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PRELIMINARY PHYTOCHEMICAL AND ANTIOXIDANT EVALUATION OF A POLYHERBAL FORMULATION *(MADHUMI)*

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ABSTRACT

Free radical and reactive oxygen species (ROS) are highly reactive molecules that are generated by normal cellular processes, environmental stresses and U.V radiation. ROS reacts with cellular components damaging DNA, carbohydrates, proteins and lipid causing cellular and tissue injury. Excess production of reactive oxygen species can also lead to inflammation, premature ageing disorder and several diseased states including cancer, diabetes and atherosclerosis. Organisms have developed complex antioxidant systems to protect themselves from oxidative stress. However, excess ROS can overwhelm the system and cause severe damage. Large number of antioxidants from different system of medicines includes Ayurveda, homeopathy, allopathic were being used and are still in great demand for the treatment of several chronic diseases. In present study, we have evaluated a polyherbal formulation -*Madhumi* powder for its anti-oxidant and free radicals scavenging activity by using various in-vitro methods. This particular polyherbal formulation was extracted with water and assessed for its phytochemical evaluation, total phenolic, total flavanoid, reducing power. The free radical scavenging activity was performed by using different in vitro antioxidant assays viz. DPPH, NO, OH, SO, ABTS and TBARS assays. The polyherbal formulation showed presence of alkaloids, glycosides, saponins, phenolic compounds, tannins, protein, amino acids and flavanoids. The phenolic and flavanoid content was found to be 11.83±0.037mg gallic acid equivalent/ gm of extract and 20.06±0.013 mg rutin equivalent/gm of extract respectively. The aqueous extract of Madhumi powder showed significant inhibition in all different anti-oxidant assays.

Key words: Antioxidant, Reducing Power, Phytochemical evaluation.

INTRODUCTION

Human body undergoes an important process known as oxidation and it leads to cause release of free radicals which has severe ill effects on the body (Ren J *et al*., 2008). When the free radicals are excessively produced they gives rise to oxidative stress (Huang D *et al*., 2005). Oxidative stress is a major cause for a large number of diseases such as neurodegenerative disorders, inflammation, viral infection, autoimmune gastrointestinal inflammation, gastric ulcer (Repetto MG and Llesuy SF, 2002), cancer, cardio-vascular diseases, diabetes and other age related illnesses found these days (Kris-Etherton *et al*., 2002). Nitric oxide radicals, hydroxyl radicals, superoxide anions and hydrogen peroxide are some of the reactive oxygen species which play a prominent role in the development of some major diseases (Wang H *et al*., 1996).

In order to quench and terminate the ill effects of the reactive species in the human body antioxidants are used (Huang D *et al*., 2005). It has been reported that a wide range of antioxidant species and phenolic compounds are found in fruits and vegetables (Ames BN *et al*., 1993; Ertan Y *et al*., 2001). Antioxidants hinder the process of lipid oxidative reactions and also scavenge free radicals produced (Manju V Subramanian & TJ James, 2010). The conventional antioxidants being used are butylated hydroxyanisole (BHA) and butylated hydroxyl toluene (BHT) which is of chemical origin. As these antioxidants are chemically synthesized they have their

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limitations (Assimopoulou AN *et al*., 2004). There have been several instances where the use of BHA and BHT has caused toxicity, carcinogenesis and liver damage (Grice H C, 1988). As a result, there is a need to synthesize better antioxidants of natural origin for non-toxic and effective use (Ferreira A *et al*., 2006).

It has been found that plants rich in flavanoids, polyphenols, alkaloids, terpenoids, carotenoids and vitamins shows good antioxidant activity (Yan-Fang Sun *et al*., 2006) which are helpful to abate the oxidative stress in the body to a considerable level (Adeyemi O *et al*., 2011). Flavanoids and other secondary plant metabolites which were found in ample quantities in plants and various vegetables (D'Archivio, M *et al*., 2007) have great hydrogen or electron donors capacity which showed great free radical scavenging activities. Flavanoids also have the property to interact with the free radicals to form products which are less reactive (Bena-Marie Lue *et al*., 2010).

Many medicinal plants show different level of antioxidant activity due to the varying amounts of free radical quencher ability. Plants with high level of antioxidant activity are used in combination so they can reinforce the potency of antioxidants to get a desired therapeutic action and suppress any undesirable effects. Hence to achieve an intense desired effect numerous plants are selected accordingly for the final product (Prashant gupta *et al*., 2013).

In this study, we have assessed the antioxidant activity of a polyherbal formulation known as *Madumi* powder which is a mixture of seven plants i.e *Swertia Chirata, setaria italica, lanatana camara, trachyspermium ammi, ficus exasperate, cyperus scariosus* through various antioxidant assays such as total phenol, total flavanoid, DPPH, ABTS, hydroxyl, superoxide, nitric oxide, TBARS and reducing power.

MATERIALS AND METHODS

Collection of Material

The *Madhumi* powder was acquired from the market of Mumbai, Maharashtra, India.

Preparation of extract

The extraction process was done by Hot Water Extraction method. 20 gm of the powdered sample was soaked in 100 ml of the solvent contained in a 500 ml of sterile conical flask and covered with a cotton wool. It was then plugged and wrapped with aluminum foil and shaken vigorously. The mixture was left to stand overnight (24 hours). The mixture was filter and filtrate was evaporated to get dry solid mass and this dried extract was used for further studies.

Chemicals and Reagents

2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, sodium carbonate, acetic acid glacial, nicotinamide adenine dinucleotide (NADH), thiobarbituric acid (TBA), nitroblue tetrazolium (NBT), Phenazine methosulphate (PMS), 2-deoxy-2-ribose, FeSO4, EDTA, ascorbic acid, Trichloro acetic acid (TCA), sodium dodecyl sulphate (SDS), sodium nitroprusside, naphthyl ethylenediamine dihydrochloride, sulphanilamide, phosphoric acid, trichloro acetic acid (TCA), Potassium ferricyanide, ferric chloride, hydrogen peroxide were obtained from Merck, SD fine chemicals, Himedia or Sigma. All other reagents used were of analytical grade.

Preliminary phytochemical screening

The extract of the powdered plant material was subjected to preliminary phytochemical evaluation using qualitative chemical tests for detecting the presence of the phytoconstituents like alkaloids, glycosides, tannins, phenolic compounds, phytosterols, carbohydrates, proteins and amino acids etc (Kokate CK, 1986).

Determination of total phenolic

The total phenol content in the extracts was estimated by using a modified Folin–Ciocalteu method (Budini *et al*., 1980). Sample extract (100µL) was mixed with 2.5mL of water in a 10mL volumetric flask. Folin–Ciocalteu reagent (0.5 mL) was added and allowed to react for 5min. Then, 1.5mL Na2CO3 solution (20 g/100 mL) was added and the mixture was made up to 10mL with water. After 120 min of incubation at room temperature, the absorbance was measured at 760 nm by using a spectrophotometer (UV- 1650, shimadzu, Kyoto, Japan) and was then compared to Gallic acid calibration curve. Total phenols were determined as Gallic acid equivalents (mg of Gallic acid/gm. of extract), and the total values presented as means of triplicates analyses.

Determination of total flavanoid

Aluminium chloride colorimetric method was used for flavanoid determination. The extract (0.5 ml of 1:10g/ml) in water was separately mixed with 1.5 ml of water, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8ml of distilled water. It remained at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm by using a spectrophotometer (UV-1650, Shimadzu, Kyoto, Japan). Three replicates were made for each test sample. The total flavanoid contents were expressed as rutin equivalents mg RE/ g extract (Singleton *et al*., 1990).

ANTIOXIDANT ACTIVITY

Reducing power assay

The reducing power of extracts was determined as per the reported method (Atmani *et al*., 2009). Different concentrations of the extract (100-1000 µg/ml) in 1 ml of water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferrocyanide (2.5 ml, 1%). The mixture was incubated at 50°c for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%). The absorbance was measured at 700 nm and compared with standards. Increased absorbance of the reaction mixture indicated increased reducing power.

DPPH radical scavenging activity

Antioxidant activity was determined based on the ability of the antioxidants to act as radical scavengers towards the stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). As described previously (F.G.K. Vieira *et al*., 2011), 1 mL of antioxidant solution (solubilised in water) was added to 3 mL of a 0.1 mM methanolic solution of DPPH. After 30 min at ambient temperature in darkness, absorbance readings were taken at 517 nm. Inhibition (%) was calculated using the equation:

$$[1-(A_s-A_0)/A_b]\times 100$$

whereby A_s was the absorbance reading for samples containing antioxidant, A_o was the absorbance of the antioxidant in pure methanol and A_b corresponded to the absorbance of the DPPH solution. Rutin, rutin laurate, rutin palmitate and BHT were investigated within the 0–200 µM concentration range, with all samples run in duplicate.

ABTS radical scavenging assay

The ABTS radical scavenging assay was done according to the method (Re *et al*., 1999). The ABTS radical was generated by the oxidation of ABTS with potassium persulphate. The ABTS radical cation solution was obtained as follows: Five millilitre of ABTS (7 mM) was mixed with 88 µl of potassium persulphate (140 mM) and then incubated in the dark at room temperature for 12–16 h. The working solution was prepared by diluting the previous solution with phosphate buffered saline (100 ml) pH 7.4 PBS containing 81.0 ml Na_2HPO_4 (0.2 M) and 19.0 ml NaH_2PO_4 (0.2 M) until the absorbance at 734 nm was 0.70± 0.02. The solution was kept for 30 min in the dark before being used. One hundred and fifty micro litres of each sample was mixed with 2.85 ml of the working solution, shaken vigorously, and left to stand for 10 min at room temperature. The absorbance of the reaction mixture was determined at 734 nm. The controls contained the extraction solvent instead of the antioxidant solution. The ABTS radical scavenging capacity of the sample was calculated by the following formula:

ABTS radical scavenging activity (%) =
[(Abs control – Abs sample)]/ (Abs control)] × 100

Hydroxyl radical scavenging Assay:

The hydroxyl radical scavenging of different fractions of the extract was evaluated by (Nagmoti *et al*., 2012). One ml of the reaction solution consisted of aliquots (500µl) of various concentrations of the fractions, 1mM $FeCl_3$, 1mM EDTA, 20mM H_2O_2, 1mM l-Ascorbic acid and 30mM deoxyribose in potassium phosphate buffer (pH 7.4). This was then incubated for 1hr at 37°c after addition of 1ml of 2.8% (w/v) trichloroacetic acid and 1ml of 1% (w/w) 2-thiobarbituric acid. Further it was heated in a boiling water bath for 15mins and absorbance was measured at 532nm against a blank.

Nitric oxide scavenging activity

The nitric oxide scavenging activity was assessed by previously described method (Ganapaty *et al*., 2007). A volume of 2 mL of sodium nitroprusside prepared in 0.5 mM phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of plant extract or BHT or rutin at various concentrations (0.2-1.0 mg/mL). The mixture was incubated at 25°c for 150 min. An aliquot of 0.5 mL of the solution was added to 0.5 mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride).. The mixture was incubated at room temperature for 30 min. The absorbance was then measured at 540 nm. The amount of nitric oxide radical was calculated using the equation:
NO radical scavenging activity =
[(Abs control – Abs sample)]/ (Abs control)] × 100

Superoxide radical-scavenging assay

Measurement of superoxide radical scavenging activity was done by using method (Khatri *et al*., 2013). The reaction mixture contained 1 ml of NBT (nitroblue tetrazolium) solution (150 µM prepared in phosphate buffer, pH 7.4), 1 ml of NADH (nicotinamide adenine dinucleotide) solution (468 µM prepared in phosphate buffer, pH 7.4) and methanol diluted sample extracts at various concentrations (5-1000 µg/ml) were added. Finally reaction was accelerated by adding 100 µL PMS (Phenazine methosulphate) solution (60 µM prepared in phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min and absorbance was measured at 560 nm against methanol as control. Percentage inhibition of scavenging of superoxide radical was calculated as follows:

% inhibition of Superoxide radical =
[(Abs control – Abs sample)]/ (Abs control)] × 100

Inhibition of microsomal lipid peroxidation

The lipid peroxidation level is measured as the thiobarbituric acid reactive substance (TBARS), by using

rat liver homogenate for induction of lipid peroxidation, mediated by FeSO4 as pro-oxidant and assessed (Okhawa *et al*., 1979). Reaction mixture containing rat liver homogenate 0.1 ml (25% w/v in Tris–HCl buffer (20 mM, pH 7.0); 0.1 ml of FeSO4.6H2O (0.16 mM); 0.1 ml ascorbic acid (0.06 mM) and various concentrations of aqueous extracts (100–1000 µg/ ml) in water were incubated at 37°C for 1 h. After the incubation period, reaction mixture was treated with 0.2 ml SDS (8.1%); 1.5 ml TBA (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4 ml with distilled water and then kept in a water bath at 95–100 °C for 60 min. After cooling, 1.0 ml of distilled water, 5.0 ml of n-butanol and pyridine mixture (15:1, v/v) was added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The organic layer was removed and its absorbance was measured. The inhibition of *in vitro* lipid peroxidation by the measurement of thiobarbituric acid reactive substances (TBARS) in the extracts was measured spectrophometrically at 532 nm. The assay was performed in triplicates. Ascorbic acid was taken as reference standard. The percentage of inhibition of lipid peroxidation was calculated using the following equation:

% Inhibition of lipid peroxidation =

[(Abs control – Abs sample)]/ (Abs control)] × 100

RESULTS

Preliminary phytochemical screening

The phytochemical screening of aqueous extract of *Madhumi* powder has shown presence of alkaloids, glycosides, saponins, tannins, proteins, amino acids, flavanoids and phenolic compounds.

Total Phenolic & flavanoid Content

In the present study, total phenolic content present in extract was estimated using modified Folin-phenol method. The total phenolic contents of the extracts is expressed as gallic acid equivalents (mg gallic acid/g dried extract).The total phenolic contents of the aqueous extract was found to be 11.83±0.037mg gallic acid equivalent/ gm of extract. The flavanoid content of the aqueous extract in terms of rutin equivalent was found to be 20.06±0.013 mg rutin equivalent/gm of extract.

Antioxidant Activity

Reducing power assay

The reducing power of aqueous the extract of polyherbal formulation was estimated using potassium ferric cyanide method. Like the antioxidant activity, the reducing power of polyherbal formulation increased with increasing amount of sample.

DPPH radical scavenging activity

The effect of aqueous extract of the polyherbal formulation on 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) radical scavenging activity is shown in Table 2 and Figure 2. The extract showed the DPPH radical scavenging activity in a concentration dependent (100-1000 µg/ml) manner. The aqueous extract showed IC_{50} value of 32.02 ± 0.42µg/ml.

ABTS radical scavenging assay

The effect of aqueous extract of the polyherbal formulation on 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity is shown in Table and Figure. These extracts showed the 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity in a concentration dependent (100-1000µg/ml) manner. The aqueous extract showed IC_{50} value of 157.84 ± 0.24µg/ml.

Hydroxyl radical scavenging Assay

In the present investigation, the effect of aqueous extract of the polyherbal formulation on Hydroxyl Radical Scavenging Activity is shown in Table no. 2 and Figure no. 2. These extracts showed the Hydroxyl Radical Scavenging Activity in a concentration dependent (100-100µg/ml) manner. The aqueous extract showed IC_{50} value of 33.77 ± 0.468 µg/ml. The hydroxyl radicals quenching capacity of the polyherbal extract seems to be directly related to scavenger of active oxygen species, hence dropping the velocity of chain reaction

Nitric oxide scavenging activity

Suppression of NO• release may be attributed to a direct NO• scavenging effect of the extracts decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro* as shown in Figure 2. The results show that polyherbal extract (100-1000µg/ml) had scavenging activity in concentration dependent manner. The IC_{50} value of extract was found to be of 64.45 ± 0.30µg/ml.

Superoxide radical-scavenging assay

The effect of aqueous extract of the polyherbal formulation on Superoxide Radical Scavenging Activity is shown in Table 2 and Figure 2. The extracts showed the Superoxide radical scavenging activity in a concentration dependent (100-1000µg/ml) manner. The aqueous extract showed IC50 value of 52.60 ± 0.94µg/ml.

Inhibition of microsomal lipid peroxidation

Table 1 shows the antilipid peroxidation activity of aqueous extracts of *Madhumi* powder by suppressing TBARS formation in rat liver homogenate. The extract shows concentration dependent inhibition (Fig 2). The IC_{50} value of aqueous extracts of *Madhumi* powder was found to be 376.43 ± 0.71 (Table 2).

Table 1. Extraction yield, total phenols and flavonoid contents of aqueous extract of *Madhumi* powder.

Extraction yield (%)	Total phenols (mg GAE/g)	Total flavonoids (mg RE/g)
14.52%	11.83 ± 0.037*	20.02 ± 0.013*

*Each Value represents Mean ± SEM (n=3), GAE - Gallic acid equivalents; RE- Rutin equivalents

Table 2. IC_{50} values of aqueous and methanolic extract of *Indigofera cordifolia* with their standard drug

Sr. No.	Type of assay	IC_{50} value (µg/ml)
1	DPPH radical scavenging	32.02 ± 0.42
2	Super Oxide (SO) scavenging	52.60 ± 0.94
3	Hydroxyl radical (OH) scavenging	33.77 ± 0.468
4	Nitric oxide radical (NO) scavenging	64.45 ± 0.30
5	ABTS radical scavenging	157.84 ± 0.24
6	Lipid peroxidation (TBARS)	376.43 ± 0.71

Fig 1. Reducing power of aqueous extract of *Madhumi* powder. Each values represents mean ±SD(n=3)

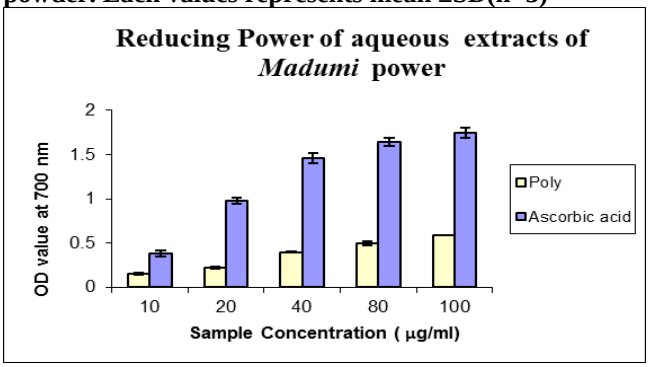
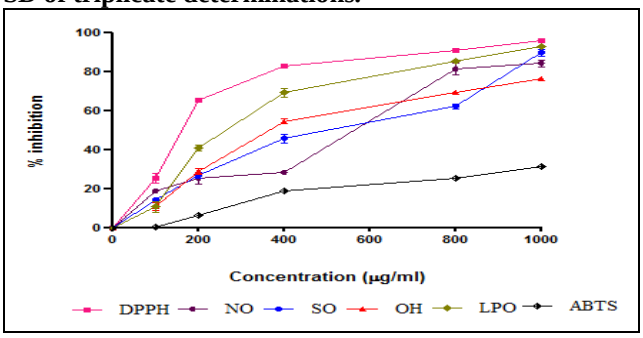


Fig 2. Effect of aqueous extract of *Madhumi* powder on different *in vitro* free radical models. Values are Mean ± SD of triplicate determinations.



DISCUSSION

Secondary metabolites like Phenolic compounds are largely distributed in plant kingdom and their ability to act as both efficient radical scavengers and metal chelator has gain much attention as potential natural antioxidant. Reports reveals that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers (Gupta. *et al*. 2013).

Flavonoids are an important, most diverse, pervasive group of natural compounds and are of mainly essential natural phenolics. The medicinal plants rich in flavonoids are continuously evaluated for their antioxidant potential. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging activity. Antioxidant activity of phenolic compounds is based on their ability to donate hydrogen atoms to free radical (Malairaj *et al*., 2012). Reductones possesses the reducing properties and exert antioxidant activity by breaking the free radical chain by donating a hydrogen atom. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al*., 1995).

Small antioxidant molecules are tested by the DPPH test which is a most suitable method because the reaction can be observed visually using common TLC and dot-blot techniques, and also its intensity can be analyzed by simple spectrophotometric or chromametric assays. The DPPH radical is scavenged by antioxidants through the donation of hydrogen to form the stable reduced DPPH molecule. The antioxidant radicals formed are stabilized through the formation of nonradical products. (Argolo *et al*., 2004). The ABTS radical cation decolourisation test is another method widely used to assess antioxidant activity. Reduction in colour indicates reduction of ABTS radical (Zhu *et al*., 2011).

The hydroxyl radical is an enormously reactive free radical created in biological systems and has been concerned as a extremely harmful species in free radical pathology, able to damage nearly each molecule found in living cells. This radical has the power to bond nucleotides in DNA and cause strand rupture, which leads to cause cytotoxicity carcinogenesis, mutagenesis (Manian *et al*., 2008). Nitric oxide engaged in the regulation of different physiological process. No is a free radical generated by endothelial cells, macrophages, neurons, etc and their overproduction leads to cause several diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and proxy nitrite anions, which act as free radicals. (Baskar *et al*., 2008).

Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. Superoxide anion is an initial free radical formed from mitochondrial electron transport systems. Mitochondria generate energy using 4-electron chain reactions, reducing oxygen to water. Some of the electrons escaping from the chain reaction of mitochondria directly react with oxygen and form superoxide anion. It plays an important role in the formation of other reactive oxygen species, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems (Siddhuraju and Becker *et al*., 2007). Thiobarbituricacid reactive species (TBARS), the byproducts of lipid peroxidation that occur in non-polar region of the biological membranes , involve in the free radical induced cellular damage that lead to many human diseases.

CONCLUSION

The aqueous extract of *Madhumi* powder showed considerable amount of polyphenols like phenolic and flavanoid content which is excellent antioxidants help in controlling the free radical production. The strong reducing power of extract also contributed in its free radical scavenging property. The aqueous extract of *Madhumi* powder has shown potential antioxidant properties which were assessed in different antioxidant assays like DPPH, NO, OH, SO, ABTS and TBARS assays. The results of the present study would surely help to establish the potency of the *Madhumi* powder extracts as potential source of natural antioxidants.

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