FREE RADICAL SCAVENGING ACTIVITY OF POLYHERBAL FORMULATION

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Summary

The free radical scavenging potential of Madhuhari a polyherbal antidiabetic formulation was studied for its *in vitro* scavenging activity by different methods *viz.* DPPH radical scavenging activity, Nitric oxide radical scavenging activity, lipid peroxidation scavenging activity and superoxide radical scavenging activity. The results were analyzed statistically by regression method. The percentage scavenging and IC_{50} values were calculated for all the methods. The maximum inhibitory concentration (IC_{50}) in all the methods were found to be 24.85, 25.42, 26.35, and 67.14 µg/ml respectively. In all the methods, the Madhuhari showed its ability to scavenge free radicals in a concentration dependent manner. With the above results we can say that formulation used in this study possess significant antioxidant potential and it is possible that this high antioxidant potential could contribute in their folk antidiabetic and medicinal properties.

Keywords: DPPH model, antioxidant activity, Madhuhari, lipid per oxidation.

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Introduction

Free radicals are generally very reactive molecules possessing an unpaired electron. These free radicals, because of their relatively unstable nature, are highly reactive and damage number of biomolecules viz. lipids, proteins, enzymes, nucleic acids by oxidizing them. The free radicals are either oxygen derived or nitrogen derived called as a proxidant (1). The oxygen derived species include superoxide, hydroxyl, hydroperoxyl, peroxyl and alkoxyl as free radicals; and hydrogen peroxide, hypochlorous acid, ozone and singlet oxygen as non-radicals. Similarly, the nitrogen derived species include nitric oxide, nitrogen dioxide, peroxynitrite and dinitrogen trioxide (2).

However, when the generation of free radicals (FR)/reactive oxygen species (ROS) far exceeds the levels of antioxidant defenses, oxidative damages can lead to the functional impairment in cells and even cause its death and the prevailing condition is termed as oxidative stress (3). Oxidative stress is implicated in several clinical conditions of various diseases such as diabetes (4), cataracts (5), muscular degeneration (6), impaired wound healing (7), gastrointestinal inflammatory diseases (8), atherosclerosis, inflammatory injury, aging (9), cancer (10), cardiovascular diseases (11), neurodegenerative diseases, including Parkinson's and Alzheimer's diseases (12,13). Hyperglycemia aggravates endothelial ROS generation by a variety of mechanisms. ROS increases the generation of TNF-a expression and aggravates oxidative stress. Suppression of intracellular mitochondrial ROS over-production by use of low molecular weight inhibitors and antioxidants prevents glucose-induced activation of PK-C, formation of advanced glycation end products, sorbitol accumulation and activation of cytokines. This study has opened a new avenue to make a radical approach for the treatment of diabetic complications (14). Anti-oxidants and polyphenolic compounds have been shown to scavenge free radicals, reduce oxidative stress and decrease the expression of TNF-a. Therefore, phytochemicals appear to manipulate by various indirect mechanisms, the complications of diabetes mediated through oxidative stress, ROS or TNF-α.(15)

Madhuhari is polyherbal antidiabetic formulation which contains Karela-10%w/w (16); jamun-10%w/w (17); Babul ki chhal-10%w/w (18); Amba haldi-10%w/w (19); Gulvel-10%w/w (20); Bilwa patra-10%w/w (21); Neem-5%w/w (22); Kutki-5%w/w (23); Shilajit-2.5%w/w; Excipients-Q.S The present study is designed to investigate the free radical scavenging activity of madhuhari to generate scientific data as a proof of bioefficacy for finished herbal formulation.

Materials and methods

Chemicals: DPPH (1, 1- Diphenyl-2-Picryl Hydrazyl), haemoglobin was obtained from Sigma Chemicals, USA. Ferrous sulphate, dimethyl sulphoxide (DMSO), ethylene diamine tetra acetic acid (EDTA), sodium hydroxide were obtained from Ranbaxy Fine Chemicals Ltd. India. TBA (Thiobarbituric acid), TCA (Trichloro acetic acid), NBT (Nitro blue tetrazolium chloride) and BHT (Butylated hydroxytoluene) were obtained from Himedia Laboratories Ltd. Mumbai, India. All other chemicals and solvents used in the study were of analytical grade.

Estimation of Total Phenolic Content : Total Phenolics concentration was estimated by Folin-Ciocalteu method described by Singleton and Rossi. 1 ml aliquots of sample (1g/100ml) were mixed with 5 ml folin-ciocalteu reagent (diluted tenfold) and 4 ml (7.5%w/v) sodium carbonate. The absorption was read after 30 minute at 765 nm, for the determination of phenolics content in sample. All determinations were performed in triplicate (24,25). Total content of phenolic compounds in sample in gallic acid equivalent [GAE], was calculated by formula $C=(cV/m)\times100$ where, C=Total content of phenolics compound mg/gm sample in GAE, c-the concentration of gallic acid established from calibration curve (mg/ml), V-the volume of the sample (ml), m-the weight of the sample taken (g)

Estimation of Flavonoids Content: Flavonoids were estimated by using Quercetin as reference compound. 1 ml of sample (1g/100ml) (stock solution SS) was mixed with 1 ml aluminum trichloride ($AlCl_3$) in ethanol (2g/100ml) and diluted with ethanol to 25 ml. The absorption at 415 nm was read after 40 minute, in the spectrophotometer (26). Blank were prepared from 1ml of SS and diluted to 25 ml with methanolic acetic acid (0.5%v/v). All determinations were carried out in triplicate. The amount of flavonoids in sample as Quercetin equivalent was calculated.

***In vitro* antioxidant study:**

Determined by using DPPH radical scavenging activity, Nitric oxide radical scavenging activity, lipid peroxidation scavenging activity and Superoxide radical scavenging activity methods.

DPPH radical scavenging activity: The antioxidant activity of the sample, on the basis of scavenging activity of the stable DPPH free radical, was determined by the method describe by (27) Bracu et al. To 1 ml of various concentrations of sample, 3 ml of solution of DPPH 0.1 mM was added to the test tube. An equal amount of methanol was added to the control. Ascorbic acid was used as the standard for comparison. After 20 minutes incubation in the dark, absorbance was recorded at 517 nm. Experiment was performed in triplicate. In the entire method % Inhibition was calculated by using the formula . % Inhibition= [Control-Test/ Control]x 100

Nitric Oxide scavenging activity: To the 1 ml of various concentrations of the sample (10-100 µg/ml), 0.3 ml of 5 mM sodium nitroprusside was added in the test tubes. The test tubes were incubated at 25ºC for 5 h. After 5 h, 0.5 ml of Griess reagent was added. The absorbance was measured at 546 nm (28). The experiment was performed in triplicate

Lipid peroxidation scavenging activity: The lipid peroxidation in rat brain homogenate was measured *invitro* in terms of formation of thiobarbituric acid reactive substance (TBARS). Different concentration of the sample was added to the brain homogenate (0.5 ml) and the mixture were incubated with 0.15M KCl (100 µl). Lipid peroxidation was initiated by adding 100 µl of 15mM $FeSo_4$ solution and the reaction mixture was incubated at 37 ^{0}C for 30 minute. An equal volume of TBA:TCA (1:1 ml) was added to the above solution followed by addition of 1 ml BHT. This final mixture was heated on the water bath for 20 minute at 80^{0}C, cooled centrifuged and the absorbance read at 532 nm using spectrophotometer (29,30).

Superoxide radical scavenging activity: Superoxide radical scavenging activity was based on the capacity of the sample to inhibit formazon formation by scavenging the superoxide radicals generated in riboflavin light-NBT system. The reaction mixture contains 50mM phosphate buffer pH 7.6, 20mcg riboflavin, 12mM EDTA, NBT 0.1mg/3ml, added in that sequence. Reaction was started by illumination the reaction mixture containing different concentration of sample extract for 90 sec and absorbance was measured immediately at 590nm (31,32). Ascorbic acid was used as positive control.

Statistical Analysis: All results are expressed as mean ± S.E.M. Linear regression analysis was used to calculate the IC_{50} values.

Results and discussion

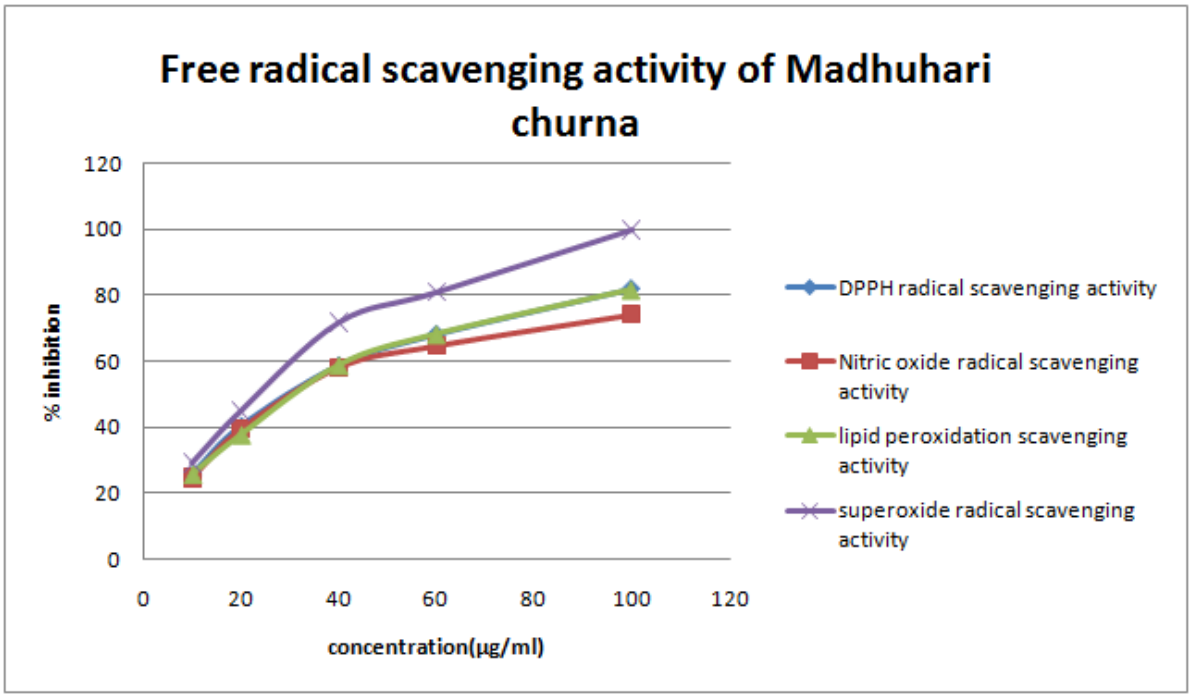
Total phenolic content of sample was determined according to the Folin-Ciocalteu method and expressed as mg GAE/g dry weight of plant material. It is found to be 68.19 (0.19 µg/mg Gallic equivalents. The total flavonoids concentration was measured as quercetin equivalents and is found to be 30.67 ± 0.28 µg/mg quercetin equivalents. It is well known that plant phenolic compounds and flavonoids are highly effective free radical scavengers. The Folin-Ciocalteu phenol method is actually not an antioxidant test but a replaceable assay for the quantity of oxidizable substance, i.e. phenolic compounds. Phenol compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the reagent (33).

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The DPPH free radical scavenging activity of Madhuhari is nearly similar to that of the standards used and is indicated in table 1 and fig.1. The model of scavenging the stable DPPH radical is widely used for relatively rapid evaluation of antioxidant activities. The essence of the DPPH method is that the antioxidants react with the stable free radical, i.e. 1, 1-diphenyl-2-picrylhydrazyl (deep violet colour) and convert it to 1,1-diphenyl-2-picrylhydrazine with discoloration (34). The antioxidant power is indicated by the degree of discoloration which could be determined by measuring of a decrease in the absorbance at 517 nm.

Table 1: Comparison of IC50 values of extracts with standard

Sr. No.	Model	Madhuhari Churna (µg/ml)	Ascorbic acid (µg/ml)
1	DPPH radical scavenging activity	24.85	16.60
2	Nitric Oxide scavenging assay	25.42	20.34
3	Lipid peroxidation scavenging activity	26.35	16.68
4	Superoxide radical scavenging activity	67.14	24.03

Figure 1: Free radical scavenging activity of Madhuhari churna by different antioxidant methods. Each value represents mean ± S.E.M



The nitric oxide scavenging activity is also quantitatively equivalent to that of the standards used and is indicated in the table1 and fig1. These activities are also found to increase with increase in concentration. Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as NO_2, N_2O_4, N_3O_4, NO_3-and NO_2- are very reactive. These compounds are responsible for altering the structural and functional behaviour of many cellular components. Nitric oxide is also implicate inflammation, cancer and other pathological conditions (35). The procedure is based on the method, where sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions.

In lipid peroxidation, hydroxyl radicals are generated which are considered to be the most reactive species of all, initiating the peroxidation of the cell membrane which breaks down to aldehyde such as malonaldialdehye, which are known to be mutagenic and carcinogenic. The decrease in malonaldialdehye formation with antioxidants thus indicates the consumption of hydroxyl radicals in the reaction mixture (36). Madhuhari protect cells from damage due to lipid peroxidation, induced by ferric-ascorbate in rat brain homogenate in a dose dependent manner as indicated by reduction in malondialdehyde formation with IC_{50} values as 26.35, to that of standard 16.68.

Super oxide radical is known to be very harmful to cellular as a precursor of more reactive species. The assay for studying superoxide scavenging activity is based on the capacity of the sample or test compounds to inhibit the formazan formation by scavenging the superoxide radical generated in riboflavin-light-NBT system. Illumination of either UV or visible light on riboflavin in the presence of either UV or visible light on riboflavin in the presence of EDTA will generate superoxide radicals react with nitro blue tetrazolium to form blue colored complex, which can be measured at 590 nm (37). When the superoxide radicals are scavenged by the sample, there is decrease in the intensity of test solution. The results indicated that Madhuhari possess significant antioxidant potential.

Conclusions

Many of herbs present in madhuhari are rich in polyphenols and flavonoid have been reported to have strong antioxidant activity. And also our study has shown that Madhuhari possess significant antioxidant potential and it is possible that this high antioxidant potential could contribute in their folk antidiabetic and medicinal properties. The studies offer impetus to need of evaluation of marketed polyherbal formulation in order to generated confidence both among the practitioner and patient.

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