Niger. J. Physiol. Sci. 37 (June 2022): 9 – 19
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full-Length Research Article

A Novel Gedunin-2-hydroxypropyl-β-cyclodextrin Inclusion Complex Improves Anti-nociceptive and Anti-inflammatory Activities of Gedunin in Rodents

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Summary: Gedunin is a bioactive compound, obtained from *Entandrophragma angolense* (EA), which has limited therapeutic usefulness due to poor aqueous solubility and first-pass effects. Cyclodextrins are cyclic oligosaccharides that form complexes with poorly soluble compounds, thus enhancing their pharmacological activity. In this article, we evaluated the pharmacological activities of gedunin-2-hydroxypropyl-β-cyclodextrin complex (GCD) in rodents. The antinociceptive activity of GCD (50, 100, 200 mg/kg) and Gedunin (50mg/kg) was tested in acetic acid-induced writhing and formalin-induced paw licking in mice. The anti-inflammatory activity was investigated in carrageenan-induced paw oedema and air pouch inflammation models in rats. Leucocyte counts, Tumour Necrosis Factor-alpha (TNF-α) level, nitric oxide, malondialdehyde, reduced glutathione, and myeloperoxidase enzyme activities were assessed in the air pouch exudate. The GCD (200mg/kg) significantly decreased writhing response, reduced licking duration and decreased oedema compared with gedunin and control. Exudate volume and leucocyte count were significantly reduced by GCD (200 mg/kg), it decreased myeloperoxidase activity and inhibited TNF-α release. The carrageenan-induced GSH depletion, increased malondialdehyde and nitrite levels were significantly reversed by GCD (200 mg/kg) relative to gedunin and control. The GCD complex demonstrated significant antinociceptive and anti-inflammatory activities relative to gedunin alone via mechanisms associated with inhibition of oxidative stress and inflammation in rodents.

Keywords*: anti-inflammatory, anti-nociceptive, gedunin, gedunin-2-hydroxypropyl¬-β-cyclodextrin*

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Manuscript received- February 2022; Accepted- April 2022

DOI: https://doi.org/10.54548/njps.v37i1.2

INTRODUCTION

Cyclodextrins are structurally related natural compounds produced when cellulose is digested by bacteria. Chemically, they are non-toxic macrocyclic biodegradable oligosaccharides with at least 6 D-(+) glucopyranose connected by α-(1, 4) glucosidic linkages. They are shaped like a truncated cone rather than ideal cylinders and have a lipophilic center cavity and a hydrophilic outside surface (Brewster and Loftsson, 2007; Cusola *et al*., 2013; Gidwani andVyas, 2015). Cyclodextrins are capable of interacting with a vast variety of guest molecules to produce non-covalent inclusion complexes (Wankar *et al*., 2020). The synthesis of inclusion complexes improves the physical, chemical, and biological characteristics of guest compounds in the cyclodextrin cavity's hydrophobic environment (Carneiro *et al*., 2019). The most common use of cyclodextrins in pharmaceuticals is to improve solubility of drugs in aqueous solutions (Ueda *et al*., 2021). A reduction in variability of oral drug absorption can then be achieved through enhancement of drug dissolution rate as a result of improved aqueous drug solubility by cyclodextrin complexation (Wankar *et al*., 2020). This leads to an improvement in drug bioavailability. Furthermore, complexation can increase the stability of the therapeutic substance preventing degradation or bioconversion at the absorption site (Otero-Espinar *et al*., 2010). Other benefits of cyclodextrin complexation include the ability to mask unpleasant taste or color, fewer side effects, and the possibility of a drug release system. These have been thoroughly researched and documented in literature. Several investigations have shown that improved bioavailability associated with cyclodextrin complexation usually leads to an increase in the guest compound's pharmacological activity.

The *in vitro* physico-chemical characterization and *in vivo* analgesic and anti-inflammatory studies performed showed that the complexation process enhanced the aqueous solubility and dissolution of meloxicam. In addition, the meloxicam-cyclodextrin complex had greater gastrointestinal tolerability, much faster onset of action, and better efficacy (Bandarkar *et al*., 2013). The evaluation of the antinociceptive effect of tetracaine complexes, using the intraorbital nerve blockade test in rats, revealed that complexation with β-cyclodextrin (β-CD) and 2-hydroxypropyl-β- cyclodextrin (HBD) increased the

analgesic duration and intensity induced by tetracaine (Franco de Lima *et al*., 2012). Indomethacin, etoricoxib and etodolac cyclodextrin complexes exhibited improved activity in the tail-flick and hot plate analgesic tests (Sinha and Amita Goel, 2010; Singh *et al*., 2011; El-Feky *et al*., 2013). The onset of action and hypoglycaemic effect was greater for β-CD and HBD complexes of gliquidone than gliquidone alone due to improved biopharmaceutics (Miro *et al*., 2004). The inclusion of bupivacaine and ropivacaine in HBD lowered myotoxicity and improved the anesthetic effect following injection in the subarachnoid space or after sciatic nerve blockade, compared to free uncomplexed anaesthetic solutions at the same concentration (Dollo *et al*., 1998; Dollo *et al*., 2000; Araujo *et al*.,2005; Araujo *et al*., 2006; Araujo *et al*., 2008; Cereda *et al*., 2012).

Gedunin is a limonoid found in the Meliaceae family of plants (Braga *et al*., 2020). These plants have historically been utilized as fever remedies in Africa, India, and South America. Gedunin is highly effective against *Plasmodium falciparum in vitro* (Khalid et al., 1989; Bray *et al*., 1990; Bickii *et al*., 2000). It has shown anti-inflammatory and antiproliferative effects *in vitro* (Brandt *et al*., 2008; Ravangpai, *et al*., 2011). Poor solubility in water and short half-life have hampered gedunin's pharmacological efficacy following oral administration. Spectrophotometric studies of a novel gedunin-2-hydroxypropyl-β-cyclodextrin binary system revealed the formation of a stable inclusion complex between gedunin and HBD at room and body temperatures (Ologe *et al*., 2016). Inclusion complex formation was supported by the physico-chemical analysis of this binary system (Ologe *et al*., 2021). The formation of an inclusion complex between gedunin and HBD is expected to improve the solubility of gedunin in aqueous solutions as well as increase its bioavailability. The projected increase in bioavailability should improve gedunin's pharmacological activity *in vivo*. Thus, the present study was aimed at evaluating the antinociceptive and anti-inflammatory activities of gedunin (Ged) and gedunin-2-hydroxypropyl-β-cyclodextrin (GCD) in rodents.

MATERIALS AND METHODS

Drugs and Reagents: Gedunin (Ged) was isolated from the heartwood of *Entandrophragma angolense* Welwitsch C.D.C (Meliaceae) (Okhale *et al*., 2012). Sigma-Aldrich Chemical Company (Japan) supplied 2-hydroxypropyl-β-cyclodextrin. Sodium nitrite, carrageenan (type 1), sodium nitroprusside, O-dianisidine, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, indomethacin and 5,5′–dithio-bis-(2-nitrobenzoic acid) were manufactured by Sigma Aldrich (Steinheim, Germany). Ethanol analytical grade and sodium nitrite were produced by BDH Chemicals Ltd, Poole, England. Mouse TNF-α ELISA MAX™ Deluxe set from Biolegend (San Diego, USA). Other reagents used were analytical-grade.

Preparation of solid inclusion complex: Ged and HBD solid complex was produced by freeze drying at a molar ratio of 1:1 based on the stoichiometric ratio determination results (Ologe *et al*., 2016). The HBD was dissolved in distilled water and an equimolar amount of Ged, dissolved in 95% ethanol, was added. The suspension was shaken at 37ºC for 6 h. The resulting solution was kept in a -20ºC freezer and lyophilized in a freeze-dryer (LTE Lyotrap Plus, UK) for 24 h (Ologe *et al*., 2021). The freeze-dried powder was kept in a dessicator in a sealed glass vial.

Experimental animals: Male and female Swiss albino mice (Vom strain, 25–30 g), as well as male and female Wistar rats (150–200 g), were obtained from the Animal House at the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Nigeria. The animals were allowed to acclimatize for one week at room temperature of 28 ± 2 °C, relative humidity of 60%–70%, 12:12 h light: dark cycle. All animals were given unlimited access to water and commercial rat food pellets (Ladokun Feeds Ltd., Ibadan, Nigeria. All experiments were done in line with the principles of laboratory animal care" (NIH publication No. 85-23) guidelines and protocols, which were approved by the Animal Care and Use Research Ethics Committee of the University of Ibadan (UI-ACUREC/App/2015/074).

Antinociceptive and anti-inflammatory tests

Acetic acid-induced writhing test: Acetic acid-induced writhing in mice was done as previously reported (Koster *et al*., 1959). Mice (25-30 g) were assigned randomly into six groups of five mice each. Group 1 received 10% propylene glycol (10 ml/kg). The gedunin-2-hydroxypropyl-β-cyclodextrin inclusion complex (GCD) were administered to Groups 2, 3 and 4 at 50, 100, and 200 mg/kg respectively and group 5 received Ged alone (50 mg/kg). Group 6 received indomethacin (10 mg/kg). The vehicle, standard and test drugs were administered orally 1 h before nociception was induced with intraperitoneal injection of 10 ml/kg of 0.6% acetic acid. The number of writhes were counted for 15 min five minutes after the administration of acetic acid.

Formalin-induced paw licking test: Formalin test was conducted using recognized methods (Hunskaar and Hole, 1987). Mice (25-30 g) were randomly assigned to groups and treated orally as described for the acetic acid-induced writhing test above. Nociception was induced 1 h later by injecting 20 µl of 2.5% formaldehyde solution in the sub-plantar region of the left posterior paw. The duration of paw licking and/or biting the injected paw was a measure of the painful response and it was determined at 0-5 min (early phase, neurogenic) and 20-30 min (late phase, inflammatory) after injecting the noxious agent.

Tail immersion test: Hot water-induced tail withdrawal reflex was also used as a model of nociception (D'Amour and Smith, 1941; Barrot, 2012). Mice (25-30 g) were assigned into groups and treated orally as described in acetic acid-induced writhing test above. The tail of each animal (up to 5 cm) was immersed in water at 55.0 ± 0.2ºC. The latency time (in seconds) for removal of the animal's tail from the stimulus was taken as the reaction time to pain. A cut-off time of 20 seconds was applied to avoid tissue damage. The tail withdrawal latencies were determined at 0, 1, 1.5 and 2 h after the drug administration.

Hot plate test: A thermostatically controlled heated metal plate (Ugo Basile, Italy) within a restraining perspex

cylinder was used for the hot plate test (Eddy and Leimbach, 1953; Franzotti *et al.*, 2000). The hot plate apparatus temperature was kept at 55± 0.5ºC. Mice (25-30 g) were randomly allotted into six groups of five mice each and treated orally as described earlier. The reaction time of each mouse was assessed at 0, 60, 90 and 120 minutes after drug administration using a 15-second cut-off period time. The differences in reaction time between treated and control groups were compared.

Carrageenan-induced rat paw edema inflammation model: Rats (weighing 150 – 200g) were randomly allotted into six groups and pre-treated orally, with vehicle (10 ml/kg, 10% propylene glycol), GCD (50, 100 and 200 mg/kg), Ged 50 mg/kg and indomethacin (5 mg/kg) for three days. After 1 h of last treatment, 100 µl of 1% w/v carrageenan (Sigma, Type 1) was injected into the rat hind paw under the subplantar aponeurosis, to induce acute pedal inflammation (Winter *et al*., 1962). The volumes of the injected and contralateral paws were measured using a Ugo Basile (7150 model) digital plethysmometer (Comerio, VA, Italy) at 1, 3, and 5 hours after inducing edema. For each animal, the edema volume was calculated as the difference between the contralateral and carrageenan-injected paws.

Carrageenan-induced air pouch inflammation model: The formation of air pouches was induced in rats with minor modifications (Sedgwick and Lees, 1986; Martin et al., 1994). The air pouches were formed by injecting 20 ml of sterile air subcutaneously into the intra-scapular area of each rat's shaved back after anaesthesia with intraperitoneal ketamine (100 mg/kg). The created air pouches were preserved by re-injection of 10 ml of sterile air into the cavity 72 h later, followed by oral administration of vehicle (10 ml/kg) to Groups 1 and 2, GCD (50, 100 and 200 mg/kg) to groups 3, 4 and 5, Ged 50 mg/kg to group 6 and indomethacin (5 mg/kg) to group 7. The vehicle, GCD, Ged, and indomethacin were administered to the rats orally (like described) for three consecutive days before inflammation was induced. On day 6, 1 h after the oral administration of the test agents, standard drug and vehicle, 2 ml of sterile normal saline was injected into the air pouch of the saline group (group 1) while 2 ml of 1% carrageenan (Sigma, Type I) was injected into the pouch of groups 2–7 to generate an inflammatory response.

The rats were subjected to ether inhalation anaesthesia 24 h after saline or carrageenan injection. Normal saline (2 ml) was injected into the pouch, of each animal, before a small incision was gently made in the pouch wall, and the contents of the air pouch was carefully collected into a sterile tube. The total volume of collected exudate was calculated. The exudate cells were separated in a centrifuge at 10,000 rpm for 10 min at 4ºC. The leukocytes were counted after staining with Turk solution in a Neubauer chamber under a light microscope with a ×40 objective lens (Nikkon Eclipse E200, USA). Analyses for tumor necrosis factor (TNF), reduced glutathione (GSH), nitrites, thiobarbituric acid reactant substance (TBARS/MDA) and nitrite were done on the cell-free exudate supernatant that had been aliquoted and stored at -80ºC. A part of the pouch lining was fixed in 10% neutral buffered formalin, processed into 4 µm section and stained with hematoxylin and eosin (H & E) for light microscopic examination. Another portion of the pouch lining was assayed for myeloperoxidase (MPO) activity.

Biochemical assays

Myeloperoxidase assay: The MPO activity in pouch lining tissue was assessed using established procedure (Bradley *et al*., 1982). The tissue was homogenized in 50 mM potassium phosphate buffer (pH 6.0) and centrifuged at 10,000 rpm for 15 min at 4ºC. The pellet was suspended in extraction buffer (0.5% hexadecyltrimethyl ammonium bromide in 50 mM potassium phosphate buffer, pH 6.0) and frozen at 20ºC. The frozen suspension was thawed and sonicated (for 10 seconds). The freezing, thawing and sonicating process was repeated thrice before centrifuging it at 15,000 rpm at 4ºC for 15 min. The supernatant (0.2 ml) was added to 2.8 ml of mixed solution (containing 0.167 mg/ml O-dianisidine in 50 mM potassium phosphate buffer and 0.15 mM H_2O_2). The change in absorbance at 450 nm was monitored for 3 min using a UV/VIS spectrophotometer (752N INESA, China). A unit of MPO was defined as a change in absorbance of 0.001 per minute with the specific activity represented as MPO/mg protein.

Reduced glutathione (GSH) in pouch exudate: The amount of reduced glutathione (GSH) amount in pouch exudates was quantified using Ellman's reagent, with minor modifications (Moron *et al*.,1979; Sin *et al*., 1997). Cell free exudate supernatant (0.1 ml) was diluted ten times and mixed with 1 ml Trichloroacetic acid (20%) for de-proteinization; the mixture was centrifuged at 10,000 rpm at 4ºC for 10 mins. The supernatant (0.25 ml) was mixed with 0.75 ml sodium phosphate buffer (0.1 M, pH 7.4) and 2 ml of 0.0006 M DTNB. The absorbance was read within 5 min at 412 nm in a UV/VIS spectrophotometer (INESA 752N, China). The glutathione concentration which was calculated using a standard curve generated with standard glutathione (0-200 µM) was expressed as a function of the volume of pouch exudates (µM GSH/ml of pouch exudates).

Thiobarbituric acid-reacting substances (TBARS) in pouch exudates: The extent of lipid peroxidation was determined using the TBARS assay (Ohkawa *et al*., 1979). The sample (0.1 ml) was diluted twenty times in 0.15 M Tris-KCl buffer, and mixed with 0.5 ml TCA (30%), and 0.5 ml thiobarbituric acid (0.75%). The mixture was incubated in a water bath at 80ºC for 45 min. The test tubes were dipped in ice-cold water for 10 min to halt the reaction, and thereafter the reaction mixture was centrifuged at 4000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm in a UV/VIS spectrophotometer. The majority of TBARS are malondialdehydes (MDA), thus the result was calculated using an index of absorption for MDA (molar extinction coefficient 1.56 x 10^5/M/cm). The concentration of TBARS in pouch exudate was expressed as nmol MDA/mg protein. Total protein content in pouch exudates for TBARS and MPO analyses was estimated by the biuret method (Gornall *et al*., 1949). Protein concentration was determined using a standard curve generated with Bovine serum albumin (0–100 µM).

Nitrite assay: Nitric oxide production in pouch exudates was assessed by Nitrite assay using Griess reagent. Griess

reagent was freshly prepared from a mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% of N-1-naphthyl ethylenediamine dihydrochloride) at a ratio of 1:1. The samples were incubated with Griess reagent in the dark for 30 min at room temperature, absorbance was read with a spectrophotometer at 540 nm. The nitrite concentration was determined from a standard curve of sodium nitrite (0 – 100 µM).

TNF-α assay: The amount of TNF-α generated in the carrageenan-induced air pouch exudate was estimated with a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kit (Biolegend, USA), according to the manufacturer's instruction.

Histopathological analysis of air pouch lining: A portion of the pouch tissue linings was fixed in 10% neutral buffered formalin, dissected longitudinally, placed in embedding cassettes, embedded in paraffin, and then cut into 4-µm sections. Tissue sections were stained with hematoxylin and eosin (H & E) for light microscopic examination.

Statistical Analysis: Results were presented as Mean ± SEM. Statistical analysis between groups was performed by one-way analysis of variance and Student's-Newman-Keuls multiple comparison tests using Graph Pad Prism 5.0 software (Graph Pad Prism Software Inc., San Diego, CA, US). The level of significance for all tests was set at $p<0.05$

RESULTS

GCD reduced nociceptive reaction in mice: GCD decreased ($p < 0.05$) the number of writhes in the acetic acid-induced writhing tests compared to control although the decrease was not dose-dependent. The effect of GCD was highest at 100 mg/kg (72.6% inhibition) compared to 46% inhibition at 50 mg/kg and 65.7% inhibition at 200 mg/kg. Ged 50 mg/kg, equivalent to GCD 200 mg/kg, inhibited the writhing response by 58%. The writhing response was reduced by 58% with Ged 50 mg/kg. Ged 50 mg/kg is equivalent to GCD 200 mg/kg. Thus, GCD (200mg/kg) showed a significantly ($p < 0.05$) higher response than Ged (50 mg/kg). Indomethacin decreased the writhing response by 89% (Figure 1).

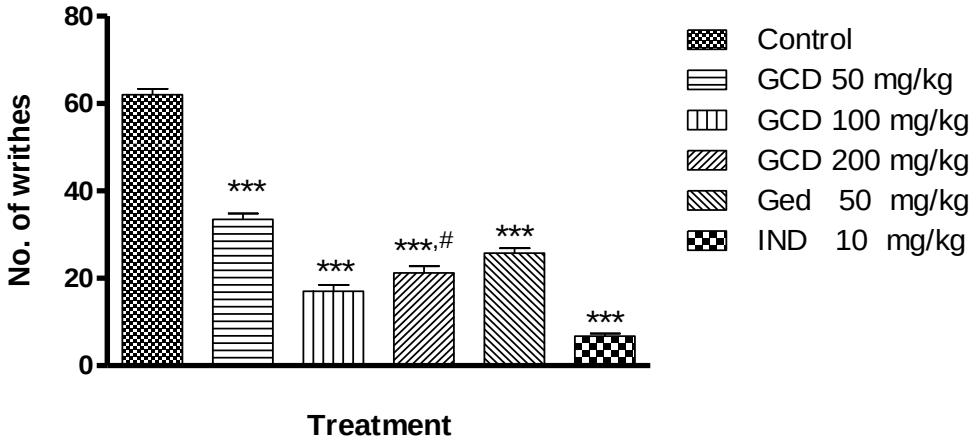

Figure 1:
Gedunin-cyclodextrin inclusion complex attenuates acetic acid–induced writhing in mice. The results are expressed as Mean ± SEM (n=5). ***$p < 0.001$ vs control, #$p < 0.05$ vs Ged 50 mg/kg. GCD: gedunin – cyclodextrin; Ged: gedunin; IND: Indomethacin.

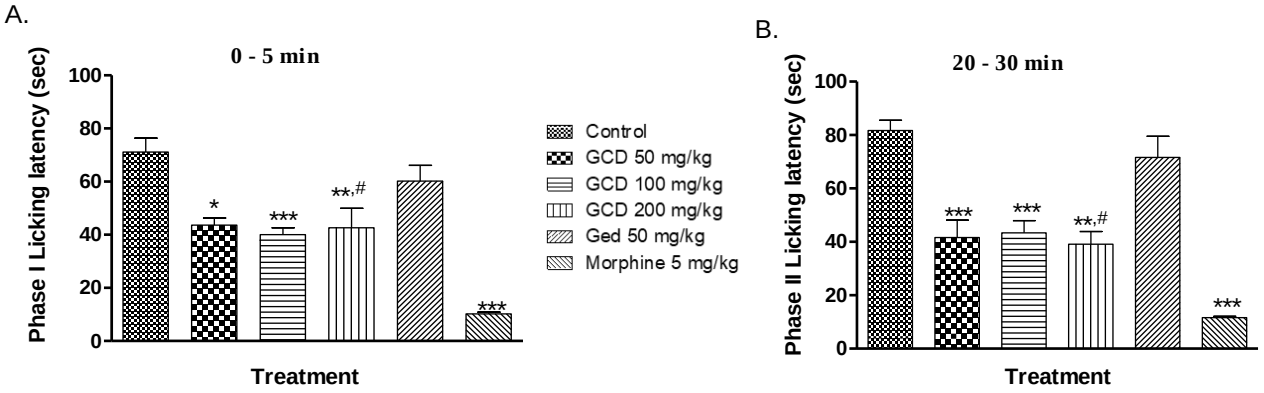

Figure 2:
Gedunin-cyclodextrin inclusion complex inhibits formalin-induced nociception in mice (A) Phase 1 and (B) Phase II. The results are expressed as Mean ± SEM (n=5). *$p < 0.05$ vs control, #$p < 0.05$ vs GCD 200 mg/kg. GCD: gedunin – cyclodextrin; Ged: gedunin.

GCD inhibited nociceptive reaction significantly ($p < 0.05$) compared to control in both phases of the formalin test while the effect of gedunin (50 mg/kg) was comparable to control. The inhibition of nociceptive reaction due to GCD at 200 mg/kg was significantly ($p < 0.05$) higher than that of gedunin at 50 mg/kg in both phases. In the first phase (neurogenic pain), GCD produced 38.7%, 43.7% and 40.1% inhibition at 50, 100 and 200 mg/kg respectively. However, these values are significantly ($p < 0.05$) less than 85.7% pain inhibition produced by morphine (Figure 2a). In the second phase (inflammatory pain), GCD 200 mg/kg decreased biting and licking responses by 52.1%; this percentage reduction was highest when compared with Ged 50 mg/kg and GCD 50 and 100 mg/kg but it was lower than 85.8% inhibition of pain by morphine (Figure 2b).

Table 1:
Effect of gedunin-cyclodextrin inclusion complex, gedunin and morphine on reaction time in tail immersion test in mice

		Reaction time (secs)			
Group	**Dose (mg/kg)**	**0 min**	**60 min**	**90 min**	**120 min**
Control	10 ml/kg	0.60 ±0.04	0.61 ±0.06	0.61 ±0.06	0.63 ±0.04
GCD	50	0.61 ±0.08	0.86 ±0.15*	0.83 ±0.05	0.71 ±0.04
	100	0.61 ±0.04	0.72 ±0.04*	0.66 ±0.08	0.93 ±0.26
	200	0.67 ±0.04	0.71 ±0.10*	0.72 ±0.05	0.74 ±0.04
Ged	50	0.57 ±0.03	0.67 ±0.05	1.06 ±0.13	0.77 ±0.09
Morphine	5	0.71 ±0.07*	3.00 ±0.22*	3.01 ±0.12*	2.37 ±0.15*

*The results are expressed as Mean ± SEM (n=5), *p < 0.05 vs control.*
GCD: gedunin – cyclodextrin; Ged: gedunin

Table 2:
Effect of gedunin-cyclodextrin inclusion complex, gedunin and morphine on reaction time in hot plate test in mice

		Reaction time (secs)			
Group	**Dose (mg/kg)**	**0 min**	**60 min**	**90 min**	**120 min**
Control	10 ml/kg	4.40 ±0.61	4.43 ±0.35	4.37 ±0.85	3.47 ±0.33
GCD	50	5.24 ±0.85	4.52 ±0.57	4.86 ±0.58	4.06 ±0.37
	100	5.64 ±0.46	4.52 ±0.92	3.72 ±0.33	4.08 ±0.33
	200	3.92 ±0.39	5.04 ±0.34	5.58 ±0.84	6.20 ±0.79
Ged	50	5.45 ±0.24	5.53 ±1.01	4.78 ±0.46	5.80 ±0.59
Morphine	5	5.33 ±0.73*	13.23 ±0.97*	12.60 ±0.93*	10.90 ±1.15*

*The results are expressed as Mean ± SEM (n=5). *p < 0.05 vs control*
GCD: gedunin – cyclodextrin; Ged: gedunin

The oral administration of GCD (50, 100 and 200 mg/kg) to mice, in the tail immersion test, caused analgesia by significant ($p < 0.05$) increase in reaction time to thermal stimulus of hot water relative to the control 60 min post-administration (Table 1). However, Morphine (5 mg/kg) induced significant ($p < 0.05$) analgesia relative to the control at 60, 90 and 120 min post-drug administration.
In the hot plate test, GCD 200 mg/kg and Ged 50 mg/kg increased the reaction time to pain while the lower doses of GCD showed no analgesic effect compared to controls. Morphine showed an increase in reaction time that was significant ($p < 0.05$) with respect to the controls (Table 2).

Effect of Ged and GCD on inflammation in carrageenan-induced rat paw edema: The administration of GCD (50 - 200 mg/kg) led to significant ($p < 0.05$) suppression of oedema three hours post carrageenan injection (Figure 3). A significant dose-dependent increase in GCD effect; 40.0%, 61.6% and 68.8% at 50, 100 and 200 mg/kg respectively, was observed five hours post carrageenan injection. Gedunin (50 mg/kg) produced a lower oedema suppression of 60.0% than GCD (200 mg/kg), although these values were not significantly different. Indomethacin exhibited a significant ($p < 0.05$) suppression of oedema by 61.0% and 64.0% at the third and fifth-hour post carrageenan injection respectively.

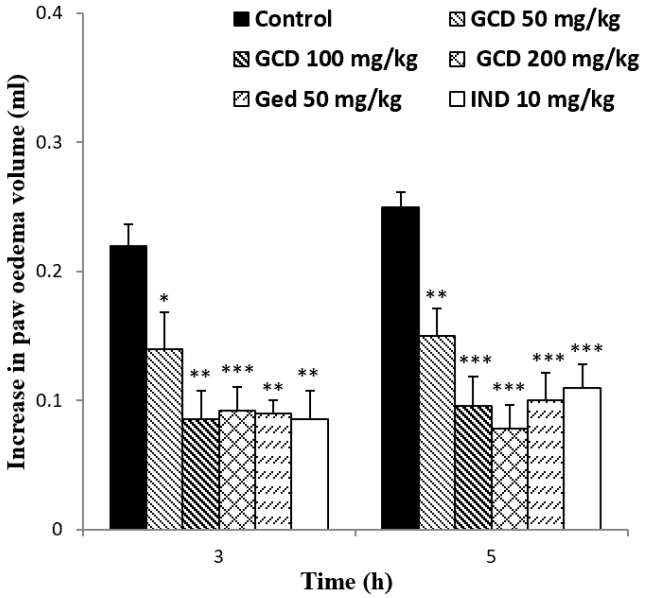

Figure 3:
Gedunin-cyclodextrin inclusion complex reduces carragenaan-induced rat paw oedema. The results are expressed as Mean ± SEM (n=5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs carrageenan control group.

GCD decreased cellular migration and exudate formation in carrageenan induced air pouch: The volume of the air pouch exudate was significantly ($p < 0.05$) reduced in GCD and gedunin-treated animals compared to the carrageenan control group (Figure 4). The effect of GCD (200 mg/kg) on exudate volume was significantly ($p<0.05$) higher compared to that of Ged (50 mg/kg). Indomethacin caused a significant ($p < 0.05$) decrease exudate formation.

The total number of leucocytes in the carrageenan control air pouch exudates was significantly ($p < 0.05$) increased by about 80% of that of the saline control (Figure 5). Pre-treatment with GCD (50 mg/kg) reduced the leucocyte count by 55% while 100 mg/kg and 200 mg/kg lowered it by 51% and 43% respectively ($p < 0.05$). Gedunin (50 mg/kg) reduced the migration of leucocytes by 16%.

The activity of GCD at the three doses was comparable to Indomethacin reduction of 41%.

Suppression of myeloperoxidase activity by GCD: Infiltration of neutrophil to acute inflammatory sites is induced by carrageenan thus neutrophil activation was evaluated by the myeloperoxidase (MPO) enzyme activity in the pouch tissue lining. There was a significant ($p < 0.05$) reduction of 25.3%, 55.8%, 57.4% and 49.5% in MPO activity in the pouch lining of animals treated with GCD 50, 100 and 200 mg/kg and indomethacin respectively. The activity of MPO activity was not reduced by gedunin compared to carrageenan control. GCD 200 mg/kg caused a significant ($p < 0.05$) reduction in MPO activity compared to Ged 50 mg/kg (Figure 6).

Nitrite and tumor necrosis factor-α levels reduction in pouch exudate by GCD: The effect of GCD, Ged and Indomethacin on release of markers of inflammation is shown in Figures 7A and B. The concentration of TNF- α was lower in pouch exudates of Ged and GCD pre-treated animals. GCD (50, 100 and 200 mg/kg) showed a significant ($p < 0.05$) TNF-α inhibition by 33.7%, 27.3% and 49.9% respectively compared to carrageenan control (Figure 7A); Ged 50 mg/kg inhibited TNF-α by 25.4%. There was a significant difference ($p<0.05$) between the effect of GCD 200 mg/kg and Ged 50 mg/kg on TNF-α concentration.
Pre-treatment with GCD reduced Nitrite level (Figure 8B). GCD 50, 100, 200 mg/kg and Ged 50 mg/kg produced a significant ($p < 0.05$) reduction in nitrite values by 67.4%, 71.3%, 70% and 86.1% respectively compared to carragenaan control. However, the higher reduction in nitrite brought about by Ged was not statistically significant with respect to GCD 200 mg/kg. Indomethacin (5 mg/kg) decreased nitrite (64%) and TNF- α (24.5%) levels in the exudates.

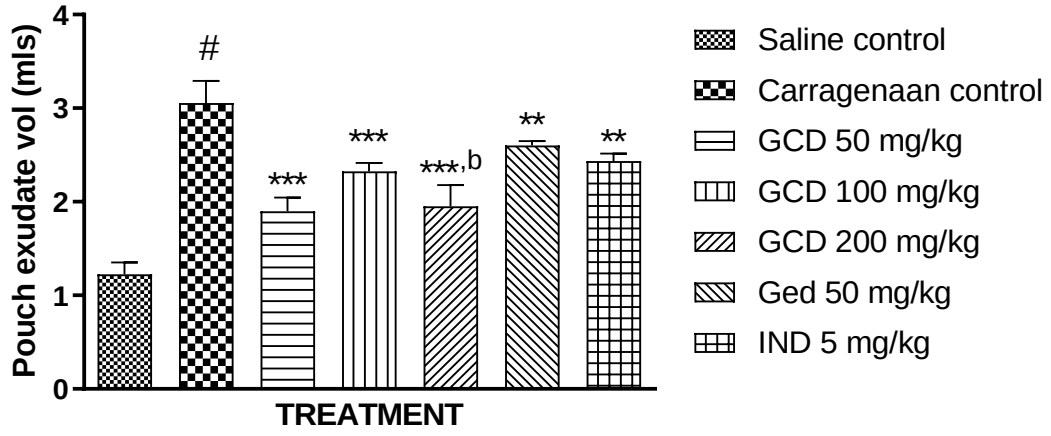

Figure 4:
Gedunin-cyclodextrin inclusion complex reduces exudate volume in carragenaan-induced air pouch model in rats. The results are expressed as Mean ± SEM (n=5). #$p<0.001$ vs saline control, **$p < 0.01$, ***$p < 0.001$ vs carrageenan control group, $^b p < 0.05$ vs Ged 50 mg/kg.

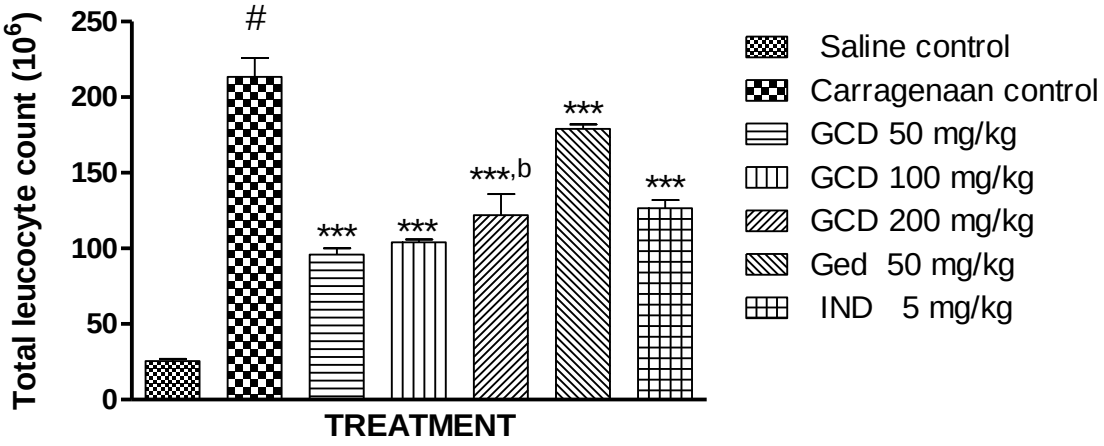

Figure 5:
Gedunin-cyclodextrin inclusion complex reduces total leucocyte count in carrageenan-induced air pouch cellular migration in rats. The results are expressed as Mean ± SEM (n=5). #$p < 0.001$ vs normal saline control, ***$p < 0.001$ vs carrageenan control, $^b p< 0.001$ vs Ged 200 mg/kg.

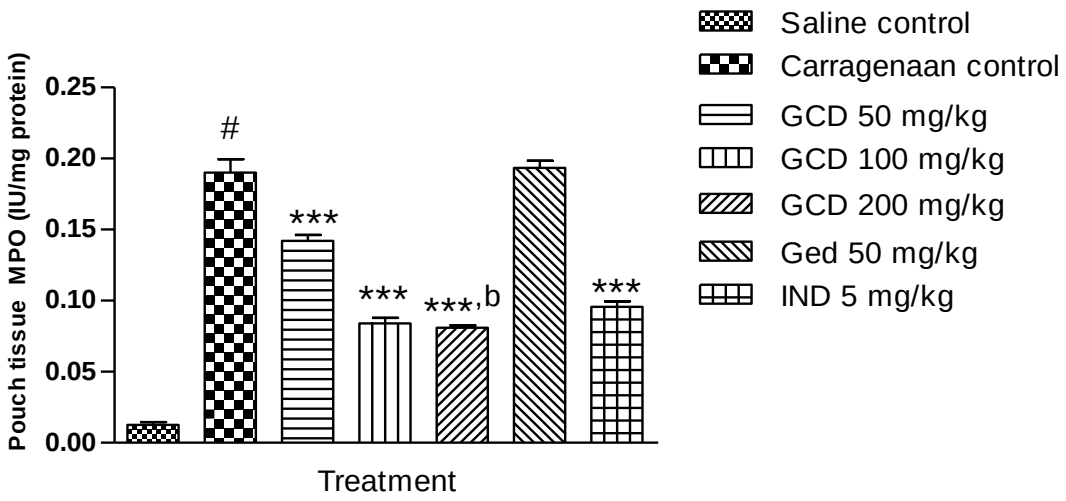

Figure 6:
Gedunin-cyclodextrin inclusion complex attenuates myeloperoxidase activity in carrageenan-induced air pouch in rats. The results are expressed as Mean ± SEM (n=5). $^{\#}p < 0.001$ vs normal saline control. $^{*}p < 0.001$ vs carrageenan control, $^{b}p < 0.05$ vs Ged 200 mg/kg

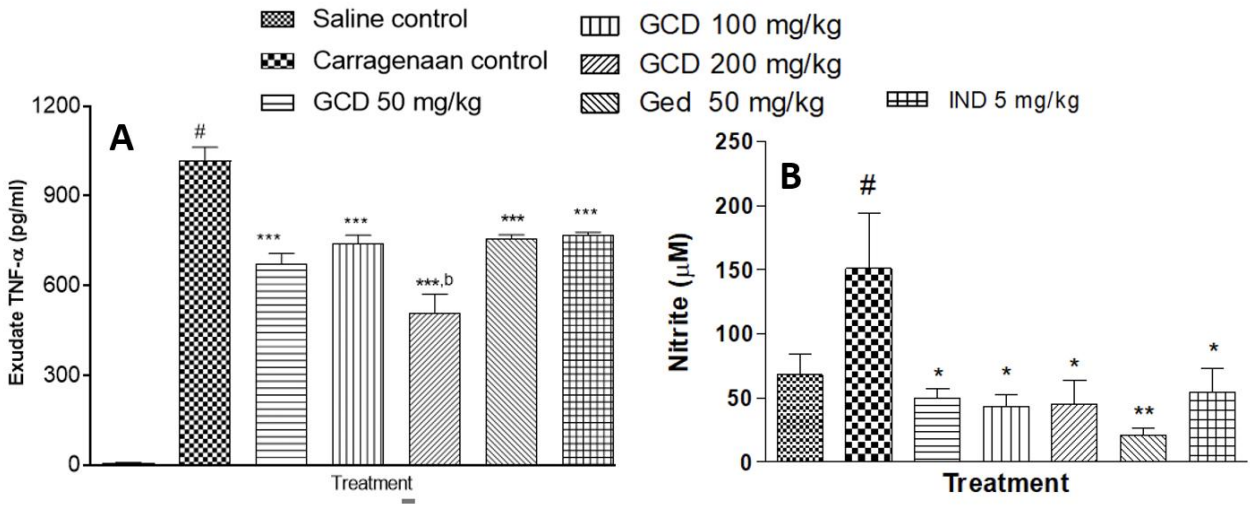

Figure 7:
Gedunin-cyclodextrin inclusion complex inhibits carrageenan-induced release of on the release of (A) tumor necrosis factor-α and (B) Nitrite concentration in rats. The results are expressed as Mean ± SEM (n=5). #$p < 0.001$ vs saline control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs carrageenan control group, $^{b}p < 0.05$ vs Ged 50 mg/kg.

Table 3:
Effect of GCD on the level of reduced glutathione (GSH) and malondialdehyde levels in pouch exudate

Experimental groups	GSH (μM)	TBARS (μM of MDA/mg protein)
Saline control	4.39±0.17	229.60±37.14
Carrageenan control	3.45±0.21$^{\#}$	412.00±9.15$^{\#}$
GCD 50 (mg/kg)	5.62±0.21***	288.90±13.90**
GCD 100 (mg/kg)	6.35±0.17***	205.70±14.18***
GCD 200 (mg/kg)	5.48±0.31^{***,b}	246.00±11.18^{***,b}
Ged 50 (mg/kg)	4.24±0.35*	346.10±6.58*
IND 5 (mg/kg)	6.17±0.16***	349.70±13.18*

*The results are expressed as Mean ± SEM (n=5). $^{\#}p < 0.05$ vs normal saline control. *p<0.05, ***p<0.001 vs carrageenan control, $^{b}p < 0.01$ vs Ged 50 mg/kg. GCD: gedunin – cyclodextrin; Ged: gedunin, IND: Indomethacin*

GCD altered carrageenan-induced glutathione depletion and lipid peroxidation: Pre-treatment with GCD (50 – 200 mg/kg) and indomethacin reversed the depletion of GSH observed in the carragenaan-injected pouch (Table 3). The reversal effect of Ged 50 mg/kg on GSH depletion was less ($p < 0.05$) compared to GCD 200 mg/kg.

The level of thiobarbituric acid reacting species (TBARS) was raised in carragenaan-injected pouches compared to saline group (Table 3) but administration of GCD, Ged and indomethacin reduced lipid peroxidation ($p < 0.05$) compared to controls but reduction was higher in GCD treated groups. GCD 200 mg/kg achieved a higher and significant ($p < 0.05$) reduction compared to Ged 50 mg/kg.

Histological changes in the air pouch tissue lining : The infiltration (severe, diffuse) of inflammatory cells was

observed in the pouch wall, and the tissue oedema contributed to the dispersed connective tissue fibers observed in the vehicle-treated carrageenaan animal (Figure 8). The GCD-treated air pouch, on the other hand, presented signs of very mild to moderate acute inflammatory response. Ged (50 mg/kg) air pouch revealed considerable cellular infiltration. These findings imply that pre-treatment with GCD suppressed the acute inflammatory response in the pouch wall better than Ged alone.

DISCUSSION

The antinociceptive and anti-inflammatory properties of gedunin were improved by complexation with 2-hydroxypropyl-β-cyclodextrin (HBD) in this study.

Acetic acid-induced writhing test is a common inflammatory pain model which has high sensitivity and low specificity (Braggio *et al*., 2002). The intraperitoneal administration of acetic acid causes local irritation which indirectly triggers the release of nociceptive endogenous mediators (e.g. bradykinin, serotonin, and prostaglandin) and proinflammatory cytokines (e.g. TNF-α, IL-1β and IL-8); chemosensitive nociceptors which contribute to the development of inflammatory pain are activated by these mediators (Manjavachi *et al*., 2010). Acetic acid-induced writhing can be inhibited by peripherally acting drugs like indomethacin, a non-steroidal anti-inflammatory drug because the test identifies peripheral analgesics. The antinociceptive action of GCD (50–200 mg/kg), Ged 50 mg/kg and indomethacin in this test was probably due to inhibition of release and/or activity of inflammatory mediators. No particular mediator was targeted, however, the test agents act more on the peripheral pain induced in the peritoneal cavity.

The formalin test was performed to determine whether GCD and/or Ged suppressed pain centrally or peripherally. The formalin test is more precise and has a biphasic response. The first phase is the neurogenic nociceptive response which occurs during the first five minutes after formalin injection, while the second phase is the inflammatory nociceptive response which occurs between twenty and thirty minutes post formalin injection (Ribeiro *et al*., 2010). Centrally acting analgesics can inhibit both phases, peripherally acting analgesics inhibit the second phase only (Liao *et al*., 2012). GCD (50 - 200 mg/kg) and morphine (5 mg/kg) decreased the nociceptive response significantly ($p< 0.05$) in both phases induced by formalin but Ged (50 mg/kg) did not diminish the response in any of the phases. GCD may have central and peripheral antinociceptive action because it inhibited nociception in both phases. This confirms the possibility of GCD's anti-inflammatory property as observed in the acetic acid–induced writhing. The greater nociceptive inhibition by GCD in the acetic acid-induced writhing and formalin tests is possibly due to the improved bioavailability of the orally administered GCD compared to Ged alone (free, uncomplexed gedunin). This enhanced effect may be attributed to the high aqueous solubility of HBD resulting in an improved availability of the hydrophobic gedunin molecules aiding them in reaching and/or remaining at their site of action. In a model of articular inflammation, gedunin suppressed and diminished hyperalgesia (Conte *et al*., 2015). This supports the effect of GCD in reducing inflammation-associated pain as seen in the antinociceptive tests results.

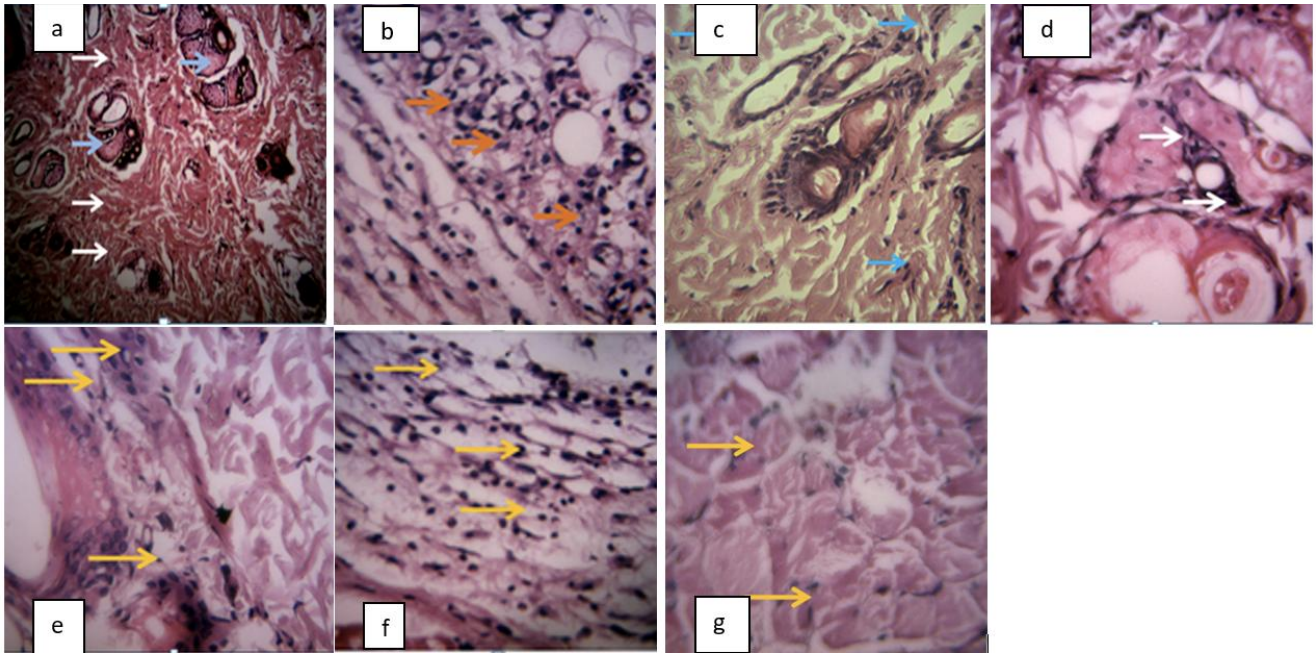

Plate 1
Gedunin-cyclodextrin inclusion complex attenuates carrageenan-induced pathohistological changes in the pouch lining of rats.**A**. saline control showing dermal connective tissue (white arrows) surrounding sebaceous glands (blue arrows). **B**. carrageenan control showing severe diffuse neutrophilic cellular infiltration of the dermis. The connective tissue fibers are dispersed. **C**. GCD 50 mg/kg group; mild/moderate cellular infiltration of the dermis is seen, the connective tissues are slightly dispersed. **D**. GCD 100 mg/kg; a very mild (periglandular) cellular infiltration with normal dense dermal connective tissue fibers. **E**. GCD 200 mg/kg; very mild dermal cellular infiltration, dermal connective tissues are slightly dispersed. **F**. Ged 50 mg/kg group with severe diffuse cellular infiltration by neutrophils. **G**. IND 5 mg/kg, very low infiltrative cell population. *H&E: Heamotoxylin-Eosin stain (magnification x400)*

Hot plate and tail immersion tests were performed for a further assessment of GCD and Ged central antinociceptive activity. The hot-plate method is a common and selective test for evaluating centrally-mediated analgesic action. Morphine increased the reaction time to pain in the hot plate and tail immersion tests, when compared with the control, while GCD 200 mg/kg and the equivalent dose of Ged 50 mg/kg prolonged the reaction time in hot plate test although the lower doses of GCD showed no analgesic effect. This implies that GCD and Ged may have central analgesic action that is not opioid mediated. The complexation of analgesic compounds with cyclodextrins can improve their efficacy (Brito *et al*., 2015). Cyclodextrin complexation enhanced the central analgesic property and half-life of (*R*)-(–)-linalool, a monoterpene alcohol (Quintans-Junior *et al*., 2013). pCM (p-cymene) and pCM-β-CD (the cyclodextrin complexed p-cymene) inhibited nociceptive behavior for 2 h and 8 h respectively in the acetic acid-writhing test (Quintans et al., 2013).. The findings of this study corroborate these reports.

Carrageenan-induced paw edema model is frequently used to evaluate the anti-edematogenic effect of natural products. The induced paw edema is a biphasic event; bradykinin, histamine, and 5-hydroxytryptamine (5-HT) are released in the first phase of edema (0-1 h) while cyclooxygenase-2 (COX-2), prostaglandins (PGs), TNF-*α*, IL-1*β* are produced min the second phase (1–6 h) (Ribeiro *et al*., 2010).. The increased production of PGs in the latter phase is through the activation of COX-2 and release of nitric oxide (NO). The inflammatory edema is highest at the third hour before it starts to reduce (Kirkova *et al*., 1992). In the carragenaan-induced rat paw edema test, GCD at all doses and Ged exerted significant edema inhibition comparable to indomethacin 3 hours post-carragenaan injection. There was a significant improvement in the anti-inflammatory activity of the complexed gedunin. This implies that anti-inflammatory activity of gedunin was still significantly preserved in the complexes and complexation with cyclodextrin can improve the edema inhibitory activity of gedunin. This observation correlates well with carragenaan rat paw edema test results of cyclodextrin inclusion complexes of some drugs with anti-inflammatory action. Free celecoxib and celecoxib-β-cyclodextrin achieved significantly higher inhibition of the inflammation induced by carrageenan compared to control group but the inclusion complex was more effective than celecoxib alone in edema inhibition (Sensoy *et al*., 2009). In another study, dexamethasone-β cyclodextrin complexation did not decrease the edema inhibitory and anti-inflammatory action of dexamethasone (Rodrigues *et al*., 2014).

The anti-inflammatory activity of GCD was examined further with the carragenaan air pouch model of inflammation. Carrageenan injection into an air pouch triggers an inflammatory reaction which is associated with cellular infiltration, increased exudate, and pronounced release of pro-inflammatory mediators like prostaglandins, leukotrienes, and cytokines. These markers can be quantified and used to determine degree of inflammation, resolution of inflammation or anti-inflammatory activity of drugs (Kim *et al*., 2006). The oral pre-treatment with GCD reduced exudate volume and number of leucocytes in the air pouch. The reduction achieved by GCD 200 mg/kg was significantly higher than the equivalent dose of Ged 50 mg/kg. This is an indication that the formation of the inclusion complex between gedunin and HBD improved the bioavailability of gedunin from the oral route.

The margination of neutrophils, an early cellular event in inflammation, can be measured using the neutrophil-specific enzyme myeloperoxidase (MPO) (Esmat *et al*., 2012). Increased tissue and plasma MPO levels are a marker of neutrophil proliferation and degranulation (Kothari *et al*., 2011) and intracellular MPO activity measurement can be used as a surrogate marker for the number of neutrophils contained in a biological sample (Pulli et al., 2013). Thus, the decreased MPO activity due to the administration of GCD was an indication that GCD inhibited neutrophil activation and infiltration induced by carrageenan. This was confirmed by histological examination of the pouch tissue. GCD (50 - 200 mg/kg) mitigated the inflammatory histopathological modifications.

The TBARS (an index of lipid peroxidation) was increased in carrageenan injected pouches, as compared to the normal saline injected pouches, as a result of released neutrophilic reactive oxygen species triggered by carragenaan. This agrees with previous reports of increased exudates and tissue LPO that was partly mediated via increase in ROS/RNS radical production in carrageenan injected animals (Tanas *et al*., 2010; Jain and Parmar, 2011). The end product of cell membrane decomposition, malondialdehyde (MDA), is an indicator of the inflammatory process (Chou et al., 2012). Also, lipid peroxidation is an oxidative injury marker in disease conditions. This was the basis for assessing glutathione (GSH), TBARS (measured as MDA), TNF-α and NO levels in the pouch exudate. Pretreatment with GCD 50, 100, 200 mg/kg and Ged 50 mg/kg inhibited the increased TBARS levels significantly. However, GCD 200 mg/kg brought about a significantly higher inhibition of TBARS increase than the equivalent free Ged 50 mg/kg. Injection of carragenaan triggers an increase in glutathione level. Glutathione reacts with free radicals to generate thiol radicals. This reaction depletes GSH level over time in animals exposed to inflammatory stress (Conner and Grisham, 1996). Administration of GCD and Ged 50 mg/kg blocked carragenaan-induced GSH depletion in the air pouch. However, GCD 200 mg/kg exerted a significantly higher effect than Ged 50 mg/kg.

In conclusion, Gedunin-2-hydroxypropyl-β-cyclodextrin complex demonstrated significant anti-nociceptive and anti-inflammatory activities relative to gedunin by inhibition of oxidative stress and inflammation in rodents. The improved pharmacological activities of the complex might partially be due to reduced first pass effect and increased bioavailability.

Acknowledgment

The authors appreciate the Staff Development Award from the University of Ilorin, Ilorin, Nigeria which led to the execution of part of the study.

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