecule contains four iron atoms; the oxygen taken through lungs is transported into blood as dissolved oxygen which is bound to haemoglobin. Therefore iron ions are required for both the process of red blood cells and also the haemoglobin component of the cells. At the same time so many enzymes also require Fe ions for aerobic metabolism in human. Fe also plays an important role in plant metabolism especially in Photosystem I and II. In plant P680⁺ also contains manganese is the core redox centre, which has four oxidation states of Mn²⁺, Mn³⁺, Mn⁴⁺, Mn⁵⁺ co-ordinates tightly to oxygen containing species. The human ferritin protein is very crucial in iron and oxygen metabolism. When Fe²⁺ ions enter the ferritin protein pores, with in milliseconds the ferroxidase site is buried within each human ferritin subunit. This ferroxidase site activity to oxidise the Fe²⁺ and forms diferroxo-mineral precursors (Fe³⁺-O- Fe³⁺) which is the best characterized function of ferritin in humans [40].

CONCLUSION

The EPR, ICP-OES and IR spectral investigation has been carried out on three different dark green leafy vegetables *Hibiscus sabdariffa* (Gongura), *Spinacia oleracea* (Palakura) and *Amaranthus gangeticus* (Thotakura) to understand the presence of multivalent paramagnetic metal ions and mechanisms involved in metal complexes. The EPR spectral investigations at different temperatures indicate the presence of Fe³⁺ ions in rhombic symmetry and also the presence of Mn²⁺ ions in divalent state. These ions play a important role in oxygen metabolisms in humans and also in photosynthetic reactions of plants. The temperature dependent EPR spectra of the leafy vegetables clearly explained the redox mechanism involved in the metal complexes, Such as Mn-O-Mn type of complex and also Fe³⁺-O- Fe³⁺ complexes. The EPR and IR spectra's of heated and unheated green leafy vegetable samples showed characteristic peaks of Fe³⁺ ion and peaks for the C=C, C-C, C-H, C-O-C and N-H stretching, respectively in all the leafy vegetables. The results of this study clearly indicate the metal-metal interactions and metal-carboxylic interactions. The spin state mechanisms of the multivalent manganese and Iron complexes will give a better understanding of nutritional metabolisms absorption of nutrients therapeutically. The presence of Mn and Fe was also confirmed by ICP-OES analysis. The IR spectra give an evidence for the protein matrix bands corresponding to carboxylic C=O bonds. The present work will be useful for future work, in the direction of nutritional approach to understand the metabolism, absorption of nutrients therapeutically.

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