International Journal of Pharmaceutical Science Invention
ISSN (Online): 2319-6718, ISSN (Print): 2319- 670X
www.ijpsi.org Volume 9 Issue 2 ǁ Feb 2020 ǁ PP.01-07

Anti Oxidant Property of Madhulai Manappagu – A Siddha Herbal Syrup for Iron Deficiency Anaemia in Children

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Abstract:
Madhulai Manappagu, a well known Siddha syrup formulation commonly used in day today Siddha clinical practices across the country. This formulation is proven haematinic syrup prescribed for iron deficiency anaemia in which juice of Madhulai (Pomegranate – Punica granatum) is the main ingredient. Madhulai manappagu is also used by the pregnant women during first trimester to combat morning sickness. Also, this syrup is prescribed along with certain cancer treatment. The antioxidant property of Madhulai(Pomegranate) fruit is attributed to the phytochemicals present in it. It is indeed necessary to assess the antioxidant property of this syrup formulation.
This study is aimed to screen the antioxidant effect of Madhulai Manappagu. In this study DPPH, Hydrogen peroxide, Nitric Oxide and ABTS radical scavenging studies were performed. The presently analyzed herbal formulation Madhulai Manappagu was found to exhibit remarkable antioxidant activity in terms of DPPH radical scavenging, nitric oxide radical scavenging, hydrogen peroxide inhibition and ABTS radical scavenging activities.

Key Words: *Madhulai Manappagu, Anti Oxidant Property, Siddha Medicine, Pomegranate, Punica granatum*

Date of Submission: 20-03-2020 Date of acceptance: 06-04-2020

I. INTRODUCTION:

Siddha system of Medicine is a traditional system of Medicine widely practised in Southern India, particularly in Tamilnadu. The system is part and parcel of the culture and tradition of Tamil people, taking them to live their healthy life. Mostly the medicines are formulated mainly with the drugs from plants. The therapeutic values of each and every drug from plant kingdom are described in Siddha literatures by the Siddhars who are considered to be spiritual scientists. The medicinal values of these drugs are evaluated by the present day preclinical and clinical research parameters.

Among the plant based drugs, almost each and every plant of both natural and cultivated vegetation of the region are in practice [(1)]. Among these drugs, Madhulai (pomegranate – Punica granatum) is widely used in various formulations in Siddha[(2)]. The therapeutic uses of Madhulai are more and is indicated for a no.of diseases of the human kind[(3)]. Madhulai Manappagu, a well known Siddha syrup formulation commonly used in day today Siddha clinical practices across the country, is one of the highly valued syrup preparation[(4)]. Out of 32 types of Internal Medicines described in Siddha Literatures, Manappagu is a type of Internal Medicine that can be correlated with the syrup preparation[(5)]. As per the statement "Prevention is better than Cure[(6)]", the syrup finds a place in prescription for any nutritional disorders. A syrup prepared with the pomegranate fruit juice as a main ingredient along with honey, jiggery, rosewater is a good haematenic. The syrup is used to stop morning sickness in first trimester of gestation, burning sensation in palms and soles, nausea, vomiting[(7,11)]. Pomegranate seeds are rich in iron and thus, help to decrease the anaemic symptoms like fatigue, dizziness and weakness and hair loss[(8)]. Madhulai manappagu is also used by the pregnant women during first trimester to combat morning sickness. The antioxidant property of Madhulai(Pomegranate) fruit is attributed to the phytochemicals present in it. In addition to this, the syrup is prescribed in certain cancer treatments also. Various scientific evidences to substantiate the antioxidant activity of the fruit pomegranate are available. But, the antioxidant property of Madhulai Manappagu, is to be established. The main objective of this study is to evaluate the antioxidant activity of Madhulai Manappagu which can throw scientific values upon the usage of this syrup for a quiet longer period.

II. MATERIALS AND METHODS :

Preparation of Madhulai Manappagu[7]

Ingredients:

1.	Madhulam Pazham Saru (Pomegranate Juice)	- 500 ml
2.	Thaen (Honey)	- 500 ml
3.	Panneer (Rose Water)	- 500 ml
4.	Kalkandu (Sugar Candy)	- 500 gms

Process:

Pomegranate fruit juice was taken and filtered. Equal quantities of rose water and sugar candy were added to the juice, mixed well and filtered. The filtrate was boiled and stirred frequently. When aroma aroused, fire was put down and was allowed to cool. After it had cooled, honey was added to the syrup and was collected and stored in a clean glass container[9]. The container was sterilised with boiling hot water and then dried up in hot air oven.

Dose and dispensing:

5 – 7.5 ml twice a day with water. Dispensed in clean and dry containers.

Indications:

Velluppu Noi[10]. (Pandu), peripheral neuritis.

Shelf life : 6 Months

Anti-oxidant Profiling by Free Radical Scavenging Assay:

DPPH (2, 2-Diphenyl 1-2 picrylhydrazyl) Assay:

The antioxidant activity of the test sample Madhulai Manappagu was determined using the 2,2-diphenyl 1-2 picrylhydrazyl (DPPH) free radical scavenging assay . The sample was mixed with 95% methanol to prepare the stock solution in required concentration (10mg/100ml or 100µg/ml). From the stock solution 1ml, 2ml, 4ml, 6ml 8ml and 10ml of this solution were taken in five test tubes and by serial dilution with same solvent were made the final volume of each test tube up to 10 ml whose concentration was then10 µg/ml, 20 µg/ml, 40µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml respectively. Ascorbic acid were used as standard was prepared in same concentration as that of the sample extract by using methanol as solvent. Final reaction mixture containing 1 ml of 0.3 mM DPPH methanol solution was added to 2.5 ml of sample solution of different concentrations and allowed to react at room temperature. Absorbance in the presence of sample extract at different concentration of (10 µg, 20 µg, 40 µg, 60 µg, 80 µg and 100 µg/ml) was noted after 15 min incubation period at 37^0C. Absorbance was read out at 517 nm using double-beam U.V Spectrophotometer by using methanol as blank.

% scavenging = [Absorbance of control - Absorbance of test sample/Absorbance of control] X 100

The effective concentration of sample required to scavenge DPPH radical by 50% (IC_{50} value) was obtained by linear regression analysis of dose-response curve plotting between %inhibition and concentrations.[12]

Hydrogen peroxide scavenging (H_2O_2) assay

H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals ($OH_{\bullet}$) that can initiate lipid peroxidation and cause DNA damage in the body. The ability of test sample to scavenge hydrogen peroxide can be estimated. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (50 mM pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. The sample Madhulai Manappagu was prepared in different concentrations (10-100 µg/ml) was added to hydrogen peroxide and absorbance at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging was calculated as follows:

$$\%\ \text{scavenged}\ (H_2O_2) = [(A_i - A_t)/A_i] \times 100$$

where A_i is the absorbance of control and A_t is the absorbance of test.[13]

Nitric Oxide Radical Scavenging Assay[14]

The concentrations of test sample Madhulai Manappagu was made into serial dilution from 10−100 µg/mL and the standard gallic acid. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylenediaminedihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different concentrations of the extracts (10−100 µg/mL) and incubated at 25°C for 180 mins. The test drug was mixed with an equal volume of freshly prepared Griess reagent. Control samples

without the extracts but with an equal volume of buffer were prepared in a similar manner as was done for the test samples. The absorbance was measured at 546 nm using a Spectra Max Plus UV-Vis microplate reader (Molecular Devices, GA, USA). Gallic acid was used as the positive control. The percentage inhibition of the test sample and standard was calculated and recorded. The percentage nitrite radical scavenging activity of the Madhulai Manappagu and gallic acid were calculated using the following formula:

percentage nitrite radical scavenging activity:

$$\text{nitric oxide scavenged } (\%) = \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100,$$

where A_{control} = absorbance of control sample and A_{test} = absorbance in the presence of the samples extracts or standards.

ABTS Assay[15]

This assay carried out for the purpose of evaluating the anti-oxidant potential of Madhulai Manappagu against 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS radicals

The ABTS radical cation method was modified to evaluate the free radical-scavenging effect of one hundred pure chemical compounds. The ABTS reagent was prepared by mixing 5 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate. The mixture was then kept in the dark at room temperature for 16 h to allow free radical generation and was then diluted with water (1 : 44, v/v). To determine the scavenging activity, 100 µL ABTS reagent was mixed with 100 µL of test sample (10-100µg/ml) and was incubated at room temperature for 6 min. After incubation, the absorbance was measured 734 nm. 100% methanol was used as a control. Gallic acid with same concentrations of extract was measured following the same procedures described above and was used as positive controls. The antioxidant activity was calculated using the following equation: The ABTS scavenging effect was measured using the following formula:

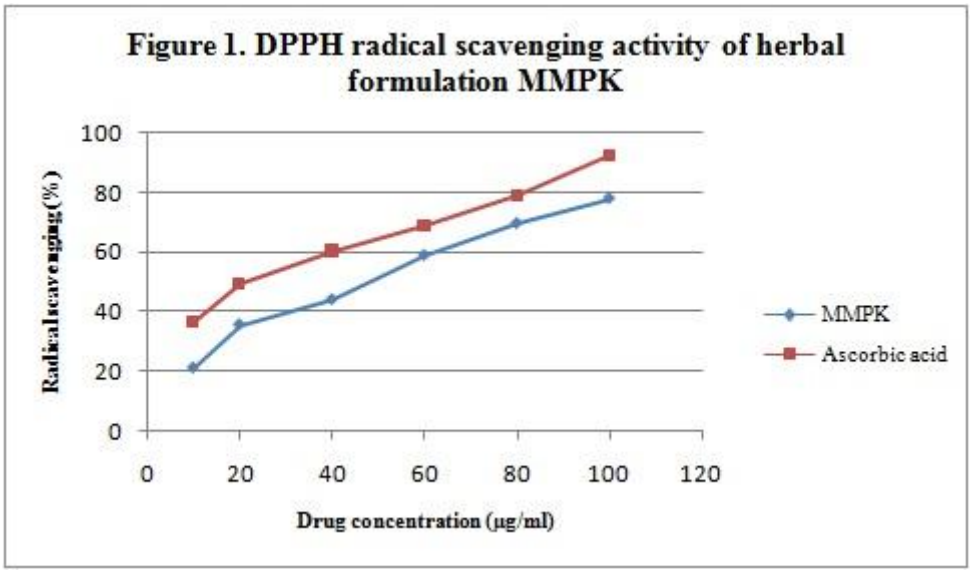
$$\text{Radical scavenging } (\%) = \left[\frac{(A)_{\text{control}} - (A)_{\text{sample}}}{(A)_{\text{control}}}\right] \times 100.$$

III. RESULTS AND DISCUSSION :

Table 1. DPPH radical scavenging activity of herbal formulation Madhulai Manappagu.

Drug concentration (µg/ml)	DPPH Inhibition (%) by Madhulai Manappagu	DPPH Inhibition (%) by Ascorbic Acid
10	21.43 ± 7.96	36.79 ± 4.61
20	35.81 ± 3.10	49.32 ± 3.58
40	44.19 ± 9.07	60.42 ± 2.36
60	58.9 ± 8.94	68.69 ± 2.21
80	69.51 ± 3.52	78.68 ± 1.03
100	77.82 ± 4.99	92.18 ± 1.93
IC-50 value	50.93	20.27

Data are given as Mean ± SD of three separate analysis (n=3).

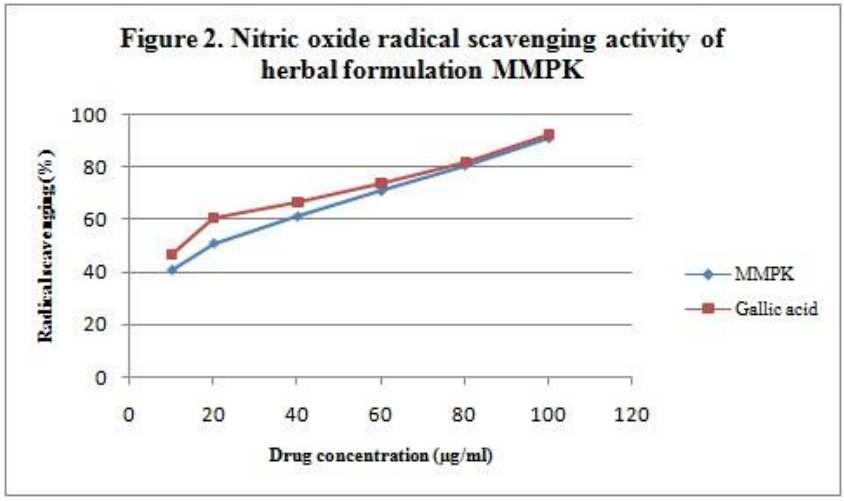


DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in methanol. This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to colorless ethanol solution. The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry. In the present study, the herbal formulation Madhulai Manappagu exhibited maximum DPPH radical scavenging activity of 77.82% at 100 µg/ml concentration, which is comparable to that of standard ascorbic acid (92.18%) (Table 1 & Figure 1). However, the IC-50 value of Madhulai Manappagu (50.93 µg/ml) indicated that it has lower antioxidant power when compared to synthetic ascorbic acid (IC-50 value 20.27 µg/ml).

Table 2. Nitric Oxide radical scavenging activity of herbal formulation Madhulai Manappagu.

Drug concentration (µg/ml)	Nitric Oxide Inhibition (%) by Madhulai Manappagu	Nitric Oxide Inhibition (%) by Gallic Acid
10	40.9 ± 2.28	46.95 ± 1.55
20	51.1 ± 2.23	60.64 ± 2.22
40	61.37 ± 2.39	66.73 ± 4.64
60	71.16 ± 4.14	73.7 ± 6.46
80	80.94 ± 2.20	81.86 ± 7.74
100	91.32 ± 1.69	92.19 ± 2.26
IC-50 value	19.56	10.64

Data are given as Mean ± SD of three separate analysis (n=3).

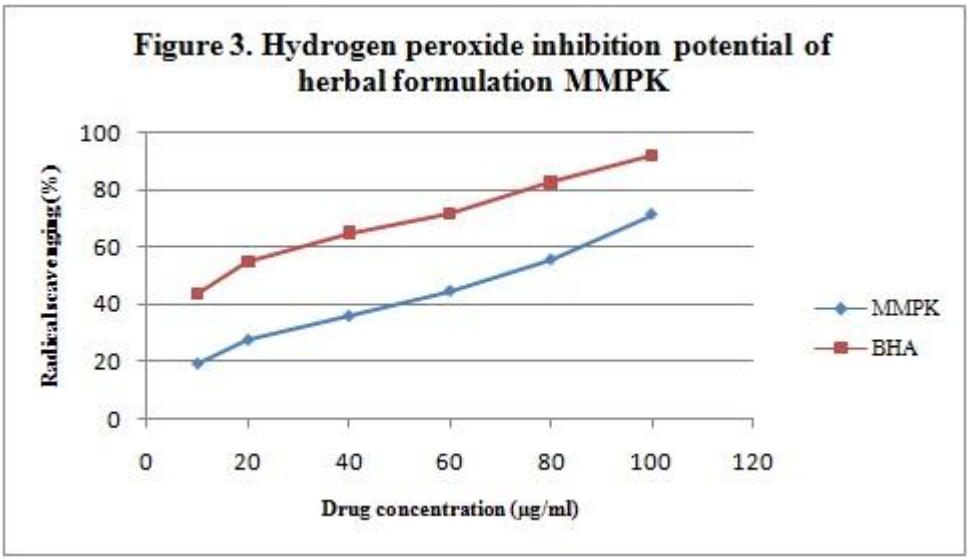


Nitric oxide is a free radical that is derived from the interaction of NO with oxygen or reactive oxygen species. Nitric oxide is classified as a free radical because of its unpaired electron and displays important reactivity with certain types of proteins. Low concentrations of Nitric oxide are sufficient in most cases to effect the physiological functions of the radical. Nitric oxide is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation, and antimicrobial and antitumor activities. In the present work, herbal formulation Madhulai Manappagu exhibited remarkable Nitric oxide scavenging activity (91.32%) at a concentration of 100 µg/ml, which is similar to that of synthetic compound, gallic acid (92.19%) (Table 2 & Figure 2). However, the IC-50 value of Madhulai Manappagu (19.56 µg/ml) indicated that it has lower antioxidant power when compared to gallic acid (IC-50 value 10.64 µg/ml).

Table 3. Hydrogen peroxide inhibition potential of herbal formulation Madhulai Manappagu.

Drug concentration (µg/ml)	Hydrogen Peroxide Inhibition (%) by Madhulai Manappagu	Hydrogen Peroxide Inhibition (%) by BHA
10	19.32 ± 1.45	43.53 ± 1.62
20	27.55 ± 1.27	54.83 ± 2.44
40	35.87 ± 2.96	64.86 ± 1.48
60	44.64 ± 1.35	71.73 ± 1.41
80	55.5 ± 2.02	82.53 ± 5.44
100	71.28 ± 2.97	91.89 ± 1.10
IC-50 value	72.07	18.23

Data are given as Mean ± SD of three separate analysis (n=3).

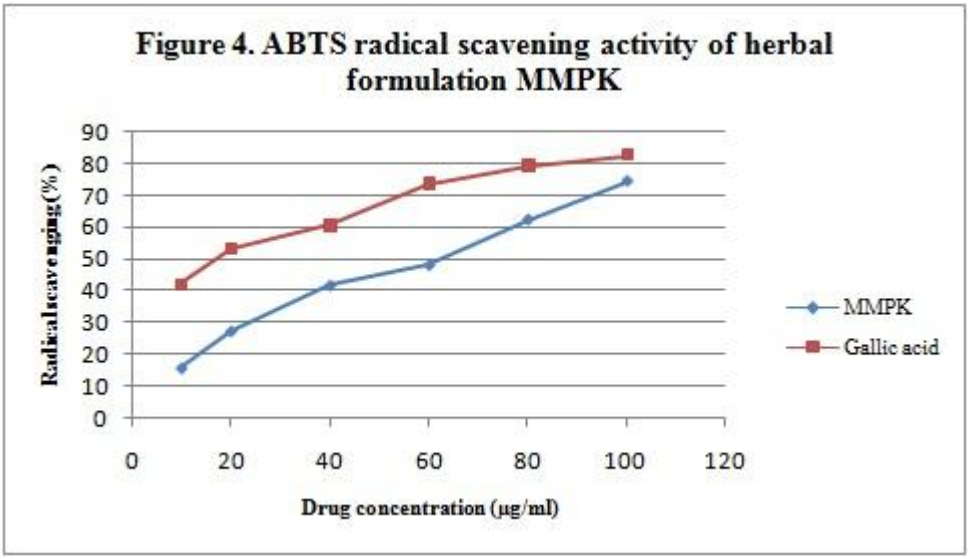


Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because of it may give rise to hydroxyl radical in the cells. Thus, the removal / inhibition of H2O2 is very important for antioxidant defense in cell or food systems. Antioxidants from plant extracts could inhibit / destroy the H2O2 and therefore terminate the free radical formation thereof. In the present work, the herbal formulation Madhulai Manappagu at 100 µg/ml revealed 71.28% of H2O2 inhibition potential. But the IC-50 values indicated that Madhulai Manappagu has lower H2O2 inhibition potential (72.07 µg/ml) when compared to the synthetic standard BHA (18.23 µg/ml) (Table 3 & Figure 3).

Table 4. ABTS radical scavenging activity of herbal formulation Madhulai Manappagu.

Drug concentration (µg/ml)	ABTS radical Inhibition (%) by Madhulai Manappagu	ABTS radical Inhibition (%) by Gallic Acid
10	15.76 ± 2.38	42.24 ± 3.71
20	27.34 ± 2.33	53.3 ± 3.72
40	41.64 ± 0.32	60.56 ± 6.29
60	47.99 ± 3.86	73.53 ± 2.04
80	62.26 ± 3.17	79.2 ± 4.14
100	74.29 ± 2.06	82.48 ± 3.93
IC-50 value	62.51	18.76

Data are given as Mean ± SD of three separate analysis (n=3).



ABTS assay measures the relative ability of antioxidants to scavenge the synthetic free radical (ABTS), which is generated in aqueous phase. The ABTS free radical is generated by reaction of ABTS salt with a strong oxidizing agent (potassium persulfate). When the ABTS radicals are reduced by the antioxidant compounds, blue green coloured ABTS radical solution become colour-less and decrease in the colour of the ABTS solution corresponds to the antioxidant power of test substance. In the present work, the herbal formulation Madhulai Manappagu exhibited moderate ABTS radical scavenging activity (74.29%) at higher concentration of 100 µg/ml. However, when we looked into the IC-50 values, Madhulai Manappagu formulation possesses lower antioxidant power (IC-50 value 62.51 µg/ml) when compared to the synthetic antioxidant, gallic acid (IC-50 value 18.76 µg/ml).

IV. CONCLUSION :

The presently analyzed herbal formulation Madhulai Manappagu was found to exhibit remarkable antioxidant activity in terms of DPPH radical scavenging, nitric oxide radical scavenging, hydrogen peroxide inhibition and ABTS radical scavenging activities. Based on IC-50 values, it is understood that Madhulai Manappagu formulation might act as a strong antioxidant, especially against nitric oxide radicals and moderately effective against DPPH and ABTS radicals, but weak antioxidant against hydrogen peroxide. The results of presently conducted *in vitro* experiments must be confirmed through suitable *in vivo* animal models in order to develop Madhulai Manappagu formulation as efficient antioxidant drug.

ACKNOWLEDGEMENT

The authors wish to thank National Institute of Siddha, Chennai, Ministry of AYUSH, New Delhi and The Tamilnadu Dr.M.G.R.Medical University, Chennai for granting of permission and registration of this study to be carried out in National Institute of Siddha, Tambaram Sanatorium, Chennai respectively.

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Dr M Meenakshi Sundaram "Anti Oxidant Property of Madhulai Manappagu – A Siddha Herbal Syrup for Iron Deficiency Anaemia in Children". *International Journal of Pharmaceutical Science Invention(IJPSI),* vol. 09(02), 2020, pp. 01-07.