Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(3): 474-478
Received: 03-03-2017
Accepted: 04-04-2017

Khumukcham Nongalleima
a) Department of Botany, Nagaland University, Lumami, Nagaland, India
b) Institute of Bioresources and Sustainable Development (IBSD), Takyelpat, Imphal, Manipur, India

T Ajungla
Department of Botany, Nagaland University, Lumami, Nagaland, India

Chingakham B Singh
Institute of Bioresources and Sustainable Development (IBSD), Takyelpat, Imphal, Manipur, India

Correspondence
Chingakham B Singh
Institute of Bioresources and Sustainable Development (IBSD), Takyelpat, Imphal, Manipur, India

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-IC_{50} 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

(300mL) and 500 mL aqueous methanol (1:1 volume/volume) at room temperature for 2 days with occasional stirring. After filtration, the filtrate was evaporated at 40^0C under reduced pressure in a rotary evaporator (Buchi, Switzerland).

Antioxidant assay

The antioxidant property was assayed using the following three methods:

Reducing power assay

100 µL of sample with different concentrations (10-100µg/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 fericyanide. The reaction mixture was incubated at 50^0C for 20 minute. After incubation, 100 µL of 10 % trichloro acetic acid (w/v) were added. It was then centrifuged at 5000 rpm for 10 min (Eppendorf centrifuge 5430 R). The upper layer (200 µL) was mixed with 200 µL deionized water and 40 µL of 0.1 % ferric chloride. The absorbance was read at 700 nm in a 96 well microplate reader (Thermo Scientific) [6]. Higher absorbance indicates higher reducing power. The assays were carried out in triplicate and the results are expressed as mean values ± standard error mean. Ascorbic acid was used as standard. Percentage inhibition was calculated and this activity was expressed as an inhibition concentration 50 (IC_{50}).

The percent increase in reducing power was calculated using the following equation.

% Reduction= [1- (1-As/Ac)] X 100

As=maximum absorbance of max concentration of standard,

Ac- absorbance of sample

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. 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 25^0 C for 150 min. Griess reagent (500 µL) was added and incubated at 25^0 C for 30 minute. 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– 100µg/mL). The mixture were shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517nm in Thermo Multiscan Spectrum. 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 (IC 50). The percentage inhibition was calculated by using the formula.

$$\% \text{ Inhibition} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

RP- HPLC analysis

Instrument

The high performance Liquid Chromatography (HPLC) equipment used was UFLC Shimadzu, CBM-20A communication bus module, 2 solvent delivery system, pump LC-20 AD (A), LC -20 (B), a SPD-M20 photodiode array detector, a 2 chamber in-line degasser. The analysis was carried on a Purospher STAR RP-18 endcapped column (Milipore), 5µm particle size, 250mm X 4.6 mm

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^{-1} minute. 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 IC_{50}. 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 extraction of the plant. In i*n 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 36.71 µg/mg, 59.4 µg/mg, and 926 µg/mg respectively as their IC50s. In Nitric oxide assay NC1, NC2,

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.

	DPPH assay IC_{50} µg/mg ± SEM*	**Reducing power assay IC_{50} µg/mg ± SEM***	**Nitric oxide assay IC_{50} µg/mg ± SEM***
Ascorbic acid	7.64 ± 0.005	8.43 ± 0.01	7.6 ± 0.7
Aqueous (NC1)	87.83 ± 0.012	36.71 ± 0.01	94.35 ± 0.008
Methanol (NC2)	237.95 ± 0.005	59.4 ± 0.05	78.11 ± 0.101
Aqueous methanol (NC3)	276.11 ± 0.101	926 ± 0.33	95.82 ± 0.090

* 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 regession obtained were y=33275x + 4E+06 for Quercetin, y=54358x + 6E+06 for rutin and y=23564x + 2E+06 for kaempferol.

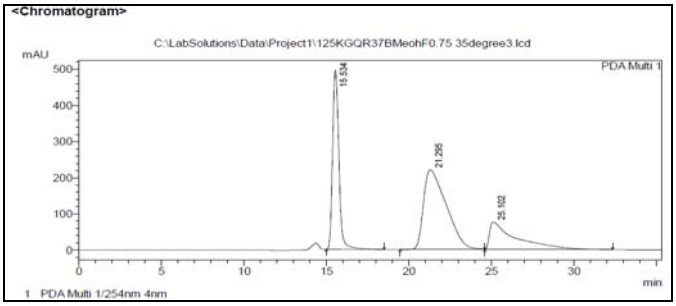

Fig 1: Representative HPLC profile of Quercetin, Rutin and Kaempferol

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

Compounds	**Concentration range (µg/mL)**	**Standard curves**	**LOD (µg/mL)**	**LOQ (µg/mL)**
Quercetin	31.25 – 500	y=33275x + 4E+06	0.011	0.032
Rutin	31.25 – 500	y=54358x + 6E+06	0.007	0.020
Kaempferol	31.25 – 500	y=23564x + 2E+06	0.0148	0.045

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.

Compounds	**Aqueous extract (µg/mL)**	**Methanol extract (µg/mL)**	**Aqueous methanol (µg/mL)**
Quercetin	17.69	431.1	232.03
Rutin	67.91	108.35	260.38
Kaempferol	31.65	59.50	17.66

LOD and LOQ

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

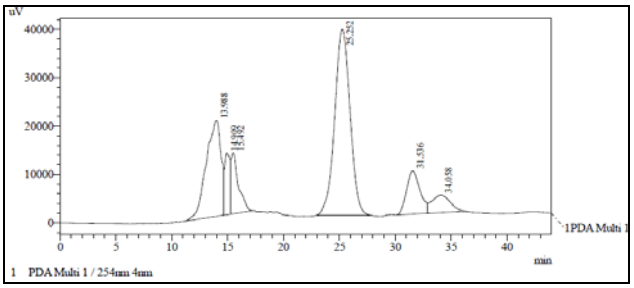

Fig 2: HPLC profile of Aqueous extract

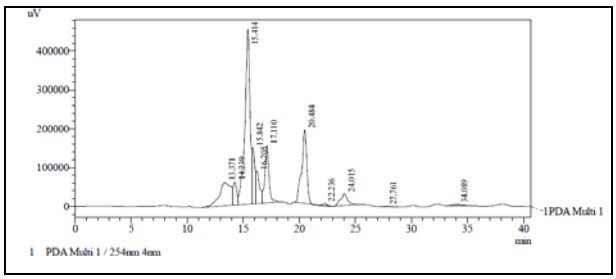

Fig 2: HPLC profile of methanol extract

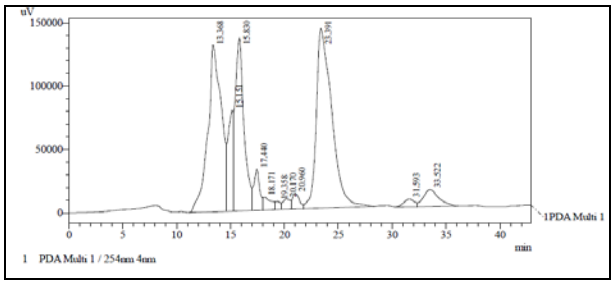

Fig 2: HPLC profile of Aqueous methanol extract

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].

Ethanolic 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^{-1} 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 anti-carcinogenic 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 hapatoprotective 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* Montruz. The results illustrated that the extracts exhibited concentration and solvent dependent bioactivies. 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 from the selected medicinal plant for which might be accountable for the activity is under progress.

Acknowledgements

Authors are thankful to Department of Biotechnology, Govt. of India for financial support to carry out the study, and also to Director, IBSD, Imphal for providing the laboratory facilities for carrying out the studies.

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

Reference

1. Stone BC. Rutaceae. in: Dassanyake MD, Fosberg FR, (Eds.), A Revised Handbook of the Flora of Ceylon, Vol. V. Smithsonian Institution and the National Science Foundation, Washington, DC. 1985,
2. Dugo G, Di Giacomo A. Citrus: The Genus Citrus, Medicinal and Aromatic Plants- Industrial Profiles, Taylor & Francis group, London, 2002.
3. Silalahi J. Anticancer and health protective properties of *Citrus* fruit components, Asia Pacific J. Clinical Nutri., 2002; 1(1):79-84.
4. Sidana J, Saini V, Dahiya S, Nain P, Bala S. A Review on Citrus – The Boon of Nature, Int. J. Pharm. Sci. Rev. Res., 2013; 18(2):20-27.
5. Albishi T, John JA, Al-Khalifa AS, Shahidi F. Phenolic content and antioxidant activities of selected potato varieties and their processing by-products. J. Funct. Foods, 2013; 5:590-600.
6. Gangwar M, Gautam MK, Sharma AK, Tripathi YB, Goel RK, Nath G. *et al.* Antioxidant Capacity and Radical Scavenging Effect of Polyphenol Rich *Mallotus philippenensis* fruit extract on Human Erythrocytes: An *in Vitro* Study. The Scientific World J., 2014; http://dx.doi.org/10.1155/2014/279451
7. Sakthivel G, Dey A, Nongalleima Kh, Chavali M, Rimal Isaac RS, Surjit Singh N, *et al.* Deb L. *In Vitro* and *In Vivo* Evaluation of Polyhedral Formulation against Russell's Viper and Cobra Venom and Screening of Bioactive Components by Docking Studies, Evidence-Based Compl.Alt. Med., 2013, 2013; 1-12.
8. Marin FR, Martinez MT, Uribesalgo SC, Frutos MJ. Changes in nutraceutical composition of lemon juices according to different industrial extraction systems. Food Chem., 2002; 78:319-324.
9. Sawalha SMS, Arráez-Román D, Segura-Carretero A.

Quantification of main phenolic compounds in sweet and bitter orange peel using CE−MS/MS. Food Chem., 2009; 116:567-574.
10. Li S, Lo C, Ho C. Hydroxylated Polymethoxyflavones and Methylated Flavonoids in Sweet Orange *(Citrus sinensis)* Peel. J. Agric. Food Chem., 2006; 54:4176-4185.
11. Gope PS, Dutta AK, Makhnoon S, Banik S, Siddiquee MA, Kabir Y. *et al.* Effect of solvent and extraction time on the antioxidant properties of *Citrus macroptera*, Int.J. Drug Dev. Res., 2014; 6(2):28-35.
12. Chowdhury SA, Hossain SM, Datta BK, Choudhury MH. Chemical and Antioxidant Studies of *Citrus macroptera*. Bangladesh J. Sci. Ind. Res., 2008; 43(4):449-454.
13. Rahman H, Eswaraiah MC, Dutta M. *In-vitro* Anti-oxidant Activity of *Citrus macroptera* (Var. Annamensis) Fruit Peels Extracts. Int. J. Pharm. Pharmaceut. Sci., 2014; 6(1):364-371.
14. Ding M, Zhao JS, Bowman L, Lu YJ, Shi XL. Inhibition of AP-1and MAPK signaling and activation of Nrf2/ARE pathway by quercitrin. Int. J. Oncol. 2010; 36:59-67.
15. Tapas AR, Sakarkar DM, Kakde RB. Flavonoids as nutraceuticals: A review. Trop. J. Pharm. Res., 2008; 7:1089-1099.
16. Hanasaki Y, Ogawa S, Fukui S. The correlation between active oxygen scavenging and antioxidative effects of flavonoids. Free Radic. Biol. Med., 1994; 16: 845-850.
17. La Casa C, Villegas I, de la Lastra C, Motilva V, Calero MJ. Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions. J. Ethnopharm., 2000; 71:45-53.
18. Jiang P, Burczynski F, Campbell C, Pierce G, Austria JA, Briggs CJ. *et al.* Rutin and flavonoid contents in three buckwheat species *Fagopyrum esculentum, F. tataricum,* and *F. Homotropicum* and their protective effects against lipid peroxidation. Food Res. Int., 2007; 40:356-364.
19. Cailet S, Yu H, Lessard S, Lamoureux G, Ajdukovic D, Lacroix M *et al.* Fenton reaction applied for screening natural antioxidants. Food Chem., 2007; 100:542-552.
20. Peng X, Zheng Z, Cheng KW, Shan F, Ren GX, Chen F. *et al.* Inhibitory effect of mung bean extract and its constituents vitexin and isovitexin on the formation of advanced glycation endproducts. Food Chem., 2008; 106:475-481.