academicJournals

Vol. 9(31), pp. 829-837, 17 August, 2015
DOI: 10.5897/JMPR2015.5733
Article Number: 55D6D4554892
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

An aqueous *Citrillus colocynthis* peel extract inhibits neutrophil reactive oxygen species production and attenuates lung inflammation in mice

Fatma Zioud[1], Amira Ben Mahmoud[1], Tarak Boussetta[2,3], Jamel El-Benna[2,3] and Rafik Bachoual[1]*

[1]Faculté des Sciences de Gabès, Université de Gabès, Tunisia.
[2]Inserm, U1149, CNRS, ERL8252, Centre de Recherche sur l'Inflammation (CRI), Paris, France.
[3]Université Paris Diderot, Sorbonne Paris Cité, Laboratoire d'Excellence Inflamex Faculté de Médecine, Site Xavier Bichat, Paris, France.

Received 12 January, 2015; Accepted 12 August, 2015

Citrillus colocynthis **peel aqueous extract (CCPAE) is widely used to treat disorders such as inflammation, ulcers and infections, but its pharmacological target is not known. The objectives of this work were to study the effect of *C. colocynthis* peel aqueous extract, on human neutrophil reactive oxygen species (ROS) production *in vitro,* and to evaluate its protective effect on lipopolysaccharide (LPS)-induced lung inflammation *in vivo* in mice. Neutrophils were isolated from blood of healthy volunteers. ROS generation was measured by luminol-amplified chemiluminescence. Superoxide anion generation was detected by the cytochrome c reduction assay. H_2O_2 was detected by horseradish peroxidase (HRP)-amplified chemiluminescence assay. Myeloperoxidase (MPO) activity was measured by the tetramethylbenzidine oxidation method. Lung inflammation was induced in mice by LPS instillation. CCPAE inhibited luminol-amplified chemiluminescence of resting neutrophils and N-formyl-methionyl-leucyl-phenylalanine (fMLF)- or phorbolmyristate acetate (PMA)-stimulated neutrophils, in a concentration-dependent manner. CCPAE also inhibited superoxide anion generation; and did not scavenge H_2O_2 and superoxide anions nor inhibited MPO activity *in vitro* suggesting that it inhibits nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation. *In vivo* studies showed that CCPAE attenuated LPS-induced lung inflammation in mice. This study shows that CCPAE inhibits neutrophil ROS production and attenuates LPS-induced lung inflammation in mice. Inhibition of NADPH oxidase activation by CCPAE could explain its anti-inflammatory action.**

Key words: *Citrullus colocynthis*, colocynth, inflammation, neutrophils, reactive oxygen species (ROS), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.

INTRODUCTION

Citrullus colocynthis (L.) Schrad. (Cucurbitaceae), commonly known as "bitter apple" is a plant that grows abundantly in Tunisia (Pottier-Alapetite, 1981), and widely in other parts of the world (Abbes el al., 2006). In the Tunisian traditional medicine, this plant has been used to treat various diseases including, hypertension and rheumatism (Le Flock, 1983; Boukef et al., 1982), while in other countries it is used to treat constipation,

oedema bacterial infections, cancer and diabetes, and as an abortifacient (Abbes el al., 2006). The ethnobotanical uses of this plant include its use as cathartic, purgative and vermifuge, and for the treatment of fever, cancer, amenorrhea, jaundice, leukemia, rheumatism and tumour (Abbes el al., 2006). The ethnobotanical use efficiency of this plant was consolidated by a number of studies which demonstrated that *C. colocynthis* Schrad has a potent anti-tumour (Tannin-Spitz et al., 2007), anti-microbial (Marzouk et al., 2009, 2010a) and antioxidant activity (Marzouk et al., 2010b). Many secondary metabolites from *C. colocynthis*, including cucurbitacins, flavonoids, caffeic acid derivatives and terpenoids, have been previously reported (Yankov and Hussein, 1975; Hatam et al., 1989; Maatooq et al., 1997) and could explain the biological activity of this plant.

Inflammatory disorders are due to excessive production of pro-inflammatory mediators, such as tumor necrosis factor (TNF)α, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1, IL-6, IL-8, leukotriene B4 and platelet-activating factor (PAF), to the activity of inflammatory cells such as neutrophils, monocytes and macrophages, and to the excessive production of reactive oxygen species (ROS) (Ley, 2002; Nathan, 2006). Polymorphonuclear neutrophils play a key role in host defenses against invading microorganisms (Hampton et al., 1998), but excessive neutrophil activation participates in tissue damage associated with inflammatory disorders (Babior, 2000). In response to a variety of agents neutrophils migrate to inflammatory sites, where they release proteases, bactericidal peptides, and large quantities of ROS, in a process known as the respiratory burst (Babior, 1984). Oxygen reduction by neutrophil NADPH oxidase, a multicomponent enzyme system, yields superoxide anion ($O_2^{-\cdot}$) (El-Benna et al., 2005), while myeloperoxidase (MPO) produces hypochloric acid from hydrogen peroxide (Klebanoff, 2005).

This study was undertaken to analyze the effect of *C. colocynthis* peel aqueous extract (CCPAE) on ROS production by human neutrophils; and to evaluate the effect of this product on intratracheal lipopolysaccharide (LPS)-induced lung inflammation in mice.

MATERIALS AND METHODS

Chemicals and reagents

Luminol, cytochrome c, fMLF, PMA, zymosan, superoxide dismutase (SOD), catalase and HRPO *Escherichia coli* (O55:B5) lipopolysaccharide (LPS) were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Ficoll and Dextran T500 were from GE Healthcare. phosphate buffered saline (PBS), Hank's balanced salt solution (HBSS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and glucose were from Gibco. 2',7'-dichlorofluoroscein-diacetate (DCFH-DA) was from Acros Fine Chemicals. Stock solutions of fMLF (10^{-2} mol/L) and PMA (1 mg/ml) were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C. The different solutions were diluted in PBS immediately before use.

Preparation of CCPAE

In this study, three batch of Tunisian *C. colocynthis*, collected from the island of Jerba in South of Tunisia were used. The colocynth peels were, dried at 37°C, blended and suspended in sterile 0.9% NaCl, then centrifuged at 2000 rpm for 3 min. From each batch, the supernatants of different preparations of CCPAE were used for the experiments. The results obtained with different preparations from different batches are reproducible and the same dose effect responses were found.

Isolation of human neutrophils

Venous blood was collected from healthy adult volunteers and neutrophils were isolated by Dextran sedimentation and density gradient centrifugation as previously described (El Benna and Dang, 2007). Erythrocytes were removed by hypotonic lysis. Following isolation, the cells were resuspended in appropriate medium, such as HBSS. The cells were counted and their viability was determined with the Trypan Blue exclusion method.

Measurement of ROS production by chemiluminescence

Isolated neutrophils were resuspended in HBSS at a concentration of 1 million per ml. Cell suspensions (5×10^5) in 0.5 ml of HBSS containing 10 µM luminal in the presence or absence of CCPAE were preheated to 37°C in the thermostatted chamber of a luminometer (Berthold-Biolumat LB937) and allowed to stabilize. After a baseline reading, cells were stimulated with 10^{-6} M fMLF or 100 ng/ml PMA. Changes in chemiluminescence were measured over a 30-min period.

Measurement of superoxide production

Isolated cells were resuspended in HBSS at a concentration of 1 million/ml. Cell suspensions in 1 ml of HBSS containing 1 mg/ml cytochrome c in the presence or absence of CCPAE were preheated to 37°C in the thermostated chamber of a spectrophotometer (Uvikon) and allowed to stabilize. After a baseline reading, cells were stimulated with 10^{-6} M fMLF or 100 ng/ml PMA. Changes in absorbance were measured at 550 nm over a 15-min period.

Detection of H_2O_2

In order to investigate whether CCPAE reacts directly with H_2O_2 CCPAE was incubated in PBS with H_2O_2 (80 µM) for 15 min in the presence of luminol (10 µM) and the reaction was initiated by adding HRPO (5U). Changes in chemiluminescence were

*Corresponding author. E-mail: Rafik.Bachoual@fsg.rnu.tn. Fax: (00) (216) 75 392 421.

measured over a 15 min period.

Preparation of azurophilic granules and measurement of MPO activity

Neutrophils were lysed by nitrogen cavitation and the granule fraction was purified by Percol gradient centrifugation (Udby and Borregaard, 1989). The granules were sonicated in 0.2 cetyltrimethylammonium bromide (CTAB) and MPO activity was assessed using the H_2O_2-dependent tetramethylbenzedine (TMB) oxidation assay at 650 nm.

LPS-induced lung inflammation in mice

These experiments were approved by our Institutional Committee on Animal Care and Use and the experimental protocol complied with Tunisian legal requirements for animal studies. Male Balb C mice aged 7 weeks and weighing 22 to 26 g were purchased from SIFAT (Société des industries pharmaceutique de Tunisie) and housed in standard wire-topped cages and the temperature-controlled units. Food and water were supplied *ad libitum*. The mice received an intraperitoneal injection of 200 mg/kg CCPAE on day 0 (D0), followed by 200 mg/kg on day 1 (D1). Three hours after the second injection, the mice received a cocktail of anesthetics [75 mg/kg ketamin (Virbac Santé Animale) plus 1 mg/kg medetomidine (Pfizer)], before intratracheal LPS instillation (5 µg/mouse). The mice were aroused by an intraperitoneal injection of 1 mg/kg atipamezol (Pfizer), a medetomidine antagonist, and were killed 24 h later.

Bronchoalveolar lavage (BAL) and lung sampling

The mice were anesthetized by an intraperitoneal injection of 50 mg of urethane (sigma) and killed by exsanguination. The lungs were lavaged twice with 1 ml of physiological saline, removed from the chest cavity, and immediately placed at -80°C until use. The lavage fluid (1.8 ml) was immediately placed on ice. Free alveolar cells were recovered from the lavage fluid by centrifugation at 400 *g* for 15 min at 4°C. The total protein concentration in the supernatant was measured with the Quick-Start Bradford assay (Bio-Rad, Marnes-la-Coquette, France). The cell pellet was suspended in 150 µl of physiological saline and an aliquot was used to determine the total white cell count with a hemocytometer. For differential counts, the cell suspension was cytospun (Cytospin-2, Shandon Products Ltd.), fixed in methanol, and stained with Diff Quick solution (Medion Diagnostics, Plaisir, France). One hundred cells were counted with an oil immersion lens (1000×).

Statistical analysis

Data were reported as mean ± standard error. The Newman-Keuls multiple comparisons test was used, and P values <0.05 were considered to denote significant differences.

RESULTS

CCPAE inhibits luminol-amplified chemi-luminescence in human neutrophils, independently of the stimulus

To investigate the effect of CCPAE on neutrophil ROS production, human neutrophils were incubated with different CCPAE concentrations and ROS were detected by luminol-amplified chemiluminescence. Results show that CCPAE inhibited luminol-amplified chemiluminescence in resting neutrophils (Figure 1A) and in neutrophils stimulated with fMLF (Figure 1B) or PMA (Figure 1C). The effect of CCPAE shows an inhibitory effect starting at a concentration between 20 and 40 µg/ml. As fMLF and PMA activate neutrophils through different transduction pathways, these results suggested that CCPAE decreases neutrophil ROS production by either inhibiting a final common target, such as the NADPH oxidase or MPO, or by scavenging ROS.

CCPAE inhibits fMLF- and PMA-stimulated superoxide anions production by human neutrophils

Luminol-amplified chemiluminescence technique used above allows the detection of several ROS molecules (such as superoxide anions, H_2O_2) and several neutrophil functions (such as NADPH oxidase activation, degranulation and MPO activity). To investigate precisely the effect of CCPAE on neutrophil NADPH oxidase activation, superoxide anion production was measured using cytochrome c reduction assay. Since superoxide production is not detectable in resting neutrophils, the effect of CCPAE was tested only on PMA- and fMLF-stimulated cells. Results show that CCPAE inhibited superoxide anion production by neutrophils stimulated with PMA or fMLF (Figure 2). The effect of CCPAE shows an inhibitory effect starting at a concentration as low as 20 µg/ml. As fMLF and PMA activate the NADPH oxidase through different transduction pathways, these results suggested that CCPAE directly inhibits a final common target, such as the NADPH oxidase, or scavenges superoxide anions.

CCPAE does scavenge superoxide anions nor H_2O_2 nor inhibit MPO activity

Firstly, to investigate whether CCPAE scavenges superoxide anions, xanthine/xanthine oxidase was used to produce superoxide anions, which were then detected by the cytochrome c reduction assay. Results show (Figure 3A) that CCPAE had no effect on superoxide anions production by this system. Secondly, to investigate whether CCPAE scavenges H_2O_2, commercial H_2O_2 which was detected by the luminol-amplified chemiluminescence assay was used. Results show (Figure 3B) that CCPAE had no effect on H_2O_2. Thirdly, to investigate the effect of CCPAE on MPO activity, azurophilic granules extracts were incubated with different concentrations of CCPAE, and MPO activity was measured using H_2O_2-TMB oxidation assay. Results

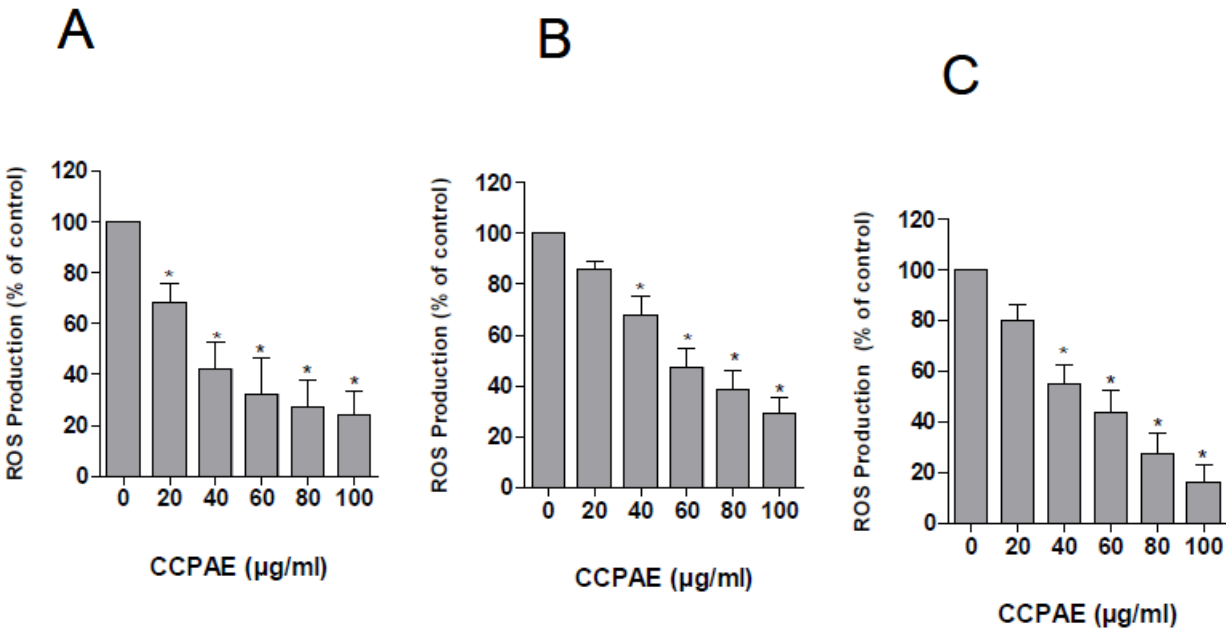

Figure 1. Effect of CCPAE on ROS production by human neutrophils. Human neutrophils (5×10^5) were incubated in the presence or absence of different CCPAE concentrations in resting conditions (A), and stimulated with fMLF (10^{-6} M) (B), or PMA (100 ng/ml) (C). Luminol-amplified chemiluminescence was measured for 30 min (mean ± SEM of 5 experiments, *p<0.05).

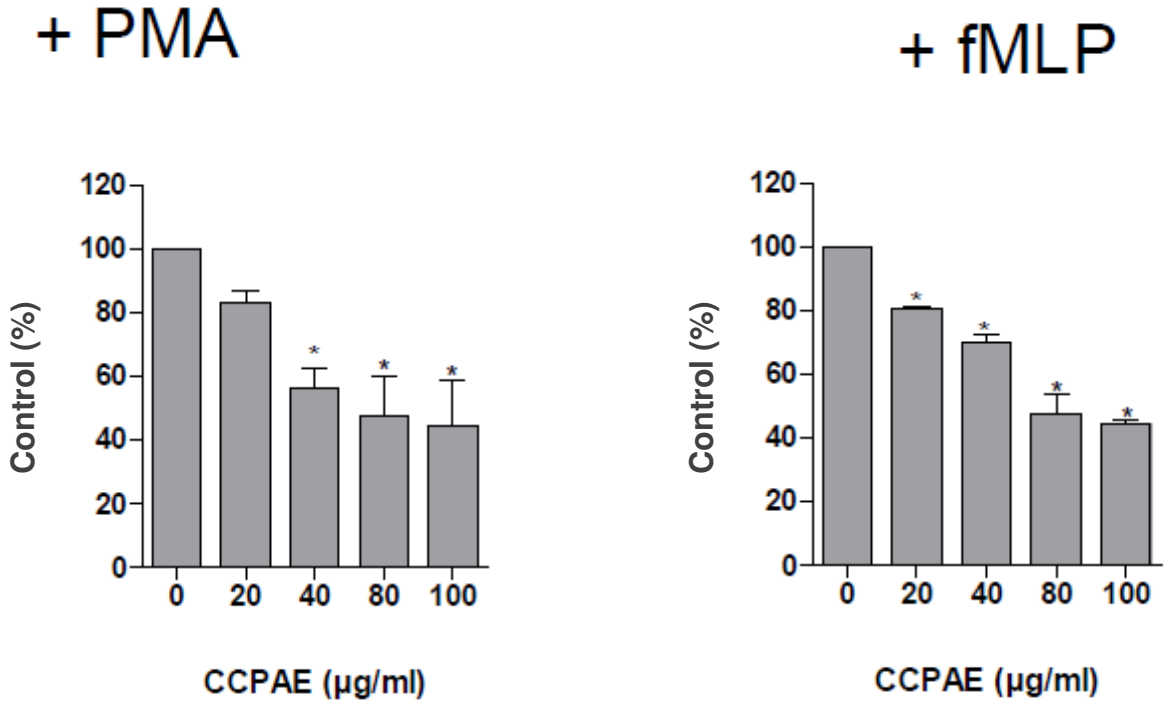

Figure 2. Effect of CCPAE on superoxide anion production by neutrophils. Human neutrophils (1×10^6) were incubated in the presence or absence of CCPAE, and stimulated with fMLF (10^{-6} M) or PMA (100 ng/ml). Cytochrome c reduction was measured at 550 nm in a spectrophotometer for 10 min (mean +/- SEM of 5 experiments, *p<0.05).

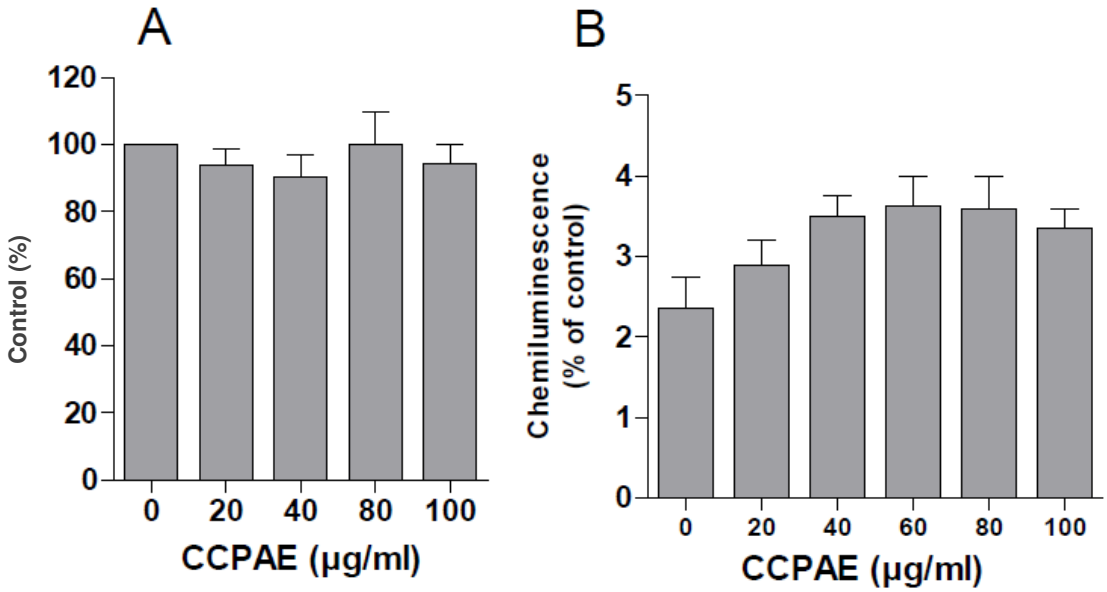

Figure 3. Effect of CCPAE on superoxide anions and H_2O_2 *in vitro*. (A) Xanthine oxidase was incubated in the presence or absence of CCPAE, xanthine was added and superoxide anions was detected by the cytochrome c reduction assay. (B) H_2O_2 was incubated in the presence or absence of CCPAE and detected using HRP-amplified chemiluminescence (mean +/- SEM of 3 experiments, *p<0.05).

show (Figure 4) that CCPAE had no effect on MPO activity.

Effect of CCPAE on BAL fluid protein content and cellularity in LPS-treated mice

Intratracheal administration of 5 µg of LPS to mice induced a significant increase in BAL protein content after 24 h, compared with animals treated with either the vehicle or CCPAE alone (Figure 5). Interestingly, the BALF protein content after intratracheal LPS challenge was significantly lower when animals were pretreated with 200 mg/kg CCPAE (Figure 5).

Moreover, intratracheal LPS administration induced a significant increase in both the BALF total cell count ($p < 0.05$ vs. vehicle or CCPAE alone, Figure 6A and B) and the BALF neutrophil count after 24 h ($p < 0.05$ vs. vehicle or CCPAE alone, Figure 6A and C). Neither the vehicle nor CCPAE at 200 mg/kg modified the BALF cell count. However, intraperitoneal CCPAE injection at 200 mg/kg significantly reduced both the BALF total cell count after intratracheal administration of LPS ($p < 0.05$ vs. LPS alone, Figure 6A and B), and the neutrophil recruitment when compared with LPS alone ($p < 0.05$ vs. LPS, Figure 6A and C).

DISCUSSION

In this study, it was shown that CCPAE inhibited neutrophil ROS production, as measured by luminol-amplified chemiluminescence in both resting and stimulated neutrophils. However, CCPAE did not scavenge superoxide anions or H_2O_2, but markedly inhibited superoxide anions production by neutrophils. In addition, CCPAE attenuated LPS-induced lung inflammation in mice.

CCPAE inhibited luminol-amplified chemiluminescence in human neutrophils stimulated with the chemotactic peptide fMLF or the protein kinase C activator PMA. As fMLF and PMA induce NADPH oxidase activation through different transduction pathways, these results suggest that CCPAE does not affect a specific transduction pathway, but directly inhibits a final common biochemical target, such as the NADPH oxidase or MPO, or that it scavenges reactive oxygen species. Besides, it was found out that CCPAE had an inhibitory effect on cytochrome c reduction, a specific technique for superoxide anion detection, suggesting that CCPAE could react either by inhibiting NADPH oxidase activity or by scavenging superoxide anions.

Luminol-amplified chemiluminescence can be used to assay both intracellular and extracellular ROS production by neutrophils, as luminol is a membrane-permeable molecule. Luminol-amplified chemiluminescence is dependent on superoxide anions, H_2O_2, and on peroxidases, such as cytosolic peroxidases and MPO (Dahlgren and Karlsson, 1999). To determine whether CCPAE reacted with H_2O_2, a more specific technique was used to detect H_2O_2 *in vitro*. CCPAE did not affect

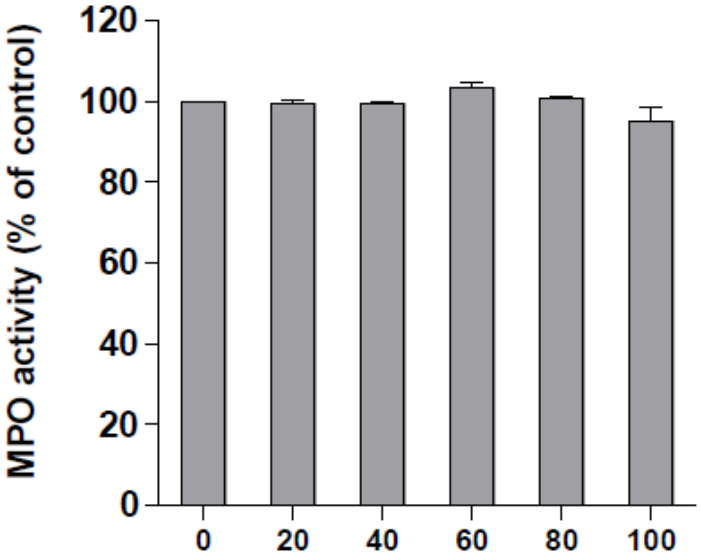

Figure 4. Effect of CCPAE on MPO activity. MPO was incubated with or without CCPAE and its activity was measured in terms of tetra-methylbenzidine oxidation at 655 nm (mean +/- SEM of 5 experiments, *p<0.05).

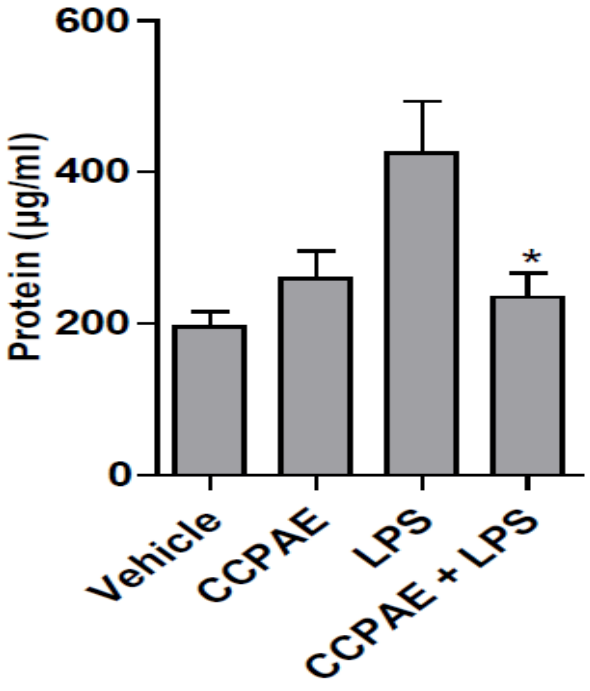

Figure 5. Effect of CCPAE on the protein concentration of mouse bronchoalveolar lavage fluid (BALF). Total content of protein was measured in BALF 24 h after intratracheal instillation of lipopolysaccharide (LPS 5 µg/mouse) or in controls. LPS induced a massive increase in the protein content, which was significantly attenuated by CCPAE (200 mg/kg) (n=8, mean ± SEM, *p<0.05).

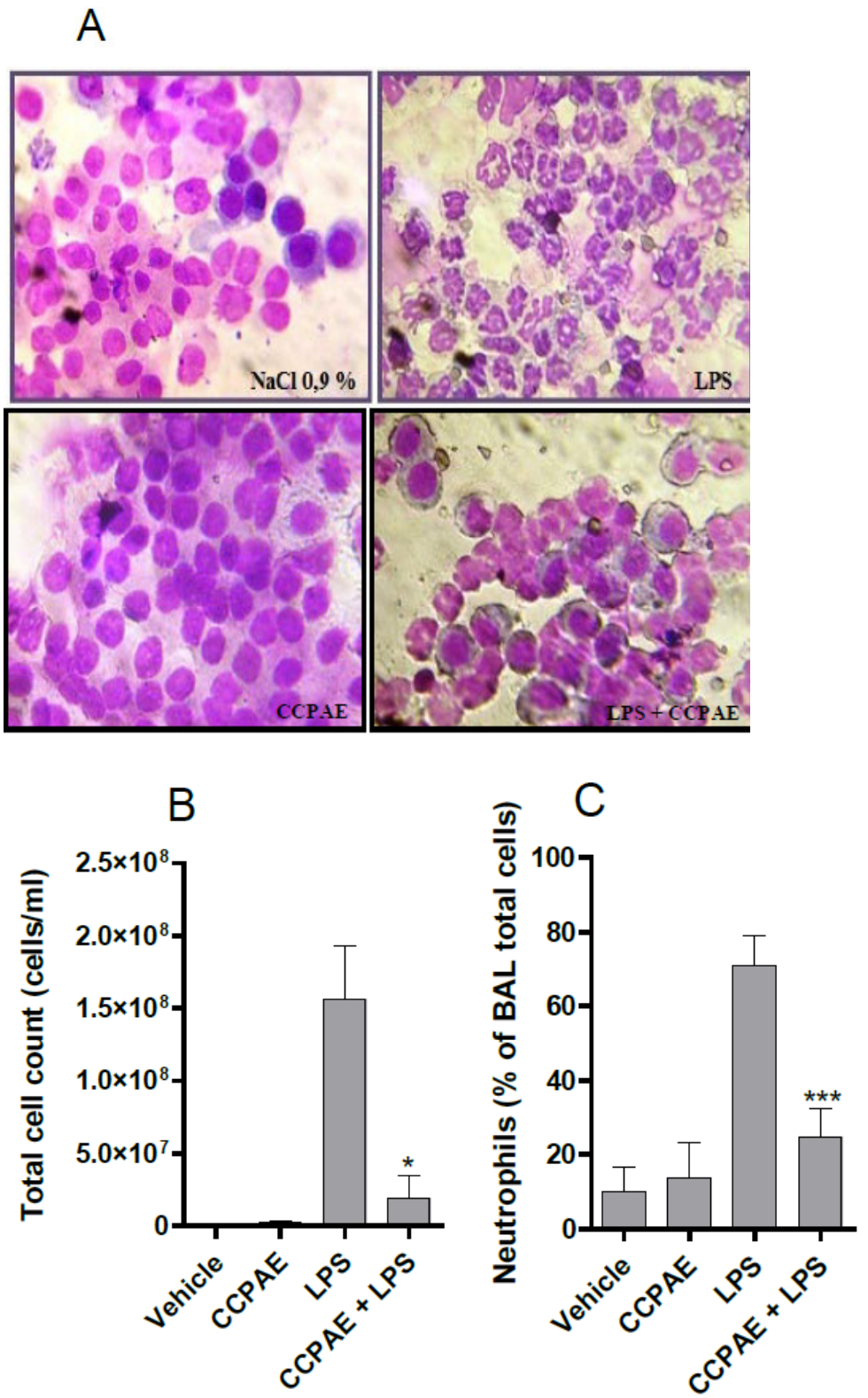

Figure 6. Effect of CCPAE on BALF cell content. BALB/c mice were treated with vehicle, CCPAE (200 mg/kg), LPS (5 µg/mouse) or CCPAE (200 mg/kg) plus LPS (5 µg/mouse), and cells were counted in BALF. A, Total cell counts; B and C, Neutrophil counts (n=8, mean ± SEM *p<0.05).

the amount of H_2O_2, suggesting that CCPAE does not react with H_2O_2. However, CCPAE inhibited NADPH oxidase activity *in vitro* and exerted a potent anti-inflammatory effect in the lungs of mice exposed to LPS, reducing the BALF protein content, total cell number and neutrophil count. The inhibitory effect of CCPAE on NADPH oxidase activity could thus explain its anti-inflammatory action *in vivo.*

Persistent asthma, chronic obstructive pulmonary disease (COPD) and emphysema are chronic inflammatory lung diseases (Lemanske and Busse, 1997; Ward, 1997). COPD and asthma involve several types of inflammatory cells and soluble mediators (Barnes et al., 2003). COPD is associated with destruction of the alveolar epithelium and flooding of the alveolar spaces with proteinaceous exudates containing abundant neutrophils (Di Stefano et al., 1998). In our study, BALF from mice exposed to LPS contained a large amount of protein, reflecting high-permeability pulmonary oedema. The BALF protein concentration was significantly reduced by CCPAE treatment, suggesting that CCPAE reduces lung vascular permeability and oedema, and might therefore protect the integrity of the alveolocapillary membrane. The reduction in neutrophil infiltration could explain these beneficial effects, as neutrophils are considered a primary cellular effector of alveolocapillary damage in COPD and asthma (Pesci et al., 1998; Fabbri et al., 2003).

It was found out that CCPAE inhibited NADPH oxidase activity *in vitro*, an effect possibly explaining the anti-inflammatory action observed *in vivo*. NADPH oxidase stimulation triggers murine macrophages to produce ROS (Lincoln et al., 1995; Gelderman et al., 1998). Secreted ROS enhances the secretion of TNFα, IL-8 and other proinflammatory cytokines (Nelson et al., 1998). In particular, alveolar macrophage-derived TNFα and IL-8 recruit neutrophils to sites of inflammation (Gibson et al., 2001). NADPH oxidase inhibition by CCPAE could attenuate these inflammatory reactions.

Fruit and vegetables are important sources of antioxidants, including ascorbic acid, carotenoids, flavonoids and hydrolysable tannins. Epidemiological studies indicate that populations that consume foods rich in specific polyphenols have a lower incidence of inflammatory disorders, such as cardiovascular and cerebrovascular disease, as well as certain cancers (Huxley and Neil, 2003; Temple and Gladwin, 2003). Several studies have demonstrated the high antioxidant activity of colocynth. This activity is attributed especially to a number of plant secondary metabolites including cucurbitacins, flavonoids, caffeic acid derivatives and terpenoids (Flavone C-glycosides) and cucurbitacin glycosides from *C. colocynthis*. (Delazar et al., 2006). In carrageenan-induced rat paw edema model, Marzouk et al. (2013) showed that *C. colocynthis* also has a potent anti-inflammatory action. The intraperitoneal administration of aqueous extracts of seeds and fruits of *C. colocynthis*, significantly reduced the paw edema induced by the noxious agent (Marzouk et al., 2013).

ROS are important contributors to tissue injury, inflammation, cancer and many other diseases. The antioxidant properties of flavonoids especially the flavones glucoside (isosaponarin, isovitexin and isoorientin 3'-*O*-methyl ether) and the cucurbitacin glucosides (2-*O*-β-D-glucopyranosylcucurbitacin and 2-*O*-β-D-glucopyranosyl cucurbitacin L), probably contribute, at least to some extent, to the pharmacological and traditional medicinal uses of the *C. colosynthis* (Abbas el al., 2009). These compounds scavenge free radicals and inhibit lipid oxidation *in vitro* (Gil et al., 2000; Noda et al., 2002). Further studies are needed to identify the precise phenolic compounds responsible for the NADPH oxidase inhibition observed in this study.

Conclusion

As concluded from this study, CCPAE inhibited neutrophil luminol-amplified chemiluminescence *in vitro*, by inhibiting NADPH oxidase. CCPAE also attenuated inflammation induced by intratracheal endotoxin instillation in mice, leading to a decrease in the BALF protein concentration, total cellularity and neutrophil content.

Conflict of interest

The authors declare that they have no conflict of interest.

Abbreviations

fMLF, N-formyl-methionyl-Leucyl-phenylalanine; **MPO,** myeloperoxidase; **PMA,** phorbolmyristate acetate; **ROS,** reactive oxygen species.

REFERENCES

Babior BM (2000) Phagocytes and oxidative stress. Am. J. Med. 109:33-44.

Barnes PJ, Shapiro SD, Pauwels RA (2003). Chronic obstructive pulmonary disease: molecular and cellular mechanisms. Eur. Respir. J. 22:672-688.

Boukef K, Souissi HR., Balansard G (1982). Contribution to the study of plants used in traditional medicine in Tunisia. Plant Med. Phytother. 16:260-279.

Dahlgren C, Karlsson A (1999). Respiratory burst in human neutrophils. J. Immunol. Methods 232:3-14.

Delazar A, Gibbons S, Kosari AR, Nazemiyeh H, Modarresi M, Nahar L, Sarker SD (2006). Flavone C-glycosides and cucurbitacin glycosides from Citrullus colocynthis. DARU J. Pharm. Sci. 14(3):109-114.

Di Stefano A, Capelli A, Lusuardi M, Balbo P, Vecchio C, Maestrelli P,

Mapp CE, Fabbri LM, Donner CF, Saetta M (1998). Severity of airflow limitation is associated with severity of airway inflammation in smokers. Am. J. Respir. Crit. Care Med. 158(4):1277-1285.
El-Benna J, Dang PM, Gougerot-Pocidalo MA, Elbim C (2005). Phagocyte NADPH oxidase: a multicomponent enzyme essential for host defenses. Arch. Immunol. Ther. Exp. (Warsz) 53:199-206.
Fabbri LM, Romagnoli M, Corbetta L, Casoni G, Busljetic K, Turato G, Ligabue G, Ciaccia A, Saetta M, Papi A (2003). Differences in airway inflammation in patients with fixed airflow obstruction due to asthma or chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. 167(3):418-424.
Gelderman MP, Lefkowitz DL, Lefkowitz SS, Bollen A, Moguilevsky N (1998). Exposure of macrophages to an enzymatically inactive macrophage mannose receptor ligand augments killing of *Candida albicans*. Proc. Soc. Exp. Biol. Med. 217(1):81-88.
Gibson PG, Simpson JL, Saltos N (2001). Heterogeneity of airway inflammation in persistent asthma: evidence of neutrophilic inflammation and increased sputum interleukin-8. Chest 119(5):1329-1336.
Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA (2000). Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J. Agric. Food Chem. 48(10):4581-4589.
Hampton MB, Kettle AJ, Winterbourn CCHampton MB, Kettle AJ, Winterbourn CC (1998). Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 92(9):3007-3017.
Hatam NA, Whiting DA, Yousif NJ (1989). Cucurbitacin glycosides from Citrullus colocynthis. Phytochemistry 28(4):1268-1271.
Huxley RR, Neil H (2003). The relationship between dietary flavonol intake and coronary heart disease mortality: a metaanalysis of prospective cohort studies. Eur. J. Clin. Nutr. 57:904-908.
Klebanoff SJ (2005). Myeloperoxidase: friend and foe. J. Leukoc. Biol. 77(5):598-625.
Le Flock E (1983). Contribution à une étude Ethnobotanique de la flore tunisienne, Ed. Imprimerie officielle de la république tunisienne, Tunis, Tunisia.
Lemanske RF Jr, Busse WW (1997). Asthma. JAMA 278:1855-1873.
Ley K (2002). Integration of inflammatory signals by rolling neutrophils. Immunol. Rev. 186:8-18.
Lincoln JA, Lefkowitz DL, Cain T, Castro A, Mills KC, Lefkowitz SS, Moguilevsky N, Bollen A (1995). Exogenous myeloperoxidase enhances bacterial phagocytosis and intracellular killing by macrophages. Infect. Immun. 63(8):3042-3047.
Maatooq GT, El-Sharkawy SH, Afifi MS, Rosazza PN (1997). C-PHydroxybenzoyl-glycoflavanones from Citrullus colocynthis. Phytochemistry 44:187-190.
Marzouk B, Marzouk Z, Décor R, Edziri H, Haloui E, Fenina N, Aouni M (2009). Antibacterial and anticandidal screening of Tunisian Citrullus colocynthis Schrad. from Medenine. J. Ethnopharmacol. 125(2):344-349.
Marzouk B, Marzouk Z, Décor R, Mhadhebi L, Fenina N, Aouni M (2010a). Antibacterial and antifungal activities of several populations of Tunisian Citrullus colocynthis Schrad. immature fruits and seeds. J. Med. Mycol. 20(3):179-184.
Marzouk B, Marzouk Z, Haloui E, Turki M, Bouraoui A, Aouni M, Fenina N (2013). Anti-inflammatory evaluation of immature fruit and seed aqueous extracts from several populations of Tunisian Citrullus colocynthis Schrad. Afr. J. Biotechnol. 10(20):4217-4225.
Marzouk Z, Marzouk B, Mahjoub MA, Haloui E, Mighri Z, Aouni M, Fenina N (2010b). Screening of the antioxidant and the free radical scavenging potential of Tunisian Citrullus colocynthis Schrad. from Mednine. J. Food Agric. Environ. 8:261-265.
Nathan C (2006). Neutrophils and immunity: challenges and opportunities. Nat. Rev. Immunol. 6:173-182.
Nelson S, Summer WR (1998). Innate immunity, cytokines, and pulmonary host defense. Infect. Dis. Clin. North Am. 12:555-567.
Noda Y, Kaneyuki T, Mori A, Packer L (2002). Antioxidant activities of pomegranate fruit extract and its anthocyanidins: delphinidin, cyanidin, and pelargonidin. J. Agric. Food Chem. 50(1):166-171.
Pesci A, Balbi B, Majori M, Cacciani G, Bertacco S, Alciato P, Donner CF (1998). Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. Eur. Respir. J. 12(2):380-386.
Pottier-Alapetite G (1981). Flore De La Tunisie, Angiospermes-Dicotylédones: Gamopétales. Ed. Imprimerie officielle de la république tunisienne, Tunis, Tunisia. p 930.
Tannin-Spitz T, Grossman S, Dovrat S, Gottlieb HE, Bergman M (2007). Growth inhibitory activity of cucurbitacinglucosides isolated from *Citrulluscolocynthis*on human breast cancer cells. Biochem. Pharmacol. 73(1):56-67.
Temple NJ, Gladwin KK Temple NJ, Gladwin KK (2003). Fruit, vegetables, and the prevention of cancer: research challenges. Nutrition 19:467-470.
Udby L, Borregaard N (2007). Subcellular fractionation of human neutrophils and analysis of subcellular markers. Methods Mol. Biol. 412:35-56.
Ward PA (1997). Phagocytes and the lung. Ann. NY Acad. Sci. 832:304-310.
Yankov LK, Hussein SM (1975). Fatty acid from the oil of the seeds of *Citrulluscolocynthis*, Dokl. Bolg. Akad. Nouk 28:209-12.