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Protective action of *Crateva nurvala* Buch. Ham extracts against renal ischaemia reperfusion injury in rats *via* antioxidant and anti-inflammatory activities

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ABSTRACT

Ethnopharmacological relevance: *Crateva nurvala* stem bark is commonly used in *Ayruveda* in treatment of many renal injuries, e.g., urinary lithiasis, diuretic and nephroprotective. However, its protective effect against renal ischaemia/reperfusion, the major cause of acute kidney injury, has never been studied. Moreover, no comprehensive chemical profiling of its extracts was recorded.

Aim of the study: Assessment of the protective effect of the plant extracts against renal ischaemia/reperfusion and elucidation of the possible mechanism of action. Then, to determine its bioactive constituents using modern UPLC-HRMS technique.

Material and methods: Unilateral ischaemia was induced by clamping the left renal artery for 1 h then reperfusion for 24 h. Rats were divided in 4 groups: i) sham-operated group, ii) ischaemia/reperfusion, I/R group, iii) I/R protected by previous administration of *Crateva* leaves extract, CLE group and iv) I/R protected by previous administration of *Crateva* bark extract, CBE group. At the end of reperfusion, blood samples were analyzed for renal function biomarkers. Kidneys were examined histopathologically and their homogenates were used in determining the intracellular levels of oxidative stress, inflammatory, and apoptosis markers.

Results: Leaves and bark extracts attenuated the deleterious effects of I/R apparent in reducing LDH, creatinine and blood urea nitrogen levels. The extracts reduced the oxidative stress by replenishing the glutathione levels and Nrf2 factor levels. Moreover, extracts decreased levels of pro-inflammatory TNF-α, NF-κβ and IL-6; which ultimately resulted in reducing the pro-apoptotic caspase-3.

Bark and leave extracts have quite similar chemical profile where 42 compounds of various chemical classes were identified. Flavonoids are the major class of the bioactive phytochemicals

Conclusion: *C. nurvala* extracts had effectively ameliorated the deleterious effects of renal I/R by mainly counteracting oxidative stress and presumably inflammation. Consequently, it can be used as a complementary treatment with other agents. In this aspect, leaves stand as a sustainable alternative to bark. The presented chemical profiling can be used in future standardization and quality control of the drug.

1. Introduction

Crateva nurvala Buch-Ham is a tropical tree indigenous to India where it is widely used in *Ayruveda* (Bhattacharjee et al., 2012). Although different tree parts are used in treatment of different aliments, bark extracts are used predominantly in alleviating different urinary tract diseases (Bhattacharjee et al., 2012; Bopana and Saxena, 2008; Khattar and Wal, 2012). These activities are the most clinically and pharmacologically verified activities (Bopana and Saxena, 2008; Deshpande et al., 1982). The bark extract and its constituent, lupeol, had antiurolithiatic by diminishing the formation of urinary stones and had protective activity against hyperoxaluria toxic complications (Anand et al., 1994; Malini et al., 2000; Varalakshmi et al., 1991; Vidya and Varalakshmi, 2000). Moreover, it ameliorated urinary tract infections either alone or in herbal combination (Deshpande et al., 1982; Oneal and White, 2004). Recently, the protective activities of the bark

Abbreviations: ACN, acetonitrile; AKI, acute kidney injury; CLE, *C. nurvala* leaves extract; CBE, *C. nurvala* bark extract; DGF, delayed graft function; GSH, glutathione; I/R, ischaemia/reperfusion; IL-6, interleukin-6; MDA, malondialdehyde; Nrf-2, nuclear factor erythroid 2 (NFE2)-related factor 2; NF-κβ, nuclear factor kappa-light-chain-enhancer of activated B cells; ROS, reactive oxygen species; TNF-α, tumor necrosis factor alpha

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extract and lupeol against cisplatin-induced nephrotoxicity were verified (Shelkea et al., 2011; Shirwaikar et al., 2004a, 2004b). Despite these beneficial effects in different renal aliments, no report has studied their protective effect in renal ischaemia/reperfusion injury (I/R).

Renal I/R is an injury that occurs after sepsis, shock or renal trauma where the temporary reduction in blood supply and subsequent reperfusion and tissue reoxygenation lead to acute kidney injury (AKI), previously, known as acute kidney failure; this is associated with high mortality rates (Malek and Nematbakhsh, 2015). Furthermore, renal I/R is an unavoidable procedure during kidney transplantation especially if the transplant comes from deceased donor. Lesions caused by I/R may cause delayed graft function (DGF) and AKI (Li et al., 2015; Ponticelli, 2014). Renal I/R is a complex process that involves an acute inflammatory response, infiltration of macrophages and neutrophils, production of reactive oxygen species (ROS) and mitochondrial dysfunction leading to proximal tubule apoptosis (Li et al., 2016; Malek and Nematbakhsh, 2015). Several synthetic and natural drugs were tested for their protective effect against I/R (Malek and Nematbakhsh, 2015; Ponticelli, 2014).

Despite of the efficacy and safety of *C. nurvala* in treating different renal injuries, its efficacy in combating I/R was not evaluated. On the other hand, like many ethnobotanicals, full chemical characterization and exploitation of *C. nurvala* is underdeveloped. Only some triterpenes and few flavonoids were isolated and identified in *C. nurvala* extracts (Bhattacharjee et al., 2012; Khattar and Wal, 2012). No comprehensive phytochemical analysis using modern techniques, e.g., UPLC-MS/MS, was performed. This knowledge is essential to perform further standardization of herbal products. The current study has two aims: i) to determine the possible protective action of *C. nurvala* extracts against I/R making advantage of its safe application in *Ayruveda,* ii) to perform metabolic profiling of leaves and bark extracts of the *C. nurvala* growing in Egypt using UPLC-DAD-qTOF-MS technique in order to provide a comprehensive profile of its active constituents.

2. Material & methods

2.1. Ethical statement

The study was conducted following the Medical Research Ethics Committee of the National Research Centre, Cairo, Egypt and following the recommendations of the National Institutes of Health Guide for Care and Use of laboratory Animals (NIH Publications no. 8023, revised 1978).

2.2. Material

2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich, Bromocresol green from (Loba Chemie, India) while all other chemicals from (El-Nasr for chemicals, Egypt). Solvents were of LC-MS grade (Sigma-Aldrich, Germany).

2.3. Plant material collection and preparation of extracts

Leaves and stem bark of *C. nurvala* (Capparaceae) were collected in June 2016 from a private garden in the North West of Egypt. The plant was authenticated by Dr. Mohamed El-Gibaly, Plant taxonomy department, National Research Centre, Giza, Egypt. A voucher specimen (20.6.16) of the leaves were registered and deposited in the herbarium of Faculty of Pharmacy, Cairo University. *C. nurvala* Buch.-Ham, the plant list (http://www.theplantlist.org), last access on 30 July 2017.

Shade-dried leaves (2 kg) and dried bark (200 g) were powdered and extracted by maceration in ethanol (70%). the extracts were filtered and evaporated to dryness under reduced pressure. The maceration was repeated till exhaustion. The obtained residue was used in the pharmacological experiments and subjected to different quantitative chemical analyses. Leaves and bark extracts (5 mg each) were extracted in methanol containing umbelliferone (10 μg/ml), as internal standard, by sonication for 20 min. Each extract was then vortexed vigorously and centrifuged at 10,000 g for 5 min, 1 ml is aliquoted and filtered through 22 μm Millipore filter; eluent was injected directly in the UPLC instrument.

2.4. Biological study

2.4.1. Animals and experimental design

The study was performed on male Wistar albino rats (n = 24), 8–10 weeks old and weighed ranging 150–200 g. They were bred and obtained from Animal House Colony, National Research Centre, Cairo, Egypt. All animals were housed in cages in a controlled temperature (24 ± 1 °C) with a 12 h light/dark cycle and 60 ± 5% humidity and were provided with standard laboratory diet, water ad libitum.

The study was planned to assess the ability of *Crateva* leaves and bark extracts to attenuate I/R consequences in rats. After acclimatization for 14 days, rats were randomly divided into four groups containing six animals each. Group I (sham operated control) was treated with vehicle alone for 14 days. Group II (I/R group) was also treated with vehicle for 14 days. Group III received 200 mg/kg of *Crateva* leaves extract (CLE) for 14 days. Group IV received 200 mg/kg of *Crateva* bark (CBE) for 14 days. For the last three groups, I/R was induced on the 14th day. Doses (200 mg/kg) were selected based on previous literature (Moniruzzaman and Imam, 2014; Vijayabhaskar et al., 2016). Extracts and vehicle were delivered by oral administration.

2.4.2. Induction of I/R

Rats were anesthetized by intraperitoneal injection of chloral hydrate (300 mg/kg). A midline abdominal incision was made to expose the left kidney. Blood supply to the kidney was interrupted by clamping the left renal artery using artery clamp for 1 h. Ischaemia was confirmed by the blanching of the kidney. After 1 h, the clamp was removed and reperfusion was confirmed visually. The wound was then closed in two layers with silk suture and the animals were allowed to recover with free access to food and water (Savransky et al., 2006). The sham-operated group was subjected to the same conditions but without I/R induction. At the end of reperfusion period (24 h), blood and kidney samples were collected for further assessment. Blood samples were withdrawn from the retro orbital venous plexus for estimating serum parameters, namely, blood urea nitrogen (BUN) and serum creatinine levels. Left kidneys were removed and rinsed thoroughly with saline and 10% neutral buffered formaline (pH 7.4) was used for preservation of portions of tissue for subsequent histopathological examination. Remaining tissue was placed at − 80 °C for other biochemical measurements.

2.4.3. Assessment of Kidney function biomarkers

To determine the kidney function and possible injury, serum creatinine, blood urea nitrogen (BUN) and lactate dehydrogenase (LDH) levels were determined. Serum creatinine levels were determined according to the method developed by Spencer (Spencer, 1986), using kit purchased from Biodiagnostic Co. (Cairo, Egypt). The red color developed from interaction of creatinine with picric acid in alkaline medium was measured at 492 nm. Blood urea nitrogen levels were determined by the method developed by Fawcett and Scott (Fawcett and Scott, 1960), using kit purchased from Biodiagnostic Co. (Cairo, Egypt). It based on the interaction with Berthelot's reagent, the developed green color was measured at 578 nm. Spectrophotometric measurements were performed using double beam spectrophotometer (Shimadzu, UV-PC160, Japan). LDH was assayed in serum samples for the evaluation of generalized tissue damage. Its serum levels were determined spectrophotometrically using an automated analyzer.

2.4.4. Histopathology examination

Representative kidney samples from each group were reserved for histopathology, where it was fixed in 10% formol saline for twenty four hours. Samples were washed in tap water then different strengths of alcohol for dehydration. The specimens were cleared in xylene and embedded in paraffin at 65 °C for 24 h. Sections of 4 μm were cut using microtome. The obtained sections were stained by hematoxylin & eosin stain and examined using light microscope.

2.4.5. Assessment of oxidative stress

Reduced glutathione (GSH) levels in renal tissue were determined with Ellman's reagent according to Beutler et al. (1963). Malondialdehyde (MDA), a major product of lipid peroxidation, was determined as thiobarbituric acid-reactive substance (Mihara and Uchiyama, 1978). The nuclear factor erythroid 2–related factor 2 (Nrf2) is the key regulator of cellular defense against oxidants. NRf2 protein levels were determined by ELISA according to manufacturer's instructions (Cusabio Life Science, USA).

2.4.6. Assessment of pro-inflammatory markers

Tumor necrosis factor-alpha (TNF-α), nuclear factor-kappa B (NF-κβ) and interleukin-6 (IL-6) contents were measured in kidney tissue by ELISA technique using ELISA kits provided by Sunlong, (China), following the manufacturer's instructions. Catalogues' number were SL0722Ra, SL0537Ra and SL0411Ra, respectively.

2.4.7. Assessment of pro-apoptotic markers

Caspase-3 activity in the kidney tissue was measured using a caspase-3 colorimetric assay kit, according to the manufacturer's instructions (Biovision Co. USA).

2.5. Phytochemical profiling

2.5.1. UPLC-Orbitrap HRMS analysis

Both negative and positive high resolution ESI modes and collision-induced dissociation (CID) MS^n spectra were acquired from an Orbitrap Elite mass spectrometer (Thermo Fischer Scientific, Darmstadt, Germany) equipped with a heated electronspray ion source adjusted at 3 kV and 4 kV in negative and positive modes, respectively, capillary voltage of 300 °C, source heater temperature of 250 °C, FTMS resolution of 30.000. The MS spectrometer was coupled to an UHPLC system (Dionex UltiMate 3000, Thermo Fischer Scientific), equipped with a RP-18 column (30 mm × 2.1 mm × 1.8 μm), Acquity HSS T3, Waters, column temperature of 40 °C. DAD (220–600 nm, Thermo Fischer Scientific). Mobile phase consisted of water (A) and ACN (B) supplied with 0.1% formic acid. The following gradient was used: 0–1 min at 5% B, followed by linear increase to 100% B till 11 min; B 100% was kept from 11 to 19 min, then B reduced to 5% from 19 to 30 min. Flow rate was 150 μl/min and injection volume was 2 μl. The CID mass spectra were recorded using normalized collision energy (NCE) of 35%. The instrument was externally calibrated by the Pierce ESI negative ion calibration solution (Product no. 88324) and Pierce ESI positive ion calibration solution (Product no. 88323) from Thermo Fisher Scientific. The data were evaluated using the software Xcalibur 2.2 SP1. Metabolites were characterized by their UV–vis spectra (220–600 nm), retention times relative to external standards, mass spectra and comparison to phytochemical dictionary of natural products database (CRC) and reference literature.

2.5.2. Preliminary quantitative chemical analyss

Total phenolics were determined using Folin-Ciocalteu reagent and gallic acid as standard (Ainsworth and Gillespie, 2007). Briefly, 100 μl of the tested extract, methanol or standard gallic acid solutions (10-100 μg/ml) is mixed with 200 μl Folin-Ciocalteu reagent (10%) and vortexed, then 800 μl of 700 mM Na_2CO_3 were added. The mixture was left in dark at room temperature for 1 h. Finally, 200 μl of extract, standard and control is transferred to a clear 96-well plate; absorbance was measured at 690 nm.

Total flavonoids were determined by measuring color intensity developed by mixing samples with $AlCl_3$ in acidic medium using quercetin as standard (Pękal and Pyrzynska, 2014). Briefly, 250 μl of aqueous 2% $AlCl_3$ is mixed with 500 μl of extract, methanol or quercetin (5-100 μg/ml), then 1 M HCl (250 μl) was added and the mixtures were vortexed thoroughly. Aliquotes (200 μl) were transferred to a clear 96-well plate; absorbance was measured at 420 nm. All measurements were performed using microplate reader (Infinite F50, Tecan, Switzerland).

Total alkaloids were determined by measuring the yellow color intensity extracted by dicholomethane after mixing samples with bromocresol green reagent and using quinine as standard (John et al., 2014). Briefly, solution of extracts in 2 M HCl (1 ml) were transferred to a separating funnel and mixed with 5 ml of 0.2 M citrate/phosphate buffer (pH 4.7) and 5 ml bromocresol green solution (70 mg/l). The mixture was extracted four times successively with 2 ml dichloromethane. Dichloromethane extracts were collected in 10 ml volumetric flask and volume was completed. Same procedures were applied to quinine sulphate solution, in concentration range (50-200 μg/ml). Absorbance was recorded at 405 nm using Jenway 6051 Benchtop Colorimeter (Jenway Co., UK). Standard curves which were set up for the aforementioned authentics are shown in Fig. S1.

2.5.3. In vitro antioxidant activities

Two methods were applied, namely, scavenging the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and iron reducing capacity (Saeed et al., 2012). Briefly, 180 μl of methanolic DPPH solution is mixed with 20 μl of tested extracts, gallic acid or methanol. Absorbance was recorded at 540 nm after 15 min incubation in dark at room temperature. IC_{50} was calculated from graph of concentrations versus absorbance. The tested concentrations fell in range (0.4-50 mg/ml) for CBE, (0.5-65 mg/ml) for CLE and (5-400 μg/ml) for gallic acid.

The capacity to reduce ferric ion in converting ferricyanide to ferrocyanide and then measuring the Prussian blue color intensity formed after addition of ferric chloride was performed. Briefly, tested extract, ascorbic acid or methanol is mixed with potassium ferrocyanide solution (2.5 ml) and 2 ml phosphate buffer (0.2 M, pH 6.6). the mixture was shaken and incubated at 50 °C for 20 min. Reaction was stopped by addition of 2.5 ml trichloroacetic acid (10% solution) then 2.5 ml water was added followed by 500 μl of ferric chloride hexahydrate (0.1%). The developed color is measured after 10 min at 710 nm using Jenway 6051 bench colorimeter. Concentration produced absorbance (0.4, half the maximum 0.8) was selected as EC_{50}, calculated from graph of concentrations versus absorbance. The tested concentrations fell in range (25–200 mg/ml) for CBE, (30–260 mg/ml) for CLE and (0.5–10 mg/ml) for ascorbic acid.

Graphs used in calculation of IC_{50} and EC_{50} are shown in Fig. S2.

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism v6 software. Data were expressed as mean ± SEM. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by Tukey post hoc test. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. C. nurvala extracts attenuated kidney damage induced by I/R

Kidney tissue damage was manifested by significant increase in renal LDH levels (3 folds) (Fig. 1A); this damage hampered kidney function as detected by tremendous increase in serum creatinine and BUN levels, 5 and 3.5 folds; respectively (Fig. 1B, C). Administration of both *Crateva* leaves and bark extracts (CLE, CBE) prior to I/R attenuated kidney damage as observed in significant reduction of LDH levels.

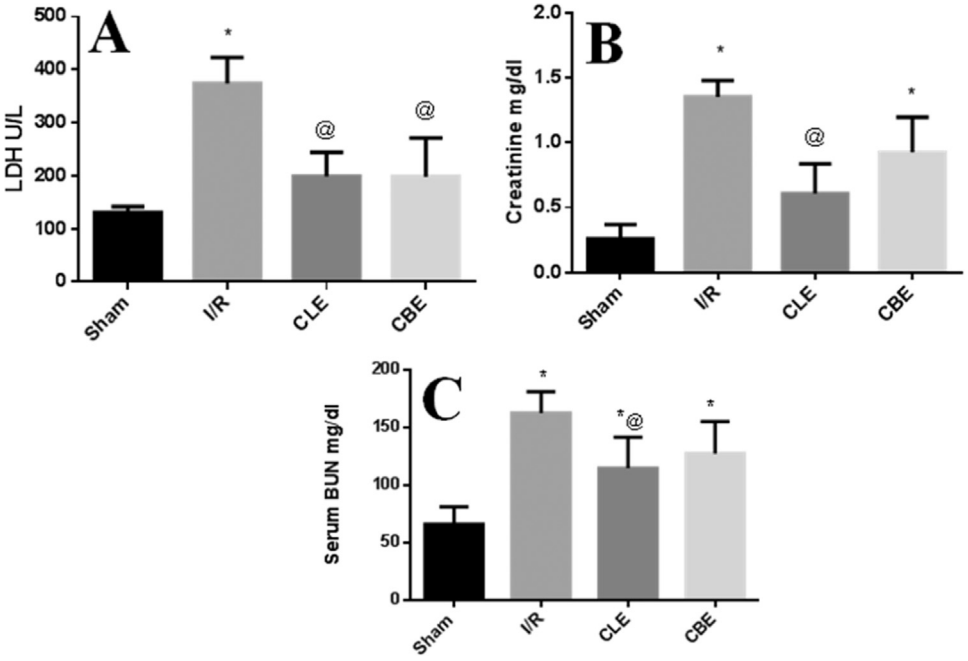

Fig. 1. Effect of *C. nurvala* extracts on serum levels of kidney function biomarkers following I/R. A) Lactate dehydrogenase (LDH), B) Serum creatinine, C) Serum blood urea nitrogen (BUN). I/R, ischaemia/reperfusion; CLE, *C. nurvala* leaves extract; CBE, *C. nurvala* bark extract. Data represented with S.E. (n = 6) and compared with sham (*) and I/R (@) (one way ANOVA followed by Tukey post hoc test) at $P < 0.05$.

However, CLE caused a significant improvement in kidney function as indicated by significant reduction in serum creatinine and BUN levels, in contrary to CBE which did not reduce those parameters significantly. The renoprotective effects of the tested extracts were confirmed further through the histopathological study.

3.2. Histopathological examination of kidney tissues

In I/R group, there was a massive necrosis and degeneration of renal tubular epithelium accompanied with congested glomeruli (Fig. 2b; Table 1). Focal hemorrhage was observed in between degenerated and necrosed tubules in the medulla. CLE and CBE ameliorated these effects; neither glomeruli congestion nor tubular necrosis was observed. However, tubular degeneration was mild but with eosinophilic casts in

Table 1
The severity of histopathological alterations in the kidney of rats in different experimental groups.

	Normal	I/R	CLE	CBE
Tubular degeneration	–	+ + +	+ +	+ +
Tubular necrosis	–	+ + +	–	–
Focal hemorrhage	–	+ + +	–	–
Congestion of glomerular tuft	–	+ + +	–	–
Vacuolization in endothelial cells lining the glomeular tuft	–	–	–	+

I/R, ischaemia/reperfusion; CLE, *C. nurvala* leaves extract; CBE, *C. nurvala* bark extract. -: no effect (0–25%); +: mild, 25–50% were affected; + +: moderate, 50–75% were affected; + + +: severe, 75–100% of the examined tissue were affected.

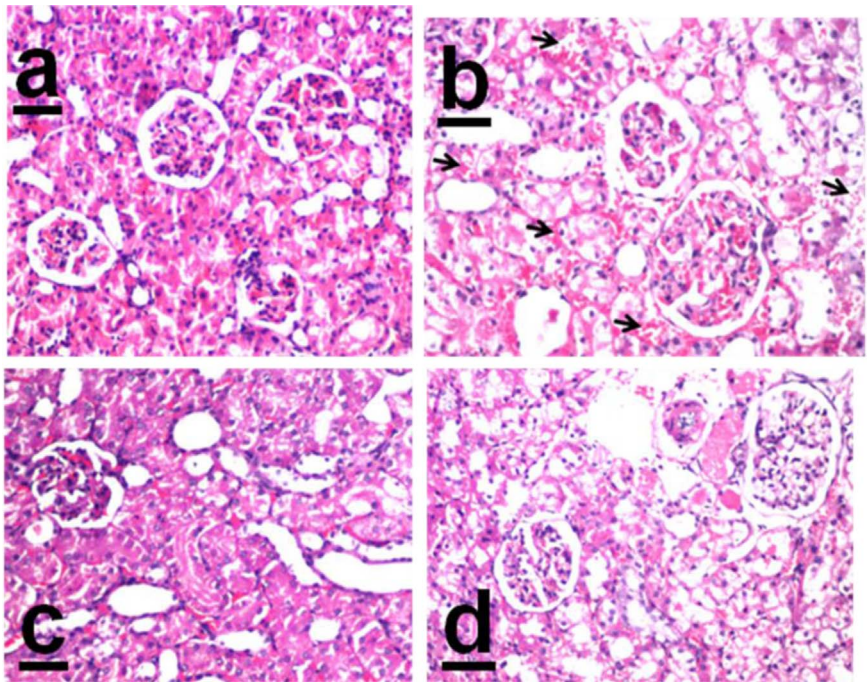

Fig. 2. *C.nurvala* extracts ameliorated kidney tissue damage induced by I/R. (2a) Sham group showing normal hitstological structure, (2b) I/R group showing glomerular congestion, arrows indicate the focal hemorrhage in between degenerated and necrosed tubules, (2c) CLE treated group showing mild degeneration of in the lining epithelium of few tubules and eosinophilic cast, (2d) CBE treated group showing similar histological structure to 2c but with vacuolization of endothelial cells lining the glomerular tuft. Sections were stained by hematoxylin & eosin.

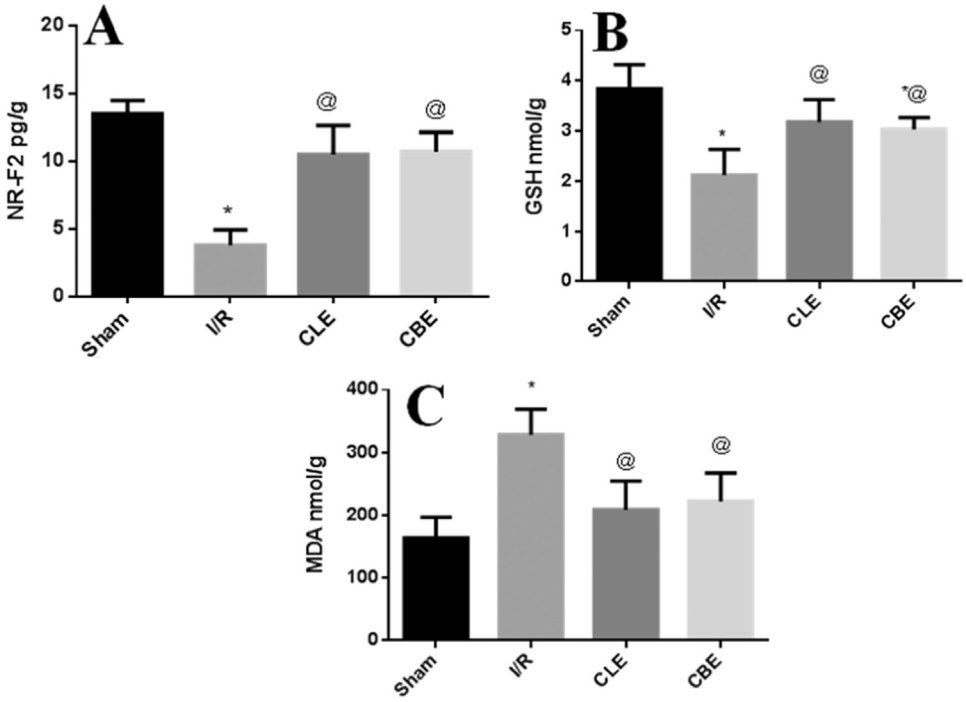

Fig. 3. *C. nurvala* extracts attenuated elevated oxidative stress markers in tissue homogenate of I/R model. A) Nrf2 level, B) Glutathione level (GSH), C) Malondialdehyde (MDA). Data are represented with S.E. (n = 6) and compared with sham (*) and I/R (@) (one way ANOVA followed by Tukey post hoc test) at $P < 0.05$.

tubular lumen (Fig. 2c, d; Table 1). In contrast to CBE treated group, CLE treated group showed no vacuolization in the endothelial cells lining the glomerular tuft indicating the greater protective effect of CLE.

3.3. C. nurvala extracts abated oxidative stress induced by I/R

Oxidative stress is one of the major domains responsible for kidney tissue damage during I/R through depletion of natural antioxidants and subsequent oxidation of cell membrane lipids, cellular proteins and cellular DNA. Nrf2 is a key transcription factor which activates expression of antiodixative enzymes and production of natural antioxidants. I/R significantly reduced the Nrf2 transcription factor levels in kidney tissues (75% reduction), this effect was manifested in depletion of glutathione levels in kidney tissues (50% reduction) and elevation of MDA (2 folds), which is a marker of lipid peroxidation (Fig. 3). Both CLE and CBE had significantly increased the tissue content of Nrf2 and replenished the glutathione content in kidney tissues. Ultimately, MDA levels were significantly reduced indicating the abatement of oxidative stress.

3.4. C. nurvala extracts reduced pro-inflammtory and pro-apoptotic levels

Pro-inflammatory mediators play a major role in I/R pathophysiology by guiding the infiltration of macrophages and neutrophils to kidney tissues. I/R caused a significant increase in intracellular levels of the key transcription factors, TNF-α and NF-κβ, which partially contribute to production of inflammatory cytokines, e.g. IL-6 (Fig. 4). Both CBE and CLE reduced intracellular levels of TNF-α and IL-6 significantly. However, the reduction in NF-κβ was not significant. Inflammation and oxidative stress are predisposing factors for mitochondrial dysfunction and are triggers for cell apoptosis. Caspase-3 is produced by mitochondria and plays central role in apoptosis. Caspase-3 was significantly elevated in I/R tissue and remarkably reduced by treatment of both CLE and CBE (Fig. 4C).

It is observable that the antioxidant activity of the tested extracts was more pronouncing than their anti-inflammatory action. Furthermore, CLE performed slightly better than CBE in reducing malicious signal molecules and biomarkers as well as boosting the good ones.

3.5. Chemical profiling

Crateva extracts were profiled qualitatively and quantitatively. Modern UPLC-HRMS analysis gave a holistic picture of the extracts' constituents. Preliminary quantifications of total phenolics, flavonoids and alkaloids were useful in ranking the extracts, especially that the bioactive and the bioavailable constituent(s) of the extract is/are still not known. Two different in vitro antioxidant assays were performed to complement the characterization of the extracts and to investigate possible linkages to other chemical and biological parameters.

Chemical profiling is essential for the characterization of the bioactive compound. Bark and leaves extracts used in the pharmacological studies were analyzed using UPLC-HRMS in both positive and negative ESI modes. Constituents of the extracts were tentatively identified based on their accurate mass, isotopic distribution, UV/Vis spectrum, fragmentation pattern and comparing the data with those reported in literature and the database, dictionary of natural products (http://dnp.chemnetbase.com). Chromatograms produced by TIC were quite similar in terms of number of major peaks and their retention times (Fig. 5). Different chemical classes were identified including lignans, megastigmane, phenolics and triterpenes (Table 2). This indicated that the extracts were more versatile than ever studied. Alkaloids were detected in positive ion mode and were detected only in bark extracts. Cadabicine, (peak 3) was the major identified alkaloid, previously isolated from the bark (Ahmad et al., 1987). Its molecular ion at *m/z* (436.22314) produced three characteristic daughter ions, namely, at *m/z* 419 $[M+H-17\ (NH_3)]^+$, 348 $[M+H-88\ (1,4\text{-diaminobutyl})]^+$ and 265 $[348-56\ (\text{aminopropyl})]^+$ as previously stated (Khanfar et al., 2003). The minor peaks 1 and 2 had the same fragmentation pattern and were identified as cadabicine-*O*-hexoside and cadabicine-*O*-deoxyhexoside. The sugar part was identified from characteristic loss of 162 amu (hexose) and 146 amu (deoxyhexose). This is the first report about occurrence of cadabicine glycosides in *Crateva*, although cadabicine-*O*-glucoside was isolated from *Capparis spinosa* (Fu et al., 2008). Minor fatty acid amides, *viz.*, palmitamide (peak 6) and oleamide (peak 7) were identified. All these alkaloids and amines are detected in bark only which displays an interesting biosynthetic insight.

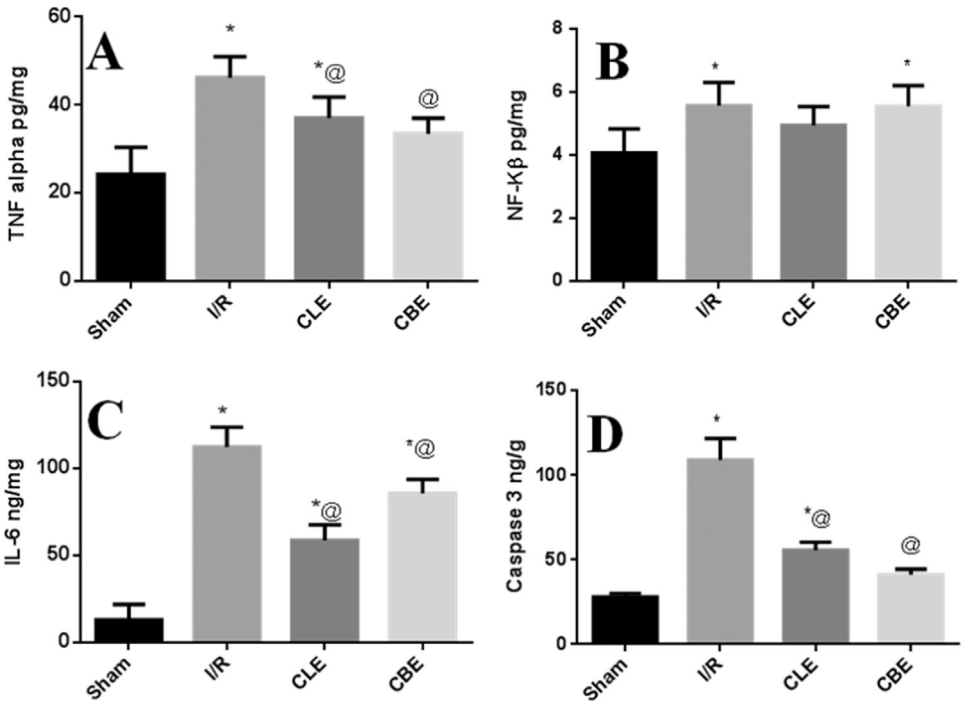

Fig. 4. Effect of *C. nurvala* extracts on pro-inflammatory and pro-apoptotic mediators levels in tissue homogenate of renal I/R model. A) TNF-α, B) NF-κβ, C) IL-6, D) Caspase-3. Data are represented with S.E. (n = 6) and compared with sham (*) and I/R (@) (one way ANOVA followed by Tukey post hoc test) at $P < 0.05$.

Major flavonoids belong to kaempferol-o-glycosides. The aglycone portion was characterized from the characteristic UV/Vis spectrum and presence of mass signal at *m/z* 285 ($C_{15}H_9O_6^-$, Kaempferol) and 301 ($C_{15}H_9O_7^-$, quercetin). Kaempferol-di-*O*-deoxyhexoside (peak 13) was identified from its mass fragmentation pattern. The base peak at *m/z* 431 $(M\text{-}H\text{-}146)^-$ and the weak signal of the aglycone at *m/z* 285 are indicative of disubstituted aglycone, i.e., the sugar units are not in a chain (Pandey et al., 2014). The same fragmentation pattern was observed in MS spectra of peak 12 which is assigned as kaempferol-di-*O*-glycoside(-*O*-hexoside, -*O*-deoxyhexoside). The last two compounds are the major flavonoids in both CLE and CBE. The same fragmentation pattern was manifested in peak 11 but with the aglycone mass signal at *m/z* 301 $(M\text{-}H)^-$ characteristic for quercetin; peak 11 was identified as quercetin-di-*O*-glycoside(-*O*-hexoside, -*O*-deoxyhexoside). It cannot be assigned as quercetin-*O*-rutinoside because the aglycone signal at 301 had weak intensity and the mass signal $(M\text{-}H\text{-}hexose)^-$ at *m/z* 447 is the base peak (Pandey et al., 2014). These previous three flavonoids represent the major signals in both bark and leaves extracts (Fig. 5), they were previously isolated from *C. nurvala* but with different glycosidation pattern (Bhattacharjee et al., 2012; Gagandeep and Kalidhar, 2006; Ghani, 1998; Khattar and Wal, 2012). Flavonoid-*C*-glycosides were readily identified from the characteristic cross ring cleavage of the sugar moiety, i.e., (− 120 amu) and (− 90 amu) for *C*-hexosides. Naringenin-6,8-di-*C*-hexoside (peak10) showed loss of two *C*- hexose unites $(M\text{-}H\text{-}2\times90)^-$ and $(M\text{-}H\text{-}2\times120)^-$ at *m/z* 415 and 355, respectively and the same UV spectral data as previously stated (Mortimer et al., 2015). Peaks 9 and 10 exhibited the same fragmentation loss of (− 120 amu) and dehydration (− 18 amu) and the same UV/Vis spectra. However, the signal $(M\text{-}18)^{+/-}$ was more intense in peak 10 which accordingly assigned as eriodictyol-6-hexoside, hence, peak 9 was assigned as eriodictyol-8-hexoside (Otify et al., 2015). Eriodictyol is biosynthetically related to quercetin. Flavonoid-*C*-glycosides were not previously reported in *Crateva* spp.

Peak 33, 34 were identified as β-sitosterol and lupeol, respectively. They exhibited the same fragmentation pattern reported for these compounds in positive ESI mode (Martelanc et al., 2007). Remarkably, the molecular ion peaks at *m/z* 397 and 409 for β-sitosterol and lupeol, respectively, are indicative for [M + H-18]. Lupeol and sitosterol were isolated from *C. nurvala* bark (Parvin et al., 2011; Shirwaikar et al., 2004a). Other previously isolated triterpenes, e.g., friedelin and betulinic acids were not traced. Their absence could be because of improper ionization technique or variation of habitat.

Minor peaks correspond to lignans, megastigmane and sesquiterpene derivatives were tentatively identified from their molecular formula, UV spectra and structure similarity to other compounds reported in the database. However, exact identification was not possible because they were not reported in *Crateva* spp. before and because of lack of data discussing their MS^n fragmentation. However, they are reported in other genera of Capparaceae (Afifi, 2014; Dhakad et al., 2016; Morgan et al., 2015).

Preliminary quantitative chemical analyzes are useful in ranking different extracts. CBE had higher total phenolics and flavonoids contents than CLE, around 1.4 folds higher (Table 3). This could explain the greater DPPH scavenging potential of CBE (smaller IC_{50}) compared to CLE (greater IC_{50}). However, both extracts had quite equal iron reducing capacity. Alkaloids were detected and quantified only in bark; a finding which complies with literature and UPLC-MS analysis.

4. Discussion

Renal I/R is a multifactorial injury where oxidative stress and inflammation are the main factors responsible for the development and progress of the disease. Protection using plant extracts benefit from their multi-targeting mechanism of action because of its multitudinous constituents (Brusotti et al., 2014). Furthermore, ethnobotanicals have been being used safely for long time now. Therefore, it was incentive to test the renoprotective effect of *C. nurvala* extracts, a plant which is commonly used in *Ayurveda* for treatment of different renal aliments. Both leaves and bark extracts had effectively ameliorated I/R deleterious effects as evidenced by histopathological examination and assessment of kidney function. These effects were reflected in reducing renal tubular necrosis and ameliorating glomerular degeneration (Fig. 2, Table 1) and improving kidney function parameters (Fig. 1). Serum levels of LDH enzyme, a marker for renal disease and renal cells' damage, were reduced. CLE was more effective than the CBE in restoration of the kidney function as indicated in significant reduction of serum creatinine and blood urea nitrogen levels (Fig. 1). This is the first experimental verification for such an activity.

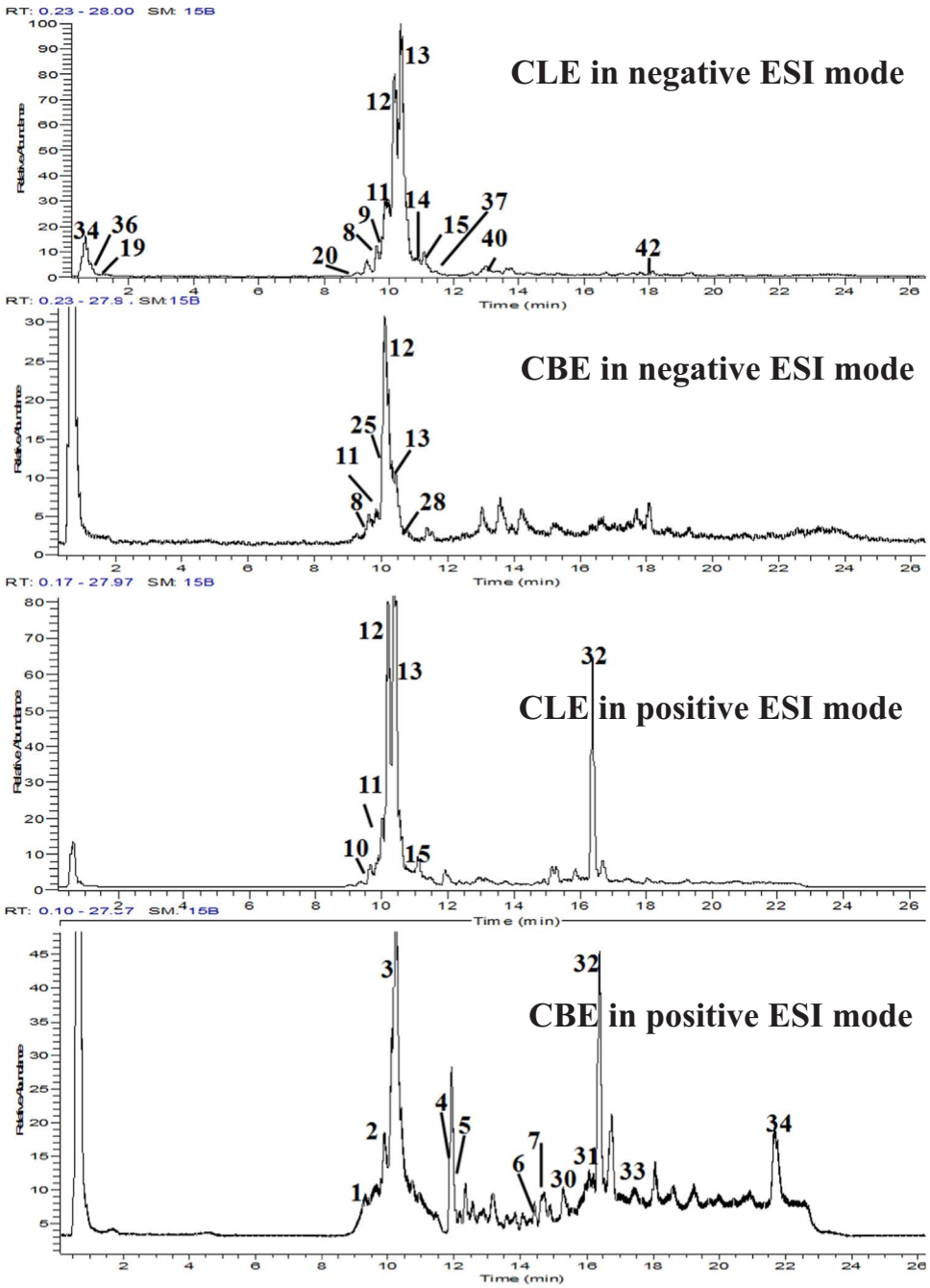

Fig. 5. TIC chromatograms of UPLC-Orbitrap-HRMS of *C. nurvala* extracts in positive and negative ion modes. CLE, *C. nurvala* leaves extract; CBE, *C. nurvala* bark extract. Numbers refer to identified compounds listed in Table 2.

Oxidative stress and inflammation interact mutually to aggravate the deleterious effects of I/R injury. Ischaemia lead to ATP depletion, cellular degeneration and initiation of an inflammatory response (Malek and Nematbakhsh, 2015). Moreover, ischaemia is accompanied by metabolic aberration and accumulation of succinate; the later metabolite drives the massive mitochondrial production of ROS upon reperfusion (Chouchani et al., 2014). Upon reperfusion massive production of ROS occurs. This is associated with lipid peroxidation, infiltration of inflammatory cells and increase in endothelial permeability. These events eventually leads to tubular obstruction, production of inflammatory cytokines and further worsening of the conditions (Malek and Nematbakhsh, 2015). Reducing lesions produced in this phase reduce the DGF and increases chances of graft survival (Ponticelli, 2014). ROS play a central role in progression of inflammatory diseases (Mittal et al., 2013). CLE and CBE extracts provided protection against I/R by counteracting oxidative stress and inflammation.

The renoprotective effect of *C. nurvala* extracts is predominantly mediated by attenuation of oxidative stress through i) boosting intracellular levels of Nrf2 (Fig. 3) ii) direct interaction with ROS. Nrf2 is a redox-sensitive transcription factor which regulates the expression of many antioxidant defense genes, e.g., haem oxygenase-1 (HO-1), NADPH-quinone oxidoreductase-1 (NQO-1), glutathione S-transferase. Nrf2-knockout mice exhibited more severe kidney dysfunction, inflammation and apoptosis than normal mice after I/R (Liu et al., 2009). Activation of Nrf2 signaling pathway using N-acetylcysteine or ischaemic pre-conditioning resulted in attenuation of inflammatory response and apopotosis triggered by I/R (Nezu et al., 2017; Shokeir et al., 2014; Zhang et al., 2014). The increment in intracellular Nrf2 level could be attributed to kaempferol and quercetin, the main flavonoids in CLE and CBE. Their antioxidant activities are partly mediated by increasing mRNA and protein levels of Nrf2, by decreasing its

Table 2
Constituents of *Crateva nurvala* leaves and bark extracts identified using UPLC-Orbitrap-HRMS.

Peak	R_t (min)	UV	$M^{+/-}$	Molecular formula	Error	MS^n	Identification	Occurence		Reference
								Leaves	Bark	
Alkaloids & Amides										
1	9.31	307	598.27667	$C_{31}H_{40}O_9N_3^+$	1.3	581, 436, 419, 348,265	Cadabicine-*O*-hexoside	−	+	(Fu et al., 2008)
2	9.94	286	582.28125	$C_{31}H_{40}O_8N_3^+$	0.4	565, 436	Cadabicine-*O*-deoxyhexoside	−	+	
3	10.17	219, 289	436.22314	$C_{25}H_{30}O_4N_3^+$	− 0.007	419, 348, 265	Cadabicine	−	+	(Ahmad et al., 1985;
			434.20767	$C_{25}H_{28}O_4N_3^-$	− 1.9	391, 314		−	+	Ahmad et al., 1987; Khanfar et al., 2003)
4	11.92	nd	274.27399	$C_{16}H_{36}O_2N^+$	− 0.2	256, z30, 106, 88	An alkaloid or amine	−	+	
5	11.97	nd	318.30054	$C_{18}H_{40}O_3N^+$	0.8	300, 256	Phytosphingosine	−	+	
6	14.43	nd	256.26346	$C_{16}H_{34}ON^+$	− 0.12	256, 214, 102, 88	Palmitamide	−	+	
7	14.71	nd	282.27936	$C_{18}H_{36}ON^+$	0.8	256, 247	Oleamide	−	+	
Flavonoids										
8	9.63	218, 292	595.1649	$C_{27}H_{31}O_{15}^-$	− 3.2	577, 505, 415, 457, 385, 355	Naringenin-6,8-*C*-diglucoside	+	+	(Mortimer et al., 2015)
			597.1817	$C_{27}H_{33}O_{15}^+$	0.5	579, 459		+	+	
9	9.78	294	449.10779	$C_{21}H_{21}O_{11}^-$	− 2.5	359, 329, 431, 287	Eriodictyol-8-*C*-hexoside	+	−	(Otify et al., 2015)
10	9.93	230, 311	451.12384	$C_{21}H_{23}O_{11}^+$	0.8	433, 331	Eriodictyol-6-*C*-hexoside	+	−	
11	10.05	326	609.14343	$C_{27}H_{29}O_{16}^-$	5.2	463, 447, 301	Quercetin–di-*O*-glycoside (-*O*-	+	+	(Pandey et al., 2014)
			611.1640	$C_{27}H_{31}O_{16}^+$	9	449, 303	deoxyhexose, -*O*-hexoside).	+	+	
12	10.18	265, 349	593.14984	$C_{27}H_{29}O_{15}^-$	− 2.3	447, 28, 431	Kaempferol-di-*O*-glycoside (-*O*-hexoside, -*O*-deoxyhexoside)	+	+	
13	10.34	265, 343	577.15515	$C_{27}H_{29}O_{14}^-$	− 1.9	431, 285	Kaempferol-di-*O*-deoxyhexoside	+	+	
			579.17078	$C_{27}H_{31}O_{14}^+$	− 0.09	433, 287				
14	10.9	287(sh), 313	305.06619	$C_{15}H_{13}O_7^-$	− 1.6	287	hexahydroxyflavan	+	−	
15	11.04	271, 318	431.0975	$C_{21}H_{19}O_{10}^-$	− 0.87	285, 284, 151	Kaempferol-*O*- hexoside	+	−	
			433.11246	$C_{21}H_{21}O_{10}^+$	− 1.07	287				
16	10.79	292	595.14398	$C_{30}H_{27}O_{13}^-$	− 2.9	431, 311, 577, 295	Unknown acylated flavonoid	+	−	
			597.16064	$C_{30}H_{29}O_{13}^+$	0.6	579, 477, 459, 459, 289	glycoside	+	−	
17	11.21	310	305.066	$C_{15}H_{13}O_7^-$	− 2.2	287, 261, 243, 199, 163,159, 119	hexahydroxyflavan	+	−	
Other phenolics and glycosides										
18	0.63	272	381.07947	$C_{17}H_{17}O_{10}^+$	− 5.6	219, 201	Furocoumarin –*O*-glycoside	−	+	
19	1.74	nd	315.07117	$C_{13}H_{15}O_9^-$	− 3.1	153, 109	Dihydroxybenzoic acid –*O*-hexoside	+	−	
20	9.03	298	325.09213	$C_{15}H_{17}O_8^-$	− 2.3	163, 119, 145	Coumaroylglucose	+	−	
21	9.18	215, 336	361.095	$C_{18}H_{17}O_8^-$	6.9	281, 237	Lignan derivative	+	−	
22	9.67	218, 301	581.22382	$C_{28}H_{37}O_{13}^-$	− 0.3	277	Lignan derivative	−	+	
23	9.92	270 (sh), 311	323.07666	$C_{15}H_{15}O_8$	− 1.6	159, 177, 305	Unknown phenolic	+	−	
24	10.11	287(sh), 325	161.02435	C9 H5 O3$^-$	− 0.06	133	Coumarin derivative	−	+	
25	10.19	218, 286	581.22211	$C_{28}H_{37}O_{13}^-$	− 1.1	566, 419, 401, 233	Lignan derivative	−	+	
26	10.85	310	351.10794	$C_{17}H_{19}O_8^-$	− 1.7	205, 333	3-*O*-p-Coumaroylquinic acid; 1-Me ether	+	−	
27	9.75	291, 330 (sh)	385.18695	$C_{19}H_{29}O_8^-$	5.5	223, 285, 205	Megastigmane –*O*-glycoside	+	−	
28	10.45	288(sh), 297	547.2384	$C_{25}H_{39}O_{13}$	− 2.2	501, 311, 293	Megastigmadien-di-*O*- glycoside	−	+	
Terpenoids										
29	14.9	nd	425.28641	$C_{24}H_{41}O_6^+$	5.9	365, 281	Unidentified acytelated diterpene	−	+	
30	15.33	nd	621.27100	$C_{28}H_{45}O_{15}^+$	− 8.6	593, 561, 533, 487	Acylated taxadiene	−	+	
31	15.87	nd	623.28571	$C_{35}H_{43}O_{10}^+$	1.02	605, 545	Acylated terpenoid	+	−	
32	16.38	nd	607.29102	$C_{35}H_{43}O_9^+$	1.4	547, 575	Acylated terpenoid	+	−	
33	17.06	nd	397.38275	$C_{29}H_{49}^+$	− 0.32	397, 243, 257, 161, 315, 287	β-sitosterol	−	+	
34	21.66	nd	409.38284	$C_{30}H_{49}^+$	− 0.09	409, 271, 257, 285, 229, 215, 203	Lupeol	−	+	
Acids										
35	0.67	nd	133.01437	$C_4H_5O_5^-$	0.9	115	Malic acid	+	−	
36	1.08	nd	205.03	$C_7H_9O_7^-$	− 2.7	179	Methylcitrate	+	−	
37	11.61	nd	329.23264	$C_{18}H_{33}O_5^-$	− 0.7	311, 293, 229, 171, 211	Trihydroxyoctadecenoic acid	+	−	
38	11.72	nd	287. 2223	$C_{16}H_{31}O_4^-$	− 0.5	269, 241	Dihydroxyhexadecanoic acid	+	−	
39	12	nd	307.19006	$C_{18}H_{27}O_4^-$	− 4.6	289, 235, 185	Octadecatrienoic acid	+	−	
40	12.91	nd	277.21613	$C_{18}H_{29}O_2^+$	− 4.2	259, 241, 221, 195, 163, 149, 135, 121, 107, 93	Octadecadienoic acid	+	−	
41	17.7	nd	341.26904	$C_{20}H_{37}O_4^-$	− 2.3	313, 269	Eicosanedioic acid	−	+	
42	18.32	nd	341.26917	$C_{20}H_{37}O_4^-$	− 1.6	313, 269, 253, 190	Eicosanedioic acid	+	−	

nd. not defined.

ubiquitination and boosting expression of its target genes (Kumar et al., 2016; Saw et al., 2014; Xiao et al., 2011). Boosting intracellular Nrf2 levels results in upregulation and expression of antioxidant genes which would ultimately reduce peroxidation of lipids, DNA and proteins. Nrf2 hyperactivation in early phases of I/R results in diminishing tubular damage because of ROS (Nezu et al., 2017).

Reduction in MDA levels, a biomarker of lipid peroxidation and the replenishing of intracellular glutathione levels could be attributed not

Table 3
Preliminary quantitative determination of different phytochemical classes and *in vitro* antioxidant activity of *C. nurvala* extracts[a].

Assay	*C. nurvala* leaves extract (CLE)	*C. nurvala* bark extract (CBE)	Standard	Units
Total phenolics	33.04 ± 2.2	45.5 ± 5.7	–	µg gallic acid equivalent/mg extract
Total flavonoids	38.5 ± 1.5	53.5 ± 3.1	–	µg quercetin equivalent/mg extract
Alkaloids	Nil.	0.64 ± 0.1	–	µg quinine equivalent /mg extract
DPPH	3.02 ± 0.07	1.83 ± 0.04	Gallic acid 0.038 ± 0.002	IC_{50} mg/ml
Iron reducing capacity	94.3 ± 1.5	87.66 ± 1.1	Ascorbic acid 1.5 ± 0.1	EC_{50} mg/ml

[a] Data and SE are average of triplicates.

only due to Nrf2 activation but also to the direct antioxidant activities of phenolics and flavonoids of *Crateva* extracts. Phenolics interact directly with ROS to save and spare intracellular antioxidant, e.g. GSH and to protect the targets of ROS, e.g, DNA and proteins. Such direct activities were observed when quercetin and kaempferol contributed in the reduction of tubular injury during cold preservation (Ahlenstiel et al., 2003).

The anti-inflammatory action of CLE and CBE is not as pronouncing as their antioxidant activities (Figs. 3 and 4). TNF-α levels increased following I/R either due to NF-κβ activation or secretion from dendritic cells and macrophages (Dong et al., 2007; Kezić et al., 2017). Like other pro-inflammatory mediators it mediates neutrophil infiltration (Kezić et al., 2017) and differentiation of monocytes (Chomarat et al., 2003). Agents reduce its level favors kidney protection (Patil et al., 2016) as observed in the present study. On the other hand, IL-6 is a biomarker for acute kidney injury (AKI) (Akcay et al., 2009). It is produced by both leucocytes and tubular epithelium. It is one of the early chemokines released in I/R (Nechemia-Arbely et al., 2008; Vries et al., 2009). It was significantly reduced by both CLE and CBE. However, a recent report assumes that it trans-signaling could play a protective role in AKI (Nechemia-Arbely et al., 2008; Vries et al., 2009).

CLE and CBE failed to reduce the elevated intracellular levels of NF-κβ which is a key player in renal I/R injury (Markó et al., 2016; Sung et al., 2002). It gets activated by ischaemia with or without reperfusion. NF-κβ is responsible for transcription and production of many other chemokines and cytokines responsible for progression of inflammation, oxidative stress and for leucocytes and macrophages infiltration and migration (Kezić et al., 2017). NF-κβ mediates the expression of monocyte chemoattractant protein-1 (MCP-1), responsible for monocytes recruitment. Genetic suppression of NF-κβ resulted in improved kidney function, reduced renal apoptosis and attenuated neutrophil and macrophage infiltration (Markó et al., 2016). Therefore, it is a therapeutic approach to target NF-κβ and/or suppress its activation (Kezić et al., 2017; Wan et al., 2011).

Although both CBE and CLE had reduced IL-6 and TNF-α, which act downstream to NF-κβ, they failed to suppress NF-κβ (Fig. 4). This suggests that the anti-inflammatory activity of the extracts could be mediated by targeting mediators other than NF-κβ. This agrees with a recent study on the anti-inflammatory activity of *C. nurvala* bark. *C. nurvala* extract counteract lipopolysaccharide- stimulated inflammation in murine macrophage culture by suppressing MAPK/ERK (mitogen-activated protein kinases/ extracellular signal-regulatedkinase) pathway rather than suppressing NF-κβ whose levels were not affected (Cho et al., 2015). Other studies are needed to decipher the mechanism of the anti-inflammatory activity of *C. nurvala* extracts.

Beneficial effects of *Crateva* extract in reducing oxidative stress and inflammation were reflected in attenuating the elevated levels of the apoptotic marker caspase-3. Suppression of caspase-3 utilizing siRNA or peptide led to significant attenuation in I/R injury (Chen et al., 2015).

Flavonoids are so frequently reported to have an antioxidant and anti-inflammatory activities. These activities are beneficial in counteracting oxidative stress and inflammation produced by I/R (Zhao et al., 2016). Generally, flavonoids are active in counteracting I/R deleterious effects (Lv et al., 2017; Testai et al., 2013; Zhang et al., 2013). Kaempferol, the aglycone of the main flavonoids of *Crateva,* is reported to protect heart muscles against I/R through its antioxidant activity and inhibition of GSK-3β, MAPK activities (Suchal et al., 2017; Zhou et al., 2015). Terpenoids activate Nrf2 which counteract the oxidative stress (Ding et al., 2015; Liu et al., 2014). Moreover, kaempferol plays many roles in counteracting inflammation, either by inhibiting the activation of pro-inflammatory mediator, modulating the pro-inflammatory enzymes, e.g COX-2, modulating the proinflammatory genes, e.g. MAPK or repressing the expression of adhesion molecules, e.g. VCAM-1, ICAM-1 which are essential for adhesion of leucocytes to endothelial cells of blood vessels or renal tubules (Devi et al., 2015).

To best of our knowledge, cadabicine was not involved in any pharmacological study as a single agent to determine its possible mechanism of action or activity.

Chemicals profiling gives insights in the bioactive constituents of extracts as a preliminary step to establish quality assessment of these extracts. Both CLE and CBE have so similar chemical profile except that bark contains cadabicine alkaloids. Biological data show that presence of alkaloids show no additive action to the bark extract. Actually, it can partly be inferred that alkaloids may antagonize the antioxidant activities of flavonoids or phenolics present in bark extract. On the other hand, CLE performed better than CBE in most of the analyzed pharmacological markers, therefore, leaves are highly recommended to be administered in case of I/R than bark especially that leaves are more sustainable source than bark. Trees need time to recover from bark collection.

Kaempferol glycosides are the major constituents of the tested extracts and previous studies have correlated the antioxidant and anti-inflammatory activities to them. Therefore, Kaempferol can serve as a biomarker for *C. nurvala* extracts.

In vitro antioxidant analyses can be correlated neither to each others nor to *in vivo* biological activity (Tan and Lim, 2015). This applies here, CLE and CBE performed similarly in iron reducing capacity, but CBE performed better in DPPH scavenging activity (Table 3). Different assays have different mechanisms of action. CLE and CBE had similarly attenuated the oxidative stress induced by I/R, although CBE had higher flavonids and phenolics content. This discrepancy can be attributed to other factors e.g., bioavailability, or presence of antagonizing constituents.

Finally, the present study gave insights in the chemical versatility of *C. nurvala* extracts, its main bioactive constituents and verified its potential renoprotective effect. However, other studies testing other mechanisms of action and/or studying the pharmacokinetics of the extract will complement the ethnopharmacological applications of the plant.

5. Conclusion

Crateva nurvala extracts are effective in alleviating I/R condition mainly through the antioxidant and the anti-inflammatory activities of their phytochemicals. Flavonoids, especially kaempferol are responsible for the renoprotective action. In contrast to the ethnoknowledge, leaves are as effective as bark and can be utilized as renoprotective because of its sustainable nature. Chemical profiling using modern techniques pave the way for adequate quality control of *C. nurvala* extracts and

help in elucidating the main bioactive phytochemicals.

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Conflict of interest

None.

Authors contribution

Design of the experiment and writing of the article: M.A. Choucry, M. N.A. Khalil, S. A. El Awdan.

Performing the chemical study: M.A. Choucry, M. N.A. Khalil.

Performing the pharmacological Study: S. A. El Awdan.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2017.11.034.

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