BALNEO RESEARCH Balneo and PRM Research Journal English Edition
e ISSN 2734 - 8458 p ISSN 2734 - 844X ROMANIAN ASSOCIATION OF BALNEOLOGY

In vivo antinociceptive and anti-inflammatory potential of hesperidin and its cyclodextrin inclusion compounds

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Balneo and PRM Research Journal DOI: http://dx.doi.org/10.12680/balneo.2021.417 Vol.12, No.1, March 2021 p: 40–45

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Abstract

Introduction. This study aims to evaluate the antinociceptive activity on inflammatory and non-inflammatory nociception models, as well as the anti-inflammatory action of hesperidin and its inclusion compounds with beta-cyclodextrin and hydroxypropyl-beta-cyclodextrin. **Material and method.** For these experiments, we employed nociception models using thermal, chemical and pressure stimuli and an inflammation model for the evaluation of inflammatory edema by plethysmometer test. **Results and discussions.** The obtained results demonstrate that the HES-βCD inclusion compounds exhibited antinociceptive action predominantly on experimental non-inflammatory nociception models, while HES-HP-βCD exhibited anti-inflammatory and antinociceptive activities predominantly in inflammatory nociception models. **Conclusions.** This research may be the starting point for future studies regarding the improvement of biopharmaceutical qualities of HES by encapsulation in cyclodextrins.

Keywords: *hesperidin, cyclodextrin inclusion compunds, antinociceptive, anti-inflammatory activity,*

Introduction

Research regarding reduction of side effects and optimization of medications have led to the development of new compounds with improved therapeutic potential. One of the problems encountered in the formulation of oral drugs is substance solubility, the dissolution capacity and dissolution rate greatly influencing the absorption process through the gastrointestinal mucosa. The solubility of certain substances can be improved using one of the most modern methods, complexation, which consists in inducing a reversible association between a substrate and a ligand to form new chemical species.

Cyclodextrins (CDs) are obtained through the enzymatic degradation of starch and have the shape of a truncated cone with a lipophilic cavity and a hydrophilic outer surface. Consequently, CDs can form inclusion compounds in which the "guest" molecule is partially or completely included in the "host" molecule, the most important conditions being steric and thermodynamic factors (1, 2).

First generation CDs are natural and contain 6-8 units of glucopyranose, having a limited solubility in water, such as β-cyclodextrin (β-CD) with a solubility in water of 18.5 mg/L. Therefore, chemical or enzymatic modifications of CDs have been used. One such example consists of substituting hydrogen and hydroxyl groups with various substituents, such as alkyl, hydroxyalkyl, carboxyalkyl, glucosyl, that lead to the formation of CDs with increased solubility, such as hydroxypropyl-β-cyclodextrin (HP-β-CD), that has a water solubility of more than 600 mg/mL (3).

CDs have a wide range of applications in the pharmaceutical industry, such as increasing the solubility, stability and dissolution rates of active substances, reducing the unpleasant taste and smell of substances, preventing interactions between active substances or between active substance and excipients, as well as controlled release of the active substance (4).

Flavonoids were the subject of a great number of researches in the plant physiology field, being involved in complex biological processes in the cell and cell membrane. Worldwide, statistics show a large number of papers that record data about their structure, synthesis and pharmacodynamic actions. Since flavonoids represent the majority of plant polyphenols, they were relatively rapidly isolated, being major candidates in research. For the current study, hesperidin was chosen from this class of compounds for inclusion in cyclodextrins.

Hesperidin (HES) is a flavanone glycoside, which is sparingly soluble in water and therefore has a limited bioavailability. However, it possesses important pharmacological actions such as: antioxidant, antitumoral (5), improving capillary status by permeability reduction and capillary resistance enhancement (6), lowering

cholesterol, lipid levels and blood pressure, diuretic effect and anti-replicative activity against some viruses (7).
Moreover, it has anti-inflammatory and analgesic effects through the inhibition of eicosanoid synthesis or of histamine release (8). It has also been shown that HES can be an effective therapeutic agent in improving the chondrogenesis of human mesenchymal stem cells by inhibiting inflammation and thus facilitating connective tissue repair (9).
In the last years, HES has also been shown to have important neuroprotective properties in neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's diseases (7).
The motivation of our study was driven by the therapeutic importance and the physicochemical properties of HES, one main objective of the current research being the improvement of its biopharmaceutical qualities by encapsulation in cyclodextrins. Therefore, our research has included the preparation of inclusion compounds with β-CD and HP-β-CD by different methods. The inclusion in CDs was demonstrated by various methods such as: solubility studies, UV-Vis spectroscopy, TLC, NMR, FTIR, thermal methods. Afterwards, the *in vitro* dissolution kinetics of inclusion compounds compared to free HES was studied. The results confirmed an increase in the dissolution of inclusion compounds in simulated gastric fluid at a pH of 1.2. Further *in vitro* studies have focused on assessing the antimicrobial and antioxidant activities of the inclusion compounds compared to free HES. The tests demonstrated an increase of these properties for inclusion compounds (10-12).
To the best of our knowledge, this is the first study that aims to evaluate the antinociceptive activity of these inclusion compounds (HES-βCD and HES-HP-βCD) on inflammatory and non-inflammatory nociception models, as well as their anti-inflammatory action compared to free HES.

Materials and methods

Samples: HES and its inclusion compounds with β-CD and HP-β-CD were administered in 1 % CMC-Na suspension p.o. All tests included a sequence of dose values in geometrical progression, in the range of 100-400 mg/kg HES and amounts of inclusion compounds with an equivalent HES content.
Chemicals: sodium carboxymethyl cellulose (CMC-Na) (Sigma); Zymosan A (Sigma) and λ-carrageenan (Sigma) suspended in physiological saline solution (Zentiva).
Animals: The study was carried out on adult male Swiss albino mice, weighing 20-25 g, that were purchased from the Cantacuzino Institute (Bucharest) and transported according to the current legislation. The proper housing conditions were established by the Experimental Pharmacodynamics Laboratory, Department of Pharmacodynamics and Clinical Pharmacy, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy Iasi. These conditions consisted of: laboratory enclosure under constant and controlled temperature (21 ± 2 ^{0}C) and humidity, and a 12 hours light-dark cycle (7.00 am - 7.00 pm); Mini Duna plexiglas cages provided with water and food bowls, which allowed feeding with standard diet (Biobase Baneasa) and water *ad libitum.*
Since the tests examine the behavioral reactions of animals, a period of 10 days for acclimatization was established prior to the beginning of experiments, in which observations regarding their behavior in terms of water and food consumption, neurological signs, etc., were made. The animals were randomly allocated to treatment groups of 6-10 mice/group and 3 hours before each test, the access to food and water was stopped.
All experiments were conducted in accordance with the specific regulations approved by "Grigore T. Popa" University of Medicine and Pharmacy Iași, European bioethical regulations (13) and International Association for the Study of Pain (IASP) guidelines.
Apparatus: thermostatic system Hot Plate Ugo Basile 7280 model, Ugo Basile Analgesimeter 37215 model, Ugo Basile plethysmometer 7200 model.
Methods: In order to evaluate the antinociceptive action of HES and its inclusion compounds the following tests on inflammatory and non-inflammatory nociceptive models were used: by thermal stimulus: the hot plate test and the tail immersion test; by chemical stimulus: the constrictive abdominal response test; by mechanical stimulus: Randall-Selitto test. The anti-inflammatory action was evaluated using the inflammatory edema test.

The hot plate test

This test was carried out according to the method established by Woolfe and Mac Donald 1994 and modified by Eddy and Leimbach 1953; O'Callaghan and Holzman, 1975 (14). The animals were subjected to a preliminary test, after which the samples were administered. Each mouse was placed into a closed cylindrical space with a metal hot plate maintained at a constant temperature of 52.5 ± 0.1 ^{0}C, for 30 seconds (cut-off time). Pain response latency was determined at 30, 60, and 90 minutes after sample administration. This experiment produces two types of behaviors (paw-licking and jumping), for which reaction times can be measured (14-18). The evaluation was based on a graded response and the antinociceptive effect was calculated using the following formula:

% Inhibition = $(T_x-T_0) / (T_m-T_0)$ x 100	**(1)**

where:
T_0 – response latency measured before sample administration
T_x – response latency measured after sample administration, at different periods of times
T_m – *cut-off* time, set in order to avoid injury in animals.

Tail immersion test

In this case, the method of Ben-Bassat et al., 1959 and Janssen et al., 1963, modified by Grotto and Sulman, 1967 (14) and Bild et al. 2009 was used (19). The experiment was conducted by immersing the animal's tail in a thermostated water bath at 52.5 ± 0.1 ^{0}C for 15 seconds (cut-off time), at 30, 60 and 90 minutes after sample administration (14, 17, 19). Pain reaction time was monitored by tail retraction. The evaluation was based on a quantal response and the antinociceptive effect was calculated using the following formula:

% Inhibition = (no. non-responders / total no. of animals/group) x 100	**(2)**

The constrictive abdominal response test

The method of Siegmund et al., 1957, Koster et al., 1959, modified by Domer, 1971; Tallarida et al., 2003; Turner and Hebborn, 1965 (16) and Bild et al. 2017 was applied (20). 60, 90 and 120 minutes after the administration of test samples, the groups received a Zymosan A saline suspension 20 mg/kg i.p. (intra-peritoneally). When administered i.p., Zymosan A has the capacity to produce a characteristic response called abdominal constriction response, characterized by elongation and constriction of the animal, abdominal retraction and opisthotonos. The number of abdominal constriction responses was recorded for 12 minutes after administration of Zymosan A (17, 20).

The evaluation was based on a quantal response and the antinociceptive effect was calculated using the following formula:

% Inhibition = (no. non-responders / total no. of animals/group) x 100	**(3)**

The Randall-Selitto test and inflammatory edema test

The experiment was performed according to a method modified by Bild et al. 2011 (21). After administration of test samples, an irritant agent capable of producing edema (λ-carrageenan, 3 % saline suspension) was injected s.c. (subcutaneously) into the plantar region. A mechanical stimulus (a cut-off pressure of 250 g) was applied on the inflamed paw, followed by the measuring of its withdrawal latency, 4 hours after administration of the inflammatory agent (14, 17, 21, 22). The evaluation was based on a graded response and was made by comparison with the contralateral paw, in which only saline solution was injected. The antinociceptive effect was calculated using the following formula:

% Inhibition = $(g_x - g_0) / (g_m - g_0)$ x 100	**(4)**

where:

g_x – response latency measured after sample administration

g_0 – response latency measured before sample administration

g_m – *cut-off* time.

Further testing was done in order to assess the anti-inflammatory action by evaluating the capacity of samples to inhibit λ-carrageenan-induced edema. This test was completed by measuring the volume of the paw using the plethysometric method, 4 hours after administration of the inflammatory agent. The degree of the inflammatory edema inhibition was calculated according to the following formula:

% Inhibition = (M – T)/M x 100	**(5)**

where:

M – value of the degree of inhibition of the control group

T – value of the degree of inhibition of the treatment group.

Results and discussion

The *in vivo* experiments performed in this study aimed to test the antinociceptive and anti-inflammatory actions of HES inclusion compounds with β-CD and HP-β-CD, as well as of free HES. Consequently, the response to various thermal, mechanical, chemical nociceptive and inflammatory stimuli was tested.

After oral administration of samples (HES-β-CD, HES-HP-β-CD, free HES, β-CD and HP-β-CD), the ED_{50} values (mg/kg) were determined. The results are presented in Tables 1-4.

For β-CD and HP-β-CD, the 50 % activity level could not be demonstrated for the studied doses in any of the performed tests.

The hot plate test

The behaviors noticed during this test, that implies a thermo-algesic mechanism, are considered to be supraspinally integrated responses, which allow the evaluation of a compound as an opioid or non-opioid analgesic, considering the experimental conditions and the animal's type of reaction (14).

Table 1. ED_{50} values (mg/kg body weight/p.o) for samples analyzed by hot plate test

Time	HES	HES-βCD	HES-HP-βCD
30 minutes	*	*	*
60 minutes	225.46 ± 15.34 *TLC 168.36-853.05*	165.89 ± 12.98 *TLC 138.36-720.10*	*
90 minutes	192.22 ± 23.11 *TLC 110.25-890.25*	*	*

* level of activity < 50 %,

TLC - True Confidence Limits

According to the data presented in Table 1, the ED_{50} values for free HES showed comparable antinociceptive action at 60 and 90 minutes. Among the inclusion compounds, for that with HP-β-CD, the 50 % activity

level could not be achieved. However, HES-β-CD inclusion compounds showed 50 % antinociceptive action at 60 minutes and showed superior potency compared to free HES.

Tail immersion test

This test completes the hot plate test for confirmation of the involvement of spinal structures for pain perception and integration, both measuring animal nociceptive response latencies to thermal stimuli (23).

Table 2. ED_{50} values (mg/kg body weight/p.o) for samples analyzed by tail immersion test

Time	HES	HES-βCD	HES-HP-βCD
30 minutes	*	90.35 ± 32.52 *TLC 20.29 – 198.6*	*
60 minutes	240.21 ± 66.12 *TLC 138.36-853.05*	91.85 ± 28.63 *TLC 17.29 – 176.4*	*
90 minutes	203.58 ± 44.83 *TLC 123.65-398.53*	122.90 ± 36.67 *TLC 30.61 – 312.13*	*

* level of activity < 50 %,
TLC - True Confidence Limits

For free HES, the antinociceptive action was over 50 % after 60 minutes and was maintained at 90 minutes. Administration of free HES and its inclusion compounds containing an equivalent amount of HES lead to similar results as those obtained in the previous test, regarding the difference between the two types of studied CDs (Table 2). For HES-β-CD, the action was assessed for all studied temperature ranges, while HES-HP-β-CD compounds didn't modify the reactivity of animals to painful thermal stimulation in a percentage that allows to obtain a level of activity over 50 %. The ED_{50} values for the HES-β-CD inclusion compounds were lower compared to the values obtained for the group treated with free HES, which demonstrates their superior potency.

Thermal nociceptive models are sensitive to opioid drugs and the analgesic activity is mediated by μ, κ and δ receptors, which are located only in the central nervous system, but not by receptors located in the peripheral nervous system (23).

HES is a lipophilic substance, that has shown central analgesic and anxiolytic-sedative effects, suggesting that it may cross the blood-brain barrier (24). Loscalzo et al. demonstrated that the effects of HES are completely blocked by naltrexone, which is a non-selective opioid antagonist, and thus supports the idea that opioid receptors are involved in the antinociceptive effects of HES (8). Therefore, the lack of reactivity for compounds with HP-β-CD to such tests that imply the thermo-algesic mechanism could be explained by the lack of involvement in the mechanism dependent on opioid receptors located in the central nervous system, because these compounds have minimal access at this level. By inclusion in cyclodextrins, especially HP-β-CD which has a high water solubility compared to β-CD, the HES-HP-β-CD complex became less lipophilic than the HES-β-CD complex, which caused a decreased crossing of the blood-brain barrier and implicitly a lack of reactivity during this tests.

The constrictive abdominal response test

This test is usually considered to be relevant to the pathogenesis of inflammatory pain, since no cell necrosis occurs and it allows the evaluation of central and peripheral analgesia (15).

Table 3. ED_{50} values (mg/kg body weight/p.o) for samples analyzed by constrictive abdominal response test

Time	HES	HES-βCD	HES-HP-βCD
60 minutes	194.29 ± 55.68 *TLC 28.24 – n/a*	217.73 ± 67.86 *TLC 113.38-1156.4*	133.11 ± 36.93 *TLC 75.5-306.84*
90 minutes	220.11 ± 42.34 *TLC 24.44-1300.18*	223.34 ± 86.22 *TLC 64.22-1021.45*	128.34 ± 31.27 *TLC 64.45 - 280.38*
120 minutes	224 ± 26.22 *TLC 48.22-1267.32*	231.68 ± 51.84 *TLC 3.38-1312.34*	140.22 ± 38.54 *TLC 44.52 - 342.84*

TLC - True Confidence Limits,
n/a – not available

As seen in Table 3, all compounds showed antinociceptive action on the Zymosan chemical nociceptive model for the studied sequence of doses. The ED_{50} values demonstrate a comparable action between free HES and HES-β-CD, but inclusion in HP-β-CD lead to superior potency.

The inflammatory response to administration of Zymosan is characterized by abdominal constriction response, plasma extravasation, leukocyte infiltration and biosynthesis of hyperalgesic eicosanoids (25). Moreover, it has been shown that inflammatory agents don't directly stimulate the release of primary hyper-nociceptive mediators, but this is in fact preceded by a cascade of cytokines that act simultaneously and synergistically (15, 16). Therefore, the antinociceptive action by chemical mechanism can be explained by inhibition of

proinflammatory mediators such as IL-1, IL-6, IL-8, TNF-α and PGE2.

The Randall-Selitto test and inflammatory edema test

The Randall-Selitto test allows the assessment of inflammatory pain by applying pressure to the inflamed area. The development of edema allows the evaluation of mechanical antinociceptive action (hyperalgesia produced by chemical stimulus and pressure stimulus) and of the anti-inflammatory action by measuring the paw volume.

Table 4. ED_{50} values (mg/kg body weight/p.o) for samples analyzed by Randall-Sellito test and inflammatory edema test

Test	HES	HES-βCD	HES-HP-βCD
Randall Selitto	228.16 ± 66.23 *TLC 122.61 – 988.08*	211.28 ± 43.27 *TLC 113.38 – 1156.4*	148.84 ± 41.64 *TLC 35.23 – 886.42*
Inflammatory paw edema	200.80 ± 38.21 *TLC 68.23 – 1133.89*	188.86 ± 60.21 *TLC 52.24 – 1123.15*	160.22 ± 43.20 *TLC 38.44 – 880.26*

TLC - True Confidence Limits

The animals presented a significant reduction in pain perception for all administered samples. In both tests, the ED_{50} values could be determined for all analyzed compounds.

HES-β-CD compounds showed similar antinociceptive action to that of free HES, but a superior potency for the anti-inflammatory action.

HES-HP-β-CD compounds showed superior potency for both types of actions compared to free HES and HES-β-CD compounds, the antinociceptive action in inflammatory conditions being more relevant.

Cunha et al. demonstrated that s.c. administration of carrageenan into the plantar region in mice causes hyperalgesia by locally stimulating the production and release of inflammatory cytokines such as TNF-α, keratinocytes and IL-1β, which could represent the link between the release of primary hypernociceptive mediators and injury (26, 27). Moreover, carrageenan can trigger an acute inflammatory process involving the sequential release of pro-inflammatory mediators, especially histamine, serotonin, prostaglandins and thromboxanes (23).

Pinho-Ribeiro et al. demonstrated that HES reduces inflammatory pain and inflammation by suppressing cytokine production, NF-κB activity and oxidative stress (28). Thus, it is reconfirmed that the analyzed samples can reduce carrageenan-induced hypernociception by interfering with cytokine production. Furthermore, by inhibiting cellular influx of carrageenan and reducing proinflammatory cytokines, the inflammatory process will be diminished.

The anti-inflammatory activity proved to be stronger when the complex was obtained using a more hydrophilic cyclodextrin (HP-β-CD), which also lead to the formation of a more easily soluble complex. In this case, the involvement of the peripheral opioid system is important in clinical practice, the adverse effects of opioids on the central nervous system (e.g. respiratory depression or addiction) being suppressed or reduced (23).

Conclusions

Free HES has shown antinociceptive and anti-inflammatory activities on all studied models, with a higher potency for non-inflammatory nociceptive models by thermal stimulus (90-minute testing) compared to chemical stimulus. For inflammatory nociception and inflammatory edema models, the inhibition potency is comparable.

Inclusion compounds with β-CD showed a superior potency compared to free HES on non-inflammatory nociceptive models by thermal stimulus at 60 minutes, but not on chemical stimulus models. Moreover, for these compounds the potency was comparable for inflammatory nociception models and inflammatory edema model.

HP-β-CD inclusion compounds had no activity on non-inflammatory nociceptive models by thermal stimulus. However, for non-inflammatory nociceptive model by chemical stimulus, inflammatory nociceptive model, and for inflammatory edema model, the potency was higher than that of β-CD inclusion compounds and of free HES.

In conclusion, the inclusion compounds showed superior potency compared to free HES. For HES-βCD, the antinociceptive action in experimental models of non-inflammatory nociception by thermal stimulus prevailed, while for HES-HP-βCD the antinociceptive action on models of non-inflammatory nociception by chemical stimulus, the antinociceptive action in inflammatory conditions and the anti-inflammatory action prevailed.

This research may be the starting point for future studies regarding the improvement of some pharmacological activities of HES through inclusion in CDs, so as to find better therapeutic options, which is of great importance in the pharmaceutical field.

Author contributions.

All authors had the same contribution.

Conflict of interests. The authors declare that there is not conflict of interest.

Acknowledgments: Scientific research funded by "Grigore T. Popa" University of Medicine and Pharmacy Iasi, based on contract no. 4872/18.03.2013.

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