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FREE REDICALS SCAVENGING ACTIVITY OF POLYHERBAL FORMULATION (*DIASWETA*)

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ABSTRACT

Many oxidative stress related diseases are as a result of accumulation of free radicals in the body. A lot of researches are going on worldwide directed towards finding natural antioxidants of plants origins. *Diasweta* a polyherbal formulation have been used as a healthy food and traditional drug in India. The present study investigated the phenolic compounds and antioxidant activity of ethanolic extract from Polyherbal formulation *Diasweta*. The antioxidant activity was measured by Reducing power, Hydrogen peroxide and Nitric oxide inhibition activities. Total phenol content was determined as Gallic acid equivalents by the Folin-Phenol method. *Diasweta* exhibited its scavenging effect in concentration dependent manner on nitric oxide radical, hydrogen peroxide, and reducing power. The total phenolic content was higher in the ethanol fraction (57.47 ± 1.74 g GAE). The ethanol extract (OD_{700} = 1.96) also exhibited the strongest reducing power. Our findings provide evidence that the crude ethanolic extract of *Diasweta* is a potential source of natural antioxidants.

Key words: Reducing power assay, Hydrogen peroxide assay, Antioxidant activity, Free radicals scavenging.

INTRODUCTION

Reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion (O_2-) and hydroxyl (OH) radical and hydrogen peroxide (H_2O_2) are often generated as by products of biological reactions or from exogenous factors. These reactive species exert oxidative damaging effects by reacting with nearly every molecule's found in living cells. Free radicals have been implicated in the causation of several diseases such as liver cirrhosis, atherosclerosis, cancer, diabetes etc. and compounds that can scavenge free radicals have great potential in ameliorating these disease processes (Wilson RL, 1998). Antioxidants thus play an important role to protect the human body against damage by reactive oxygen species (Lollinger J, 1981). Radicals and other reactive oxygen species are formed constantly in the human body and are removed by the enzymic and non-enzymic antioxidant defense systems (Sundaram R and Mitra SK, 2007). There is an increasing interest in natural antioxidants e.g. polyphenols, present in medicinal and dietary plants, which might help prevent oxidative damage (Rajeshwar Y *et al*., 2005). In modern period, hub on medicinal plant research has augmented all over the world. Man is turning to natural products; predominantly those derived from plants for his as well as animal's health care due to the growing recognition that the natural products are non-toxic, lesser side effects, and are accessible at affordable prices (Chandrasekaran CV *et al*., 2010). Ayurveda extensively uses the plant derived compound formulation for the treatment of various ailments. Plants are complex mixtures of compounds and no single compound can provide the desired activity. Some compounds potentiate a desired therapeutic action, while others reinforce the same and yet others interact to neutralize and counteract any possible side effect that may exist. Therefore, several plants with the common desired activities are selected so that the final formulation will have a concentrated desired activity (Jagetia GC *et al*., 2004).

In the traditional system of Indian medicine, plant formulation and combined extracts of plants are used as drug of choice rather than individual and these herbal formulations are used for the treatment of a wide variety of diseases (Ansarullah Jadeja RN *et al*., 2009).

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Diasweta, a polyherbal formulation, is a mixture of several herbal extracts including garlic (*Allium sativum*), onion (*Allium cepa*), ginger (*Zingiber Officinale*), bitter melon (*Momordica charantia*). In this study an attempt is made to evaluate the antioxidant activity of *Diasweta*, those ingredients is listed in indigenous medicine as having high therapeutic value & is even now used in remedies for various diseases.

MATERIAL AND METHODS

Collection of Plant material

All the plants used in formulation were collected from local market of Bhopal. They were identified and authenticated by Dr. Zia ul Hassan and a voucher specimen was deposited at the Herbarium of the Department of Botany, Saifia College of science, Bhopal (Voucher no. 135/Bot/Saifia/2010).

Preparation of Plant extract

The different plant parts were air dried, reduced to coarse powder, macerated with ethanol for 72hr, filtered and the filtrate was evaporated under reduce pressure to obtain dry extract (ethanolic extractive value 4.8% w/w). The extract was stored in cool and dry place and was used for pharmacological evaluation.

Chemicals & Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Ascorbic acid, Gallic acid, Folin-Ciocalteau reagent, potassium ferricyanide, $FeCl_3$, sodium bicarbonate, trichloroacetic acid (TCA), sodium carbonate, hydrogen peroxide, sodium nitroprusside, sulphanilamide, H_3PO_4, napthylethylenediamine dihydrochloride, tribromoacetic acid (TBA). All chemicals used including solvents were of analytical grade.

Reducing Power Assay

The reducing power of the *Diasweta* was determined according to the method of Oyaizu (1986) (Gupta M *et al*., 2007). Different concentrations of the *Diasweta* (10–100 μg/ml) in 1.0 ml of deionized water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium Ferro cyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion of tri chloro acetic acid (2.5 ml, 10%) was added to the mixture, which was 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%) and the absorbance was measured at 700 nm and compared with standards. Increased absorbance of the reaction mixture indicated increased reducing power.

Scavenging Of Hydrogen Peroxide

The ability of the *Diasweta* to scavenge hydrogen peroxide was determined according to the method of Ruche, Cheng and Klaunig (Ruch RJ *et al*., 1989). A solution of hydrogen peroxide (2 mmol/l) (Fine Chem. Industries, Mumbai) was prepared in phosphate buffer (pH 7.4). *Diasweta* (10–100 µg /ml) were added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for background subtraction. The percentage scavenging activity of hydrogen peroxide by *Diasweta* was calculated using the following formula,

% scavenging activity [H_2O_2] = [Abs (control) – Abs (standard) / Abs (control)] × 100.

Where, Abs (control): Absorbance of the control and

Abs (standard): Absorbance of the extract/standard.

Nitric Oxide Radical Scavenging Activity

Nitric oxide was generated from nitroprusside and measured by the Greiss reaction. Sodium Nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, (Green LC *et al*., 1982) which interacts with oxygen to produce nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (Ruch RJ *et al*., 1989). Sodium nitroprusside (5 mM) in phosphate–buffered saline (PBS) was mixed with 3.0 ml of different concentrations (10-100 µg /ml) of the drugs dissolved in the suitable solvent systems and incubated at 25^{oC} for 150 min. The samples from the above were reacted with Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm and referred to the absorbance at standard solutions of potassium nitrite, treated in the same way with Greiss reagent.

NO scavenged (%) = [Abs (control) – Abs (standard) / Abs (control)] × 100.

Where, Abs (control): Absorbance of the control reaction and

Abs (standard): Absorbance of the extract/standard.

Total Phenolic Content

The total soluble phenolic in the *Diasweta* was determined with using Folin's phenol reagent according to the method of Singleton (Singleton VL and Rossi JA, 1965; Kim D *et al*., 2003). 1 ml of extract solution containing 1g extract in a volumetric flask was diluted with 46 ml of distilled water. 1 ml of Folin's phenol reagent (SRL Pvt. Ltd, Mumbai.) was added and the

content of the flask mixed thoroughly. 3 min later 3 ml of 2% sodium carbonate was added and the mixture was allowed to stand for 2h with intermittent shaking. The absorbance of the blue color that developed was read at 760 nm. Gallic acid (Lobe Chemie Pvt. Ltd., Mumbai) was used as a standard. The concentration of total phenols in the *Diasweta* was expressed as µg/g of dry extract. The concentration of total phenolic compounds in the gallic acid was determined as µg of gallic acid equivalent using an equation obtained from the standard gallic acid graph.

$$y = 0.0082 x + 0.0546.$$

Ferric Thiocyanate Method (FTC)

FTC method was used to determine the amount of peroxide at initial state of lipid per oxidation (Hakimoglu Fidan *et al*., 2007). The peroxides react with ferrous chloride ($FeCl_3$) dye. In this method, the concentration of peroxides decreases as the antioxidants activity increases. A mixture of 4mg sample was placed in 4ml of absolute ethanol; 4.1mg of 2.52% linoleic acid in absolute ethanol, 8ml of 0.05 M phosphate buffer (pH 7.0) and 3.9ml of water was placed in a vial with a screw cap and then placed in an oven at 40°C in the dark. To 0.1 ml of this solution, 9.7 ml of 75% ethanol & 0.1ml 30% HCl to the reaction mixture, the absorbance was measured at 500nm every 24 h until the absorbance of the control reached maximum. The control & standard were subjected to the same procedures as the sample, except that for control, only the solvent was added and for the standard, 4mg sample was replaced with 4mg of vitamin E.

Statistical analysis

All the analysis were carried out in triplicate and the results were expressed as the mean ±SD. Correlation co-efficient (R) to determine the relationship between two or more variables among Radical Scavenging activity tests and content of total phenolic compounds were calculated using MS Excel software (CORREL statistical function). Regression analysis was used to calculate IC50, defined as the concentration of inhibitor necessary for 50% inhibition of the enzyme reaction.

RESULTS & DISCUSSION

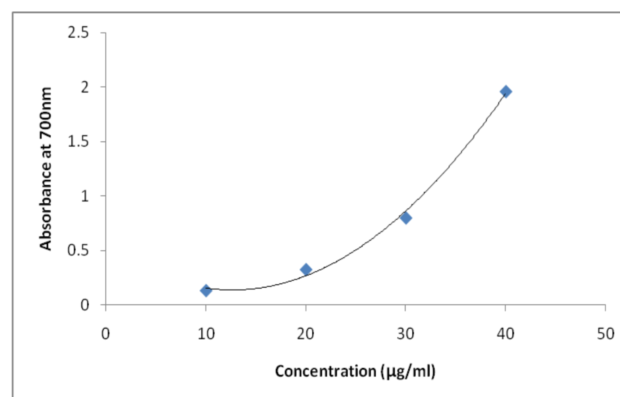
Compelling evidence indicates that increased consumption of dietary antioxidants or fruits and vegetables with antioxidant properties may contribute to the improvement in quality of life by delaying onset and reducing the risk of degenerative diseases associated with it.

Reducing power activity

For the measurements of the reducing ability, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of *Diasweta*. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Yıldırım A *et al*., 2000). Figure 1 depicts the reductive effect *Diasweta*. Similar to the antioxidant activity, the reducing power of *Diasweta* increased with increasing dosage. The result shows that *Diasweta* consist of hydrophilic polyphenol compounds that cause the greater reducing power.

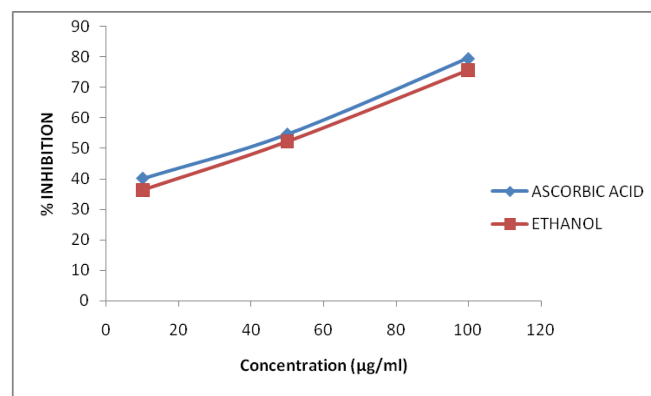
Figure 1. Reducing power of *Diasweta* ethanol extract at different concentrations.

Each value represents means ± SD (n=3).
High absorbance at 700 nm indicates high reducing power.



Hydrogen peroxide scavenging activity assay

Figure 2. H_2O_2 scavenging activity of *Diasweta* ethanol extract at different concentrations.
Each value represents means ± SD (n=3).



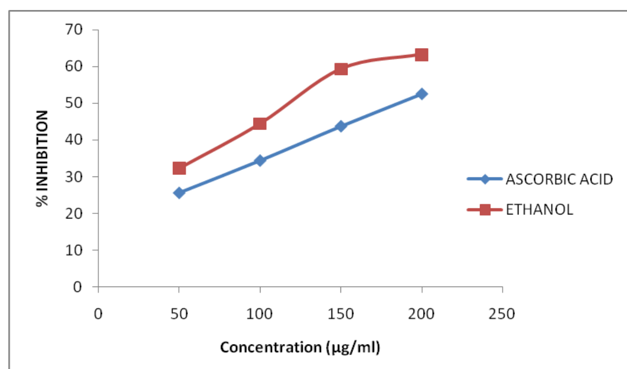
As shown in Figure 2, *Diasweta* also demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner with an IC50 of

1.642 mg mL^{-1}. Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects (Halliwell B and Gutteridge JMC, 1989). It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The decomposition of H_2O_2 by *Diasweta* may at least partly result from its antioxidant and free radical scavenging activity.

Nitric oxide scavenging activity

Nitric oxide (NO) is a potent pleiotropic mediator of physiological process such as smooth muscle relaxant, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities (Miller MJ *et al*., 1993). Although nitric oxide and superoxide radicals are involved in host defense, over production of these two radicals contributes to the pathogenesis of some inflammatory diseases (Guo X *et al*., 1999). Moreover in the pathological conditions, nitric oxide reacts with superoxide anion and form potentially cytotoxic molecules, peroxynitrite. Nitric oxide inhibitors have been shown to have beneficial effects on some aspect of inflammation and tissue damage seen in inflammatory diseases. *Diasweta* significantly inhibited nitric oxide in a dose dependent manner (Fig 3) with the IC 50 being 0.428 mg mL^{-1}. The result indicated that the extract might contain compounds able to inhibit nitric oxide and offers scientific evidence for the use of the rhizomes in the indigenous
system in inflammatory condition.

Figure 3. Nitric oxide scavenging activity of *Diasweta* ethanol extract at different
concentrations. Each value represents means ± SD (n=3).

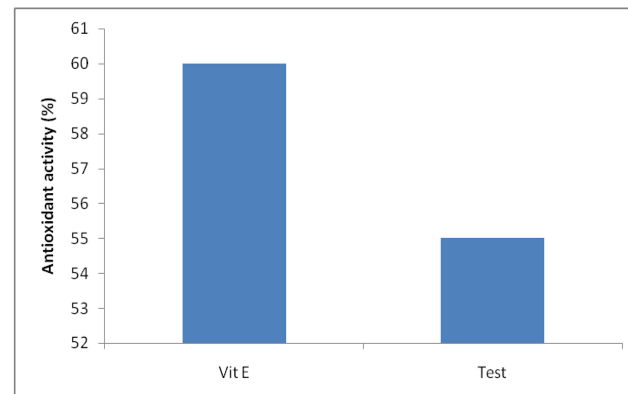


Amount of total phenolic compounds

Phenolics are the most wide spread secondary metabolite in plant kingdom (Kiritikar KR *et al*., 1988). These diverse groups of compounds have been received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers (Rice-Evans CA *et al*., 1995). Therefore, in the present study, total phenolic content present in extract was estimated using modified Folin-phenol method. Values are expressed as (57.47 ± 1.74 g GAE) of fresh *Diasweta* extract.

Ferric thiocyanate method

Figure 4. The total antioxidant activity of ethanol extract of *Diasweta* and Vitamin E by using FTC method. Values are mean ± S.D.(n=3)



The total antioxidant activity of ethanol extract of *Diasweta* was determined by using FTC method and compared with Vitamin E. Fig 4. Shows the total antioxidant activity of ethanol of *Diasweta* by FTC method. Vitamin E had the highest activity (60%) followed by ethanol extract of *Diasweta* (55%) by using FTC method. No significant difference was found between the total antioxidant activity of ethanol extract of *Diasweta* compared with Vitamin E.

CONCLUSION

Diasweta showed strong antioxidant activity by inhibiting Hydrogen peroxide and nitric oxide radicals, and reducing power activities when compared with standard L-ascorbic acid. In addition, the *Diasweta* was found to contain a noticeable amount of total phenols, which play major role in controlling oxidation. The results of this study show that the *Diasweta* can be used as easily accessible source of natural antioxidant. Therefore, it is suggested that in-vivo antioxidant activity should be performed.

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