Determination of antioxidant activity and simultaneous RP-HPLC analysis of quercetin, rutin and kaempferol in *Citrus macroptera* Montruz

Khumukcham Nongalleima, T Ajungla and Chingakham B Singh

**Abstract**

*Citrus macroptera* Montruz. fruits were collected, its rind was dried and powdered. Three different organic extracts, aqueous (NC1), methanol (NC2) and aqueous methanol (NC3) were prepared. *In vitro* antioxidant and reverse phase High Performance Liquid Chromatography (RP-HPLC) was done to quantify the quercetin, rutin and kaempferol content in three extracts of *Citrus macroptera* Montruz. NC1 showed highest antioxidant (DPPH-IC₅₀ 87.83 µg/mg, reducing power assay 36.71 µg/mg). Quercetin and kaempferol content was highest in NC2 extract, was quantified as 431.1 µg/mL and 59.50 µg/mL respectively. However rutin content was highest in aqueous methanol extract (260.38 µg/mL). The designed studies indicate remarkable potential of *C. macroptera* as dietary source of antioxidant and also the presence of important flavonoids. Studies on isolation of lead compound attributing the studied bioactivity is under progress.

**Keywords:** DPPH, antioxidant, RP-HPLC, quercetin, rutin and kaempferol

**Introduction**

Citrus are rich in antioxidants and vitamins. Their flavonoids have potential antioxidant (prevents aging), anti-cancer, antiviral, anti-inflammatory activities, effects on capillarity, and cholesterol-lowering ability [1]. Citrus fruits are well-known for their dietary, nutritional, medicinal and cosmetic properties and are also good sources of citric acid, flavonoids, phenolics, pectins, limonoids, ascorbic acid, etc [2]. Citrus fruits, including oranges, lemons, limes and grapefruits, are a principal source of such important nutrients, which are suggested to be responsible for the prevention of degenerative disease. These include vitamins C, folic acid, carotenoids, dietary fibres, potassium, selenium and a wide range of phytochemicals[3]. Sidana [4] reviewed on Citrus and reported that polymethoxylated flavones of Citrus have been shown in numerous *in vitro* studies to exert strong anti-proliferative action against cancer cells, antigen activated T lymphocytes, gastric cancer cells, prostate cancer cells, squamous cell carcinoma, and ant metastatic actions against human breast cancer cells, protective cardiovascular, ant hyperglycemic, anti-inflammatory, anti-allergic, analgesic, anti-feedent, antioxidant, antibacterial, antifungal, antiviral activities. Citrus Peel is a good source of phenolic compounds which may potentially be used in food formulations or when extracted can be used as natural antioxidants to prevent oxidation of selected foods [5].

Among the Citrus plant’s rich information on antioxidants and curative medicinal properties, there is less reports on *Citrus macroptera* species. Till date, there has been no published report on the antioxidant property of *Citrus macroptera* locally called as “Heiribob”, found in Manipur, North East India. In our present study we report free radical scavenging activity of the selected citrus species and simultaneous determination of kaemferol, rutin and quercetin using reverse phase HPLC.

**Experimental**

**Plant material**

Fruits of *Citrus macroptera* were collected from Kwatha Village, Chandel District, Manipur, North East India. It was identified by taxonomist of the institute, Imphal and faculty of Botany Department, Nagaland University (Fig. S1A). A voucher specimen was deposited at IBSD with voucher number IBSD/M-1031A.

**Preparation of extract**

Rinds of the fruits were peeled off; dried and coarse powder was made using a commercial blender. 60 g powdered sample each was macerated in aqueous (500mL), methanol
Antioxidant assay
The antioxidant property was assayed using the following three methods:

Reducing power assay
100 µL of sample with different concentrations (10-100 µg/mL) of the extract were mixed with 100 µL of 0.2 M sodium phosphate buffer (pH 6.6) and 100 µL of 1% potassium ferrocyanide. The reaction mixture was incubated at 50°C for 20 minutes. After incubation, 100 µL of 10% potassium ferricyanide was added, and the reaction mixture was incubated at 100°C for 10 minutes. The absorbance was read at 700 nm in a 96 well microplate reader (Thermo Scientific). The percent increase in reducing power was calculated using the following equation:

\[
\% \text{ Reduction} = \left[1 - \frac{A_{\text{sample}}}{A_{\text{blank}}}\right] \times 100
\]

Higher absorbance indicates higher reducing power. The percent increase in reducing power was calculated and this activity was expressed as an inhibition concentration 50 (IC50).

Nitric oxide reducing assay
Under aerobic conditions, nitric oxide reacts with oxygen to produce stable products (nitrates and nitrite). The quantities of which can be can be determined using Griess reagent. The scavenging effect of the plant extract on the nitric oxide was measured according to the modified method of [6]. 500 µL of test sample with different concentration (10-100 µg/mL) was mixed with 2 mL of SNP 10mM SNP, 500 µL of 50 mM phosphate buffer saline pH 7.4. They were incubated at 25°C for 150 minutes. Griess reagent (500 µL) was added and incubated at 25°C for 30 minutes. The absorbance was read at 540 nm. A phosphate buffer saline served as blank [6].

DPPH free reducing assay
The free radical scavenging activity of the extract were measured by 1, 1-Diphenyl-2-picryl hydrazil [7]. Briefly, 0.1mM solution of DPPH in ethanol was prepared. Then, 1mL of this solution was added to 3mL of test sample and L Ascorbic acid (positive control) solution at different doses (10–100 µg/mL). The mixture were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517nm in Thermo Multiscan Spectrum. The lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Percentage DPPH free radical inhibition was calculated and this activity was expressed as an inhibition concentration 50 (IC50). The percentage inhibition was calculated by using the formula:

\[
\% \text{ Inhibition} = \frac{OD_{\text{blank}} - OD_{\text{sample}}}{OD_{\text{blank}}} \times 100
\]

Preparation of standard
Stock solution of standards (Quercetin, Rutin and Kaempferol) were prepared by dissolving 1 mg of each compound in 1 mL of methanol or methanol water (1:1 v/v). The stock solutions of each standards were diluted to five different concentrations viz. 31.25, 62.5, 125, 250, 500 µg/mL with methanol.

Chromatographic condition
Solvent system used was water with 0.2% acetic acid (Solvent A) and methanol (solvent B). The elution was conducted at 37% solvent B. The flow rate was kept at 0.75 mL/min. All standards and samples were filtered through 0.25 µm Axiva syringe filter and 20 µL loop was used in the study.

Quantification of Quercetin, Rutin and Kaempferol by RP-HPLC
Kaempferol, Rutin and Quercetin quantification determined by RP-HPLC was performed for each extracts of Citrus macroptera Montruz as per the above optimized chromatographic conditions. The retention time of the peaks of the samples were compared with retention time of the tested flavonoids’ peak.

Each of the standard solutions with five different concentration containing 31.25-500 µg/mL were injected on to the HPLC and elution was carried out following the above chromatographic conditions. The analyses were done in triplicate with injection volume of 10 µL. The calibration curve was prepared by plotting peak area (average of three runs) versus concentration of the standard analyte. The amount of quercetin, Rutin, kaempferol were quantified from the linear regression equation of the calibration curve. Limit of detection and limit of quantification was also calculated.

Statistical analysis
The results were expressed as the mean ± SEM for three replicates. Linear regression was used to calculate IC50. Results were considered significant at ***P<0.001, or **P < 0.01 or * P<0.05 when compared test groups v/s control group. For numerical results, one-way analysis of variance (ANOVA) with Tukey-Kramer Multiple Comparisons post tests were performed using GraphPad InStat Version 3 (GraphPad Software). All the graphs and figures were drawn using GraphPad Prism.

Results
Anti-oxidant assay
Antioxidant activity varied over the organic solvent used for the extraction of the plant. In *in vitro* antioxidant activity, ascorbic acid showed IC50 7.64 ± 0.005 µg/mg in DPPH assay, 8.43 ± 0.01 µg/mg in reducing power assay, and 7.56 ± 0.7 µg/mg in nitric oxide assay.

Aqueous extract (NC1), methanol (NC2), aqueous methanol (NC3) showed IC50 of 87.83, 237.95, 276.11 µg/mg respectively in DPPH assay. In reducing power assay NC1, NC2, NC3 showed 31.25, 62.5, 125, 250, 500 µg/mL with methanol.
NC3 showed 94.35, 78.11, and 95.82 µg/mg respectively as their IC50s. Antioxidant activity (IC50) of all the three extracts are concisely presented in Table 1.

### Table 1. In vitro antioxidant assay of extracts (NC1-NC3) of *Citrus macroptera* Montruz.

<table>
<thead>
<tr>
<th></th>
<th>DPPH assay IC50 (µg/mg ± SEM)</th>
<th>Reducing power assay IC50 (µg/mg ± SEM)</th>
<th>Nitric oxide assay IC50 (µg/mg ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>7.64 ± 0.005</td>
<td>8.43 ± 0.01</td>
<td>7.6 ± 0.7</td>
</tr>
<tr>
<td>Aqueous (NC1)</td>
<td>87.83 ± 0.012</td>
<td>36.71 ± 0.01</td>
<td>94.35 ± 0.008</td>
</tr>
<tr>
<td>Methanol (NC2)</td>
<td>237.95 ± 0.005</td>
<td>59.4 ± 0.05</td>
<td>78.11 ± 0.101</td>
</tr>
<tr>
<td>Aqueous methanol (NC3)</td>
<td>276.11 ± 0.101</td>
<td>926 ± 0.33</td>
<td>95.82 ± 0.090</td>
</tr>
</tbody>
</table>

*mean ± standard error mean, µg Ascorbic acid equivalent per gram od DW (dryweight), p< 0.05, n= 5 analyses

**RP-HPLC Linearity**

The graph was plotted with concentration (µg/mL) against area. It was found linear in the concentration range of 31.25-500 µg/mL (31.25, 62.5, 125, 250, 500). The regression obtained were $y=33275x + 4E+06$ for Quercetin, $y=54358x + 6E+06$ for rutin and $y=23564x + 2E+06$ for kaempferol.

### Table 2: Chromatographic parameter of the method used for quantification of compounds in extracts of *C. macroptera* Maontruz.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration range (µg/mL)</th>
<th>Standard curves</th>
<th>LOD (µg/mL)</th>
<th>LOQ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>31.25 – 500</td>
<td>$y=33275x + 4E+06$</td>
<td>0.011</td>
<td>0.032</td>
</tr>
<tr>
<td>Rutin</td>
<td>31.25 – 500</td>
<td>$y=54358x + 6E+06$</td>
<td>0.007</td>
<td>0.020</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>31.25 – 500</td>
<td>$y=23564x + 2E+06$</td>
<td>0.0148</td>
<td>0.045</td>
</tr>
</tbody>
</table>

**Quantification of Kaempferol, Rutin and Quercetin**

Kaempferol, rutin and quercetin HPLC profile is presented in Fig.1. Their content were determined in different extracts of *Citrus macroptera* using linear regression. Quercetin content was found to be 17.69 µg/mL, 431.1 µg/mL, and 232.03 µg/mL respectively in NC1, NC2 and NC3 extracts (Fig.2-4).

Rutin content found in NC1, NC2 and NC3 extracts were 67.91, 108.35, 260.38 µg/mL respectively while kaempferol content was found to be 31.65, 59.50 and 17.66 µg/mL in NC1, NC2 and NC3 extracts respectively. The results are presented in table 3.

### Table 3: Quercetin, rutin and kaempferol content in three extracts of *C. macroptera* Montruz.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Aqueous extract (µg/mL)</th>
<th>Methanol extract (µg/mL)</th>
<th>Aqueous methanol (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>17.69</td>
<td>431.1</td>
<td>232.03</td>
</tr>
<tr>
<td>Rutin</td>
<td>67.91</td>
<td>108.35</td>
<td>260.38</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>31.65</td>
<td>59.50</td>
<td>17.66</td>
</tr>
</tbody>
</table>

**LOD and LOQ**

LOD and LOQ value for Quercetin, Rutin, Kaempferol are presented in table 2.

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*Fig 1: Representative HPLC profile of Quercetin, Rutin and Kaempferol*

*Fig 2: HPLC profile of Aqueous extract*

*Fig 2: HPLC profile of methanol extract*
Discussion

Citrus by-products could be of use as functional ingredients in the production of functional foods, since they are good sources of dietary fiber and bioactive compounds [8]. The citrus peel and seeds are very rich in phenolic compounds, such as phenolic acids and flavonoids; the peel is richer in flavonoids than seeds [9]. In contrast with other types of fruits, around 34% of the fruit is used for juice production, yielding about 44% of peels as byproducts [10].

Both time and solvent extractions played a vital role in the extraction of phenolic contents and their antioxidant properties of *C. macroptera* [11]. We also found that different solvents used for extraction has different potential on quenching the free radicals. The hot methanol extract of the stem bark of *Citrus macroptera* showed potential antioxidant activity with the IC50 value of 178.96 μg/mL whereas the cold methanol and the dichloromethane extracts showed moderate activity with the IC50 of 242.78 μg/mL and 255.78 μg/mL respectively. The n-hexane extract showed mild activity (IC50: 422.94 μg/mL) against DPPH free radical. It is evident that all possess antioxidant activity [12].

Ethanol extract of *Citrus macroptera* fruit peels (EECM) in DPPH scavenging activity (IC50 281.11 μg/mL), Nitric Oxide scavenging activity (IC50 182.89 μg/mL) were comparable with standard Ascorbic acid [13]. In our study, the order of antioxidant activities was aqueous> methanol> aqueous methanol extracts of *Citrus macroptera* fruit peels. IC50 of DPPH scavenging assays range from 7.9-276.11 μg/mL which reveal a promising value as compared to earlier reported value. *C. macroptera* has wide range of uses viz. the dried rind of the fruit as flavouring spice in preparation of meat dishes, the juice of the fruit as medicine for treatment of stomach ailments as well as digestive enzyme. Quercetin has been reported to have antioxidant and anticarcinogenic qualities via its inhibition of neoplastic transformation by blocking activation of the mitogen-activated protein kinase (MAPK) pathway and stimulation of cellular protection signaling [14]. Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hepatoprotective activities [15]. Selected flavonoids can directly scavenge superoxides, whereas other flavonoids can scavenge the highly reactive oxygen-derived radical called peroxynitrite. Epicatechin and rutin are also powerful radical scavengers [16].

The scavenging ability of rutin may be Antioxidants 2015, 4 500 due to its inhibitory activity on the enzyme xanthine oxidase. Rutin is also known for its anti-inflammatory and vasoactive properties, as well as for its capability to diminish capillary permeability and to reduce the risk of arteriosclerosis, whereby reducing coronary heart disease, possibly through the diminishing of platelet aggregation [17, 18]. There are also studies that show a dose-response effect of rutin in inhibiting low-density lipoprotein (LDL) peroxidation [19] and the antioxidant activity of rutin in Fenton reaction [20].

Conclusion

With varying solvent type, the magnitude of activity of the extracts and fractions also varies. The extracts of *Citrus macroptera* showed antioxidant activity. It can be used for drug discovery and development to battle those diseases which are induced by oxidative stress. Bioactive compounds will be targeted for isolation from the active fractions and structure will be identified using NMR, mass spectrometry etc. Thus, the present study reveals the antioxidant activity in *Citrus macroptera* Montraz. The results illustrated that the extracts exhibited concentration and solvent dependent bioactivities. Thus our findings serves to exemplify the potential of this wild orange as natural dietary antioxidants.

Isolation and identification of lead molecule for drug discovery and development to battle those diseases which are induced by oxidative stress. Bioactive compounds will be targeted for isolation from the active fractions and structure will be identified using NMR, mass spectrometry etc.

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Conflict of interest: There is no conflict of interest among the authors in carrying out the work.

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