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Research Article

ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF DIOSGENIN DETERMINED BY USING DIFFERENT IN VITRO METHODS

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ABSTRACT

Objective: Targeting antioxidants for treatment of oxidative stress mediated disease is a potential new therapeutic strategy. Antioxidants from phytochemicals ameliorating oxidative stress-mediated diseases possess their future promise for treatment. Present study was to assess antioxidant and free radical scavenging activities ofDiosgenin by using various in-vitro scavenging assays. **Methods:** 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ABTS, hydroxyl, superoxide, hydrogen peroxide, nitric oxide radicals and reducing power scavenging assays. **Results:** The results of the study were showed that Diosgenin exhibited substantial inhibition of free radical which was calculated as IC_{50} value, compared with standard ascorbic acid. IC_{50} values of Diosgenin and ascorbic acid were found to be 46.14µg/mL and 39.27µg/mL in DPPH radical, 35.17µg/mL and 39.14µg/mL in $ABTS^{\bullet+}$, 34.21µg/mL and 38,24µg/mL in hydroxyl radical, 29.17µg/mL and 34.19µg/mL in superoxide anion radical, 32.12µg/mL and 36.17µg/mL in hydrogen peroxide radical, 35.19µg/mL and 39.14µg/mL in nitric oxide radical, and the reducing power of Diosgenin is 41.15µg/mL we compared to ascorbic acid 43.51 µg/mL.**Conclusion:** We conclude that above results showed Diosgenin has more potent antioxidant and free radical scavenging properties.

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INTRODUCTION

Free radicals are chemical species that possess unpaired electrons, which are highly reactive, also referred to as reactive oxygen species (ROS). The ROS includes hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and superoxide anions (O_2^-) which are essential and natural byproducts of our body's metabolism [1]. They are dangerous, however, when present in excess, results in oxidative stress that cause the structure and functions of biological molecules such as DNA, proteins, lipids, enzymes and RNA [2]. Human body has certain defence mechanisms to combat and reduce the oxidative damage. The beneficial effects of antioxidants on promoting health is believed to be achieved through several possible mechanisms, such as direct reaction with and quenching free radicals, chelation of transition metals, reduction of peroxides, and stimulation of the antioxidative enzyme defense system [3]. The antioxidant activity of this saponin is mainly due to their redox properties, which allow them to act as reducing agents or hydrogen-atom donors. There are frequent articles in newspapers or scientific literature citing the usefulness of the free-radical scavenging properties of antioxidants and their general benefits to human health [4].

Several methods have been developed to measure the free radical scavenging capacity and antioxidant activities in vitro studies. Natural and synthetic entities have major role in chemopreventive studies [5]. The scavenging activities of Diosgenin on superoxide anions, $DPPH^+$, $ABTS^+$ (2,2'-azinobis[3-ethylbenzothiazoline-6-sulphonate), H_2O_2, NO^- and reducing power were determined. Medicinal plants contains a wide variety of free radical scavenging molecules, such as saponin, phenolic, flavonoids, anthocyanins, carotenoids, dietary glutathione, vitamins and endogenous metabolites are rich in antioxidant activities [6]. These plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, peroxide decomposer, enzyme inhibitors and synergists [7]. There is no clear report about Diosgenin in cancer and the present study is mainly focused to evaluate the

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free radical scavenging properties of Diosgenin on DPPH, $ABTS^{\bullet+}$, hydroxyl, superoxide, hydrogen peroxide, nitric oxide radicals and reducing power assays. Thus in the present study, therefore investigated the invitro antioxidant and free radical scavenging potential of Diosgenin.

MATERIALS AND METHODS

Chemicals: Thiobarbituric acid (TBA), Phenozinemethosulphate (PMS), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS●+), nitro blue tetrazolium (NBT), 5,5-dithiobis 2-nitrobenzoic acid (DTNB), potassium ferricyanide, ferric chloride, nicodinamide adenine dinucleotide (NADH), glacial acetic acid, hydrogen peroxide (H_2O_2), ethanol, phosphate buffered saline (PBS), and ascorbic acid were purchased from Sigma-Aldrich, and HiMedia Pvt, India. Diosgenin was also purchased from Sigma-Aldrich, Pvt, India.

DPPH radical scavenging assay: The effects of Diosgenin and positive control (ascorbic acid) on 1, 1-diphenyl-2-picrylhydrazyl radicals (DPPH) were estimated according to the method of Lee et al[8]. The sample solution was prepared in absolute ethanol and the resulting concentration was 10 to 50µg/mL. Different concentrations of (10-50µg/ml) Diosgenin were added to 2mL of methanolic DPPH solution. The mixture was shaken vigorously with a Vortex Mixer and the absorbance was measured by Spectrophotometrically at 517nm immediately and recorded at 5min intervals until the absorbance reached a stable state. The mixture without the addition of sample served as the control. All the tests were performed in triplicate and the (%) of inhibition was calculated according to the equation compared with standard ascorbic acid.

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

$ABTS^{\bullet+}$ radical scavenging assay: This method determine the ability of Diosgenin to scavenge the 2, 2 -azino-bis-3-ethylbenzothiazoline-6-sulphonic acid radical cation ($ABTS^{\bullet+}$) by the method of Arnao et al. [9] The antioxidant activity was measured by reaction mixture (0.5mL of 15µM H_2O_2, 0.5mL of 7mM ABTS and 50mM sodium phosphate buffer, pH 7.5) and different concentrations of Diosgenin (10-50µg/mL). Set blank contained without Diosgenin replaced water. The absorbance was read in spectrophotometer at 734nm and compared with standard ascorbic acid. We calculated IC_{50} value is the expected concentration of test sample to inhibit 50% of $ABTS^{\bullet+}$ production followed formula.
% $ABTS^{\bullet+}$ scavenging = [(Abs control – Abs sample) / Abs control] ×100

Hydroxyl radical scavenging activity: The % of hydroxyl radicals scavenging ability of Diosgenin is measured by the method of Kunchandy and Rao[10]. The 1.0mL of reaction mixture containing 100µL of 2-deoxyribose (28mM in 20mM KH2PO4-KOH buffer, pH 7.4), different concentration of the test sample (10-50µg/mL), 200µL EDTA (1.04mM) and 200µM FeCl3 (1:1 v/v), 100µL of H2O2 (1.0mM) and 100µL ascorbic acid (1.0mM) which is incubated at 37°C for 1 hr. 1.0mL of thiobarbituric acid (1%) and 1.0mL of trichloroacetic acid (2.8%) are added and incubated at 100°C for 20min. After cooling, absorbance is measured at 532nm, set blank reaction mixture water place in test sample.
% of hydroxyl radical scavenging = [(Abs control – Abs sample) / Abs control] ×100

Superoxide radical scavenging assay: The superoxide anion scavenging activity was measurement by method of Fontana et al.[11] Superoxide radical is generated in phenazinemethosulfate-nicotinamide adenine dinucleotide (PMS-NADH) systems by oxidation of NADH. It measured by the reduction of nitrobluetetrazolium (NBT) to a purple formazan. The 1mL reaction mixture contained phosphate buffer (20mM, pH 7.4), NADH (73µM, NBT (50µM), PMS (15µM) and various concentrations of test sample solution (10 to 50µg/mL). After incubation for 5min at ambient temperature, the absorbance at 562nm was measured against an appropriate blank to measure the formazan generated. The experiment was repeated thrice. The results were compared with standard ascorbic acid.

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

Hydrogen peroxide scavenging assay: Hydrogen peroxide scavenging potential of the Diosgenin was determined using the method of Jayaprakasha et al.[12] A solution of hydrogen peroxide (20mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Diosgenin and standard ascorbic acid at different concentrations (10 to 50µg/mL) in ethanol (1mL) was added to 2mL of H_2O_2 solution in PBS. After 10min the absorbance was measured at 230nm against a blank solution that contained hydrogen peroxide solution without any substance. The percentage of H_2O_2 scavenging of the Diosgenin (IC_{50}) values was calculated by following formula.
% Hydrogen peroxide scavenging = [(Abs control – Abs sample) / Abs control] ×100

Nitric oxide radical scavenging assay: The determine nitric oxide radical scavenging activity of Diosgeninto screening various method of Garrat, [13]. 2mL of sodium nitroprusside (10mM) prepared in phosphate buffer saline (pH 7.4) was mixed with 0.5mL of Diosgenin at various concentrations at a ranging from 10 to 50 µg/mL and ascorbic acid at various concentrations depend manner from 10 to 50µg/mL. The mixtures were incubated at 25°C for 150 min. After incubation withdrawn 0.5mL solution and mixed with 0.5mL of Griess reagent [1.0mL sulfanilic acid reagent (0.33% prepared in 20% glacial acetic acid at room temperature for 5min with 1mL of naphthylenediaminedihydrochloride (0.1% w/v)]. The mixtures were incubated at room temperature for 30min, followed by the measurement of absorbance at 540nm using spectrophotometer. The results were calculated IC_{50} values following the formula.
% Nitric oxide radical scavenging = [(Abs control – Abs sample) / Abs control] ×100

Reducing power assay: The measured Diosgenin reducing power was determined by the method of Yen and Duh [14]. Sample dissolved in ethanol at a different concentration (10-50µg/mL) were 2.5mL of phosphate buffer (200mM, pH 6.6) and 2.5mL of 1 % potassium ferricyanide. The mixtures were incubated at 50°C for 20min. After incubation was added 2.5mL of 10% trichloroacetic acid (TBA) these mixture followed by centrifugation at 3000g for 10min.

The collect upper layer of the supernatant (5mL) was mixed with 5mL of distilled water (1:1) and added 1mL of 0.1% ferric chloride and the resolved absorbance of the mixed solution were measured at 700nm. Ascorbic acid was used as the standard.

RESULTS

The present study demonstrates the free radical scavenging activity of Diosgenin was evaluated by different scavenging assays compared with standard ascorbic acid. Diosgenin as well as standard ascorbic acid IC_{50} values were calculated and summarized in graphically presented in figure 1-7. The free radical scavenging consequence activities were increased with the increasing concentrations of Diosgenin.

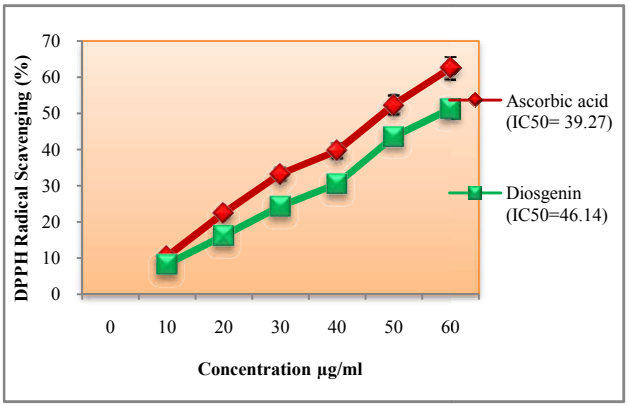

Figure 1 DPPH radical scavenging effect of Diosgenin compared to that of standard Ascorbic acid

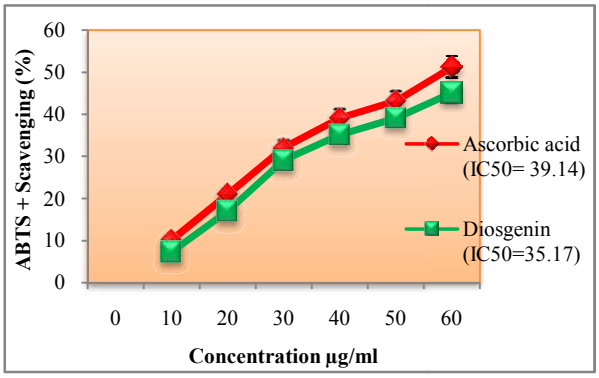

Figure 2 ABTS+radical scavenging effect of Diosgenin compared to that of standard Ascorbic acid

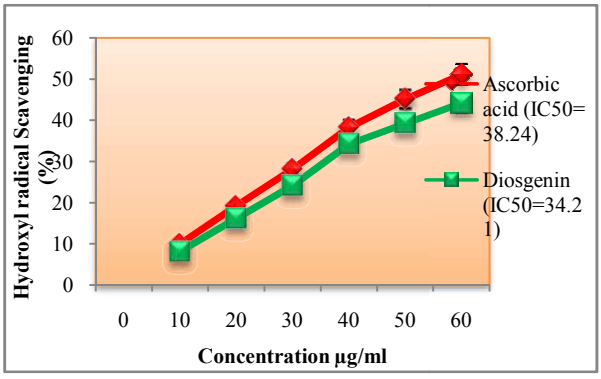

Figure 3 Radical scavenging effect of Diosgenin compared to that of standard Ascorbic acid

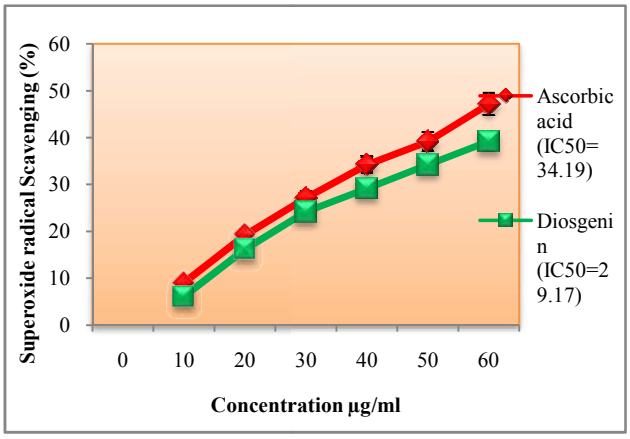

Figure 4 Superoxide radical scavenging effect of Diosgenin compared to that of standard ascorbic acid

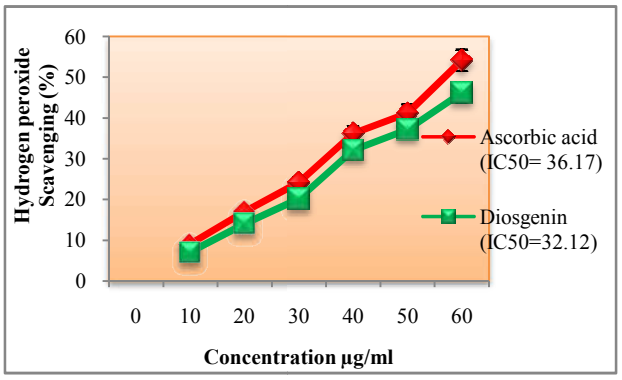

Figure 5 Hydrogen peroxide radical scavenging effect of Diosgenin compared to that of standard Ascorbic acid

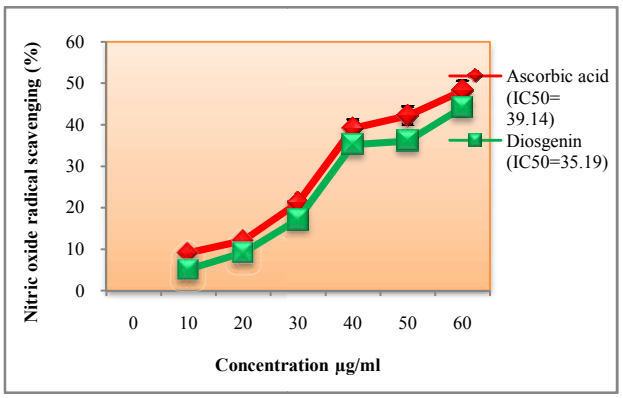

Figure 6 Nitric oxide radical scavenging effect of Diosgenin compared to that of standard ascorbic acid

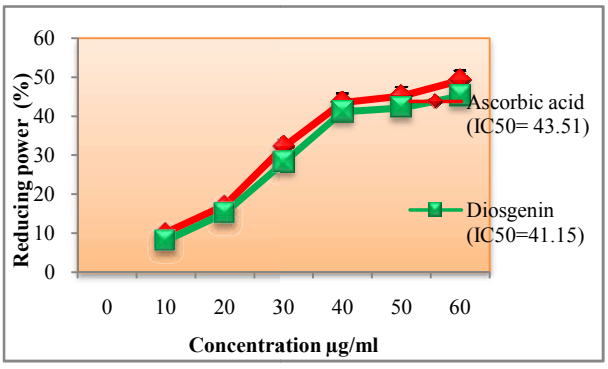

Figure 7 Reducing power inhibition effect of Diosgenin compared to that of standard Ascorbic acid

DPPH radical scavenging assay

Figure 1 shows the % of DPPH radical scavenging inhibition of Diosgenin. The IC_{50} values of Diosgenin (46.14µg/mL) were nearby standard ascorbic acid IC_{50} value (39.27µg/mL). DPPH is unstable nitrogen centered free radical, react with suitable reducing substrate of Diosgenin that can donate a hydrogen atom become paired off forming the consequent hydrazine with the loss of violet color. In the present study support, Diosgenin has good antioxidant and scavenges DPPH radicals.

$ABTS^{\bullet+}$ radical scavenging assay

$ABTS^{\bullet+}$ radical scavenging measured based on the reduction of blue/green $ABTS^{\bullet+}$chromophore generated from the reaction between $ABTS^{\bullet+}$ and potassium persulphate by an electron donating antioxidant. The $ABTS^{\bullet+}$ radical scavenging ability of Diosgenin showed as IC_{50} values were presented in Figure 2. Diosgenin significantly inhibit at dose dependent manner inhibition of $ABTS^{\bullet+}$ radical activity with IC_{50} values of 35.17µg/mL. The IC_{50} value of Diosgenin was compared with the standard antioxidant ascorbic acid (39.14µg/mL).

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of Diosgenin was shows in fig. 3. Hydroxyl radical is singlet electron that scavenging by test sample of Diosgenin donating free electron to form stable molecules. Our result shows that Diosgenin potent scavenging hydroxyl radical on IC_{50} value (32.12µg/mL) to compare with ascorbic acid IC_{50} value (36.17µg/mL). The result obtained emphasized the strong antioxidant capacity Diosgenin.

Super oxide free radical scavenging activity

Figure 4: Shows the % of super oxide radical scavenging of Diosgenin. Super oxide anions are generated in the formation PMS-NADH system by the oxidation of NADH and determine by the reduction of NBT in resulting the formation of blue coloredformazan that can be measured at 560nm. From results, super oxide radicals scavenged by Diosgenin in a dose depend manner, IC_{50} value of Diosgenin (29.17µg/mL) showed potent free radical scavenging activity compared to the standard ascorbic acid IC_{50} value (34.19µg/mL).

Nitric oxide scavenging activity

The % of Nitric oxide scavenging ability was performed with Diosgenin compared with standard ascorbic acid were shown in figure 5. From the results, Diosgenin exhibited most effective dose dependent inhibition of nitric oxide radicals with the IC_{50} values of 395.19µg/mL. It was significantly similar to the scavenging effect of ascorbic acid IC_{50} value at a concentration of 39.14µg/mL.

Reducing power

The reducing power of Diosgenin was shown in figure 6. In this study, strong antioxidant compounds of Diosgenin donating electron to convert the oxidation form of iron (Fe^{+3}) in ferric chloride to ferrous (Fe^{+2}). From the result reducing power activity of Diosgenin effectively inhibition of oxidation form of free radical at the concentration Diosgenin 41.15µg/mL as compared to standard ascorbic acid 43.51µg/mL.

DISCUSSION

The propagation of free radicals can bring about many adverse reactions leading to extensive tissue damage [15,16]. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals. Antioxidants are compounds that can reduce or inhibits the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions. The antioxidant activity of the compounds is mainly due to their redox properties in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [17]. Among the various natural antioxidants, saponins are very important constituents because of their multiple biological effects and direct contribution to antioxidative activity. According to this hypothesis, the present study designed to examine the antioxidants and free radical scavenging activity of Diosgenin. The results of our study reveal that there is a strong coincidence between antioxidant activity and saponin content.

In the present study, the free radical scavenging and antioxidant potential of Diosgenin was assessed by subjecting the radicals and oxidants namely DPPH, $ABTS^{+}$, H_2O_2, NO^{-} and $OH^{\bullet}$. The Diosgenin exhibited strong radical scavenging activity against $DPPH^{+}$, $ABTS^{+}$, $H_2O_2^{-}$, $OH^{\bullet}$ and Fe^{2+} assay. Many studies have been reported on the use of medicinal plants as radical scavengers [18]. Diosgenin exhibited strong reducing power and free radical scavenging effect on free radical, hydroxyl, superoxide and hydrogen peroxide radicals.

Ascorbic acid is a very good reducing agent and free radical scavenger. It is also reported to prevent the damage to RBC membrane by interacting with superoxide and hydroxyl radical [19]. DPPH is a relatively stable free radical and usually used as a substrate to evaluate the antioxidant assay. This assay determines the ability of Diosgenin to reduce DPPH radical to the corresponding hydrazine by converting the unpaired electrons to paired ones. Antioxidants and other radical scavenger can act by converting the unpaired electrons to paired ones. The maximum concentration (50µM) of Diosgenin exhibited the highest percentage of inhibition which indicates that Diosgenin causes reduction of DPPH radical in a strohometric manner.

ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{+,}$ which has a characteristic long wavelength absorption spectrum [20]. ABTS, a protonated radical has characteristic absorbance maximum at 734nm which decreases with the scavenging of proton radicals [21]. The results obtained imply the activity of the Diosgenin either by inhibiting or scavenging the $ABTS^{+}$ radicals. Superoxide anion is produced from molecular oxygen due to oxidative enzymes [22] of body by non enzymatic reaction such as auto-oxidation by catecholamines[23]. Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals could be generated and it also has the ability to change to other harmful reactive oxygen species and free radicals within the living cells. The probable mechanism of scavenging the superoxide anions may be due to inhibitory effect of the Diosgenin towards generation of superoxides an*in vitro* reaction mixture.

The hydroxyl radical (OH^-) thus produced may attack the sugar of DNA bases causing sugar fragmentation, base loss and DNA strand breakage [24]. This radical has the capacity to induce carcinogenesis, mutagenesis and which rapidly initiates lipid peroxidation [25]. From the present results, it is inferred that the Diosgenin have better hydroxyl radical scavenging activity as reflected in terms of percentage inhibition. Nitric oxide (NO) is a reactive free radical produced by phagocytes and endothelial cells, to yield more reactive species such as peroxy nitrite which can be decomposed to OH radical. Excess concentration of NO is associated with several diseases [26]. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions which acts as free radicals. The level of nitric oxide was significantly reduced in this study by the effect of Diosgenin indicating the free radicals scavenging properties in a concentration dependent manner.

The reducing power of an antioxidant may therefore serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe3+/ferricyanide complex to the ferrous form [27]. Duh [28] also stated that reductones are efficient reducing agents and their efficiency is attributed to their hydrogen-donating ability. The reducing power of Diosgenin and the reference compounds (ascorbic acid) were determined. Increasing absorbance indicates an increase in reductive ability by the Diosgenin. From the above results, it can be concluded that Diosgenin showed the most potent in vitro antioxidant activity with high percentage inhibition. This may be attributed to the presence of acetogenein and tetra hydro furan portion in the molecules which probably play a role as an effective free radical scavenger and have an effective anticancer agent.

CONCLUSION

In conclusion, etiological factors of several clinical disorders could be traced to a deficient natural antioxidant defence in an individual. These disorders can be prevented or delayed by supplementing the body's natural antioxidant defence. The overall finding of the present study concluded that bioactive principle of Diosgenin proved by antioxidant and free radical scavenging activities. These in vitro assays indicate that Diosgenin has a significant source of antioxidant, which might be useful in preventing the progress or various oxidative stresses. Diosgenin shows as a promising natural source of antioxidants suitable for application in nutritional/pharmaceutical fields, and in the prevention of free radical mediated diseases.

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