



## Article

# Improving Sexual Dysfunction with Cinnamon Leaf Extract and Nanoemulsion by Using a Rat Model

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## Abstract

**Background/Objectives:** Taiwan cinnamon leaves have been reported to be effective in improving chronic diseases. Herein, cinnamon leaf extract (CLE) and nanoemulsion (CLEN) were prepared to explore their effects in improving sexual dysfunction in rats. **Methods:** Following extraction with 80% ethanol and analysis by UPLC-MS/MS, CLEN was prepared using an optimal ratio of soybean oil, lecithin, Tween 80, deionized water, and CLE. A total of 48 male rats and 48 female rats were used, with the former being induced with erectile dysfunction, followed by treatment with CLEN or CLE at two doses (100 mg/kg and 50 mg/kg) for 4 weeks. After conducting the penile reflex test, male rats were paired with female rats for measurement of sexual behavior and ICP/MAP. Following sacrifice,  $\alpha$ -SMA, nNOS, and  $\beta$ -III tubulin expression areas were measured by histochemical analyses; SMC/collagen ratio by Masson's trichrome staining; and NO, cGMP, and PDE5 levels by ELISA kits. **Results:** CLEN was more effective than CLE in increasing intromission frequency, decreasing intromission and ejaculation latency, and recovering erectile response for improving copulatory and ejaculatory performances. A higher maximum ICP/MAP ratio was shown for CLEN through elevation of neurovascular function and erectile capacity. Additionally, CLEN efficiently reduced fibrosis, enhanced neuronal marker expression, and increased the SMC/collagen ratio, leading to penile tissue protection and neural regeneration. Both treatments showed elevated levels of NO and cGMP with a reduction in PDE5, probably through modulation of the NO-cGMP signaling pathway. **Conclusions:** CLEN was more effective than CLE in restoring erectile function in rats. Some more clinical trials are needed to verify this finding.



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**Keywords:** cinnamon leaves; cinnamaldehyde; CLE; CLEN; sexual dysfunction; rat model

## 1. Introduction

Cinnamon, a traditional medicinal herb used worldwide for thousands of years, has been reported to possess important biological functions, including anti-inflammation, anti-cancer, anti-obesity, anti-cardiovascular disease, anti-diabetes, anti-Parkinson's disease, anti-Alzheimer's disease, anti-depression, anti-osteoarthritis, and anti-osteoporosis [1]. Of the various parts in cinnamon, bark is frequently used as raw material for the production

of essential oil, cinnamon powder, spice, and some other types of products [2]. However, cinnamon bark was found to contain a higher level of coumarin, a carcinogenic compound to the liver, than cinnamon leaves [3]. Furthermore, cinnamon leaves (*Cinnamomum osmophloeum* ct. cinnamaldehyde) grown in Taiwan were shown to have similar bioactive compounds compared with commercial cinnamon barks [4].

Currently, the major cinnamon products sold worldwide are dried powder of bark from different species such as Cassia cinnamon and Ceylon cinnamon, with the former mainly from species such as Indonesian Cassia (*Cinnamomum burmannii*), Chinese Cassia (*Cinnamomum cassia* J.S. Presl), and Saigon cassia (*Cinnamomum loureiroi* Nees), and the latter from Sri Lanka and southern India (*Cinnamomum verum* J.S. Presl) [2]. In addition, Cassia cinnamon was found to contain higher levels of coumarin than Ceylon cinnamon [2]. By comparison, Taiwan's indigenous cinnamon leaves with trace levels of coumarin should be a more optimal raw material for the production of functional food or botanic drugs than Cassia and Ceylon cinnamons [2,4]. Moreover, obtaining tree bark with a knife can be more time-consuming and less environmentally friendly. Thus, Taiwan's cinnamon leaves can be an optimal raw material for the production of cinnamon-derived products.

Sexual dysfunction, referring to the inability to achieve normal sexual intercourse, includes erectile dysfunction (ED), premature ejaculation, hypoactive sexual desire disorder, and orgasmic disorder [5]. Of these disorders, ED and premature ejaculation are the most common ones, with the former affecting 5–10% of men under 40 and 52% of men aged 40–70, while the latter is prevalent among 30% of men [6–8]. Most importantly, ED fails to achieve an erection sufficient for satisfactory vaginal intercourse, typically for a period of >6 months, with the probability increasing significantly with age [7,9]. It is estimated that at least 150 million men globally and 30–50 million men in the USA have ED, which is actually an underestimation due to reporting bias and cultural factors, as previously projected data estimates 322 million men can be affected by ED by 2025 [8,10]. Although age is the predominant factor for ED, several medical conditions, including diabetes, cardiovascular disease, obesity, depression/anxiety/stress, neurological disorder, low testosterone level, hypogonadism, prostatic hyperplasia, and kidney disease, as well as lifestyle habits such as smoking, alcohol consumption, and drug abuse, can also significantly contribute to ED occurrence [7,11,12]. The current drugs used for ED management, such as sildenafil, vardenafil, and tadalafil, can have side effects including headache, feeling sick, indigestion, and dizziness [11,13]. Therefore, it is pivotal to develop botanical products instead for treating ED. Many medicinal plants, particularly cinnamon, have been reported to possess great potential in the treatment of sexual dysfunction through boosting sexual desire, increasing penis blood flow, reducing the risk of sexual dysfunction, and improving fertility [14,15].

In an earlier study, Goswami et al. [16] reported that the *Cinnamomum cassia* methanol extract was effective in improving sexual dysfunction of young male Wistar rats through an increase in smooth muscle level and inhibition of arginase activity, which is important for arginine synthesis for subsequent NO production, thereby improving blood circulation and relaxing blood vessels for ED management. Likewise, the *Cinnamomum cassia* methanol extract was shown to improve ED through inhibition of Rho-kinase 2 (ROCK-II), a vital enzyme involved in vascular resistance (hypertension), cell motility, and cytoskeleton, as well as through relaxation of isolated rat corpus cavernosum smooth muscle [17]. In addition to arginase and ROCK-II, cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE5) inhibitor is also imperative in the treatment of ED, as it can inhibit the PDE5 enzyme, leading to a rise in cGMP level, blood vessel widening (vasodilation), an increase in penile blood flow, and relaxation of penile tissue smooth muscle (corpus cavernosum smooth muscle) [16]. While a few studies demonstrated the improvement of

ED in aged and diabetic rats by cinnamon bark extracts [18–21], the effects of cinnamon leaf extract and nanoemulsion on improving ED in rats remain unexplored. Thus, this is the first study dealing with the effects of cinnamon leaf extract and nanoemulsion on improving sexual dysfunction in rats. Compared with cinnamon bark, in this study, we demonstrated that cinnamon leaves contained a higher level of CA and a lower level of coumarin, and thus cinnamon leaves can be a much safer raw material for functional food development and a possible botanical drug candidate in the future.

Nanotechnology, defined as the technology for the production and application of nanomaterials and nanoproducts with a size from 1 to 100 nm, can result in a substantial modification of the chemical and physical properties of a material for improvement in bioavailability and biological activity in vivo [22]. Among the various nanosystems, nanoemulsion is one of the most frequently used techniques because of ease in preparation and capability in encapsulating unstable bioactive compounds through utilization of oil, water, surfactant, and cosolvent/cosurfactant in an optimal ratio for formation of a transparent or semi-transparent dispersion, with both oil-in-water (o/w) and water-in-oil (w/o) systems being frequently prepared [23].

In our previous studies Wang et al. [22] prepared a nanoemulsion containing cinnamon leaf extract, soybean oil, Tween 80, lecithin and deionized water in an appropriate proportion with a mean particle size being '30.1' nm, the Parkinson's disease in rats was improved substantially following administration with this nanoemulsion at a dose of 60 mg/kg bw, as evident by a pronounced rise in the levels of tyrosine hydroxylase and dopamine in the striatum, and antioxidant enzymes including catalase, glutathione peroxidase and superoxide dismutase in the midbrain, as well as a distinct decline in levels of  $\alpha$ -synuclein in the striatum and malondialdehyde in the midbrain. Similarly, Huang and Chen [4] prepared a nanoemulsion with a similar composition with a mean particle size of 36.58 nm and reported a substantial improvement in type II diabetic rats following intake of this nanoemulsion at a dose of 60 mg/kg bw, through a reduction in fasting blood glucose, oral glucose tolerance test value, serum insulin, and insulin resistance index. Additionally, the contents of serum triglyceride, cholesterol, creatinine, uric acid, urea nitrogen, alanine aminotransferase, and aspartate aminotransferase were decreased, demonstrating a possible improvement of cardiovascular, kidney, and liver functions after consumption of cinnamon leaves-derived nanoemulsions. Apparently, the encapsulation of bioactive compounds from cinnamon leaves in a nanoemulsion system can elevate stability and bioavailability in vivo, thereby achieving a higher therapeutic efficiency. Specifically, the major bioactive compounds in cinnamon leaf extract include cinnamaldehyde (CA), cinnamic acid, eugenol, and cinnamyl alcohol, all of which exhibit antioxidant, anti-dementia, and anti-inflammation activities as mentioned above [4,22]. Therefore, it is feasible to develop a similar nanoemulsion system derived from cinnamon leaves for the possible treatment of sexual dysfunction.

For some other types of nanoemulsions prepared from natural plants and shown to improve sexual dysfunction, El-Shimi et al. [24] developed a Panax ginseng extract nanoemulsion, which was found efficient in improving male infertility defects by secreting more male sex hormones, decreasing oxidative stress, and restoring the structural organization of the testes in rats. In another study dealing with the neuroprotective effect of docosahexaenoic acid (DHA) nanoemulsion on erectile function in a rat model of bilateral cavernous nerve injury, Liao et al. [25] reported that the DHA nanoemulsion was effective in elevating maximum intracavernous pressure (ICP), ratio of area of expression of neuronal nitric oxide synthase (nNOS)/ $\beta$ -III tubulin, numbers of axon in the dorsal penile nerve and smooth muscle cell content in the corpus cavernosum, as well as smooth muscle cell/collagen ratio. Obviously, the improvement in male infertility and sexual

dysfunction can be attributed to the presence of major bioactive compounds, particularly ginsenosides, also known as ginseng saponins, in *Panax ginseng* extract and nanoemulsion, as well as DHA in DHA nanoemulsion, as both ginsenosides and DHA have been demonstrated to possess antioxidant, anti-inflammatory, immunomodulatory, and neuroprotective activities [26,27].

The objectives of this study were to prepare cinnamon leaf extract (CLE) and nanoemulsion (CLEN) for comparison in improving sexual dysfunction using a rat model. The success of this study can form a basis for possible future development into a functional food or botanical drug with Taiwan's cinnamon leaves as raw material for the treatment of sexual dysfunction with minimal side effects.

## 2. Results and Discussion

### 2.1. Analysis of Cinnamon Leaf Powder by UPLC-MS/MS

The quantitative data of CA and the other bioactive compounds in the cinnamon leaf powder were identified and quantified according to the methods of Huang and Chen [4] and Wang et al. [22] (Table 1 and Figure 1), with CA showing the highest content (16,060.51 µg/g), followed by *trans*-cinnamic acid (278.11 µg/g), eugenol (152.81 µg/g), cinnamyl alcohol (53.52 µg/g), kaempferol 3-β-D-glucopyranoside (19.25 µg/g), benzoic acid (9.88 µg/g), coumarin (3.74 µg/g), kaempferol (2.38 µg/g), quercetin-3-O-galactoside plus quercetin-3-O-glucoside (1.36 µg/g), rutin (0.72 µg/g), caffeic acid (0.53 µg/g), quercetin (0.50 µg/g), 5-O-caffeoylquinic acid (0.19 µg/g), and *p*-coumaric acid (0.09 µg/g). It is worth mentioning that the overlap of quercetin-3-O-galactoside and quercetin-3-O-glucoside peaks shown in Figure 1 is mainly method-dependent, and their quantification as a combined value is due to chromatographic conditions such as column type, mobile phase, flow rate, and detection mode instead of analytical limitations.

**Table 1.** Identification and quantification data of bioactive compounds in cinnamon leaf powder by UPLC-MS/MS.

| Peak No.       | Compound <sup>a</sup>                                | Retention Time<br>(min) | MS/MS ( <i>m/z</i> ) |             | Content<br>(µg/g) |
|----------------|------------------------------------------------------|-------------------------|----------------------|-------------|-------------------|
|                |                                                      | Sample                  | Precursor Ion        | Product Ion |                   |
| 1              | Quercetin                                            | 7.18                    | 301                  | 151         | 0.50              |
| 2              | Coumarin                                             | 5.66                    | 147                  | 91          | 3.74              |
| 3 <sup>b</sup> | Quercetin-3-O-galactoside<br>Quercetin-3-O-glucoside | 7.34                    | 463                  | 300         | 1.36              |
| 4              | Rutin                                                | 7.26                    | 609                  | 300         | 0.72              |
| 5              | Caffeic acid                                         | 4.25                    | 179                  | 134         | 0.53              |
| 6              | Benzoic acid                                         | 6.64                    | 121                  | 77          | 9.88              |
| 7              | 5-O-Caffeoylquinic acid                              | 2.13                    | 353                  | 179         | 0.19              |
| 8              | <i>trans</i> -Cinnamic acid                          | 9.15                    | 147                  | 103         | 278.11            |
| 9              | Cinnamaldehyde                                       | 8.45                    | 132                  | 55          | 16,060.51         |
| 10             | Kaempferol                                           | 8.42                    | 287                  | 153         | 2.38              |
| 11             | Eugenol                                              | 10.82                   | 165                  | 137         | 152.81            |
| 12             | Kaempferol 3-β-D-glucopyranoside                     | 8.40                    | 447                  | 284         | 19.25             |
| 13             | <i>p</i> -Coumaric acid                              | 5.62                    | 164                  | 90          | 0.09              |
| 14             | Cinnamyl alcohol                                     | 8.25                    | 117                  | 115         | 53.52             |

<sup>a</sup> Peaks were positively identified by comparison of retention time and mass spectra of unknown peaks with standards. <sup>b</sup> Both peaks were overlapped with the content being 1.36 µg/g for quercetin-3-O-galactoside plus quercetin-3-O-glucoside.

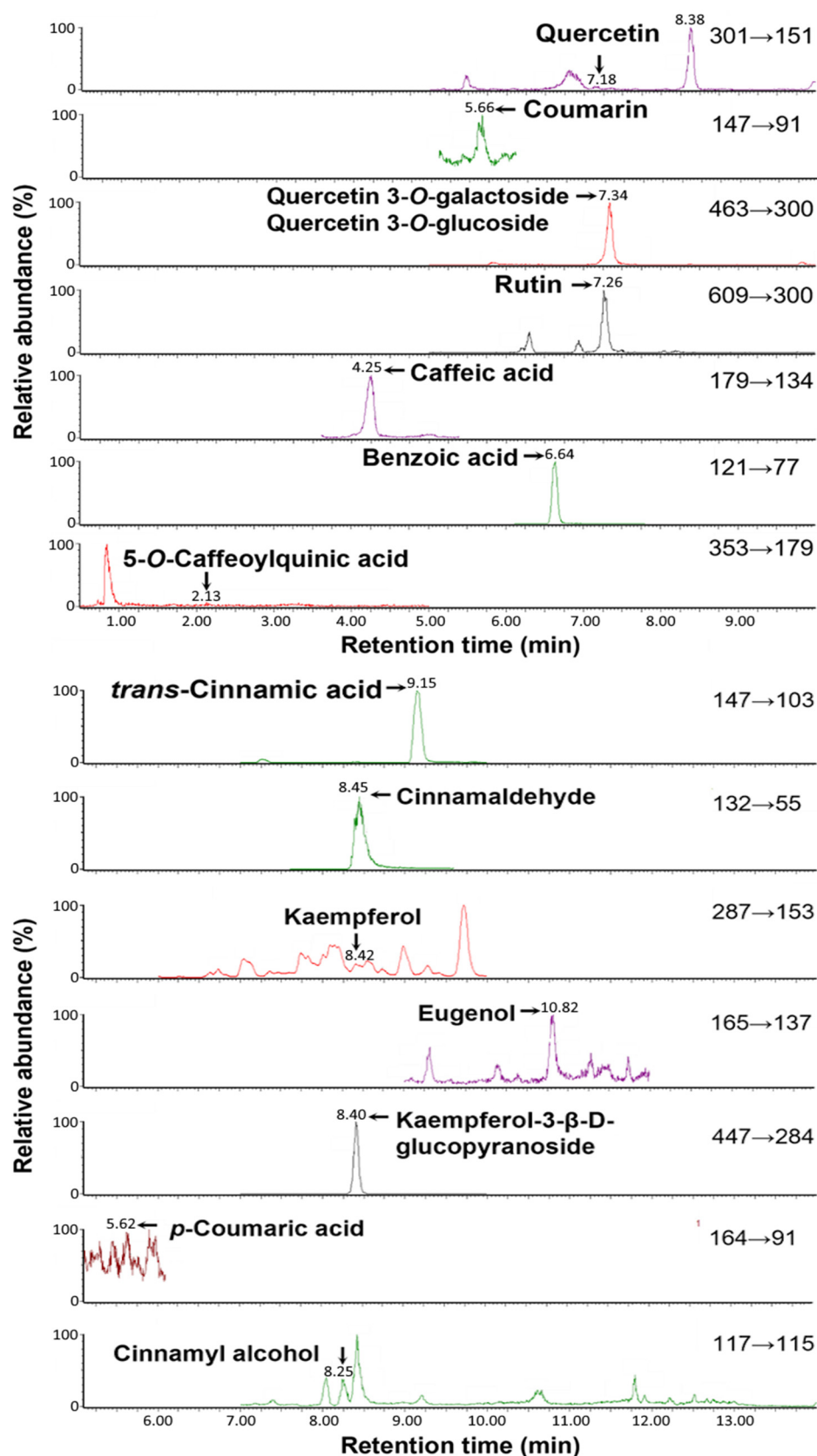


**Figure 1.** Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) chromatogram of various bioactive compounds in cinnamon leaves detected by multiple reaction monitoring (MRM) mode.

A similar result was reported by Wang et al. [22], studying the effect of cinnamon leaf extract and nanoemulsion on improving Parkinson's disease using a rat model, with CA

being present at the highest level, followed by trans-cinnamic acid and eugenol. The presence of phytochemicals in cinnamon leaf powder may be responsible for the prevention or improvement of various chronic diseases such as cardiovascular impairment, neurological dysfunction, diabetes, and cancer [1].

As noted above, the method validation data for this UPLC-MS/MS method have been reported in our previous study [4]. In brief, the relative standard deviation (RSD) value of the intra-day and inter-day variability for precision ranged from 1.93% to 8.21% and from 1.81% to 8.93%, respectively, while the recovery data (accuracy) ranged from 90.21% to 107.30% with the RSD ranging from 0.03% to 9.63%, demonstrating a high reliability of this method. Furthermore, both peaks of quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside were overlapped with a level of 1.36 µg/g, accounting for only 0.008% of the total amount of the 15 bioactive compounds (16,583.59 µg/g), which should not impact the interpretations and results of this study, as the dominant compounds such as CA, cinnamyl alcohol, and cinnamic acid should play a more important role in improving sexual dysfunctions in male rats.

## 2.2. Characteristics Analysis of CLEN

The characteristics data of CLEN are shown in Figure 2A–E and Table 2, with the average particle size, PDI, and zeta potential being 17.0 nm, 0.230, and −30.6 mV, respectively, based on DLS analysis (Figure 2C). A low PDI of 0.230 also implies a uniform distribution of nanoparticles in CLEN, as a PDI value should be controlled at <0.3 [4,22]. A zeta potential of −30.6 mV also indicates a high stability of CLEN, as it should be controlled at >+30 mV or <−30 mV to promote electric repulsion for prevention of nanoparticle aggregation during storage of nanoemulsion [28,29]. The TEM image shows well-dispersed nanoparticle distribution (Figure 2D), with a mean particle size of 18.67 nm (Figure 2E), which is similar to that obtained by DLS. For storage stability of CLEN, Table 2 shows a slight change in mean particle size, PDI, and zeta potential over a 12-week storage period at 4 °C as evident by a range of mean particle size, zeta potential, and PDI from 17.0 to 23.5 nm, −30.3~−35.7 mV, and 0.122–0.241, respectively, revealing a high stability of the CLEN prepared in the present study. Additionally, the encapsulation efficiency of CA in CLEN was determined to be 91.6%, which is similar to that (92.9%) reported by Chen et al. [30], using a similar composition for CLEN preparation. This outcome further demonstrated a high stability of CA in CLEN during animal experiments.

**Table 2.** The changes in mean particle size, zeta potential, and polydispersity index (PDI) over a 12-week storage period at 4 °C.

| Week | Mean Particle Size (nm) <sup>a,b</sup> | Zeta Potential (mV) <sup>a,b</sup> | Polydispersity Index (PDI) <sup>a,b</sup> |
|------|----------------------------------------|------------------------------------|-------------------------------------------|
| 0    | 17.0 ± 0.6 <sup>B</sup>                | −30.6 ± 0.9 <sup>A</sup>           | 0.230 ± 0.092 <sup>A</sup>                |
| 2    | 19.3 ± 2.0 <sup>A</sup>                | −31.5 ± 1.2 <sup>A</sup>           | 0.218 ± 0.075 <sup>A</sup>                |
| 4    | 17.8 ± 1.3 <sup>B</sup>                | −33.4 ± 1.8 <sup>A</sup>           | 0.198 ± 0.034 <sup>A</sup>                |
| 6    | 21.5 ± 2.2 <sup>A</sup>                | −30.3 ± 0.5 <sup>A</sup>           | 0.122 ± 0.018 <sup>A</sup>                |
| 8    | 20.9 ± 1.8 <sup>A</sup>                | −35.7 ± 2.1 <sup>A</sup>           | 0.215 ± 0.023 <sup>A</sup>                |
| 10   | 23.5 ± 3.1 <sup>A</sup>                | −32.9 ± 1.2 <sup>A</sup>           | 0.227 ± 0.019 <sup>A</sup>                |
| 12   | 22.8 ± 2.3 <sup>A</sup>                | −31.6 ± 0.9 <sup>A</sup>           | 0.241 ± 0.095 <sup>A</sup>                |

<sup>a</sup> Data shown as mean ± standard deviation (*n* = 3). <sup>b</sup> Data with different capital letters (A,B) in a column are significantly different at *p* < 0.05.

Upon incubation with simulated gastric fluid, the CLEN showed only a slight variation in mean particle size (Figure 3A), while it varied significantly, ranging from 29.1 to 33.2 nm in the simulated intestinal fluid at different incubation times. Conversely, the changes in zeta potential were significant under both gastric and intestinal conditions, with the values

ranging from  $-30.8$  to  $-34.2$  mV and from  $-38.1$  to  $-40.8$  mV, respectively (Figure 3B). Furthermore, after incubating CLEN separately with gastric and intestinal fluids for 2 h, the encapsulation efficiency of CA in CLEN declined to 88.4% and 82.4%, accounting for a CA loss of only 3.5% and 10%, respectively. Thus, the above variations in physical and chemical stability should be minimal, implying a high stability of CLEN under both storage and gastrointestinal conditions.

The percentages of *in vitro* CA release from CLEN under simulated gastric and intestinal conditions are shown in Figure 3C. Following incubation of CLEN with simulated gastric fluid, a 24.9% release of CA was observed after 1 h incubation, with only a minor change thereafter until 4 h. On the contrary, a time-dependent rise in CA release was shown under intestinal condition, attaining a maximum of 53.7% after 4 h incubation. Compared to gastric fluid, the release of CA in intestinal fluid was significantly higher, revealing a high stability of CLEN under highly acidic stomach conditions, with a subsequent higher release in the intestine for absorption through intestinal epithelial cells. A similar outcome was shown by Amani et al. [31], who prepared a cinnamon essential oil nanoemulsion by a complex coacervation method using gelatin and tragacanth gum, reporting a cinnamon essential oil release of 34.91% and 42.63%, respectively, under simulated gastric and intestinal conditions. Thus, through encapsulation of unstable and poorly absorbed bioactive compounds into a nanoemulsion system, their stability could be greatly enhanced under *in vivo* conditions.

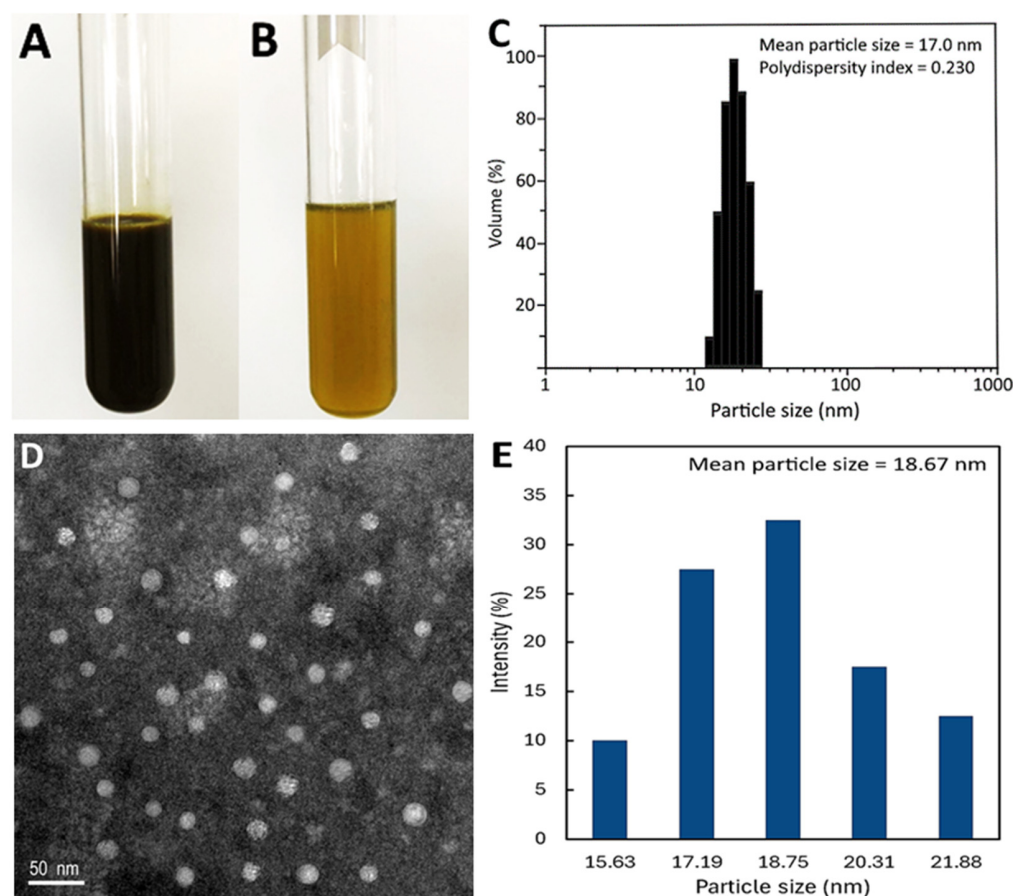


**Figure 2.** Appearance of cinnamon leaf extract nanoemulsion (A) and diluted cinnamon leaf extract nanoemulsion (B), particle size distribution and polydispersity index as determined by DLS (C), and TEM image of cinnamon leaf extract nanoemulsion (D) with particle size distribution histogram showing the mean particle size at 18.67 nm (E). CA, cinnamaldehyde; DLS, dynamic light scattering; TEM, transmission electron microscopy.

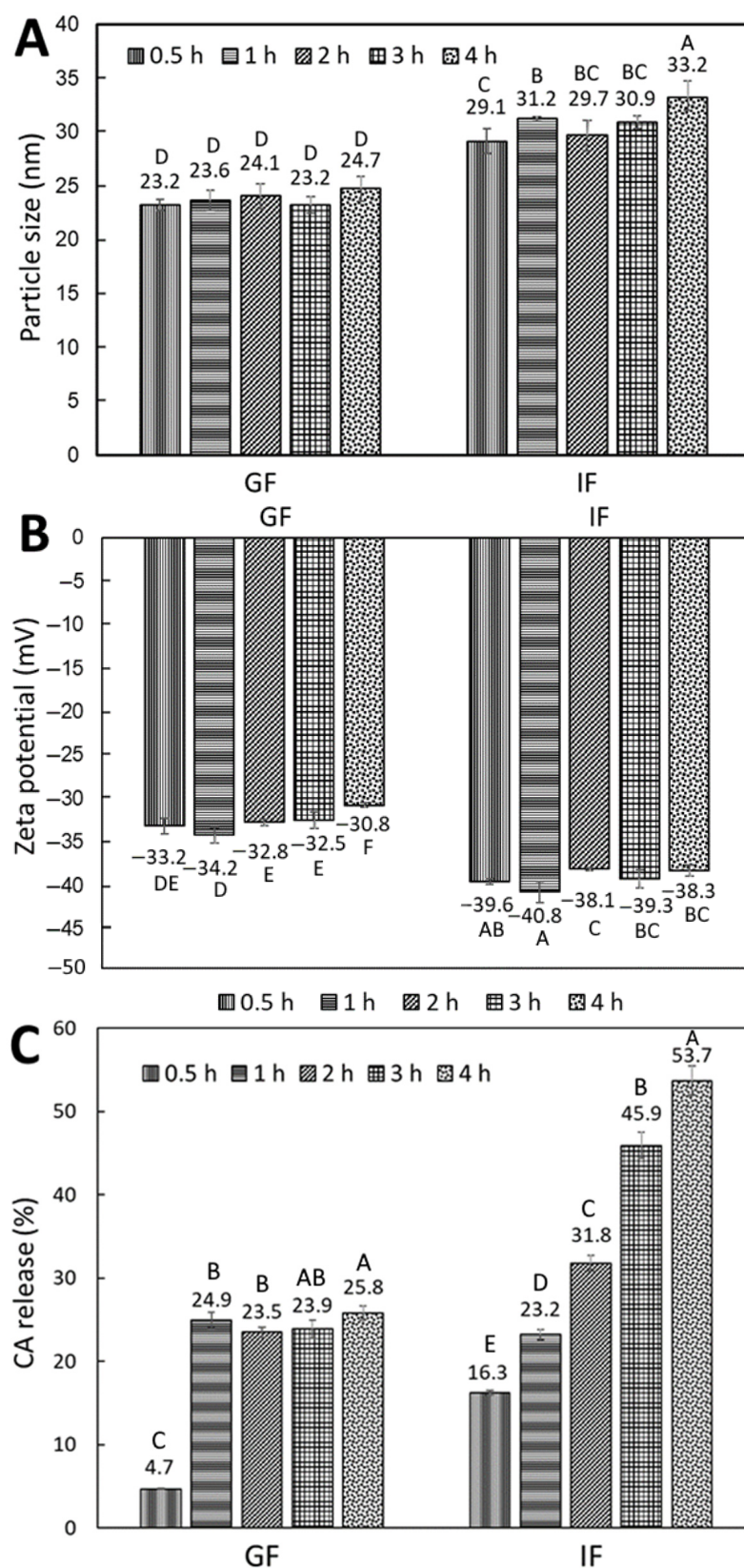


**Figure 3.** Changes in mean particle size (**A**) and zeta potential (**B**) of CLEN, as well as CA release from CLEN (**C**), as affected by incubation time in simulated gastric and intestinal fluids. Data shown as mean  $\pm$  standard deviation ( $n = 3$ ), and data bearing different capital letters (A–E) at varying incubation times for GF or IF are significantly different at  $p < 0.05$ . CA, cinnamaldehyde; CLEN, cinnamon leaf extract nanoemulsion; GF, gastric fluid; IF, intestinal fluid.

Several studies have shown that encapsulation of CA into microemulsion and nanoemulsion could substantially improve its stability and bioavailability. For instance, Yong et al. [32] prepared a CA microemulsion with a mean particle size of 257.23 nm, PDI at 0.157, and zeta potential at  $-24.78$  mV by a high-pressure homogenization method through mixing 2.5% CA with 1.5% of Tween-80/Span-80 (1:1), 1.5% of medium-chain triglyceride, 1.5% Poloxamer, 1.5% lecithin, and 91.5% water. Dialysis of CA microemulsion and CA solution separately through a 10–12 kDa MWCO dialysis bag against PBS solution (pH 6.8) demonstrated a CA release of 29.79% and 64.74%, respectively, after the initial 10 min, as well as 100% and 85% after 4 h under intestinal conditions, with a sustained and higher release fitting first-order kinetics for the former and a fast CA release through Fickian diffusion fitting the Ritger-Peppas model for the latter. For pharmacokinetic study, the administration of CA microemulsion (50 mg/kg bw) via oral gavage revealed a 3-, 3.5-, and 2.3-fold increase in  $T_{\max}$  (time to attain maximum concentration in plasma),  $C_{\max}$  (maximum concentration in plasma), and  $AUC_{0-\infty}$  (area under the concentration-time curve) compared with the administration of CA solution at the same dose. In a later study, Dong et al. [33] also showed an elevation of both stability and bioavailability after encapsulation of CA into a nanoemulsion system. More elaborately, CA nanoemulsion with a mean particle size of 33.13 nm, PDI of 0.047, and zeta potential of  $-3.77$  mV was prepared by a water titration method using CA (7.5%), vitamin E oil (5%), Tween 80 (24.3%), 1,2-propanediol (4.8%), and water (58.4%). The in vitro release study was conducted in a dialysis bag (10 kDa MWCO) against simulated intestinal fluid (pH 6.8) at 37 °C, and the amount of CA released from CA nanoemulsion and solution was 41.3% and 30.2%, respectively, after 0.17 h, but after 24 h, the CA release reached 90% for both. The release kinetics of CA from both nanoemulsion and solution fit the Ritger-Peppas model, indicating that the release mechanism was governed by the Fickian diffusion. After administration of CA nanoemulsion or solution at a dose of 375 mg/kg bw by oral gavage into rats, the CA levels in plasma were determined by HPLC at different time intervals (5–480 min) to obtain  $T_{\max}$  (0.504 h),  $C_{\max}$  (171.4 mg/L),  $AUC_{0-\infty}$  (452.5 mg/L·h), and MRT (maximum residence time, 2.289 h) for CA nanoemulsion, which were 3-, 1.3-, 2.6-, and 1.5-fold higher than that for CA solution. Collectively, the incorporation of CA into a microemulsion/nanoemulsion system can enhance the stability and intestinal absorption for improvement in oral bioavailability.

### 2.3. Measurement of Sexual Behavior

Based on the recovery formula provided in Section 3.17, the recovery percentage shown in the following sections denotes a statistical return towards the normal values at  $p < 0.05$ .

#### 2.3.1. Penile Reflex Test

Total penile reflexes (TPR), an indicator of penile erection and function, are shown in Figure 4A. Using the contact sexual function assessment model, the TPR of normal rats (group N) averaged 11.8, while rats with ED (group I) were significantly reduced to 4.1 ( $p < 0.05$ ), concomitant with a reduction in the numbers of erections (Figure 4B), long flips (LF) (Figure 4C) and quick flips (QF) (Figure 4D), indicating that bilateral cavernous nerve compression resulted in a significant impairment of the erection reflex. In contrast, compared with group I, the high-dose CLEN treatment (group HN) was effective in improving the frequency of erectile reflexes, as evident by a TPR of 10.1 and a recovery of 78%, while a TPR of 7.4 and a recovery of 57% was shown for the low-dose CLEN treatment (group LN) (Figure 4A). Additionally, the number of erections, long flips, and quick flips was significantly higher ( $p < 0.05$ ) in both HN and LN groups than in group I (Figure 4B–D). Furthermore, for both treatments of high-dose CLE (group HE) and low-dose CLE (group

LE), the TPR were 6.1 and 4.6, respectively, implying an improvement in recoveries for the former (25%) and the latter (6%). Collectively, the TPR assessment of various treatments follows the  $N > HN > LN > HE > LE > I$  order, demonstrating that CLEN possesses a much better penile erection and function than CLE.

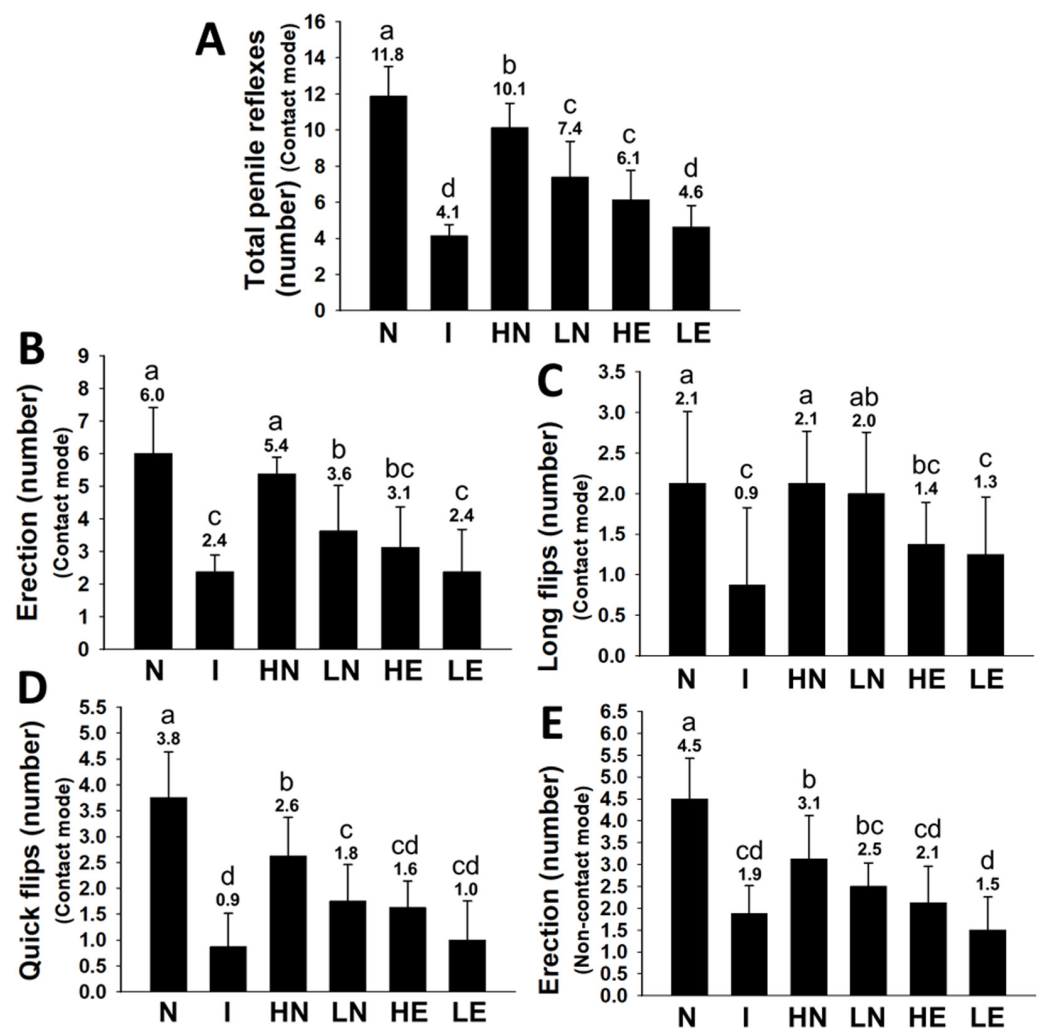


**Figure 4.** Effects of feeding cinnamon leaf extract nanoemulsion (CLEN) and cinnamon leaf extract (CLE) on total penile reflexes (A), erection number in contact mode (B), long flips number in contact mode (C), quick flips number in contact mode (D), and erection number in non-contact mode (E) in rats with ED. Data are represented as the mean of octuplicate analyses  $\pm$  standard deviation ( $n = 8$ ). Data bearing different small letters (a–d) in the same parameter are significantly different at  $p < 0.05$ . Group N, normal; group I, induction; group HN, high-dose cinnamon leaf extract nanoemulsion (100 mg/kg bw); group LN, low-dose cinnamon leaf extract nanoemulsion (50 mg/kg bw); group HE, high-dose cinnamon leaf extract (100 mg/kg bw); group LE, low-dose cinnamon leaf extract (50 mg/kg bw); ED, erectile dysfunction.

During non-contact stimulation, the mean number of erections was 4.5 in group N but decreased significantly to 1.9 ( $p < 0.05$ ) in group I, revealing that cavernous nerve damage significantly inhibited the erectile response in rats (Figure 4E). Compared with group I, the number of erections increased to 3.1 (53% recovery) and 2.5 (23% recovery) for the HN and LN groups, respectively. However, for the CLE treatment, only a high dose was effective in raising the number of erections to 2.1 (8% recovery), but a low dose failed to elevate the number of erections. Taken together, the TPR assessment of various treatments for non-contact stimulation follows the order:  $N > HN > LN > HE > I > LE$ .

Currently, there is a lack of data regarding the improvement of sexual behavioral parameters in rats with ED by cinnamon-derived products. However, there are several studies dealing with the effects of plant extracts on improving sexual behavior in male rats. For instance, Tang et al. [34] investigated the effects of horse-teethed quince (*Allium tuberosum*) seed extract on sexual behavior and libido efficiency in male Wistar rats. Compared with the induction group, the administration of 400 mg/kg of *Allium tuberosum* seed extract for 45 days significantly increased erection, long flip, quick flip, and TPR by 2.15-, 2.60-, 2.30-, and 2.15-fold, respectively, following a dose-dependent response. In another study dealing with the effect of feeding narrow-leaved geranium (a medicinal plant) on the sexual behavior of male Wistar rats, Fouche et al. [35] reported that following administration with geranium extract at 300 mg/kg bw for 7 days, an improvement in erection, long flip, quick flip, and TPR by 1.5-, 2.25-, 5-, and 2.54-fold was found, respectively, compared with the induction group. Similarly, the daily oral administration of 1200 mg/kg extracts from burdock root significantly ( $p < 0.05$ ) increased erection, long flip, quick flip, and TPR by 69.7%, 23.9%, 88.5%, and 104%, respectively [36]. These outcomes indicated that plant extracts possess great potential in elevating sexual function and behavior in humans.

As mentioned earlier, contact sexual function assessment can reflect the local penile reflexes and smooth muscle coordination, while non-contact and contact sexual behavioral assessment can reflect the modulation of erectile function by central sexual stimuli (visual, olfactory) and peripheral penile reflexes (tactile), respectively. In our study we observed that damage to cavernous nerves significantly reduced degree of erectile reflexes, with CLEN exhibiting a significant protective effect on sexual function of rats after nerve damage, particularly at high dose (100 mg/kg bw based on CA-equivalent content), which may be attributed to the presence of high level of CA and the other bioactive compounds in cinnamon leaf powder, as they have been demonstrate to possess anti-oxidation, NO production, PDE5 reduction and neuronal inflammation activities [1,37]. In addition, the encapsulation of CLE into CLEN can enhance the stability and bioavailability of bioactive compounds in vivo, thereby improving ED.

### 2.3.2. Sexual Behavior Test

ED is a male sexual dysfunction defined as “the inability of a man to achieve and maintain an erection for satisfactory sexual intercourse” [38]. The normal male sexual response cycle is functionally divided into five interrelated events: libido or sexual desire, erection, ejaculation, orgasm, and detumescence, with libido being defined as the physiological need for sexual activity (the sexual urge), which is usually manifested as a desire for sex [39]. The frequency and latency of mounting, intromission, and ejaculation are commonly used in animal experiments to test male animal sexual behavior [40]. In this study, a video camera was used to monitor sexual behavior for 30 min to measure sexual behavior parameters. According to a recent study by Ogunro and Yakubu [41], rats with at least a 25% reduction in the sexual behavior parameters of mounting frequency (MF) and insertion frequency (IF) and at least a 25% lengthening of the mounting latency (ML), insertion latency (IL), and ejaculation latency (EL) were considered to have sexual dysfunction and ED. As shown in Figure 5A,B, in the sexual function mating experiment, the sexual behavior of group N was normal, while there was no significant difference ( $p > 0.05$ ) between ML and MF in all groups, probably due to the fact that ED caused by bilateral spinal nerve compression did not affect sexual desire [42]. Collectively, for all the treatments, ML follows the LN > HN > HE > I > N > LE order, while MF follows the LE > N > HN > LN = HE = I order. Another sexual function parameter (IL) assessed was 309 s in group N and 1003 s in group I, whereas CLEN at high dose was 420 s (84% recovery) and 486 s at low dose (74% recovery) (Figure 5C). Additionally, the high-dose

CLE was 588 s and 694 s at low dose, with recoveries at 59% and 44%, respectively, when compared with the induction treatment. For IF, group N had 14, while both groups I and HN had 3 and 11 (72% recovery), respectively. But for group LN, an IF value of 10 with a 63% recovery was shown, which was much higher than the HE and LE groups (Figure 5D). This outcome implied that postoperative ED in rats with bilateral spinal nerve compression resulted in penetration difficulty but was improved by continuous administration with CLEN and CLE, particularly for high-dose CLEN. Thus, the sexual function assessment of various treatments follows the  $N > HN > LN > HE > LE > I$  order for IL and  $N > HN > LN > HE > LE > I$  order for IF. Similarly, for EL, 486 s was shown in group N, which was significantly lower ( $p < 0.05$ ) than group I (748 s), but a reduction to 543 s and 580 s was found for HN and LN groups, respectively, accompanied by an increase in the recoveries to 77% and 63%, respectively (Figure 5E). For CLE treatment, the recoveries of 33% and 13% were shown for high-dose and low-dose, respectively. Similar to the results of MF, ML, IF, and IL, the EL of various treatments follows the  $N > HN > LN > HE > LE > I$  order. This result suggests that CLEN administration can improve erectile function, but rats may fail to ejaculate during the 30 min copulation period. This can be judged based on observation of ejaculation during rat copulation and vaginal plug in female rats the next day, and thus the proportions of ejaculating rats in each group can then be determined as follows: 87.5%, 50%, 75%, 62.5%, 75%, and 62.5% for N, I, HN, LN, HE, and LE groups, respectively. This finding demonstrated that CLEN was able to improve sexual dysfunction caused by nerve damage, especially in the penetration (IL, IF) and ejaculation (EL) behaviors. In a similar study, Goswami et al. [20] explored the effect of cinnamon bark methanol extract on erectile and sexual function in aged and young male rats, which were tube-fed with the extract at a dose of 100 mg/kg bw for 28 days. The sexual function of aged rats was found to be significantly worse than that of young male rats. But following feeding aged male rats with the methanol extract, the mounting and penetration latencies decreased by 46% and 43%, respectively. Furthermore, the mounting frequency, penetration frequency, and ejaculation latency rose by 1.4-, 2.0-, and 1.0-fold, respectively, revealing a substantial improvement in sexual dysfunction of aged male rats.

The mating test is an important indicator of sexual desire, sexual function, and erectile efficiency in male animals. In general, excessive prolongation of IL is often associated with ED or neurotransmission abnormalities. In the present study, we found that after cavernous nerve damage, male rats showed the most pronounced IL prolongation and IF reduction, representing impaired sexual function and mating ability, while EL prolongation indicated a decrease in the ejaculatory reflex efficiency [43,44]. These changes are in line with ED commonly seen in patients after clinical adenocarcinoma surgery [41]. However, following feeding with high doses or low doses of CLEN, rats showed a significant decline in IL and an increase in IF, suggesting a positive effect on the restoration of mating ability. In addition, a decrease in EL represented the improvement of ejaculatory efficiency, which may be associated with the following factors: (1) promotion of NO production and reduction in PDE5 level via improvement of cavernous smooth muscle relaxation and increase in blood supply [37]. (2) antioxidant and anti-inflammatory effects of CA and the other bioactive compounds via reduction in oxidative stress and protection of neural function [1]. (3) CLEN results in a substantial improvement in bioavailability, allowing CA and the other bioactive compounds to exert a significant absorption effect in vivo even at low doses [25]. This finding suggests that CLEN exhibits great potential to be used as a functional food or adjuvant therapy for sexual dysfunction treatment.

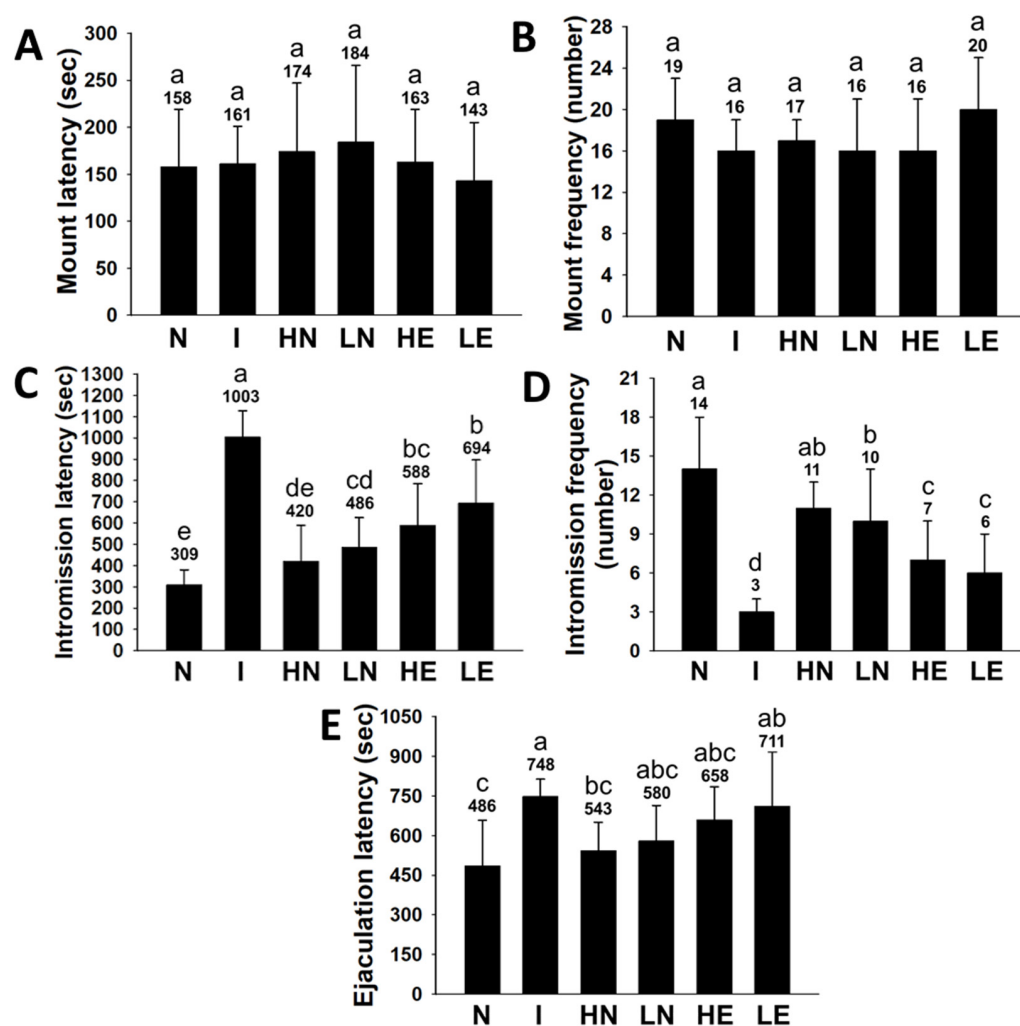


**Figure 5.** Effects of feeding cinnamon leaf extract nanoemulsion (CLEN) and cinnamon leaf extract (CLE) on sexual behavior parameters, including mount latency (A), mount frequency (B), intromission latency (C), intromission frequency (D), and ejaculation latency (E) in rats with ED. Data are represented as the mean of octuplicate analyses  $\pm$  standard deviation ( $n = 8$ ). As some rats with ED fail to ejaculate, the number of ejaculation latency data in (E) for each group is as follows: N,  $n = 7$ ; I,  $n = 4$ ; HN,  $n = 6$ ; LN,  $n = 5$ ; HE,  $n = 6$ ; LE,  $n = 5$ . Data bearing different small letters (a–e) in the same parameter are significantly different at  $p < 0.05$ . Group N, normal; Group I, induction; Group HN, high-dose cinnamon leaf extract nanoemulsion (100 mg/kg bw); Group LN, low-dose cinnamon leaf extract nanoemulsion (50 mg/kg bw); Group HE, high-dose cinnamon leaf extract (100 mg/kg bw); Group LE, low-dose cinnamon leaf extract (50 mg/kg bw); ED, erectile dysfunction.

### 2.3.3. Changes in Intracavernous Pressure (ICP)

The use of electrical stimulation of spinal nerves induces a stable and reproducible erectile function response in the penis, and the measurement of intracavernous pressure (ICP) allows for assessment of erectile function, which is a vital technique for studying ED [45]. The effects of feeding CLEN and CLE on changes in ICP in rats with ED measured by a Biopac Physiograph are shown in Figure 6A. Additionally, we also measured ICP-related parameters (Figure 6B), including maximum ICP (Figure 6(B1)), ICP change ( $\Delta$ ICP) (Figure 6(B2)), ICP area under curve (Figure 6(B3)), minimum ICP (Figure 6(B4)), mean arterial pressure ratio (maximum ICP/MAP) (Figure 6(B5)),  $\Delta$ ICP and MAP ratio ( $\Delta$ ICP/MAP) (Figure 6(B6)). Following spinal nerve injury for 28 days, the maximum ICP/MAP of group N was 0.96, which was higher than that of group I by 44.8%. Compared with group I, both high and low doses of CLEN showed a significant rise in maximum

ICP/MAP ratio at 0.84 and 0.69, with recoveries at 72% and 37%, respectively. Likewise, both high and low doses of CLE showed an increase in maximum ICP/MAP ratio at 0.66 and 0.56, with recoveries at 30% and 6%, respectively. This result implied that CLEN was more effective than CLE in improving sexual dysfunction. Comparatively, the high-dose CLEN showed the most distinct effect in improving maximum ICP, maximum ICP,  $\Delta$ ICP, ICP area under curve, minimum ICP, and  $\Delta$ ICP/MAP, followed by low-dose CLEN, high-dose CLE, and low-dose CLE. Apparently, the encapsulation of bioactive compounds into a nanoemulsion system could elevate stability and bioavailability in vivo, thereby achieving a high recovery of sexual function.

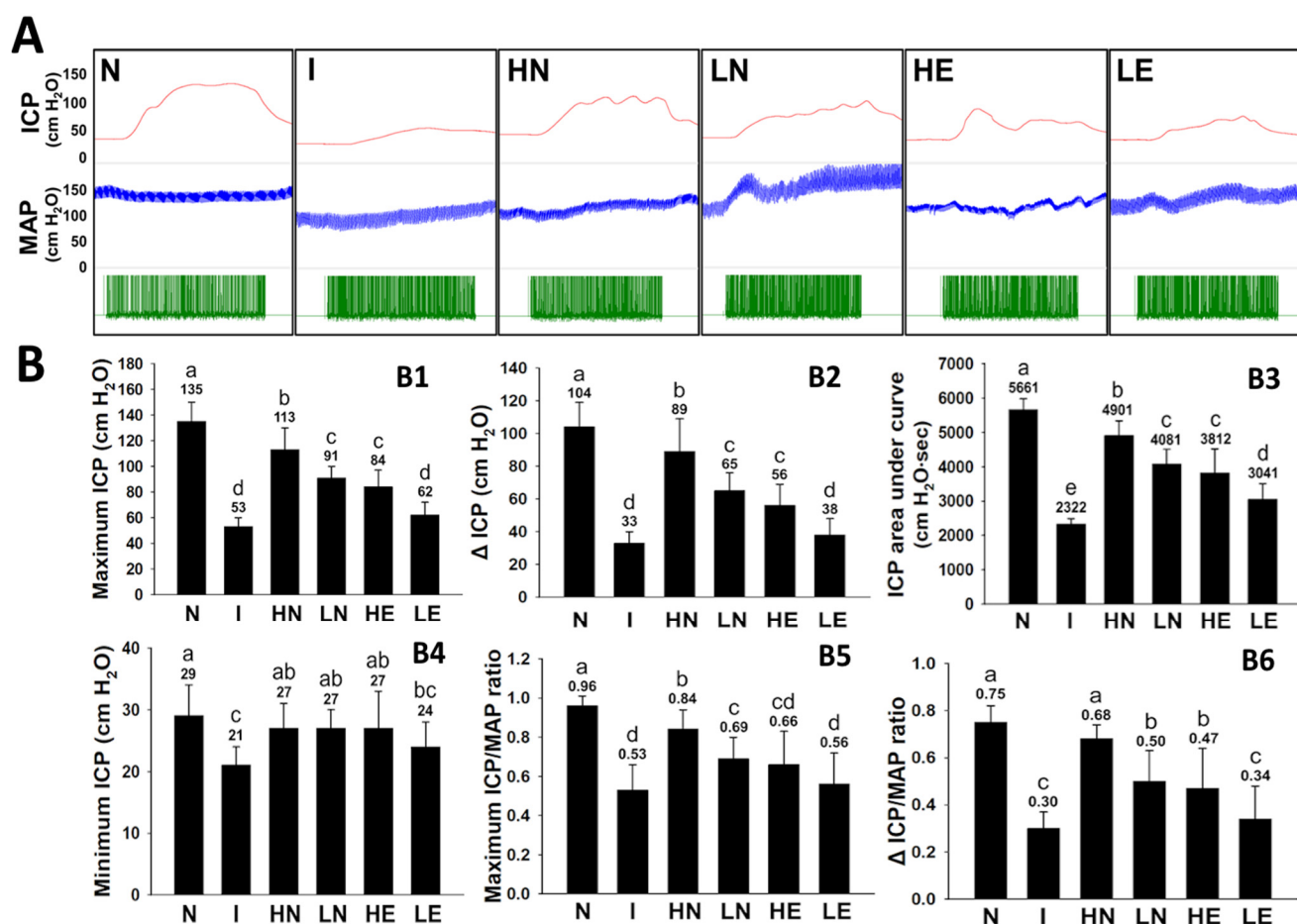


**Figure 6.** Effects of feeding cinnamon leaf extract nanoemulsion (CLEN) and cinnamon leaf extract (CLE) on changes in ICP shown in a Biopac Physiograph in rats with ED (A), along with the measured ICP-related parameters (B), including maximum ICP (B1), ICP change ( $\Delta$ ICP) (B2), ICP area under the curve (B3), minimum ICP (B4), mean arterial pressure ratio (maximum ICP/MAP) (B5), and  $\Delta$ ICP and MAP ratio ( $\Delta$ ICP/MAP) (B6). The red and blue colors in (A) indicate ICP and MAP curves, with a green bar denoting electrical stimulus for 60 s values of representative rats in each group. Data are represented as the mean of octuplicate analyses  $\pm$  standard deviation ( $n = 8$ ). Data bearing different small letters (a–e) in the same parameter are significantly different at  $p < 0.05$ . Group N, normal; group I, induction; group HN, high-dose cinnamon leaf extract nanoemulsion (100 mg/kg bw); group LN, low-dose cinnamon leaf extract nanoemulsion (50 mg/kg bw); group HE, high-dose cinnamon leaf extract (100 mg/kg bw); group LE, low-dose cinnamon leaf extract (50 mg/kg bw); ICP, intracavernous pressure; MAP, mean arterial pressure; ED, erectile dysfunction.

In a previous study, Onder et al. [46] evaluated the effect of cinnamon essential oil rich in CA and CA on the penile sponges of diabetic rats; the ICP area under the curve for essential oil treatment shows a high recovery at 89% ( $p < 0.05$ ), while the maximum ICP/MAP ratio was significantly restored by 74% ( $p < 0.05$ ). Similarly, compared to the

diabetes-induced group, the ICP area under curve and maximum ICP/MAP ratio for CA treatment were significantly restored by 71% and 62%, respectively, indicating that both essential oils and CA could increase the internal pressure of sponges and enhance sponges' blood congestion, thereby prolonging the duration of erectile response and elevating the erection strength, which may be attributed to the effects of CA in inhibiting reactive oxygen species (ROS) induced by hyperglycemia, possibly through activation of Nrf2 (nuclear factor erythroid 2-related factor) transcription factor, enhancement of antioxidant activity, and attenuation of cytotoxicity in diabetic mice through inhibition of inducible NOS and activation of NF- $\kappa$ B [47,48].

Additionally, Salman et al. [49] demonstrated that the maltodextrin-encapsulated cinnamon oil nanoformulation rich in CA (85%) could dose-dependently exert protective effects on titanium dioxide nanoparticles (TiO<sub>2</sub> NPs)-induced oxidative stress, DNA damage, chromosomal aberration, and reproductive disturbances in male mice. This nanoformulation with a mean particle size of 321 nm and zeta potential at −17.35 mV was prepared by the spray drying method using maltodextrin as the wall material. Following co-administration of this nanoformulation (50 mg/kg bw or 100 mg/kg bw) with TiO<sub>2</sub> NPs (25 mg/kg bw) into male mice, the liver and kidney functions (ALT, AST, albumin, total protein, creatinine, urea/uric acid) and antioxidant enzyme activities (SOD and CAT) were improved in a dose-dependent manner, accompanied by inhibiting DNA fragmentation as well as alleviating oxidative stress markers (NO and MDA), serum cytokines ( $\alpha$ -fetoprotein, TNF- $\alpha$  and carcinoembryonic antigen), chromosomal aberrations in bone marrow/germ cells and sperm shape abnormalities when compared to that in mice treated with only TiO<sub>2</sub> NPs. More recently, Ranganathan et al. [50] have shown that cinnamon oil nanoemulsion could preserve the sperm quality more effectively than clove oil and eugenol oil nanoemulsions, as well as some other common semen preservatives, including glycerol and egg yolk citrate. Cinnamon oil nanoemulsion was prepared by the titration method using Tween 80 and coconut oil, followed by treatment with sperm samples collected from 44 infertile human subjects with smoking habits and 42 fertile human subjects without smoking habits. After cryopreservation (−196 °C) for 24 h, cinnamon oil nanoemulsion was demonstrated to improve oxidative stress and standard semen parameters, such as morphology, viability, and integrity, much better than other nanoemulsions and semen preservatives, implying that cinnamon nanoformulations possess great potential in improving oxidative and reproductive effects in male subjects.

In addition to cinnamon, many studies have also investigated the effects of plant extracts on ED in male rats. For example, Mao et al. [51] evaluated the effect of isorhamnetin on improving ED induced by type I diabetes in rats. Following oral administration of high-dose (20 mg/kg) and low-dose (10 mg/kg) of isorhamnetin for 4 weeks, the maximum ICP/MAP was shown to be significantly restored by 67% and 72%, respectively. This finding suggests that isorhamnetin exerts protective effects on erectile function in diabetic rats, possibly through inhibition of inflammatory response, attenuation of oxidative stress and cavernosal fibrosis, improvement of endothelial function, and inhibition of cell apoptosis, with the mechanism being associated with activation of the PI3K/AKT/eNOS signaling pathway. In another study, Ye et al. [52] evaluated the effect of Hong-Jing I, a mixture of several traditional Chinese medicines including *Rhodiola rosea*, *Radix astragali*, and *Codonopsis pilosula*, on ED using a rat model of bilateral cavernous nerve injury, and the results showed that following administration for 28 days, the medium-dose (5.67 g/kg) and high-dose (11.34 g/kg) significantly increased maximum ICP compared with ED group, as evident by elevation of recoveries to 50% and 65%, respectively, while for the ICP/MAP ratio, it was improved with recoveries at 41% and 59%, respectively. The effects of *Ginkgo biloba* extract (GBE) on ED recovery in rats with bilateral cavernous nerve injury were conducted

by Wu et al. [53], reporting that medium-dose (4%, 5 mL/kg/day) and high-dose (10%, 5 mL/kg/day) of GBE could improve the maximum ICP, with recoveries at 44% and 83%, respectively, as well as the ICP/MAP ratio with recoveries at 43% and 84%, respectively. Like CA, this outcome revealed the improvement of erectile function following GBE intake, probably due to the elevation of antioxidant activity for subsequent enhancement of neuroregeneration and neuroprotection. The effects of DHA nanoemulsions on ED in male rats were investigated by Liao et al. [25], showing that low-dose (10 µg/kg), medium-dose (50 µg/kg), and high-dose (250 µg/kg) of DHA nanoemulsions could significantly improve the maximum ICP, with recoveries at 42%, 82%, and 51%, respectively, as well as the ICP/MAP ratio with recoveries at 71% and 59% for the medium-dose and high-dose of DHA nanoemulsions, respectively.

Collectively, the above results and discussions indicate that CLEN and some other plant extracts effectively promote the recovery of erectile function in rats with cavernous nerve injury through improvement of penile vascular dilation and cavernosal smooth muscle relaxation, with the high dose of CLEN at 100 mg/kg bw yielding the most favorable effect. This finding is in accordance with a previous study by Onder et al. [46], reporting that the administration of cinnamon essential oil could improve ED in diabetic rats.

#### 2.3.4. Histochemical Staining (Immunofluorescence Staining and Masson's Trichrome Staining)

As mentioned above, the reduction in neuronal nitric oxide synthase (nNOS) and NO, along with increased apoptosis of penile smooth muscle cells, is associated with ED [25]. In this study, penile tissues were subjected to immunofluorescence staining, including rabbit anti-nNOS and mouse anti-neuron-specific  $\beta$ -III tubulin staining. The ratio of nNOS to  $\beta$ -III tubulin fluorescence expression (quantification of fluorescence area) and anti- $\alpha$ -smooth muscle actin (anti- $\alpha$ -SMA) staining was used to measure  $\alpha$ -SMA fluorescence expression for analysis of the content of smooth muscle actin in the corpus cavernosum, with the data shown in Figure 7A–D. In group N,  $\alpha$ -SMA staining of corpus cavernosum tissue revealed a fluorescence expression area of smooth muscle actin at 12.0%, but it decreased to 6.0% for group I, indicating a reduction in smooth muscle actin content in the penile tissue of rats with bilateral cavernous nerve compression surgery (Figure 7B). In contrast, compared with group I, a high dose and a low dose of CLEN increased the fluorescence expression area to 9.5% (58% recovery) and 7.4% (23% recovery), respectively. However, for high-dose and low-dose of CLE, it only showed an increase in the fluorescence expression area by 10% and 5%, respectively. This outcome indicates that continuous oral administration with CLEN could restore smooth muscle actin content in the corpus cavernosum of rats to normal levels. Taken together, the  $\alpha$ -SMA fluorescence expression area (smooth muscle actin content) of various treatments follows the N > HN > LN > HE > LE > I order (Figure 7B).

Figure 7D shows the nNOS/ $\beta$ -III tubulin ratio in penile tissue, with groups N and I showing the ratio at 0.5 and 0.18, respectively, indicating that bilateral cavernous nerve compression impaired the neural function of the corpus cavernosum in rats. Following administration with high-dose and low-dose of CLEN, the nNOS/ $\beta$ -III tubulin ratios were raised to 0.42 (78% recovery) and 0.41 (71% recovery), respectively. Likewise, both high and low doses of CLE increased the nNOS/ $\beta$ -III tubulin ratios to 0.33 (46% recovery) and 0.23 (15% recovery), respectively. Overall, the nNOS/ $\beta$ -III tubulin fluorescence expression ratio of various treatments follows the N > HN > LN > HE > LE > I order (Figure 7D).

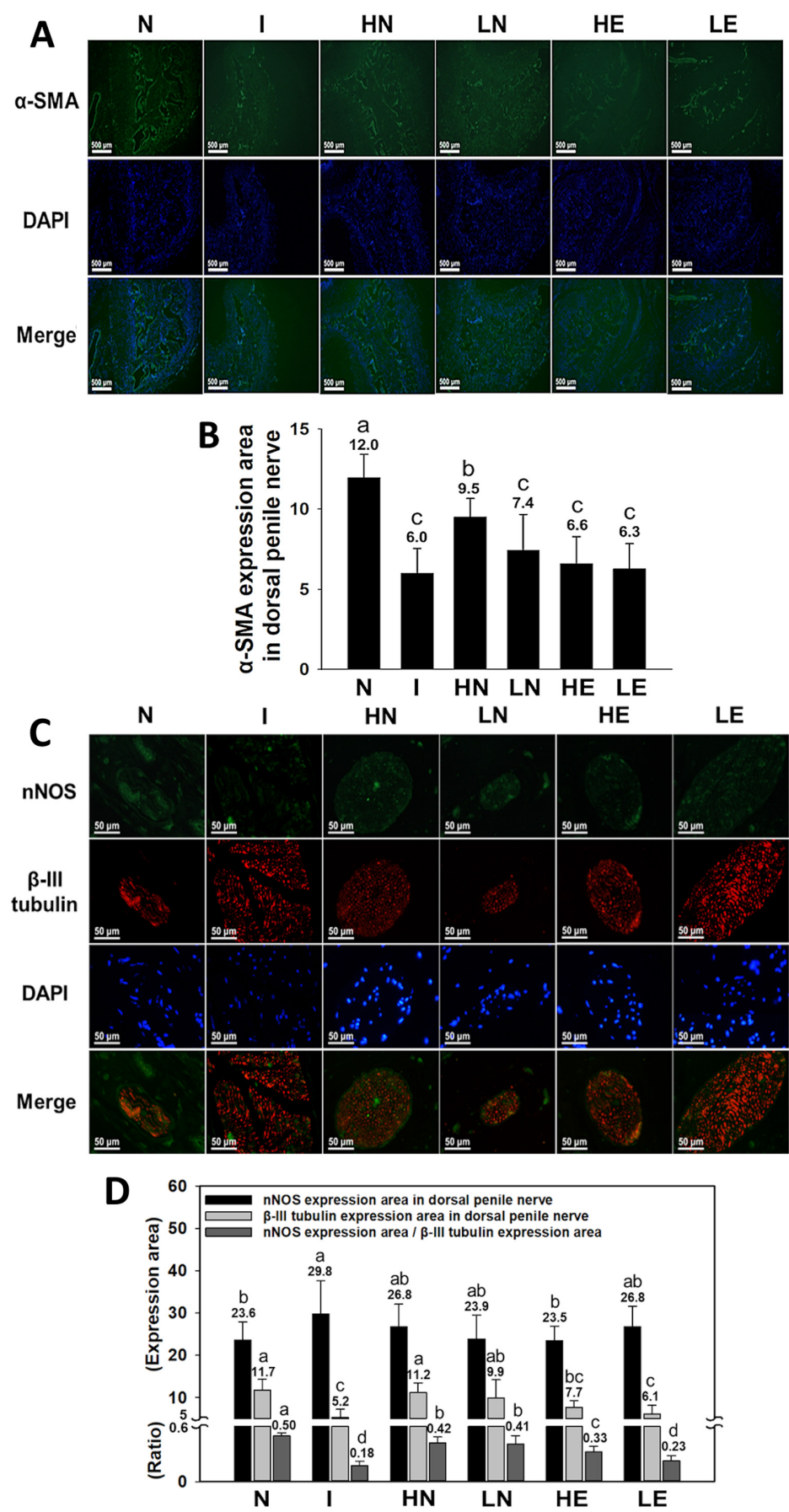


**Figure 7.** Effects of feeding cinnamon leaf extract nanoemulsion (CLEN) and cinnamon leaf extract (CLE) on histochemical immunofluorescence images (40 $\times$ ) of  $\alpha$ -SMA-positive areas obtained by

staining of corpus cavernosum with Alexa Flour 488 and Texas Red (A), along with  $\alpha$ -SMA expression area (B) and immunofluorescence images ( $400\times$ ) of nNOS and  $\beta$ -III tubulin (C), along with nNOS and  $\beta$ -III tubulin expression area and ratio of nNOS/ $\beta$ -III tubulin expression area (D) in dorsal penile nerve of rats with ED. Green and blue color in (A) indicates smooth muscle and nuclei, respectively, while green and red color in (C) indicates nNOS and  $\beta$ -III tubulin. Data are represented as the mean of octuplicate analyses  $\pm$  standard deviation ( $n = 8$ ). Data bearing different small letters (a–d) in the same parameter are significantly different at  $p < 0.05$ . Group N, normal; group I, induction; group HN, high-dose cinnamon leaf extract nanoemulsion (100 mg/kg bw); group LN, low-dose cinnamon leaf extract nanoemulsion (50 mg/kg bw); group HE, high-dose cinnamon leaf extract (100 mg/kg bw); group LE, low-dose cinnamon leaf extract (50 mg/kg bw); nNOS, neuronal nitric oxide synthase;  $\beta$ -III tubulin, tubulin beta-3 chain;  $\alpha$ -SMA, alpha smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole.

Additionally, some other factors, such as a reduction in elastic fibers, can contribute to ED as well, as it decreases the penile elastic capacity and erectile rigidity, thereby leading to ED [54]. Consequently, fibrosis of the corpus cavernosum is a vital pathological process causing ED [55], and it can be assessed using Masson's trichrome staining. The quantitative data of Masson's trichrome-stained sections are shown in Figure 8A,B, with the ratio of smooth muscle/collagen area in penile tissue in groups N and I being 0.30 and 0.15, respectively. However, for high-dose and low-dose CLEN, this ratio rose to 0.24 (60% recovery) and 0.19 (26% recovery), respectively (Figure 8B). But for high-dose and low-dose CLE, this ratio was only raised to 0.17 (13% recovery) and 0.16 (6% recovery), respectively. These results demonstrated a restorative effect on smooth muscle actin and a protective effect on the structure of corpus cavernosum tissue following administration with CLEN and CLE, with CLEN showing a more pronounced effect.

Currently, only a few studies have explored the effects of cinnamon on immunofluorescence and Masson's trichrome staining of penile tissue in rats with ED. In an earlier study, Goswami et al. [20] examined the effects of methanolic extracts of cinnamon bark on erectile and sexual activity in young male rats by administration of 100 mg/kg bw extract daily for 28 days, with the ratio of smooth muscle to collagen in the penile corpus cavernosum being 1:10 in young rats, while aged rats showed a reduction in the smooth muscle/collagen ratio in the corpus cavernosum. However, following administration with cinnamon extract (100 mg/kg bw), the smooth muscle actin content in the penile tissue of aged rats rose substantially, implying that cinnamon extract could elevate the smooth muscle/collagen ratio, thereby improving sexual function in aged rats.

Some other studies have explored the effects of plant extracts on ED using animal models with immunofluorescence and Masson's trichrome staining. For instance, Ye et al. [52] studied the effects of Hong-Jing I on ED in rats; an increase in smooth muscle content and reduction in collagen area were found for both medium-dose (5.67 g/kg/day) and high-dose (11.34 g/kg/day) groups following 28-day administration, concomitant with a rise in the smooth muscle/collagen ratio and recoveries of 57% and 72%, respectively, revealing an improvement in ameliorating corpus cavernosum fibrosis. In another study dealing with the effects of *Ginkgo biloba* extract (GBE) on ED in rats, Wu et al. [53] reported that after 4-week administration with 3 doses of GBE (10%, 4%, and 2%) at 5 mL/kg/day, the nNOS/ $\beta$ -III tubulin ratios were restored by 54%, 35%, and 17%, respectively, with the high-dose group showing the most prominent effect. Moreover, a rise in  $\alpha$ -SMA expression was also observed for GBE at doses of 10% (73% recovery), 4% (67% recovery), and 2% (20% recovery), and restoration in smooth muscle/collagen ratios at 63%, 47%, and 10%, respectively, with the high-dose group showing the most significant increase. Obviously, GBE could prevent smooth muscle atrophy in the corpus cavernosum through neuroprotective, neuroregenerative, and anti-apoptotic cell effects in a dose-dependent manner.

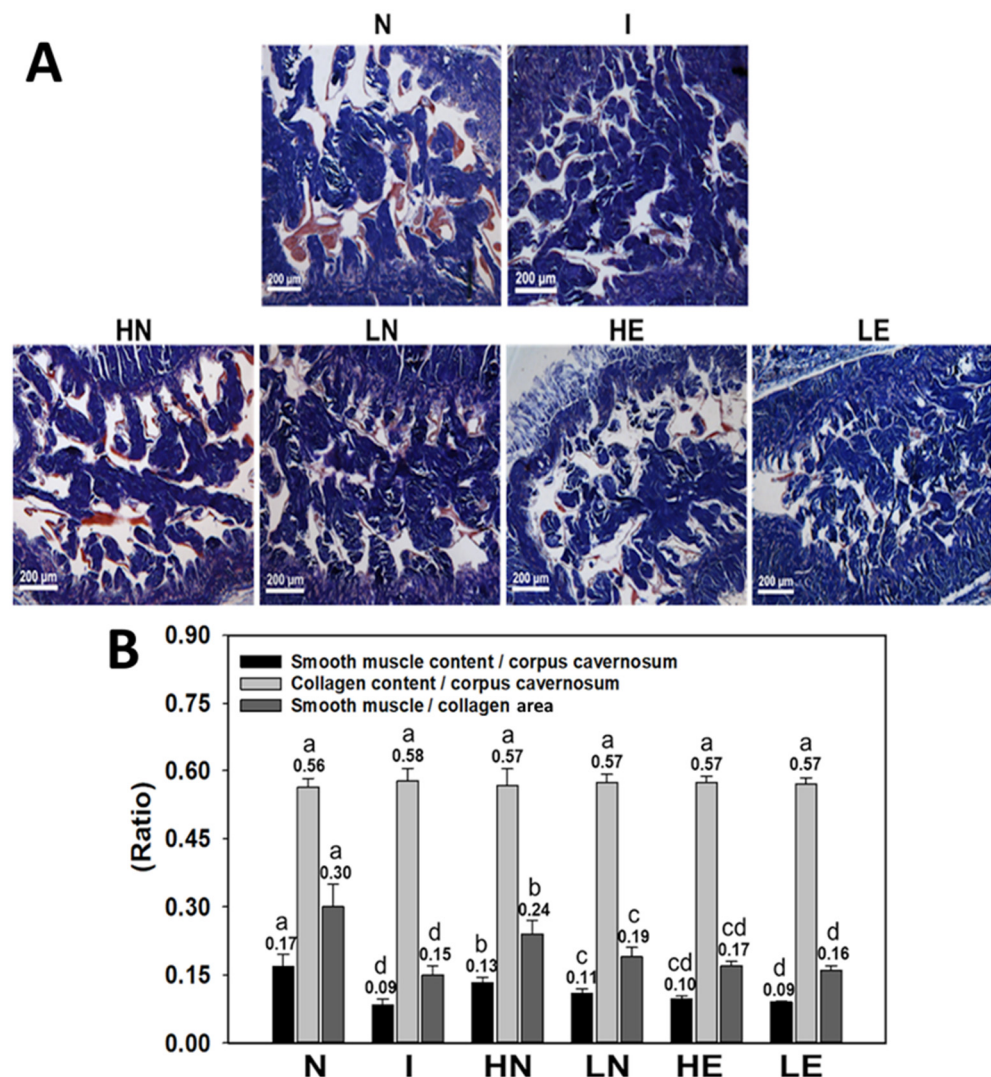


**Figure 8.** Effects of feeding cinnamon leaf extract nanoemulsion (CLEN) and cinnamon leaf extract (CLE) on Masson's trichrome staining images (40×) of corpus cavernosum (**A**), along with ratios of smooth muscle to corpus cavernosum, collagen to corpus cavernosum, and smooth muscle to collagen (**B**) in rats with ED. Red, blue, and light pink color indicates staining of smooth muscle, collagen/connective tissue, and cytoplasm, respectively. Data are represented as the mean of octuplicate analyses ± standard deviation ( $n = 8$ ). Data bearing different small letters (a–d) in the same parameter are significantly different at  $p < 0.05$ . Group N, normal; group I, induction; group HN, high-dose cinnamon leaf extract nanoemulsion (100 mg/kg bw); group LN, low-dose cinnamon leaf extract nanoemulsion (50 mg/kg bw); group HE, high-dose cinnamon leaf extract (100 mg/kg bw); group LE, low-dose cinnamon leaf extract (50 mg/kg bw); ED, erectile dysfunction.

Regarding the effects of nanoemulsions on ED in male rats, Liao et al. [25] evaluated the neuroprotective effects on ED in rats; the nNOS/ $\beta$ -III tubulin ratios were restored by 46%, 96%, and 62% for three doses at 10, 50, and 250 µg/kg, respectively, following 28-day administration, accompanied by recoveries of 66%, 99%, and 38% for the  $\alpha$ -SMA ratio. Similarly, for the smooth muscle/collagen ratio, it was restored by 33%, 60%, and 47%, respectively, but the difference is insignificant ( $p > 0.05$ ). This finding verified that DHA nanoemulsions effectively improved ED in rats caused by bilateral cavernous nerve injury.

Collectively, the results of Masson's trichrome staining,  $\alpha$ -SMA, and nNOS/ $\beta$ -III ratios demonstrated that cavernous nerve injury leads to decreased smooth muscle content and impaired neural function of the corpus cavernosum, thereby causing ED. Thus, maintaining an optimal smooth muscle/collagen ratio helps preserve cavernosal elasticity,

restore  $\alpha$ -SMA expression, increase smooth muscle content, promote vasodilation and engorgement, and enhance nNOS expression, thereby increasing nitric oxide production and neurotransmission, ultimately leading to restoration of erectile reflexes. As mentioned earlier, CLEN prepared in this study was shown to improve sexual function in male rats, presumably through antioxidant effects of CA and polyphenolic compounds in cinnamon leaves, improvement of stability and bioavailability of these compounds in vivo, as well as elevation of antifibrotic and neuroregenerative effects, thereby strengthening its potential clinical value.

#### 2.3.5. Biochemical Parameters of Penile Tissue

The primary mechanism of penile erection involves the release of NO from cavernous nerves upon sexual stimulation for subsequent diffusion into the smooth muscle cells of penile cavernosal arteries, where it activates guanylate cyclase, catalyzing the conversion of guanosine triphosphate (GTP) to cGMP. The resulting accumulation of cGMP induces smooth muscle relaxation, arterial dilation, and subsequent engorgement of the corpora cavernosa by opening potassium channels and inhibiting calcium channels [16]. Next, cGMP can promote cGMP-dependent protein kinase for stimulation of calcium ion flow out of the cell or store calcium ions in the cell to inhibit entry of calcium ions into the cell for subsequent decrease of calcium ions in the cytoplasm, leading to penile erection. Additionally, the arginine/NO/cGMP pathway has been demonstrated to relax corpus cavernosum smooth muscle, which is pivotal for penile erection [16]. In addition to CA, the major bioactive compound cinnamic acid in CLEN and CLE has been reported to activate the arginine/NO/cGMP pathway and inhibit the Rho/Rho-kinase pathway for relaxation of corpus cavernosum smooth muscle through production of vascular endothelial growth factors (VEGF) and fetal liver kinase-1/kinase insert domain receptor, the VEGF receptor involved in proliferation of endothelial cells [16,56]. Termination of erection occurs when cGMP is degraded by PDE5, thereby reducing smooth muscle relaxation and causing penile detumescence [57]. Consequently, PDE5 inhibitors such as sildenafil are widely used for ED treatment [58]. However, clinical trials of PDE5 inhibitors have shown adverse side effects, including visual disturbances and other ocular complications [59]. Thus, increasing attention has been directed toward the development of natural herbal products with PDE5 inhibitory activity. Furthermore, several studies have demonstrated that NO is the principal mediator of cavernosal smooth muscle relaxation [60], and elevating penile NO levels represents a therapeutic approach for both prevention and treatment of ED. In the present study, NO, cGMP, and PDE5 levels in penile tissue were measured using commercial assay kits. As shown in Figure 9A–C, group N exhibited NO and cGMP levels at 0.670  $\mu\text{mol/g}$  and 0.220 nmol/g, respectively (Figure 9A,B), with PDE5 content being 1116 pg/g (Figure 9C). However, in the bilateral cavernous nerve compression injury group, both NO and cGMP levels were significantly reduced to 0.360  $\mu\text{mol/g}$  and 0.102 nmol/g, respectively, accompanied by a marked increase in PDE5 content (2488 pg/g), indicating the impairment of the NO-cGMP signaling pathway caused by nerve injury, thereby leading to insufficient smooth muscle relaxation and compromised erectile function. In contrast, the oral administration of high-dose and low-dose CLEN significantly increased NO levels to 0.596  $\mu\text{mol/g}$  (76% recovery) and 0.508  $\mu\text{mol/g}$  (47% recovery), respectively (Figure 9A). Comparatively, both high and low doses of CLE showed lower NO levels, which amounted to 0.485  $\mu\text{mol/g}$  (40% recovery) and 0.403  $\mu\text{mol/g}$  (13% recovery), respectively. For the cGMP level, it was raised to 0.195 nmol/g (78% recovery) and 0.162 nmol/g (50% recovery) after CLEN treatment at high dose and low dose, respectively. But for CLE treatment, the cGMP levels were only raised to 0.137 nmol/g (29% recovery) and 0.104 nmol/g (1.6% recovery), respectively (Figure 9B). Like cGMP, PDE5 levels were significantly reduced to

1292 pg/g (87% recovery) and 1323 pg/g (84% recovery) following high-dose and low-dose CLEN treatments, respectively (Figure 9C). But for CLE treatments at high dose and low dose, PDE5 levels decreased to 1575 pg/g (66% recovery) and 1931 pg/g (46% recovery), respectively. Overall, the biochemical parameters of NO, cGMP, and PDE5 in penile tissues of various treatments follow the same  $N > HN > LN > HE > LE > I$  order.

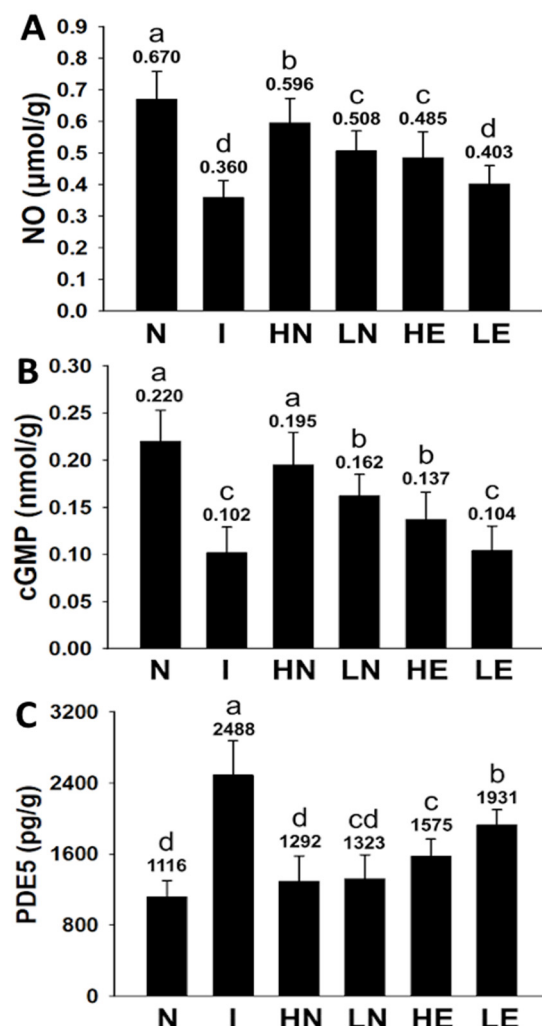


**Figure 9.** Effects of feeding cinnamon leaf extract nanoemulsion (CLEN) and cinnamon leaf extract (CLE) on penile biochemical values, including NO (A), cGMP (B), and PDE5 (C) in rats with ED. Data are represented as the mean of octuplicate analyses  $\pm$  standard deviation ( $n = 8$ ). Data bearing different small letters (a–d) in the same parameter are significantly different at  $p < 0.05$ . Group N, normal; group I, induction; group HN, high-dose cinnamon leaf extract nanoemulsion (100 mg/kg bw); group LN, low-dose cinnamon leaf extract nanoemulsion (50 mg/kg bw); group HE, high-dose cinnamon leaf extract (100 mg/kg bw); group LE, low-dose cinnamon leaf extract (50 mg/kg bw); NO, nitric oxide; cGMP, cyclic guanosine monophosphate; PDE5, phosphodiesterase type 5; ED, erectile dysfunction.

In an early study, Dell'Agli et al. [58] examined several plant extracts traditionally used for the treatment of male impotence, including *Epimedium brevicornum* and *Cinnamomum cassia*, with the former showing a stronger inhibitory activity against PDE5A1 at a dose of 50  $\mu\text{g/mL}$  with  $\text{IC}_{50}$  at 5.9  $\mu\text{M}$ , which can be ascribed to the dominant bioactive compound icariin. More recently, Wang et al. [37] determined the composition of hydrosol and essential oil of Taiwanese cinnamon (*Cinnamomum osmophloeum* Kanehira) leaves and evaluated its effect on PDE5 reduction, with CA being the major compound in hydrosol and essential oil, accounting for 65.03% and 63.38%, respectively. By comparison, hydrosol was more

efficient in reducing PDE5 levels than essential oil at doses of 0.025, 0.05, and 0.1 µg/mL, as evident by the inhibition percentages of 35.4%, 49.9%, and 70.7% for hydrosol, respectively. As PDE5 inhibitors have been well established as effective therapeutic agents for ED, this finding further underscores the potential of hydrosol from *C. osmophloeum* leaves as a natural therapeutic candidate for ED management.

Several other studies have also investigated the effects of plant extracts on ED in male rats. For example, Muritala et al. [61] examined the effect of *Rauwolfia vomitoria* bark extract on sexual function in male Wistar rats with paroxetine-induced ED. Following administration with paroxetine for 21 days and then *R. vomitoria* bark extract at 3 doses of 12.5, 25, and 50 mg/kg for 7 days, the PDE5 activities were significantly restored by 138%, 163%, and 175%, respectively, compared with the ED group, while the penile NO levels were significantly restored by 157%, 200%, and 229%, respectively, implying that *R. vomitoria* bark extract ameliorated paroxetine-induced ED and elevated sexual performance. In another study dealing with the effects of *Lespedeza cuneata* extract on sexual function in aged male Sprague–Dawley rats with ED, Mo et al. [62] reported that following administration with the extract at doses of 150 and 300 mg/kg for 4 weeks, the latter significantly increased penile NO level by 45.5% and cGMP level by 40%, compared with the aged group. This outcome suggests that *L. cuneata* extract improves erectile function in aged male rats, possibly through modulation of the NO-cGMP signaling pathway by promoting endothelial nitric oxide synthase (eNOS) phosphorylation, thereby boosting vasodilation and alleviating endothelial dysfunction.

Both neuronal NOS (nNOS) and endothelial nitric oxide synthase (eNOS) have been demonstrated to play a vital role in NO formation during erection, with the former contributing to the initiation of smooth muscle relaxation in the nitrergic nerves and the latter responsible for the maintenance of penile erection [63]. Many factors, including smooth muscle loss, endothelial dysfunction, low testosterone level, nerve damage, and reduced penile arterial blood flow, have been reported to be closely associated with ED [64]. Regarding the molecular mechanisms involved in the regulation of endothelial NO bioavailability and eNOS activity, both the production of high-level reactive oxygen species and the decrease in NO bioavailability have been reported to inhibit eNOS through the activation of Ras homolog gene family member A (RhoA)/ROCK signaling, leading to enhanced cavernosal smooth muscle contraction for subsequent ED development [65]. As noted above, PDE5 inhibitors are the first-line drugs used for ED treatment due to their high efficiency and safety, which can target the NO/cGMP/PKG pathway. Additionally, many other pathways, including phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β)/Smad, nuclear factor erythroid 2-related factor (Nrf2), Janus kinase (JAK)/signal transducer and activator of transcription (STAT), mitogen-activated protein kinase (MAPK), adenosine monophosphate-activated protein kinase (AMPK), and nuclear factor kappa-B (NF-κB) have been reported to be involved in ED progress [65]. Nevertheless, the NOS/NO signaling was recognized as a key pathway involved in physiological erection [63,65]. Moreover, all these pathways affect cellular processes such as hypoxia, apoptosis, autophagy, oxidative stress, inflammation, angiogenesis, and fibrosis greatly, and thereby can be crucial factors in ED pathogenesis [65].

In line with these findings, our study demonstrated that cavernous nerve injury suppressed NO and cGMP formation while increasing PDE5 level, ultimately leading to ED. In contrast, oral administration with high-dose and low-dose of CLEN significantly increased NO and cGMP levels while reducing PDE5 levels. Collectively, our study demonstrates that CLEN exerts a significant protective effect against ED via modulation of the NO-cGMP signaling pathway, thereby offering a promising natural therapeutic option for ED manage-

ment. It is worth pointing out that the CLEN prepared in this study is mainly composed of 91% deionized water, 6% Tween 80, 2% lecithin, and 1% soybean oil, along with CLE. Lecithin, rich in phosphatidylcholine, is a well-known surfactant and antioxidant widely used for functional food production. Furthermore, lecithin may play an indirect role in improving sexual dysfunction, as the release of choline from phosphatidylcholine in the intestine for subsequent synthesis of a pivotal neurotransmitter, acetylcholine, in the brain within cholinergic neurons through choline acetyltransferase and acetyl-CoA, can maintain brain function [22,30]. Additionally, acetylcholine was shown to be involved in penile erection when the smooth muscles of the corpus cavernosum relax, permitting increased blood flow into the penile tissue, as the corpus cavernosum is innervated by cholinergic nerves and contains cholinergic receptors, suggesting endogenous activity of acetylcholine in the penile tissue [66]. Interestingly, in a study dealing with the analysis of bioactive compounds in cinnamon leaves and the preparation of nanoemulsions and byproducts for improving Parkinson's disease in rats, a substantial rise in dopamine content of 49.39% and 28.38% was observed in the rat striatum following administration of high-dose and low-dose of CLEN for 5 weeks, respectively [22]. Like lecithin, dopamine may be involved in improving sexual function as well. Depending on its level, dopamine in the medial preoptic area at low levels was reported to disinhibit genital reflexes, facilitate parasympathetically mediated erections and copulatory behavior at moderate levels, and promote sympathetically mediated ejaculation but inhibits erections at high levels [66]. Namely, dopamine is imperative from the motor aspect during copulation, but not for sexual motivation. Significantly, the presence of acetylcholine was reported to promote dopamine release, while the absence of acetylcholine may impair sexual function [67]. Thus, the optimal level of dopamine and the mechanism for improving ED need to be further investigated. Another important neurotransmitter, serotonin, mainly released by the neurons of the raphe nuclei, was also found to increase substantially following administration of rats with CLEN [68]. At an optimal level, serotonin could act on the smooth muscles of the vascular system of the genitals and other sexual organs to induce vasodilatation and vasoconstriction, and thereby affect sexual behavior [66]. But this needs further exploration.

In addition, soybean oil, rich in essential fatty acids including linoleic acid (49–53%) and linolenic acid (5–9%), has been demonstrated to possess anti-inflammatory, immune system-enhancing, and anti-cancer functions [30]. However, the effect of essential fatty acids on improving sexual dysfunction has been less explored. Several studies have revealed that  $\omega$ -3 fatty acids such as linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) may play a significant role in improving sexual dysfunction and the integrated functions of the central nervous system, such as anxiety and mood, caused by an increase in dopamine and serotonin levels [69,70], as well as a rise in uterine blood flow [71]. In a double-blind randomized controlled clinical trial performed on 124 pregnant women at 16–22 weeks of gestation, the omega-3 supplementation once a day (300 mg) for 8 weeks was shown to improve sexual function in pregnant women by preventing increased pregnancy anxiety [72]. In another study dealing with the effect of omega-3 fatty acid (EPA plus DHA at 3:2) on male sexual performance in rats, Odetayo and Olayaki [67] reported that omega-3 fatty acid could ameliorate bisphenol F-induced sexual dysfunction in rats by upregulating NO/cGMP signaling and steroidogenic enzyme activities, resulting in vasodilation of smooth muscle of corpora cavernosa and thereby improving sexual function. This finding was supported by a study including a total of 3730 participants from the National Health and Nutrition Examination Survey 2001–2004 to explore the association between polyunsaturated fatty acid intake and the prevalence of ED, with the results showing that the arachidonic acid (a polyunsaturated fatty acid with 20 carbons and 4 double bonds) intake was found to be negatively correlated with the

prevalence of ED, probably caused by the anti-inflammatory and anti-endothelial injury effects [73]. But for linoleic acid in a cell model, it was shown to promote testosterone production by activating the Leydig cell GPR120/ERK pathway and restore bisphenol A-impaired testicular toxicity [74]. As testosterone plays a vital role in regulating sperm production and sex drive, the effect of linoleic acid on improving sexual function using an animal model needs to be further investigated. Regarding the safety issue associated with cinnamon, it is generally recognized as a safe food additive according to the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the United States Food and Drug Administration (USFDA) [30]. Most importantly, no adverse effects have been reported in human studies following intake of cinnamon and its extracts [30].

Collectively, the above discussion reveals that multiple mechanisms, including NO-cGMP signaling, PDE5 reduction, dopaminergic, serotonergic, and cholinergic pathways, may be involved in the protective effects of CLEN on ED. However, these mechanistic interpretations are only hypothesis-generating, and, thus, some more in-depth experiments are necessary to validate the actual mechanistic pathways. Furthermore, while rodent models have been instrumental in elucidating ED mechanisms and discovering potential therapeutic targets, they cannot comprehensively simulate human physiological complexity; that is, the complex nature of human sexual function cannot possibly be replicated in animal models [75]. For example, the penile structure in mice/rats is different from that in humans, with the former possessing a higher ratio of collagen fibers and less smooth muscle in the corpus cavernosum [76]. Additionally, animal studies mainly focus on the conditions causing ED, while human ED often involves complex psychological factors such as stress, depression, and relationship issues, which are difficult to reproduce in rodents [77]. Arousal mechanisms in rodents are primarily driven by olfactory cues, whereas human sexual function is heavily driven by visual and auditory stimuli [78]. Moreover, the short lifespan of rodents limits the ability to study chronic age-related development of ED and induction of ED by unnatural methods such as severe nerve crush injury, which does not accurately reflect the gradual degeneration seen in human clinical cases [76]. Mating tests, often used to evaluate ED in conscious animals, can be highly subjective and influenced by the animal's sexual desire and not just penile vascular function [79]. Experiments with animal models require anesthesia for measuring ICP, which can interfere with the erection control, potentially causing artifacts in data [79]. Consequently, clinical trials are needed to validate the research findings and directly assess the translation of these animal models to humans, ensuring safety risks and long-term efficiency [77]. Most importantly, they could confirm if the therapeutic effect observed in animal models holds true in multiple coexisting conditions such as diabetes, hypertension, and aging in humans while establishing the safety, dosage, and side effects of tested therapeutic agents/formulations [80]. Additionally, by utilizing standardized and validated questionnaires with International Index of Erectile Function (IIEF) scores along with physiological measurements, a more comprehensive evaluation is possible with clinical trials [77].

Nevertheless, the cinnamon leaf extract and nanoemulsion are investigated for the first time to explore their effects on improving sexual dysfunction in rats, demonstrating the feasibility of cinnamon leaves as a raw material for functional food development and a possible botanic drug candidate in a future clinical trial.

### 3. Materials and Methods

#### 3.1. Materials

A total of 2.5 kg of Taiwan cinnamon leaves grown in Pinglin District, New Taipei City, was provided by Fu-Tai Co. (New Taipei City, Taiwan). Following harvesting, cinnamon leaves were transported to our lab, Fu Jen Catholic University (New Taipei City, Taiwan),

for subsequent drying at 60 °C for 2 h, grinding into powder, pouring into several vacuum packaging bags separately, and then storing at −20 °C until use. Prior to processing, the voucher specimen of *C. osmophloeum* leaves was deposited in the herbarium of the Taiwan Forestry Research Institute (TAIF, Taipei, Taiwan) and identified by Dr. Wen-Liang Chiou (TAIF) based on the accession number 11097.

Standards including rutin, quercetin-3-*O*-glucoside, quercetin, quercetin-3-*O*-galactoside, kaempferol, kaempferol-3- $\beta$ -D-glucopyranoside, *p*-coumaric acid, and eugenol were procured from Sigma-Aldrich Co. (St Louis, MO, USA). Cinnamaldehyde (CA), caffeic acid, benzoic acid, *trans*-cinnamic acid, and cinnamyl alcohol were obtained from Chem Service Inc. (West Chester, PA, USA), while coumarin was from AccuStandard Inc. (New Haven, CT, USA), and 5-*O*--caffeoylquinic acid from Chendu Biopurity Phytochemicals Inc. (Chendu, China).

HPLC-grade solvents, including acetonitrile and methanol, were from Merck Co. (Darmstadt, Germany). Dimethyl sulfoxide, potassium dihydrogen phosphate, and ethanol (99%) were from Sigma-Aldrich Co. Glacial acetic acid was from Thermo Fisher Scientific Inc. (San Jose, CA, USA), while deionized water was obtained using a Milli-Q water purification system from Millipore Co. (Bedford, MA, USA). Soybean oil was from Taiwan Sugar Corporation (Tainan, Taiwan), while lecithin was from Chen-Fang Co. (Taipei, Taiwan) and Tween 80 from Yi-Pa Co. (Taipei, Taiwan). Progesterone,  $\beta$ -estradiol-3-benzoate, anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), mouse anti-neuron-specific  $\beta$ -III tubulin, and rabbit anti-neuronal nitric oxide synthase (nNOS) were from Sigma-Aldrich Co. Potassium chloride was from Nacalai Tesque Co. (Kyoto, Japan). Formalin (10%) and phosphate-buffered saline (PBS) were from Uni-Onward Corp. (New Taipei City, Taiwan), while heparin was from Tai-Yu Chem Co. (Hsin-Chu, Taiwan) and a winged infusion set (24 G) from Unimed Healthcare Inc. (Taipei, Taiwan). Zoletil 50 was from Virbac Co. (Carros, France), while rompun was from Elanco Co. (Greenfield, IN, USA). The feeding needle was from Tai-Ho Instrument Co. (New Taipei City, Taiwan), while the surgical instruments were from Long-Time Lab Services Co. (Taichung, Taiwan), and the sterilized cotton swab was from Team Power Medical Instrument Ltd. (New Taipei City, Taiwan), sindine solution from Siphar Pharmaceutical Co. (I-Lan, Taiwan), and mercury-free saline from Nang-Kuang Pharmaceutical Co. (Tainan, Taiwan).

### 3.2. Extraction of Bioactive Compounds from Cinnamon Leaves

For analysis of bioactive compounds in cinnamon leaves, the cinnamon leaf extract (CLE) was prepared by mixing 6.6 g of cinnamon leaf powder sample with 80% ethanol (33 mL) at a ratio of 1:5, after which this mixture was stirred and then sonicated for 2 h in a sonicator (model DC 400H) from Mandarin Scientific Co. (New Taipei City, Taiwan) for subsequent filtration through a filter paper, evaporation using a rotary evaporator (N-1) from Eyela Co. (Tokyo, Japan) and redissolving in 9.6 mL of deionized water to obtain CLE. Then the CLE was filtered through a 0.22  $\mu$ m membrane filter for UPLC-MS/MS analysis. The extraction yield based on the dry weight of CLE was 17% (*w/w*), with CLE concentration being 0.117 g CLE dry powder/mL containing 10,000  $\mu$ g/mL of CA.

The preparation of high-dose cinnamon leaf nanoemulsion (CLEN) or CLE (100 mg/kg bw) and low-dose CLEN or CLE (50 mg/kg bw) for animal experiments was based on CA concentration following analysis by UPLC-MS/MS. For preparation of CLE for animal experiments, 100 g of cinnamon leaf powder was mixed with 80% ethanol (500 mL), and the remaining steps were the same as described above, and a total of 145 mL of extract containing 10,000  $\mu$ g/mL of CA was prepared. This extraction process was repeated 20 times using 2 kg of cinnamon leaf powder to prepare CLE with a total of 2900 mL, and 1450 mL was collected for CLEN preparation using the same procedure described in Section 3.4,

while the remaining 1450 mL was directly used for CLE treatment. For administration of high-dose CLEN or CLE (100 mg/kg bw), each rat was fed with 4 mL per day for 8 rats, accounting for a total of 896 mL for 4 weeks, but for low-dose CLEN or CLE (50 mg/kg bw), each rat was fed with 2 mL per day for 8 rats, accounting for a total of 448 mL for 4 weeks. The surplus 106 mL of CLEN or CLE was used in case of any accidental loss.

### 3.3. UPLC-MS/MS Analysis

A Luna omega C18 column (100 mm × 2.1 mm ID, particle size 1.6 µm) coupled with UPLC-MS/MS (Water ACQUITY ultra performance LC system, Water Co., Milford, MA, USA) using a TQS triple quadrupole MS/MS system from Water Co. A mobile phase of 0.025% acetic acid in water (A) and 0.025% acetic in methanol (B) with column temperature at 30 °C and flow rate at 0.3 mL/min, in a gradient mode was used: 83% A and 17% B initially, rose to 20% B in one min, 40% B in 5 min, 55% B in 10 min, 99% B in 12 min and then returned to the original ratio, with the column equilibrium time being 8 min. A total of 15 compounds, including rutin, caffeic acid, quercetin, quercetin-3-O-glucoside, quercetin-3-O-galactoside, benzoic acid, coumarin, *p*-coumaric acid, *trans*-cinnamic acid, eugenol, cinnamaldehyde (CA), cinnamyl alcohol, kaempferol-3-β-D-glucopyranoside, kaempferol, and 5-O-caffeoylquinic acid, were separated within 9 min. Detection was carried out using multiple reaction monitoring (MRM) mode with ESI negative mode. Namely, the cone voltage was 30 V, source offset voltage 30 V, capillary voltage 2.5 kV, nebulizer pressure 7 bar, cone gas flow 150 L/h, desolvation gas flow 800 L/h, and vaporizer temperature 200 °C. Identification was performed by comparing retention time and *m/z* of precursor and product ions of unknown peaks with reference standards, while quantitation was conducted using an external calibration method as described by Huang and Chen [4] and Chen et al. [30]. Briefly, the standard curve of each compound was prepared using 8 concentrations, including 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.02, and 0.01 µg/g for subsequent plotting of concentration against peak area, with the linear regression equation from each standard curve being used for calculation. As a detailed method validation procedure was performed previously by Huang and Chen [4], the method validation was omitted in this study.

### 3.4. Preparation of Cinnamon Leaf Extract Nanoemulsion (CLEN)

For the preparation and characterization of CLEN, CLE was initially prepared by mixing 6.6 g of cinnamon leaf powder and 33 mL of 80% ethanol, sonicating for 2 h, and filtering through a filter paper following the same procedure as shown above in Section 3.2. Next, a mixture composed of 0.6 g of Tween 80 (6%), 0.1 g of soybean oil (1%), and 0.2 g of lecithin (2%) was prepared and poured into a round-bottom flask, followed by adding 1 mL of 99% ethanol, mixing thoroughly, adding 30 mL of CLE, evaporating to remove ethanol, and redissolving in 9.1 mL of deionized water for subsequent sonication at 40 kHz for 30 min to obtain CLEN with a deep-green appearance.

For preparation of CLEN for animal experiments, a total of 1450 mL of CLE sample obtained as described above was divided into 10 portions, and each portion (145 mL) was poured into a round-bottom flask and mixed thoroughly with 8.7 g of Tween 80 (6%), 1.45 g of soybean oil (1%), 2.9 g of lecithin (2%), and 15 mL of 99% ethanol. The mixture was then evaporated to remove ethanol and redissolved in 132 mL of deionized water and sonicated for 30 min to obtain CLEN containing CA at a concentration of 10,000 µg/mL. This procedure was repeated 10 times to prepare a total of 1450 mL CLEN.

### 3.5. Determination of CLEN Characteristics

A total of 100 µL CLEN was collected and diluted 50 times with potassium dihydrogen phosphate buffer (25 mM), after which this mixture was filtered through a 0.22 µm

membrane filter and poured into a polystyrene colorimetric tube for measurement of mean particle size and polydispersity index (PDI) by a dynamic light scattering instrument (DLS, 90 plus particle size analyzer, Brookhaven Instruments, Holtsville, NY, USA) at 25 °C, with the upper limit value of particle velocity being 300–500 kcps. Similarly, 100 µL of CLEN was collected and diluted 50 times with deionized water, after which 300 µL was collected and poured into a sample analysis cell for measurement of zeta potential using an SZ-100 Nanoparticle Size and Zeta Potential Analyzer (HORIBA Scientific Co., Kyoto, Japan) at 25 °C with a range from −200 mV to 200 mV.

The measurement principle of DLS is based on illumination of suspended nanoparticles with a laser beam for detection of the intensity of fluctuations of scattered laser light over time as the nanoparticles move, with the fluctuations being caused by Brownian motion (random motion) and analyzed to measure diffusion coefficient of nanoparticles by a correlator, along with the sample solution's temperature and viscosity for calculation of mean particle size and particle size distribution using the Stokes-Einstein equation, as the diffusion coefficient is related to the hydrodynamic diameter of nanoparticles.

For TEM image analysis, a 100 µL CLEN was collected, and a 20 µL sample was dripped on a carbon-coated copper mesh for settling for 90 s, followed by eliminating superfluous sample with a glass filter paper, negative staining for 30 s with 2% phosphotungstic acid (PTA), removing excess PTA with a filter paper, and placing in a moisture-proof cabinet (desiccator) for complete drying. A transmission electron microscope (TEM) (model JEM-1400, JEOL, Tokyo, Japan) was used to measure the shape and particle size distribution of CLEN by enlarging sample  $3 \times 10^5$  times with a voltage of 120 kVa.

Principally, TEM can generate high-resolution image by shooting a beam of high-energy electrons with very short wavelength through an ultra-thin specimen, with electromagnetic lenses focusing on this electron beam for subsequent interaction with the sample for transmitting and scattering electrons based on the sample's thickness and density, followed by magnification by additional lenses and projection onto a digital detector for creating an image revealing the sample's internal structure (nanoparticle size and shape) at atomic levels, with the denser areas of the sample appearing darker on the image as more electrons are blocked, and lighter areas indicating more electrons have passed through.

For the determination of encapsulation efficiency of CA in CLEN, a method based on Chen et al. [30] was used. Briefly, a 100 µL sample of CLEN was collected and mixed with 400 µL n-hexane, after which this mixture was shaken for subsequent collection of free CA in the upper layer, while another CLEN sample (100 µL) was mixed with 400 µL ethanol for 2 h ultrasonication to release total CA. Then, HPLC was performed using the same separation condition as shown above with UV detection at 280 nm for quantitation and determination of CA encapsulation efficiency using a formula as described by Wang et al. [22].

### 3.6. Stability of CLEN During Storage and Under Gastrointestinal Conditions

For the storage stability study, a portion (2 mL) of CLEN was collected and poured into a tube in triplicate for storage for 12 weeks at 4 °C, with a total of 18 tubes being used, during which 3 tubes were collected every two weeks for determination of mean particle size, PDI, and zeta potential of CLEN.

According to a method reported by Hsu and Chen [81] and Ye et al. [82], the stability of CLEN under gastrointestinal conditions was determined. Gastric fluid was formulated by preparing a mixture containing sodium chloride (2 g/L) and pepsin (3.2 g/L) in deionized water, followed by adjusting the pH to 1.5 using HCl (1 M). Likewise, the intestinal fluid was simulated by mixing  $\text{KH}_2\text{PO}_4$  (8.09 g/L) and bile salts (5.16 g/L) in deionized water and adjusting the pH of the mixture to 7.5 using NaOH (0.1 M). The stability of CLEN under gastric conditions was determined by mixing CLEN (4 mL) with simulated gastric fluid

(16 mL) in a glass beaker and stirring (100 rpm at 37 °C) for 2 h, followed by collecting 1 mL of solution at every 30 min interval, centrifuging (1000 rpm at 4 °C) for 10 min, and diluting the supernatant 10-fold with  $\text{KH}_2\text{PO}_4$  buffer for particle size measurement, while diluting the supernatant 50-fold with deionized water for zeta potential measurement. Similarly, the CLEN stability under intestinal conditions was tested by mixing a gastric-digested CLEN sample with simulated intestinal fluid in a glass beaker and stirring (100 rpm at 37 °C) for 2 h. A 1 mL portion was collected every 30 min and centrifuged (1000 rpm at 4 °C) for 10 min for measurement of particle size after diluting the supernatant 5-fold with  $\text{KH}_2\text{PO}_4$  buffer, as well as zeta potential after diluting the supernatant 10-fold with deionized water. In addition, the encapsulation efficiency of CA in CLEN was determined by collecting a 1 mL sample after 2 h incubation of CLEN separately with gastric fluid and intestinal fluid, followed by repeating the same procedure as described above.

### 3.7. *In Vitro Release of CA Under Gastrointestinal Conditions*

Based on a report by Hsu and Chen [81], the *in vitro* release of CA from CLEN under gastrointestinal conditions was determined by preparing simulated gastric and intestinal fluids as shown above. For *in vitro* release under gastric conditions, a 2 mL sample of CLEN was mixed with 8 mL of gastric fluid in a glass beaker and stirred at 100 rpm for 4 h at 37 °C. A 0.5 mL sample was collected every 30 min and centrifuged (1000 rpm, 4 °C), and the supernatant was diluted 100-fold with methanol for subsequent CA analysis by HPLC. On the other hand, the *in vitro* release under intestinal conditions was determined by mixing CLEN (2 mL) with intestinal fluid (8 mL) in a glass beaker and stirring at 100 rpm for 4 h at 37 °C, followed by collecting 0.5 mL sample every 30 min, centrifuging (1000 rpm at 4 °C), collecting the supernatant, and diluting 100-fold with methanol for subsequent CA analysis by HPLC.

### 3.8. *Animal Experiment*

The Institutional Animal Care and Use Committee (IACUC) of Fu Jen Catholic University (New Taipei City, Taiwan) approved the animal experiment protocols (Approval no. A11312), which were based on standard experimental animal operation procedures. A total of 48 male and 48 female Sprague-Dawley rats of 10 weeks old, each weighing 300–450 g, were purchased from BioLASCO Co. (Taipei, Taiwan). All rats were raised in the Fu Jen Catholic University Experimental Animal Center with a temperature of  $21 \pm 2$  °C, relative humidity at  $55 \pm 10\%$ , and an illumination cycle at 12 h. Both standard rodent chow diet (LabDiet Co., St Louis, MO, USA) and water were provided *ad libitum* during the experiment. Moreover, environmental conditions, including air quality, temperature, humidity, lighting, and noise levels, were standardized across all cages throughout the experiment, along with environmental enrichment in the form of wooden sticks and bedding materials to promote natural behavior and reduce stress. One rat was considered as one experimental unit, and the rats were randomly assigned to individual standard polycarbonate cages with stainless steel wire lids (one rat per cage) by the animal center personnel for subsequent numbering, with the control group first, followed by the induction group and treatment groups. Both position and numbering order of cages were maintained in an open rack, and all researchers involved in this study were informed of group allocation and order of cages during different stages of the experiment, including allocation, conduct of experiment, outcome assessment, and data analysis to minimize potential confounders on experimental outcome. Consequently, the blinding method was not followed in this study. The overall health of rats was monitored once every two days by the animal center personnel. To reduce stress, rats were gently grasped by the tail and supported with the opposite hand while being removed from the cage and subsequently calmed by gentle stroking before

feeding. In addition, the apparent signs of pain in rats, including vocalization, aggressive or defensive behavior, social withdrawal, or self-isolation, were effectively managed with subcutaneous injection of ketoprofen (2–3 mg/kg). It was also established to euthanize rats by CO<sub>2</sub> inhalation if the humane endpoints were encountered, including rapid weight loss, severe weakness with an inability to eat/drink, paralysis, and visible damage to vital organs. However, no expected or unexpected adverse events occurred during the study.

After an adaptation period of one week with each rat weighing ~400 g (11-week-old), 48 male rats were randomly divided into 6 groups ( $n = 8$ ), including group N (normal control), group I (induction), group HN (high-dose CLEN), group LN (low-dose CLEN), group HE (high-dose CLE), and group LE (low-dose CLE), with body weight, food intake, and water consumption being measured every week. To maintain homogeneity of the analyzed parameters, the baseline parameter values between groups prior to treatment were averaged for statistical significance. At the end of the first week, groups I, HN, LN, HE, and LE were induced with ED by a 5 min squeezing of the bilateral cavernous nerves, followed by feeding groups HN with 100 mg/kg bw of CLEN, LN with 50 mg/kg bw of CLEN, HE with 100 mg/kg bw of CLE, and LE with 50 mg/kg bw of CLE daily for 4 weeks. At the end of the 5th week, the penile reflex test of rats in all groups was conducted, followed by pairing with female rats and measuring the sexual behavior. Finally, the male rats were separated from the female rats, and the male rats were further subjected to ICP/MAP measurements and subsequently sacrificed by CO<sub>2</sub> inhalation for the collection of penises for biochemical and histochemical analyses. The selection of high-dose (100 mg/kg bw) and low-dose (50 mg/kg bw) for CLEN and CLE was based on our preliminary tests with rats and several published reports [4,22,30]. Furthermore, as there were no rats died and no weight or behavior change in rats during the experiment, both CLEN and CLE should be non-toxic at these two doses. Figure 10 shows the schematic diagram illustrating the animal experiment design for the improvement of sexual dysfunction by CLEN and CLE in a rat model.

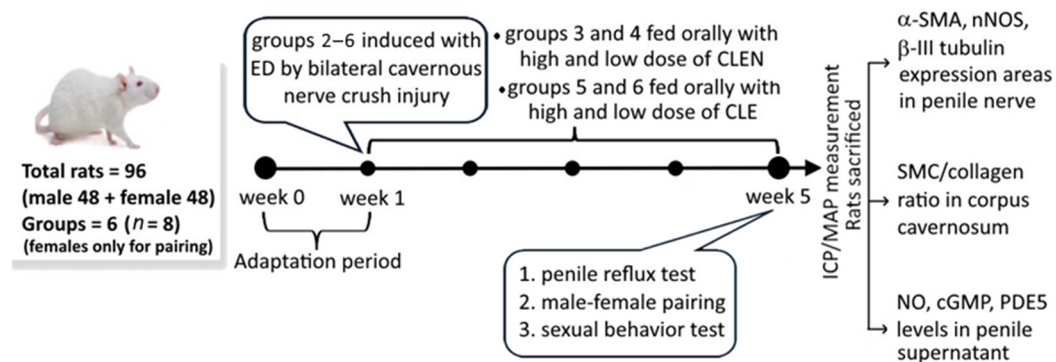


**Figure 10.** A schematic diagram illustrating the study design of animal experiments for improvement of sexual dysfunction by CLEN and CLE in a rat model. CLEN, cinnamon leaf extract nanoemulsion; CLE, cinnamon leaf extract; ED, erectile dysfunction; ICP, intracavernous pressure; MAP, mean arterial pressure; α-SMA, alpha-smooth muscle actin; nNOS, neuronal nitric oxide synthase; β-III tubulin, tubulin beta-2 chain; SMC, smooth muscle cells; NO, nitric oxide; cGMP, cyclic guanosine monophosphate; PDE5, phosphodiesterase type 5.

### 3.9. Establishment of a Rat Model with Erectile Dysfunction

A method based on Wu et al. [83] and Cong et al. [84] was used to establish a rat model for induction with ED. Initially 11-week-old rats were injected separately with a mixture of zoletil 50 and rompun (3:2) at a dose (1 µg/g bw) intraperitoneally for anaesthetization, followed by removing abdomen hair, wiping with iodine solution, midline incision in lower abdomen for prostate exposure, finding posterolateral cavernous nerves and ganglia

(pelvic and sacral) to distinguish injury location, and using non-jagged hemostatic forceps to squeeze bilateral cavernous nerves for 5 min for induction of rats with severe ED. But for group 1 rats, their abdomens were dissected to expose cavernous nerves, and then abdominal wounds were sutured without nerve compression.

### 3.10. Sexual Behavior Test

A method based on Tang et al. [34] and Akhigbe et al. [85] was used to determine the sexual behavior of rats during the fifth week. Prior to pairing with male rats, female rats were individually injected with progesterone (0.5 mg/100 g bw) at the 4th hour and estradiol benzoate (10 mg/100 g bw) at the 48th hour intraperitoneally to promote the estrus state of female rats. Then a male rat and a female rat in estrus state were placed into a transparent acrylic cage (50 cm × 30 cm × 25 cm) under red light. Sexual behaviors, including the climbing of the male rat over the female rat from the rear and catching of the female rat's flank by the front paw of the male rat, vaginal penetration by the male rat's penis, and deeper pushing action of the male rat for ejaculation with the appearance of cross posture, were carefully monitored. Furthermore, the male rat's ejaculation can be judged by the presence of a vaginal plug (suppository) in the female rat. However, if ejaculation failed to occur within 15 min, this experiment should be terminated.

Specifically, the evaluation of penis erection can be divided into non-contact and contact stimulations, with the former simulating the sense of smell and vision and the latter simulating tactility. More elaborately, for the non-contact method, a transparent plastic cage was divided into two halves by a plastic fiber web, and a male rat was placed in a clean compartment to block contact but allow stimulation of hearing, vision, and sense of smell, while a mirror could be used to observe the ejaculation situation. At the same time, a female rat in an estrus state was placed in a compartment on the other side for recording the erection function. Generally, a stick-up of the penile sheath can be regarded as an erection, and a score can be given based on the frequency of penile erection. For the contact evaluation method, a male rat was in a supine position for retracting the preputial sheath (penile sheath) behind the gland, maintaining it for 15 min to induce genital reflex. Total penile reflexes (TPR), a vital index of penile erection and function, are a series of genital reflexes elicited by specific stimulation, including erections (E), quick flips (QF), and long flips (LF). Thus, a TPR formula can be as follows:  $TPR = E + QF + LF$ .

In this study, we used a video recorder to monitor sexual behavior for 30 min by measuring parameters including mount latency (ML), intromission latency (IL), mount frequency (MF), intromission frequency (IF), and ejaculation latency (EL). A minimum decline of 25% for MF and IF and a minimum rise of 25% for ML, IL, and EL are considered as having sexual dysfunction with difficulty in erection [41]. ML can be defined as the time a male rat takes to initiate mounting behavior after a female rat is introduced into a cage, while IL is the time interval from when a male rat first achieves intromission into a female rat during sexual intercourse. For MF, it is the number of mounting times before ejaculation by the male rat, and IF is the number of intromission times by the penis of the male rat into the vagina of the female rat, while EL is the time from the initiation of sexual intromission to ejaculation.

### 3.11. Analysis of Intracavernous Pressure (ICP)

Following surgery for 4 weeks, the cavernous nerves were separated from the abdominal midline incision to distinguish the cavernous roots. A needle (24 G) containing heparin solution at 50 U/mL was inserted into the right cavernous root, connected to a polyethylene 50 tube for measurement of ICP and mean arterial pressure (MAP) using a pressure sensor (MP 36) (BIOPAC Systems, Inc., Goleta, CA, USA). Cavernous nerves were stimulated by

using a bipolar stainless-steel electrode through the formation of a single-phase square pulse from a calculator containing a stimulus isolator with constant current (DS 3), with stimulus parameters being 7.5 mA for amplitude, 20 Hz for frequency, 0.2 ms for pulse width, and 60 s for duration time. Erection response was evaluated based on the maximum ICP, ICP change ( $\Delta$ ICP), total ICP area under curve, minimum ICP, ratio of the maximum ICP to the mean MAP (Max ICP/MAP), and ratio of  $\Delta$ ICP to MAP ( $\Delta$ ICP/MAP).

### 3.12. Measurement of Key Biomolecules in Rats

Following measurement of the erection response in male rats, rats were sacrificed by asphyxiation with carbon dioxide for subsequent anatomy. A method based on Wu et al. [83] was used to isolate penile middle tissue for the immunofluorescence experiment, while the remaining tissues were used to prepare penile supernatant following a method by Ogunro and Yakubu [41] for the determination of key biomolecules associated with erectile dysfunction and oxidative stress. For penile supernatant preparation, the penile tissues were washed with potassium chloride (0.15%) and blot dried for weighing, followed by homogenizing in potassium phosphate buffer, centrifuging at 3500 rpm for 10 min using a microcentrifuge (Thermo Fisher Scientific, Waltham, MA, USA), and collecting the supernatant for measurement of NO, cGMP, and PDE5. For immunofluorescence, the penile middle tissues were fixed with formaldehyde (10%) for 12 h and then dehydrated, post-fixed, and embedded. Next, the cross-section tissues of the penis were dewaxed in xylene and repeated twice, followed by hydration in ethanol, incubation of the slide in blocking buffer for 1 h at room temperature, and subsequent incubation with rabbit anti-neuronal nitric oxide synthase (nNOS), mouse anti-neuron-specific  $\beta$ -III tubulin, and anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) for 1 h at room temperature separately. Then penile tissues were immersed in an antibody bead containing Alexa Flour 488 and Texas Red for 1 h incubation for subsequent observation by a fluorescence microscope. Both nNOS and smooth muscle cells were quantified by calculating the ratio of positive cell area of nNOS to  $\beta$ -III tubulin area of neurons and nerve fibers, and area of  $\alpha$ -SMA under 400 $\times$  and 40 $\times$ , respectively. All nerve tissue morphologies were analyzed using the ImageJ Software (version 1.54r).

### 3.13. Masson Trichrome Staining

Similarly, for Masson trichrome staining, the penile middle tissues were fixed with 10% formaldehyde for 12 h, followed by dehydration, postfixing, embedding, dewaxing the cross-section tissues of the penis in xylene and repeating twice, hydration with ethanol, and staining using a Masson's Trichrome Stain kit (TRM-2, ScyTek Lab., Logan, UT, USA). Initially the penile tissue was fixed with formalin and then with Bouin solution at 56 °C for 1 h to raise staining quality, followed by: washing with tap water to remove yellow color; staining the nucleus with Weigert's iron hematoxylin solution; washing again with tap water; washing again with distilled water; staining with Biebrich scarlet-acid fuchsin; washing with distilled water; and reacting with phosphomolybdic-phosphotungstic acid; washing with distilled water; staining with aniline blue solution; washing with distilled water; dehydration with ethanol; clarification with xylene; fixing with resin fixative; and taking a photograph by a microscope with the collagen color being blue and smooth muscle cell color being red following staining. The tissue morphology was analyzed under 40 $\times$  by ImageJ software (version 1.54r).

### 3.14. Measurement of NO

A commercial sandwich ELISA kit from Sunlong Biotech (Hangzhou, China) was used to determine NO in penile supernatant. Standards and samples were added to the appropriate Microelisa stripplate wells, combined with a specific antibody. Then a horseradish

peroxidase (HRP)-conjugated antibody specific for NO was added to each Microelisa strip-plate well and incubated, while free (uncombined) components were washed away. Then TMB substrate was added to each well, and wells containing NO and HRP-conjugated NO antibody appeared blue, turning yellow after the addition of the stop solution. The optical density (OD) was measured spectrophotometrically at 450 nm, and the NO concentration in samples was calculated based on the standard curve.

### 3.15. Measurement of cGMP

Similarly, a sandwich ELISA kit (Sunlong Biotech, Hangzhou, China) was used for the determination of cGMP in penile supernatant, which is the same as that of NO, with the exception that the Microelisa stripplate is conjugated with a specific antibody of cGMP and the addition of HRP-conjugated antibody specific for cGMP to each Microelisa stripplate well for reaction. Quantitation was carried out using a cGMP standard curve.

### 3.16. Measurement of PDE5

Likewise, a sandwich-ELISA kit (Sunlong Biotech, Hangzhou, China) was used for the determination of PDE5 in penile supernatant, which is the same as that of NO, with the exception that the Microelisa stripplate is conjugated with a specific antibody of PDE5 and the addition of HRP-conjugated antibody specific for PDE5 to each Microelisa stripplate well for reaction. Quantification was performed using a PDE5 standard curve.

### 3.17. Statistical Analysis

All the data were subjected to statistical analysis using Statistical Product and Service Solutions (SPSS, version 30, IBM, Armonk, New York, USA), followed by conducting analysis of variance (one-way ANOVA) and Duncan's multiple range test for significance in mean comparison ( $p < 0.05$ ). One-way ANOVA is usually used to determine the significant difference for mean values of three or more independent sample groups, but it fails to provide information on specific groups showing the significant difference. Therefore, in addition to one-way ANOVA, we used the Duncan post hoc test (multiple range test) as it provides a more powerful multiple comparison between groups with ease and statistical integrity. Data were expressed as mean  $\pm$  standard deviation, and histograms were made using SigmaPlot software (version 14.0, Grafitti LLC, Palo Alto, CA, USA). The recovery was calculated using the following formula:

$$\text{Recovery} = \frac{\text{average value of HN, LN, HE or LE group} - \text{average value of I group}}{\text{average value of N group} - \text{average value of I group}} \times 100$$

## 4. Conclusions

A bilateral cavernous nerve crush injury rat model was employed to evaluate the therapeutic potential of CLEN and CLE in ameliorating ED. Both high and low doses of CLEN markedly restored erectile responses, shortened intromission latency and ejaculation latency, and increased intromission frequency, demonstrating improvements in copulatory performance and ejaculatory capacity. Furthermore, a significant increase in the maximum ICP/MAP ratio was observed, suggesting enhanced neurovascular function after nerve injury for CLEN treatment. Histochemical analysis revealed that CLEN treatment raised the smooth muscle-to-collagen ratio, reduced fibrosis, and enhanced the expression of neuronal markers, highlighting its dual protective effects on neural regeneration and penile tissue architecture, while biochemical assays demonstrated elevated levels of NO and cGMP, accompanied by a significant reduction in PDE5 level. These findings suggest that CLEN was more efficient than CLE in ED improvement through modulation of the NO-cGMP signaling pathway. Thus, CLEN, particularly at high dose (100 mg/kg bw based

on CA-equivalent content), effectively restores erectile function impaired by cavernous nerve injury, underscoring its potential as a natural therapeutic agent for neurogenic or postoperative ED. However, future clinical trials are warranted to verify CLEN treatment in improving human sexual function.

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## Abbreviations

|               |                                              |
|---------------|----------------------------------------------|
| ANOVA         | analysis of variance                         |
| BBB           | blood–brain barrier                          |
| β-III tubulin | tubulin beta-3 chain                         |
| BSA           | bovine serum albumin                         |
| CA            | cinnamaldehyde                               |
| CCSM          | corpus cavernosum smooth muscle              |
| cGMP          | cyclic guanosine monophosphate               |
| CN            | cavernous nerve                              |
| DAPI          | 4',6-diamidino-2-phenylindole                |
| DHA           | docosahexaenoic acid                         |
| DLS           | dynamic light scattering                     |
| DMSO          | dimethyl sulfoxide                           |
| E             | erectile                                     |
| ED            | erectile dysfunction                         |
| EL            | erectile latency                             |
| ELISA         | enzyme-linked immunosorbent assay            |
| GTP           | guanosine triphosphate                       |
| HN            | high-dose cinnamon leaf extract nanoemulsion |
| HE            | high-dose cinnamon leaf extract              |

|               |                                                                  |
|---------------|------------------------------------------------------------------|
| EPA           | eicosapentaenoic acid                                            |
| HPLC          | high-performance liquid chromatography                           |
| HRP           | horseradish peroxidase                                           |
| I             | induction                                                        |
| IACUC         | Institutional Animal Care and Use Committee                      |
| ICP           | intracavernous pressure                                          |
| IF            | intromission frequency                                           |
| IIEF          | International Index of Erectile Function                         |
| IL            | intromission latency                                             |
| JECFA         | Joint FAO/WHO Expert Committee on Food Additives                 |
| kcps          | kilo counts per second                                           |
| LF            | long flips                                                       |
| LN            | low-dose cinnamon leaf extract nanoemulsion                      |
| LE            | low-dose cinnamon leaf extract                                   |
| MAP           | mean arterial pressure                                           |
| MF            | mount frequency                                                  |
| ML            | mount latency                                                    |
| MRM           | multiple reaction monitoring                                     |
| N             | normal/control group                                             |
| ND            | not detected                                                     |
| nNOS          | neuronal nitric oxide synthase                                   |
| NO            | nitric oxide                                                     |
| O/W           | oil in water                                                     |
| PBS           | phosphate-buffered saline                                        |
| PDE5          | phosphodiesterase type 5                                         |
| PDI           | polydispersity index                                             |
| PTA           | phosphotungstic acid                                             |
| QF            | quick flips                                                      |
| ROCK-II       | Rho-associated, coiled-coil containing protein kinase 2          |
| SD            | standard deviation                                               |
| $\alpha$ -SMA | alpha-smooth muscle actin                                        |
| SPSS          | Statistical Product and Service Solutions                        |
| SSRI          | selective serotonin reuptake inhibitors                          |
| STZ           | streptozotocin                                                   |
| TAIF          | Taiwan Forestry Research Institute                               |
| TEM           | transmission electron microscopy                                 |
| TMB           | 3,3',5,5'-tetramethyl-benzidine                                  |
| TPR           | total penile reflexes                                            |
| TQS           | triple quadrupole mass spectrometer                              |
| UPLC-MS/MS    | ultra-performance liquid chromatography-tandem mass spectrometry |
| USFDA         | United States Food and Drug Administration                       |
| W/O           | water in oil                                                     |

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