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Antinociceptive Pentacyclic Triterpenoids from the Cameroonian Brown Propolis

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ABSTRACT

Background: Propolis is a resinous substance that honeybees collect from different plant exudates and use to fill gaps and to seal parts of the hive. It possesses many biological activities: antinociceptive, anti-inflammatory, antibacterial, antiviral, fungicidal, antitumoral, etc. The present study was designed to evaluate the antinociceptive effects of three pentacyclic triterpenoids derivatives isolated from the Cameroonian brown propolis: lup-20(29)-en-3-one, erythrodiol palmitate and 18-iso-olean-12-ene-3,11-dione. **Materials and Methods:** The antinociceptive effects of the pentacyclic triterpenoids were investigated in animals employing acetic acid induced abdominal constrictions, formalin-induced nociception and the mechanical hypernociception induced by prostaglandin E_2. Mice were submitted to the open-field test in order to assess any motor dysfunction and sedation or alteration in locomotor activity. The ability of these pentacyclic triterpenoids to induce cytotoxicity were further investigated by using prostate cancer (PC-3) or mouse fibroblast (3T3) cells lines and a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) bioassay. **Results:** The pentacyclic triterpenoids isolated from propolis administered intraperitonealy produced significant antinociceptive effects in the acetic acid and formalin tests. All the triterpenoids elicited dose-dependent antinociceptive effects in mechanical hypernociception induced by intraplantar injection of prostaglandin E_2. These antinociceptive effects were significantly attenuated by pretreatment with naloxone. The pentacyclic triterpenoids did not alter the locomotion of animals in the open-field tests which suggest a lack of a central depressant effect. As shown, incubation for 24 h of the PC-3 or 3T3 cells lines with the pentacyclic triterpenoids up to a concentration of 30 μM produces no cell toxicity. **Conclusion:** Taken together the results of this study suggest that the pentacyclic triterpenoids derivatives isolated from propolis (lup-20(29)-en-3-one, erythrodiol palmitate and 18-iso-olean-12-ene-3,11-dione) produced dose related antinociception in models of chemical nociception and mechanical hypernociception through mechanisms that involve an interaction with opioidergic pathway.

Key words: Pentacyclic triterpenoids, antinociceptive, opioidergic, prostaglandine E_2

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INTRODUCTION

Pain is one of the most important health problems because of its prevalence and the disabilities it can induce. In fact, every one experiences pain at least once in his lifetime. It is believed that acute pain serves as alarm and deserves to protect the organism against noxious stimuli while chronic pain, in contrast, is an entire disease and may result from tissue injuries. Indeed, chronic pains may be a consequence of sustained inflammatory diseases or tissue damages such as nerve injury in the case of neuropathic pain[1]. The detrimental

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effects of pain to our society are overwhelming and affect many different aspects of the quality of our daily life[2]. Moreover, chronic pain is often resistant to existing therapy. So, there is great to need to search for new and better drugs[3].

Propolis is a complex material produced by honeybees, containing mainly wax and resins collected from buds or plant secretions of different tree species. It can be considered as a complex mixture of chemicals, whose composition depends on the constituents of the plant material and the time of collection. Its colour varies form yellow, green to dark brown depending on its source and age[4,5]. Propolis has a long history of use in folk medicine and is a popular remedy today to treat a variety of ailments[6].

At least 200 compounds were identified in different propolis samples, with more than 100 in each one, including: fatty and phenolic acids and esters, substituted phenolic esters, flavonoids (flavones, flavanones, flavonols, dihydroflavonols, chalcones), terpenes, β-steroids, aromatic aldehydes and alcohols, sesquiterpenes, naphtalene and stilbene derivatives[7,8,9,10,11,12]. However, although of different chemical composition, propolis always demonstrates considerable biological activity, especially antimicrobial activity[13,14] antiviral activity[15], antibacterial; fungicidal[16], antiulcer and anti-tumor etc[9]. It had been proven to be 100% effective against some lethal protozoa and would also decrease inflammation associated with parasite infection[17]. For this reason, the chemical diversity of propolis has the potential to provide valuable leads to active components and new types of propolis from unexplored regions continue to attract growing interest among scientists searching for new bioactive molecules[5].

An extensive search of the literature reveals no reports on the antinociceptive activity of Cameroonian brown propolis and it isolated compounds. In order to contribute to the ongoing research on the isolation and pharmacological characterization of bioactive secondary metabolites from the honeybee propolis, we have studied the brown propolis, one of the many varieties indigenous to Meiganga (Adamaoua-Cameroon), where potions made from this propolis are popularly used in village communities for the traditional treatment of dysentery, stomach pains, asthma, female sterility, ulcers, dental caries, fever, boils and various types of inflammations. The present study aimed at evaluating the antinociceptive properties and also the possible mechanisms which underlie the antinociceptive action of three pentacyclic triterpenoids derivatives isolated from the Cameroonian brown propolis; triterpenoids lup-20(29)-en-3-one, erythrodiol palmitate and 18-iso-olean-12-ene-3,11-dione.

MATERIALS AND METHODS

Animals: The experiments were conducted using male Swiss mice (25-30 g) and male Wistar rats (160-180 g). All animals were housed in a controlled environment, with free access to food, water and were maintained on a 12 h light-dark cycle. Each animal was used only once. All experiments were performed according to the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Additionally, the study protocol was approved by the Cameroon National Ethical Committee (Ref No. FW-IRB00001954) for animal handling and experimental procedure.

Propolis collection: Propolis sample was collected in Meiganga (Adamaoua-Cameroon), in April 2006. The sample was dried in the shade and ground.

Extraction and isolation: The dried powder (950 g) was extracted three times with methanol at room temperature for 48 h. The methanolic extract was concentrated *in vacuo* and extracted successively with ethyl acetate (3 times). The ethyl acetate extract was evaporated *in vacuo* to dryness to give 118.7 g dry residue. The ethyl acetate dry residue was chromatographed over silica gel column with hexane and increase polarity of methylene chloride and then with methylene chloride and increase polarity of methanol and 14 fractions were obtained. Fraction 1 was eluted with hexane/CH_2Cl_2 (90:10) on silica gel column chromatography to yield compound (1) (180.7 mg). Fraction 5 was purified by repeated silica gel column chromatography and eluted with hexane/CH_2Cl_2 (60:40) to yield finally purified compounds (2) (11.79 mg) and (3) (31.56 mg). Compound (1) (Fig. 1a) was identified as lup-20(29)-en-3-one (DB-PR-01)[18]. Compound (2) (Fig. 1b) and (3) (Fig. 1c) are, respectively identified as lupeol (DB-PR-02)[19] and erythrodiol palmitate (DB-PR-04)[20]. Fraction 7 was chromatographed on silica gel with hexane/CH_2Cl_2 (20:80) to obtain compound (4) (Fig. 1d) which is 18-iso-olean-12-ene-3,11-dione (DB-PR-14[21].

^{1}H-NMR experiments were performed on a bruker AM400 and AMX 500 NMR (Advanced) instruments using the UNIX data system at 400 and 500 MHZ, respectively. The ^{13}C-NMR spectrum was recorded at 300 and 500 MHZ, respectively, using $CDCL_3$ and CD_3OD as solvent. ^{1}H-^{13}CHMBC and HSQC spectra were recorded as mentioned above. EI-MS spectra were recorded on a finnigan MAT 312. Fab mass measurements were on Joel JMS HX 110 mass spectrometer. Column chromatography was carried out on silica (M and N), 70-230 and 230-400 meshes. Compounds on the TLC were employed to detect compounds 254 and 266 nm using ceric sulphate as spraying reagent.

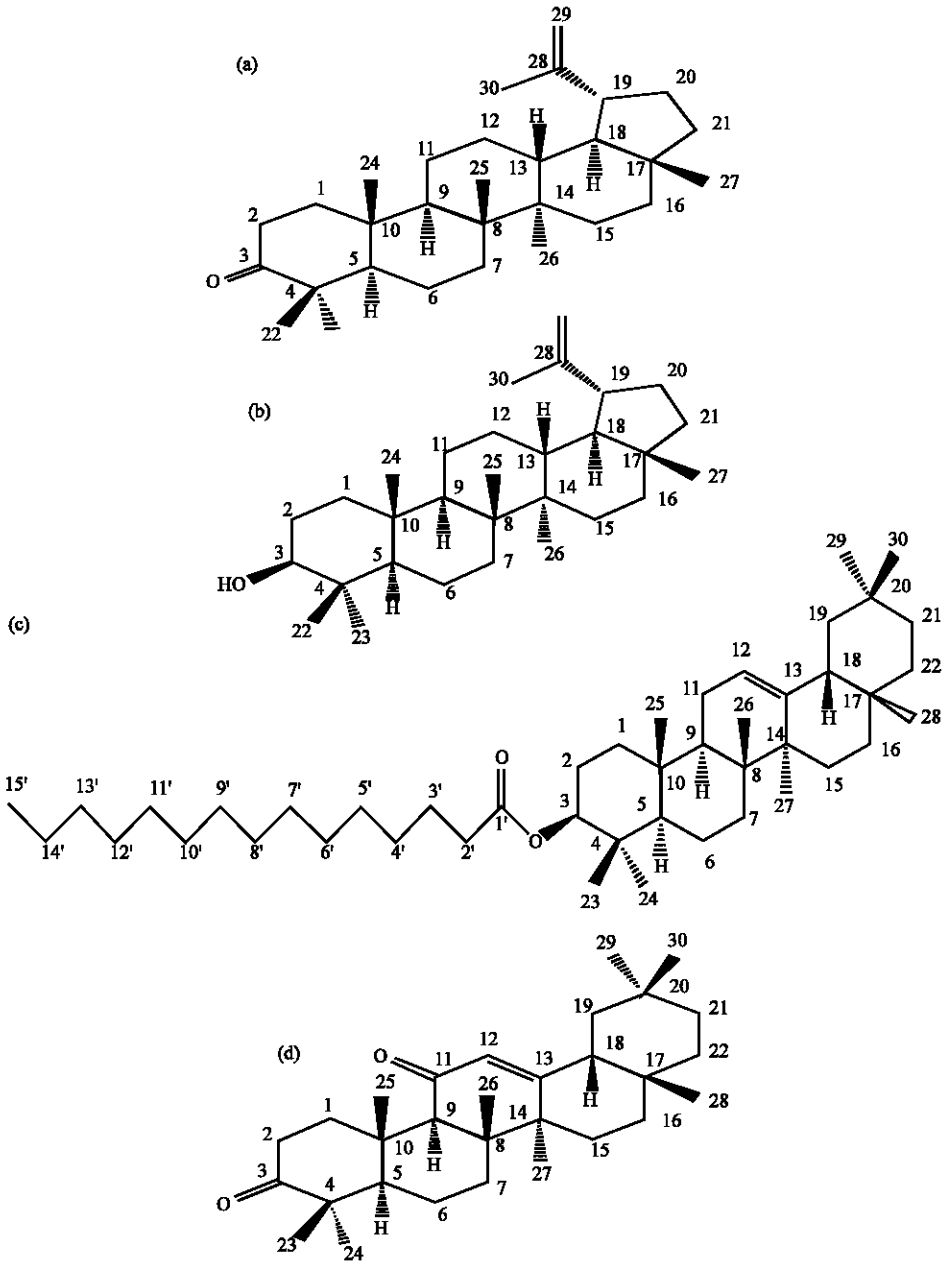

Fig. 1(a-d): The chemical structure of the pentacyclic triterpenoids: (a) Lup-20(29)-en-3-one, (b) Lupeol, (c) erythrodiol palmitate and (d) 18-iso-olean-12-ene-3,11-dione

Drugs and chemicals: Acetylsalicylic Acid (ASA) is from Laboratory 3M, France. Acetic acid, formalin, morphine, naloxone, prostaglandin E_2, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and all other reagents were purchased from Sigma chemical Co. (St. Louis, MO, USA). Formalin stock solution was prepared in phosphate buffer solution (PBS concentration in mM: NaCl 137, KCl 2.7 and phosphate buffer, 10). Acetic acid was prepared in saline (0.9% NaCl). Morphine was prepared in saline (0.9% NaCl) and contained 2% DMSO. Prostaglandine E_2 (PGE_2) stock solution (10^{-3} M) was

prepared in 10% ethanol and further diluted 200-fold to a final solution of 1.76 mg mL^{-1} using a 0.9% saline solution. DB-PR-01, DB-PR-04 and DB-PR-14 were dissolved in 0.9% saline containing 2% DMSO (vehicle) and subjected to the following pharmacological studies.

Pharmacological analysis

Abdominal constrictions induced by acetic acid: Acetic acid (0.6%, 10 mL kg^{-1}) was injected intraperitonealy and the number of abdominal constrictions (writhings) during the following 30 min period was observed. The reaction time was recorded 1 h after administration of various doses of DB-PR-01 (2.5, 5, 7.5 and 10 mg kg^{-1}; i.p.), DB-PR-04 (2.5, 5, 7.5 and 10 mg kg^{-1} i.p.), DB-PR-14 (2.5, 5, 7.5 and 10 mg kg^{-1} i.p.), Acetylsalicylic acid (ASA; 5 mg kg^{-1} p.o.) or vehicle (10 mL kg^{-1} i.p.). A significant reduction in the number of abdominal constrictions by any treatment compared with vehicle treated mice was considered as an antinociceptive response[22]. In order to investigate the participation of the opioid system in the antinociceptive action of the pentacyclic triterpenoids isolated from propolis, mice were pretreated with naloxone (5 mg kg^{-1} i.p.) and after 15 min the animals received an injection of one of the three pentacyclic triterpenoids. The antinociceptive response was recorded 1 h after pentacyclic triterpenoids treatment using acetic acid assay.

Formalin-induced nociception: The formalin test was carried out as described by Tjolsen *et al.*[23]. Each test compound; DB-PR-01 (2.5, 5, 7.5 and 10 mg kg^{-1}; i.p.), DB-PR-04 (2.5, 5, 7.5 and 10 mg kg^{-1}; i.p.), DB-PR-14 (2.5, 5, 7.5 and 10 mg kg^{-1}; i.p.), morphine (5 mg kg^{-1}; s.c.) or vehicle (10 mL kg^{-1}; i.p.) were administered by intraperitoneal route to groups of six mice 1 h before injecting formalin. Pain was induced by injecting subcutaneously in the right hind paw 20 µL of 2.5% formalin (0.9% formaldehyde). The amount of time spent licking the injected paw was measured and considered as an indication of pain. The first phase o f the nociceptive response normally peaks 0-5 min after injection and the second phase 15-30 min after formalin injection. These two phases correspond to the neurogenic and inflammatory pain responses, respectively[24].

Measurement of mechanical hypernociception induced by prostaglandin E_2: Hypernociception was induced by a subcutaneous injection of prostaglandin E_2 (20 µL paw^{-1}) into the plantar surface of mouse's hindpaw. The mechanical hyperalgesia was measured as described before[25], as the withdrawal response frequency to 10 applications of 0.4 g von Frey filaments. Rats were further acclimatized in individual clear Plexiglas boxes on an elevated wire mesh platform to allow access to the ventral surface of the hind paws. The frequency of withdrawal was determined before and after or prostaglandin injection. In order to verify whether the various treatments act on the PGE_2 receptors or on PGE_2 receptors downstream, DB-PR-01 (2.5, 5, 7.5 and 10 mg kg^{-1} i.p.), DB-PR-04 (2.5, 5, 7.5 and 10 mg kg^{-1} i.p.), DB-PR-14 (2.5, 5, 7.5 and 10 mg kg^{-1} i.p.), morphine (5 mg kg^{-1} s.c.) or vehicle (10 mL kg^{-1} i.p.) were given 1 h after intraplantar injection of prostaglandine E_2 and hypernociception was evaluated at 1, 2, 4, 6 and 8 h post-treatment. Frequency response to von Frey hair stimulation was measured at 1, 2, 4, 6 and 8 hours post-prostaglandin E_2 injection.

Measurement of locomotor activity: The study of ambulatory behavior was carried out on mice according to a slightly modified method[26]. The open field used was a wooden square box 40×40×45 cm; the floor was divided into 16 smaller squares of equal dimensions (10×10 cm). During all the experiments, the laboratory room was dark. The mouse was placed individually into the centre of the arena and allowed to explore freely. The ambulations (the number of crossing sector lines with all four paws) were recorded, over 2 consecutive 30 min periods, 1 h after administration of DB-PR-01 (2.5, 5, 7.5 and 10 mg kg^{-1} i.p.), DB-PR-04 (2.5, 5, 7.5 and 10 mg kg^{-1} i.p.), DB-PR-14 (2.5, 5, 7.5 and 10 mg kg^{-1} i.p.), morphine (5 mg kg^{-1} s.c.) or vehicle (10 mL kg^{-1} i.p.).

Cell viability essay: PC-3 or 3T3 cells were maintained at 37°C in 5% CO_2 in F-12K nutrient medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum and 10,000 units mL^{-1} streptomycine and penicillin. PC-3 or 3T3 cells were seeded into 96 well micro plates at density of approximately 8×10^8 cells/well. After 2 days of culture, the cells were incubated for period of 4 or 24 h at 37°C with various concentrations of DB-PR-01 (1-100 µM), DB-PR-04 (1-100 µM) or DB-PR-14 (1-100 µM). Control wells containing cell culture medium alone or with cells, both without compounds addition, were included in each experiment. Doxorubucin (1-100 µM) are used in this assay as positive controls. All assays were run in triplicates. The cells were then incubated MTT for 30 min. Conversion of MTT into purple colored MTT formazan by the living cells indicated the extent of cell viability[27]. The extent of MTT reduction to formazan within cells was calculated by measuring the absorbance at 570 nm, using a microplate ELISA reader (Biotek Elx-800, Mandel

Scientific Inc.). The cytotoxicity was recorded as concentration causing 50% growth inhibition for PC-3 or 3T3 cells.

Data analysis and statistics: Data were expressed as Mean±SEM per group. Statistical differences between control and treated groups were tested by two-way repeated measures analysis of variance (ANOVA), followed by Newman-Keuls post hoc test. Results obtained in cell viability assay were plotted as percent of cytotoxicity and concentration-response were fitted in order to determine the 50% effective concentration (EC_{50}).

RESULTS

Effects of DB-PR-01, DB-PR-04 and DB-PR-14 on abdominal constrictions induced by acetic acid: The mean number of abdominal constrictions after intraperitoneal injection of acetic acid was 87.5±7.3 in control animals. A significant reduction ($p<0.01$) in abdominal constrictions was observed in acetylsalicylic acid treated mice and the mean value being 43.6±1.6 (Fig. 2). Intraperitoneal administration of the three pentacyclic tripernoids (DB-PR-01, DB-PR-04 or DB-PR-14) did not produce any irritation action "*per se*", but caused a dose-related and significant inhibition ($p<0.001$) of acetic acid-induced abdominal constriction in mice. Nearly 50% inhibition of nociception was observed with 5 mg kg^{-1} for all the three pentacyclic triterpenoids and further increase in doses up to 10 mg kg^{-1} resulted in a maximum inhibition of nociception ranging from 60 to 65% (Fig. 2). Pre-treatment of mice with an naloxone (5 mg kg^{-1} i.p.), a non-selective opioid receptor antagonist, completely and significantly reversed the antinociceptive effects of all the three pentacyclic tripernoids (DB-PR-01, DB-PR-04 and DB-PR-14) in acetic acid-induced abdominal constriction in mice (Fig. 3).

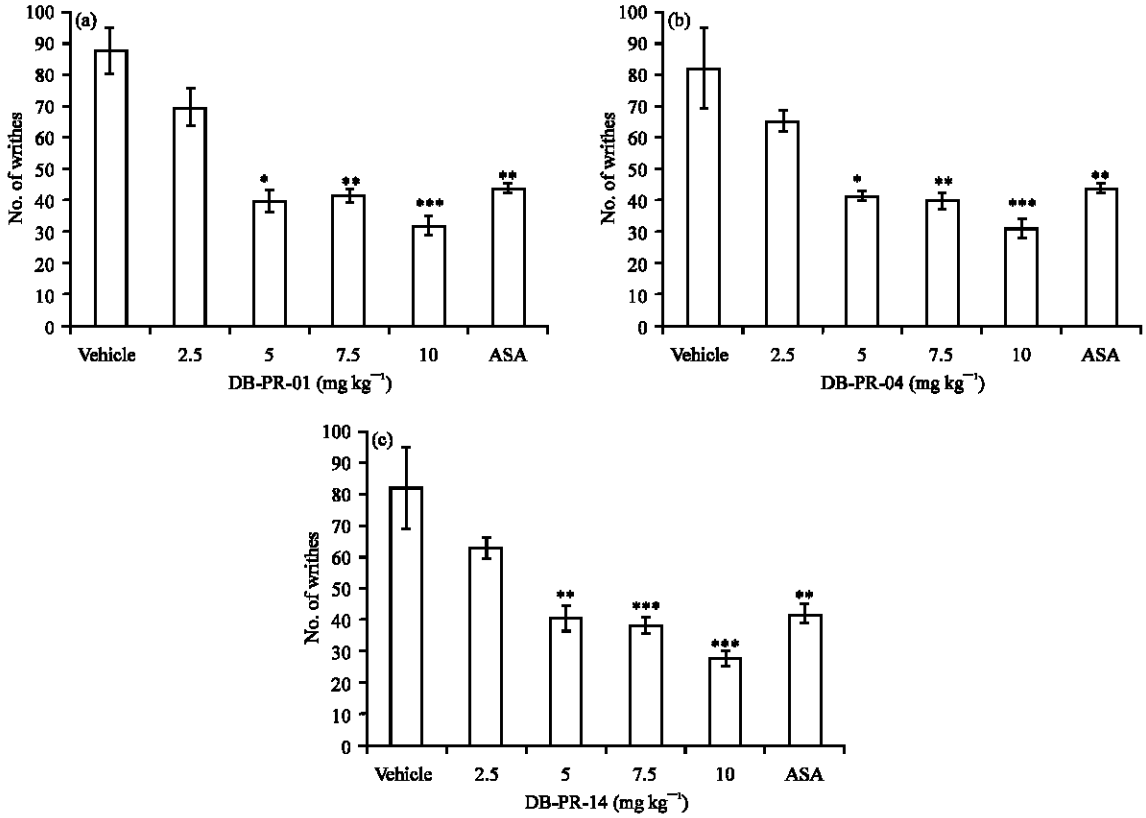

Fig. 2(a-c): Effects of the treatment with pentacyclic triterpenoids isolated from propolis (panel (a) DB-PR-01, panel (b) DB-PR-04 and panel and (c) DB-PR-14) or acetyl salicylic acid (ASA) on acetic acid-induced writhing. Results are Mean±SEM, for 6 animals, The compouds at all doses used began manifesting its assuaging effect on the writing reflex 1 h following the administration. Data were analysis by two-way ANOVA, followed by Newman-Keuls *post hoc* test, *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different compared to the vehicle

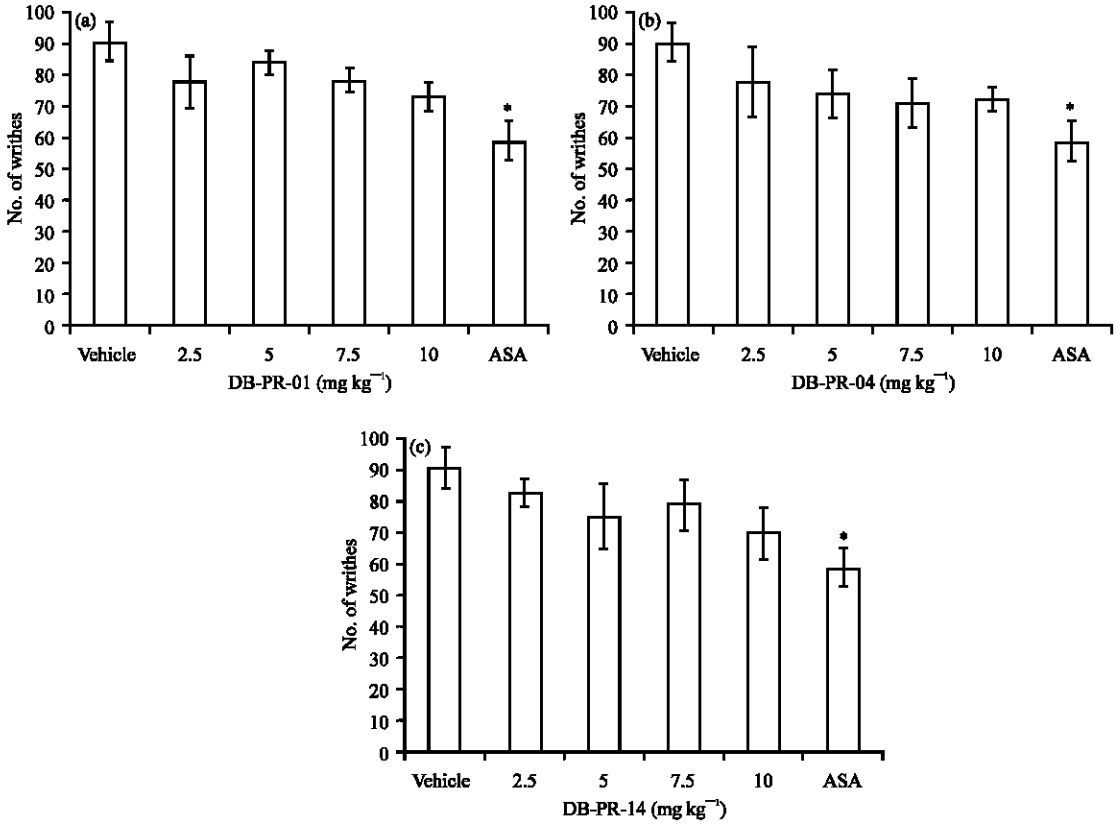

Fig. 3(a-c): Effects of the pretreatment with naloxone (5 mg kg^{-1} i.p.) on pentacyclic triterpenoids isolated from propolis (panel (a) DB-PR-01, panel (b) DB-PR-04 and panel (c) DB-PR-14) or acetyl salicylic acid (ASA) induced inhibition of acetic acid writhing in mice, Results are Mean±SEM, for 6 animals. Data were analysis by two-way ANOVA, followed by Newman-Keuls *post hoc* test, *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different compared to the vehicle

Effects of DB-PR-01, DB-PR-04 and DB-PR-14 on formalin-induced nociception: In vehicle treated control animals the mean paw licking response time was 65.8±1.2 seconds in the acute phase and 71.6±1.3 seconds in the chronic phase. Morphine treatment resulted in a marked reduction ($p<0.001$) of response time to 24.3±2.7 seconds and 23.8±2.4 seconds in the acute and chronic phases, respectively (Fig. 4). All the tested pentacyclic triterpenoids in varying doses showed a dose dependent and statistically significant reduction ($p<0.001$) in biting and licking response time after formalin injection compared to vehicle treatment. Out of the three tested pentacyclic triterpenoids, two compounds viz DB-PR-04 and DB-PR-14, inhibited the response time to an extent of 50% in acute phase compared to 65% inhibition in chronic phase (Fig. 4). Even though a significant reduction in response time was seen in both the acute and chronic phases, it was evidently more in the acute phase. However, under the same conditions, DB-PR-01 produced 35.6 and 51.2% inhibition of nociceptive response in the acute and chronic phases, respectively.

Effects of DB-PR-01, DB-PR-04 and DB-PR-14 on measurement of mechanical hypernociception induced by prostaglandin E_2: Intraplantar injection of PGE2 (0.1 nmol paw^{-1}) induced a marked mechanical hypersensitivity. This hypernociception was significantly reduced ($p<0.001$) by the three tested pentacyclic triterpenoids; DB-PR-01 (10 mg kg^{-1}, i.p.), DB-PR-04 (10 mg kg^{-1}, i.p.) or DB-PR-14 (10 mg kg^{-1}, i.p.) when given as preventive treatment with an inhibition of 80% ($p<0.001$), 70% ($p<0.001$) and 70% ($p<0.001$), respectively, 2 to 4 h after administration (Fig. 5). The compounds effect's started 1 h after PGE2 injection and was maintained for up to 6 h with inhibitions of 70%

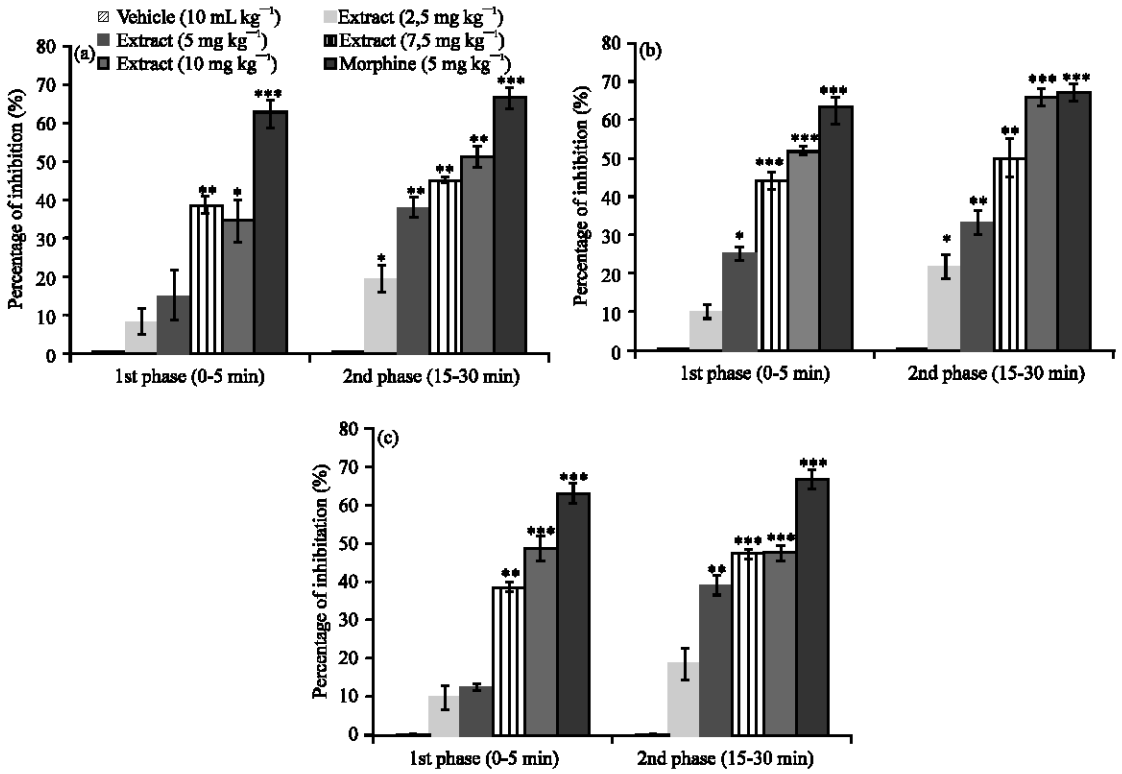

Fig. 4(a-c): Effects of the treatment with pentacyclic triterpenoids isolated from propolis (panel (a) DB-PR-01, panel (b) DB-PR-04 and panel and (c) DB-PR-14) or morphine on formalin-induced nociception. Results are Mean ± SEM, for 6 animals. The amount of time spent licking and biting the injected paw was measured in the first and second phases of the formalin test in each group, Data were analysis by two-way ANOVA, followed by Newman-Keuls *post hoc* test, *p<0.05, **p<0.01, ***p<0.001, significantly different compared to the vehicle

Table 1: Effects of the treatment with pentacyclic triterpenoids isolated from propolis (DB-PR-01, DB-PR-04 and DB-PR-14) or morphine on locomotor activity in the open field test

		Locomotor activity		
Treatments	Dose (mg kg^{-1})	0 min	30 min	60 min
Vehicle	-	18.5±2.1	46.9±1.4	71.5±2.7
DB-PR-01	2.5	18.3±1.7	43.7±2.4	72.1±2.1
DB-PR-01	5	17.8±1.8	42.4±1.4	71.3±2.3
DB-PR-01	7.5	18.7±2.7	45.1±2.8	76.2±2.5
DB-PR-01	10	17.8±1.6	44.6±3.3	73.4±3.1
DB-PR-04	2.5	17.9±2.1	45.7±3.4	74.8±8.6
DB-PR-04	5	18.1±2.7	44.6±2.7	78.5±9.4
DB-PR-04	7.5	18.3±2.4	43.5±3.4	79.6±8.7
DB-PR-04	10	17.9±3.2	44.5±5.3	82.1±9.2
DB-PR-14	2.5	18.1±2.3	44.7±3.1	79.1±4.1
DB-PR-14	5	17.9±1.9	44.6±2.5	77.2±3.1
DB-PR-14	7.5	17.7±1.7	46.5±3.9	75.9±2.8
DB-PR-14	10	18.2±2.1	47.6±3.2	78.7±3.5
Morphine	5	18.1±1.9	21.6±3.4**	34.3±4.7***

Results are Mean ± SEM of the No. of crossing in a cumulative way, for 6 animals. Data were analysis by two-way ANOVA, followed by Newman-Keuls *post hoc* test, **p<0.01, ***p<0.001, significantly different compared to the vehicle

(p<0.001) approximately. Given subcutaneously morphine (5 mg kg^{-1}, s.c.) produced significant inhibition of 82% (p<0.001) of the prostaglandine-induced hypernociception in rat (Fig. 5).

Effects of DB-PR-01, DB-PR-04 and DB-PR-14 on motor coordination: Mice treated with the pentacyclic triterpenoids; DB-PR-01, DB-PR-04 or DB-PR-14 failed to display any detectable alteration in the locomotor activity, when compared to the cumulative value recorded from control vehicle-treated animals over 0 (p>0.69), 30 (p>0.57) and 60 (p>0.81) minutes (Table 1).

Cell viability essay: The ability of pentacyclic triterpenoids DB-PR-01 (1-100 μM), DB-PR-04 (1-100 μM) and DB-PR-14 (1-100 μM) to induce cytotoxicity was further investigated by using PC-3 or 3T3 cells and a standard MTT bioassay (Table 2). As

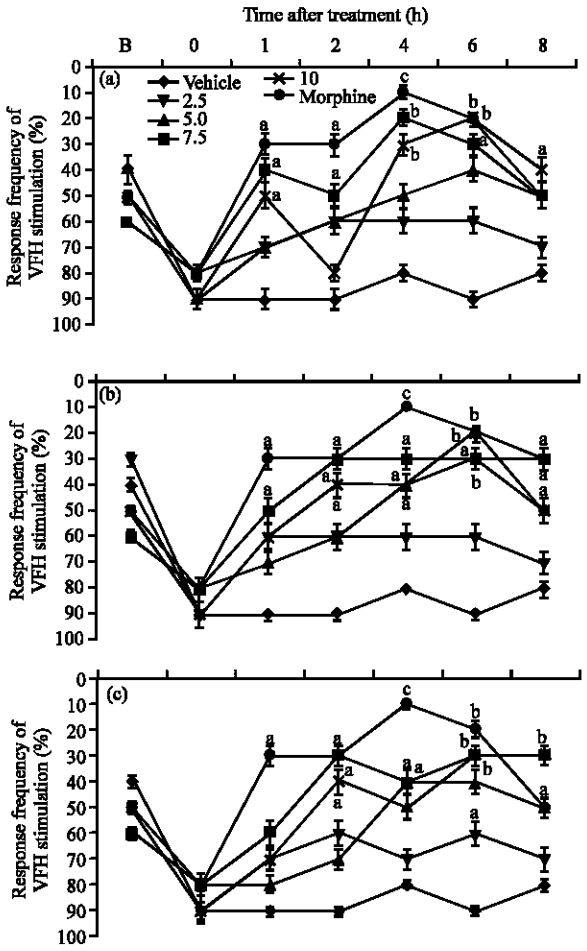

Fig. 5(a-c): Effects of the treatment with pentacyclic triterpenoids isolated from propolis (panel (a): DB-PR-01, panel (b): DB-PR-04 and panel (c): DB-PR-14) or morphine on the mechanical hypernociception assessed with von Frey hair (0.4 g) in mechanical hypernociception induced by prostaglandine E_2 (20 µL paw^{-1}), Results are Mean±SEM, for 6 animals, After subcutaneous injection of prostaglandine E_2 into the plantar surface of mouse's hindpaw, the von Frey paw withdrawal threshold was measured in each group of mice. Data were analysis by two-way ANOVA, followed by Newman-Keuls *post hoc* test, *p<0.05, **p<0.01, ***p<0.001, significantly different compared to the vehicle. B: basal response to mechanical stimulation

shown, incubation for 24 h of these cells lines with various concentration of DB-PR-01, DB-PR-04 or DB-PR-14, up to a concentration of 30 µM, produces no cell toxicity. The toxicity values observed were not significantly ($p<0.001$) different from baseline. However, incubation of PC-3 or 3T3 cells with a higher concentration of doxorubicin (1-100 µM) produced higher cell death with IC_{50} of 0.912±0.120.912±0.12 and 3.1±0.2 µM, respectively.

Table 2: IC_{50} values of DB-PR-01, DB-PR-04, DB-PR-14 or doxorubicin towards PC3 and 3T3 line cells at 24 hours incubation times as determined by using the MTT assay

Treatments	Concentration (µM)	PC3 line cells IC_{50}	3T3 line cells IC_{50}
Control	-	-	-
DB-PR-01	1-100	39.18±0.29***	37.26±2.31***
DB-PR-02	1-100	38.31±0.41***	39.12±2.25***
DB-PR-04	1-100	38.15±0.26***	38.32±0.49***
DB-PR-14	1-100	38.64±0.17***	37.84±0.68***
Doxorubicin	1-100	0.91±0.12	3.10±0.20

Results are expressed as Mean±SEM of the number of cell, Data were analysis by two-way ANOVA, followed by Newman-Keuls *post hoc* test, ***p<0.001, significantly different compared to the vehicle

DISCUSSION

The result of the current study show that the pentacyclic tripernoids DB-PR-01, DB-PR-04 and DB-PR-14, produced dose-related and marked antinociceptive effects when assessed in different animal model of nociception.

In general, acetic acid-induced abdominal constriction is used to evaluate the compounds for peripheral antinociceptive activities[28]. Acetic acid injection produces peritoneal inflammation which triggers a response characterized by writhing[29]. The writhing test is useful to discriminate central and peripheral nociception[28]. Related studies have demonstrated that acetic acid indirectly induces the release of endogenous mediators of pain (such as prostaglandins, kinins, histamin, etc.) that stimulate the nociceptive neurons which are sensitive to non-steroidal anti-inflammatory drugs and opioids[30]. Our results indicated that the pentacyclic triterpenoids can reduce the number of writhings in animal models, implying that it had a powerful antinociceptive effect.

The present results reveal that naloxone was able to significantly attenuate the antinociceptive activity of the triterpenoids. This observation indicated a role of opioid mechanism in the antinociceptive action of all the three pentacyclic triterpenoids isolated from Cameroonian propolis.

The nociceptive behavior after formalin injection was distinctly recorded in two phases. The first phase of paw licking/biting response started immediately after the injection and was due to direct stimulation of nociceptors[31]. The second phase which appears little later is considered to be due to a combination of an inflammatory reaction in the peripheral tissue and changes in central processing[23]. The advantage of the

formalin model of nociception is that it can discriminate pain in its central and/or peripheral components. It has been reported that formalin-induced persistent pain in mice paws produced a distinct biphasic nociception[23,24]. Central analgesics, such as narcotics, inhibit both phases while peripherally acting drugs, such as steroids (hydrocortisone, dexamethasone) and NSAIDs suppress mainly the late phase[24]. A significant and dose related antinociceptive effect was clearly evident for all the three pentacyclic triterpenoids against both neurogenic (early phase) and inflammatory (late phase) pain behavior caused by formalin injection in mice.

The tripernoids DB-PR-01, DB-PR-04 and DB-PR-14 produced a well-defined dose-dependent peripheral antinociceptive effect in the rat paw prostaglandin E_2 induced hyperalgesia test. Given after intraplantar injection of PGE_2, the triterpenoids exhibited significant antinociceptive activity, suggesting that their effect is not only at the level of cyclooxygenase but also involved of the prostaglandine E_2 pathway. These results further demonstrate that the three triterpenoids act downstream prostaglandin receptors since they were able to reduce nociception at post-treatment. Our results are in agreement with a previous study[32] that supports that the analgesic effect of the antinociceptive drugs results from a direct blockade of hyperalgesia and not only from prevention of the release of prostaglandin E_2 in inflamed tissues.

The present study further demonstrates that, the systemic administration of the pentacyclic triterpenoids; DB-PR-01, DB-PR-04 and DB-PR-14 did not produce any motor dysfunction, sedation or alteration in locomotor activity of animals. These results suggest that inhibition of pain is not related to the reduction of spontaneous locomotor activity and the central nervous system depressant[22].

As seen, using the more sensitive MTT assay, no cytoxicity has been observed on PC-3 or 3T3 cells lines incubated with various concentration of the pentacyclic triterpenoids DB-PR-01 (30 μM), DB-PR-04 (30 μM) or DB-PR-14 (30 μM). MTT assay measured the metabolism of 3- (4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide to form formazan precipitate by mitochondrial dehydrogenase which only present in viable cells[33]. Formazan accumulation directly reflected mitochondrial activity which was an indirect measure of cell viability[27].

In summary, the present study demonstrated the antinociceptive activity of all the three pentacyclic triterpenoids in the test models of chemical nociception and mechanical hypernociception and further suggested that antinociceptive activity of these pentacyclic triterpenoids; lup-20(29)-en-3-one, erythrodiol palmitate and 18-iso-olean-12-ene-3,11-dione might be related to the involvement of the opioïdergic system which merited further studies regarding the precise site and the mechanism of action.

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