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In Vitro Antioxidant Properties of Naringi*Crenulata*(Roxb.)Nicolson(Rutaceae)

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ABSTRACT

The *invitro*antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of leaf and bark of*Naringicrenulata*have been tested using various antioxidant model systems *viz.*, DPPH, hydroxyl, superoxide, ABTS and reducing power. Methanol extract of *Naringicrenulata*is found to possess higher DPPH, hydroxyl radical scavenging activity, while ethanol extract is found to possess higher superoxide & ABTS radical cation scavenging activity. Methanol extract of leaf and barkof *Naringicrenulata*shows the highest reducing ability. The study indicates the significant free radical scavenging potential of *Naringicrenulata*leaf and bark extract which can be experimented for the treatment of various free radicals mediated aliments.

Keywords:
Naringicrenulata, Ethanol, DPPH, antioxidant.

Introduction

Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called “Free radicals”. Free radicals are capable of attacking the healthy cells, causing them to lose their structure and function. Cell damage caused by free radicals appears to be a major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular disease, cataracts and immune system decline and brain dysfunction [1].Radical formation is controlled naturally by various beneficial compounds known as “antioxidants”.Free radicals are electrically charged molecules, ie., they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to neutralize themselves. Although the initial attachment causes the free radical to become neutralized, another free radicals forms in the process, causing a chain reaction to occur. And until subsequent free radicals one deactivated, thousands of free radical reactions can occur within records of the initial reaction. Antioxidants one capable of stabilizing or deactivating, the fadicals before they attack cells [2]. Vegetables and Fruits are sources of different antioxidants such as tocopherol, glutathione, ascorbic acid [3].The present study aims to study the antioxidant activities of NaringiCrenulata by using different models.

MATERIALS & METHODS:

Plant sample extraction

Leaf and bark of *N. crenulata*were cleaned, shade dried and pulverized to powder in a mechanical grinder. Required quantity of powder was weighed and transferred to Stopperd flask and treated with petroleum ether, benzene, ethyl acetate, methanolamd ethanol separately until the powder is fully immersed. The flasks were shaken every hour for the first six hours and then it was kept aside and again shaken after 24hours. This process was repeated for three days and then the extracts were filtered. The extracts were collected and evaporated to dryness by using vacuum distillation unit. The final extracts were thus obtained were used for *in vitro* antioxidants activity.

DPPH radical scavenging activity

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in

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the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H.

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method [4]. Briefly, an 0.1mM solution of DPPH in methanol was prepared, and 1mL of this solution was added to 3 mL of the solution of all extracts at different concentration (50,100, 200,400 & 800µg/mL).The absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition) = $\{(A_0 - A_1)/A_0)*100\}$

where A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Hydroxyl radical scavenging activity

The scavenging capacity forhydroxyl radical was measured according to the modified method of Halliwell *et al.* (1987) [5]. Stock solutions of EDTA (1 mM), FeCl3 (10 mM), Ascorbic Acid (1 mM), H_2O_2 (10 mM) and Deoxyribose (10 mM), were prepared in distilled deionized water.

The assay was performed by adding 0.1 mL EDTA , 0.01 mL of $FeCl_3$**,** 0.1 mL H_2O_2, 0.36 mL of deoxyribose, 1.0 mL of the extract of different concentration (50, 100, 200, 400 & 800 µg/mL)dissolved in distilled water,0.33 mL of phosphate buffer (50 mM , pH 7.9), 0.1 mL of ascorbic acid in sequence . The mixture was then incubated at 37^0C for 1 hour. 1.0 mL portion of the incubated mixture was mixed with 1.0 mL of 10% TCA and 1.0 mL of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532 nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation is calculated by using the following equation

Hydroxyl radical scavenging activity= $\{(A_0 - A_1)/A_0)*100\}$

where A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Superoxide radical scavenging activity

The superoxide anion scavenging activity was measured as described by Srinivasan*et al*. (2007) [6]. The superoxide anion radicals were generated in 3.0 mL of Tris – HCL buffer (16 mM, pH 8.0), containing 0.5 mL of NBT (0.3mM), 0.5 mL NADH (0.936mM) solution, 1.0 mL extract of different concentration (50,100,200,400 & 800µg/mL), and 0.5 mL Tris – HCl buffer (16 mM, pH 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12mM) to the mixture, incubated at 25^oC for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by using the following equation

Superoxide radical scavenging activity= $\{(A_0 - A_1)/A_0)*100\}$

where A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Antioxidant Activity by Radical Cation (ABTS. +)

ABTS assay was based on the slightly modified method of Huang *et al*.,(2011) [7]. ABTS radical cation (ABTS+) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS + Solution were diluted with ethanol to an absorbance of 0.70+0.02 at 734 nm.

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After addition of 100µL of sample or trolox standard to 3.9 mL of diluted ABTS+ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC).

ABTS radical cation activity = $\{(A_0 - A_1)/A_0)*100\}$

where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Reducing Power

The reducing power of the extract was determined by the method of Kumar and Hemalatha(2011) [8]. 1.0 mL of solution containing 50,100,200,400 &800 µg/mL of extract was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH6.6) and potassium ferricyanide (5.0 mL, 1.0%). The mixture was incubated at 50^{o}C for 20 minutes. Then 5 mL of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5^{o}C) in a refrigerator centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride and absorbance read at 700 nm. The experiment was performed thrice and results were averaged.

REULTS

DPPH radical scavenging activity

DPPH radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of leaf and bark of *N. crenulata*shown in Figures 1 and 2. Among the solvent tested, methanol extract exhibited highest DPPH radical scavenging activity. At 800µg/mL concentration, methanol extract of leaf and bark of *N. crenulata* possessed 118.41% and 99.25% scavenging activity on DPPH respectively.

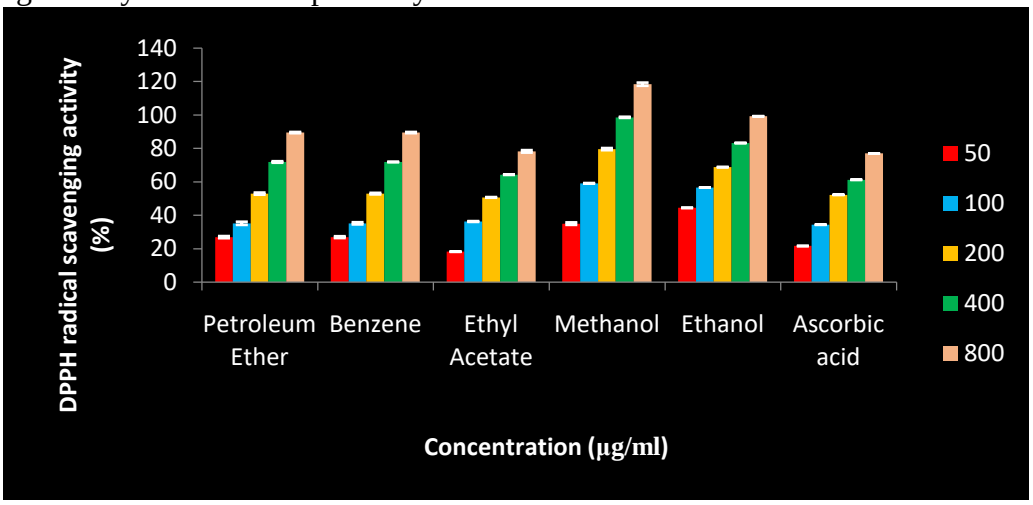

Fig 1: DPPH radical scavenging activity of different solvent extracts of leaf of *N. crenulata*

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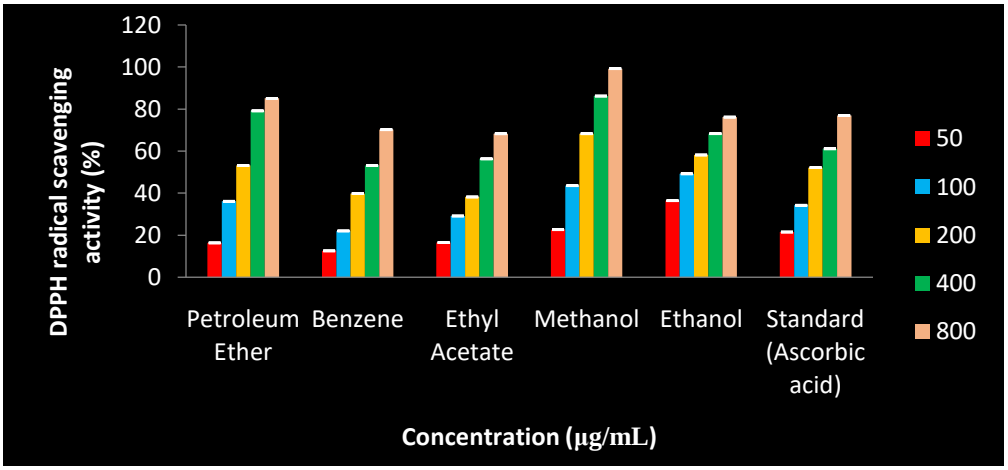

Fig 2:DPPH radical scavenging activity of different solvent extracts of bark of *N. crenulata*

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of leaf and bark of *N. crenulata*are presented in figure 3 and 34. Petroleum ether extract of bark of *N. crenulata*(800µg/mL) exhibited the maximum hydroxyl scavenging activity (98.24%). At 800µg/mL concentration, methanol extract of leaf of *N. crenulata*exhibited 78.13% scavenging activity on hydroxyl radical.

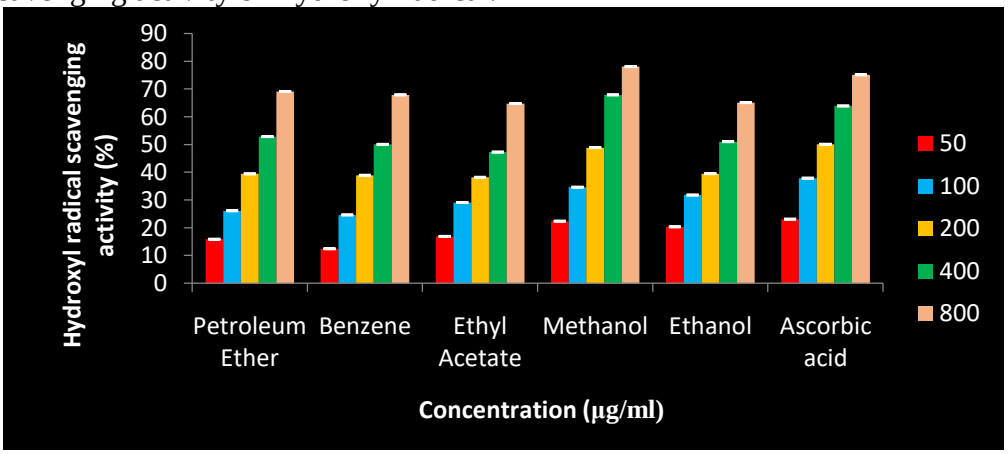

Fig 3: Hydroxyl radical scavenging activity of different solvent extracts of leaf of *N. crenulata*

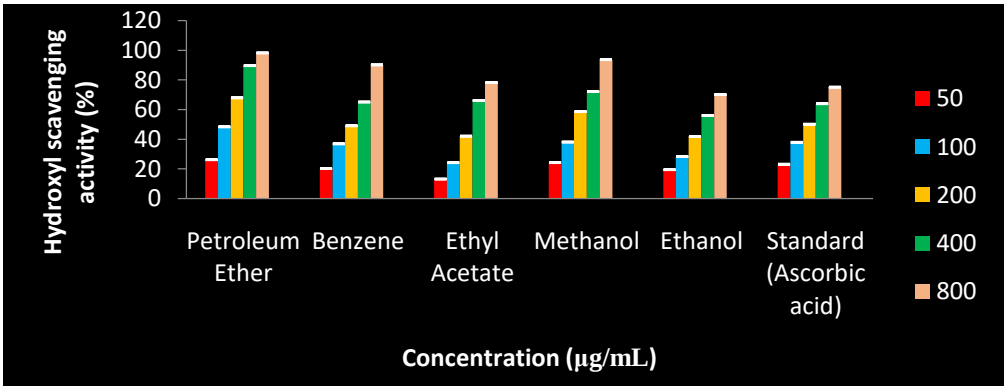

Fig 4 : Hydroxyl radical scavenging activity of different solvent extracts of bark of *N. crenulata*

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Superoxide radical scavenging activity

The *N. crenulata*leaf and bark extracts were subjected to the superoxide scavenging activity and the results were given in Figure 5 and 6. Among the solvent tested, ethanol extracts of leaf and bark of *N. crenulata*exhibited the maximum superoxide radical scavenging activity. At 800 µg/mL concentration, ethanol extracts of *N. crenulata*showed 101.16% and 108.54% scavenging activity on superoxide radical respectively.

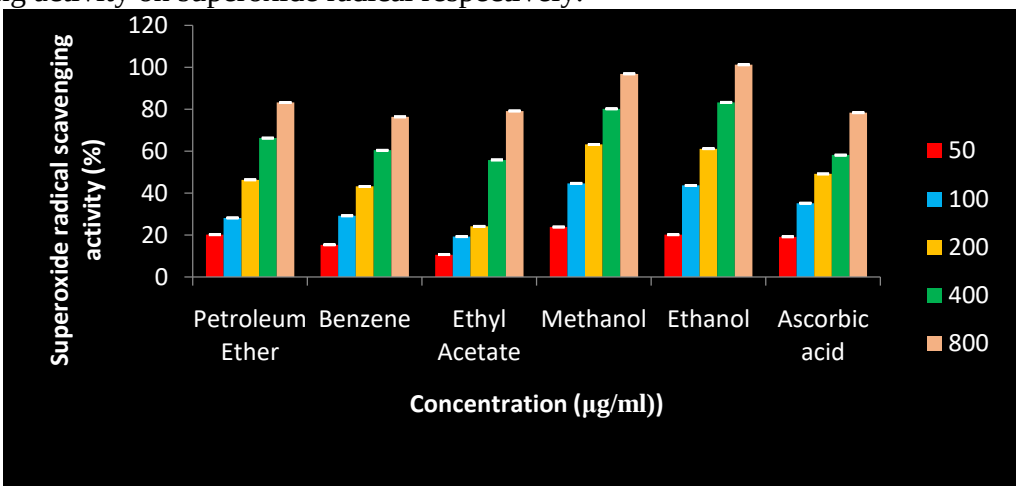

Fig 5: Superoxide radical scavenging activity of different solvent extracts of leaf of *N. crenulata*

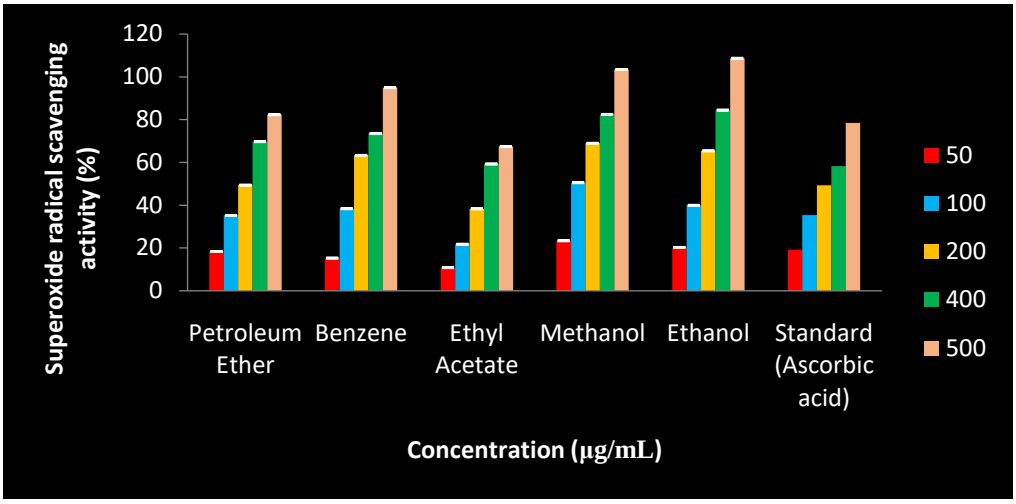

Fig 6: Superoxide radical scavenging activity of different solvent extracts of bark of *N. crenulata*

ABTS radical scavenging activity

The *N. crenulata*leaf and bark extracts were subjected to the ABTS radical cation scavenging activity and the results were presented in Figure 7 and 8. The methanol extracts of leaf and bark of *N. crenulata*exhibited potent ABTS radical cation scavenging activity in concentration dependent. At 800µg/mL concentration, *N.crenulata*leaf and bark extracts possessed 95.30% and 89.13% scavenging activity on ABTS which is higher than the standard trolox whose scavenging activity is 70.16%.

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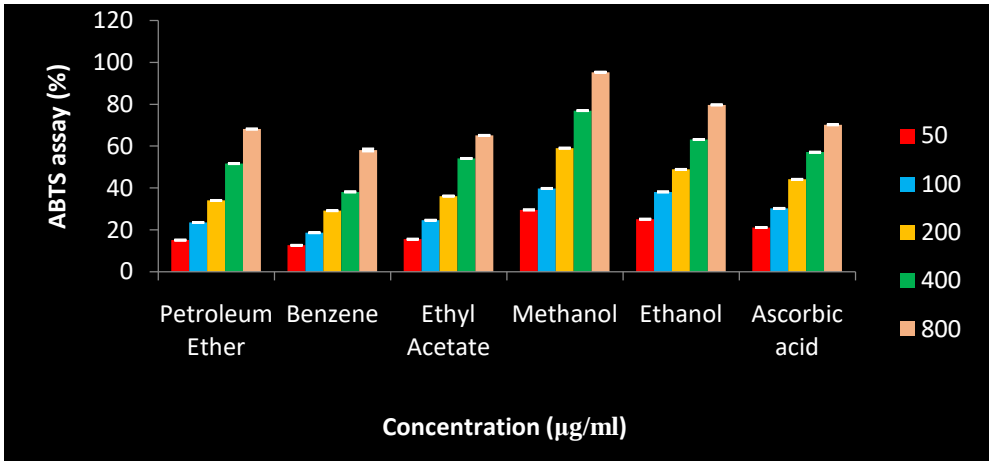

Fig 7: ABTS radical cation scavenging activity of different solvent extracts of leaf of *N. crenulata*

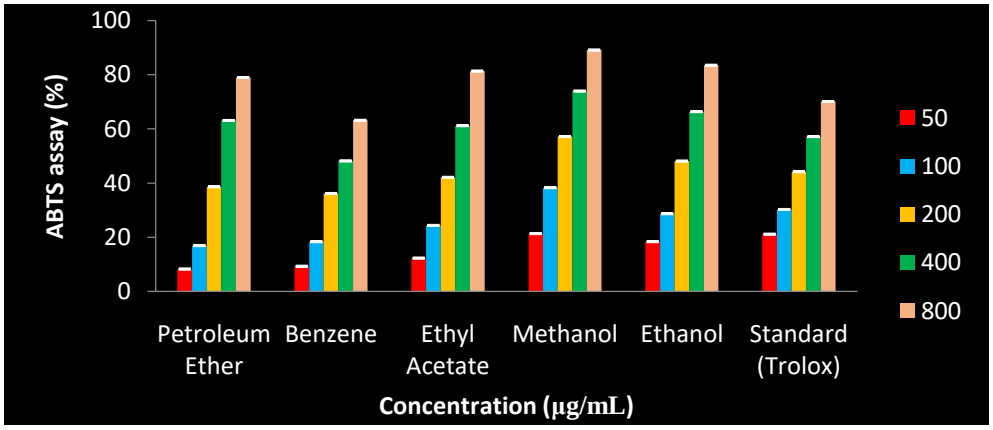

Fig 8: ABTS radical cation scavenging activity of different solvent extracts of bark of *N. crenulata*

Reducing power

Figure 9 and 10 show the reducing ability of different solvent extracts of *N. crenulata*leaf and bark compared to standard ascorbic acid. Absorbance of the solution was increased when the concentration increased. Among the plant parts studied, bark extracts possessed higher reducing ability.

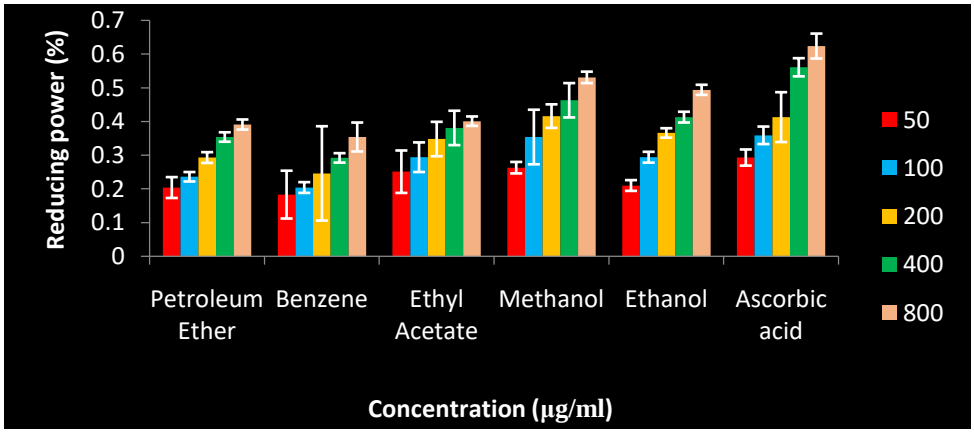

Fig 9: Reducing power ability of different solvents extracts of leaf of *N. crenulata*

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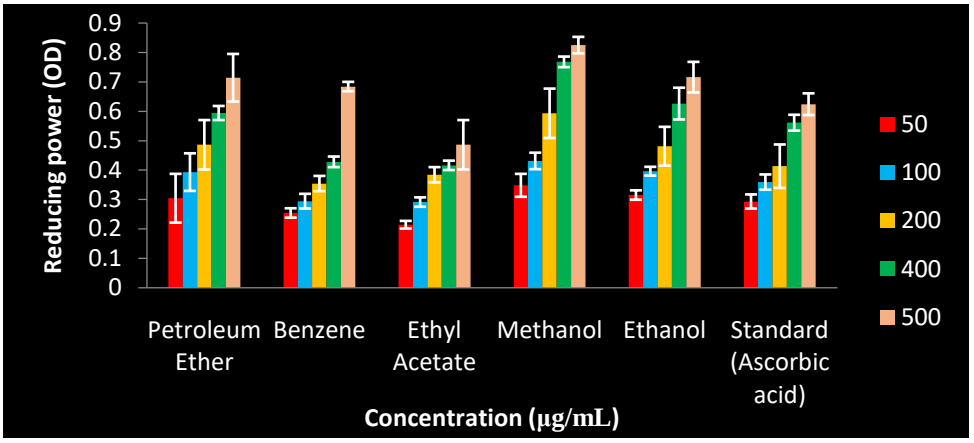

Fig 10: Reducing power ability of different solvents extract of bark of *N. crenulata*

Table 1: IC_{50} values of different solvents extract of leaf & bark of *N. crenulata*

Solvent Extract	IC50 (µg/mL)							
	DPPH		Hydroxyl		Superoxide		ABTS radical	
	Leaf	Bark	Leaf	Bark	Leaf	Bark	Leaf	Bark
Petroleum ether	21.84	20.16	17.14	20.84	21.67	24.13	17.04	21.54
Benzene	22.15	18.41	16.11	19.05	20.84	25.64	15.81	19.34
Ethyl acetate	19.12	18.05	16.46	18.16	21.16	18.16	17.54	21.84
Methanol	32.11	24.17	19.24	19.24	25.13	28.11	23.16	23.22
Ethanol	30.84	21.54	16.62	16.24	28.16	29.14	19.68	22.93
Ascorbic acid	**19.11**		**18.98**		**24.39**		**20.13**	

DISCUSSION:

In the present study, the different solvent extracts of *N.Crenulata* possess free radical scavenging activity with different invitro models viz DPPH , hydroxyl , superoxide and ABTS radical cation scavenging activity.

DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [9]. With an absorption maximum band around 515-528 nm and thus it is a useful reagent for evaluation of antioxidant activity of compounds [10]. In the DPPH test , the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenypierylhydrazine, and the extent of the reaction will depend on the hydrogen donating ability of the antioxidants. In the present study, methanol extract exhibited more DPPH radical scavenging activity for both leaf & bark (118.41% and 99.25%). A higher DPPH radical scavenging activity is associated with the lower IC50 value (32.11 &24.17µg/ml). From the results, in the present study a dose dependent relationship was observed in the DPPH radical scavenging activity. Among the five extracts, methanol extract showed maximum scavenging activity followed by ethanol extract.

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Hydroxyl radical scavenging capacity of an extract is directly related to its anti-oxidant activity. Among the reactive oxygen species the hydroxyl radical is the most reactive and induces severe damage to the adjacent biomolecules [11]. In the present study, IC50 values were found to be 20.84µg/ml for bark petroleum ether exract and 19.24µg/ml for leaf methanol extract .

Although superoxide anion is a weak oxidant, it gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress [12].Superoxide anions are the most common free radicals *in vitro* and are generated in a variety of biological systems, either by auto-oxidation processes or by enzymes. The concentration of superoxide increases under conditions of oxidative stress and related situations [13]. Moreover, superoxide anions produce other kinds of cell damaging free radicals and oxidizing agents[14]. The IC50 values were found to be 28.16µg/ml and 29.14µg/ml for leaf & bark respectively for ethanol extract. The results clearly indicate that the *N. crenulata leaf & bark* extracts have a noticeable effect as scavenging superoxide radical.

Another screening method for antioxidant activity is the ABTS radical cationdecolourization assay. The ABTs radical cation is generated from the reaction of ABTs with potassium persulfate overnight in water [15].The ABTS chemistry involves direct generation of ABTS radical mono cation with no involvement of any intermediary radical. The IC50 values of methanol extract of leaf & bark were 23.16µg/mL and 23.22µg/ml respectively.

Reducing power activity is often used to evaluate the ability of natural antioxidant to donate electron [16, 17]. The extracts of *N. crenulata* leaf & bark showed strong antioxidant activity of inhibiting DPPH, hydroxyl and superoxide scavenging activity when compared with standard ascorbic acid..

CONCLUSION:

The leaf & bark extracts of *N. crenulata* plant showed strong antioxidant activity of inhibiting DPPH, hydroxyl and superoxide scavenging activity when compared with standard ascorbic acid. Although the antioxidant activities found *in vitro* experiments were only indicative of the potential health benefit, these results remain important as the first step in screening antioxidant activity of *N. crenulata* plant.

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