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# Study of antioxidant and free radical scavenging activities of methanolic extract of *Rumex acetosella* roots and its fractions in different solvents

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## Abstract

The context and purpose of the study: In view of the wide ethnomedicinal applications of *Rumex acetosella*, and as part of our quest for natural antioxidants, the present research was designed to evaluate antioxidant and free radical scavenging activities of the methanolic (MeOH) extract of its roots and its sub-fractions in solvents of different polarity employing1,1-dipicryl-2-phenyl hydrazyl (DPPH) free radical scavenging, 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Ferric reducing antioxidant potential (FRAP), phosphomolybdate, reducing power and lipid peroxidation assays.

Main findings: The MeOH extract and its fractions exhibited considerable antioxidant potential. The n-butanol fraction, having highest phenolic and flavonoid contents, 252.19μg/mL of Gallic acid equivalent and 891.34μg/mL of Rutin equivalent respectively, was most potent. All the fractionsefficiently scavenged the DPPH free radical; n-butanol fraction was most powerful and had the lowest $EC_{50}$(concentration of a sample required to scavenge 50% DPPH), which was 212.36μg/mL, and its$TEC_{50}$(time taken to scavenge 50% of DPPH) was 4 min. The phosphomolybdate antioxidant activity of the plant extracts ranged from 325.41-82.47μg/mL of Ascorbic Acid Equivalent (AAE). The n-butanol fraction had the highest FRAP, or ferric reducing antioxidant potential, value (569.52μg/mL of AAE) and the highest Trolox equivalent antioxidant capacity, or TEAC, value (1747.71 mM) in ABTS assay.The chloroform fraction that was least active in all the assays showed the lowest TEAC value (638.87 mM).

Brief summary and potential implications: The polar fractions of the MeOH extract of the roots of *R. acetosella*, havinghigher phenolics and flavonoids, displayed noteworthy antioxidant properties, the n-butanol fraction being the most powerful.Results present the plant as a potential source of natural antioxidants.

**Keywords:** Rumex acetosella roots, antioxidants, free radical scavenging, flavonoids

## Introduction

Production, in the body, of the reactive oxygen species (ROS) such as hydroxyl ($OH^{\bullet}$),superoxide ($O_2^{\bullet}$), nitric oxide ($NO^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$) radicals may cause severeimpairment to biomolecules and result in diseases like cancer, diabetes, cardiovascular disorders, neurodegenerative syndromes, and others [1-4]. Antioxidants and substances capable of scavenging ROS are required in the form of nutrients or medicines to inhibit the production or propagation of these toxic species in the body [5]. A number of synthetic antioxidants are available including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylated hydroquinone, and gallic acid esters, but they have harmful side effects [6,7]. Safer natural antioxidants are, thus, most desirable and their quest is a hot subject of extensive research the world over [8,9].

Plants constitute a virtually unending reservoir of bioactive principles [10-13]. The species of genus Rumex (family: Polygonaceae)are noted for their pharmacological propertiesincluding antioxidant [14,15]. Phytochemical investigations on different species of the genus have resulted in the isolation of various flavonoids, phenoliccompounds, and terpenoids [16-20]. R. acetosella var. acetosella is a widely distributed plant of the genus and is known for its diverse folkloric applications. It is used for the treatment of ailments pertaining to liver and digestive system,inflammatory diseases, tumors and urinary/kidney disorders [21].R. acetosella, which mainly grows in hilly grasslands and moist valleys, occurs in Pakistan in the northern hilly areas. It is a herb and can grow up to 1 m in height with long,green leaves [22]. The roots of the plant are small and woody. Theliterature, as long as we can explore, does not report any study of the antioxidant activity on the roots of R. acetosella. The objective of this research was, therefore, to evaluate the antioxidant and free radical scavenging activities of the methanolic extract of the roots of R. acetosella and its fractions in different solvents.

## Materials and Methods

### Chemicals

Sodium nitrite, Rutin, Folin-Ciocalteu reagent, ammonium molybdate, ferric chloride, potassium thiosulfate, iron(II) sulfate, iron(II) chloride, Tween-20, dipotassium phosphate, potassium thiocyanate, butylated hydroxyanisole (BHA) and all solvents used were of analytical grade and were purchased from Merck (Germany). Aluminum chloride was obtained from BDH Labs (England), Gallic acid from Scharlau (Switzerland), and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), ascorbic acid, 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) from MP Biomedicals (France). 2,4,6-Tripyridyl-s-triazine (TPTZ), linoleic acid and Trolox were obtained from Sigma-Aldrich (Germany), potassium ferricyanide and trichloroacetic acid were of Unichem (China).

### Plant Material Preparation

*Rumex acetosella* was collected from the hills of Abbottabad, Pakistan. The roots were separated from the aerial parts, washed with distilled water to remove dust particles, and chopped into small pieces. After drying in shade for two weeks, the roots were ground to obtain a fine powder. The powder (200 g) was extracted in MeOH (900 mL) for 15 days at room temperature. The extract was then filtered and concentrated by evaporating the solvent in rotary evaporator under reduced pressure.

### Preparation of different solvent extracts

The crude methanolic extract was suspended in distilled water, and extracted with n-hexane, chloroform, ethyl acetate and n-butanol respectively. In this way,n-hexane, ethyl acetate, chloroform, n-butanolic and residual aqueous fractions were obtained. Each fraction was concentrated on rotary evaporator under reduced pressure at 30 C, weighed, and kept in a refrigerator until further used.

### Total Phenolic Content

The total phenolic content was estimated according to the previously reported method using Folin-Ciocalteu reagent [23-24]. Briefly, 40 µL (0.3 mg/mL of MeOH) of the plant extract (or standard Gallic acid solution) was mixed with 3.16 mL of distilled water, and 200 µL of Folin–Ciocalteu reagent in a glass cuvette. After an interval of 8 min, 600 µL of sodium carbonate solution (7% w/v) was mixed. The samples were incubated at 40 C for 30 min before measuring the absorbance at 765 nm. The blank was prepared by using the same procedure but adding 40 µL of methanol in place of sample. The total phenolic content was calculated in term of micrograms per milliliter of Gallic Acid Equivalents (µg/mL of GAE).

### Total Flavonoid Content

The total flavonoid content was evaluated as per a known method [25]. In a glass cuvette, 300 µL (0.3 mg/mL of MeOH) of plant extract (or standard Rutin solution) was mixed with 3.4 mL of 30% aqueous methanol. Then 150 µL of $NaNO_2$ (0.5M) solution was added. After an interval of 5 min, 150 µL of $AlCl_3$ (0.3M) solution, and after lapse of further 5 min 1 mL of NaOH (1M) solution was added and mixed. Absorbance was measured at 506 nm. Blank contained 300 µL of aqueous methanol in place of the sample. The total flavonoid content was expressed as micrograms per milliliter of Rutin Equivalents (µg/mL of RE).

### DPPH Radical Scavenging Assay

The radical scavenging activity of the plant samples was determined using stable DPPH free radical [26]. Each of the plant samples was prepared by dissolving 10 mg of its extract in 10 mL of methanol. The stock solution od DPPH was prepared bydissolving 24 mg of DPPH in 100 mL of methanol and kept in a refrigerator until used. The working solution of DPPH was obtained by diluting the DPPH stock solution with methanol to obtain an absorbance of about 0.98 (±0.02) at 517 nm. In a glass cuvette, 3 mL of the DPPH working solution was mixed with 100 µL of the plant sample (or the standard solution). After incubating the mixture at 37 C for 30 min, absorbance was noted at 517 nm.Negative control was prepared by adding 100 µL of methanol to 3 mL of DPPH working solution. Percent antioxidant activity of each sample was calculated with the following formula:

%Antioxidant Activity = [(1 – (Sample absorbance/control absorbance)] x 100

Effect of the samples on DPPH with time was measured as follows [22]. In a glass vial, 3 mL of the working solution was mixed with 100 µL of the plant extract (or the standard solution) and its absorbance was measured at 517 nm for a period of 30 min. The percent scavenging activity, (percent DPPH remaining) was calculated as per the following formula:

$$\%DPPH_{rem} = \frac{DPPH_{T=t}}{DPPH_{T=0}} \times 100$$

Where $\%DPPH_{rem}$ is the percent of unreacted DPPH at any time t, $[DPPH]_{T=0}$ is the concentration of DPPH before its reaction with the sample, while $[DPPH]_{T=t}$ is its concentration at time t after its reaction with the sample. Ascorbic acid was used as a standard.
For each sample, $EC_{50}$ value was also determined thatis the effective concentration that inhibits 50% of the DPPH free radicals. $TEC_{50}$ or the time taken by the sample to scavenge 50% of the DPPH radicals was also determined.

### Phosphomolybdate Assay

The phosphomolybdate antioxidant assay was conducted using standard protocol [27]. Plant sample was prepared by dissolving 2.5 mg of the extract/fraction in 10 mL of methanol. Phosphomolybdate reagent was prepared by mixing sulfuric acid (100 mL, 0.6 M), ammonium molybdate (100 mL, 4 mM) and

sodium phosphate (100 mL, 28 mM) solutions. In a test tube, 3 mL of phosphomolybdate reagent was mixed with 300 µL of the plant sample (or standard Ascorbic acid solution). After capping the test tube with silver foil, it was incubated in water bath at 95 C for 90 min. Then, it was cooled to room temperature and absorbance was measured at 765 nm. The blank was prepared by using the same procedure except for replacing the sample with 300 µL of the solvent. Ascorbic acid was used as a standard and the antioxidant activity of the MeOHextract and its fractions were expressed as micrograms per milliliter of Ascorbic Acid Equivalents (µg/mL of AAE).

## Ferric Reducing Antioxidant Potential(FRAP) Assay

The total antioxidant activity of each plant sample was measured by FRAP (ferric reducing antioxidant potential) assay [28]. Each plant sample was prepared by dissolving 10mg of the extract/fraction in 10 mL of methanol. The FRAP reagent was prepared by dissolving 2.5 mL of each of the TPTZ and ferric chloride solutions in 25 mL of acetate buffer. The mixture was then incubated at 37 C for 15 min before use. In a cuvette, 2.85 mL of the FRAP reagent was mixed with 150 µL of a sample (or standard Ascorbic acid solution) and, after incubating for 30 min in dark, its absorbance measured at 593 nm. For blank, 3 mL of the FRAP reagents was mixed with 100 µL of methanol. The FRAP value of the samples were expressed as micrograms per milliliter of Ascorbic Acid Equivalents (µg/mL of AAE).

## Reducing Power Assay

The Reducing Power Assay was performed according to the protocol described by Oyaizuand coworkers [29].In a test tube, 2.5 mL of a plant sample (or standard Gallic acid solution) was mixed with 2.5 mL of sodium phosphate buffer (0.2 M) and 2.5 mL of 1% potassium ferricyanide. After incubating the mixture for 20 min at 50 C, 2.5 mL of trichloroacetic acid (10% w/v) was added, and the mixture was centrifuged at 650 rpm for 10 min. The supernatant (5 mL) was mixed with 5 mL of distilled water and 1 mL of ferric chloride solution. The absorbance was measured at 700 nm. For blank, the same procedure was used except that 2.5 mL of the solvent replaced the sample.

## ABTS Antioxidant Assay

The ABTS antioxidant assay was conducted using standard protocol [30]. The ABTS radical cation (ABTS•+) was produced by reacting 9.5 mL of ABTS solution with 245µL of potassium persulfate (100 mM), and making up the volume up to 10 mL by adding distilled water. Hence, the final concentration of ABTS stock solution was 7 mM of ABTS and 2.45 mM of potassium persulfate. The solution was allowed to stand in the dark, at room temperature, for 18 h before further use.The ABTS stock solution was diluted with PBS (pH 7.4) to an absorbance of 0.70 (±0.02) at 745 nm at 30 C. Each plant sample was prepared by dissolving 10mg of plant extract / fraction in 20 mL of methanol.In a cuvette, 10 µL of the sample (or standard Trolox solution) was mixed with 2.99 mL of working solution. The absorbance was measured at 734 nm, after an interval of 8 min. The same procedure was used to prepare the control except for replacing the sample with 10 µL of the solvent. The percentage antioxidant activity of each sample was calculated by the following formula.

%Antioxidant Activity = [(1 – (sample absorbance/control absorbance)] x 100

Where the control absorbance is the absorbance of ABTS radical in the absence of the sample and the sample absorbance is the absorbance 8 min after the addition of sample in the ABTS solution. The antioxidant activity of plant samples were expressed as Trolox Equivalents

## Lipid Peroxidation Assay

The lipid peroxidation values of various samples were estimated according to a standard protocol [31]. An emulsion of linoleic acid was prepared by mixing 155 µL of linoleic acid and 175 µg Tween-20 in potassium phosphate buffer (pH 7, final volume 50 mL). Each plant sample was prepared by dissolving its 10mg in 2 mL of methanol.In a test tube, 100 µL of a sample (or standard BHA solution) was mixed with 2.4 mL of potassium phosphate buffer and 2.5 mL of linoleic acid emulsion. The mixture wasthen placed for incubation at 37°C, and 100 µL of this solution was taken at regular intervals of 24 h, dissolved in 3.7 mL of ethanol and allowed to react with ferrous chloride (100 µL). Absorbance was measured at 500 nm after mixing the solution with 100 µL of potassium thiocyanate solution. Blank contained 2.5 mL of linoleic acid emulsion in 2.5 mL of potassium phosphate buffer. Butylated hydroxyanisole (BHA), a known food preservative, was used as a standard.

## Percept Yield of the Extracts

The percent yield of the crude methanolic extract (based on the dried plant material) of *Rumex acetosella*rootsand its sub-fractions (based on MeOH extract) are shown in Table 1. Amongst all the fractions, n-butanolic showed the highest yield (43.75%), while the chloroform showed the minimum (4.22%). The % yield of n-hexane, ethyl acetate and aqueous fractions was 8.18%, 12.45% and 24.48% respectively. The higher yield of the polar fractions indicates the presence of higher amounts of polar substances in the plant.

## Total Phenolic Content

The phenolic content of different fractions of *R. acetosella* was determined in terms of Gallic acid equivalents and the results are shown in Table 1. As reported earlier, the n-butanolic fraction showed the highest phenolic content (252.19 µg/mL of GAE), while the chloroform fraction displayed the lowest value (34.44 µg/mL of GAE). The presence of higher phenolic contents in polar solvents is in agreement with the general trend discovered in other studies too [32-34].

## Total Flavonoid Content

The total flavonoid contents of the MeOH extract and its fraction were estimated in the units of Rutin equivalents and the results are exhibited in Table 1.

Here again the n-butanolic fraction showed the highest value and the chloroform fraction exhibited the lowest, a trend also found in other studies [33,34]. Polar solvents in general have higher flavonoid and phenolic contents owing to the polar nature of these compounds.

**Table 1:** % Yield and total flavonoid and phenolic contents of different MeOH extract and fractions of *R. acetosella* roots.

| Plant Extract/Fraction | % Yield* | Total Phenolic Content (µg/mL) of GAE | Total Flavonoid Content (µg/mL) of RE |
|---|---|---|---|
| Crude Methanolic | 10.35 | 108.88 ±2.65 | 724.13 ±2.46 |
| n-Hexane | 08.18 | 43.70 ±1.90 | 511.65 ±1.34 |
| Chloroform | 04.22 | 34.44 ±2.30 | 431.98 ±1.98 |
| Ethyl acetate | 12.45 | 230.71 ±1.78 | 667.53 ±2.98 |
| n-Butanol | 43.75 | 252.19 ±2.32 | 891.34 ±1.45 |
| Aqueous | 24.88 | 94.07 ±2.25 | 812.49 ±3.10 |

%Yield of MeOH is based on dried plant material, while that of fractions is based on MeOH extract.

## DPPH Radical Scavenging Assay

The free radical scavenging activities of the MeOH extract of the roots of *R. acetosella* and its fractions in different solvents were determined using the well-known DPPH assay. The DPPH is a stable free radical and under the reaction conditions reacts with free radical scavengers present in the samples and form DPPH-H, the formation of which is monitored spectrophotometrically. The results are exhibited in Table 2.

**Table 2:** DPPH radical scavenging activities of MeOH extract of *R. acetosella* roots and its fractions in different solvents, and their comparison with ascorbic acid.

| Plant Fraction | %Activity | $EC_{50}$ (µg/mL) | $TEC_{50}$ (min) |
|---|---|---|---|
| Crude Methanolic | 81.44±0.61 | 400.14 | 17 |
| n-Hexane | 62.22±1.71 | 1499.75 | 24 |
| Chloroform | 35.29±0.19 | 885.13 | 21 |
| Ethyl acetate | 83.09±0.91 | 379.35 | 15 |
| n-Butanol | 94.56±0.24 | 212.36 | 04 |
| Aqueous | 81.54±0.45 | 398.89 | 15 |
| Ascorbic Acid | 93.35±0.38 | 230.18 | 20 |

The polar fractions showed higher free radical scavenging property than the nonpolar ones. The n-butanolic fraction displayed the highest activity. The trend can be explained on the basis of higher phenolic and flavonoid contents in the polar fractions. The change of antioxidant potential with time was determined by calculating the decrease in %DPPHrem as a function of time (Figure 1). There was a sharp decrease in absorbance in the beginning after the addition of the sample but then became moderate for the rest of the time. This indicated the presence of both the slow reacting and fast reacting antioxidants in the samples. The $EC_{50}$ and $T_{EC50}$ values are shown in Table 2. The free radical scavenging potential of the samples in general had an inverse relationship with $EC_{50}$. The trend was in agreement with that found in other similar studies[35-38, 43].

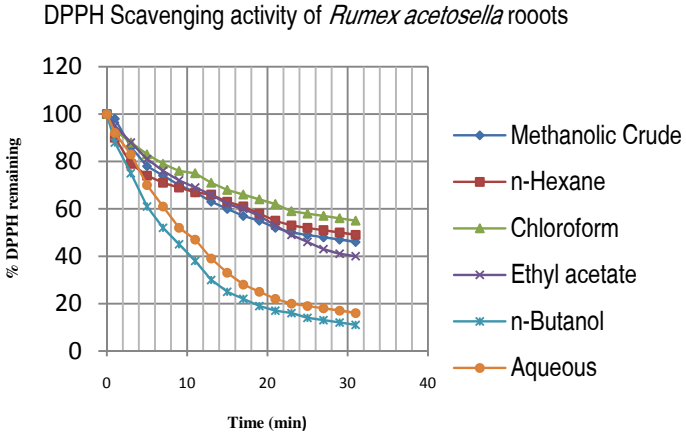


**Figure. 1:** The radical scavenging activity in terms of %DPPHrem by MeOH extract of *R. acetosella* roots and its fractions in different solvents.

## Phosphomolybdate Assay

This assay involves the reduction of Mo(VI) to Mo(V) by an antioxidant sample, which is detected by the formation of green molybdenum(V) complex. The results of the assay are shown in Table 3. The n-butanolic fraction that had the highest phenolic and flavonoid contents exhibited the highest antioxidant activity in this assay. The trend is in consonance with the findings of other studies [39, 43].

Table 3: Antioxidant activities determined by phosphomolybdate and FRAP assays of MeOH extract of *R. acetosella* roots and its fractions in different solvents.

| Plant Extract/Fraction | Antioxidant Activity | |
|---|---|---|
| | Phosphomolybdate Assay(µg/mL) of AAE | FRAP Assay (µg/mL)of AAE |
| Crude Methanolic | 118.45 ±2.05 | 444.32 ±4.06 |
| n-Hexane | 098.99 ±0.86 | 213.12 ±2.98 |
| Chloroform | 082.47 ±0.434 | 194.32 ±1.04 |
| Ethyl acetate | 253.56 ±4.57 | 546.72 ±2.98 |
| n-Butanol | 325.41 ±3.59 | 569.52 ±3.10 |
| Aqueous | 218.02 ±0.97 | 524.32 ±2.45 |

## FRAP Assay

The MeOH extract of *Rumex acetosella* roots and its fractions in different solvents were subjected to the FRAP,or Ferric Reducing Antioxidant Potential, assay and the results are shown in Table 3.The MeOH extract showed notable antioxidant activity in this assay which was 444.32 µg/mL of ascorbic acid equivalent. Expectedly, the n-butanolic fraction displayed the highest antioxidant potential in this assay also (569.52 µg/mL AAE). In contrast to the polar fractions, the non-polar fractions exhibited very poor FRAP value [40].

## Reducing Power Assay

This assay is based on the reduction, by antioxidant present in a sample, of $Fe^{3+}$ionsto $Fe^{2+}$ ions which are detected as ferricyanide complex at 700 nm (Table 4).The antioxidant potential of the fractions of *R. acetosella* in this assay was in the following order: n-butanolic > ethyl acetate >n-hexane > aqueous > chloroform. The n-butanolic fraction again showed the highest antioxidant potential in this assay indicating the presence of compounds that can easily transfer an electron to ferric and convert it into ferrous [41].

**Table 4:** The reducing power of MeOH extract of *R. acetosella* roots and its fractions and their comparison with Gallic acid standard expressed as absorbance at 700 nm.

| S.# | Extract/Fractions | Absorbance |
|---|---|---|
| 1. | Crude Methanolic | 3.225 ±0.61 |
| 2. | n-Hexane | 3.051 ±0.98 |
| 3. | Chloroform | 2.098 ±1.04 |
| 4. | Ethyl acetate | 3.288 ±0.01 |
| 5. | n-Butanol | 3.426 ±0.04 |
| 6. | Aqueous | 2.335 ±0.15 |
| 7. | Gallic acid | 3.385 ±0.01 |

## ABTS Assay

The ABTS antioxidant potentials for MeOH extract of *R. acetosella* roots and its fractions were measured in terms of Trolox equivalents and are displayed in Table 5.The TEAC, or Trolox equivalent antioxidant activity, values of the samples were estimated by using the following equations:

TEAC= (%Antioxidant potential - 0.256) / 0.0464, $R^2$ =0.992

The TEAC values showed, in general, a direct relationship with the polarity of the solvents. The highest TEAC value was, thus, exhibited by n-butanol fraction (1747.71 mM), while the lowest was shown by chloroform fraction (638.87 mM). This can be explained on the basis of the polar nature of polyphenols and other natural products having higher antioxidant properties [42,43].

**Table 5:** Trolox Equivalent Antioxidant Capacity of MeOH extract of *R. acetosella* roots and its fractions.

| Plant Fraction | Trolox Equivalent Antioxidant Capacity (TEAC) (mM) |
|---|---|
| Crude Methanolic | 672.28 ±1.87 |
| n-Hexane | 879.86 ±2.65 |
| Chloroform | 638.87 ±0.63 |
| Ethyl acetate | 1204.18 ±2.76 |
| n-Butanol | 1747.71 ±3.21 |
| Aqueous | 1446.85 ±4.77 |

## Lipid Peroxidation Assay

Under the assay conditions, linoleic acid undergoes oxidation producing peroxideradicals that oxidise $Fe^{2+}$ions to $Fe^{3+}$ ions. The reaction of $Fe^{3+}$ ions with thiocyanate ions forms a coloured complex that can be detected at 500 nm. The presence of an antioxidant in the medium slows down the process. The MeOH extract of the roots of *R. acetosella* and its sub-fractions in different solvents exhibited remarkable ability to inhibit peroxidation process in linoleic acid (Fig. 2). They in general showed slightly better free radical scavenging potential than standard BHA. The samples continued to be effective even after 96 h. Since the plant is edible, further studies may prove its suitability for its addition to lipids as preservatives against rancidity [22].

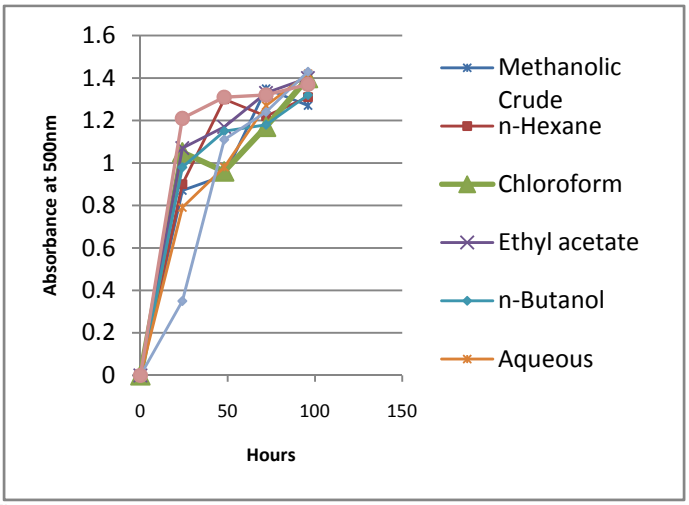


**Figure. 2:** Lipid peroxidation inhibitory activity of different fractions of *Rumex acetosella* roots.

## Conclusion

The methanolic extract of the roots of*Rumex acetosella* and its sub-fractions showed promising antioxidant and free radical scavenging properties. The polar fractions invariably proved to be most potent

in all assays. There was a significant correlation between the antioxidant properties and the total phenolic and flavonoid contents of the fractions. Assay-guided phytochemical investigation on different fractions, particularly the polar ones, may result in the isolation of natural products, which can be used as future preservatives for edible materials and vegetable oils.

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