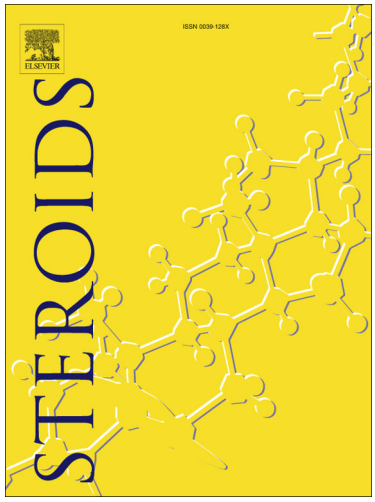

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In search of safe pain relief: The analgesic and anti-inflammatory activity of phytosteryl ibuprofenates

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ABSTRACT

β-Sitosteryl (*S*)-ibuprofenate (**2**), stigmasteryl (*S*)-ibuprofenate (**3**), ergosteryl (*S*)-ibuprofenate (**4**), and cholesteryl (*S*)-ibuprofenate (**5**) were prepared in 70–75% yields by Steglich esterification and were characterized by 1D and 2D NMR, as well as by MS. The new esters were evaluated in *in vivo* pain models of antinociception and anti-inflammation using the writhing, formalin, and carrageenan tests, in mice and rats, and the results were compared with those of (*S*)-ibuprofen (**1**). Damage to the gastric mucosa of animals was also assessed. The results indicated that **2**–**5** have comparable or eventually better activity than **1** at the same mg/kg doses. Since the molecular weight ratio of esters **2**–**5** to ibuprofen is about 3 to 1, the amount of truly incorporated ibuprofen was roughly one third to achieve similar effects. This resulted in minimal gastrointestinal damage in the stomach of the animals, in contrast to the large gastric injury caused by (*S*)-ibuprofen.

Keywords:

Phytosteryl (*S*)-ibuprofenates; Spectroscopy properties; *In vivo* pain models, Antinociception, Anti-inflammation, Gastric injury

1. Introduction

Studies on the search for non-addictive analgesics for safe pain relief evidence that 1.5 billion people have chronic pain worldwide [1]. In the USA some 100 million people face this problem, of which 20% suffer pain that affects their sleep a few nights a week. The most common types of chronic pain being low back pain (28%), neck pain (15%), and severe headaches or migraines (15%). The annual cost of chronic pain in the USA health care system involving lost wages and productivity is estimated in the $560–635 billion range, and people with chronic pain report that their quality of life decreases considerably, according to the following percentages: 77% report feeling depressed, 70% report trouble concentrating, and 59% report an impact on their overall enjoyment of life [1].

Based on the above reality, the need to find better and safer pain relief treatments is evident. A strategy to improve the quality of a drug is to increase its bioavailability to reduce the intake dose, which results in a reduction of side effects. This can be achieved by binding the drug to a transporter molecule that releases the drug into the cell to exert its effect. If additionally, the transporter molecule has beneficial action and contributes to the desired therapeutic action, then the drug will be more effective, with less undesirable effects, and safer.

Ibuprofen is one of the non-steroidal anti-inflammatory drugs (NSAIDs) with the highest prescription for the control of inflammation and pain, both for acute and chronic conditions. Its mechanism of action, pharmacological effects, therapeutic applications, and adverse reactions have been reported [2]. Administration doses for adults usually range from 400 to 800 mg three times a day, which results in an important ulcerating effect of the gastrointestinal tract, and the decrease in life quality of the consumers.

Phytosterols are typical components of cell walls of plants and their metabolism and action on the organism is well known [3, 4]. They are ingested with vegetable foods in the daily diet and are reported to have health benefits, such as reduction of cholesterol absorption [5] due to structure similarity and an associated decrease in colon cancer risk [6]. Likewise, moderate nociception inhibitions have been reported for *β*-sitosterol and stigmasterol in the formalin-induced pain test [7]. In addition, *in vivo* studies, including humans, have also demonstrated that consumption of dietary phytosterols produces reduction of plasma levels of proinflammatory cytokines and that these effects may be mediated through beneficial alterations in the membrane composition, affecting membrane fluidity, sensitivity, and signaling pathways, which may alter the immune response by influencing the synthesis of leukotrienes and prostaglandins [8].

Ibuprofen contains a carboxylic acid group, while phytosterols have the alcohol functionality, so if an ester derived from both substances is formed, susceptible to being hydrolyzed by an esterase inside the cell, it could release both ibuprofen and the sterol to exert their effect synergistically. Based on the above consideration, the current paper describes the preparation and characterization of new esters of (*S*)-ibuprofen (**1**) and the natural sterols *β*-sitosterol, stigmasterol, and ergosterol, as well as their antinociceptive and anti-inflammatory activities, in addition to gastric injury, in *in vivo* models, as compared with those of (*S*)-ibuprofen. The cholesteryl ester of ibuprofen was also prepared and evaluated for comparative purposes.

2. Experimental

2.1 General

(+)-(*S*)-Ibuprofen (≥ 98%), *β*-sistosterol (≥ 70%, containing campesterol and *β*-sitostanol), stigmasterol (≥ 95%), ergosterol (≥ 95%), cholesterol (99%), and other chemicals were commercially available (Aldrich). Except for *β*-sitosterol, they were used as purchased. Melting points were determined on a Büchi capillary apparatus and are uncorrected. The ^{1}H and ^{13}C NMR spectra, including COSY, HSQC, and HMBC experiments, were recorded on a Bruker Ascend instrument (400 MHz for ^{1}H; 100 MHz for ^{13}C) from $CDCl_3$ solutions using the remaining proton signal of the solvent centered at 7.26 ppm or TMS as internal standard. Chemical shifts are given in ppm, multiplicity abbreviations are s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, and b = broad, while coupling constants are in Hz. Optical rotations were recorded in $CHCl_3$ on a Perkin-Elmer 341 polarimeter. Column chromatography was developed on Merck silica gel 60 (Aldrich, 230–400 mesh ASTM). ESI-HRMS were determined on a Waters Synapt G2 spectrometer at the Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO. USA. All reactions were routinely checked by TLC on silica gel Merck 60 F254.

2.2 Purification of β-sitosterol

To a solution of *β*-sitosterol ≥ 70% (3 g) in pyridine (60 mL) was added *p*-toluenesulfonyl chloride (6.0 g), and the mixture was stirred for 72 h at room temperature. The reaction mixture was poured over 300 mL of ice-water and the precipitate was collected by filtration, washed with water and dried. The ^{1}H NMR analysis of this product showed that the total sterols mixture was tosylated. The tosylates mixture was dissolved in a mixture of 60 mL of acetone and 40 mL of water, and refluxed for 4 h. The acetone was removed with

an air stream to obtain a precipitate which was separated by filtration. Separation of the precipitate on a short silica gel column using CH_2Cl_2 (300 mL) and then EtOAc (300 mL) as eluents allowed to obtaining *β*-sistostanol *p*-toluenesulfonate [9] (123 mg) in the CH_2Cl_2 fraction, while from the fraction eluted with EtOAc a 91:9 mixture (1.89 g) of *β*-sitosterol and campesterol was recovered. This sample showed mp 136–138 °C and $[\alpha]_D = -35$ (*c* 0.2, $CHCl_3$). Their spectroscopic data were identical to those reported [9–12].

*2.3 General procedure for the preparation of **2**–**5***

To a stirred solution of (+)-(*S*)-ibuprofen (0.5 g, 2.42 mmol) in 10 mL of CH_2Cl_2 was added 4-(dimethylamino)pyridine (90 mg), *N*,*N*′-dicyclohexylcarbodiimide 1 M in CH_2Cl_2 (5 mL, 4.84 mmol), and the sterol (2.42 mmol) in 15 mL of CH_2Cl_2. The reaction mixture was stirred at 30 °C in a water bath for 24 h, filtered, and the solvent was removed under an air stream at room temperature. Waxy solid products **2**–**5** were obtained in pure form after filtration through a short silica gel column using hexanes–CH_2Cl_2 (1:1) as eluent.

*2.4 β-Sitosteryl (S)-ibuprofenate (**2**)*

The title compound was formed in 70% yield using purified *β*-sitosterol (a 91:9 mixture of *β*-sitosterol and campesterol), mp 81–84 °C; $[\alpha]_D = +10.5$ (*c* 0.2, $CHCl_3$); HRMS (ESI+) *β*-Sitosteryl (*S*)-ibuprofenate: calculated for $C_{42}H_{66}O_2$ + Na $[M + Na]^+$, 625.4955; found, 625.4960; Campesteryl (*S*)-ibuprofenate: calculated for $C_{41}H_{64}O_2$ + Na $[M + Na]^+$, 611.4798; found, 611.4799; ^1H and ^{13}C NMR, see Tables 1 and 2.

*2.5 Stigmasteryl (S)-ibuprofenate (**3**)*

Compound **3** was obtained in 75% yield starting from stigmasterol, mp 90–93 °C; $[\alpha]_D$ = +26.0 (*c* 0.2, $CHCl_3$); HRMS (ESI+): calculated for $C_{42}H_{64}O_2$ + Li $[M + Li]^+$, 607.5061; found, 607.5067; ^1H and ^{13}C NMR, see Tables 1 and 2.

*2.6 Ergosteryl (S)-ibuprofenate (**4**)*

Ester **4** was obtained in 73% yield starting from ergosterol, mp 134–136 °C; $[\alpha]_D$ = –17.3 (*c* 0.2, $CHCl_3$); HRMS (ESI+): calculated for $C_{41}H_{60}O_2$ + Li $[M + Li]^+$, 591.4748; found, 591.4745; ^1H and ^{13}C NMR, see Tables 1 and 2.

*2.7 Cholesteryl (S)-ibuprofenate (**5**)*

Ester **5** was prepared in 75% yield starting from cholesterol, mp 91–93 °C; $[\alpha]_D$ = +10.0 (*c* 0.2, $CHCl_3$); HRMS (ESI+): calculated for $C_{40}H_{62}O_2$ + Li $[M + Li]^+$, 581.4904; found, 581.4913; ^1H and ^{13}C NMR, see Tables 1 and 2.

2.8 Purity determination

The test was carried out by quantitative 1D ^1H NMR experiments using 1,4-dinitrobenzene (98% pure, Aldrich) as internal calibration standard. The ^1H NMR spectra were recorded on a Bruker Ascend (400 MHz) spectrometer. Before each determination, the ^1H 90° pulse was calibrated and documented (see Supporting Information). Thus, samples of compounds **2**–**5** (8–11 mg), and 1,4-dinitrobenzene (2–3.5 mg) were carefully weighed, mixed, dissolved in 0.6 mL of $CDCl_3$ and filtered into 5 mm standard NMR sample tubes. The 1D ^1H spectra were obtained with the following controlled parameters: Pulse program:

'zg' with 90° pulse; sample temperature: 24.4 °C (297.55 K, regulated ± 0.1 K); data points (TD): 96152; zero filling (SI) to 256K; dummy scans: 4; relaxation delay (D1): 60 s; acquisition time (AQ): 4 s; spectral window (SW): 30 ppm; transmitter offset: 7.5 ppm; number of scans (NS): 64. The post-acquisition processing and measurement of integrals were the follows: Line broadening (LB): 0.1 Hz; zero filling: to 254K real data; phasing: manual; baseline correction: 5^{th} order polynomial. Signals of interest of each compound were selected, integrated, and averaged to obtain the quantitative measurements (see Supporting Information).

2.9 Pharmacological evaluation

For the animal assays, all experiments were conducted in compliance to the Guidelines of Ethical Standards for Investigations of Experimental Pain in Animals [13] and with the official Mexican regulations [14] regarding technical specifications for production, care, and use of laboratory animals. All animals were obtained from the vivarium of the Universidad Autonoma del Estado de Hidalgo, Pachuca, Mexico. The experimental protocol was approved by the local Institutional Animal Care and Use Committee (CIECUAL) in accordance with the Mexican Ministry of Health and international guidelines. Wistar female rats (180–200 g) and $CD1^+$ male mice (30–35 g), were housed in plastic cages in a well-ventilated room maintained at 23–25 °C and 45 ± 5% humidity and in a 12 h light/12 h dark cycle (light 8:00–20:00 h). Animals had free access to standard pellets (Laboratory Chow 5001 lab rodent diet; PMI Nutrition International, Inc., MO) and drinking water ad libitum. All experiments were performed between 8:00 and 16:00 h. The groups consisted of a least six animals for all the experimental procedures. Animals were fasted by 12 h before the experiments. Each animal was used only one time, and after the experiment, these were

euthanized in a CO_2 chamber. (*S*)-Ibuprofen, carrageenan, formaldehyde, and acetic acid were dissolved in a physiologic saline solution for the biological tests. Compounds **2**–**5** were suspended in vehicle dissolved 1% Span 20 in distilled water.

2.9.1 Antinociceptive activity in the writhing test

The acetic acid-induced abdominal writhing test was performed as previously described [15]. Mice (n = 6) were treated with vehicle alone or different doses of (*S*)-ibuprofen (**1**) and esters **2**–**5** (18, 30 and 45 mg/kg) by intragastric route, 30 min before 0.6% acetic acid (10 mL/kg) by ip route. The animals were then placed in an observation box, and the total number of writhes (abdominal constrictions) produced in treated animals over 30 min was counted. Antinociceptive activity was expressed as the percentage change in writhing rate concerning controls, and it was expressed as the area under the curve (AUC) of writhing movements against time.

2.9.2 Antinociceptive activity in the formalin test

The rat paw 1% formalin test was used to assess nociceptive effects [16, 17]. The formalin test consists of placing each rat in an open, transparent observation chamber for 30 min to allow it to accommodate to its surroundings. Mirrors were placed behind it to enable unhindered observation, and then the rat was removed for formalin administration. The nociceptive behavior showed a biphasic pattern [18]. A formalin solution (1% in 0.9% saline, 50 μL/paw) was injected into the right hind paw plantar surface of rats (n = 6) with a 30-gauge needle, the animals were individually placed in observation chambers and flinching behavior quantified as previously described [19]. To assess the systemic antinociceptive effect, oral treatments with the vehicle alone (1% Span 20 in distilled water), increasing doses

of **1**–**5** (10, 30 and 100 mg/kg) were administrated 20 min before formalin injection. The nociceptive behavior showed a biphasic pattern. The time of flinching of the injected paw was defined as a nociceptive response, which was recorded in 5 min periods during 60 min after injection. The number of flinches yielded a biphasic curve, the initial acute phase (0–5 min) is followed by a relatively short quiescent period, which was then followed by a lengthy tonic response (20–60 min). The area under the curve (AUC) was calculated for both phases.

2.9.3 Anti-inflammatory activity using the carrageenan paw edema test

The inflammation was evaluated using the carrageenan-induced paw edema test [20]. Paw edema was measured with a plethysmometer (Ugo Basile Model 7140 Comerio, Italy). The basal volume of the right hind paw was determined before any drug administration. Then, animals (n = 6) were divided into experimental groups in such a way that the mean volumes were similar among groups. Subsequent, vehicle or different doses of **1**–**5** were orally administered (40, 80, and 160 mg/kg), 20 min before intraplantar injection of carrageenan 1% (100 μL/paw). The paw volumes were measured at 1, 2, 3, 4, and 5 h after inflammatory stimulus. The inflammatory response was quantified by the increase of the paw size (edema) which, as has been described [20], is maximal around 5 h post carrageenan injection and is modulated by inhibitors of specific molecules within the inflammatory cascade. Data were expressed as area under the curve (AUC) of inflammation.

*2.9.4 Acute toxicity of compounds **2**–**5***

The acute toxicity was studied according to the modified Lorke method in mice [21]. The animals were treated orally with 10, 100, 1600, and 2000 mg/kg of esters **2**–**5** and were observed for 14 days. During this time, no mortality or toxic effects were detected.

2.9.5 Statistical analysis

Curves were constructed by plotting the time of flinching of the injected paw as a function of time in the formalin test or the time between the presentations of writhing behavior as a function of time on the writhing test. The percentage activity was obtained from the area under the curve (AUC) from the temporal courses using the trapezoidal rule. All results are presented as the mean values ± S.E.M. for at least six animals per group. One-way analysis of variance (ANOVA) followed by Tukey´s test was used to compare the differences among treatments. Differences were considered statistically significant when $p < 0.05$. All statistical analyses were performed using the GraphPad Prism® version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

2.10 Analysis for interference compounds

Esters **2**–**5** were tested for pan assay interference compounds (PAINS) or colloidal aggregators [22] using the publicly available filter ZINC (http://zinc15.docking.org/patterns/home) and no problems were found.

3. Results and discussion

The *β*-sitosteryl (*S*)-ibuprofenate (**2**), stigmasteryl (*S*)-ibuprofenate (**3**), ergosteryl (*S*)-ibuprofenate (**4**), and cholesteryl (*S*)-ibuprofenate (**5**) esters (Figure 1) were prepared in 70–75% yields by reaction of (*S*)-ibuprofen (**1**) with *β*-sitosterol, stigmasterol, ergosterol, and cholesterol, respectively, under Steglich [23] esterification conditions. In the case of *β*-sitosterol (70%, Aldrich), containing campesterol and *β*-sitostanol, the sterol mixture was

subjected to a known procedure to remove *β*-sitostanol. Thus, selective solvolysis [24] of the corresponding tosylates [9] mixture, taking advantage of the intermediate *i*-steroids, gave a 91:9 mixtures of *β*-sitosterol and campesterol which was esterified with (*S*)-ibuprofen and the resulting mixture was used as compound **2**. Compounds **2**–**5** were purified by column chromatography and characterized by 1D and 2D ^{1}H and ^{13}C NMR, as well as by mass spectrometry. The ^{1}H and ^{13}C NMR chemical shifts, multiplicities, and coupling constants of the ibuprofenate residue were essentially the same in all compounds. Their ^{1}H NMR spectra showed an AA'BB' system at δ 7.20 and 7.08 ppm (d, J = 8.0 Hz) for the aromatic H-5'/H-9' and H-6'/H-8' signals, respectively, two signals at δ 3.65 (q, J = 7.2 Hz) and 1.47 ppm (d, J = 7.2 Hz) were assigned to H-2' and Me-3', the signals of the H-10' methylene and the H-11' methine were observed at δ 2.44 and 1.86 ppm, correspondingly, and both Me-12' and Me-13' (d, J = 6.6 Hz) signals occurred at δ 0.89 ppm. The ^{13}C NMR spectra of the ibuprofenate residues showed the ester carbonyl at δ 174.3, the C-2' methylene at δ 45.5, while C-3' appeared at δ 18.8. In addition, two signals at δ 140.5 and 138.2 were assigned to non-protonated carbons C-7' and C-4', respectively, whereas C-6'/C-8' and C-5'/C-9 occurred at δ 140.5 and 138.2, respectively. The sp^3 carbons C-10' and C-11', and C-12'/C-13', were observed at δ 45.2, 30.3, and 22.5, correspondingly. The ^{1}H and ^{13}C NMR signals of the sterol moieties showed great similarity with those of the sterols, [10–12, 25] except for C-2, C-3 and C-4 that are close to the ester function, as C-3 (δ_C 73.1–74.3) is shifted 2–3 ppm to higher frequencies, while C-2 (δ_C 27.9–28.2) and C-4 (δ_C 36.5–38.8) are shifted around 3 ppm to lower frequencies. The shift of H-3 at δ_H 4.59 to higher frequencies, about 1 ppm relative to the natural sterol, along with that of the C-3 shifted to higher frequencies (2–3 ppm) with respect to the sterol, and the characteristic signal for the carbonyl of the ester group at δ_C 174.2–174.4 in their ^{13}C NMR spectra, confirmed the esters formation. The ester

linkage was further corroborated by HMBC experiments which showed correlation of H-3 and the carbonyl group signal. Finally, the molecular formulas of esters **2**–**5** were corroborated by high-resolution mass spectrometry, in which the molecular ions at *m*/*z* 625.4960 (calculated for $C_{42}H_{66}O_2$ + Na = 625.4955), 607.5067 (calculated for $C_{42}H_{64}O_2$ + Li = 607.5061), 591.4745 (calculated for $C_{41}H_{60}O_2$ + Li = 591.4748), and 581.4913 (calculated for $C_{40}H_{62}O_2$ + Li = 581.4904), correspondingly, were determined. Before subjecting esters **2**–**5** to the antinociceptive and anti-inflammatory tests, their purity was determined by qHNMR using 1,4-dinitrobenzene as the internal calibration sample (see Supporting Information) and those samples with a purity greater than 95% were used for the biological study.

The acetic acid writhing test was used as an inflammatory pain model stimulus for acute nociception. Acetic acid causes tissue damage and releases pain-producing substances, including PGs of the E series, thus resulting in activation of visceral peripheral nociceptive afferents but also results in release of excitatory glutamate, principally in the spinal cord [7]. These specific behaviors are considered as reflexes and to be evidenced as visceral pain, although it would probably be wiser to call it peritoneo-visceral pain, as the pain is probably similar to that resulting from peritonitis [7]. The intraperitoneal injection of 0.6% acetic acid produced a typical pattern of writhing behavior in mice; (*S*)-ibuprofen (**1**), and esters **2**–**5** (18, 30 and 45 mg/kg) significantly reduced the number of abdominal constrictions induced by acetic acid, with an inhibition rate 69.2% using **2** at 45 mg/kg which was very close to that shown by ibuprofen (71.4%) (Figure 2). Esters **2**, **3**, and **5** exhibited dose dependence as that of ibuprofen (**1**) although less pronounced. At 18, 30 and 45 mg/kg, **1** showed an inhibition rate in abdominal constrictions of 12.5, 39.8, and 71.4%, while the esters showed: **2** (34.1, 43.3, and 69.2%), **3** (30.0, 38.6, and 52.5%), and **5** (36.7, 49.3, and 55.2%). In

contrast, ester **4**, derived from ergosterol, displayed inhibition of the contractions but not in a dose dependent manner (32.8, 29.9, and 26.6%) perhaps since the B-ring of the sterol contains two double bonds. The inhibition of writhing response indicates the peripheral antinociceptive effect of **1**–**5**, in addition to its central effect. The results suggest that esters **2**–**5** may be involved in the inhibition of the release or functions of endogenous nociceptive mediators. Previous studies reported that *β*-sitosterol at 10 and 20 mg/kg reduced the number of writhing in 50.3 and 60.8% [26]. Other studies reported that stigmasterol, its acetate, *β*-sitosterol, and aspirin (3–100 mg/kg, ip) inhibited, in a dose-related manner, acetic acid-induced abdominal constriction in mice with ID_{50} of 16, 11, 9, and 24 mg/kg, respectively [7]. Given orally, stigmasterol and stigmasterol acetate at 50–200 mg/kg also exhibited significantly, although in less extent the analgesic action against both acetic acid and formalin-induced nociception in mice. Besides, stigmasterol (up to 100 mg/kg, ip), in contrast to morphine (10 mg/kg, sc), had no analgesic effect in either tail-flick or hot-plate models [7].

Formalin-induced nociception results in typical animal behavior such as flinching and licking the paw injected with this algesic substance. The model evaluates two phases of nociception, the first of neurogenic origin, is due to direct stimulation of the nociceptors of afferent fibers, mainly peripheral C-fibers, and therefore this phase is sensitive to local anesthetics. The second phase is associated with formalin-induced release of peripheral mediators such as prostaglandins (PGs), nitric oxide (NO), histamine (His), and bradykinin that causes the lower intensity of C fiber accompanied by facilitation of dorsal horn neuronal responses, reflecting the integration of peripheral and central (spinal/supraspinal) signaling [27]. The second phase responds to various drugs such as opiates, steroids and non-steroidal anti-inflammatory analgesic drugs, *N*-Methyl-D-aspartate antagonists or gabapentin [28].

The administration of formalin produced a typical pattern of flinching behavior. The initial phase started immediately after the administration and then diminished gradually for the next 10 min. The second phase started after 15 min and lasted until 1 h-post administration. In this test, **1**–**5** at doses of 10, 30 and 100 mg/kg, produced antinociceptive response (about 50%) during phase two ($p < 0.05$; Figure 3) but not in phase one ($p > 0.05$; data not shown). The higher antinociceptive response was obtained with **2** at 100 mg/kg. In this case, a dose-dependence was observed for **1** and esters **2** and **5**. At 10, 30, and 100 mg/kg, ibuprofen (**1**) showed antinociceptive responses of 39.6, 43.2, and 53.2%, while the esters showed: **2** (51.7, 58.6, and 61.4%) and **5** (38.2, 49.1, and 54.6%). Esters **3** and **4** displayed important effects in the formalin-induced nociception test with respect to ibuprofen but not in a dose dependent manner showing 55.1, 44.1, and 47.4% for **3** and 41.2, 41.9, and 44.9% for **4**. These results suggest that the antinociceptive effects produced by (*S*)-ibuprofen could be due to inhibition of prostaglandins synthesis, and agree with those reported [29] showing that NSAIDs only demonstrated antinociceptive activity in phase 2. In this regard, it has been found that the systemic and intrathecal administration of ibuprofen suppressed the formalin-induced behavior, PGE2, glutamate and aspartate release [26]. Likewise, it is proposed that the antinociception induced by esters could be produced by the same mechanism. Similarly, 52 and 58% nociception inhibitions have been reported when *β*-sitosterol was administered at 10 and 20 mg/kg [30]. Other reports [7] show that stigmasterol and its acetate (10–100 mg/kg, ip) caused graded inhibition of both the neurogenic (first phase) and inflammatory phases (second phase) of formalin-induced pain. However, both compounds were more effective in the second phase of this test with ID_{50} values of 26 and 41 mg/kg, respectively. In addition, *in vivo* studies, including humans, have demonstrated that consumption of dietary phytosterols produces reduction of plasma levels of proinflammatory cytokines such as C-

reactive protein, IL-6, TNF-α, phospholipase A1, and fibrinogen. These effects may be mediated through beneficial alterations in the membrane composition, affecting membrane fluidity, sensitivity, and signaling pathways. These changes may subsequently alter the immune response by influencing the synthesis and secretion of eicosanoids, leukotrienes, and prostaglandins [8].

Inflammation induced by carrageenan is acute, nonimmune, well-researched, and highly reproducible. It has been described that in this test cardinal signs of inflammation such as edema, hyperalgesia, and erythema, develop immediately following subcutaneous injection, resulting from the action of proinflammatory agents, bradykinin, histamine, tachykinins, complement and reactive oxygen, and nitrogen species. Neutrophils readily migrate to sites of inflammation and can generate proinflammatory reactive oxygen and other species [20]. The final phase of the model is attributed to the local production of PGs, especially those of the E series. The precursor of both PGs and thromboxanes is PGH2, derived from arachidonic acid by the action of cyclooxygenase (COX) enzymes. A role for neutrophil-derived reactive oxygen species, nitric oxide, and peroxynitrite in carrageenan-induced inflammation has also been identified, and some specific inhibitors have been identified which have a potential clinical use.

A saline solution of carrageenan injected subcutaneously in rats induced an acute swelling that becomes maximal 3–5 h after injection. At the last hour, esters **2**–**5** at doses of 40, 80, and 160 mg/kg demonstrated anti-inflammatory responses resembling the effect of (*S*)-ibuprofen (**1**) (Figure 4). Their percentages of paw edema inhibition were **1** (13.2, 40.2, and 38.3%), **2** (16.9, 21.4, and 46.6%), **3** (6.1, 42.4, and 38.3%), **4** (19.2, 37.8, and 38.7%), and **5** (17.9, 16.8, and 46.5%).

Previous studies [30] reported the use of *β*-sitosterol in the carrageenan test at doses of 50, 100, and 200 mg/kg. The three tested doses of this sterol were found to induce a significant dose-dependent inhibition of the edema in comparison with the result obtained in the control group, and the inhibition was 51, 63, and 70%, respectively. These results support the suggested beneficial effects of *β*-sitosterol for some diseases, which include benign prostatic hyperplasia, colon and breast cancer, atherosclerosis, and gastrointestinal ulceration. Another work [11] describes that *β*-sitosterol at 10 and 20 mg/kg significantly ($p < 0.05$) inhibits inflammation by 40.2 and 50.7% after 4 h, respectively, while the reference drug, ibuprofen, at 40 mg/kg inhibits inflammation by 60.9% after the same time.

Gastric ulcer is a prevalent disorder characterized by mucosal lesions caused by an imbalance between aggressive factors, and the defensive mechanism factors that work towards the maintenance of mucosal integrity such as mucus, bicarbonate, prostaglandins, blood flow, and nitric oxide [31]. NSAIDs are among the most widely used medications in the world and generally produce extensive gastrointestinal injury. It has been estimated that 1–2% of continuous NSAID users may have a severe gastrointestinal side effect, such as perforation, ulceration, or bleeding. Patients taking NSAIDs experience a relative risk of upper gastrointestinal bleeding and perforations of up to 4.7 compared with nonusers [32]. The introduction of selective COX-2 inhibitors has not fulfilled all the expectations of safety, and there is evidence that some COX-2-selective and non-selective NSAIDs are associated with a moderately increased risk of cardiovascular events [33]. Therefore, strategies to diminish the risks include educational methods aimed to reduce prescribing or reducing the NSAID dose, co-prescribing gastro-protective agents such as misoprostol or omeprazole, switching to NSAIDs that are perceived to be less toxic, and the use of paracetamol as an alternative analgesic [32, 34].

Evidence from animal models shows that certain dietary phytosterols have a protective action against gastroduodenal ulceration, both singly and in combination, due to NSAIDs [35]. Other studies have demonstrated that stigmasterol produces anti-nociceptive effects without causing gastric ulcers or altering the gastrointestinal transit in mice [36].

Four hours after administration of **2**–**5**, at 10, 30, 40, 80, and 100 mg/kg, female rats were euthanized in a CO_2 chamber. The stomach was removed and opened along the greater curvature. An observer, blinded to the experimental treatment status of the animals, measured the area (mm^2) of each gastric lesion in the corpus of the stomach using the ImageJ software. In contrast, after 4 h of administrating **1**, the animals showed severe hemorrhagic erosions in the gastric mucosa. Gastric damage caused by **1** was significantly different from that measured in the vehicle-treated animals. Consistent with the earlier evidence [37] we found that **1** was able to produce significant gastric injury at 3 h. However, one of the more surprising observations from the present study was that **2**–**5** do not produce gastric injury in animals at doses of 10, and 30 mg/kg, whereas at 40, 80, and 100 mg/kg produce minimal gastric damage and significantly different ($p < 0.05$) from (*S*)-ibuprofen at 4 h (Figure 5). In light of this result, it is evident that the sterol plays an important role in the protection of the gastric mucosa of the stomach of the animals used in this test.

Acute toxicity of esters **2**–**5** was evaluated in mice, to which doses of 10, 100, 1600, and 2000 mg/kg of each compound were administrated orally and were observed for 14 days. During this time, no animal deaths or toxic effects were detected, which suggests that esters **2**–**5** seem safe for consumption.

In order to get a preliminary idea about the stability of esters **2**–**5** under acidic hydrolysis conditions as in the human stomach, *β*-sitosteryl ibuprofenate (**2**) (6.6 mg) was dissolved in a mixture of 0.1 M acetic acid in D_2O (pH 2.8)-tetrahydrofuran-d_8 5:7 (0.8 mL) and stirred

in an NMR spectrometer at 37 °C for 3 h. After this treatment, ester **2** remained intact and no detectable amounts of ibuprofen and *β*-sitosterol seemed to be present. To confirm that the signals for the hydrolysis products were clearly visible under these measurement conditions, a spectrum of a 1:1 mixture of ibuprofen and *β*-sitosterol was also determined. Therefore, it may be assumed that esters **2**–**5** could reach the intestine and be absorbed there, thereby increasing their bioavailability in the bloodstream. Further pharmacokinetic studies are beyond the limits of the present study.

4. Conclusions

Evaluation of *in vivo* pain models showed that steryl (*S*)-ibuprofenates **2**–**5** possess comparable or, in some cases, better activity than (*S*)-ibuprofen (**1**) at the same administered doses in mg/kg. Since the molecular weight ratio of the esters and ibuprofen is about 3 to 1, the ibuprofen administered amount was three times lower to achieve similar effects. This resulted in minimal gastrointestinal damage to the stomach of the test animals in contrast to the large gastric injury observed when (*S*)-ibuprofen was administered, thus revealing information about the safety of these esters. It is therefore suggested that esters **2**–**5** are safer regarding gastric damage. However, the efficacy and safety of **2**–**5** in human patients in clinical situations awaits additional validation.

Acknowledgments

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FIGURE AND TABLE LEGENDS

Figure 1. Formulas of (*S*)-ibuprofen (**1**) and steryl (*S*)-ibuprofenates **2**–**5**.

Figure 2. Systemic antinociceptive effect of vehicle (**V**), (*S*)-ibuprofen (**1**), and esters **2**–**5** on writhing test. (A) 18 mg/kg, (B) 30 mg/kg, (C) 45 mg/kg. Male mice (n = 6 animals) were orally administered either with vehicle (Span 20), and **1**–**5**, 30 min before test. The number of writhes was counted over a 30 min period following the injection of 0.6% acetic acid. Data are expressed as area under curve (AUC) of the number of writhing by 30 min. *Significantly different from the vehicle group ($P < 0.05$) as determined by the analysis of variance followed by Tukey's test.

Figure 3. Systemic antinociceptive effect of vehicle (**V**), (*S*)-ibuprofen (**1**), and esters **2**–**5** on the 5% formalin test. (A) 10 mg/kg, (B) 30 mg/kg, (C) 100 mg/kg. Female rats were administered either with vehicle (Span 20), and **1**–**5** 20 min before stimuli. Data are expressed as the area under the number of flinches versus time curve (AUC) on the second phase. Bars are the mean ± S.E.M. for 6 animals. *Significantly different from the vehicle group ($P < 0.05$), as determined by the analysis of variance followed by Tukey's test.

Figure 4. Systemic anti-inflammatory effect of vehicle (**V**), (*S*)-ibuprofen (**1**), and esters **2**–**5** on the 1% carrageenan test. (A) 40 mg/kg, (B) 80 mg/kg, (C) 160 mg/kg. Female rats were administered either with vehicle (Span 20), and **1**–**5** 20 min before stimuli. Data are expressed as the area under curve of the inflammation (AUC). Bars are the mean ± S.E.M. for 6 animals.

*Significantly different from the vehicle group ($P < 0.05$), as determined by the analysis of variance followed by Tukey's test.

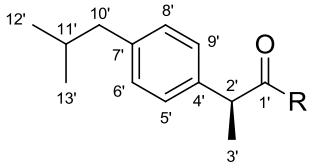
Figure 5. Evaluation of gastric injury produced (*S*)-ibuprofen (**1**), and esters **2**–**5**. (A) 40 mg/kg, (B) 80 mg/kg, (C) 100 mg/kg. Female rats were pretreated with **1**–**5** and were sacrificed 4 h later. The stomach was removed and the extent of hemorrhagic damage was scored. Data are expressed as the score of gastric injury. Each point corresponds to the mean ± SEM of 6 animals. *Significantly different from the ibuprofen group ($P < 0.05$) as determined by the analysis of variance followed by Tukey's test.

Table 1

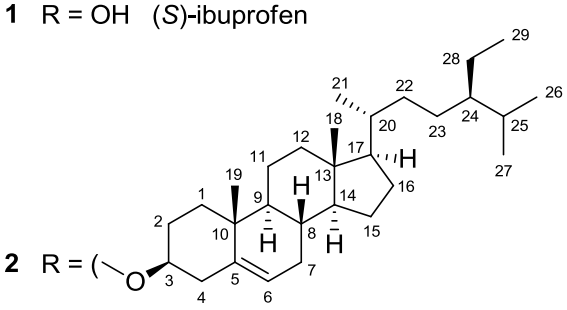
^{1}H NMR spectroscopic data of compounds **2**–**5** in $CDCl_3$ (δ in ppm, J in Hz).[a]

Table 2

^{13}C NMR spectroscopic data of compounds **2**–**5** in $CDCl_3$ (δ in ppm).[a]

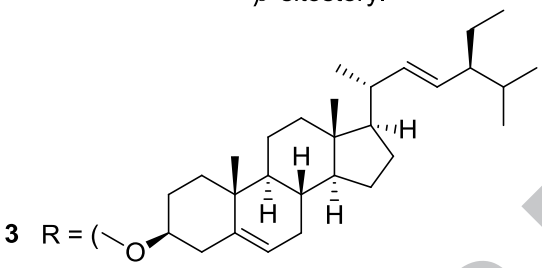


1 R = OH (*S*)-ibuprofen



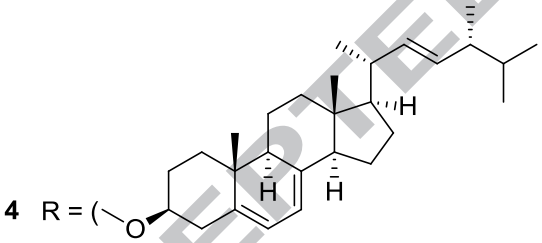
2 R = (

β-sitosteryl



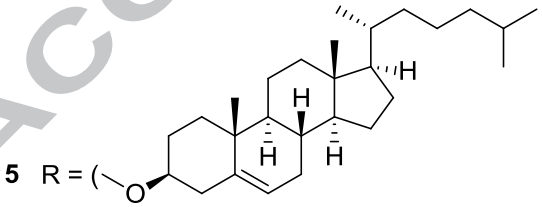
3 R = (

stigmasteryl



4 R = (

ergosteryl



5 R = (

cholesteryl

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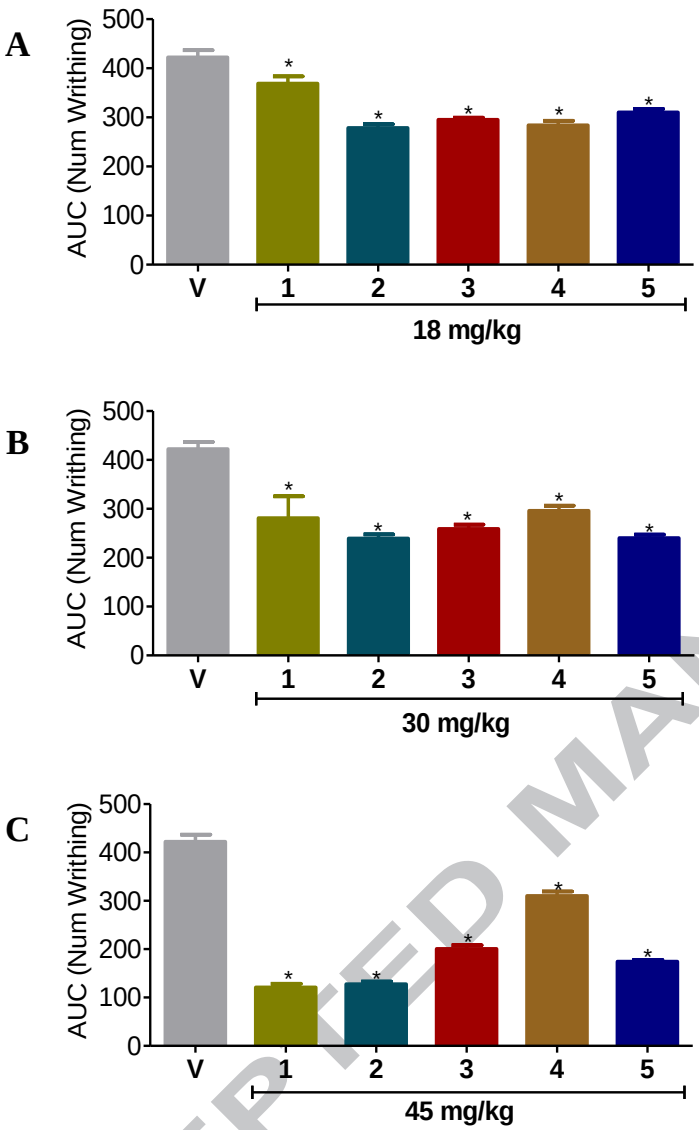

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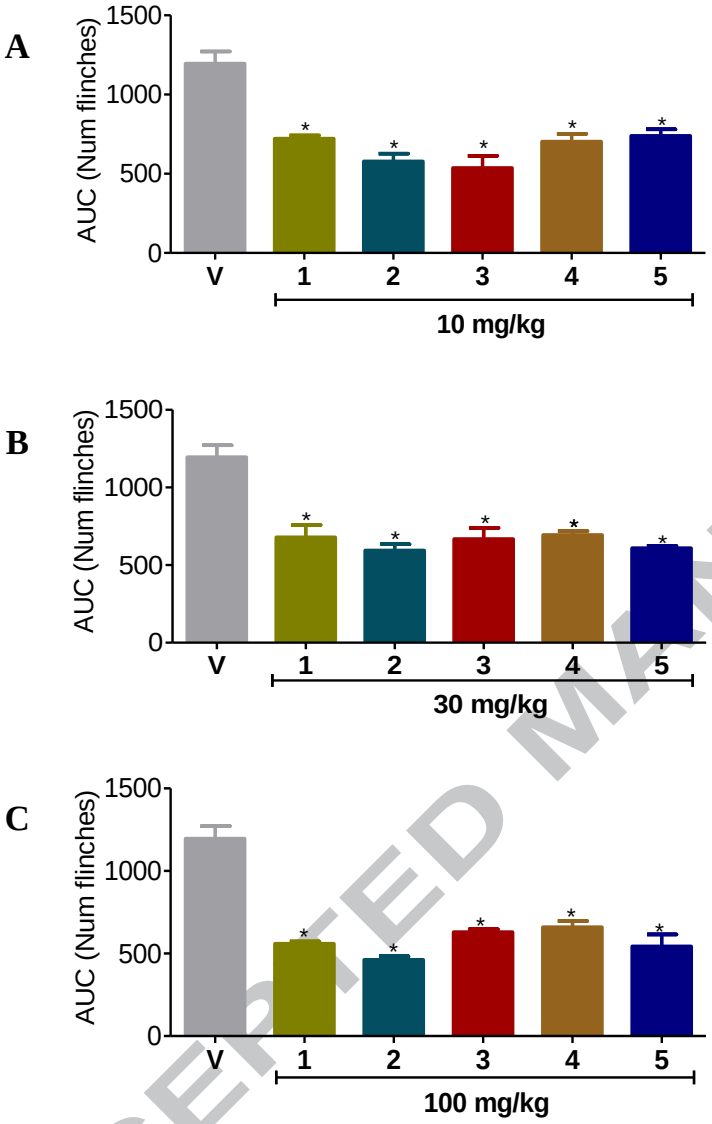

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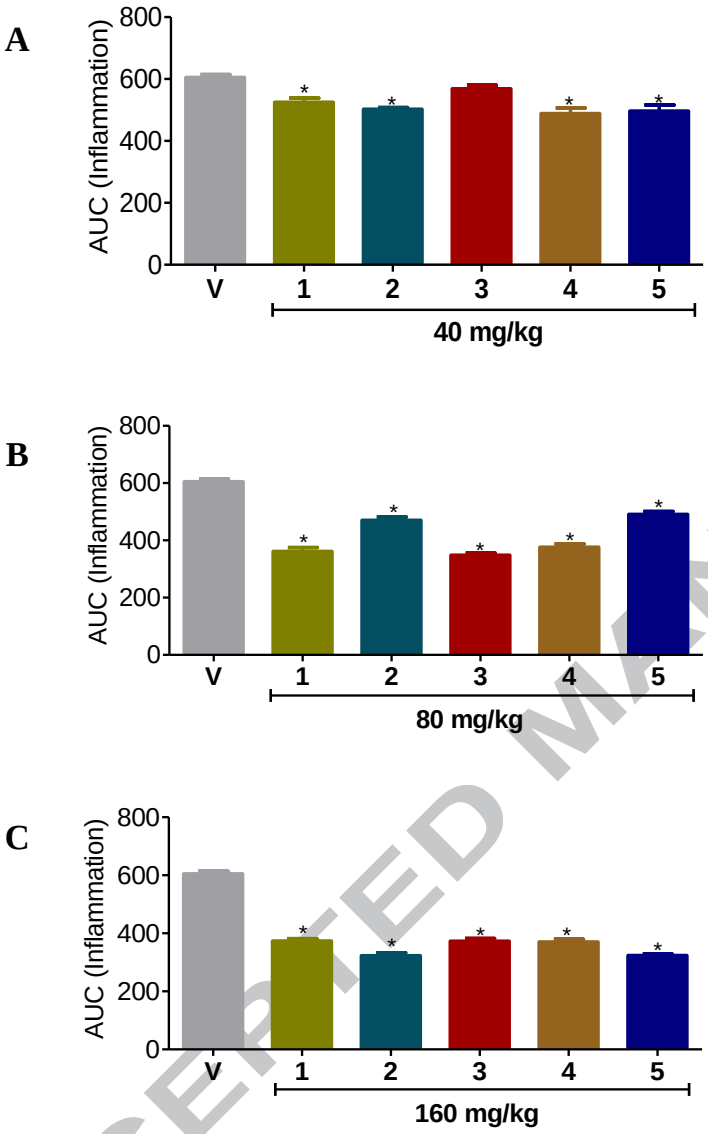

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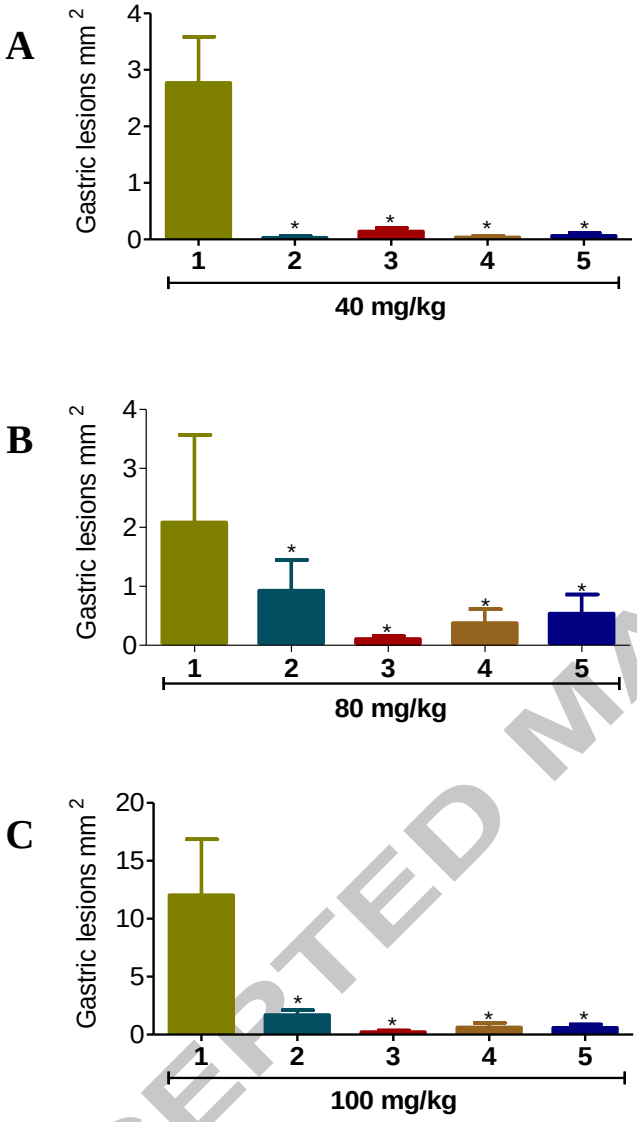

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Table 1

^{1}H NMR spectroscopic data of compounds **2**–**5** in $CDCl_3$ (δ in ppm, J in Hz).[a]

Position	**2**	**3**	**4**	**5**
3	4.59, m	4.59, m	4.40, tt (11.4, 4.5)	4.60, m
4	2.20, m	2.20, m	2.39, ddd (14.4, 4.8, 2.0); 2.24, bt (13.0)	2.20, m
6	5.32, d (4.9)	5.32, d (5.0)	5.51, dd (5.6, 2.2)	5.32, d (5.0)
7	—	—	5.36, dt (5.6, 2.4)	—
18	0.67, s	0.69, s	0.62, s	0.67, s
19	0.99, s	0.99, s	0.92, s	0.99, s
21	0.92, d (6.6)	1.02, d (6.6)	1.03, d (6.6)	0.91, d (6.6)
22	—	5.02, dd (15.1, 8.7)	5.18, dd (15.3, 7.6)	—
23	—	5.15, dd (15.1, 8.6)	5.21, dd (15.3, 7.0)	—
26	0.81, d (6.8)	0.79, d (7.2)	0.82, d (6.6)	0.87, d (6.6)
27	0.83, d (7.3)	0.85, d (6.4)	0.84, d (6.3)	0.86, d (6.6)
28	—	—	0.91, d (7.0)	—
29	0.84, t (7.6)	0.81, t (7.2)	—	—

[a]The chemical shifts and the coupling constants of the ibuprofen residue in **2**–**5** were essentially the same, as follows: δ 3.65 (q, J = 7.2 Hz, 1H, H-2'), 1.47 (d, J = 7.2 Hz, 3H, Me-3'), 7.20 (d, J = 8.0 Hz, 2H, H-5', H-9'), 7.08 (d, J = 8.0 Hz, 2H, H-6', H-8'), 2.44 (d, J = 7.2 Hz, 2H, H-10'), 1.86 (m, 1H, H-11'), 0.89 (d, J = 6.6 Hz, 6H, Me-12', Me-13').

Table 2

^{13}C NMR spectroscopic data of compounds **2**–**5** in $CDCl_3$ (δ in ppm).[a]

Position	2	3	4	5
1	37.1	37.1	38.1	37.1
2	27.9	27.9	28.2	27.9
3	74.3	74.1	73.1	74.3
4	38.0	38.0	36.5	38.0
5	139.8	139.8	138.7	139.8
6	122.7	122.7	120.3	122.7
7	32.0	32.0	116.5	32.0
8	32.0	32.0	141.6	32.8
9	50.2	50.2	46.2	50.2
10	36.7	36.7	37.2	36.7
11	21.2	21.2	21.2	21.2
12	39.9	39.8	39.2	39.9
13	42.5	42.4	43.0	42.5
14	56.8	56.9	54.7	56.8
15	24.4	24.5	23.1	24.4
16	28.4	29.1	28.4	28.4
17	56.2	56.1	55.9	56.3
18	12.0	12.2	12.2	12.0
19	19.5	19.5	16.3	19.5
20	36.3	40.6	40.6	35.9
21	18.9	21.4	21.3	18.9
22	34.1	138.5	135.7	36.3
23	26.3	129.4	132.1	24.0
24	46.0	51.4	43.0	39.7
25	29.3	32.0	33.2	28.2
26	19.2	19.1	20.1	22.7
27	20.0	21.2	19.8	23.0
28	23.2	25.6	17.8	—
29	12.1	12.4	—	—

[a]The chemical shifts of the ibuprofen residue in **2**–**5** were the same, as follows: δ 174.3 (C-1'), 45.5 (C-2'), 18.8 (C-3'), 138.2 (C-4'), 127.2 (C-5', C-9'), 129.4 (C-6', C-8'), 140.5 (C-7'), 45.2 (C-10'), 30.3 (C-11'), 22.5 (C-12', C-13').

Graphical abstract

In search of safe pain relief: The analgesic and anti-inflammatory activity of phytosteryl ibuprofenates

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Highlights for

In search of safe pain relief: The analgesic and anti-inflammatory activity of phytosteryl ibuprofenates

- Phytosteryl ibuprofenates possess comparable activity than ibuprofen at the same mg/kg doses.
- The new esters cause minimal gastric injury in comparison with free ibuprofen.
- Acute toxicity in *in vivo* models suggests that phytosteryl ibuprofenates are safe for consumption.