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An isobolographic analysis of the anti-nociceptive effect of geraniin in combination with morphine or diclofenac

Abstract

Background: Geraniin, a dehydroellagitannin, is a major component of the aqueous extract of the aerial parts of *Phyllanthus muellerianus* (Kuntze) Exell. (*Euphorbiaceae*). Several *Phyllanthus* species are traditionally used for painful disorders. The anti-nociceptive effects of the aqueous extract of the aerial parts of *P. muellerianus* and of geraniin have been scientifically established. The aim of the paper is to determine whether a combination of geraniin and diclofenac or geraniin and morphine leads to better anti-nociceptive effects.

Methods: The nature of the interactions of morphine and diclofenac with geraniin was evaluated by undertaking the isobolographic analysis. Mice were treated with geraniin (3–30 mg/kg), morphine (1–10 mg/kg), and diclofenac (10–100 mg/kg) to obtain the ED_{50} values of the agents in the formalin test. Dose-response curves were then obtained and analyzed after the co-administration of geraniin with morphine or diclofenac in fixed ratio (1:1) combinations based on specific fractions (1/2, 1/4, and 1/8) of their respective ED_{50} values for the formalin test.

Results: Geraniin was less potent than morphine but more potent than diclofenac in the formalin-induced nociception. The isobolographic analysis of geraniin/morphine (G/M) and geraniin/diclofenac combinations (G/D) at different fractions revealed the potentiation of their anti-nociceptive effects. The degrees of potentiation, which were calculated as interaction indices, showed synergism for both combinations in both phase I (G/M: 0.040, G/D: 0.017) and phase II (G/M: 0.004, G/D: 0.002) of the formalin test.

Conclusions: The present study demonstrates synergism for the co-administration of geraniin with both morphine and diclofenac.

Keywords: analgesia; nociception; synergism.

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Introduction

Combination therapy has become one of the mainstays of several disease managements, including pain. Various analgesic combinations, such as aspirin and caffeine, paracetamol and caffeine, as well as ephedrine HCL, paracetamol, and 30 mg caffeine have become the most preferred choices among clients who visit community pharmacies and hospitals. This has been a popular practice because the use of these combinations might facilitate patient compliance, simplify prescribing, and improve efficacy without increasing adverse effects. In special cases, the combination of drugs from different analgesic classes results in synergistic analgesia, but not synergistic adverse effects, thus enabling the patient to achieve increased pain control or comparable control with a lower risk for adverse events [1].

However, there is still a need to search for more effective analgesics with minimal or no side effects at therapeutic doses and possible therapeutic advantages over the existing ones. This need arises from the numerous, life-threatening side effects associated with the use of most analgesics, such as gastric irritation with non-steroidal anti-inflammatory drugs (NSAIDS); the constipation,

tolerance, dependence, and respiratory depression with opioids [2]; and the general dissatisfaction among many sufferers of pain about care. Such a demand forms the basis of multimodal analgesia, whereby individual analgesics are used in optimal doses in order to enhance efficacy and minimize side effects by employing the principle that agents with different mechanisms may have synergistic effects when used in combination to relieve or prevent acute pain [3].

Geraniin (96% w/w, MW = 952.64 g/mol), a dehydroellagitannin, is a pale amorphous compound that is isolated from several *Phyllanthus* species, including *P. muellerianus* (Kuntze) Exell. (*Euphorbiaceae*). *P. muellerianus* is a monoecious, scandent shrub that is distributed widely in tropical and subtropical countries, such as Ghana, Guinea, Sudan, Angola, and Tanzania [4]. The twigs are sucked to prevent toothache and to treat dysmenorrhea as well as a variety of other painful conditions [4]. Geraniin has also been proven to possess strong cellular proliferation effects using primary dermal fibroblasts and human adult high calcium low temperature (HaCaT) keratinocytes [5]. The anti-nociceptive effects of both geraniin and the aqueous extract of *P. muellerianus* has been recently established in chemical-induced nociception models in mice [6]. In that study, geraniin exhibited significant anti-nociceptive effects, which were reduced in the presence of the opioid antagonist naloxone [6]. Recognizing the established anti-nociceptive effects of geraniin [6], the current study seeks to determine whether a combination of geraniin with morphine or geraniin with diclofenac can produce a better (synergistic), additive, or antagonistic effect than the administration of single agents.

Materials and methods

Geraniin

Geraniin (96% w/w HPLC grade) (Figure 1), isolated from the aqueous extract of the aerial parts of *P. muellerianus* (Kuntze) Exell. (*Euphorbiaceae*), was a kind donation by Prof. Andreas Hensel of the Institute of Pharmaceutical Biology and Phytochemistry, University of Muenster, Muenster, Germany.

Animals

The ICR mice (25 ± 5 g) were obtained from the Noguchi Memorial Institute for Medical Research, Ghana, and housed in the *vivarium* of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology, KNUST. They were housed in stainless steel cages (34 × 47 × 15 cm³) in groups of five animals per cage with soft
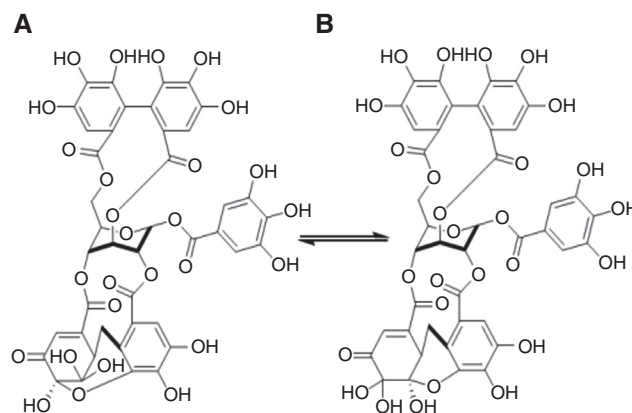

Figure 1: The chemical structures of the two isomers (A and B) of geraniin.
(Adopted from Agyare et al. [5].)

wood shavings as bedding in a 12 h light/dark cycle. Food (normal mice chow: Agricare Ltd, Kumasi, Ghana) and water (tap water) were given *ad libitum*. All procedures and techniques used in these studies were in accordance with the guidelines for the capture, handling, and care of mammals [7]. All protocols used were approved by the Departmental Ethics Committee (No.: FPPS/PCOL/0019/2013).

Drugs and chemicals

Diclofenac sodium was purchased from Troge Medical GmbH (Hamburg, Germany), morphine hydrochloride was obtained from Bodene (PTY) Limited Trading (Port Elizabeth, South Africa), and formalin was purchased from British Drug Houses (Poole, England). Normal saline was used as the vehicle for all drug preparations.

Methods

Determination of ED_{50s} in the formalin-induced nociception test: The formalin test was carried out as described by [8] to determine the ED_{50s} of geraniin, diclofenac, and morphine. Mice were acclimatized to the test chambers (15 × 15 × 15 cm³) for 30 min before formalin injection. Ten groups of mice (n = 5) were then pre-treated with vehicle, geraniin (3, 10, 30 mg/kg, p.o.), morphine (1, 3, 10 mg/kg, i.p.) or diclofenac (10, 30, 100 mg/kg, i.p.) for 60 min (p.o.) or 30 min (i.p.) before the intraplantar injection of 10 μL of 5% v/v formalin. The mice were returned individually into the testing chamber after the formalin injection and their nociceptive behaviors (licking/biting of the injected paw) were captured for 1 h for analysis using the public domain Software JWatcher™, Version 1.0. The average nociceptive score per 5 min time block was calculated by multiplying the frequency and time spent in biting/licking and data were expressed as the mean ± SEM of scores between 0 and 10 min (phase I) and 10 and 60 min (phase II) after formalin injection. Doses for 50% of the maximal effect (ED_{50}) for each drug were determined by using an iterative computer least squares method.

Isobolographic analysis of geraniin/morphine and geraniin/ diclofenac combinations: The ED_{50} values obtained initially were

selected as the equi-effective doses for the isobolographic analysis. The dose combinations of geraniin and morphine as well as geraniin and diclofenac were calculated in fixed ratio (1:1) combinations based on the following fractions: 1/2, 1/4, 1/8 of the respective ED_{50s} [9–11]. Mice (n = 5) received these combinations once, after which the formalin test was conducted and scored as previously described.

An isobologram (a cartesian plot of pairs of doses that, in combination, yield a specified level of effect) was then created by connecting the theoretical ED_{50} values of morphine or diclofenac plotted on the ordinate and geraniin plotted on the abscissa to obtain the additivity line. For each drug mixture, the experimental ED_{50} values and their associated 95% confidence intervals (CI) were determined by linear regression analysis of the log dose-response curve (and compared by a t-test to a theoretical additive ED_{50}. The value can be obtained by using the formula

$$Z_{add} = f\ (ED_{50})\ \text{of morphine or diclofenac} + (1-f)(ED_{50})\ \text{of geraniin},$$

where f is the fraction of each component in the mixture and the variance (Var) of Z_{add} is calculated as

$$VarZ_{add} = f^2(Var\ ED_{50})\ \text{of morphine or diclofenac} + (1-f)^2\ Var(ED_{50})\ \text{of geraniin}.$$

From these variances, the SEM values were calculated and resolved according to the ratio of the individual drugs in the combination. A supra-additive or synergistic effect is defined as the effect of a drug combination that is higher and statistically different (ED_{50} significantly lower) than the theoretically calculated equi-effect of a drug combination in the same proportion. If the ED_{50} values are not statistically different, the effect of the combination is additive. In this study, the degree of interaction was calculated using fractional analysis by dividing the experimental ED_{50} (Z_{mix}) by the theoretical ED_{50} (Z_{add}).

Statistical analysis

All data are presented as mean ± SEM (n = 5). The time-course curves were subjected to two-way (treatment × time) repeated measures analysis of variance (ANOVA) with Dunnett's multiple comparisons test. The total nociceptive score for each treatment was calculated in the arbitrary unit as the area under the curve (AUC). Differences in AUCs were analyzed using the Kruskal-Wallis test with drug treatment as a between-subjects factor. Further comparisons between the vehicle- and drug-treated groups were performed using Dunn's multiple comparison test. The doses for 50% of the maximal effect (ED_{50}) for each drug were determined by using an iterative computer least squares method. The method uses the nonlinear regression (three-parameter logistic) equation given by

$$Y = \frac{a+(b-a)}{(1+10^{(LogED_{50}-x)})},$$

where x is the logarithm of the dose and Y is the response. Y starts at a (the bottom) and goes to b (the top) with a sigmoid shape. The fitted midpoints (ED_{50s}) of the curves were compared statistically using the
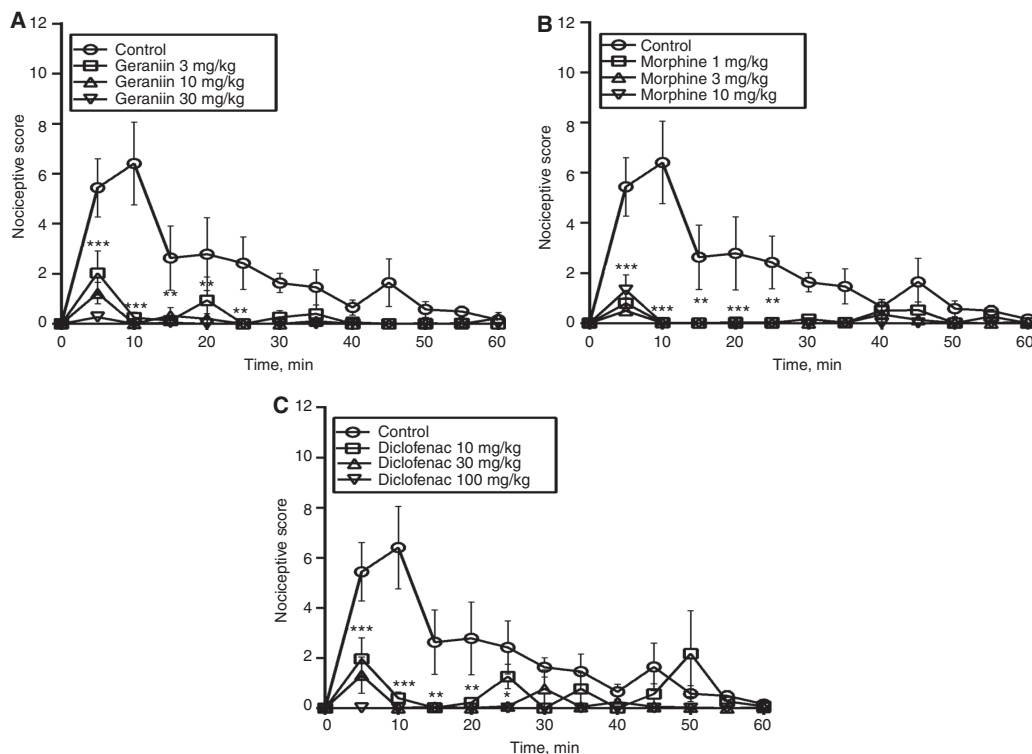

Figure 2: The time-course effects of geraniin (3–30 mg/kg) (A), morphine (1–10 mg/kg) (B) and diclofenac (10–100 mg/kg) (C) on formalin-induced nociception in mice.
**p ≤ 0.01, ***p ≤ 0.001 compared with the control at same time point (two-way ANOVA followed by the Bonferroni *post hoc* test).
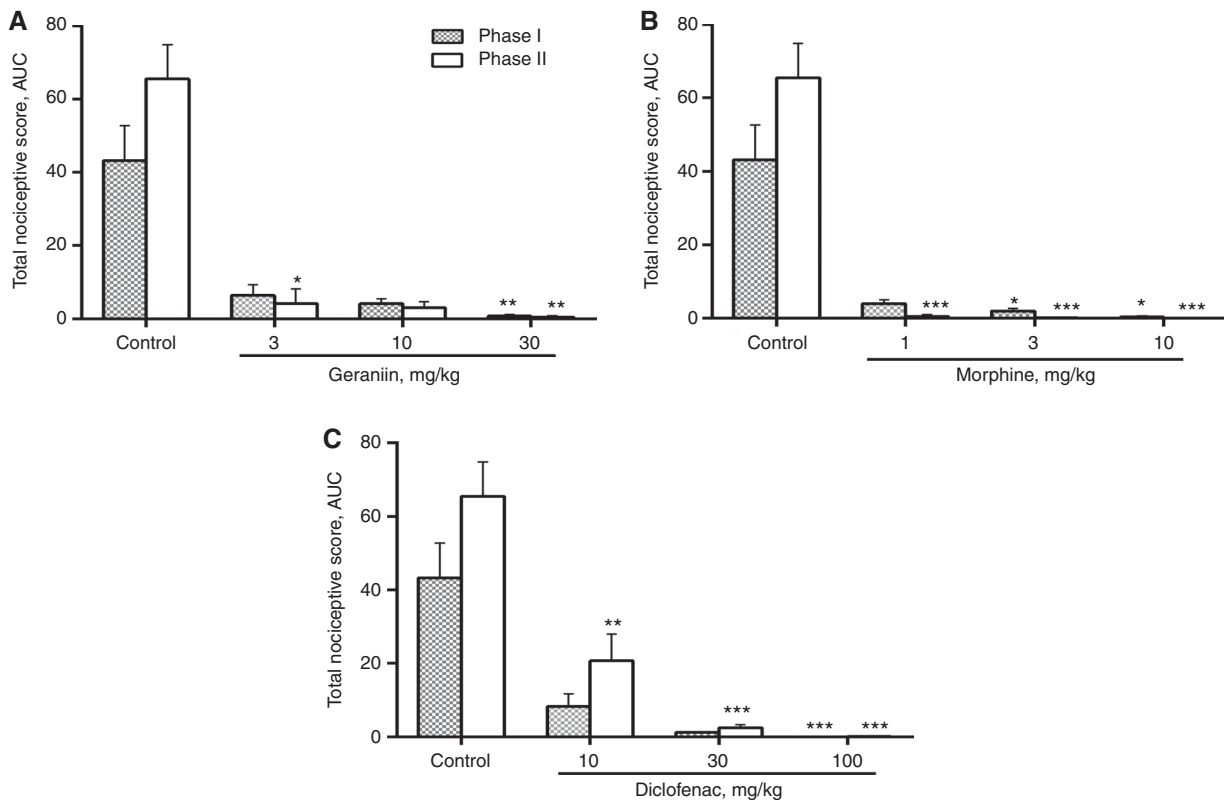
Figure 3: The total anti-nociceptive effects of geraniin (3–30 mg/kg) (A), morphine (1–10 mg/kg) (B) and diclofenac (10–100 mg/kg) (C) in phase I and phase II of the formalin-induced nociception.
*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 compared with the phase I and II control groups.

F-test. GraphPad Prism for Windows 6.0 was used for all statistical analyses and ED_{50} determinations. Here, p-values <0.05 was considered statistically significant.

The isobolographic calculations were performed with the program Pharm Tools Pro (version 1.27, the McCary Group Inc., PA, USA). Results are presented as mean ± SEM. The statistical analyses of the isobolograms were performed as described by [12] and the statistical difference between experimental and theoretical values was assessed by the student's t-test for independent means. Here, p-values <0.05 were considered significant.

Results

Determination of ED_{50s} in the formalin-induced nociception test

The administration of geraniin (3–30 mg/kg), diclofenac (10–100 mg/kg), and morphine (1–10 mg/kg) individually to the mice significantly attenuated the nociceptive response induced by formalin, as shown by the time-course curves (Figure 2). Two-way ANOVA (treatment × time) revealed the significant (geraniin: $F_{36, 204} = 2.962$, p<0.0001; morphine: $F_{36, 204} = 3.352$, p<0.0001; diclofenac: $F_{36, 204} = 2.897$, p<0.0001)

effects of the drug treatments on the formalin-induced nociception. All the agents showed significant anti-nociceptive effects in both the neurogenic phase (phase I) and inflammatory phase (phase II) of the formalin test (Figure 3). From the ED_{50} values presented in Table 1, morphine was the most potent of the three agents in both phases, followed by geraniin, and then diclofenac in that order (Table 1).

Isobologram of geraniin and morphine

Geraniin, morphine, and the fractions of geraniin and morphine combinations inhibited both neurogenic and

Table 1: The ED_{50} values of morphine, geraniin, and diclofenac in both phases of the formalin test.

Drugs	Phase I ED_{50}, mg/kg	Phase II ED_{50}, mg/kg
Morphine	0.14 ± 0.10	0.33 ± 0.19
Geraniin	0.94 ± 0.60	1.02 ± 0.67
Diclofenac	14.51 ± 10.03	21.30 ± 15.17

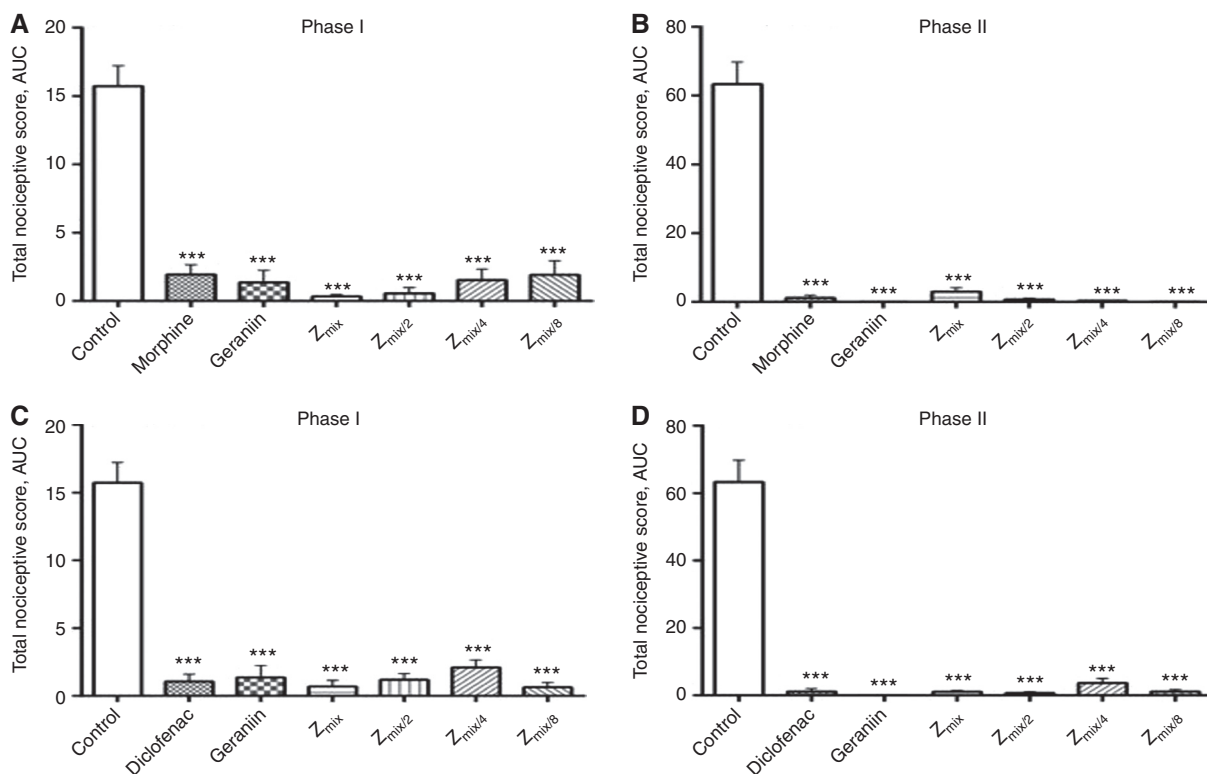Values are expressed as mean ± SEM (n = 5).

Figure 4: The total anti-nociceptive effects of geraniin (3–30 mg/kg), morphine (1–10 mg/kg) and fractions of their combination (Z_{mix} $Z_{mix/2}$ $Z_{mix/4}$ $Z_{mix/8}$) in phase I (A) and phase II (B) and of geraniin (3–30 mg/kg), diclofenac (10–100 mg/kg) and the fractions of their combination (Z_{mix} $Z_{mix/2}$ $Z_{mix/4}$ $Z_{mix/8}$) in phase I (C) and phase II (D) of the formalin-induced nociception.
$*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ compared with the respective controls (one-way ANOVA followed by Dunnett's multiple comparisons test).

inflammatory pain in the formalin test (Figure 4A and B). The theoretical additive ED_{50} (Z_{add}) was computed as 0.54 ± 0.19 mg/kg for phase I and 0.68 ± 0.14 mg/kg for phase II as shown in Table 2.

The experimental ED_{50} (Z_{mix}) obtained by non-linear regressional analysis for phase 1 (Figure 5A) was 0.022 ± 0.005 mg/kg and 0.003 ± 0.002 mg/kg for phase II (Figure 5B), both indicating the potentiation of the anti-nociceptive effect of the two drugs.

The degree of potentiation was found to be synergistic as depicted by the calculation of the interaction index by fractional analysis for phase I and phase II and as graphically displayed by Z_{mix} lying below the line of additivity (Figure 6A and B, respectively) of the isobologram.

Isobologram of geraniin and diclofenac

Geraniin, diclofenac, and the fractions of geraniin and diclofenac combinations inhibited both neurogenic and inflammatory pain in the formalin test (Figure 4C and D). The theoretical additive ED_{50} (Z_{add}) was computed as

Table 2: The theoretical and experimental $ED_{50} \pm$ SEM values of geraniin and morphine in both phases of the formalin-induced nociception with their computed interaction indices.

Combinations	Phase I	Phase II
Theoretical ED_{50}, mg/kg	0.540 ± 0.190	0.680 ± 0.140
Experimental ED_{50}, mg/kg	0.022 ± 0.005[a]	0.003 ± 0.002[b]
Interaction index	0.040	0.004
Drugs ratio	6.710:1	3.090:1

[a]$p < 0.05$, [b]$p \leq 0.01$ compared experimental ED_{50} to theoretical ED_{50}.
Values are expressed as mean $\pm$ SEM.

7.73 ± 2.00 mg/kg for phase I and 11.16 ± 3.08 mg/kg for phase II (Table 3).

The experimental ED_{50} (Z_{mix}) obtained by non-linear regressional analysis for phase I (Figure 7A) was 0.13 ± 0.044 mg/kg and 0.019 ± 0.016 mg/kg for phase II (Figure 7B), both indicating the potentiation of the anti-nociceptive effects of the two drugs. The degree of potentiation was found to be synergistic as depicted by the calculation of the interaction index by fractional analysis for phase I and phase II and as graphically displayed by
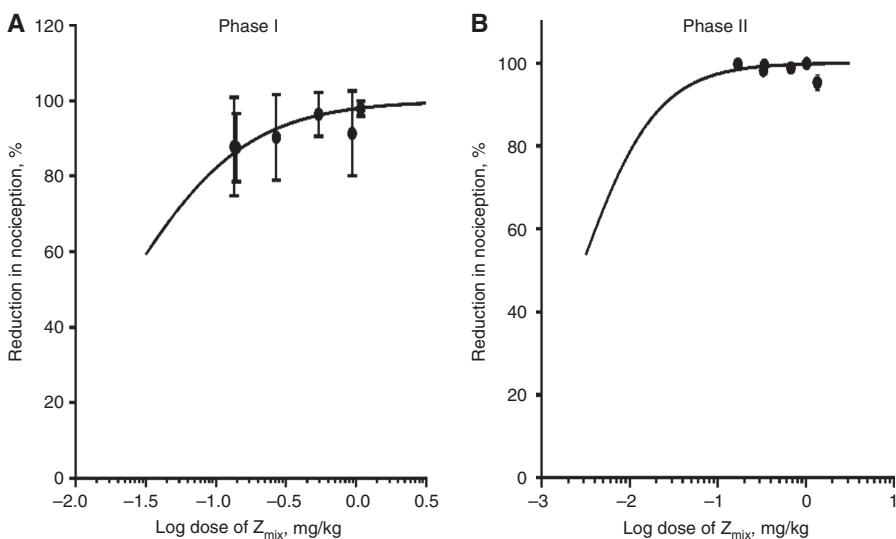
Figure 5: The dose-response curves for geraniin and morphine and the fractions of their combination for (A) phase I and (B) phase II of formalin-induced nociception, respectively.
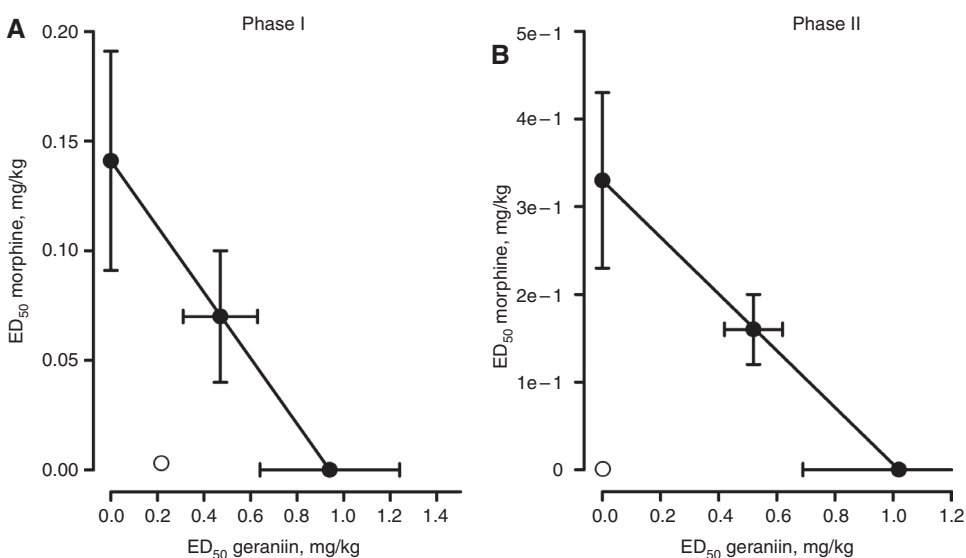

Figure 6: The isobolograms for the combination of morphine and geraniin in (A) phase I and (B) phase II of the formalin-induced nociception in mice.
Filled circles (●) are the theoretical $ED_{50} \pm SEM$ and open circles (O), the experimental $ED_{50} \pm SEM$.

Table 3: The theoretical and experimental $ED_{50} \pm SEM$ values of diclofenac and geraniin for both phases of the formalin test with their computed interaction indices.

Combinations	Phase I	Phase II
Theoretical ED_{50}, mg/kg	7.73 ± 2.00	11.16 ± 3.08
Experimental ED_{50}, mg/kg	0.13 ± 0.044[a]	0.019 ± 0.016[a]
Interaction index	0.017	0.002
Drugs ratio	1:0.065	1:0.048

[a]$p \leq 0.01$ compared experimental ED_{50} to theoretical ED_{50}. Values are expressed as mean $\pm$ SEM.

Z_{mix} lying below the line of additivity (Figure 8A and B, respectively) of the isobologram.

Discussion

Pain is usually a warning signal that provokes protective mechanisms, but sometimes, this warning system becomes faulty and the pain becomes chronic and debilitating in that case [13]. Due to the multifaceted nature of
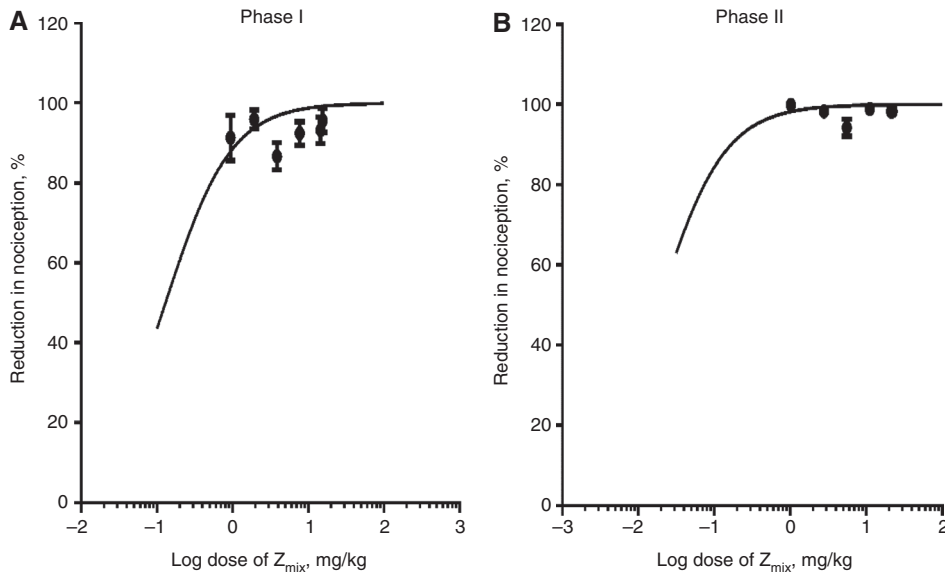
Figure 7: The dose-response curves for geraniin and diclofenac and fractions of their combination for (A) phase I and (B) phase II of the formalin-induced nociception, respectively.
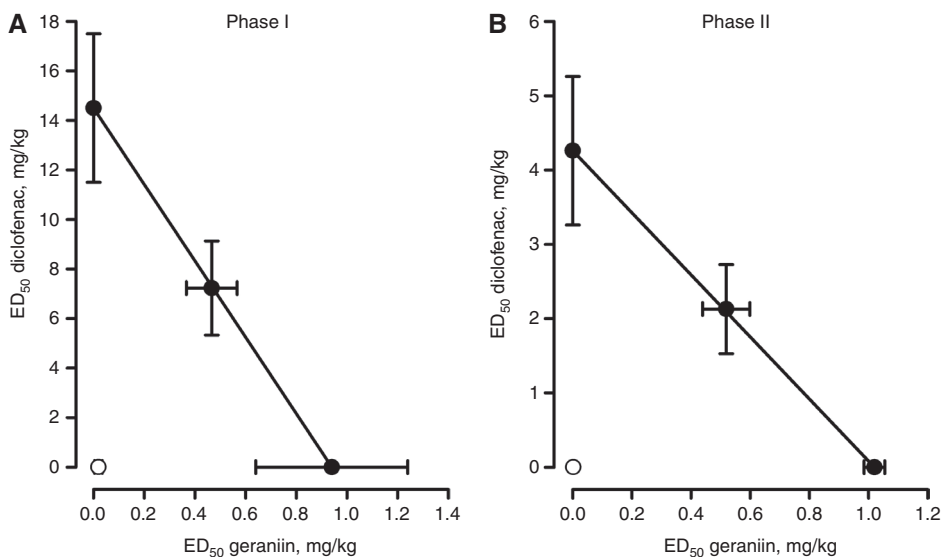

Figure 8: The isobolograms for the combination of diclofenac and geraniin in (A) phase I and (B) phase II of the formalin-induced nociception in mice.
Filled circles (●) are the theoretical $ED_{50} \pm SEM$ and open circles (O), the experimental $ED_{50} \pm SEM$.

pain, treatment can often benefit from, or necessitate, a combination of mechanistic approaches. Combining analgesic agents to achieve this goal is a rational therapy for pain [1]. The potential benefits of analgesic combinations are straightforward, such as the opportunity for better patient compliance and enhanced efficacy, as well as the potential for a reduction of undesired effects [14].

The administration of geraniin/morphine (G/M) and geraniin/diclofenac (G/D) combinations produced

anti-nociceptive effects in the formalin-induced nociception test, which were greater than what could be achieved with the administration of the individual drugs alone. The formalin test, a tonic model of continuous pain resulting from the formalin-induced tissue injury, is a useful model, particularly for the screening of novel compounds as the nociception produced in this test involves inflammatory, neurogenic, and central mechanisms [15]. Again, this test was employed to evaluate the possible interaction

between the concomitant administration of diclofenac/morphine and geraniin because it is considered the most predictive of acute pain and is believed to be a more valid model for clinical pain [8, 15]. The first phase, which is transient, is caused by the direct effect of formalin on the transient receptor potential ankyrin subtype 1 receptors (TRPA 1) [16], whereas the second prolonged phase is associated with the combination of an inflammatory reaction in the peripheral tissue. This reaction causes a release of nociceptive mediators, such as serotonin, histamine, bradykinin, and prostaglandins, which subsequently cause the sensitization of the central neurons leading to changes in the central processing of pain [17]. While it is a well-known fact that centrally-acting drugs, such as narcotics inhibit nociception in both phases equally [18], some studies have shown that diclofenac can also inhibit both phases of the formalin-induced nociception [19, 20] even though a greater effect is observed in the inflammatory phase.

The co-administration of geraniin and morphine resulted in a synergistic effect in both phases of the formalin test. However, the interaction was more effective in the inflammatory phase compared with that in the neurogenic phase. Based on the principle of independent joint action, the co-administration of geraniin and morphine may activate different pathways to produce a synergistic effect as additivity would have been realized if only a single pathway was activated by both drugs [12]. Geraniin has been previously shown to have opioidergic effects [6], which may help intensify the opioidergic effects of morphine, hence the enhanced anti-nociceptive effects. Moreover, geraniin has also been shown to exhibit significant anti-inflammatory and anti-oxidant activity [21, 22]. This, coupled with that fact that geraniin – when used alone, was more effective in the second phase (inflammatory phase) of the formalin-induced nociception test [6], may explain the reason why the combination of geraniin and morphine was more effective in the inflammatory phase. Another possible reason why the interaction was more effective in the second phase rather than the first phase could be that many more mechanisms may be involved in the alleviation of the inflammatory pain in the second phase of the formalin test. It could also be that morphine and geraniin share the majority of the common mechanisms needed to alleviate the neurogenic pain rather than the inflammatory pain [10]. Even though, the exact mechanism of the interaction has not been elucidated at present, the current study has proven that there is a lot of merit in administering the two agents together as smaller amounts of the agents are used and this could possibly lead to a better side effect profile.

Diclofenac is a non-steroidal anti-inflammatory agent known to act by the inhibition of cyclo-oxygenase enzymes and, thus, the inhibition of prostaglandin synthesis. The co-administration of diclofenac and geraniin also resulted in a synergistic effect in both phases, with a greater effect seen in the inflammatory phase. This could be a result of geraniin and diclofenac having different but efficient mechanisms of inhibiting the release and/or effect of the nociceptive mediators, such as serotonin, histamine, bradykinin, and prostaglandins, which subsequently cause the sensitization of the central neurons that, in turn, lead to changes in the central processing of pain [17].

Nevertheless, synergism was seen in both combinations, and it was observed that the G/M combination was more potent than the G/D combination. This realization was not very surprising as geraniin and morphine were far more potent than diclofenac after the individual administration of these three agents. This study has shown that geraniin at low doses may be given in combination with morphine or diclofenac to effectively alleviate both the neurogenic and inflammatory pain in mice.

Conclusions

The G/D and G/M combinations exhibited marked potentiation after the isobolographic analysis of the combination in the neurogenic and inflammatory phases of the formalin test. The degree of potentiation revealed synergism in both phases of the formalin test for both combinations.

References

1. Raffa RB. Pharmacology of oral combination analgesics: rational therapy for pain. J Clin Pharm Ther 2001;26:257–64.
2. Becker DE. Pain management: part 1: managing acute and post-operative dental pain. Anesth Prog 2010;57:67–79.

3. Buvanendran A, Kroin, JS. Multimodal analgesia for controlling acute postoperative pain. Curr Opin Anesthesiol 2009;22: 588–93.

4. Ben-Bala KD. Phyllanthus muellerianus (Kuntze) Exell. [Internet] Record from PROTA4U. Schmelzer GH & Gurib-Fakim A (Editors). PROTA (Plant Resources of Tropical Africa/Ressources végétales de l'Afrique tropicale), Wageningen, Netherlands. Available at: http://www.prota4u.org/search.asp. Accessed: 5 October 2016.

5. Agyare C, Lechtenberg M, Deters A, Petereit F, Hensel A. Ellagitannins from Phyllanthus muellerianus (Kuntze) Exell.: Geraniin and furosin stimulate cellular activity, differentiation and collagen synthesis of human skin keratinocytes and dermal fibroblasts. Phytomedicine 2011;18:617–24.

6. Boakye-Gyasi E, Kasanga EA, Biney RP, Mensah KB, Agyare C, Woode E. Anti-nociceptive effects of geraniin and an aqueous extract of the aerial parts of Phyllanthus muellerianus (Kuntze) Exell. in murine models of chemical nociception. Iran J Pharm Sci 2016;12:17–30.

7. Animal Care and Use Committee. Guidelines for the capture, handling, and care of mammals as approved by the American Society of Mammalogists. J Mammal 1998;79:1416–31.

8. Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 1977;4:161–74.

9. Miranda HF, Noriega V, Zepeda RJ, Sierralta F, Prieto JC. Systemic synergism between codeine and morphine in three pain models in mice. Pharmacol Rep 2013;65:80–8.

10. Woode E, Ameyaw EO, Abotsi WK, Boakye-Gyasi E. An isobolographic analysis of the anti-nociceptive effect of xylopic acid in combination with morphine or diclofenac. J Basic Clin Pharma 2015;6:103–8.

11. Ameyaw EO, Woode E, Kyei S, Biney RP, Boampong JN. Anti-nociceptive synergism of pregabalin and xylopic acid co-administration in paclitaxel-induced neuropathy: isobolographic analysis. Pharmacogn J 2015;7:363–68.

12. Tallarida RJ. An overview of drug combination analysis with isobolograms. J Pharmacol Exp Ther 2006;319:1–7.

13. Julius D, Basbaum AI. Molecular mechanisms of nociception. Nature 2001;413:203–10.

14. Tallarida RJ, Cowan A, Raffa RB. Anti-nociceptive synergy, additivity, and subadditivity with combinations of oral glucosamine plus non-opioid analgesics in mice. J Pharmacol Exp Ther 2003;307:699–704.

15. Tjølsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. Pain 1992; 51:5–17.

16. McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, et al. TRPA1 mediates formalin-induced pain. Proc Natl Acad Sci 2007;104:13525–30.

17. Santa-Cecília FV, Freitas LA, Vilela FC, Veloso Cde C, da Rocha CQ, Moreira ME, et al. Anti-inflammatory and anti-nociceptive effects of Garcinia brasiliensis. J Ethnopharmacol 2011;133:467–73.

18. Hunskaar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 1987;30:103–14.

19. Asomoza-Espinosa R, Alonso-López R, Mixcoatl-Zecuatl T, Aguirre-Bañuelos P, Torres-López JE, Granados-Soto V. Sildenafil increases diclofenac anti-nociception in the formalin test. Eur J Pharmacol 2001;418:195–200.

20. Santos AR, Vedana EM, De Freitas GA. Anti-nociceptive effect of meloxicam in neurogenic and inflammatory nociceptive models in mice. Inflamm Res 1998;47:302–7.

21. Boakye YD, Agyare C, Dapaah SO. In vitro and in vivo antioxidant properties of Phyllanthus muellerianus and its major constituent, geraniin. Oxid Antioxid Med Sci 2016;5:70–8.

22. Boakye YD, Agyare C, Abotsi WK, Ayande PG, Ossei PP. Anti-inflammatory activity of aqueous leaf extract of Phyllanthus muellerianus (Kuntze) Exell. and its major constituent, geraniin. J Ethnopharmacol 2016;187:17–27.