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Phytochemical, total phenolic, total flavonoid and total flavonol content estimation in *Citrus macroptera* Montruz

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Abstract
Citrus macroptera Montruz. is one of the citrus species belonging to Rutaceae family, commonly known as wild orange. The fruits were collected, its rind was dried, powdered and organic extracts; aqueous (NC1), methanol (NC2), aqueous methanol (NC3) were primed. Phytochemical screening of different extracts were performed qualitatively. The various test demonstrated the presence of alkaloid, phenolic, flavonoid, Coumarin glycosides, Saponin glycoside, Mucilage. Among the tested samples, methanol extract (NC2) showed highest phenolic content (7.47 ± 0.01 µg/g) Gallic acid equivalent. Methanolic extract also showed highest flavonoid content (7.28 ± 0.12 µg/g) Quercetin equivalent. Flavonol content was less in all the extract, the highest content was however present in aqueous methanol extract (1.07 ± 0.19 µg/g) Quercetin equivalent.

Keywords: *Citrus macroptera,* phytochemical screening, phenolic, flavonoid, flavonol

Introduction
Citrus macroptera (Var. annamensis) belongs to the Rutaceae family and it is native to the regions of Southeast Asia mainly Myanmar, Thailand, Indonesia Malaysia, Papua New Guinea, Sylhet Division of North Eastern Bangladesh and North Eastern India mainly Manipur and Assam, local in Bengali it is called "hatkora" or "shatkora" and in English known as Wild orange [1, 2]. It is called as “Heiribob” in Manipuri. It is a semi wild species and used as medicine by local tribes of Assam, India [3]. Nair and Nayar, (1997); Sharma *et al.,* (2004) [4, 5] reported that *C. macroptera* Montr. occur in the subtropical forests of North-east India and the foot hills of the East Himalayas.

In Manipur this Wild Orange (*C. macroptera*) grows well with good quality fruiting as location specific crop of Chandel District and Jiribam Sub-Division of Imphal East District, although all Citrus are primarily valued for the fruit, which is either eaten alone (sweet orange, tangerine, grapefruit, etc.) as fresh fruit, processed into juice, or added to dishes and beverages (lemon, lime, etc.); however this Wild Orange 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, the fruit pulp as washing detergent, not the least the most important is the essential oil from the leaves which otherwise wasted. (http://manipursfac.com/wild-orange-citrus-macroptera/ 1/.). This species grow in sloppy and high altitude area. It is costly species, the price ranges upto Rs.100 per fruit in off seasons. Cultivation of *C. macroptera* is the sole livelihood income generator of some tribal areas and the economy of the village entirely depended on it. The peel is dried and kept in every household as an essential spice ingredients for cooking. The juice is used for treating and flushing stone from Kidney.

Citrus flavonoids have potential antioxidant (prevents aging), anti-cancer, antiviral, anti-inflammatory activities, effects on capillarity, and cholesterol-lowering ability [6]. 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.[7]. *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 [8]. Thus, the study was designed to test the presence of phytochemicals and to estimate the total phenolic, total flavonoid and total flavonol content.

Materials and method

Plant materials, preparation of extract

Fruits of *citrus macroptera* were collected from Kwatha Village, Chandel District, Manipur. It was identified by scientists of IBSD, Imphal and faculty of Botany Department, Nagaland University (Fig. 1A). A voucher specimen was deposited at IBSD with voucher number IBSD/M-1031A. Rinds of the fruits (Fig.1B) were peeled off; dried and coarse powder was made using a commercial blender. 60 g powdered sample each was cold extracted 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 ^{0}C under reduced pressure in a rotary evaporator (Buchi, Switzerland).

Phytochemical screening

The extracts were analyzed for the presence of phytochemical constituents, qualitatively following standard procedures (9-10).

Test for alkaloid

Dragendorff's test: To 2-3 ml filtrate, few drops of Dragendorff's reagent. Formation of orange brown precipitation is formed.

Hager's test: To 2-3 ml filtrate, Hager's reagent gives yellow precipitation.

Wagner's test: To 2-3 ml filtrate, few drops of Wagner's reagent was added and observed for color change. Reddish brown precipitation indicated the presence of alkaloid.

Test for flavonoids-

Shinoda test: to dry powder or extract, add 5 ml of 95% ethanol, few drops of concentrated HCl and 0.5g magnesium turnings. Observance of pink color. To small quantity of residue, lead acetate solution was added. It was observed for yellow color precipitation. Increasing amount of sodium hydroxide to the residue shows yellow coloration which decolorizes after addition of acid.

Test for phenolic compound

Lead acetate test: To the test solution, a few drops of 10% lead acetate solution were added. Formation of white precipitate indicated the presence of phenolic compounds.

Ferric chloride test: To the test solution, a few drops of ferric chloride were added. A dark green color indicated the presence of phenolic compound.

Test for carbohydrate

Molish's test: To 2-3 ml aqueous extract, few drops of alpha-naphthol solution in alcohol was added. The solution was shaken and added conc.H_2SO_4 from sides of the test tube. A violet ring formed at the junction of two liquids indicated the presence of carbohydrates.

Test for reducing sugars:

Fehling's test

1 ml of Fehling's A and 1 ml of Fehling's B solution were mixed and boil for 1 minute. An equal volume of test solution was added. It was heated in boiling water bath for 5-10 min. Appearance of yellow precipitation followed by brick red precipitation indicate the presence of reducing sugars.

Test for non-reducing polysaccharides (starch)

a. Iodine test: 3 ml of test solution was mixed with few drops of dilute iodine solution. Blue color appearance, it disappears on boiling and reappearance on cooling indicated the presence of non-reducing polysaccharides.

Test for gum

The test solution was hydrolyzed using dilute HCl. Fehling's test was performed on it. Development of red color indicated the presence of gum.

Test for mucilage

Powdered drug material showing red color with ruthenium red indicated the presence of mucilage.

Tests for proteins

Biuret test

To 3 ml of test solution, 4% Sodium hydroxide and few drops of 1% Copper sulphate was added. Appearance of yellow and pink color indicated the presence of proteins.

Test for saponin

To the oil (10 ml) or sample solution, 25 ml if 10% Sodium hydroxide was added. They were boiled in boiling water bath for 30 min. It was cooled. Excess sodium sulphate was added, soap forms, rises to the top and it was filtered. To the filtrate, sulphuric acid was added and evaporated. The residue was collected and dissolved in ethanol. To the ethanolic residue, few crystals of potassium hydrogen sulphate was added and heated vigorously. Pungent odour of acrylic aldehyde is produced. To the ethanolic residue, few drops of copper sulphate and sodium hydroxide solution added. Observation of clear blue solution indicated the presence of saponin.

Test for steroid

Salkowski test

To 2 ml of extract, 2ml of chloroform and 2 ml of concentrated sulphuric acid was added. It was shaken well, chloroform layer appears red and acid layer shows greenish yellow fluorescence.

Test for cardiac glycosides

a. Test for deoxy-sugars (Keller-Killiani test)

To 2ml extract, glacial acetic acid, 1-2 drop of 5% Ferric chloride and concentrated sulphuric acid was added. Appearance of reddish brown color at the junction of the two liquid layers and bluish green upper layer indicated the presence of deoxy sugars.

b. Test for anthraquinone glycoside

To 3ml extract, dilute sulphuric acid was added. It was boiled and filtered. To the cold filtrate, equal volume of benzene or chloroform was added. It was shaken well and separated the organic solvent. Ammonia was added. Appearance of amonical layer turning pink or red indicated the presence of anthraquinone glycoside.

c. Test for saponin glycoside

Foam test: the extract was shaken vigorously with water. Appearance of persistent foam indicated the presence of saponin glycosides.

Test for coumarin glycosides

Moistened dry powder was taken in a test tube. The test tube was covered with filter paper soaked in dilute sodium

hydroxide. It was kept in water bath. The filter paper was exposed to UV light. Yellowish green fluorescence indicated the presence of coumarin glycosides.

Estimation of total phenolic content (TPC)

Total Phenolic content of extracts was determined by Folin–Ciocalteu method. 100µl of Extract solution (100µg/mL) was mixed with 500µl 10% (v/v) F–C reagent. 400µl of 7.5 % Na_2CO_3 was added into each tube and incubate the assay tubes at room temperature for 1 h. 300mL sample or blank was transferred from the assay tube to a clear 96 well microplate and absorbance of each well was taken at 765nm. Standard curve was calculated from standard Gallic acid at 765nm in Thermo Multiscan Spectrum and total Phenolics was obtained as Gallic acid equivalents using the regression equation [11].

Estimation of total flavonoid content (TFC)

The total flavonoid content of the extracts was determined by aluminium chloride ($AlCl_3$) colorimetric method. 0.5 mL of extract was mixed with 1.5mL methanol, 0.1mL of 10% $AlCl_3$, 0.1 mL of 1M potassium acetate and 2.8mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with Thermo Multiscan Spectrum. Using Quercetin as standard, standard curve was prepared and linearity was obtained in the range of 10-100µg/mL using the standard curve the total flavonoid content was expressed as quercetin equivalent in percentage w/w of the extracts [11].

Estimation of total flavonol content (TFoC)

Total flavonol content was estimated following Miliauskas *et al*. 2004 [12]. 500 µl of sample (100µg/mL) was mixed with 500 µl $AlCl_3$ (20g/l), 1.5 mL of sodium acetate. It was incubated at 25^0 C for 2.5 h. The absorbance was read at 440 nm. Quercetin (10-100 µg/mL) was used to generate standard curve.

Results and Discussion

The present study carried out on the extracts of *Citrus macroptera* peels revealed the presence of phytochemicals responsible for medicinal bioactivities. The studied chemical test in the extracts are presented in Table 1. The various test demonstrated the presence of alkaloid, phenolic, flavonoid, Coumarin glycosides, Saponin glycoside, Mucilage. However gum and reducing sugar were found to be present in aqueous methanol extract only.

Resin, anthraquinone glycosides, steroid, protein and Iodine were absent in all the tested extracts. Total phenolic, total flavonoid and total flavonol content varies with the varying solvent used for extraction. The contents of tested phytochemical in the extracts are presented in Fig.1. Among the tested samples, methanol extract (NC2) showed highest phenolic content (7.47 ± 0.01 µg/g) Gallic acid equivalent. Methanolic extract also showed highest flavonoid content (7.28 ± 0.12 µg/g) Quercetin equivalent (Table 2). The flavonoids in the citrus juice combine with aluminium to form a complex flavonoid-aluminium that could be measured at 430 nm [13]. Flavonol content was less in all the extract, the highest content was however present in aqueous methanol extract (1.07 ± 0.19 µg/g) Quercetin equivalent.

Various experiments have been demonstrated that phenolic compounds such as flavonoids, phenolic acids, tannins, etc. are potential antioxidant and antioxidant activity of these compounds is due to their ability to scavenge free radicals. Accumulation of free radicals can cause pathological conditions such as asthma, arthritis, inflammation, neuro-degenation, heart disease, aging effect [14]. Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties [15]. Phenolic compounds are mostly composed of flavonoids, phenolic acids, stilbenes, coumarins and tannins [16].

Flavanones, flavones and flavonols are three types of flavonoids which occur in Citrus fruit [17]. The main flavonoids found in citrus species are hesperidine, narirutin, naringi and eriocitrin [18, 19]. Epidemiological studies on dietary Citrus flavonoids improved a reduction in risk of coronary heart disease [20, 21] and is attracting more and more attention not only due to their antioxidant properties, but as anti-carcinogenic and anti-inflammatory agents because of their lipid anti-peroxidation effects [22-24].

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.

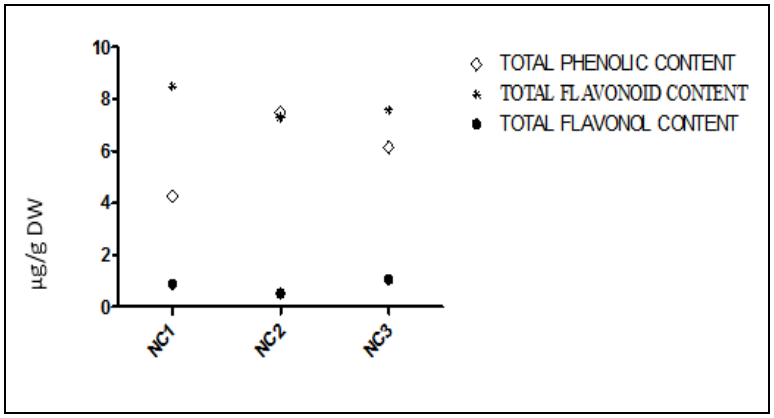

Fig 1: Total phenolic (µg of GAE/g of DW), total flavonoid (µg of QE/g of DW) and total flavonol (µg of QE/g of DW), of three extract (NC1-NC3) n=3 analyses, p<0.05.

Table 1: Phytochemical screening of NC1, NC2 and NC3 extracts of Citrus macroptera Montruz.

Phytochemical constituent	NC1	NC2	NC3
Alkaloid	+	+	+
Dragendorff's test	-	-	-
Hager's test	+	+	-
Wagner's test	+	-	-
Phenolic	+	+	+
Flavonoid	+	+	+
Resin	-	-	-
Coumarin glycosides	+	+	+
Cardiac glycoside (Keller Keliani test)	+	+	+
Anthraquinone glycoside	-	-	-
Saponin glycoside	+	+	+
Steroid	-	-	-
Mucilage	+	+	+
Protein	-	-	-
Iodine	-	-	-
Gum	-	-	+
Reducing sugar	-	-	+

Table 2: Quantification of Total phenolic, total flavonoid and total flavonol content of three extract (NC1-NC3) of *Citrus macroptera* Montruz.

	Total Phenolic content µg/mL GAE* ± SEM	Total flavonoid content µg/mL QE** ± SEM	Total flavonol content µg/mL QE ± SEM
Aqueous (NC1)	4.26 ± 0.01	5.49 ± 0.01	0.89 ± 0.2
Methanol (NC2)	7.47 ±0.01	7.28 ± 0.12	0.53 ± 0.02
Aqueous methanol (NC3)	6.14 ±0.001	6.58 ± 1.0	1.07 ± 0.19

*Gallic acid equivalent, ** Quercetin equivalent, results are expressed as µg of GAE/g of Dry weight (DW), n=3 analyses, p<0.005.

Conclusion

The content of phenolic, flavonoid and flavonol were quantified for all the extracts and fractions. With varying solvent type, the magnitude of activity of the extracts also varies.

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