

# Combination Interactive Effects of *Gongronema latifolium* Leaves and *Picralima nitida* Seeds Extracts on Glucose Tolerance

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## Abstract

This study evaluated the combination interactive effects of *G. latifolium* leaves and *P. nitida* seed extracts using a metabolic glucose tolerance test. The plant samples were extracted separately using cold maceration and their acute toxicities were determined. Dose-response glucose tolerant tests of both plants were done using a 2 g/kg glucose load monitored over 0 – 1h. A 41% effect isobologram was used to determine the needed dose combinations according to the principle of Loewe's additivity model. The glucose tolerant tests of dose pairs of the combined extracts were evaluated and their combination indices were calculated to determine the nature of their interaction. The ED<sub>41</sub> of *G. latifolium* (GL) and *P. nitida* (PN) were 180 mg/kg and 254 mg/kg respectively. The percentage reductions of GL:PN (50:160); GL:PN (100:90); and GL:PN (150:30) dose pairs were 48.23, 50.76 and 42.99 % respectively. Their combination index were calculated to be 0.91, 0.91 and 0.95 respectively - an indication of synergistic interaction. Findings from this study validate the combined use of *G. latifolium* leaves and *P. nitida* seeds in folkloric medicine. However, combining the extracts of *G. latifolium*: *P. nitida* in the dose ratios of 50:160, 100:90 and 150:30 mg/kg gave the best dose pairs with synergistic outcome.

**Keywords:** Combination studies; *Gongronema latifolium*; glucose tolerance; *Picralima nitida*.

## INTRODUCTION

Plants have evolved over millennia to address the multifactorial nature of disease pathogenesis by targeting multiple pathways associated with disease initiation, progression and complications through the combined action of structurally and functionally diverse constituents (Zaynab et al., 2018). This diversity in plants is better harnessed when they are used in crude unfractionated forms and in combination (Che et al., 2013). It is however too simplistic to assume a positive synergy from any two or more herbs combined in traditional practice as some may lead to an unexpected decrease in activity due to competition for the same site of action or through interfering with the pharmacokinetics of each other (Sun et al., 2019). Similarly, two plants when combined can exhibit both antagonism and synergism depending on the dose pair that is used (Fouquier and Guedj, 2015). The benefit of combination therapy is not simply attributed to the properties of the drugs but could also depend on the dose ratio. As the cells do not make the difference between a single dose and a combination, two drugs combined at a given dose ratio could be considered as a third agent with

its own dose-effect relation (Chou, 2010). The establishment of optimal pair of dose levels for each plant extract intended to be used in combination therapy is therefore indispensable in maximizing the treatment efficacy of combination therapy.

The use of optimal dose pairs in combination therapies exploits the chances for improved efficacy and decreased adverse effects (Podolsky & Greene, 2011). Thus, owing to these advantages, combination therapies have become a standard for the treatment of several diseases (Raskin, 2008). As such, they continue to represent a promising approach in indications of unmet medical needs.

Glucose intolerance is a general term for a group of metabolic conditions that result from hyperglycemia. It is a dysglycemic state that comprises both intermediate hyperglycemia (formerly called prediabetes) and diabetes. The major categories of glucose intolerance are as follows: Impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and diabetes mellitus (DM). IFG and IGT are intermediate conditions in the transition between normality (normal glucose tolerance, NGT) and diabetes (WHO, 2021). The development of glucose intolerance is usually initiated by insulin resistance and

worsened by compensatory hyperinsulinemia (Wang *et al.*, 2019). Pancreatic beta-cell dysfunction and/or hepatic and muscle insulin resistance are primary defects responsible for the development and progression of type-2 diabetes. Impaired glucose tolerance represents an early stage in the development of type-2 diabetes. Although it can present as the asymptomatic condition with subtle changes in fasting and/or postmeal serum glucose concentration, it is an important indicator in identifying patients at high risk of developing type-2 diabetes (Alok *et al.*, 2018).

Several studies have demonstrated the medicinal and beneficial effects of *Gongronema latifolium* and *Picralima nitida* plants. Both plants are members of the Apocynaceae family which have been used in traditional medicines for the treatment and management of malaria, abscesses, hepatitis, pneumonia, diabetes, hypertension, etc. (DeCampos *et al.*, 2020). The leaves of *G. latifolium* and seeds of *P. nitida* have been specifically reported to possess glucose lowering potential both in diabetes and normoglycemic *in-vivo* experimental models (Mohammed *et al.*, 2014). However, none of these reports have considered a combined effect of both plants, a common practice in traditional medicine. This study evaluated the combination interactive effects of *G. latifolium* leaves and *P. nitida* seed extracts using a metabolic glucose tolerance test (MGTT) in an animal model.

## MATERIALS AND METHODS

### Collection of plant materials

The seeds of *Picralima nitida* and leaves of *Gongronema latifolium* were obtained from Nsukka, Enugu State, Nigeria. The plant samples were identified by Mr Felix Nwafor, a taxonomist of the Department of Pharmacognosy and Environmental Medicine, University of Nigeria Nsukka (UNN), Enugu State, Nigeria.

### Animals

Wistar albino mice (20 – 25 g) were obtained from the Animal House of the Department of Pharmacology, Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology, Enugu State, Nigeria. The animals were housed in standard laboratory conditions of 12 h light, room temperature, 40-60% relative humidity and fed with rodent feed (Guinea Feeds Nigeria Ltd). They were allowed free access to food and water. All animal experiments were conducted in compliance with the NIH guide for the care and use of laboratory animals (National Institute of health (NIH) (2011) Pub No: 85-23).

### Preparation of plant extracts

The pods of *Picralima nitida* were opened and the seeds obtained were air-dried under a shade for 30 days. Similarly, the fresh plant sample of *Gongronema*

*latifolium* was collected from the wild fields and the leaves were detached from the unwanted parts of the plant. The leaves were air dried under a shade at room temperature for 15 days. After air-drying under a shade, the seeds and leaves were pulverized to a coarse powder using an electrical blender.

Pulverized samples of *Picralima nitida* (1kg) and *Gongronema latifolium* (1.6 kg) were macerated separately in 1.5 L each of methanol at room temperature for 72 hours with intermittent shaking. The extracts were first filtered, using a cotton wool clogged funnel and then filtered severally through Whatman No. 1 filter papers. The extracts were concentrated by evaporating the methanol to dryness under reduced temperature and pressure (below 40°C) using a rotary evaporator. The percentage yields of the extracts were determined and then transferred into air-tight containers and stored in a refrigerator until required for experimentation.

### Qualitative phytochemical analysis

The qualitative phytochemical analysis of the extracts was carried out using standard methods as described by Odoh *et al.*, 2019.

### Acute toxicity study

The acute toxicities of both samples were done using Lorke's Method (Lorke, 1983). This method has two phases which are phases 1 and 2 respectively.

Phase 1: In this phase, nine animals were randomly selected to represent the *G. latifolium* group and another nine were selected to represent the *P. nitida* group. For each group, the nine animals were divided into three sub-groups containing three animals per sub-group. Members of each of the three sub-groups were administered 10 mg/kg, 100 mg/kg and 1000 mg/kg of each of the plant samples respectively. The animals were then placed separately under 24-hour observation to monitor for any sign of behavioral changes as well as mortality that may suggest toxicity.

Phase 2: This phase involved the use of three animals per plant sample. These animals were distributed into sample groups of one animal per group. The animals were then administered higher doses (1600, 2000 and 5000 mg/kg) for *G. latifolium* and 300, 500 and 800 mg/kg for *P. nitida*. They were observed for 24h post treatment for signs of toxicity as well as mortality. Then the LD<sub>50</sub> was determined using the following equation:

$$LD = \sqrt{(D_0 * D_{100})} \quad (1)$$

D<sub>0</sub> : Highest dose that gave no mortality,

D<sub>100</sub> : Lowest dose that produced mortality.

### Dose response Glucose tolerance effect of extracts of *G. latifolium* leaves and *P. nitida* seeds

The animals were fasted overnight before the test and their fasting blood glucose levels were measured (FBG).

1hr after the extracts were given, the animals were administered 2 g/kg D-glucose in distilled water. Blood samples were taken by tail milking at 15, 30, 45 and 60 minutes after glucose administration and the glucose concentrations were determined. In comparison to the control group, the area under the curve (AUC) of the plot of blood glucose against time was used to measure metabolic glucose tolerance.

For dose-response effect of methanol extract of *G. latifolium* leaves, the animals were grouped into 5 groups of 5 mice each. They fasted for 12 h, but water was provided *ad libitum* before the experiment. The fasting blood glucose of each animal was determined before extracts were administered. Groups 1-5 were treated with 12.5, 25, 50, 100, and 200 mg/kg, respectively. Change in blood glucose levels was accessed for each animal at 15, 30, 45 and 60 minutes post-treatment. The percent change in blood glucose for each animal was calculated and the average for each group was determined.

For *P. nitida*, the animals were grouped into 4 groups of 5 mice each. They fasted for 12 h, but water was provided *ad libitum* before the experiment. The fasting blood glucose of each animal was determined before extracts were administered. Groups 1-4 were treated with 100, 200, 400, and 800 mg/kg, respectively. Change in blood glucose levels was accessed for each animal at 15, 30, 45 and 60 minutes post-treatment. The percent change in blood glucose for each animal was calculated and the average for each group determined.

The animals for the control groups were grouped into 2 groups of 5 mice each. Group 1 received 5 ml/kg distilled water and served as a negative control, and group 2 received 250 mg/kg metformin and served as the positive control. The same procedure was carried out for the control groups.

#### Combination effect of methanol extract of *G. latifolium* leaves and *P. nitida* seeds

Twenty –five (25) mice grouped into 5 groups of 5 animals per group were used for the combination interaction study. They fasted for 12 h before the experiment. The fasting blood glucose of each animal was determined before extract and drug administration. They were treated with combination doses of *G. latifolium*: *P. nitida* as follows: Group 1 (91:126.5 mg/kg), Group 2 (110:100 mg/kg), Group 3 (50:160 mg/kg), Group 4 (100:90 mg/kg) and Group 5 (150:30 mg/kg). Effects of these treatments on blood glucose were evaluated at 15, 30, 45 and 60 minutes for each animal and the average for each group was determined.

#### Evaluation of the nature of the interaction of the combination therapy

Using a dose-effect-based strategy, the interactions of various ratio combinations of *P. nitida* and *G. latifolium* extracts were determined. The percentage effect value of the extracts at different concentrations and when combined in different ratios were used in the formula to

calculate their combination index. Various ratio combination interactions between *P. nitida* and *G. latifolium* extracts were determined using a dose-effect-based strategy (Loewe Additivity). The percentage effect value of the extracts at different concentrations separately and when combined in different ratios were used for the calculation of their combination index using the formula.

$$CI = \frac{a}{A} + \frac{b}{B} \quad (2)$$

Where:

a = Dose of *G. latifolium* in the combination and A = Effective dose of *G. latifolium*; while b =Dose of *P. nitida* in the combination and B = Effective dose of *P. nitida*, CI= Combination Index. When CI= 1 (additive interaction), CI > 1 (antagonistic interaction) and CI < 1 (synergistic interaction).

#### Statistical Analysis

All values were expressed as the mean  $\pm$  standard error of the mean (SEM) of five animals per group. Data were analyzed using one-way ANOVA. P values less than 0.05 was considered statistically significant. Graphical plots were done using Microsoft Excel 2010.

## RESULTS

#### Extraction, yield and phytochemical analysis

Methanol extracts of *G. latifolium* produced a higher yield compared with *P. nitida* (Table 1) Saponins, alkaloids, tannins, steroids, and flavonoids were present in high amounts in both samples. Quinones were not detected in the sample of *Picralima nitida* but were present in high amounts in the sample of *Gongronema latifolium*. Glycosides were present in high amounts in the methanol extract of *G. latifolium* but undetected in the sample of *P. nitida*. Lastly, anthraquinones were not detected in both samples.

Table 1. Extraction yields and Phytochemical content of the extracts.

Phytochemical Constituents	<i>Gongronema latifolium</i>	<i>Picralima nitida</i>
Saponins	+++	+++
Flavonoids	+++	+++
Steroids	++	++
Alkaloids	+++	+++
Glycosides	+++	-
Quinones	-	+++
Anthraquinones	-	-
Tannins	++	++
Yield%	24.69%	15.59%

KEY: +++ = high amount of phytoconstituents present; ++ = moderate amount of phytoconstituents present; + = low amount of phytoconstituents present; - = no phytoconstituents detected

### Acute toxicity study

In the *Gongronema latifolium* study group, there was no mortality or any signs of behavioral changes or toxicity observed in the mice after oral administration of *Gongronema latifolium* up to the dose of 5000 mg/kg body weight. As such, the LD<sub>50</sub> can be said to be greater than 5000 mg/kg body weight.

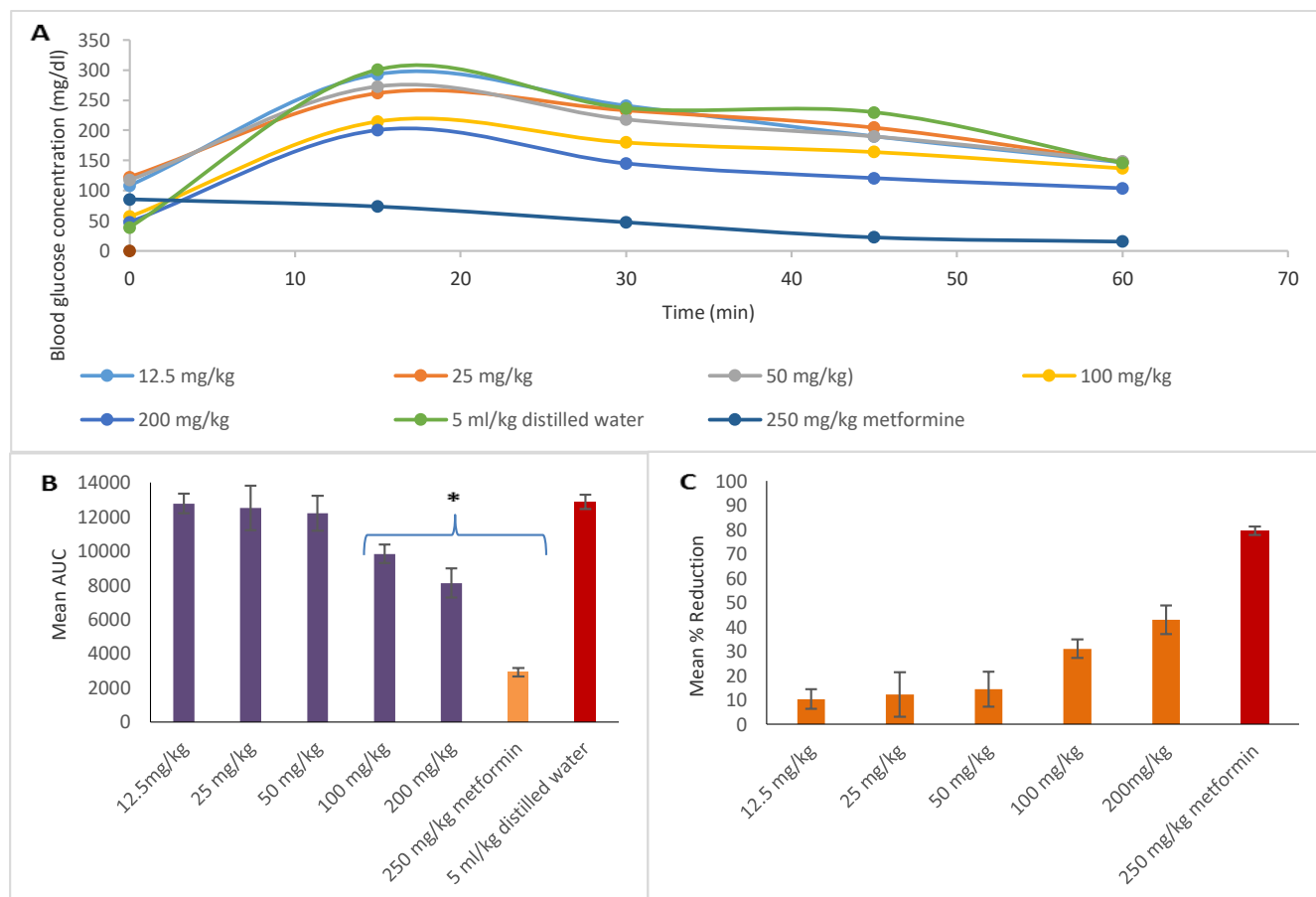
In the *Picralima nitida* study group 100% mortality was observed at 1000 mg/kg dose group in the first phase of the study. Using lower dose levels of 300 mg/kg, 500 mg/kg and 800 mg/kg in the second phase, no mortality or any sign of toxicity was observed. Hence the lethal dose was above 800 mg/kg. Furthermore, using Lorke's method (1983) to calculate the LD<sub>50</sub>, the LD<sub>50</sub> was observed to be approximately 894.4 mg/kg

### Glucose tolerance individual effects of *G. latifolium* and *P. nitida*

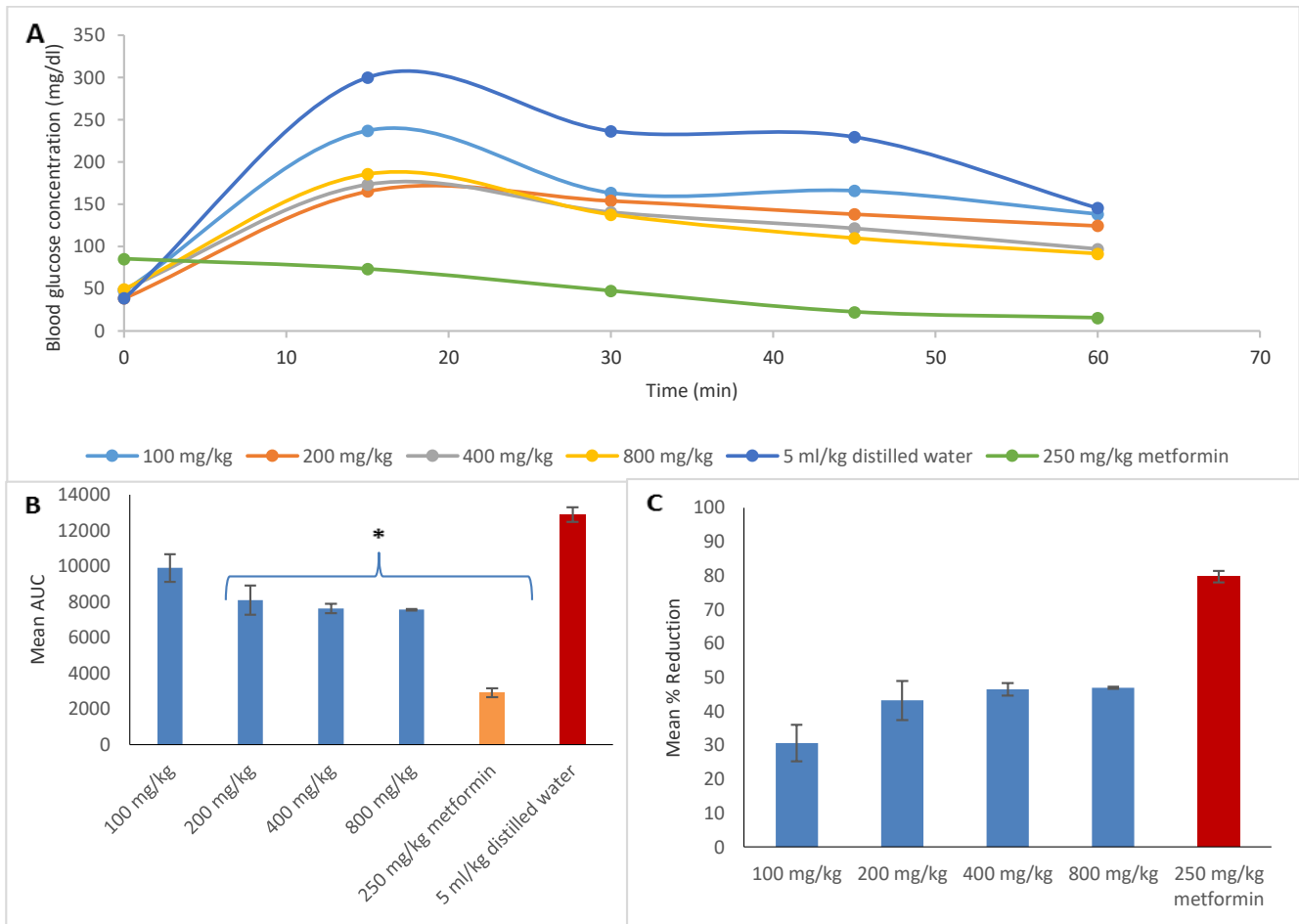
Peak hyperglycemia was observed 15 minutes post glucose load and declined continuously thereafter (Figures 1a and 2a). At 15 minutes post glucose administration, *P. nitida* at 200, 400 and 800 mg/kg produced a significant ( $p < 0.05$ ) reduction in blood glucose levels (BGL) compared to the negative control group just as was recorded for the positive control ( $p = 0.001$ ). At 30 minutes post-administration, significant

differences in BGLs were only observed with 400 mg/kg ( $p = 0.02$ ) and 800 mg/kg ( $p < 0.001$ ) of *P. nitida* as well as the positive control ( $p < 0.001$ ). At 45 minutes post-administration, a weak significant difference in BGLs was observed at 400 mg/kg ( $p = 0.05$ ) and 800 mg/kg ( $p = 0.039$ ) of *P. nitida*; as well as at 200 mg/kg of *G. latifolium* ( $p = 0.045$ ). A strong significance difference in BGL was observed for the positive control ( $p < 0.01$ ). At 60 minutes of post-administration, only the BGL of the positive control was significantly different ( $p = 0.042$ ) from the BGLs of the negative control group.

When comparing the Area Under Curve (AUC) of the negative control group with the different treatment groups, there was a significant difference in AUC of *G. latifolium* at 100 mg/kg ( $p = 0.044$ ) and 200 mg/kg ( $p = 0.038$ ) doses, as well as the positive control ( $p < 0.001$ ). There was no significant difference in the AUC of *G. latifolium* at 12.5, 25 and 50 mg/kg ( $p > 0.05$ ) (Figure 1b). There was also a significant difference in AUC between the negative control group and *P. nitida* at doses of 200 mg/kg ( $p = 0.033$ ), 400 mg/kg ( $p < 0.001$ ) and 800 mg/kg ( $p < 0.01$ ) but no significant difference at 100 mg/kg ( $p > 0.05$ ) (Figure 2b). The percentage glucose reduction in AUC of both treatments were presented in figures 1c and 2c.



**Figure 1.** Effect of *G. latifolium* on glucose tolerance. \*  $P < 0.05$  compared to 5 ml/kg distilled water vehicle control group (negative control).



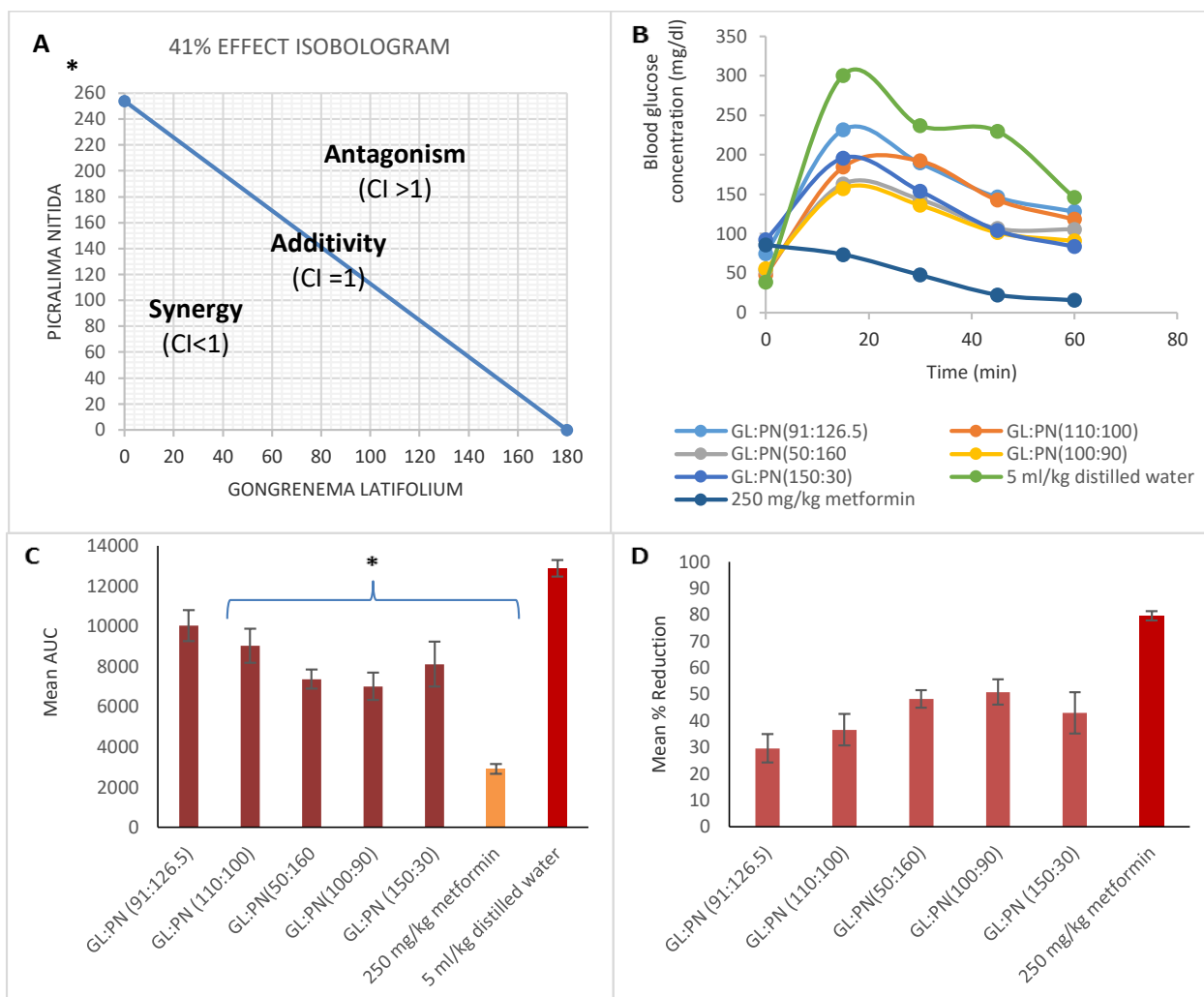
**Figure 2.** Effect of *P. nitida* on glucose tolerance. \*  $P < 0.05$  compared to 5 ml/kg distilled water vehicle control group (negative control).

### Glucose tolerance effect of the extracts in combination

Using the Loewe Additivity combination strategy (equation 2) complemented with Isobologram analysis we defined all the pairs of doses of *G. latifolium* and *P. nitida* that could lead to the combination effect EAB to form additivity and these were drawn as the line of additivity of negative slope on a graph where the x and y-axis represent the dose of *G. latifolium* and *P. nitida* (figure 3a). This representation makes clear that when *G. latifolium* is present at the selected effective dose the quantity of *P. nitida* needed to reach the specified level is 0, and that the presence of *P. nitida* reduced the need for *G. latifolium* in a quantity predicted by the model. All experimental points below the line that could give the desired effect correspond to a combination index (CI)  $< 1$  and indicate synergism.

Peak hyperglycemia was also observed at 15 minutes just like in the dose response test of the individual extracts (Figure 3b). All the combination doses exhibited

significant reduction ( $p < 0.05$ ) in blood glucose compared with the negative control group except GL:PN (91:126.5) dose pair (figure 3c). GL:PN (91:126.5) and GL:PN (110:100) combination dose pairs gave the lowest percentage reductions of  $29.57 \pm 5.4$  and  $36.59 \pm 5.93\%$  respectively while GL:PN (50:160); GL:PN (100:90); and GL:PN (150:30) combination dose pairs were  $48.23 \pm 3.28$ ;  $50.76 \pm 4.77$  and  $42.99 \pm 6.88\%$  respectively. The first two combination doses were expected to give an additivity effect based on the model. However, they could not produce the set percentage effective reduction in blood glucose AUC which was 41%. Since the effects produced from these combinations were below the effective value, these combinations were considered sub-optimal. Contrary to the behavior of the first two dose pairs, the last three dose pairs produced effects above the set value (41%) and their combination index were calculated to be 0.91, 0.91 and 0.95 respectively - an indication of synergistic interaction.



**Figure 3.** Effect of combined doses of *P. nitida* and *G. latifolium* on glucose tolerance. \*  $P < 0.05$  compared to 5 ml/kg distilled water vehicle control group (negative control).

## DISCUSSION

Control of postprandial hyperglycemia is an essential component of diabetes management. The postprandial pattern of glucose metabolism after glucose load and solid mixed meal containing carbohydrate, fat and protein have been found to be virtually the same and this validates studies like ours employing glucose loads to pertain to those observed after mixed meals (Dimitridia et al, 2021).

The liver sees the largest change in blood glucose and insulin after a meal and its role is to restrain their acute rise in the bloodstream. The spillover of glucose to the peripheral circulation requires a substantial increase in systemic insulin level and insulin secretion. In the long term, this mechanism provides insight into how a high glycemic index/load of carbohydrate diet producing sustained high insulin secretion could contribute to the development of insulin resistance – the negative consequences of hyperinsulinemia (Shanik et al., 2008). Under hyperglycemic conditions, the liver uses several

mechanisms to bring about glucose homeostasis. These include increased glucose uptake in the hepatocytes, increased activity of glucose utilizing pathways like glycolysis, pentose monophosphate shunt and glucose storage in form of glycogen by glycogenesis. Also, acetyl-CoA generated from the glycolysis-driven pyruvate pathway is used for fatty acids production which can be transported to adipose tissue for storage (Han et al., 2016). Agents enhancing these liver mediated activities have been shown to lower post-prandial high blood glucose and are useful in the management of diabetes (Rines et al., 2016).

*G. latifolium* extract has been shown to exhibit insulin like activities in the liver by increasing the flux of glucose into the glycolytic pathway and pentose monophosphate shunt in the liver of hyperglycemic animal model (Ugochukwu and Babady, 2003). These activities were reported to be mediated through increased activities of hexokinase the first enzyme of glycolysis and phosphofructokinase, the rate-determining enzyme of glycolysis. Also the activities of Glucose-6-phosphate

dehydrogenase (G6PD) a key enzyme in the pentose phosphate pathway has been shown to be increased by *G. latifolium* (Ugochukwu and Babady, 2003) in addition to its reported ability to increase liver glycogen synthesis (Ajiboye et al., 2019). Similarly, *P. nitida* extract has been shown to promote glucose uptake in the liver through the activation of glucokinase enzyme that plays a critical catalytic role in promoting the addition of a phosphate to glucose, thereby enhancing its uptake into the liver (Guzman and Gurrola-Diaz, 2019).

Glucose transport is an important step in glucose disposal by peripheral tissues because it controls the rate of glucose utilization in these tissues. In skeletal muscle and adipose tissues, the glucose transporters isoforms expressed are GLUT1, GLUT3 and GLUT4 (Shepherd et al., 1999). In the postprandial state, insulin increases the rate of glucose transport mainly by stimulating the translocation of the GLUT4 isoform from the intracellular pool to the cell membrane. Improvement in the sensitivity of tissues to this insulin action avoids marked hyperglycemia and hyperinsulinemia under postprandial conditions. *G. latifolium* extract was reported to increase muscle glucose uptake significantly in the presence of insulin, indicating an insulin-sensitizing potential as well as increased expression and translocation of GLUT4 glucose transporter (Al-Hindi et al., 2014; Ajiboye et al., 2019).

Indole alkaloids were the first set of alkaloids isolated from *P. nitida*: akuamine, pseudoakuamine, akuammidine, akuammicine, akuammigine, pseudoakuammigine, akuammiline, akuamminine and pseudoakuammicine (Mawout et al., 2020). Most of the effects of *P. nitida* including modulation of glucose metabolism have been attributed to its rich alkaloid content. Stimulation of glucose uptake is one of the known mechanisms of alkaloids containing plant's hypoglycemic effects (Mawout et al., 2020). Several alkaloids are allosteric activators of Adenosine monophosphate protein kinase – a cellular fuel sensor enzyme and glucose transporter regulator (Kumar et al., 2019; Song et al., 2021). Similarly, alkaloids have been shown to increase the translocation of GLUT4 and enhancement of glucose-stimulated insulin secretion (Kumar et al., 2019). An increase in insulin sensitivity of multiple tissues like the liver, adipose tissue and skeletal muscles by alkaloids has been reported to be mediated through the inhibition of protein tyrosine phosphatase-1B – an enzyme involved in the negative regulation of insulin signal transduction pathways (Adhikari, 2021). Akuammicine, an indole alkaloid isolated from *P. nitida* has also been shown to stimulate glucose uptake in adipocytes (Shittu et al., 2010).

The improved glucose tolerance effect of the individual plant extracts may be attributed to their phytochemicals and through hypoglycemic mechanisms already reported. The multiple targets of regulation of glucose homeostasis by both plants may be responsible for their superior effect when combined.

## CONCLUSION

Findings from this study support the known improved glucose tolerance effects of *G. latifolium* leaves and *P. nitida* seeds as well as the rationale for their combined use in folkloric medicine. However, combining the extracts of *G. latifolium*: *P. nitida* in the dose ratios of 50:160, 100:90 and 150:30 mg/kg gave the best dose pairs with synergistic outcome.

**Competing Interests:** The authors declare that there are no competing interests.

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