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In vitro antioxidant activity of *Phyllanthus niruri* leaf extracts

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Abstract
Ayurveda is considered to be the science of life, of longevity and holistic well-being. The use of plant compounds for pharmaceutical purposes has gradually increased in India. About 80% of the developed countries use traditional medicine, which involves compounds derived from medicinal plants. Of all the medicinal plant used in India, one of the important herb in Ayurveda, is *Phyllanthus niruri*, attributed to number of medicinal properties also known as stone breaker. In the present study, an effort was made to screen the free radical scavenging activity of *P. niruri* leaf extract under *in vitro* condition against a battery of free radicals such as DPPH, ABTS, H_2O_2, hydroxyl radicals and by determining its reducing property using three different extracts namely aqueous, methanol and chloroform. DPPH and ABTS scavenging activity revealed that among all the extracts, the aqueous extract exhibited strong activity while methanol and chloroform extract showed moderate scavenging activity. The hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the extract for hydroxyl radicals generated from the Fe3+/Ascorbate / EDTA / H_2O_2 both in the presence and absence of the leaf extracts. The extent of TBARS formation with deoxyribose was effectively reduced in the presence of aqueous extract of the leaves while the other two extracts showed moderate activity. The ability of the extracts to effectively scavenge non-radical oxidant H_2O_2 showed that the aqueous extracts of the leaves showed better scavenging of H_2O_2 than the other two extracts. The metal reducing activity of the leaf extracts of *P. niruri* showed that the aqueous extract exhibited the maximum activity. Among the three extracts, the aqueous extract was most effective scavenger, followed by methanol and chloroform. The extract concentration providing 50% inhibition (IC50) was calculated from the plot of inhibition (%) against extract concentration. Ascorbic acid standard was used for comparison. Thus, the results showed that *P. niruri* leaf extracts exhibited antioxidants and radical scavenging activity *in vitro.*

Keywords: *Phyllanthus niruri,* DPPH, ABTS, H_2O_2, hydroxyl radicals and reducing property

Introduction
Oxygen is essential for survival. It is very stable in the ground state. Molecular oxygen react rapidly with free radicals to become reactive oxygen species (ROS) inevitable to living cells and highly associated with wide range of pathogenesis such as diabetes, cancer, liver damage, inflammation, ageing and neurological disorder (Saha and Tamrakar, 2011) [13]. ROS cause damage to cell structures, DNA, lipids and protein. The ROS are generated continuously by both endogenous and exogenous sources. Almost all the organism possess defence mechanism against free radical induced oxidative stress, which involve preventive mechanisms, repair mechanisms, physical defences and antioxidant defences (Khatoon *et al.*, 2013) [3]. ROS including free radicals such as superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($^1O_2$) and non-free radical species such as hydrogen peroxide (H_2O_2), various form of activated oxygen are often generated by oxidation product of biological reaction or exogenous factor. There is a balance between generation of ROS and antioxidant system in organism. In pathological condition, ROS are overproduced and result in lipid peroxidation and oxidative stress. The imbalance between ROS and antioxidant defence mechanisms leads to oxidative modification in cellular membrane or intracellular molecules (Awah *et al*., 2010) [1].

The oxygen free radicals initiate lipid peroxidation and inflict damage to macromolecular components of cell (Raghavendra *et al.*, 2007) [9]. During oxidative stress and exposure to radiation, excessive free radicals are produced which are known to cause damage to biomolecules (Naik *et al*., 2008) [7]. Due to detection of many bioactive compounds in food with possible antioxidant activity, there has been increased interest in relationship between antioxidants and disease risks (Tarhan *et al.*, 2007) [15]. The antioxidant compounds may function as free radical scavengers, potential complexing of pro-oxidant metals and quenching of singlet oxygen.

Antioxidants may offer resistance against the toxic oxidative reaction inhibiting the lipid peroxidation and by other mechanism and thus prevent diseases (Umamaheswari and Chatterjee, 2008) [16].

The plant kingdom is an abundant source of phytochemicals having important properties. Plants have developed a complex antioxidative defence and many antioxidant compounds, naturally occurring from plant sources have been identified as free radical or active oxygen scavenger (Youwei *et al.*, 2008) [18]. As plant produce significant amount of antioxidants to prevent the oxidative stress they represents a potential source of new compounds with antioxidant activity. Consumption of dietary antioxidants from plant materials has been associated with a lower incidence of diseases due to oxidative stress from free radicals accordingly, dietary antioxidant have recently garnered increased research interest (Wojchikowski *et al*., 2007) [17].

Traditional herbal medicines form an important part of the health care system of India. Ayurveda, supposed to be the oldest medical system in the world provides potential leads to find active and therapeutically useful compounds from plants (Hazra *et al*., 2009) [2]. One of the most challenging pursuits in the realm of pharmaceutical and medical success is the search for newer and more potent drugs with little toxic effects, self-administrable, less expensive and completely reversible. Much of these properties are observed in the drugs of plant origin (Riaz *et al*., 2011) [10].

One such popularly used plant that is reported to have antitumor, anti-carcinogenic, hypolipidaemic, hepatoprotective, antiviral used for the treatment of urolitic disease, as a diuretic is *Phyllanthus niruri*, which is commonly known as stone breaker. Several bioactive molecules such as lignin, phyllanthin, hypophyllanthin, flavonoids, glycosides and tannins are present in *Phyllanthus niruri* (Sabir and Roacha, 2008) [12]. In all the medical preparations, it is the whole plant, fruits and leaves that areused. In order to better understand the antioxidant activity of *Phyllanthus niruri* leaves, three different extracts (aqueous, methanol and chloroform) were prepared and assessed for their radical scavenging effects using various free radicals and oxidants.

Materials and Methods

Phyllanthus niruri (Family: *Euphorbiaceae*) is a perennial herb distributed throughout India. Whole plant, fresh leaves and fruits are used to treat various ailments particularly hepatitis. It is found to possess antitumor, anticarcinogenic, hypolipidaemic, antiviral and hepatoprotective activity. *P.niruri* traditionally is claimed to be useful in the treatment of liver ailments.

Collection of plant sample

The fresh leaves of *P.niruri were* collected, washed in running tap water to remove the surface contaminants and blotted dry between folds of filter paper.

Preparation of methanol and chloroform extracts

1g of the fresh leaves was homogenized in 10 ml of methanol and chloroform using mortar and pestle. The homogenate was centrifuged at lower rpm to clarify the extract. The supernatant corresponding to the concentration of 1mg/µl was used for assay. The supernatant was transferred to a preweighed beaker and evaporated at 60ºC protected from light. The residue was weighed and dissolved in dimethyl sulphoxide (DMSO) at a concentration of 5mg/µl. The extracts were tested for their ability to scavenge the free radicals.

Preparation of aqueous extract

Aqueous extract was prepared fresh when experiments were performed.

Free radical scavenging effects of *Phyllanthus nirruri* leaf extracts

The free radical scavenging effects of *P.niruri* leaf extracts was assessed by analyzing its ability to scavenge DPPH, ABTS, H_2O_2, hydroxyl radicals and by determining its reducing property.

DPPH radical scavenging activity – spectrophotometric method

The ability of the different extracts to scavenge stable free radical DPPH (1,1 diphenyl – 2 – picrylhydrazyl) was assessed. In this method a commercially available and stable free radical DPPH, which is soluble in methanol is used. In its radical form DPPH has absorption at 517nm and can be estimated according to Mensor *et al.* (2001) [6].

ABTS radical scavenging activity

The antioxidant activity of the leaf extract was studied by the ability to scavenge the free radicals ABTS (2,2` azino-bis 3 ethyl benzthiazoline 6 sulphonic acid) and was estimated as per the procedure of Shirwaikar *et al*. (2006) [14].

Hydroxyl radical scavenging activity

The damage to deoxyribose, which is the backbone of DNA, induced *in vitro* by H_2O_2 in the presence and absence of plant extracts was quantified as TBARS and was estimated according to the procedure detailed by Kunchandy and Rao, (1990) [5].

H_2O_2 scavenging activity

The H_2O_2 scavenging ability of different extracts of leaves of *P.niruri* was determined according to the procedure of Ruch *et al*. (1989) [11].

Assay of reducing power

The reducing property was determined according to the modified method outlined by Oyaizu (1986) [8].

Determination of IC_{50} value

The extract concentration providing 50% inhibition (IC50) was calculated from the plot of inhibition (%) against extract concentration. Ascorbic acid standard was used for comparison.

Results and Discussion

DPPH spectrophotometric assay

The stable DPPH radical is widely used to evaluate antioxidant activities. The addition of extracts to the DPPH solution causes a rapid decrease in the optical density at 515nm. The degree of discoloration is indicative of the scavenging capacity of the extracts. The result of the present study is shown in Figure 1. Among the three extracts, the aqueous extract of *P. niruri* leaves exhibited strong DPPH radical scavenging activity compared to methanol and chloroform extract which shows that the leaves of *P.niruri* possess strong radical scavenging activity. The IC_{50} value represents the concentration of the extract that caused 50% inhibition in the initial DPPH concentration. The IC_{50} values

obtained for DPPH scavenging was found to be 0.4mg/20µl which were comparable for the reference standard, ascorbic acid and the results are discussed in Figure 2.

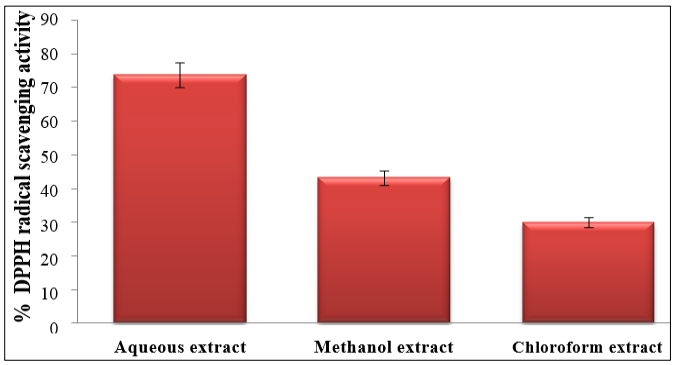

Fig 1: DPPH radical scavenging activity of *Phyllanthu niruri* leaf extracts

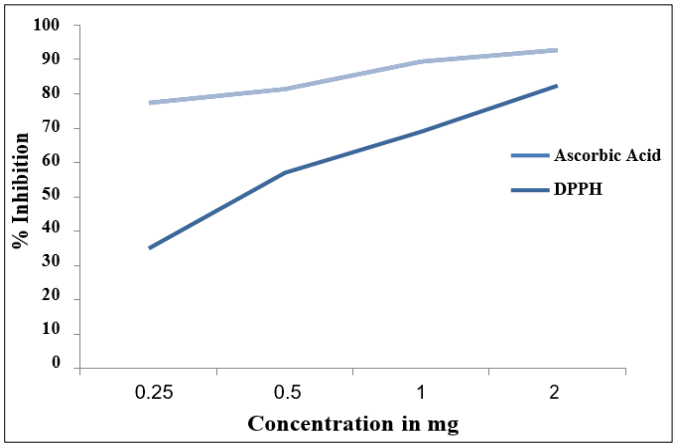

Fig 2: IC50 value of DPPH radical scavenging activity

ABTS radical scavenging activity of *Phyllanthus niruri* leaf Extracts

The radical scavenging ability of the extracts was tested with yet another radical ABTS. This assay is applicable for both lipophilic and hydrophilic antioxidants. The ABTS radical scavenging activity results are depicted in Figure 3. The IC_{50} was determined for ABTS radical scavenging activity and the results are discussed in Figure 4. The ABTS scavenging ability of the different extracts of *P.niruri* leaves were analysed using a spectrophotometric assay. Among the three different extracts, the aqueous extract was found to be a better scavenger of free radical followed by methanol and chloroform. The IC_{50} value was found to be 0.33mg/20µl for ABTS radical scavenging activity. It was compared with the standard ascorbic acid.

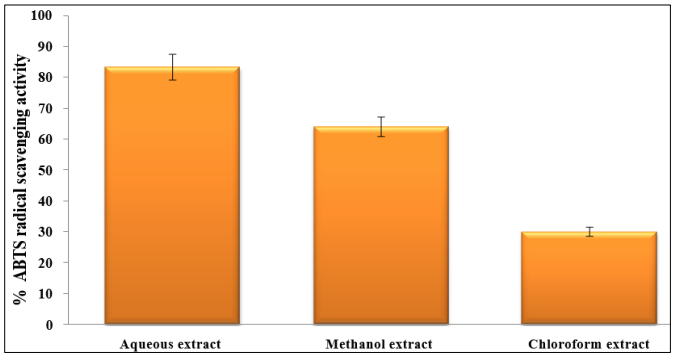

Fig 3: ABTS radical scavenging activity of *Phyllanthus niruri* leaf extracts

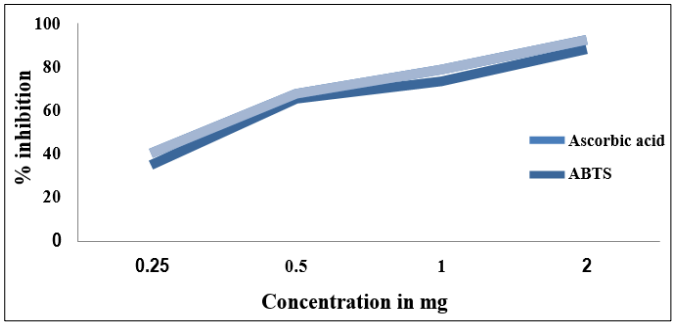

Fig 4: IC50 Value of ABTS radical scavenging activity

Hydroxyl radical scavenging activity of *Phyllanthus niruri* leaf extracts

Hydroxyl radicals are the major active oxygen species causing lipid oxidation and enormous biological damage. The effect of aqueous, methanol and chloroform extracts in scavenging hydroxyl radical is depicted in Figure 5. The exposure to H_2O_2 caused the maximum damage to deoxyribose and the damage was very effectively rectified by the treatment of the leaf extracts of *P.niruri.* The aqueous extract of leaves of *P.niruri* was more effective than the other two extracts exhibiting the ability of *P.niruri* leaves to scavenge hydroxyl radical thereby gaining lot of significance in establishing the strong antioxidant activity of the leaves. The results are supported by Kumar and Kumar (2009) [4].

Hydrogen peroxide scavenging activity of *Phyllanthus niruri* leaf extracts

The measurement of hydrogen peroxide scavenging activity is one of the useful methods for determining the ability of antioxidants to decrease the level of prooxidants such as hydrogen peroxide. The three different extracts of *P.niruri* showed considerable H_2O_2 scavenging activity. The aqueous extract of *P.niruri* showed better scavenging of H_2O_2 than the other two extracts. The values obtained are represented in Figure 6. The observations made show that the leaves of *P.niruri* can effectively scavenge H_2O_2 and possess very effective antioxidant and radical scavenging activity.

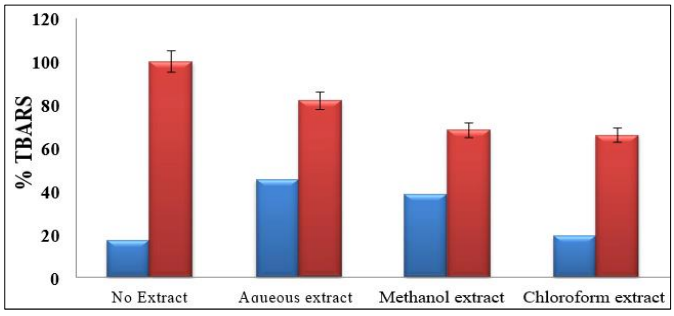

Fig 5: Hydroxyl radical scavenging activity of *Phyllanthus niruri* leaf extracts

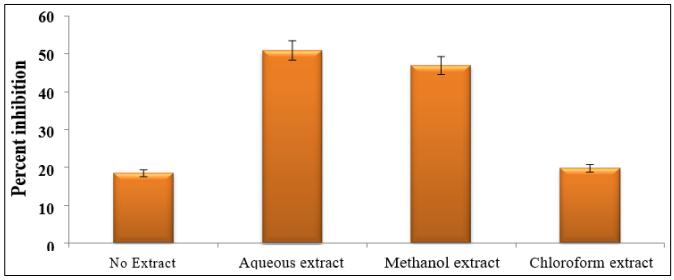

Fig 6: Hydrogen peroxide radical scavenging activity of *Phyllanthus niruri* leaf extracts

Assay of reducing property

The FRAP assay is a direct test of "total antioxidant power". Reducing power reflects the electron donating capacity of bioactive compounds, is associated with antioxidant activity. Reducing power was measured by the direct reduction of ferric cyanide to ferrous cyanide, and was determined by measuring absorbance resulting from the formation of the Perl's Prussian Blue complex followed by the addition of excess ferric ions (Fe^{3+}). In this method, higher absorbance values indicate greater reducing capacity of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions. The FRAP of the leave extracts of *P.niruri* was determined and the results are presented in Figure 7. The result indicated that the aqueous extract was very effective in reducing metal ion followed by methanol and chloroform extract.

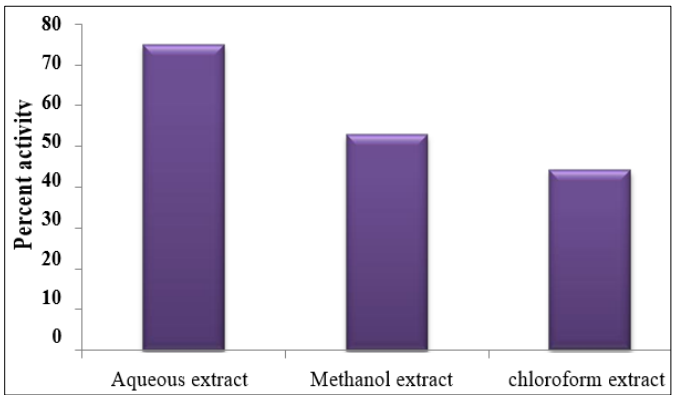

Fig 7: Reducing activities of *Phyllanthus niruri* leaf extracts

Conclusion

All the three extracts of *P.niruri* leaves effectively scavenged or inhibited all the radicals tested. Among the three extracts the aqueous extract was most effective scavenger, followed by methanol and chloroform. Thus, the results showed that *P.niruri* leaf extracts exhibited antioxidants and radical scavenging activity *in vitro.*

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