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Systematic Evaluation of Free Radical Scavenging and Antioxidative Activities In D*igera muricata* (L.) Mart.

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ABSTRACT

The present investigation was undertaken to screen the phytochemical, free radical scavenging and antioxidant activities in a wild edible plant Digera muricata (L.) Mart. (Amarathaceae). This plant is used to cure several aliments in Ayurveda. The preliminary screening and quantitative phytochemicals were estimated and recorded more positive for phenols, flavonoids, glycosides, tannins and terpenoids and minimum for saponins. The secondary metabolites are further estimated to confirm the quantitative estimations. These were further confirmed by quantitative estimations. The whole plant was used to analyze the free radical scavenging and antioxidant activities in different solvent extracts such as hexane, pet. ether, chloroform, methanol, ethanol and aqueous using successive Soxhlet extractor. Further, it was determined for ascorbic acid, reducing property, enzymes POD, SOD, and also the free radical scavenging activity was analyzed by DPPH assay. All the extracts showed promising results towards free radical scavenging and antioxidative activities. The maximum activity was recorded in methanol extract followed by methanol > aqueous > ethanol > acetone. The least activity was recorded in hexane extract. However, in the present investigation Digera muricata has shown more affinity towards both free radical scavenging and antioxidative activities.

Key word: *Antioxidants, DPPH, Free radicals, H_2O_2, Phytochemical, POD, SOD*
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INTRODUCTION

Antioxidants compounds also possess several industrial applications such as preservatives in food, cosmetics & degradation of rubber and gasoline. In food industries free radicals are found to be responsible to lower lipid peroxidation (Nunez-Delicado *et al.,* 1997; Chen and Ho 1997). The synthetic oxidant Butylated Hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT) are widely used in food industries. The toxicological and nutritional research found that the uses of these synthetic antioxidants (BHA, BHT) are toxic and harmful for human health (Sushil Chandra Sati et al., 2010). Due to this fact, considerable interest has been shown to the addition of natural based antioxidants in food and biological systems to scavenge free radicals (Ito *et al.,* 1983). They may provide safe replacement for toxic and harmful synthetic antioxidants (Sushil *et al.,* 2010). The natural antioxidants are beneficial for our health without any side effect and scavenge the free radical immediately after intake through metabolic activities. Recently various studies and research article showed that some secondary metabolites such

phenols, flavonoids and hydrolysable tannins are potent antioxidants (Hagerman *et al.,* 1998).

Many vitamins such as A, C, E, melatonin and glutathione are widely present as a potent antioxidants in plant kingdom. A predominantly plant based diet reduces the risk for development of several chronic diseases. It is often assumed that antioxidants are contributing to protect diseases. The dietary plants contain several hundreds of different antioxidants. It is useful to know the total concentration of electron donating antioxidants in individuals. We have systematically assessed for free radical scavenging and antioxidants activity in a wild edible plant *Digera muricata*.

The *Digera muricata* (L.) Mart. is a wild edible herb used by village people. It is popularly known for herbal remedy for various aliments. It is used as cooling, astringent of bowels and also used as a laxative (Parrota, 2001). The leaves are used for treatment of diabetic (Jagatha and Senthilkumar, 2011). The flower and seeds are used to treat urinary discharges (Parrota, 2001). But the scientific basis for its medicinal use especially for boiled root infusion given to mother after child birth to increase lactation purpose is to be evaluated. Therefore, the present study was undertaken to assess systematically free radical scavenging and potent antioxidant properties in this plant. The whole plant extracts of different solvents like hexane, pet ether, ethyl acetate, chloroform, acetone, methanol, ethanol and water were used to analyze the free radical scavenging and antioxidant activities.

MATERIALS AND METHODS

Chemical reagents

All the required chemical and solvents used were from SD-Fine, Hi-Media Pvt. Ltd. Mumbai and 2,2-diphenyl-1-pricrylhydrazyl (Sigma Aldrich, Pvt. Ltd. Bangalore)

Collection of Plant Materials

The Whole plant was collected from Humnabad Taluka in Bidar district, Karnataka, India. The plant specimen was authentically identified with the help of Flora of Gulbarga district (Seetharama *et al.,* 2000). The voucher specimens were deposited in the herbarium of Gulbarga University, Gulbarga (HGUG).

Preparation of the crude extracts

The collected materials were washed with alcohol (70% v/v) to remove the dust particles and microbes, then, shade dried in a tray direr at 40^0C. The dried plant material was coarsely powdered using mechanical pulverizer. The powder thus obtained was stored in an air tight container for further use. The powered material was separately extracted using Soxhlet extractor in hexane, pet. ether, ethyle acetate, chloroform, acetone, methanol, ethanol and water for 24 h (Wang and Curtis, 2006). The extracts obtained were collected and stored at 40^0 C until further use.

Preparation of crude extract

The collected materials were washed with alcohol (70% v/v) to remove the dust particles and microbes. Then materials were shade dried in a tray dried at 40^0C. The dried plant materials were pulverized to a coarse powder using a mechanical pulverizer. The obtained powder was stored in an air tight container for further use. The powder materials were separately extracted using Soxhlet extractor in absolute ethanol and methanol for 24 h (Wang and Curtis, 2006). The extracts thus obtained were dried and stored at 30^0 C for further use.

Antioxidant Activities

Analysis of Total Phenols

The amount of total phenolic content in *Digera muricata* was determined by Folin-Ciocalteu's method as described by Lister and Wilson (2001).

50µg of each plants extracts, 2.5 mL (1/10 dilution) of Folin Ciocalteu's reagent and after 3 min. 2 ml. of 7.5 % sodium carbonate solution (w/v) were added and mixed thoroughly and incubated at 45^0C for 15 min. The absorbance of all samples was measured at 765 nm using UV-Vis spectrophotometer against blank. The results were expressed as milligrams of gallic acid equivalent per gram of dry weight (GAE g/dw).

Analysis of Total Flavonoids

The total flavonoids were quantitatively determined by Swain and Hills method (1959). Each of the sample (50 µg) of both methanol and ethanol extracts of leaves, stem bark and fruit pulp were homogenate with 10 ml of methanol using pestle and mortar and centrifuged at 5000 rpm for 10 min. The obtained supernatant was collected and evaporated to dryness keeping in a hot water bath thus the residue obtained was re-dissolved in 5 ml of distilled water. 0.1 and 0.2 ml are diluted to 2ml with distilled water and 4 ml of vanillin reagent was added rapidly after 15 min. The absorbance of all samples was measured at 599 nm using UV-Vis spectrophotometer against blank. The results were expressed as milligrams of phloroglucinol equivalent per gram of dry weight.

Total Ascorbic Acid Content

Ascorbic acid content in plant material was estimated as per the method described by Sadashivam and Manikam (1992). 50 µg all plant material was homogenized in 10 ml 4% oxalic acid and centrifuged at 5000 rpm for 15 min. The supernatant were collected and bromine water was added drop wise with constant stirring to give a yellow color. The excess bromine was expelled by blowing in air with a pipette. Final volume 25 ml with 4% oxalic acid, 2 ml of brominated extract was adjusted to 3 ml with distilled water. This was allowed to react with 1 ml of 2% DNPH (2,4-Dinitro Phenol Hydrazine) filtered and used, followed by 1-2 drops of Thiourea (10%). Blank was prepared as above with distilled water in the place of Ascorbic acid or extract and incubated at 37 ^{0}C for three hours. The orange red Osazone crystals were dissolved by adding 7 ml of 80% H_2SO_4. The absorbance was measured at 540 nm using UV-Vis spectrophotometer. The results were expressed as milligrams of ascorbic acid equivalent per gram of dry weight.

Determination of Reducing Property

Reducing properties of each sample were determined by Potassium Ferricyanide method (Pulido *et al.,* 2000). 50µg of each extract made up to 2.5 ml of 200 mM of sodium phosphate buffer (pH 6.6), 1% (w/v) potassium ferricyanide and 2.5ml of Trichloroacetic acid (TCA) added, then heated at 50°C for 20min. Further, it was centrifuged at 5000 rpm for 10min. The supernatant (5 mL) was dissolved in 10 ml of distilled water. Finally 0.1% (w/v) of $FeCl_3$ (Freshly prepared) was added. The absorbance of all the samples was measured at 700 nm using UV-Vis spectrophotometer. Reducing property was expressed in terms of $OD_{700\ nm}$ and higher optical density is indicative of high reducing power.

Free radical Scavenging Assay

DPPH radical scavenging spectrophotometric assay was used in order to determine the inhibition concentration (IC_{50}) which is described as the amount of antioxidant necessary to decrease the initial concentration of the DPPH radical by 50%. Inhibition percentage (IP) descripts as the percentage of total DPPH radical which reacts with the antioxidant at the steady state of all the extract. The experiments were performed using UV-Vis spectrophotometer (Farrukh *et al.,* 2006). An

aliquots of 50µg of each samples extract (1 mg/ml) was mixed separately with 100 mM ethanolic solution of DPPH radical and the final volume was of 3 ml, ethanol served as a control. The decrease in the colour of the solution which is indicative of decrease in the concentration of DPPH radical was monitored by the decrease in the absorbance at 517nm for a period of 30min. during which time the radical stabilizes (Urishi *et al.,* 1994). The percentage of remaining DPPH (% $DPPH_{Rem}$) were calculated by formula

$$DPPH_{REM}\% = (DPPH)_t / (DPPH)_{t0} \text{ X } 100$$

Where $(DPPH)_t$ is the absorbance measured at any time 't' and $(DPPH)_{to}$ is the absorbance measured at time 'zero'. IC_{50} was calculated by plotting % $DPPH_{REM}$ at the steady state against various concentrations of each extract. The Result were expressed in mg antioxidant gm/DPPH (Argolo *et al.,* 2003).

Antioxidant of enzyme activities (SOD and POD)

POD activity was assayed according to the method of Hemeda and Klein (1990). A total of 100 mL of reaction mixture containing 10 mL of 1% guaiacol (v/v), 10 mL of 0.3% H_2O_2 and 80 mL of 50 mM phosphate buffer (pH 6.6). Enzyme extract (75 µL) was added to reaction mixture in a final volume of 3 mL. The increase in absorbance due to oxidation of guaiacol (extinction coefficient 26.6 mM/cm) was monitored at 470 nm. Enzyme activity was expressed as unit's min/mg protein.

SOD activity was measured by the photochemical method as described by Beauchamp and Fridovich. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of nitro blue tetrazolium (NBT) reduction at 560 nm in the presence of riboflavin and light. The reaction mixture contained 45 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 13 mM methionine, 0.17 mM NBT in ethanol, 0.007 mM riboflavin and enzyme aliquot. Blanks were kept in the dark and the others were illuminated for 15 min. One unit of SOD is the amount of extract that gives 50% inhibition to the rate of NBT reduction.

Statistical analysis

All the experiments were performed in triplicate and the results were expressed as mean ± SD (standard deviations). Statistical analysis was performed using SPSS 12.0 and MS-Excel 2007. The significant difference was considered at *p* values ≤ 0.05.

RESULTS AND DISCUSSION

In the present investigation various assays have been adopted to test for antioxidative and free radical scavenging activities along with phytochemical constituents. The determination of phenols, flavonoids and tannins were systematically evaluated in *Digera muricata,* a wild edible plant.

Determination of phenolic content in different extracts

Determination of phenolic content in *Digera muricata* was determined by using a different solvent extracts. From the data it was observed and recorded that the methanolic extracts were given the maximum phenolic content followed by water > acetone > ethanolic > ethyl acetate, and minimum content of phenols were recorded in hexane extract (Fig-1). Similar results were observed and supported by Padama *et al.,* (2007); Sundar and Pratima (2011) in four species of *Terminalia*.

Determination of phenolic content in different concentrations of methanolic extract

From the data, the maximum phenolic content eas observed in methanolic extracts. Therefore, the methanolic extract was to analyzed for the phenolic

content in different concentrations (*i.e.,* 10, 20, 40, 60, 80 and 100 µg/ml). The maximum content of phenols were recorded at the concentration of 100 µg/ml. here it was interesting that to record that the phenolic content increased as the concentration methanolic extract increased (Fig-2). Similar, observations were reported by Padma *et al.,* (2007) in *Terminalia species* and suggested that the ethanolic extract had the maximum activity. Concentration and dynamics of the polyphenol synthesis in plant organs depends on the plant species, type of organs and growth stage. The result of some authors also supports our observations in the present investigation Arash (2010) who have comparatively analyzed the concentration of phenolic compounds in plant parts. The fact that the highest concentrations of phenolic compounds were found in methanolic extracts of leaves.

Determination of Flavonoids content

In the literature several other studies are also found that flavonoids compounds are the main contributors of the antioxidants capacities of the medicinal plants because of their common presence in plants. Flavonoids are important components of human and animal diet. Due to the different biological activities of plant secondary metabolites, their regular consumption may have serious consequences for health, both positive and negative (Stobiecki, 2006). In the present investigation the maximum content of flavonoids were recorded in methanolic extract followed by aqueous > ethanol > ethyl acetate, the minimum content of flavonoids was recorded in hexane extract (Fig-3). The methanolic extracts were determined for further in different concentrations (*i.e.,* 10, 20, 40, 60, 80 and 100 µg/ml). The results were observed and recorded the high content of Flavonoids in higher concentration at 100 µg/ml. There is a increase in flavonoids content of *Digera muricata* as the methanolic concentrations increased (Fig-4).

Determination of reducing power

The reducing power was determined in *D. muricata* with different solvent extracts and different concentrations (*i.e.,* 10, 20, 40, 60, 80 and 100 µg/ml). The maximum reducing power was recorded in methanolic extracts followed by acetone > ethanol > water, and minimum content of flavonoids was recorded in pet. ether, followed by hexane > ethyl acetate > chloroform (Fig-5). The methanolic extracts were further determined for different concentrations. In the present investigation it was recorded that the gradual increase in the content of flavonoids as the methanolic concentrations increased (Fig-6). The maximum reducing power was recorded at 100 µg/ml (Fig-6). Similar studies were conducted by Lillian Barros (2007), suggested that the reducing properties were generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.

Determination of ascorbic acid content

The ascorbic acid is one of the leading antioxidant content *i.e.,* vitamin C, it was determined in *Digera muricata* using different solvent extracts and different concentrations (10, 20, 40, 60, 80 and 100 µg/ml). The higher content of ascorbic acid was recorded in methanolic extracts and followed by water > ethanol > ethyl acetate. Then the lower content was recorded in chloroform > hexane > pet. ether. It is interesting to record the maximum content of ascorbic acid in methanolic extracts at 100µg/ml and observed that the concentration of ascorbic acid content increased with increase in the concentrations of methanolic extract due to the solubility of the compound in methanol is very high (Fig-7). The

earlier literature reported in other plants supports our results. Ascorbic acid is water soluble which helps to work both inside and out side the cells to control the free radical damage. Vitamin C is capable of neutralizing reactive oxygen species in the aqueous phase before lipid peroxidation is initiated. The possible anticarcinogenic effect of vitamin C appears to be related to its ability to detoxify carcinogens or block carcinogenic processes through its action as an antioxidant or as a free-radical scavenger. Reports suggest that vitamin C and beta-carotene supplementation improved pulmonary function. Vitamin C may reduce the risk of chronic diseases such as cancer, cardiovascular disease and cataracts (Singh *et al.,* 2010).

Effect of enzyme POD

The *Digera muricata* were determined to peroxidase enzyme to record the effect of POD. It is interesting to record the POD enzymatic activity effected data in *D. muricata.* The maximum enzymatic effect of POD data was observed and recorded in methanolic extract followed by water > ethanol > acetone > ethyl acetate. The minimum enzymatic activity effect of POD were observed in hexane < pet. ether < chloroform extracts (Fig-8). Similar studies were observed by Abd El-Moneim (2011) in other plants Acaricidal activity of different extracts from *Syzygium cumini* L. Skeels (Pomposia) against *Tetranychus urticae* Koch. and suggested that the antioxidant defense enzymes such as superoxide distumase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), glutathione reductase (GR) and monodehydroascorbate reductase (MDAR) are the systems designed to minimize the concentrations of superoxide and hydrogen peroxide. Superoxide-dismutase (SOD) catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. (H_2O_2) is eliminated by catalase and peroxidases, which include both enzymic and non-enzymic H_2O_2 degradation. The antioxidants such as ascorbate (AsA) and glutathione (GSH) are involved in scavenging ROS primarily by the Halliwell–Asada pathway, which scavenges H_2O_2, while MDAR and GR are involved in the regeneration of ascorbate.

Determination of SOD effect

The maximum amount of enzymatic antioxidants were recorded viz., SOD and POD in *Digera muricata*. It was observed that the maximum enzymatic activities of SOD were recorded in methanolic extract followed by acetone > ethyl acetate > water > ethanol > pet.ether > Hexane. The minimum enzyme effect activity were observed and recorded in chloroform extract. It was also interesting to record the enzymatic antioxidants activity effect of SOD in *D. muricata*. The maximum enzymatic antioxidative activity effect of SOD were recorded and observed in all the different solvent extracts accept chloroform (Fig-9). This report was agreed with the studies of mushrooms extracts by Ramkumar, (2010). In the past few years, the suspected toxicity of some synthetic compounds used in food has raised interest in natural products (Stone *et al.,* 2003). Some food industries and pharmaceuticals have increased their efforts in preparing bioactive compounds from natural sources by extraction and purification. Antioxidant compounds can scavenge free radicals and increase shelf life by retarding the process of lipid peroxidation.

Determination of DPPH assays

Free radicals have been implicated in many disease conditions, the important ones being superoxide radicals, hydroxy radicals, peroxyl radicals, and single oxygen. Herbal drugs containing free radical scavengers are gaining importance in treating such

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diseases. Many plants extract exhibit efficient antioxidative properties due to their phytoconstituents, including phenolics (Milan et al., 2011). In the present experiment, free radical scavenging activity evaluated for their free radical scavenging activity using the DPPH radical assay. It was observed and recorded that the methanolic extract had the maximum activity followed by ethanol > aqueous > acetone, and minimum activity was recorded in hexane extract. Reduction of DPPH radicals can be observed by the decrease in absorbance at 517 nm. The different plant extracts reduced DPPH radicals significantly. Values of percent decolorization of DPPH radicals are shown in Fig-10. The free radical scavenging and remaining DPPH content in assay was also recorded maximum content in methanolic extract. The methanolic extracts were further determined to different concentrations (10, 20, 40, 60, 80 and 100 µg/ml) and recorded that maximum content of DPPH free radical and scavenging activity in higher concentrations of *Digera muricata* at 100 µg/ml (Fig-11). Similar, studies were observed by Padama (2007); Sundar and Pratima in four species of *Terminalia.*

CONCLUSION

The plant *Digera muricata* have been determined for their antioxidative and free radical scavenging properties and enzyme POD and SOD. From the present investigation it was clear that the phenols and flavonoids are potent antioxidative followed by ascorbic acid, reducing power, SOD, POD and free radical scavenging assay. All the activities are more active in methanol followed by ethanol > aqueous > acetone > ethyl acetate and very less was observed in hexane < pet. ether < ethyl acetate extracts. Active ingredient in many plants extract possessing antioxidant properties remains to be identified. The present investigation clearly indicated that there is a great possibility of finding potent antioxidants. Moreover, there is a need for isolation and identification of different natural antioxidants in Indian system of medicinal plants.

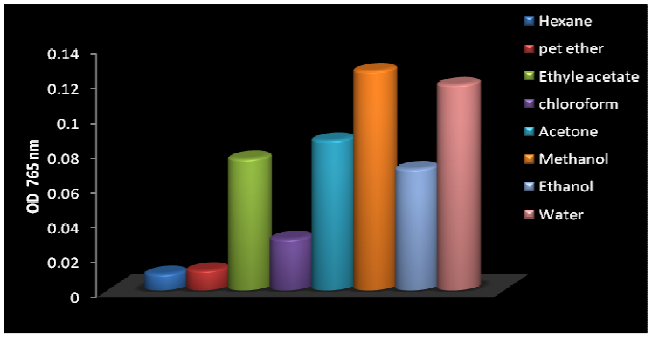

Fig-1: Determination of Phenolic content in different solvent extracts

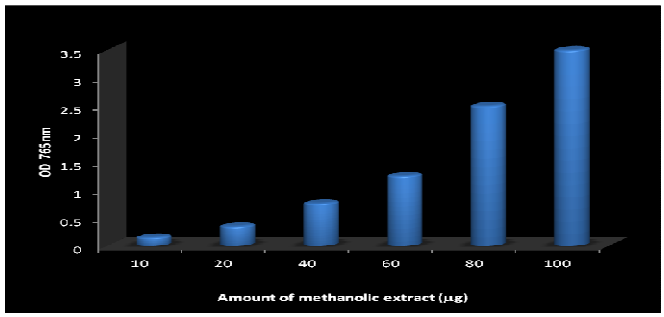

Fig-2: Determination of Phenolic content in different methanolic extract concentrations

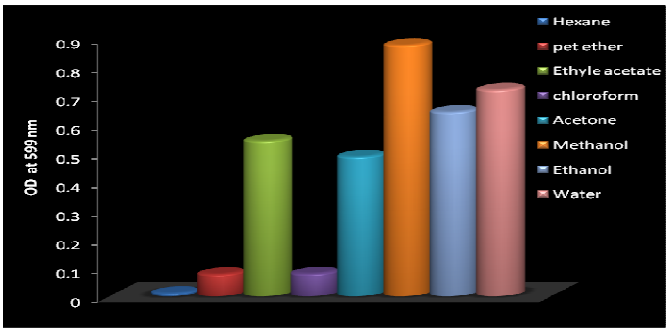

Fig-3: Determination of flavonoids content in different solvent extracts

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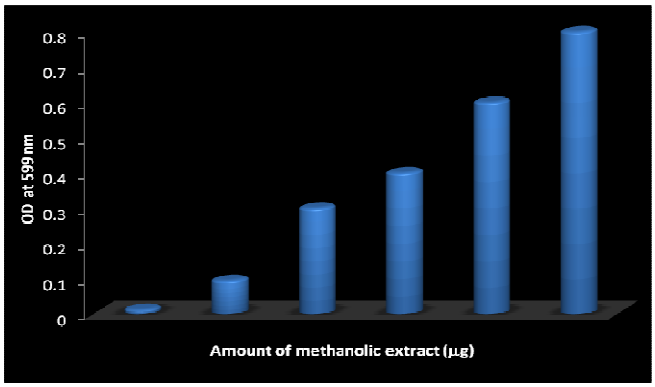

Fig-4: Determination of flavonoids content in different methanolic extract concentrations

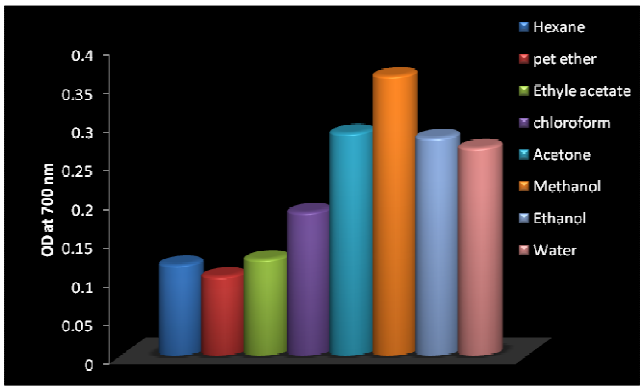

Fig-5: Determination of reducing power in different solvent extracts

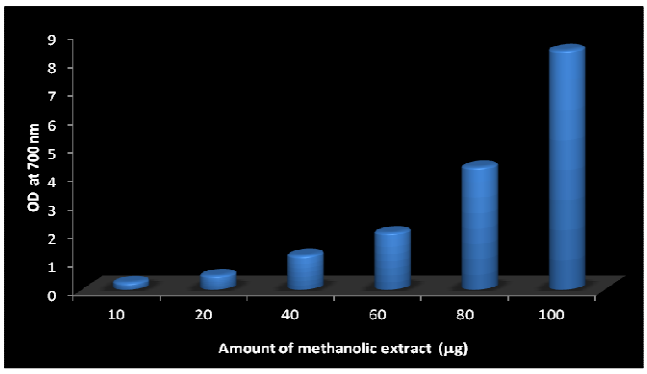

Fig-6: Determination of reducing power in different methanolic extracts concentrations

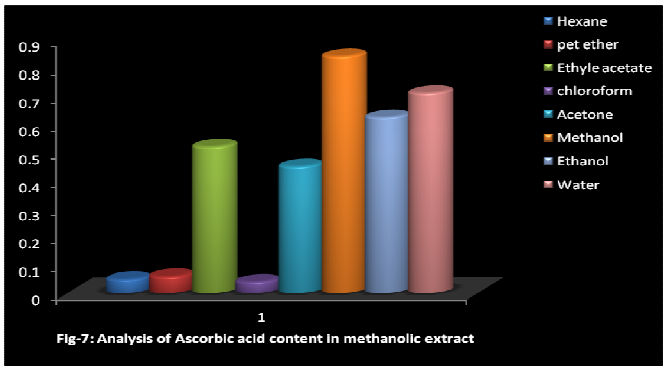

Fig-7: Determination of ascorbic acid content in different solvent extracts

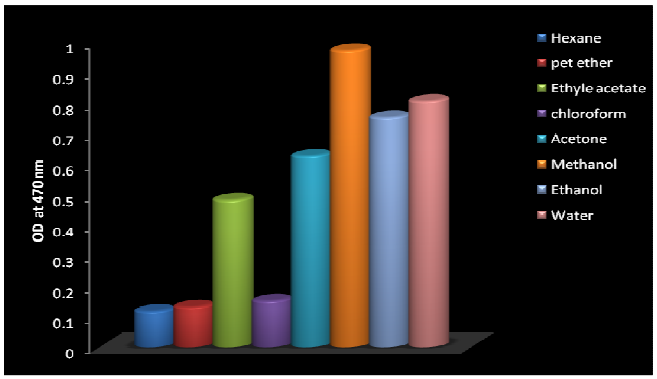

Fig-8: Determination effect of POD on *Digera muricata* in different solvent extracts

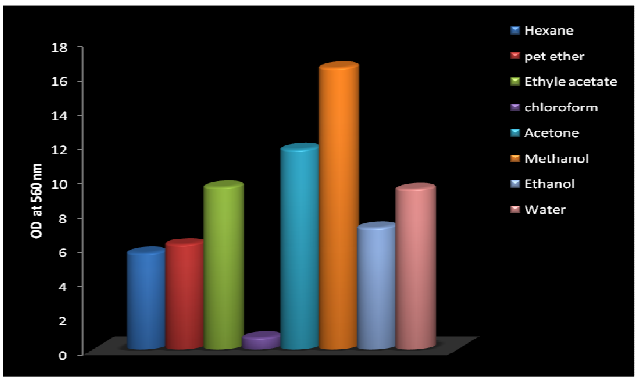

Fig-9: Determination of effect of SOD on *Digera muricata* in different solvent extracts

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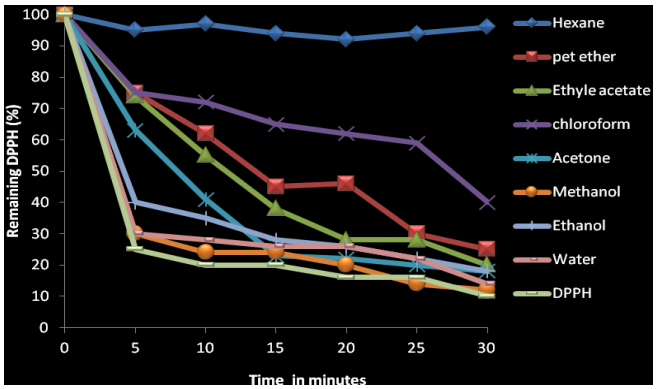

Fig-10: Determination of DPPH remaining and effect in different Solvents extracts.

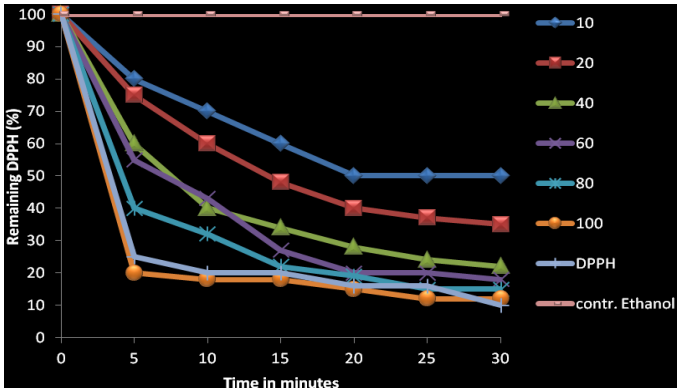

Fig-11: Determination of remaining and effect of DPPH in different methanolic extract concentrations

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