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Anti-oxidant, Anti-inflammatory and Cytotoxic activity of *Citrus macroptera* Montruz extracts

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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 anti-inflammatory activities were done followed by *in vivo* studies. NC1 showed highest antioxidant (DPPH-IC_{50} 87.83 µg/g, reducing power assay 36.71 µg/g) and highest protease inhibitory (20.44 µg/mL) activity. It showed IC_{50} 148.44 µg/mL against HeLa cells in MTT (3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphnyltetrazolium bromide) assay. In *in vivo* anti-inflammatory activity assay, the effect was almost comparable with standard drug diclofenac sodium at 4th and 6th hr after administration when compared to the control group. NC2 exhibited more amount of anti-inflammatory activity than the NC1 and NC3 extract. The designed studies indicate remarkable potential of *C. macroptera* as dietary source of antioxidant, anti-inflammatory and cytotoxic agents. Studies on isolation of lead compound attributing the studied bioactivity is under progress.
Keywords: DPPH, protease inhibitory, albumin denaturation, MTT, clonogenic activity, Carrageenan.

Introduction:

Medicinal plants and their natural products are believed to be rich sources of antioxidants. Living body system has its own oxidative defense mechanism, however, the imbalance of the reactive oxygen species and this defense system causes oxidative stre aswqaszAss which in turn provoke the body system to generate various extreme and obsessive disorders. During such oxidative stress conditions, free radicals are produced as its metabolic product which is the origin of various ailments like cardiovascular, inflammatory, cancer, neurodegenerative diseases. Phytochemicals being the rich natural sources of antioxidants, they are given prime interest for their role in uses and applications of these plants for prevention of inflammatory, cancer and related diseases.

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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.

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 and anti-inflammatory activity of *Citrus macroptera* which is locally called as "heiribob" in Manipur, NorthEast India. In our present study we report free radical scavenging and anti-inflammatory activity of the selected citrus species. *In vitro* studies were further supplemented with *in vivo* studies using mice as model animal. The designed study is the first of its kind to determine the bioactivies of this rare citrus species locally available in hill district of Manipur, NorthEast India.

Materials and Methods

Plant materials

Fruits of *citrus macroptera* were collected from Kwatha Village, Chandel District, Manipur, North East India. It was identified by scientists of IBSD, 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 (Fig.S1B) 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).

Fig S1. A. Leaves, fruits and seeds of *Citrus macroptera*. Fig. S1 B. Rind of *Citrus macroptera*

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)[5]. 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 30min. 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.

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

In-vitro Anti-inflammation

In vitro anti-inflammatory potential was assessed using the following methods:

Protease inhibitory assay

1 mL of trypsin (0.5mg. mL^{-1}) prepared in 0.1 M phosphate buffer, pH-7 was preincubated with 1 mL of sample with different concentration (10-100 ug/mL) at 37^0C for 15 minute. After incubation 2 mL of 1 % casein prepared in 0.1 M phosphate was added. It was then incubated at 37^0C for 30 minute. The reaction was terminated by adding 2.5 mL of 0.44 M Trichloroacetic acid. It was transferred to centrifuge tube and centrifuge at 10,000 rpm for 15 minute. Supernatant was taken and OD was measured at 280 nm[8].

Heat induced haemolysis assay

2 ml of reaction mixture consisting of 1 ml of test sample solution and 1 ml of 10% RBC suspension was taken in 2ml micro centrifuge tube. It was incubated at 56^0C for 30 min.in water bath. The reaction mixture was cooled and centrifuged at 2500 rpm for 5 min. The supernatant was taken and absorbance was taken at 560 nm. Saline and Diclofenac sodium was taken as control and standard reference respectively[9].

Inhibition of albumin denaturation

Reaction mixture of 1 % aqueous solution of bovine serum albumin (Sigma) and test extract at different concentration (10-100 µg/mL) was taken in a centrifuge tube and pH was adjusted to 6.8 using 1N HCl. It was incubated at 37^0 C for 20 min followed by heating at 57^0C for 20 min. The solution was cooled and absorbance was taken at 660nm[10].

in-vivo Antioxidant and Anti-inflammatory Study

Animal

Adult female Swiss albino mice (30 g ± 1.2) was used for the study. The animals were purchased from Regional Institute of Medical Sciences, RIMS, Imphal, Manipur (India). They were kept under standardized conditions (temperature 27 ± 2 ^{0} C, and light/dark cycle of 12 hrs.) and fed a normal laboratory diet. The experiments were performed based on animal ethics guidelines of Institutional Animal Ethics Committee.

Acute toxicity

Acute oral toxicity was tested. Female albino mice (n=6) were kept fasting for overnight providing only water, after which the extracts were administered orally at the dose of 50mg.kg^{-1}.p.o.$^{-1}$ and observed for 14 days. Mortality was not observed, the procedure was repeated for higher dose (100, 500, 2000 mg.kg^{-1}/p.o.$^{-1}$)

***In vivo* antioxidant**

Experimental design

After 1 week of time, mice were divided into 5 groups, n= 5. The extract was dissolved in 0.3 % Tween 80 to obtain a 15mg.mL kg^{-1} solution. Group 1 animal received 1 ml distill water and served as normal control. The following treatment pattern was adopted for the study. The extracts and standards were administered orally using a metal oropharyngeal cannula.

- Group I – Normal Control (1ml distill water p.o.)
- Group II – CCl_4 control
- Group III - CCL4 + L-ascorbic acid 10 mg.Kg^{-1}
- Group IV- CCL_4 + aqueous extract (NC1) 50mg.Kg^{-1} p.o
- Group IV - CCL_4 + methanol extract (NC2) 50mg. Kg^{-1} p.o
- Group V - CCL_4 + aqueous methanol extract (NC3) 50mg. Kg^{-1} p.o

After 24 hrs. of 7 th day treatment, blood was collected from retro-orbital plexus under ether anesthesia. The blood samples were allowed to clot, centrifuged and serum was collected. Certain volume of blood was separately transferred to a tube and treated with EDTA (plasma EDTA). The animals were then sacrificed and dissected. Liver and brain tissues were collected for *in vivo* antioxidant and other studies. The same treatment pattern was followed for two other doses (100 mg/Kg p.o. and 150 mg/Kg p.o.)

Superoxide dismutase (SOD) assay

The assay was done using the SOD assay Kit (Sigma). Briefly, 1 mL of working standard solution (WST) was diluted with 19 mL of buffer solution. The enzyme solution tube was centrifuged for 5 sec. It was mixed by pipetting and 15µl of enzyme solution was diluted with 2.5 mL of dilution buffer. 20 µl of sample solution was added to sample well and blank 2 well. 20 µl of double distill H_2O was added to each blank 1 and

blank 3 well. 200 µl of WST solution was added to each well, and they were mixed. To the blank 2 and blank 3 wells, 20 µl of dilution buffer was added. To the sample wells and blank 1 well 20 µl of enzyme working solution was added and mixed thoroughly. The plate was incubated at 37^0C for 20 min. It was then read at 450 nm.

SOD activity (% Inhibition) =

$$100X \frac{\text{(Absorbance of blank 1- Absorbance of blank 3) – (Absorbance of sample- Absorbance of blank 2)}}{\text{(Absorbance of blank 1- Absorbance of blank 3)}}$$

Catalase (CAT) assay

Catalase assay was done using catalase kit (Sigma) following the instructions provided in it. Briefly, 10 µl of serum or EDTA plasma or liver tissues, 25 µl of assay buffer was mixed with 12.5 µl 200 mM H_2O_2 and incubated for 1-5 min. 450 µl of stop solution was added. 10 µl of reaction mixture from the above was transferred to a microtube and 500 µl of color reagent was added. It was kept at room temperature for color development. OD was taken at 520 nm within 15 min.

Glutathione reductase assay

The assay was carried out using glutathione reductase assay kit (Cayman, USA). Briefly, 120 µl of assay buffer and 20 µl of GSSG was added to non-enzymatic wells. 100 µl of assay buffer, 20 µl of GSSG and 20 µl of diluted GR control was added to positive control well. 100 µl of assay buffer, 20 µl of GSSG and 20 µl of sample was added to sample well. 50 µl of NADPH was added to all the wells. The plate was shake for a few seconds. The absorbance was read at 340nm once every minute to obtain atleast 5 time points. The activity was calculated using the following

$$\blacktriangle A_{340}/\text{min.} = \frac{A_{340}\text{ (Time 2)} - A_{340}\text{ (Time 1)}}{\text{Time 2 (min.)} - \text{Time 1 (min.)}}$$

$$\text{GR activity} = \frac{\blacktriangle A_{340}/\text{min.}}{0.00373\ \mu M^{-1}} \times \frac{0.19\text{ ml}}{0.02} \times \text{sample dilution.}$$

In vivo anti-inflammatory

Carrageenan - induced paw edema

Experimental design

After 7 days of acclimatization, the animals were divided into 11 groups of 5 animals each. The treatment was done as follows.

Group I - Animals (Control) were administered 1ml distill water p.o., /animal, Group II - Aqueous extract (NC1) 50mg.Kg^{-1}p.o, Group III - Aqueous extract (NC1) 100mg. Kg^{-1}p.o., Group IV - Aqueous extract (NC1) 150mg. Kg^{-1}p.o., Group V - methanol extract (NC2) 50mg. Kg^{-1}p.o, Group VI - methanol extract (NC2) 100mg. Kg^{-1} p.o., Group VII- methanol extract (NC2) 150mg.Kg^{-1} p.o., Group VIII- aqueous methanol extract (NC3) 50mg. Kg^{-1}p.o., Group IX- aqueous methanol (NC3) extract 100mg. Kg^{-1}p.o., Group X- aqueous methanol (NC3) extract 150mg. Kg^{-1}, Group XI- Diclofenac sodium salt 50 mg. Kg^{-1} p.o.

The extracts were prepared in 0.3 % tween 80. Inflammation was induced by injecting 0.1 of 0.1% w/v carrageenan sodium salt subcutaneously in the sub-plantar region of the left hind paw in each groups. The plant extracts NC1, NC2, NC3 in three different doses, 50, 100 and 150 mg. Kg^{-1} p.o and vehicle was administered orally 60 minutes prior to injection of carrageenan. 1 hour after oral administration of reference and test drugs, carrageenan was injected. The hind paw volume was measured Plethysmometrically before and after the carrageenan injection, at hourly intervals for 6 hr.

Cytotoxicity assays

MTT assay

Cytotoxicity assay was done by using MTT (3-(4, 5- Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) assay. Cells (Hela) were plated at 5000/well/0.1 mL in complete medium.100 µl different concentration of the aqueous extract of *C. macroptera* was added and incubated at 37^0C at 95 % humidity, 5 % CO_2 ± for 72 h. At the end of 72h, 20 µl of MTT solution (5mg. Kg^{-1} mL) per well were added and incubated at 37^0C for 2 h. At the end of 2h, 0.1 mL of lysis buffer (20% W/V of SDS dissolved at 37^0C in a solution of 50 % N, N-dimethyl formamide in distilled water) was added per well and incubated at 37^0C for 4 h-overnight. The plate was read at 590 nm with just the lysis buffer as control. The assay replicated thrice.

Clonogenic assay

Cell survival was assessed using clonogenic assay of Puck and Marcus 1955[11]. 100-200 cells were inoculated in 5 cm^2 petridishes containing 5 mL media. The cells were allowed to attach for 12-16 hr prior to addition of plant extracts. After 16 hr the cultures were exposed to 5-320 µg/ mL concentration of aqueous extract. Medium containing the extract were removed, and washed with phosphate buffer saline twice. Fresh medium without any extract or drug was added and allowed the culture to grow for another 10 -11 days. After that, the colonies were stained in 1 % crystal violet in methanol for 15-20 minutes. Colonies were fixed in 3.7% Paraformaldehyde (PFA) or 10 % formalin. The culture dishes were washed in running water until the blue background of the dishes become clear. The clusters containing 50 or more cells were scored as a colony, and they were counted.

Results:

***In vitro* and *in vivo* Anti-oxidant assay**

Antioxidant and anti-inflammatory 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/g in DPPH assay, 8.43 ± 0.01 µg/g in reducing power assay, and 7.56 ± 0.7 µg/g in Nitric oxide assay. While aqueous extract (NC1), methanol (NC2), aqueous methanol (NC3) showed IC50 of 87.83 ± 0.012, 237.95 ± 0.005, 276.11 ± 0.101 µg/g respectively in DPPH assay. In reducing power assay NC1, NC2, NC3 showed 36.71 ± 0.01 µg/g, 59.4 ± 0.05 µg/g, and 926 ± 0.333µg/g respectively as their IC50s. In Nitric oxide assay NC1, NC2, NC3 showed 94.35 ± 0.008, 78.11 ± 0.101, and 95.82 ± 0.090 µg/g respectively as their IC50s. Antioxidant activity (IC50) of all the three extracts are concisely presented in Table S1.

Table S1. *In vitro* antioxidant assay of extracts (NC1-NC3) of *Citrus macroptera* Montruz.

	DPPH assay IC_{50} µg/g ± SEM*	**Reducing power assay IC_{50} µg/g ± SEM***	**Nitric oxide assay IC_{50} µg/g ± 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 of DW (dry weight), $p< 0.05$, n=5 analyses.

Reduced activities of enzymic (SOD, CAT) and non-enzymic (Glutathione reductase) were summarized in Table S2. SOD, CAT and reduced glutathione are endogenous oxidative enzymes widely found in cells and tissues that protect cells again oxidative stress. Their level is high in normal groups of treatment. The results of SOD, CAT, reduced glutathione assays which were done using serum, plasma EDTA, and liver tissues were presented in Table S2. When oxidative stress is high, there is reduced anti-oxidative enzymes. Our data showing the increase in level of such enzymes suggest the antioxidant property of the extracts.

Table S2. *In vivo* antioxidant assay (SOD, CAT, and reduced glutathione) of three extracts of *Citrus macroptera* Montruz.

Sample tissue treated with extract	SOD activity U/mL	Catalase activity U/mL	Glutathione reductase activity µmole/min./mL
Serum (S)			
Normal/control	68.93	1.03	1.743
CCl_4	53.4	0.55	0.55
Ascorbic acid	67.4	0.99	1.21
CCl_4 + NC1S	60.85	0.83	1.31
CCl_4 + NC2S	55.54	0.76	0.61
CCl_4 + NC3S	58.51	0.92	0.54
Plasma EDTA (P)			
Normal/ control	94.89	1.62	1.36
CCl_4	44.2	0.01	0.39
Ascorbic acid	95.6	1.34	1.37
CCl_4 + NC1P	50.63	0.4	0.527
CCl4 +NC2P	42.97	0.006	0.438
CCl_4 +NC3P	43.40	0.866	0.570
Liver (L)			
Normal/control	99.7	1.93	11.14
CCl_4	95.6	0.98	0.95
Ascorbic acid	98.2	1.6	10.07
CCl4 + NC1	98.29	1.76	0.89
CCl4 + NC2	96.59	1.60	1.14
CCl4 + NC3	95.78	1.52	0.88
Brain (B)			
Normal/ control	95.3	1.72	0.66
CCl_4	74.2	0.09	0.30
Ascorbic acid	95.9	1.14	1.07
CCl_4 + NC1B	99.57	1.24	0.61
CCl4 +NC2B	91.53	0.74	0.53
CCl_4 +NC3B	98.31	0.72	0.52

In vitro and *in vivo* anti-inflammatory assay

Protease inhibitory assay, albumin denaturation and heat induced haemolysis of the three extracts (NC1-NC3) were done to assess the preliminary anti-inflammatory activity. In protease inhibitory assay aqueous, methanol and aqueous methanol extract showed 20.44 ± 0.0057 µg/mL, 59.66 ± 0.0057 µg/mL, and 57.76 ± 0.005 µg/mL respectively as their IC50s. Protease inhibitor cocktail showed 114.95 ± 0.008 µg/mL IC50 in protease inhibition assay. Diclofenac sodium showed IC50 of 55.8 and 11.79 µg/g as IC50 respectively in albumin denaturation and heat induced haemolysis assay. The highest protease inhibitory activity was observed in NC1 with IC_{50} 20.44 ± 0.0057 ug/mL, heat induced hemolysis 100.36 µg/mL (Table S3). In the selected methods, the different extracts showed different level of anti-inflammatory activity.

When compared to the control group the effect was almost comparable with standard drug diclofenac sodium at 4^{th} and 6^{th} hr after administration. It is observed that the extracts at the dose level of 100 mg Kg^{-1} p.o. exhibited more degree of reduction in mice edema significantly. The effect of aqueous, methanol and aqueous methanolic extract on mice paw edema are presented (Table S4, Fig.S2). Methanolic extract (NC2) exhibited more amount of anti-inflammatory activity than the aqueous and aqueous methanolic extract.

Table S3. *In vitro* anti-inflammatory activity of three extract (NC1-NC3) of *Citrus macroptera* Montruz.

	Protease Inhibitory activity IC_{50} µg/mL^{+} ± SEM*	**Albumin denaturation IC_{50} µg /g^{++} ± SEM***	**Heat induced haemolysis IC_{50} ± SEM***
Protease inhibitor cocktail (Sigma)	11.49 ± 0.008	---	---
Diclofenac Sodium	---	55.8± 0.16	11.79± 0.01
Aqueous (NC1)	20.44± 0.008	312.31± 0.2	115.4 ± 0.05
Methanol (NC2)	59.66± 0.00	104.75± 0.00	100.36± 0.008
Aqueous methanol (NC3)	57.76± 0.01	163.98± 0.01	208.32± 0.045

+ : µg/mL equivalent of Protease inhibitor cocktail, ++ : µg/g equivalent of Diclofenac sodium, *:Standard error mean, n=5, p< 0.005

Table S4. Effect of NC1, NC2 and NC3 extracts on carrageenan induced paw edema

Name of group mg/kg,/p.o.	Before injection 0 hr	After injection (Paw volume, mean ± SEM)					
		0 hr	1 hr	2 hr	3 hr	4 hr	6 hr
Normal Control (1 mL dH_20)							
	0.29±1.01	0.36 ±1.34	0.49±1.9	0.65±1.3	0.77±0.62	0.79±0.01	0.88±.67
NC1							
50	0.21±0.98	0.24±1.2	0.29±0.78	0.33±0.01	0.48±1.34	0.34±.76	0.31±3.7
100	0.23±0.84	0.31±1.0	0.33±0.45	0.34±0.85	0.37±1.67	0.37±.32	0.30±0.99
150	0.30±0.65	0.45±0.7	0.40±0.2	0.34±1.43	0.45±.78	0.33±1.78	0.29±.78
NC2							
50	0.27±2.8	0.29±.55	0.35±3.4	0.34±5.6	0.37±.89	0.34±4.2	0.24±0.69
100	0.29±1.3	0.40±.01	0.43±0.89	0.54±0.09	0.57±2.9	0.46±1.2	0.25±2.43
150	0.19±1.9	0.41±.98	0.54±1.3	0.55±1.4	0.59±2.6	0.45±0.91	0.24±0.44
NC3							
50	0.27±0.9	0.46±1.1	0.48±0.35	0.57±0. 01	0.63±4.6	0.23±0.11	0.28±1.4
100	0.23±0.3	0.35±1.3	0.36±1.78	0.39±0.05	0.53±.78	0.46±0.78	0.32±1.9
150	0.14±1.3	0.31±0.6	0.49±.23	0.55±1.9	0.57±1.44	0.23±0.44	0.39±0.88
Diclofenac sodium							
50	0.22±0.01	0.49±1.1	0.51±0.005	0.52±1.5	0.54±3.8	0.28±0.89	0.23±1.55
100	0.24±0.44	0.39±1.2	0.45±0.01	0.46±0.09	0.48±1.4	0.29±1.45	0.20±.88
150	0.24±1.01	0.39±.99	0.45±0.8	0.39±0.81	0.33±0.69	0.29±0.67	0.24±0.23

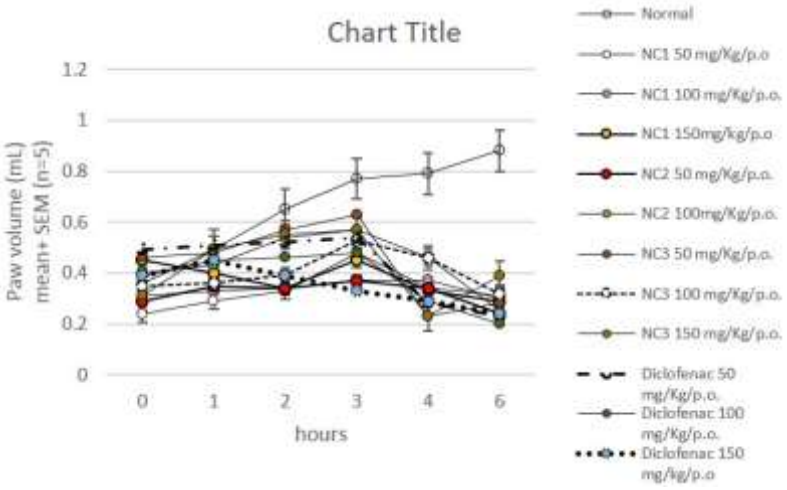

Fig. S2. Effect of aqueous (NC1), methanol (NC2) and aqueous methanol (NC3) extract on mice paw edema

Cytotoxicity Test

In MTT assay the aqueous extract showed 148.44 µg/mL as IC50. Significant inhibition of colony formation was also observed. Different concentrations of aqueous extract (5, 20, 40, 80, 160 and 320 µg/mL) when treated with the colonies of Hela cells, they show their colony inhibition potential. The number of colonies decreases as the concentration of the test extract increases (Fig. S3 A, S3 B.).

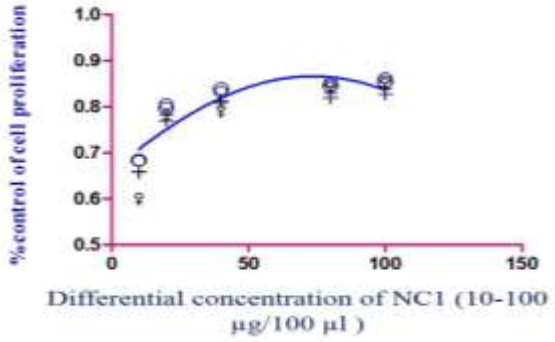

Fig. S3 A.

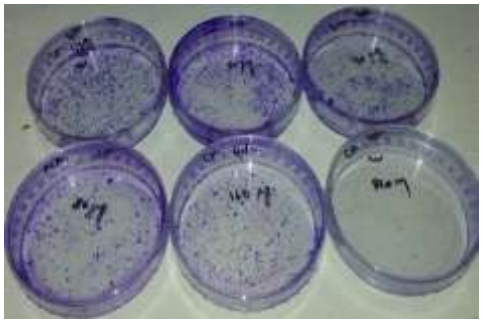

Fig. S3 B.

Fig. S3 A. Cytotoxicity assay (MTT) against Hela cells. The extract dose- response inhibit the proliferation of Hela cells. Each point represents the mean ± SD of 3 determations. *p<0.05 compared with the control (medium) Fig. S3 B. Dose response colony inhibition shown by 5-320 µg/mL of NC1 extract of *C. macroptera* in Clonogenic assay against Hela cells.

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.

Discussion

Both time and solvent extractions played a vital role in the extraction of phenolic contents and their antioxidant properties of *C.macroptera*[12]. 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 [13.]

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[14]. 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. Thus our findings serves to exemplify the potential of this wild orange as natural dietary antioxidants.

SOD is one of the chief cellular defense enzymes that dismutase superoxide radicals to water and oxygen. Catalase are heme-containing proteins that protect the cells from toxic effects of reactive oxygen species by converting hydrogen peroxide to water and molecular oxygen[15]. Catalase activity varies greatly between tissues. Hydrogen peroxide is highly deleterious to the cell and its accumulation causes oxidation of

cellular targets such as DNA, proteins, and lipids leading to mutagenesis and cell death[16]. Hence removal of H_2O_2 by catalase enzyme provide protection against oxidative stress related diseases.

Denaturation of protein is also one of the cause of inflammation. The production of auto-antigens in inflammation disease may be due to *in vivo* denaturation of protein. *C. macroptera* inhibit the denaturation of proteins and its effect was compared with the standard Protease Inhibitor cocktail. Carrageenan induced edema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1-2 hr) is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings. The late phase is sustained by prostaglandin release and mediated by bradykinin, leukotriens, polymorphonuclear cells, and prostaglandin produced by tissue macrophages[17]. The IC_{50} of antioxidant activity and anti-inflammatory activity does not correlate each other, henceforth the *in vitro* and *in vivo* readings. Of course, strong antioxidant activity *in vitro* does not necessarily translate to a significant health benefit *in vivo;* indeed, high concentrations of ingested low molecular weight antioxidants may be toxic or act as pro-oxidants in humans[18], or else be rendered inactive by metabolism before they are absorbed into the bloodstream[19].

Conclusion

With varying solvent type, the magnitude of activity of the extracts and fractions also varies. The extracts of *Citrus macroptera* showed antioxidant and anti-inflammatory activity (both *in vitro* & *in vivo*). It can be used for drug discovery and development to battle those diseases which are induced by oxidative stress. Advance cytotoxicity studies using different cell lines and blotting experiments are under study. 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 *in vitro* protease inhibitory, antioxidant, cytotoxicity and clonogenic activity in *Citrus macroptera* Montruz. The *in vivo* antioxidant and anti-inflammatory activity was also possessed by the tested extracts. The results illustrated that the extracts exhibited concentration and solvent dependent bioactivies. 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.

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