IN-VITRO EVALUATION OF ANTIOXIDANT ACTIVITY OF ISOLATED PHYTOCHEMICALS FROM RHODODENDRON ANTHOPOGON

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Abstract

Defatted methanol extract of the aerial part of *Rhododendron anthopogon* (Ericaceae) afforded Ranhuadujuanine-A (I), Cannabioricylolic acid (II), Umbliferrne (III) and Betulin (IV). The structures of the isolated compounds were confirmed by elemental analysis *i.e.* ^{1}H, ^{13}C NMR, IR and MS spectroscopy. *In vitro* antioxidant activity for all the compounds evaluated where ascorbic acid (vitamin C) treated as standard. All the analysis was made with the use of UV-Visible spectrophotometer. Result of the study suggested that all the compounds showed effective efficacy for antioxidant properties in concentration dependant manner.

Keywords: *Rhododendron anthopogon*, Phytochemicals, *In vitro*, antioxidant activity, ascorbic acid.

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1. INTRODUCTION

Rhododendron anthopogon (Ericaceae), an evergreen shrub [1] growing at an altitude of 3,000-5,000 m. This is extensively dispersed in alpine himalaya from Himanchal Pradesh, Uttrakhand, Nepal & Bhutan [2] **a**nd used as incense for its aromatic properties. It's leaves and fresh flowers are used as tea by himalayan healers to promote digestive heat, arouse hunger and relieve liver disorders [3,4]. *R. anthopogon* is also used for sore throat, common cold and lung problems [5]. Anthopogon oil obtained from aerial parts of *R. anthopogon* by steam distillation method is recognized as sunpati oil, this oil is a good natural source of sweet herbal, a faintly balsamic essence [6]. *R. anthopogon* essential oil can be used on the skin and hair [6]. According to Himalayan aromatherapy, this oil stimulates the nervous system and has been used for treating sore muscles and gouty rheumatic conditions [7]. Scarce research has been published on chemical characterization [6,8,9] and the biological properties of this plant [8,10]**.** So it is required more studies with reference to phytochemical and biological.

2. MATERIALS AND METHODS

General Experimental Procedures:
Melting points were determined in soft glass capillaries in an electrothermal melting point apparatus. The IR spectra were recorded on FTIR SHIMADZU 8400S spectrometer with KBr pellets. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 300 MHz and 75 MHz on a Brucker NMR instrument, respectively, using TMS as internal standard. FAB mass spectra were recorded on JEOL SX 102 /DA-6000 mass spectrometer using Argon /Xenon as FAB gas.

Plant Material:
The aerial parts of *R. anthopogon* were collected from Uttrakhand (India) with the help of local villagers.

Extraction and Isolation of the Constituents:
The defatted plant material (Aerial part, 1.0 kg) was extracted with methanol for 48 hours. Obtained extract was concentrated under reduced pressure to give crude extract. The methanolic extract was dissolved in the minimum amount of methanol and adsorbed on silica gel to form slurry. The dried slurry was subjected to column chromatography over silica gel. The column was eluted with different solvents in order of increasing polarity where following compounds (I to IV) were isolated, purified and characterized.

Isolation of Ranhuadujuanine-A:
When column was eluted with chloroform (100%), this compound was isolated after removal of the solvent, the product was crystallized as colourless crystals. It showed 149-151 m.p. °C. IR (KBr, cm^{-1}); 3445 (-OH stretching), 1635, 1470 (Ar. C=C stretching), 1380, 1370 (gem dimethyl group) and 1060 (C-O stretching). 1H NMR (δ ppm, $CDCl_3$); 6.05 (s, 1H, C^2), 6.30 (s, 1H, C^4), 2.61 (m, 1H, C^8), 3.01 (d, 1H, C^9), 1.68 (m, 1H, C^{10}), 1.63 (m, 2H, C^{11}), 2.37 (m, 1H, C^{12}), 1.35 (s, 3H, C^{14}), 0.85 (s, 3H, C^{15}), 1.40 (s, 3H, C^{16}), 2.20 (s, 3H, C^{17}), 5.15 (s, -OH, C^1). 13C NMR (δ ppm, $CDCl_3$); 154.0 (C^1), 106.6 (C^2), 139.0 (C^3), 112.5 (C^4), 153.3 (C^5), 106.4 (C^6), 85.2 (C^7), 36.8 (C^8), 35.5 (C^9), 37.6 (C^{10}), 25.2 (C^{11}), 43.8 (C^{12}), 36.9 (C^{13}), 35.0 (C^{14}), 17.5 (C^{15}), 28.5 (C^{16}), 20.8 (C^{17}). MS (m/z); 259 (M^++H), 258 (M^+), 243, 228, 213, 196 etc. Molecular formula; $C_{17}H_{22}O_2$.

Isolation of Cannabioricylolic Acid:
When column was eluted with chloroform and ethyl acetate in ratio 4:1, this compound was isolated after removal of the solvent, the product was yellow oily compound. IR (KBr, cm^{-1}); 3380 (-OH stretching), 1690 (Conjugated -COOH), 1380, 1370 (gem dimethyl group) and 1064 (C-O stretching). 1H NMR (δ ppm, $CDCl_3$); 6.15 (s, 1H, C^4), 2.50 (t, 1H, C^8), 3.05 (d, 1H, C^9), 1.52-1.93 (m, 4H, C^{10}, C^{11}), 1.30 (s, 2×CH3, C^{14}, C^{16}), 0.75 (s, 3H, C^{15}), 2.48 (s, 3H, C^{17}), 2.35 (t, 1H, C^{22}). 13C NMR (δ ppm, $CDCl_3$); 156.9 (C^1), 101.3 (C^2), 142.2 (C^3), 112.1 (C^4), 161.8 (C^5), 108.4 (C^6), 84.5 (C^7), 36.5 (C^8), 35.4 (C^9), 37.2 (C^{10}), 26.3 (C^{11}), 46.5 (C^{12}), 39.6 (C^{13}), 31.5 (C^{14}), 17.2 (C^{15}), 28.7 (C^{16}), 23.2 (C^{17}), 172.9 (C^{18}). MS (m/z); 291(M^++H), 290(M^+),

289(M^+-H), 274, 259, 244, 227 etc. Molecular formula; $C_{18}H_{22}O_4$.

Isolation of Umbliferrne:
When column was eluted with mixture of chloroform : ethyl acetate (3 : 2), this compound was isolated and after removal of the solvent the product was yellowish white amorphous powder crystallized with ethyl acetate as colourless crystals. It showed m.p. 225-228°C. IR (KBr, cm^{-1}); 3410 (-OH stretching), 1625, 1470 (Ar. -C=C- str.) and 1687 (>C=O stretching). 1H NMR (δ ppm, $CDCl_3$); 6.15 (d, 1H, C^2, J= 9.5 Hz.), 7.92 (d, 1H, C^4, J= 9.5 Hz.), 7.86 (d, 1H, C^5, J= 8.4Hz.), 6.78 (dd, 1H, C^6, J= 8.5, 2.3 Hz.), 6.65 (d, 1H, C^8, J= 2.3 Hz.). 13C NMR (δ ppm, $CDCl_3$); 161.5 (C^2), 110.9 (C^3), 142.3 (C^4), 131.3 (C^5), 117.6 (C^6), 158.5 (C^7), 105.4 (C^8), 151.0 (C^9), 113.9 (C^{10}). MS (m/z); 163 (M^++H), 162 (M^+), 145 etc. Molecular formula; $C_9H_6O_3$.

Isolation of Betulin:
On removal of solvent ethyl acetate (100%), Betulin was obtained as white solid which was crystallized from Petroleum ether: Benzene (1:3) to give colorless needles, m. p. 253°C. it belongs to lupine series of triterpenoids and gave positive Libermann-Burchard and Noller tests. It also produced yellow color with tetranitromethane indicative of unsaturation. IR (KBr, cm^{-1}); 3460 (-OH), 2970-2880 (C-H stretch), 1655 (C=C stretching), 1450, 1370 (>CMe2). 1H NMR (δ ppm, $CDCl_3$); 4.50 and 4.65 (=CH_2), 3.33 and 3.85(d, J=11 Hz. each –CH_2OH), 3.18 (dd, J=12, 5 Hz., H-3α), 2.44 (m, H19), 1.70 (s, =C-CH_3), 0.76 (s, 3H), 0.83 (S, 3H), 0.95 (s, 3H), 0.98 (s, 3H), 1.02 (s, 3H) for five tertiary methyl groups. 13C NMR (δ ppm, $CDCl_3$); 38.30 (C^1), 26.60 (C^2), 78.94 (C^3), 38.30 (C^4), 55.20 (C^5), 17.47 (C^6), 34.32 (C^7), 39.60 (C^8), 50.60 (C^9), 37.45 (C^{10}), 21.40 (C^{11}), 23.26 (C^{12}), 37.01 (C^{13}), 41.53 (C^{14}), 27.08 (C^{15}), 29.35 (C^{16}), 47.69 (C^{17}), 47.81 (C^{18}), 48.80 (C^{19}), 150.31 (C^{20}), 29.69 (C^{21}), 32.92 (C^{22}), 28.05 (C^{23}), 15.60 (C^{24}), 6.14 (C^{25}), 16.80 (C^{26}), 14.69 (C^{27}), 59.04 (C^{28}), 109.62 (C^{29}), 19.41 (C^{30}). MS (m/z); 442 [M] $^+$, 424 [M-H_2O] $^+$, 220, 207, 189 etc. Molecular formula ; $C_{30}H_{55}O_2$.

Evaluation of Antioxidant Activity:

The antioxidant activity of all the isolated compounds was determined by different *in-vitro* methods such as the DPPH scavenging assay, Nitric oxide radical assay, Hydroxyl radical scavenging activity and Hydrogen peroxide scavenging activity.
Concentrations of standard and different compounds were used as follows:

Standard: Ascorbic acid (Vitamin C) at 5, 10, 15, 20µg/ml

Compound I to IV at 5, 10, 15, 20µg/ml

DPPH Scavenging Activity:
DPPH method [11] of Shirwaiker et al. was performed to assess the free radical scavenging competence of the compounds. Different concentration (5, 10, 15, 20 µg/ml) of compounds prepared in methanol were mixed with 0.1mM DPPH solution dissolved in 95% methanol. After 30 minute the absorbance of the solution was measured at 517 nm using spectrophotometer.

Nitric Oxide Radical Activity:
Garret *et al*. method [12] was used to conduct nitric oxide radical activity. Different concentration (5, 10, 15, 20 µg/ml) of compounds were mixed with 5mM sodium nitro prusside dissolved in phosphate buffer saline. This solution was incubated at 250°C for 150 minute and further reacted with Griess reagent. Absorbance of the solution was read at 546 nm. Ascorbic acid (Vitamin C) at 5, 10, 15, 20µg/ml concentrations were as standard.

Hydroxyl Radical Scavenging Activity:
Salicylic acid method recommended by Smirnoff and Cumbes [13] was used. 01 ml of solution of different concentration of all the compounds was mixed with 01ml of 9 mmol/l salicylic acid, 01ml of 9mmol/l ferrous sulphate and 01ml of 9mmol/l hydrogen peroxide. This mixture was incubated at 37°C for 60 minute and absorbance measured at 510 nm by spectrophotometer.

Hydrogen Peroxide Scavenging Activity:
Scavenge hydrogen peroxide activity of all the compounds was determined by Ruch et al method [14]. 5, 10, 15, 20µg/ml concentrations of individual compound was

mixed with 0.6 ml hydrogen peroxide solution. Absorbance was measured at 230 nm.

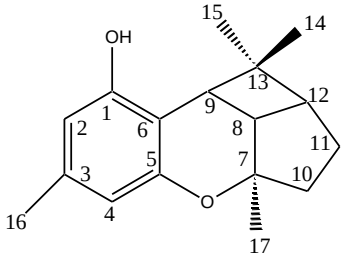
All the experiments were performed thrice. An average reading was taken for the calculation by following formula for scavenging activity.

$$\% \text{ Scavenging activity } = \frac{\text{Ao} - \text{A1}}{\text{Ao}} \text{ x } 100$$

Where Ao = Absorbance of control; A1= Absorbance of sample

3. RESULT AND DISCUSSION

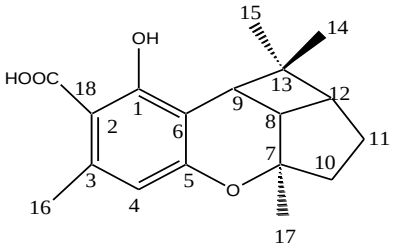
Characterization of Ranhuadujuanine-A (I):
On the basis of spectral studies the molecular formula of compound-I was assigned as $C_{17}H_{22}O_2$. The molecular ion peak was observed at 258 (M^+). The IR spectrum (KBr), of this compound confirms the presence of the hydroxyl group by showing a broad absorption at 3445. The >C=C< stretching were characterized by the absorption at 1635 and 1470. Other characteristic absorption at 1380 and 1370 confirmed the presence of gem dimethyl group [=C $(CH_3)_2$] deformation. The ^{1}HNMR spectrum (δ ppm, $CDCl_3$) of the compound-I showed sharp absorption at 1.35, 0.85, 1.40 and 2.20 for four methyl groups at C^{14}, C^{15}, C^{16} and C^{17} respectively. The proton present at C^1 position were observed at 5.15, which confirmed the presence of hydroxyl groups. Aromatic protons at C^2 and C^4 showed signals at 6.05 and 6.30 respectively. The signals observed from 1.63 to 3.01 were ascertained for remaining seven protons.



Ranhuadujuanine- A

In the ^{13}C NMR spectrum (δ ppm, $CDCl_3$), signals at 154.0 was assigned to carbon C^1 containing hydroxyl group. The signals observed at 35.0, 17.5, 28.5 and 20.8 correspond to methyl groups at C^{14}, C^{15}, C^{16} and C^{17} positions respectively. These spectral data were found to be same as reported for Ranhuadujuanine-A. On the basis of these observation the structure of compound-I was confirmed as Ranhuadujuanine-A.

Characterization of Cannabioricylolic A*cid* (II):
On the basis of spectral studies the molecular formula of compound-II was assigned as $C_{18}H_{22}O_4$. The molecular ion peak was observed at 290 (M^+). The IR spectrum (KBr), of compound-II confirms the presence of the hydroxyl group by showing a broad absorption at 3380. The conjugated -COOH stretching was characterized by the absorption at 1690. Other characteristic absorption at 1380 and 1370 confirmed the presence of gem dimethyl group [=C$(CH_3)_2$] deformation the presence of –C-O stretching was also stablished by an absorption at 1064. The ^{1}HNMR spectrum (δ ppm, $CDCl_3$) of the compound showed sharp absorption at 1.30, 0.75, 1.30 and 2.48 for four methyl groups at C^{14}, C^{15}, C^{16} and C^{17} respectively. Aromatic proton at C^4 showed signals at 6.15. The signals observed from 1.52 to 3.05 were ascertained for remaining seven protons.

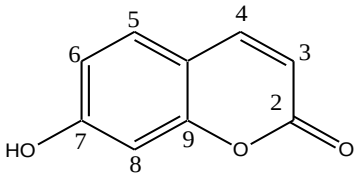


Cannabiorcicyclolic acid

In the ^{13}C NMR spectrum (δ ppm, $CDCl_3$), signals at 156.9 was assigned to carbon C^1 containing hydroxyl group. The signals observed at 31.5, 17.2, 28.7, 23.2 and 172.9 correspond to methyl groups at C^{14}, C^{15}, C^{16}, C^{17} and C^{18} positions respectively. These spectral data were found to be same as reported for Cannabiorcicyclolic acid. On the basis of observations the structure of compound-II was confirmed as Cannabiorcicyclolic acid.

Characterization of Umblliferon (III):

On the basis of spectral studies the molecular formula of compound-III was assigned as $C_9H_6O_3$. The molecular ion peak was observed at 162 (M^+). The IR spectrum (KBr), of compound-III confirms the presence of the hydroxyl group by showing a broad absorption at 3410. The aromatic >C=C< stretching were characterized by the absorption at 1625 and 1470. Presence of >C=O stretching was also established by an absorption at 1687. The ^{1}HNMR spectrum (δ ppm, $CDCl_3$) of the compound-III showed absorption at 7.86, 6.78 and 6.65 of C^5, C^6 and C^8 position respectively, which confirmed the presence of aromatic carbon. Absorption at 6.15 and 7.92 has been assigned to the conjugated olefinic proton at C^3 and C^4 position respectively.

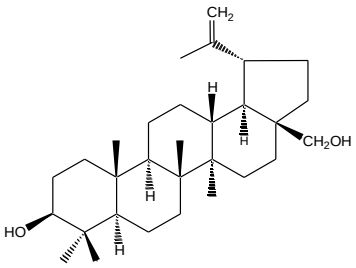


Umblliferon

In the ^{13}C NMR spectrum (δ ppm, $CDCl_3$), two signals at 110.9 and 142.3 were assigned to olefinic carbon i.e. C^3 and C^4. The signal observed at 158.5 corresponds to carbon attached to hydroxyl group at C^7. The value for carbonyl carbon at C^2 was 161.5. These spectral data were found to be same as reported for Umblliferon. On the basis of these observations the structure of compound-III was confirmed as Umblliferon.

Characterization of Betulin (IV):

It was obtained as colourless needles, m.p. 253-255°C and showed single spot on TLC. It developed pale-yellow coloration with tetranitromethane in chloroform indicative of unsaturation. It responded positively to Liebermann-Burchard and Noller tests characterstics of triterpenoids. Its infrared spectrum showed peaks at 3460 (broad, OH stretching), 2970-2880 (C-H stretching), 1655 (C=C stretching) cm^{-1}. The proton NMR spectrum (δppm, $CDCl_3$) of compound-IV showed a marked resemblance with lupeol. By analogy, a pair of broad singlets at 4.50 and 4.65 in conjugation with a singlet at 1.70 suggest the presence of isopropenyl side chain. Appearance of a multiplet at 2.44 was explicable on account of C^{19} proton of cyclopentane ring. The hydroxyl methine proton at C^3 gave a doublet at 3.18 (*J*= 12, 5 Hz.). Singlet appeared at 0.76, 0.83, 0.98 and 1.02 were on account of five tertiary methyl group. The singlet at 0.81 observed in the spectrum of compound due to methyl group at C^{28} was absent, suggesting the attachment of hydroxyl methyl group at this position.



Betulin

In the ^{13}C NMR spectrum (δ ppm, $CDCl_3$), absorptions observed at 28.05 (C^{23}), 15.60 (C^{24}), 6.14 (C^{25}), 16.80 (C^{26}), 14.69 (C^{27}) and 59.04 (C^{28}) confirmed the presence of six methyl groups. The signals observed at 109.62 and 150.31 were assigned for carbon-carbon double bond at C^{29} and C^{20} carbon atoms respectively. The absorption for methyl group at C^{30}, which is attached to olefin carbon atom, appeared at 19.41. The presence of absorption at 78.94 showed the presence of an acetoxy group attached at C^3 position. The mass spectrum showed an intense molecular peak at m/z 442 [M^+] corresponding to its molecular formula $C_{30}H_{50}O_2$ together with prominent peaks at m/z 442 [$M\text{-}H_20$]$^+$, 220, 207, 189, etc. which are characteristic for the triterpenoid of lupine series. Presence of a fragment ion peak at m/z 441 [$M\text{-}CH_20H$]$^+$ was further evidenced for the presence of satirically hindered CH_2OH group. The above spectral data were in good agreement with the structure of betulin which was confirmed by comparing with an authentic sample of betulin.

Evaluation of in-vitro Antioxidant Study

DPPH scavenging activity:

DPPH scavenging activity of compounds I, II, III and IV is shown in fig. 1, which demonstrates that the percentages of inhibitions were increased with increasing concentrations of the compound. The highest IC_{50} value for DPPH scavenging activity of standard was found 86.66 for 20µg/ml. in comparison to standard the most significant result was observed for compound IV for the same concentration. The least efficient effectiveness was shown by compound I i.e. 32.50, 45.60, 46.98 and 53.21 for concentration 5µg/ml, 10µg/ml, 15µg/ml and 20µg/ml respectively.

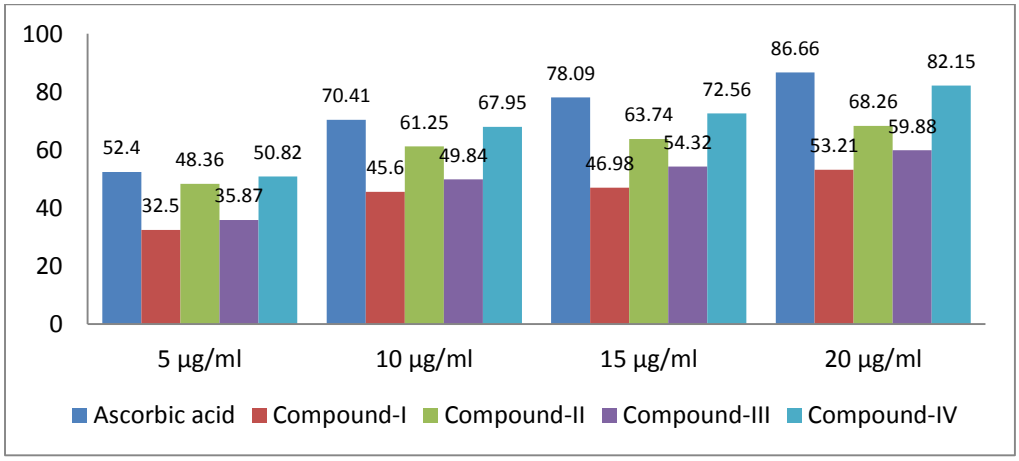


Figure 1: DPPH scavenging activity

Nitric Oxide Radical Activity:

The percentages of inhibitions were increased with increasing concentrations of compound. The IC_{50} value for scavenging of nitric oxide was found lowest 27.48 for compound I at 5µg/ml while highest 56.67 for compound IV at 20 µg/ml, while the IC_{50} value for standard was observed 40.09 and 60.93 for 5µg/ml and 20µg/ml respectively (Fig. 2).

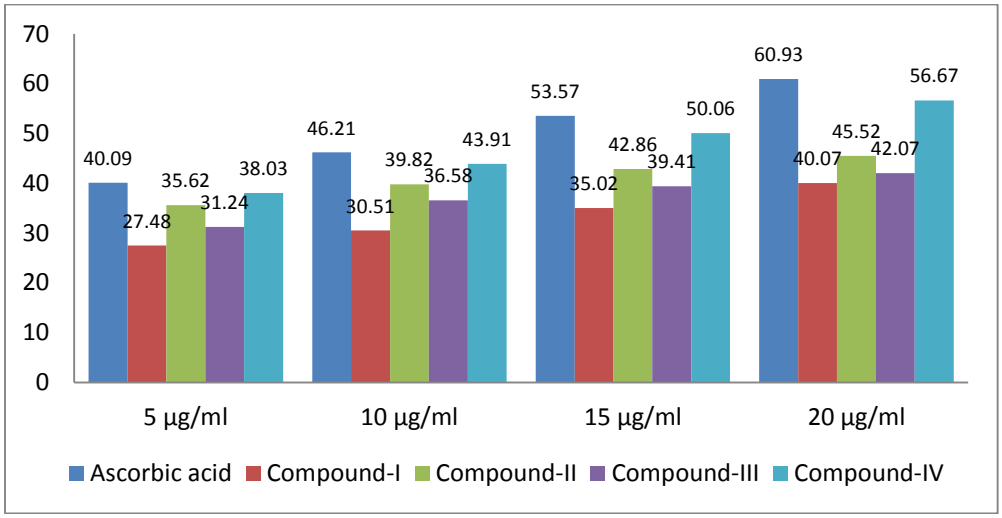


Figure 2: Nitric oxide radical activity

Hydroxyl radical scavenging activity:
The percentage inhibitions of all the compounds on hydroxyl radical was increased with increasing concentrations of compound. The IC_{50} value for Hydroxyl radical scavenging activity of compound I was found to be 18.26, 22.61, 28.09 and 32.63 for 5µg/ml, 10µg/ml, 15µg/ml and 20µg/ml concentrations respectively. For compound II it was found to be 21.38, 27.81, 36.48 and 40.07 for 5, 10, 15 and 20µg/ml concentrations respectively. For compound III it was found to be 20.06, 24.03, 27.73 and 30.38 for 5µg/ml, 10µg/ml, 15µg/ml and 20µg/ml concentrations respectively while it was 25.73, 32.42, 41.94 and 45.87 for 5µg/ml, 10µg/ml, 15µg/ml and 20µg/ml concentrations respectively for compound IV. The most effective and significant results were observed for compound IV in comparison to standard i.e. 27.67, 34.50, 42.52 and 49.29 for 5µg/ml, 10µg/ml, 15µg/ml and 20µg/ml concentrations respectively (Fig. 3).

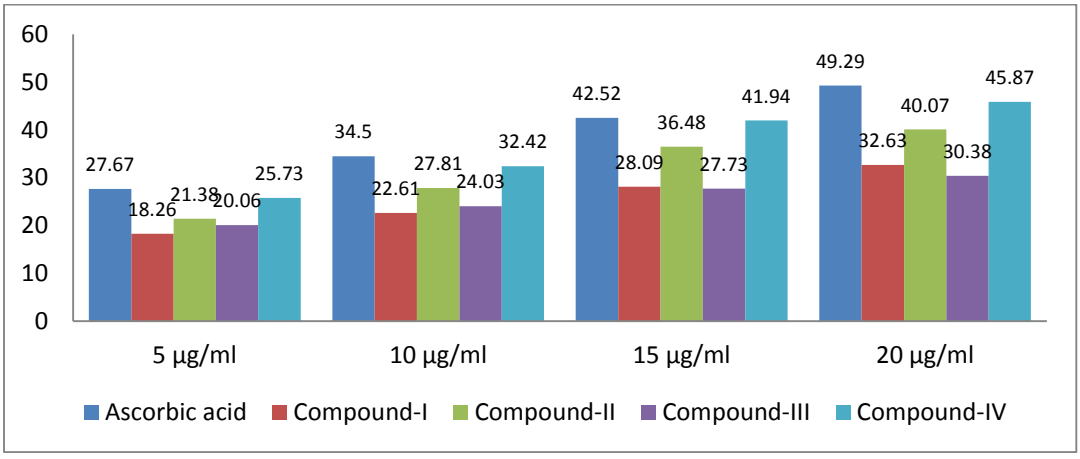


Figure 3: Hydroxyl radical scavenging activity

Hydrogen peroxide scavenging Activity:
The scavenging ability against hydrogen peroxide scavenging assay is shown in Fig. 4. Highest inhibition for all the compounds was observed in 20µg/ml concentration, 52.16, 68.81, 60.23 and 72.04 for compound I, II, III and IV respectively. While, IC_{50} value for standard 73.88 at the same concentration (Fig. 4).

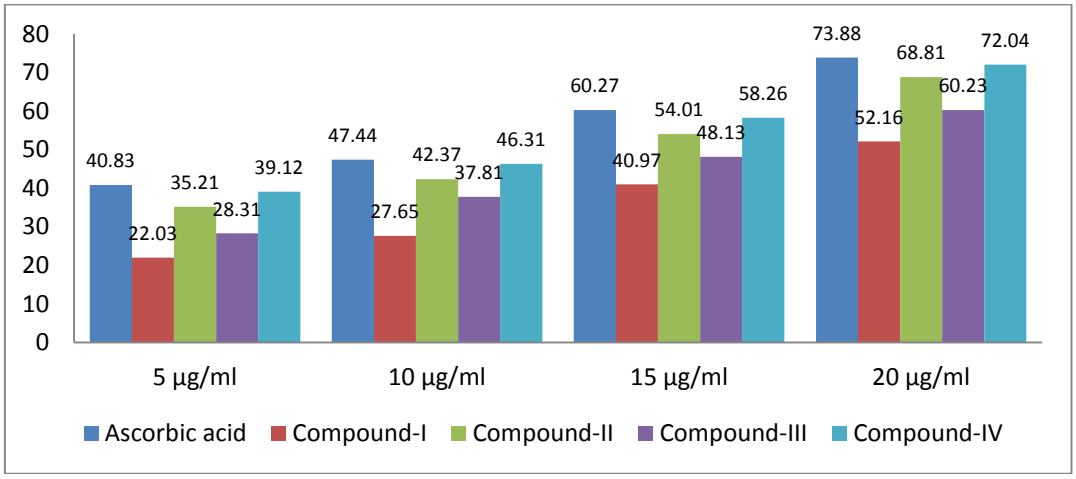


Figure 4: Hydrogen peroxide radical scavenging activity

Antioxidants act as a defence mechanism that protect against deleterious effects of oxidative reaction produced by reactive oxygen species (ROS) in a biological system. Oxidative stress is a primary cause of many disorders in humans such as neurodegenerative diseases, cancer and diabetes. Since scavenging of free radicals could inhibit the harmful effect of free radicals and stop the spreading of oxidation, antioxidants contents from plant origin through their scavenging activity are valuable for management of those diseases.

4. CONCLUSION

Many Natural chemical molecules have been isolate from plant and they are sources for new drug designing. On the basis of the findings in the present study, it is concluded that compound-IV shows high scavenging activities compared to others compounds and vitamin-C. *In-vitro* assays indicate that isolated active compounds could be a significant source of antioxidant, which might be helpful in preventing the progress of various oxidative stresses.

Conflicts of Interest: The authors declared no conflict of interest.

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