**EVALUATION OF ALPHA-AMYLASE INHIBITORY ACTIVITY OF PHENOL RICH FRACTION OF *NEWBOULDIA LAEVIS* USING TWO IN-VITRO MODELS**

**ABSTRACT**

The present study investigated the alpha-amylase inhibitory activity of the phenol-rich fraction (PRF) from *Newbouldia laevis* using two in vitro models. Leaves were extracted with methanol and fractionated using n-hexane, ethyl acetate, and butanol. The phytochemical analysis revealed the presence of saponins, flavonoids, tannins, glycosides, steroids, and terpenoids in the extract. The ethyl acetate fraction exhibited the highest total phenolic content (337.9 mg/GAE), followed by the butanol fraction (331.8 mg/GAE). The inhibitory effect of PRF on alpha-amylase activity was evaluated using starch-iodine and 3,5-dinitrosalicylic acid (DNS) methods with acarbose as a reference standard. Both PRF and acarbose showed concentration-dependent inhibition in both assay methods, with starch-iodine demonstrating higher inhibitory effects compared to DNS (IC50:533.79 vs 688.46 µg/ml for PRF; 52.98 vs 64.72 µg/ml for acarbose). Although PRF exhibited a lower potency than acarbose, it demonstrated promising enzyme inhibition. Comparative analysis revealed non-significant differences in the percentage of enzyme inhibition at lower doses, with significant differences occurring at higher concentrations. Correlation analysis showed a positive correlation between the two methods (R2:0.9994 for PRF and 0.9983 for acarbose), indicating their reliability in assessing alpha-amylase inhibitory activity. These findings suggest that the phenol-rich fraction of *N. laevis* may have potential therapeutic applications in managing postprandial hyperglycemia associated with type 2 diabetes. Further research is warranted to identify the specific phenolic compounds responsible for this activity and evaluate their efficacy and safety in vivo.

**Keywords**: Alpha-amylase inhibition; Phenol-rich fraction; *Newbouldia laevis*; Starch-iodine method; DNS method; Type 2 diabetes; Postprandial hyperglycemia

**Introduction**

One of the therapeutic targets currently used in the management of type 2 DM *is* inhibition of 𝛼-glucosidase and 𝛼-amylase enzymes to modulate the absorption of glucose in the intestine (Sim et al., 2010). The alpha-amylase (𝛼-1,4-glucan-4-glucanohydrolases) is a prominent secretory product of the pancreas and salivary gland responsible for the initial step in the hydrolysis of complex carbohydrate to a mixture of oligosaccharides and disaccharides in the intestinal mucosa. These sugars are further digested to monosaccharide by the action of alpha-glucosidase. The current alpha-amylase and glucosidase inhibitors in clinical use are associated with side effects such as hypoglycemia, diarrhea, flatulence, and bowel bloating that limit their use in the treatment of diabetes and its complications (Evans and Rushakoff, 2007). There is, therefore, an urgent need to search for complementary and alternative therapies with minimal side effects that can serve as adjunct to the management of DM (Grover et al., 2002).

The role of natural products in meeting the health needs of Nigerian populations has been emphasized in many studies (Sofowora, 1982; Osemene et al., 2013). The continued patronage and popularity of natural plant products is strongly rooted in the culture of the people, as traditional medicine practitioners may have used a particular plant to treat ailments. Treatment with medicinal plants is assumed to be safe, as the remedies are “in sync” with minimal side effects (Khan, 2016). In addition, increased interest in self-care, population increase, and economic downturn have led many Nigerians to resort to natural plant products as affordable sources of therapy for their immediate health needs (Hosseinzadeh *et al*., 2015).

*Newboldia laevis* (Bignoniacea) is a small tree approximately 7 – 8 m tall (Burkil, 1985). It is known as Ogilisi or Ogirisi in the Igbo culture of Nigeria, while the Hausas call it Aduruku, and the Yorubas, Akoko. In Ghana, it is referred to as Sesemasa but is known as Kinkin in Mali (Burkil, 1985). The roots and leaves are used to treat dysentery, malaria, elephantiasis, migraines, and seizures (Bothon *et al*., 2014). The bark and twigs are used to treat pelvic pain in women, peptic ulcer disease, ear ache, skin ulcers, epilepsy, hemorrhoids, and constipation, whereas the flowers are known for their anti-inflammatory activities (Bafor *et al*., 2009). The leaves were soaked in ethanol to treat diabetes and sickle cell disease (Kolawole *et al*., 2012). Although *N. leavis* has been traditionally used to treat diabetes, the effect of its phenol-rich fraction on alpha-amylase enzyme activity has not been determined. This study evaluated the α-amylase inhibitory activity of a phenol-rich fraction of *N. laevis* using two in vitro models.

**Materials and Methods**

**Plant materials**

The leaves of *N. laevis* were collected from Agulu in Anambra State, Nigeria, and authenticated by a trained taxonomist, Mr. Nwafor Felix, Department of Pharmacognosy and Environmental Medicine, University of Nigeria, Nsukka. A voucher specimen (no. PCG 521/A/043) was deposited at the herbarium of the Department of Pharmacognosy and Traditional Medicine, Nnamdi Azikiwe University Awka, for future reference. The plant materials were subsequently air-dried at room temperature and pulverized using a mechanical grinding machine (GX160 Delmar 5.5HP).

**Drugs**

Acarbose was purchased from Favor Spring Pharmacy, Enugu State, Nigeria. It was used as a positive control based on its mechanism of action, and several previous studies have used it as a reference drug for the inhibition of alpha amylase enzyme activity. Drug- and phenol-rich fractions were prepared in 5% Tween 20 using pyrogen-free distilled water.

**Extraction and fractionation**

 *The N. laevis* leaves powder was macerated in methanol for 72 h with intermittent shaking. The resulting solution was filtered using Whatman filter paper, and the filtrate was concentrated *in vacuo* using a rotary evaporator (RE300 Model, United Kingdom) at 40°C. Two-thirds of the extract was subjected to liquid-liquid partition successively with n-hexane, ethyl acetate, and butanol. The fractions were concentrated *in vacuo* using a rotary evaporator at 40°C to obtain the n-hexane-soluble, ethyl acetate-soluble, butanol-soluble, and water-soluble fractions. The extracts and all fractions were stored in a refrigerator at 0-4°C for further use.

**Qualitative Phytochemical analysis**

Qualitative phytochemical analysis of the extracts and fractions was performed using standard methods (Odoh *et al*. 2019).

**Determination of total phenolic content**

The total phenolic content (TPC) of the *N. laevis* methanol leaf extract and fractions was determined using Folin and Ciocalteu reagent, following the method described by Singleton and Rossi (1965) and Ajaghaku *et al*. (2025). The test sample (0.2 ml) was mixed with 0.6 ml of water and 0.2 ml–Folin-Ciocalteu’s phenol reagent (1:1). After 5 min, 1 ml of saturated sodium carbonate solution (8% w/v in water) was added to the mixture, and the volume was made up to 3 ml using distilled water. The reaction was kept in the dark for 30 min, and after centrifuging at 3000 rpm for 10 min, the absorbance of blue color from different samples was measured at 710 nM. The phenolic content was calculated as Gallic Acid equivalent GAE/g of dry plant material based on a standard curve of Gallic Acid. All determinations were performed in duplicates.

**In vitro α-amylase inhibition assay starch-iodine method.**

The Anti-diabetic activity of the phenol-rich fraction was analyzed by the starch-iodine method, as described by the standard protocol of (Ferosekhan et al., 2016). To 250 μl of each PRF concentrations in a test tube (0, 50, 100, 200, 400, 800 μg/ml) in triplicates, the following were added sequentially: 250 μl of phosphate buffer (0.02 M, pH 6.9 and containing 6 mM sodium chloride), phosphate-buffered α-amylase (250 μl, 0.05 mg/ml), and starch (250 μl, 1% w/v), and the reaction mixture was incubated for 15 min at 37°C. One molar HCl (20 μl) was added to stop the enzymatic reaction, followed by the addition of 100 μl of iodine reagent (5 mM I2 and 5 mM KI). The color change was noted, and the absorbance was read at 625 nm on a 1 ml cuvette exactly 1 min after adding the iodine/iodide solution. The control reaction, representing 100% enzyme activity, contained no test extract or reference standards. To eliminate the absorbance produced by PRF, appropriate PRF controls without enzymes were also included. The inhibition of enzyme activity was calculated as follows:

Inhibition (%) = 1-(C - S) × 100

Where S is the absorbance of the sample and C is the absorbance of the control. The concentration of the sample that resulted in a 50% inhibition of enzyme activity (IC50) was determined graphically.

**In vitro α-amylase inhibition assay by 3,5-dinitrosalicylic acid (DNS) method**

This DNS method of alpha-amylase inhibition was carried out using a standard protocol described by Kazeem et al. (2013). In brief, to 250 μl of each PRF concentration (0, 50, 100, 200, 400 and 800 μg/ml) in a test tube in triplicates, the following were added sequentially: 250 μl of phosphate buffer (0.02 M, pH 6.9 and containing 6 mM sodium chloride), phosphate-buffered α-amylase (250 μl, 0.05 mg/ml), and starch (250 μl, 1% w/v), and the reaction mixture was incubated for 15 min at 25°C. DNS (500 μl) was subsequently added, and the mixture was boiled for 5 min. The mixture was then cooled and diluted with 5 ml of dH2O. The control was prepared in the same manner as the test samples, with 5% Tween 20 replacing the extract. The absorbance of each test tube was measured at 540 nm and the percentage of inhibition was calculated as follows:

% inhibition = (Ac - At) / Ac × 100,

Where Ac and At are the absorbance of the control and tests, respectively.

The concentration of the extracts resulting in 50% inhibition of enzyme activity (IC 50) was determined graphically.

The Pearson correlation coefficient (r) of the median inhibitory concentrations (IC50) of the samples was determined by the two methods.

**Statistical analyses**

Data are expressed as mean ± SEM and analyzed by Kruskal-Wallis ANOVA. The differences between the various treatments were analyzed by multiple comparisons of the mean ranks for all groups. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance. Statistical analysis was performed using the SPSS 20 software, and graphical plots were generated using Microsoft Excel 2010.

**Results**

**Yield, Phytochemical and total phenol content**

Table 1 presents the yield of the methanol extract and fraction of *N. laevis*. The water fraction had the highest yield, followed by the butanol and ethyl acetate fractions. The n-hexane fraction yielded the lowest yield.

 Table 2 presents the phytochemical distribution of the *N. laevis leaves* extract and fractions. Saponins, flavonoids, tannins, glycosides, steroids, and terpenoids were also present in the extract.

The ethyl acetate fraction contained the highest phenolic content (337.9 mg/GAE followed very closely by the butanol fraction with 331.8 mg/GAE (Table 3). The lowest TPC was observed for the n-hexane fraction (66.4 mg/GAE).

**Table 1: Yield of methanol extract and fraction of *N. laevis* stem bark.**

|  |  |  |  |
| --- | --- | --- | --- |
| **EXTRACT/FRACTIONS** |  | **YEILDS (g)** | **PERCENTAGE YEILDS (% w/w)** |
| Extract |  | 246 | a7.45 |
| N- hexane |  | 8 | b3.25 |
| Ethyl acetate |  | 32 | b13.00 |
| Butanol |  | 59 | b23.98 |
| Water |  | 147 | b59.75 |

aYield calculated from 3300 g of powdered drug

bYield calculated from 246 g of methanol extract

**Table 2: Phytochemical analysis of extract and fractions of *N. laevis leaves***

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Phytochemical test** | **Methanol extract** | **n-Hexane fraction** | **Ethyl acetate fraction** | **Butanol fraction**  | **Water fraction** |
| **Alkaloids**  | **\_** | **\_** | **\_** | **\_** | **\_** |
| **Glycosides** | **+** | **\_** | **+** | **+** | **+** |
| **Flavonoids** | **+** | **+** | **+** | **+** | **+** |
| **Saponins** | **+** | **-** | **+** | **+** | **\_** |
| **Steroids** | **+** | **+** | **+** | **-** | **\_** |
| **Terpenoids** | **+** | **+** | **+** | **-** | **\_** |
| **Tanins**  | **+** | **-** | **+** | **+** | **+** |
|  |

**Keys = + Present, - Absent**

**Table 3: Total phenolic content of the extract and fractions of *N. laevis***

|  |  |
| --- | --- |
| Extract/fractions | Total Phenolic Content (mg/GAE) |
| Water fraction | 226.4 + 0.6 |
| Ethyl acetate fraction | 337.9 + 0.5 |
| Butanol fraction | 331.8 + 1.2 |
| n- Hexane fraction | 66.4 + 1.0 |
| Ethanol extract | 186.3 + 0.8 |

**Effect of Phenol rich fraction on alpha amylase enzyme inhibition**

The inhibitory effect of acarbose and the phenol-rich fraction of *N laevis* showed a concentration-dependent effect in both the starch-iodine and DNS assay methods (figures 1 and 2). Linear graphs with regression (R2) values above 0.95 where produced by both methods indicate a proportional increase in inhibitory activity as the concentration increases. Starch-iodine method produced higher inhibitory effect compared to DNS method with IC50 values of 52.98 vs 64.72 µg/ml for acarbose and 533.79 vs 688.46 µg/ml for PERF (figure 3) respectively. Compared to the reference standard, the effect of PERF was lower, as demonstrated by their IC50 value differences. Comparative analysis of both methods used for the assay revealed that non-significant (P>0.05) differences in percentage enzyme inhibition were recorded at lower doses, with significant (P<0.05) differences occurring at higher concentrations (figures 4 and 5). Correlation analysis of both methods showed a positive correlation of R2 0.9994 for PRF and 0.9983 for acarbose, indicating that both methods can be employed in the analysis of the alpha amylase inhibitory activity of samples.

Figure 1: Dose response curve of alpha amylase inhibitory activity of acarbose using Starch-iodine and DNS assay methods

Figure 2: Dose response curve of alpha amylase inhibitory activity of Phenol Rich Fraction using Starch-iodine and DNS assay methods

**Figure 3: Half maximum inhibitory concentration (IC50) of PRF and acarbose using Starch-iodine and DNS assay methods**

A, B

A, A

**Figure 4: Comparative inhibitory effect of Acarbose on alpha amylase enzyme activity using Starch-iodine and DNS methods.**

Where dose pair with different alphabets (A, B) = P<0.05

B

A

A, A

**Figure 5: Comparative inhibitory effect of PRF on alpha amylase enzyme activity using Starch-iodine and DNS methods.**

Where dose pair with different alphabets (A, B) = P<0.05

**Discussion**

Scientific studies have shown that strict blood glucose control is essential for minimizing the risk of type-2 diabetes and its associated chronic complications (Carillo, 2003). Elevated postprandial plasma glucose concentration is a major contributor to suboptimal glycemic control and is the earliest abnormality in glucose homeostasis associated with type 2 diabetes (Woerle et al., 2004).

Among the many determinants of the postprandial glucose profile, glucose absorption, β-cell function, and insulin sensitivity are the major control points. Abnormalities in these checkpoints are the fundamental pathophysiology of type-2 diabetes (Cerf, 2013). Inhibition of the carbohydrate-hydrolyzing enzymes present in the small intestinal brush borders is a known mechanism for the decrease in post-prandial glucose levels (Tundis et al., 2010). Although other amylolytic enzymes participate in the process of starch breakdown, the contribution of α-amylase is a prerequisite for the initiation of this process and the provision of substrates to other enzymes, such as α-glucosidase (Joshi et al., 2015). Inhibition of the α-amylase enzyme is therefore considered a useful strategy for the treatment of disorders in carbohydrate uptake, such as diabetes and obesity.

Phenolic compounds have been reported to inhibit α-amylase activity even at physiological concentrations (Funke and Melzig, 2005). Caffeic acid and its esters have also been shown to inhibit α-amylase enzyme activity and other key enzymes linked to type-2 diabetes (Oboh et al., 2015). The inhibitory activity of caffeic acid has also been shown to be enhanced by ester bond formation, and its mechanism of action has been reported to be a mixed type of inhibition involving both competitive and non-competitive effects (Narita and Inouye, 2009).

Inhibition of α-amylase activity by the phenol-rich fraction of *N. laevis* could be an additional antidiabetic mechanism in reducing post-prandial hyperglyceremia and its associated complications, including oxidative stress, beta cell malfunction, and insulin resistance.

A positive correlation between alpha-amylase activity and the increase in postprandial glucose levels has been established, demonstrating the relevance of suppressing postprandial hyperglycemia in the treatment of type 2 diabetes. The inhibitory effect of phenolic compounds on α-amylase is closely related to their molecular structure, where the substituents (hydroxyl and methoxy) on the aromatic rings contribute to their distinct molecular properties, such as polarity, stability, and binding. These factors directly affect their inhibitory and biological activities in vivo. Phenolic compounds have been reported to have a reductive ability in plasma glucose owing to the inhibition of the activities of carbohydrate-hydrolyzing enzymes in the small intestine (Hamden et al., 2011). Resveratrol, which is naturally found in grapes and red wines, was also found to inhibit carbohydrate-hydrolyzing enzymes. Dimeric stilbenoid phenolic compounds from resveratrol were more efficacious than acarbose in inhibiting pancreatic α-amylase. The inhibitory effect exhibited by the phenol-rich fraction may be connected to its structural features and may provide additional benefits in the management of post-prandial hyperglycemia in diabetic disease conditions.

**Conclusion**

The phenol-rich fraction of *Newbouldia laevis* demonstrated significant alpha-amylase inhibitory activity in both the starch-iodine and DNS assays. This inhibitory effect was concentration-dependent, with the starch-iodine method showing higher inhibition than the DNS method. Although the potency of the phenol-rich fraction was lower than that of acarbose, as evidenced by their respective IC50 values, it still exhibited promising enzyme inhibition. The positive correlation between the two assay methods suggests their reliability in evaluating the α-amylase inhibitory activity. These findings indicate that the phenol-rich fraction of N. laevis may have potential therapeutic applications in managing postprandial hyperglycemia associated with type 2 diabetes. Further research is warranted to explore the specific phenolic compounds responsible for this activity and evaluate their efficacy and safety in vivo.

**Declarations**

**Ethical approval:** Not applicable.

**Funding**: Not applicable

**Clinical trial number**: Not applicable.

**Consent for publication**: Not applicable.

**Conflicts of interest**: The authors declare that they have no conflict of interest.

**Consent to participate**: Not applicable.

**Data availability**: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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