International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 2 (2014) pp. 28-42**
http://www.ijcmas.com

Original Research Article

Bioactive compounds, radical scavenging, antioxidant properties and FTIR spectroscopy study of *Morinda citrifolia* fruit extracts

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K e y w o r d s

Antioxidant activity; Free radicals, FTIR spectroscopy; *Morinda citrifolia;* Phytochemicals

A B S T R A C T

Morinda citrifolia is one of the important medicinal plants which have been extensively used in folk medicine by the Polynesians for over 2000 years with less documentation of phytochemical screening and antioxidant activities of different fruit extracts. In the present study the fruits of *Morinda citrifolia* have been extracted with three different solvents (ethanol, water and ethyl acetate) and evaluated for their in *vitro antioxidant* activities, phytochemical screening and subjected to FTIR spectroscopy studies for functional group identification. Phytochemical screening revealed the presence of various phytochemicals in all the extracts with varying degrees with the ethyl acetate extract having maximum total phenolic content of about 187.6 mg/gm of GA equivalents. The ethyl acetate extract also had the highest antioxidant activity as revealed by total antioxidant assay, higher free radical scavenging activity as revealed by the DPPH, superoxide, and hydroxyl radical scavenging assays. FTIR spectral studies clearly indicate the presence of various functional groups which may be attributed to the antioxidant and free radical scavenging properties of these extracts. The present study clearly dictates the potential antioxidant activity and free radical scavenging properties of *Morinda citrifolia* fruit extract which may be due to the various functional compounds present in the fruit extracts as revealed by FTIR spectral studies.

Introduction

Among the two basic categories of antioxidants, natural and synthetic the main disadvantage with the synthetic antioxidants is the side effects when taken *in vivo* (Chen *et al.,* 1992). The superiority of natural antioxidants also lies in the fact that the intake of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing (Kamiya *et al.,* 2004). Hence recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants (Ito *et al.,* 1983).

Plants are the potential source of natural antioxidants. Natural antioxidants or phytochemical antioxidants are the secondary metabolites of plants (Walton and Brown 1999). Many plants have been exploited in folk medicine for centuries by most of the populations throughout the world. Plants (fruits, vegetables, medicinal herbs, etc.) may contain a wide variety of free radical scavenging molecules, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids, and some other endogenous metabolites (Cai *et al.,* 2004). Phenolic compounds are commonly found in both edible and non-edible plants, and they have been reported to have multiple biological effects, including antioxidant activity (Marja *et al.,* 1999). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa 1994).

Morinda citrifolia L. (Rubiaceae), commonly called as Noni grows widely throughout the Pacific and is one of the most significant sources of traditional medicines among Pacific island societies. The roots, barks, stems, leaves, and fruits have been used traditionally as a folk medicine for the treatment of many diseases including diabetes, hypertension, and cancer. Noni juice , which is made of the fruits of this plant, has been widely consumed for the purported prevention of lifestyle-related diseases such as diabetes, hypertension, cardiopathy and arteriosclerosis (Saludes *et al.,* 2002).

Moreover research reports clearly indicate that the plant has antitubercular effect (Hirazumi and Furusawa 1999), immune-modulatory properties (Wang and Su 2001), anti-cancer properties (MohdZin *et al.,* 2001), anti-oxidant properties (McKoy *et al.,* 2002), anti-in ammatory activity (Wang *et al.,* 2002), analgesic activity (Jain and Srivastava 1992), and cardioprotective activity (Saludes *et al.,* 2002). These effects have been attributed to the presence of several anthraquinones which have been isolated from the roots (Srivastava and Singh 1993), heartwoods (Tiwari and Singh 1977), flowers (Daulatabad *et al.,* 1989), fatty acid derivatives found in the seeds (Wang*et al.,* 1999), iridoid glycosides and flavonols glycosides found in the fruits (Sang *et al.,*2001) and in leaves (Singh and Tiwari 1976), sterol derivatives in the leavesand other volatile compounds (Farine et *al.,* 1996) in the fruits.

However, the comparative study of phytochemical analysis of different extracts of fruits and their antioxidant capacities with reference to metal chelating activity have not been well documented. Hence this preliminary study is designed to identify the phytochemical constituents and antioxidant activity of *Morinda citrifolia* fruit extracts and to analyze the functional groups present in the fruit extracts by FTIR spectroscopy.

Materials and Methods

Chemicals and Reagents

The solvents for extraction ethanol and ethyl acetate were purchased from Merck Chemical Supplies (Darmstadt, Germany). 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents used were purchased from Sisco Research Laboratories, Mumbai, India. All the

chemicals used including the solvents, were of analytical grade.

Plant Materials and Preparation of Extracts

The fruits of *Morinda citrifolia* (Noni) were collected from NCRN farm, Shalavakkam, Kanchipuram district, Tamilnadu. Before starting the extraction, the plant materials were washed twice and were shade dried for 7days. Extraction was carried out according to the method of Mohd Zin *et al* (2001) with slight modifications. The fruit samples collected were grounded to powder. The powder was extracted with ethanol, ethyl acetate and water in a shaker at room temperature for 3 days. The solvent was then removed by filtration and fresh solvent was then added to the plant material. The extraction process was thrice repeated. The samples were filtered and the filtrates were oven dried to yield a green viscous mass. The extracts were labeled as follows ethanolic extract (EtF), aqueous extract (AqF) and ethyl acetate extract (EaF).

Qualitative Phytochemical Screening

Phytochemical screening for flavonoids, tannins, and saponins were determined as described by Chew *et al.,* (2011) respectively. The presence of alkaloids was determined as described by Harborne (1973). Screening the presence of steroids and terpenoids was performed as described by Kumar *et al.,* (2009).

Quantitative Phytochemical Screening

Total Phenolic Content

The total phenolic contents of the plant extract were measured by the Folin-Ciocalteu reagent assay method determined by Slinkard and Singleton (1977). The concentration of total phenolic compounds in different extracts was expressed as mg of gallic acid equivalents (GAE)/ g of dried extract, using a standard curve of gallic acid.

Total Proanthocyanidins Content

Proanthocyanidins content of the plant extract was determined according to the procedure reported by Sun *et al* (1998). A volume of 0.5 ml of 1 mg/ml of extract solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid; the mixture was allowed to stand for 15min. The absorbance was measured at 500 nm. Total proanthocyanidins content was expressed as rutin equivalents (mg/g).

Total Tannin Content

Total tannin content was measured by Vanillin-Hydrochloride method as described by Sadashivam *et al* (2004). A volume of 1.0 ml of 0.1 mg/ml of extract solution was mixed with5 ml vanillin hydrochloride reagent; the mixture was allowed to stand for 20 min. The absorbance was measured at 500 nm. Total tannin content was expressed as rutin equivalents (mg/g).

Evaluation of Antioxidant Activity

Total Antioxidant Capacity

The antioxidant capacity of the plant extract was evaluated according to the procedure of Shirwaikar *et al* (2006). An aliquot of 0.1 ml of extract was mixed with 1 ml of reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate) in an eppendorf tube. The tubes were capped and incubated at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance was

measured at 695 nm against a reagent blank. Gallic acid was used as standard.

Ferric Reducing Antioxidant Power (FRAP) Assay

The reducing antioxidant power (FRAP) of the plant extract was determined by the method of Benzie and Strain (1996).The FRAP reagent consisted of 10 mM 2,4,6-tripyridyl- 2triazine (TPTZ) in 40mMHCl, 20 mM ferric chloride and 250mM sodium acetate buffer (pH 3.6). FRAP reagent was freshly prepared by mixing TPTZ solution, $FeCl_3$ solution and acetate buffer in a ratio 1:1:10. A 1ml of extract solution containing 1 mg extract was mixed with 1ml of FRAP reagent. After the mixture stood at 37ºC for 4 min, the absorbance at 593 nm was determined against blank. Results are expressed in µM Fe (II)/g dry mass.

Free Radical scavenging activity

2, 2-diphenyl-1-picryhydrazyl (DPPH) free radical scavenging assay

A common method of determining intrinsic free radical scavenging activity is to use a cell free assay system with the radical 2, 2-diphenyl-1-picryhydrazyl (DPPH). Scavenging activity on DPPH was assessed according to the method reported by Blois (1958). Briefly, 100 lµ of extracts (0.1 to 0.5 mg/ml) were mixed with 1 ml of methanolic solution of 0.1 mM DPPH. The mixture was shaken well and incubated at room temperature for 30 min and absorbance was measured at 517 nm in a spectrophotometer. BHT was used as standard. Experiment was performed in triplicate and averaged. Percent inhibition was calculated from control using the following equation:

Scavenging activity (%) = (1- absorbance sample/ absorbance control) ×100

Superoxide anion scavenging activity assay

The scavenging activity of the Noni fruit extract towards superoxide anion radicals was measured by the method of Liu *et al* (1997). Superoxide anions were generated in a non-enzymatic phenazinemetho sulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 mL of Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 mL of NBT (300 µM) solution, 0.75 mL of NADH (936 µM) solution and 0.3 mL of different concentrations of the extract. The reaction was initiated by adding 0.75 mL of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The super oxide anion scavenging activity was calculated according to the following equation:

% Inhibition = [(A0-A1) / A0 X 100]

Hydroxyl radical scavenging activity assay

Hydroxyl radical scavenging activity was determined according to the method of Chung *et al* (1997). The Fenton reaction mixture consisted of 200 µl 10 mM FeSO4•7H2O, 200 µl 10 mM EDTA and 200 ml 10 mM 2-deoxyribose mixed with 1.2 ml 0.1 M phosphate buffer (pH 7.4) mixed with 200 µl of Noni extract. Thereafter, 200 µl 10 mM H2O2 was added and the mixture was incubated at 37 C for 4 h. After incubation, 1 ml of 2.8% TCA and 1 ml of 1% TBA were mixed

and placed in a boiling water bath for 10 min. After cooling, the mixture was centrifuged (5 min, 395g) and the absorbance was measured at 532 nm with a UV−Vis spectrophotometer.

% Inhibition = [(A0-A1) / A0 X 100]

Metal-Chelating Activity

The chelating activity of the extract for ferrous ions (Fe2+) was measured according to the method of Sabate (2003).1 ml of extracts (0.1 to 0.5 mg/ml) was incubated with 50 µl of 2 mM ferrous chloride. The reaction was started by the addition of 200 µl ferrozine (5 mM). After 10 minutes, the absorbance of ferrous ion-ferrozine complex at 562 nm was read. EDTA served as positive control. Triplicate samples were run for each set and averaged.

% Inhibition = [(A0-A1) / A0 X 100]

Fourier Transform Infra-Red (FT-IR) analysis

The IR studies have been followed by the method described by Jagmohan (2005). The powdered fruit samples were mixed with potassium bromide (KBr pellet) and subjected to a pressure of about 5×10^6Pa in an evacuated die to produce a clear transparent disc of diameter 13 mm and thickness 1mm. IR spectra in frequency region 4000-400cm^{-1}were recorded at room temperature on a perkin- Elmer Fourier Transform Spectrometer equipped an air cooled DTGs (deuterated triglycine sulfate) detector. For each spectrum, 100scans were co-added at a spectral resolution of 4cm. The frequencies for all sharp bands were accurate to 0.01 cm. All the spectral values were expressed in (%) transmittance.

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. Where applicable, the data were subjected to one way analysis of variance (ANOVA) followed by least significant difference (LSD) test for multiple comparison and the differences between samples were determined using the SPSS software package version 16, p Values < 0.05 were regarded as significant.

Results and Discussion

It has been reported that biological activities in the selected plants are exhibited by different classes of phytochemicals (Haslam, 1989). Phytochemicals often play an important role in plant defense against prey, microorganism, and stress as well as interspecies protections, these plant components have been used as drugs for millennia. Hence, phytochemical screening serves as the initial step in predicting the types of potentially active compounds present in them and thereby substantiating its use as a traditional medicine. In the current study phytochemical screening of the *Morinda citrifolia* extracts revealed the presence of various phytochemicals such as flavonoids, tannins, saponins, terpenoids, alkaloids and steroids (Table 1). All the extracts showed the presence of these compounds with varying degree and the ethyl acetate extract of *Morinda citrifolia* showed the highest degree of flavonoids followed by saponins, terpenoids, and tannins. Steroids and alkaloids were found to be absent in this extract. In the other two extracts namely ethanolic and aqueous extracts, steroids were found to be absent and all the other phytochemicals were present.

The assay of total phenol in the three extracts revealed the presence of the

phenolic compound in the ethyl acetate fraction of *Morinda citrifolia* in highest concentration (187.6 mg/g of GA equivalents) when compared to the other two extracts (Fig 1). Tannins constitute a group of secondary metabolites widely distributed in the plant kingdom. In addition to their original use in preparation of leather, the tannins have recently become the field of interest for its potential therapeutic use in a variety of disease states (Packer *et al*., 1999). Quantitative screening of tannins (Fig 2a) and proanthocyanidins (Fig 2b) divulged their presence in all the three extracts with the ethyl acetate extract having 5.31mg/g of Rutin equivalents of tannin and 2.25mg/g of Rutin equivalents of proanthocyanidins, while ethanol and aqueous extracts have 3.6mg/g Rutin equivalents of tannin and 1.32mg/g and 2.21mg/g of Rutin equivalents of proanthocyanidins respectively. Proanthocyanidins, as a whole, cause many bioactivities that produce positive, healthful changes in the human body. It had been demonstrated that these compounds exhibit antioxidant properties and help the body ward off cardiovascular disease, various immune disorders, and neurodegenerative disease (Packer *et al*., 1999).

Many assay methods for antioxidant activity *in vitro* and *in vivo* have been developed, but only a few rapid and reliable methods applicable to antioxidant activity assay for a huge number of plant extract samples exist. The antioxidant capacity of the fractions was measured spectrophotometrically through phosphomolybdenum method. The present study demonstrated that the ethyl acetate extract of Noni fruit exhibits the highest antioxidant activity of 8.426mg/gm GAE whereas the aqueous extract and ethanolic extract exhibited the antioxidant activity of 7.32mg/gm GAE and 5.92mg/gm GAE respectively. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Luximon-Ramma et al., 2005)). The reducing power of the plant extracts is determined by FRAP assay. The ferric reducing/antioxidant power (FRAP assay) is widely used in the evaluation of the antioxidant component in dietary polyphenols. The reducing ability of the extracts was in the range of 529.57 to 667.08 µm Fe (II)/g which was lesser than that of the ascorbate, 1533.94 µm Fe (II)/g but higher than that of BHT, 263.97 µm Fe (II)/g. The results suggested that the strong antioxidant activity of extracts might be due to the presence of phenolic compounds which due to their redox properties absorb and neutralize free radicals, quench singlet, triplet oxygens and decompose peroxides (Osawa *et al.*,1994).

Free radicals and other reactive oxygen species (ROS) are mostly considered to be associated with pathogenesis and are responsible for the initiation or/and development of many diseases such as atherosclerosis, in ammation, cancer, hypertension, ischemia-reperfusion, autoimmune diseases, aging and age-related diseases as revealed from several experimental works (Emmons *et al.*, 1999). Antioxidants are biologically synthesized as defensive mechanism having an important role in preventing or

Table.1 Different classes of phytochemicals present in the Fruit extracts of *Morindacitrifolia*

Extract	Flavonoids	Tannins	Saponins	Alkaloids	Steroids	Terpenoids
EtF	++	++	++	+	-	++
AqF	++	+	+	+	-	++
EaF	+++	++	+++	-	+	++

Figure.1 Total phenolic content present in the ethanolic, aqueous and ethyl acetate extracts of *Morindacitrifolia*

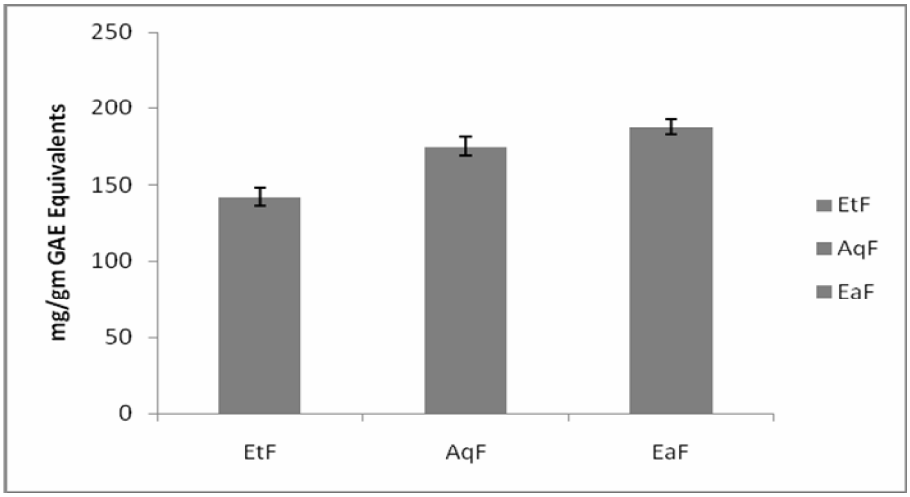
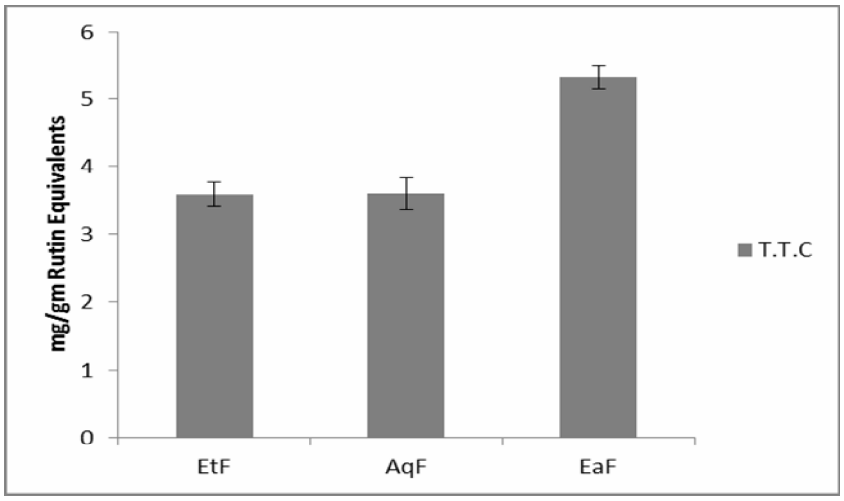


Figure.2 Total Tannin and Proanthocyanidin content of *Morindacitrifolia*fruit extracts

2a Total Tannin Content



2b Total Proanthocyanidin Content

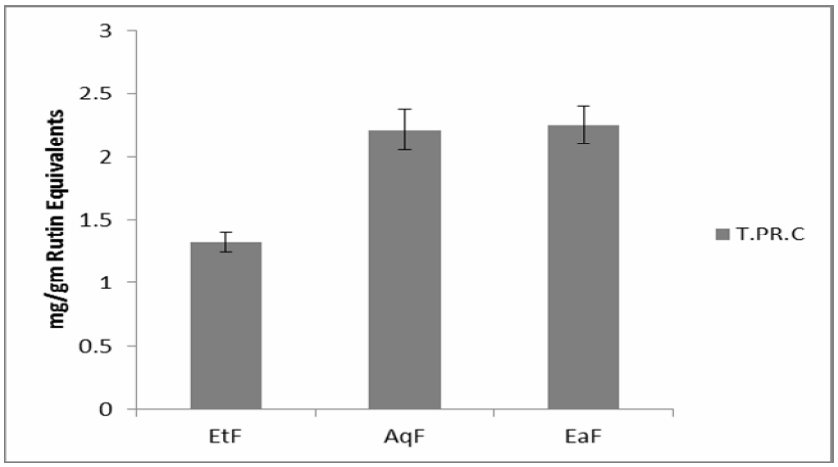
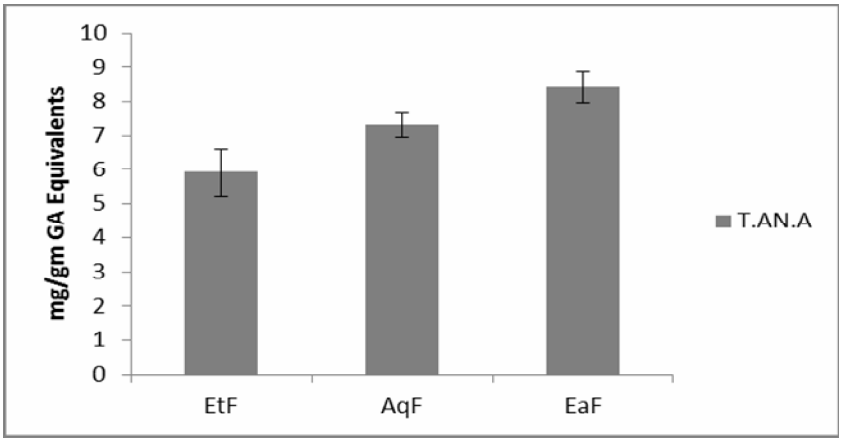


Figure.3 Antioxidant activity of the fruit extracts of *Morindacitrifolia*

3(a) Total Antioxidant activity



3(b) FRAP assay

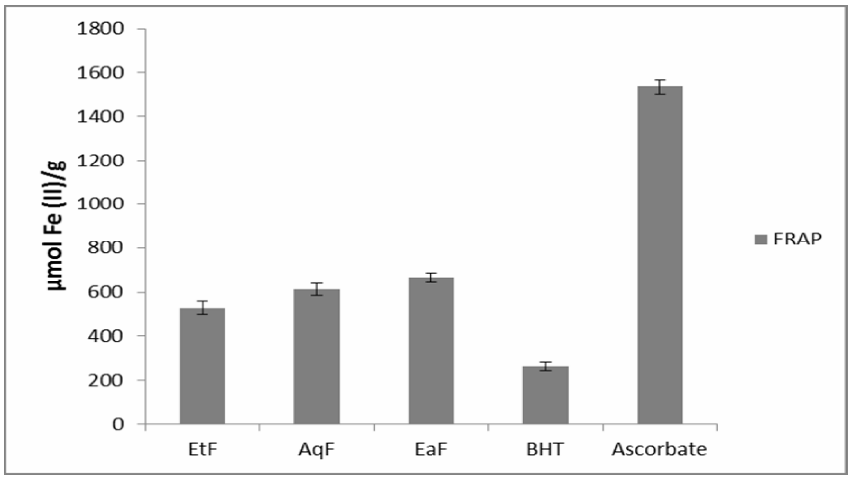
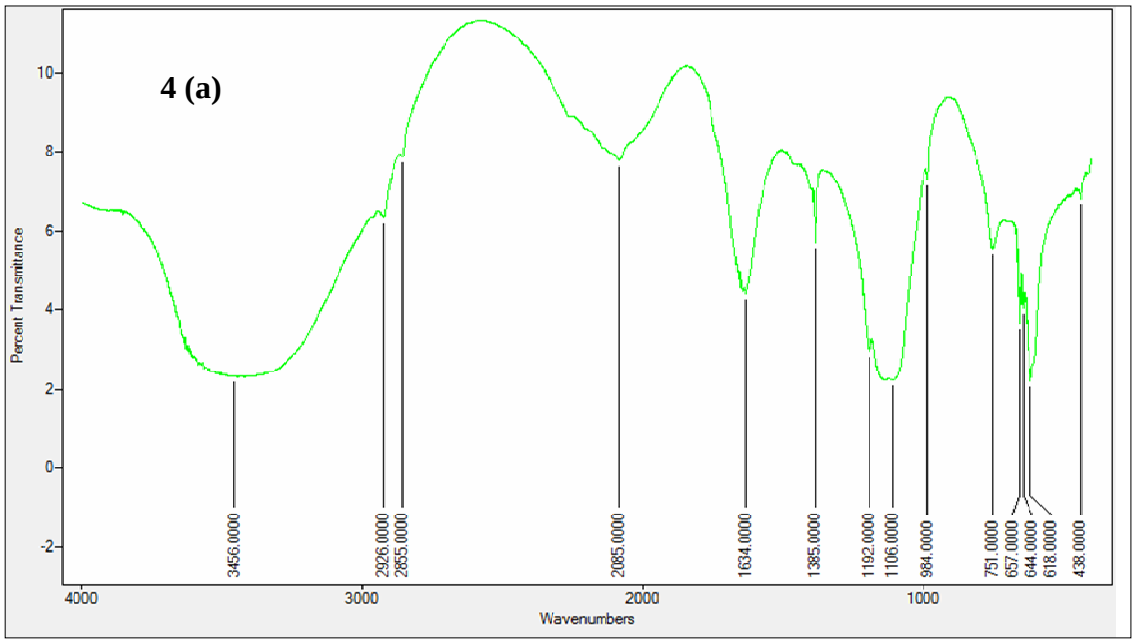
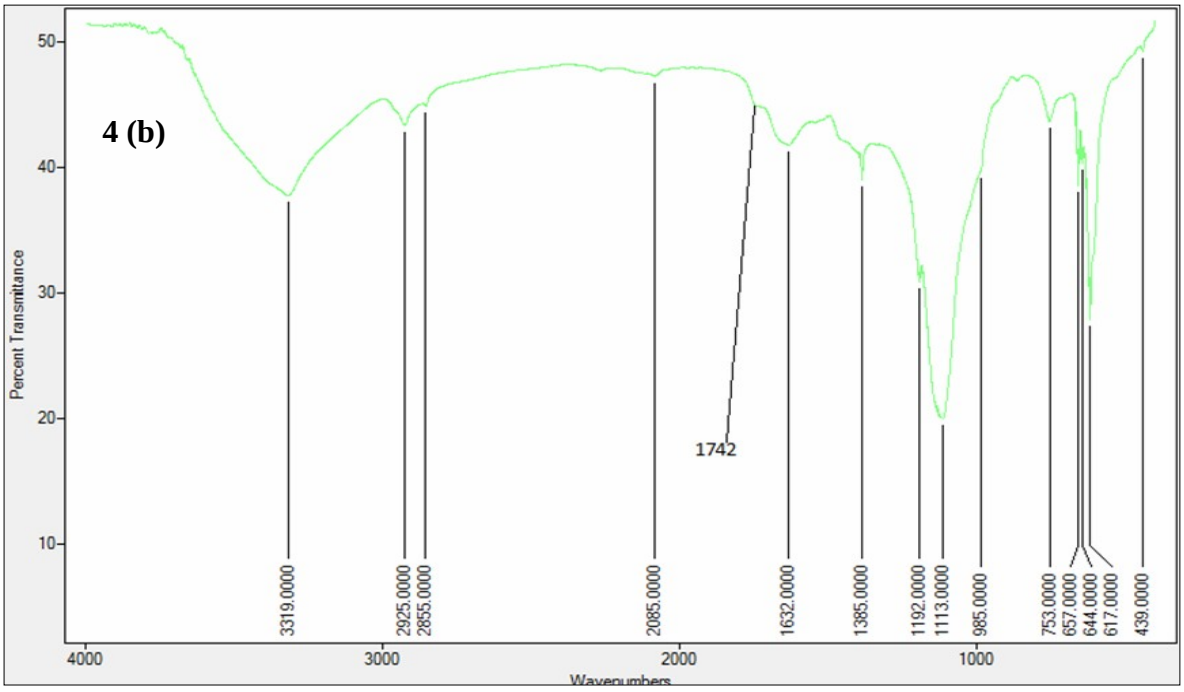


Table.2 Free radical scavenging activity and Metal chelating activity of different extracts of *Morindacitrifolia* fruit extracts. The values are the mean ± standard deviation, n=3. Significant differences at P<0.05 (*) and P<0.01 (#) are indicated with different letters.

Free Radical scavenging activity of *Morinda citrifolia* fruit extracts					
Free Radicals Scavenged	**EtF**	**AqF**	**EaF**	**BHT**	**Ascorbate**
DPPH	49.78±1.33	52.11±1.91*	53.5±1.11*	72.67±1.75	81.19±1.73
Superoxide O_{2-}	63.33±1.21	72.64±1.35*	77.32±1.06#	81.22±1.07	83.51±1.40
Hydroxyl OH^-	55.9±1.65	60.6±2.02*	61.26±2.13*	71.09±2.21	75.68±2.16
Metal chelating activity of *Morinda citrifolia* fruit extracts					
Fruit Extracts	EtF	AqF	EaF	EDTA	Ascorbate
Metal Chelating Activity	46.26±3.75	49.6±4.63*	54.13±2.77#	75.81±1.88	64.44±1.03

Figure.4 FTIR spectra for the Morindacitrifolia fruit extracts 4(a) Ethanolic extract (EtF), 4(b) Aqueous extract (AqF) and 4(c) Ethyl acetate extract




4 (b)
Percent Transmittance
50
40
30
20
10
1742
3319.0000
2925.0000
2855.0000
2085.0000
1632.0000
1385.0000
1192.0000
1113.0000
985.0000
753.0000
657.0000
644.0000
617.0000
439.0000
4000
3000
2000
1000
Wavenumbers

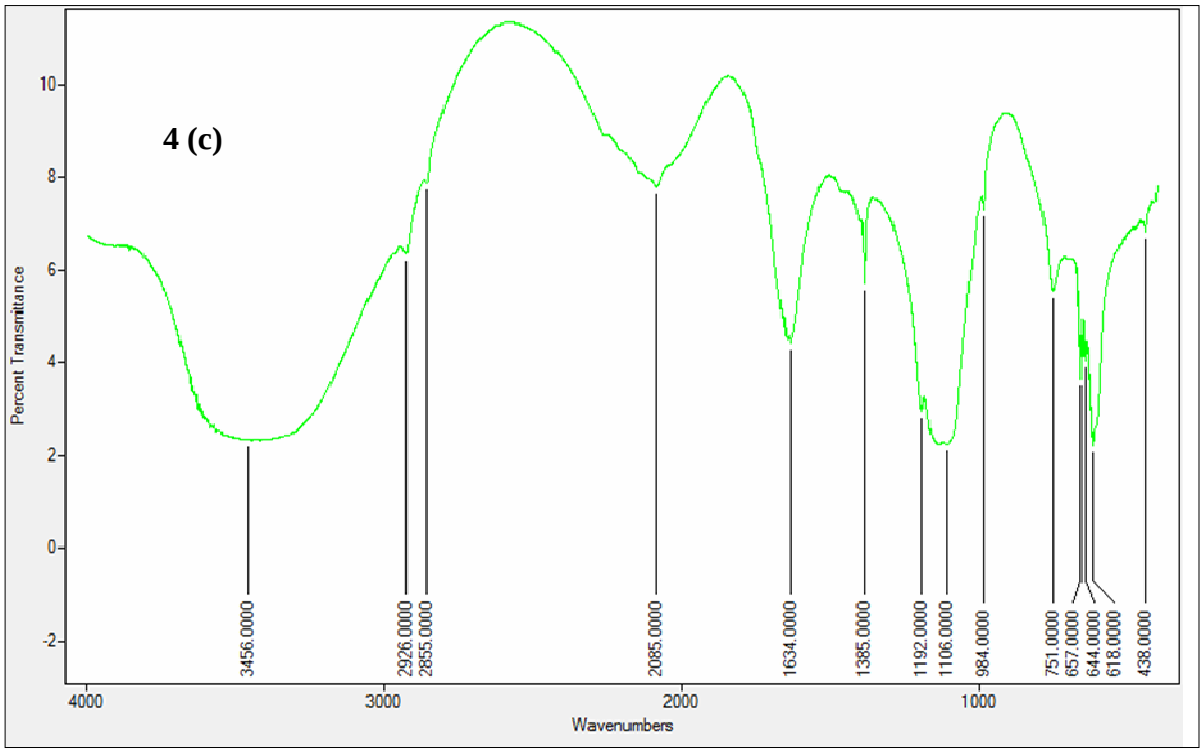

4 (c)
Percent Transmittance
10
8
6
4
2
0
-2
3456.0000
2926.0000
2855.0000
2085.0000
1634.0000
1385.0000
1192.0000
1106.0000
984.0000
751.0000
657.0000
644.0000
618.0000
438.0000
4000
3000
2000
1000
Wavenumbers

Table.3 FTIR frequency range and functional group present in the ethanolic, aqueous and ethyl acetate extract of *Morindacitrifolia*fruits.

S.NO.	Peaks values	Functional groups
1.	3400-3200	Polymeric hydroxyl compound
2.	2925-2875	Cycloalkanes (cyclo butane)
3.	2925-2855	Cycloalkanes (Cyclopentane)
4.	1745-1725	Carbonyl compound ketones (C=O)
5.	1385-1325	Methyl group
6.	1260-1150	Sulphonic acid esters (SO3)
7.	1150-1100	Aryl conjugated
8.	1220-980	Alkene
9.	835-805	Aromatic ortho di substitute
10.	730-500	Halogen compound
11.	690-550	Bromo compound
12.	620-420	Iodo compound

alleviating chronic diseases by reducing the oxidative damage to cellular components caused by free radicals and ROS. There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases (Rose *et al.*, 1982). DPPH is a free-radical generating compound and has been widely used to evaluate the free-radical scavenging ability of various antioxidative compounds (Hatano, 1995). DPPH radical assay was performed and the results are compared with standard antioxidants BHT and ascorbate. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Hatano, 1995). Though the DPPH radical scavenging abilities of the extracts were less than those of ascorbic acid (81.19%) and BHT (72.67%), the study showed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

In living organisms there are two major reactive oxygen species, superoxide radical and hydroxyl radical that are being continuously formed in a process of reduction of oxygen to water. Superoxide radical is considered a major biological source of reactive oxygen species. Superoxide radicals are formed by the one electron reduction of molecular oxygen. They may oxidize and reduce other compounds and potentially forms more reactive species via secondary reactions (Estévez, and Jordán, 2002). Free radicals, showing deleterious effects such as

superoxide radical, hydroxyl radical, and nitric oxide radical are quenched by certain synthetic antioxidants having adverse side effects. Safety is an essential consideration for antioxidants as they may be utilized in the manufacture of foods and pharmaceuticals. This let various workers on exploration for natural sources of antioxidants with multifunctional potential as alternatives for toxic synthetic antioxidants, to prevent oxidation in any metabolic pathways (Halliwell and Gutteridge 1998).The results of our study revealed the superoxide scavenging activity of the *Morinda citrifolia* fruit extracts and it was compared with the standard antioxidants. The ethyl acetate extract (77.32%) has the higher scavenging activity when compared with other two extracts; however it is inferior when compared with that of the standards ascorbate (83.51%) and BHT (81.22%). The hydroxyl radical ($OH^{\circ-}$) constitutes the chemically most reactive species of `activated oxygen' formed by successive monovalent reduction of dioxygen (O2) in cell metabolism, and is primarily responsible for the cytotoxic effects of oxygen in plants, animals and micro-organisms, living in an oxygenic atmosphere (Estévez, and Jordán, 2002). Hydroxyl radical reacts with several biological materials oxidatively by hydrogen withdrawal, double-bond addition, electron transfer and radical formation, and initiates autoxidation, polymerization and fragmentation (Chen and Schopfer, 1999). Hydroxyl radical scavenging activity of the three different extracts of *Morinda citrifolia* fruits were determined as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. Among the three, the ethyl acetate extract was found to exhibit maximum scavenging activity of about 61.26%, which is lesser when compared to the standards BHT (71.09%) and ascorbate (75.68%).

The Fourier Transform Infrared (FTIR) spectroscopy has proven to be a valuable tool for characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of plant extracts. Moreover, FTIR spectroscopy is an established time saving method to characterize and identify functional groups (Grube et al., 2008). The results of FTIR analysis confirmed the presence of polymeric hydroxyl group ($3456cm^{-1}$), cycloalkanes ($2926cm^{-1}$and $2855cm^{-1}$), ketones ($1742cm^{-1}$), aldehyde ($1634cm^{-1}$), sulphonic acid esters ($1192cm^{-1}$), alkenes ($1113cm^{-1}$), phenol ($984cm^{-1}$), aromatic compound ($657cm^{-1}$), and halogens ($618cm^{-1}$).The polymeric hydroxyl groups, aromatic compounds, phenols, aldo-ketogroup peak confirm the presence of polyphenols of which flavonoids, coumarins, anthroquinones and phenolic compounds are majorly present in *Morinda citrifolia* and attributed to their antioxidant effect.

The present study clearly dictates the potential antioxidant activity and free radical scavenging properties of *Morinda citrifolia* fruit extract which may be due to the various functional compounds present in the fruit extracts as revealed by FTIR spectral studies. However isolation and characterization of these compounds and validating their therapeutic efficacy against different pathologies is required for clinical implementation.

Acknowledgement

The authors wish to acknowledge the financial support offered by *World Noni Research Foundation (WNRF)*, Chennai, India to conduct this study. We are

grateful to Dr. P.T. Perumal, Chief Scientist & Head, Organic Chemistry Division, Central Leather Research Institute, Chennai for permitting us to work with the FTIR spectroscopy.

Conflict of interest

The authors declare that they have no conflict of interest.

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