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COMPONENT-LEVEL ANTIOXIDANT CONTRIBUTIONS AND THERMAL STABILITY OF *azadirachta indica, mangifera indica* AND *persea americana* LEAF EXTRACTS USED IN A POLYHERBAL FORMULATION

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ABSTRACT

This study investigated the component-level antioxidant contributions of *Azadirachta indica* (AI), *Mangifera indica* (MI), and *Persea americana* (PA) leaf extracts, the components of a previously studied polyherbal formulation. The aim was to delineate the contribution of each extract to the overall antioxidant capacity and to evaluate the influence of heat treatment and concentration on their activities. The samples were divided into two groups: a fresh extract and a pre-heated extract (heated for 30 min at 80°C). The antioxidant activity was evaluated using spectrophotometric assays for DPPH radical scavenging activity and total antioxidant capacity of graded concentrations (10-1000 µg/ml) and thermal treatment of 100 µg/ml solutions of the extracts for 5, 10, 15 and 20 min at 80°C. *Mangifera indica* extract demonstrated a superior antioxidant activity in a concentration-dependent manner; for DPPH, the fresh extract significantly increased from 30.53 ± 2.93% to 81.32 ± 1.22% (IC_{50} = 358.41 µg/ml) while the pre-heated extract increased from 30.53 ± 3.00% to 86.05 ± 2.29% (IC_{50} = 359.81 µg/ml). For AI, the IC_{50} values were 4329.27 µg/ml (fresh) and 2738.82 µg/ml (pre-heated), respectively. The TAC increased with the duration of heating in the fresh AI and MI extracts but varied in the pre-heated extracts. It was concentration-dependent in both fresh and pre-heated AI and MI. The findings indicate that antioxidant activity in the AI-MI-PA formulation depends on both extract composition and processing conditions, suggesting the need for optimised thermal handling to preserve bioactivity.

Keywords: *Azadirachta Indica*, *Mangifera Indica*, *Persea Americana*, Polyherbal Formulation, Antioxidant Activity

INTRODUCTION

Herbal medicine remains an essential component of primary health care in many parts of the world, particularly in regions where access to conventional therapies is limited or costly. In sub-Saharan Africa, traditional polyherbal formulations are deeply rooted in cultural practices and are often the first line of defence against common ailments. The therapeutic efficacy of polyherbal preparations is usually attributed to the synergistic interactions among multiple phytochemicals, which can confer a broader spectrum of bioactivity compared to single-plant extracts (Ebhohimen et al., 2024; Ibrahim et al., 2024). Among the bioactivities of interest in herbal medicine, antioxidant properties are especially relevant given their role in mitigating oxidative stress, a central factor in the onset and progression of several chronic diseases (Ebhohimen et al., 2021; Liu et al., 2025; Patil et al., 2022).

Oxidative stress arises when the generation of reactive oxygen species (ROS) overwhelms endogenous antioxidant defence mechanisms, leading to damage of lipids, proteins, and nucleic acids. Natural antioxidants derived from plants offer a promising strategy to restore redox balance and limit associated cellular damage (Li et al., 2023). However, a critical gap in knowledge lies in delineating the contributions of individual components to the overall activity, which is essential for rational optimisation and standardisation.

The polyherbal formulation studied in our previous work is composed of *Azadirachta indica* (AI), *Mangifera indica* (MI), and *Persea americana* (PA) leaf extracts. This combination is used by the Esan people in Ekpoma for managing uncomplicated malaria and related febrile conditions (Ebhohimen et al., 2024). Our earlier investigations demonstrated that the crude extract possesses strong in vitro antioxidant and anti-inflammatory activities, with total antioxidant capacity (TAC) and radical scavenging potentials that were modulated by heat treatment (Ebhohimen et al., 2024, 2025). These findings are consistent with the herbal medicinal uses of the polyherbal formulation, while also underscoring the need to elucidate the specific contribution of each constituent to the overall bioactivity.

Azadirachta indica (neem) is well-documented for its rich repertoire of bioactive compounds, including azadirachtin, limonoids, flavonoids, and phenolics, which exhibit antioxidant, anti-inflammatory, and antiplasmodial activities (Ahmed et al., 2023; Ousman et al., 2025; Sarkar et al., 2021). *Mangifera indica* (mango) leaves are similarly endowed with mangiferin and other polyphenols that confer potent free-radical scavenging capacity (Amaechi et al., 2024; Kumar et al., 2021; Lawrence et al., 2025; Mendonça et al., 2025). *Persea americana* (avocado) leaves, although less extensively studied, are reported to contain flavonoids and tannins with appreciable antioxidant effects (Bhuyan et al., 2019; Marra et al., 2024; Rahman et al., 2018). Despite these individual attributes, it is unclear whether the efficacy of the polyherbal extract arises primarily from one dominant component or from complementary interactions among all three.

The current study investigated the antioxidant properties of the individual components of the polyherbal formulation using two established in vitro antioxidant assays, DPPH radical scavenging activity and total antioxidant capacity (TAC).

MATERIALS AND METHODS

Collection of Plant Materials

The different plant materials, MI, AI and PA leaves, were collected freshly from a garden in Ekpoma (Latitude: 6°44' 56.904" N Longitude: 6° 4' 23.574" E), Esan West Local

Government Area of Edo State, Nigeria, in August 2025. The harvested leaves were authenticated in the Department of Plant Biology and Biotechnology, University of Benin, Edo State, Nigeria, with voucher numbers: AI=UBH-A286, MI=UBH-M257, PA=UBH-P408.

Preparation of Plant Materials

After air drying at room temperature for 18 days, the leaves were homogenised using an electric blender. The homogenates (100 g each) were macerated in 4 L of distilled water to obtain aqueous extracts. After 24 hr, the extracts were filtered with a muslin cloth. Each filtrate was divided into two parts; one part, termed 'pre-heated', was heated in a water bath for 30 minutes at 80°C to mimic the herbal medicinal preparation process. The other part, termed 'fresh' was not exposed to heat treatment. The filtrates were first concentrated using a rotary evaporator and dried by freeze-drying (Searchtech Instruments LGJ-10). All extracts were stored in airtight containers in a refrigerator at 4°C until required for analysis

Study Design

To capture the effect of further heat treatment as observed in herbal medicinal practice, heat treatment was applied to 100 µg/ml solutions of the pre-heated extract. The same heat treatment was administered to the fresh extracts. This was done in sealed tubes in a water bath (80°C) for 5, 10, 15, and 20 minutes. The start point, before heat treatment was applied to the fresh extract and before further heat treatment of the pre-heated extract, was reported as '0' under the duration of heat treatment.

The effect of concentration was evaluated using 100, 250, 500, 750, and 1000 µg/ml solutions of both fresh and pre-heated extracts. The DPPH scavenging and total antioxidant capacity assays were conducted in triplicate.

DPPH Scavenging Activity

The free radical scavenging capacity of the extracts against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined by the method of (Braca et al., 2001). A methanol solution of DPPH (2,850 µL, 0.004%) was added to 150 µL of extract. The mixture was incubated at room temperature for 30 min in the dark. The absorbance was recorded at 515 nm (Visible Spectrophotometer 721 PEC, 1 cm glass cuvette).

The radical scavenging activity was calculated using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = [(A_0 - A_1)/A_0) * 100$$

Where: A_o was the absorbance of DPPH radical + methanol; A_1 was the absorbance of
DPPH radical + sample extract or standard.

Total Antioxidant Capacity (TAC)

The phosphomolybdenum assay was used to quantify the TAC (Prieto et al., 1999). The reagent mixture (0.6 M H_2SO_4, 28 mM $NaPO_4$, 4 mM ammonium molybdate; 1:1:1; 1 ml) was used to treat 3 ml of the extracts and ascorbic acid (12.5 - 125µg/mL), which served as the standard. The absorbance was measured at 695 nm (Visible Spectrophotometer 721 PEC, 1 cm glass cuvette) after incubating the mixture for 90 min at 95°C. The TAC of the extracts were extrapolated from the ascorbic acid standard curve and reported as ascorbic acid equivalent.

Statistical Analysis

The experiments were conducted in triplicate and the data obtained were analysed using one-way analysis of variance (ANOVA) and the post hoc Tukey's test using GraphPad Prism Version 8.0 (GraphPad Software, San Diego, USA); $p < 0.05$ was considered significant. The results were presented as mean ± SD. The IC50 and EC50 for the concentration-based assays were calculated (*How to Determine an IC50 - FAQ 1859 - GraphPad*, n.d.).

RESULTS AND DISCUSSION

The study aimed to determine the component-level contributions of AI, MI and PA leaf extracts in a polyherbal formulation, by evaluating the DPPH radical scavenging activity and total antioxidant capacity across concentrations (100–1000 µg/mL) and thermal treatments (5-20 min at 80°C).

DPPH Radical Scavenging Activity

The MI fresh extract demonstrated a significantly higher ($p<0.05$) DPPH radical scavenging activity in the fresh extract compared to AI and PA extracts. Upon heating, the scavenging activity reduced significantly ($p=0.002$) but did not differ significantly from 5-20 min of heat treatment. The overall DPPH radical scavenging activity of MI was reduced in the pre-heated extract. No significant differences were observed in both the fresh and pre-heated PA extracts. The DPPH activity of the AI extract reduced with increased heating of the fresh extract, but increased significantly ($p=0.02$) in the pre-heated extract from before heat treatment (0) to 20 min of further heat treatment (Figures 1a, b).

The fresh AI and MI extracts demonstrated a concentration-dependent increase in DPPH radical scavenging activity. *Mangifera indica* (IC_{50}= 358.41 µg/ml) exhibited substantially higher activity across all concentrations compared to AI extract (IC_{50}= 4329.27 µg/ml) (Figure 1C). The pre-heating slightly increased the DPPH radical scavenging capacity but was not significantly different ($p>0.05$) across the concentrations studied. The pre-heated sample followed the same trend as the fresh extract. The radical scavenging increased significantly from 100 to 500 µg/ml, but there was no significant difference between 500, 750 and 1000 µg/ml solutions (Figure 1D).

Total Antioxidant Capacity (TAC)

The TAC of the fresh AI and PA extracts increased significantly with the duration of heat treatment. For MI, the TAC of the fresh extract reduced significantly ($p<0.05$) for 5-10 min upon heating. The capacity increased again significantly upon further heating ($p=0.01$) to 15 min. The fresh PA extract displayed a significant ($p<0.05$) upward trend following heat exposure. For the pre-heated sample that was further exposed to heat treatment, the TAC of all extracts did not follow any specific pattern, suggesting that the duration of heat treatment can affect the TAC (Figure 2a, b).

For the fresh extracts, AI and MI extracts exhibited a concentration-dependent increase in TAC, though the activity of the MI extract was higher across all concentrations (Figure 2C). For the pre-heated extracts, the overall activity of the AI extract reduced compared to the fresh extract, suggesting partial loss of activity due to heat exposure. Conversely, MI maintained a strong concentration-dependent activity (Figure 2D).

This study delineates the distinct antioxidant roles of AI, MI and PA leaf extracts for the previously reported in vitro antioxidant and anti-inflammatory properties of the crude polyherbal formulation. In the crude AI-MI-PA formulation, a substantial reduction in TAC and inhibition of albumin denaturation were observed upon prolonged heating. Although the work identified a strong overall antioxidant

capacity, it also emphasised the heat-sensitivity of the mixture (Dhakad et al., 2025; Ebhohimen et al., 2025; Sarkar et al., 2021). The present component-level data suggest that the polyherbal antioxidant profile is differentially attributable to the components, each of which can be affected by heat treatment.

Thermal processing differentially modulates activity (Narra et al., 2024). The AI extract is thermolabile, but an initial high thermal treatment has been reported to improve biological activity (Vats, 2016). While progressive heating of fresh AI modestly reduced DPPH scavenging capacity, TAC paradoxically increased slightly in the pre-heated extract, but decreased in the MI and PA extracts under the same conditions. These results suggest that processing parameters can influence the final bioactivity profile, thus the need for optimisation (Barba-Ostria et al., 2022; Wang et al., 2023).

The relatively weak and minimal variation in DPPH activity of AI extract across concentrations suggests that within the AI-MI-PA mixture, AI may only contribute a little to radical scavenging. The TAC of AI after thermal pre-treatment, though slightly higher than the fresh extract, did not increase significantly across the concentration range. This suggests that AI may be involved in other roles, which may complement pharmacological effects that are not fully captured by DPPH/TAC alone (Farahanah et al., 2025). The limitations of this study include reliance on in vitro chemical assays that do not capture cellular uptake, metabolism, or synergistic pharmacodynamics. Follow-up studies applying chromatographic profiling to identify heat-sensitive compounds, performing cell-based antioxidant and anti-inflammatory assays, and testing in vivo efficacy and safety in relevant malaria or oxidative-stress models are recommended.

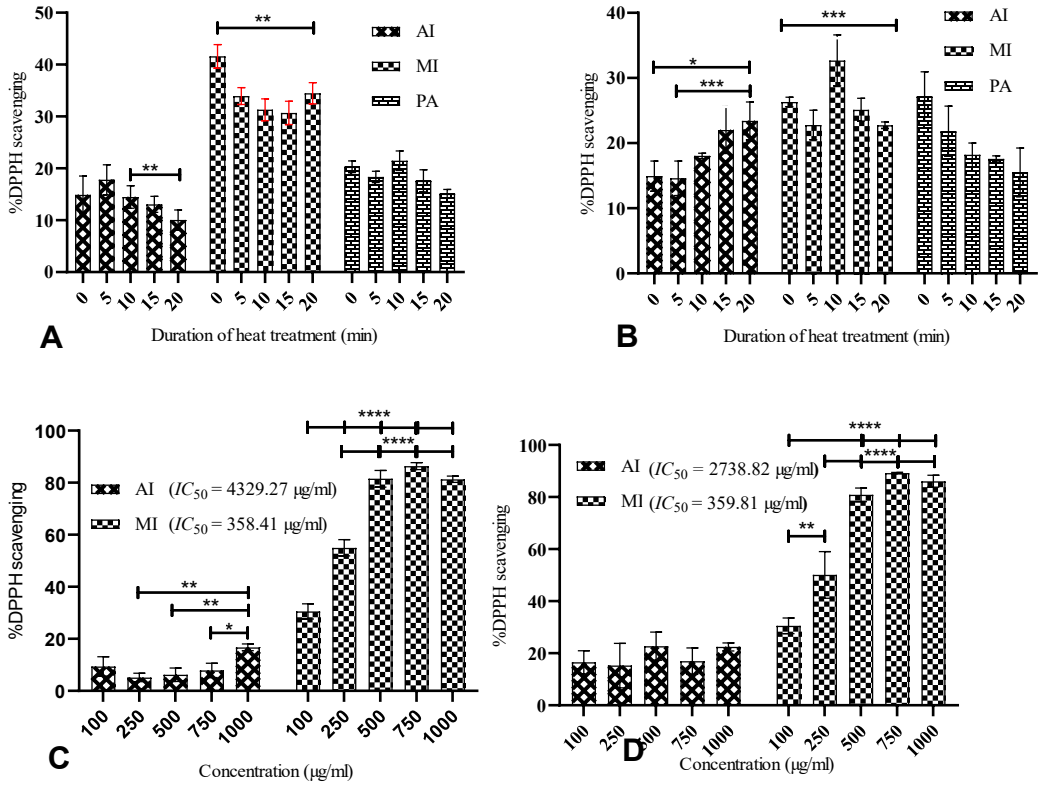

Figure 1: Percentage DPPH Scavenging activity (*n=3*, mean ± SD); (A) Effect of Heat Treatment: FRESH AI, MI and PA heat Treated up to 20 min (B) Effect of Heat Treatment: Pre-heated (30 min, 80°C) AI, MI, PA Extracts Further Heated up to 20 min. (C) Effect of Concentration: Fresh AI and MI Extracts Tested at Concentrations Ranging from 100 to 1000 µg/ml. (D) Effect of Concentration: Pre-heated (30 min, 80°C) AI and MI Extracts Tested at Varying Concentrations Ranging from 100 to 1000 µg/ml

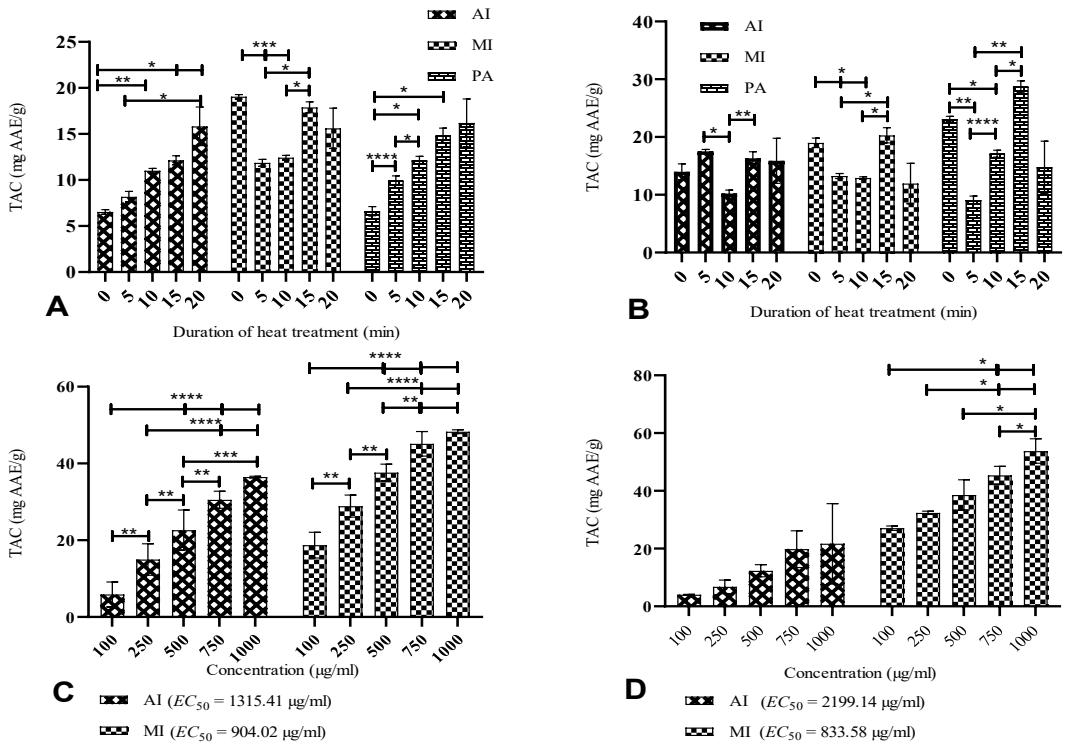

Figure 2: Total Antioxidant Capacity (*n=3*, mean ± SD); (A) Effect of Heat Treatment: fresh AI, MI, and PA Heat-Treated up to 20 min (B) Effect of Heat Treatment: Pre-heated (30 min, 80°C) AI, MI, and PA Extracts Further Heated up to 20 min. (C) Effect of Concentration: Fresh AI and MI Extracts Tested at Varying Concentrations Ranging from 100 to 1000 µg/ml. (D)Effect of Concentration: Pre-heated (30 min, 80°C) AI and MI Extracts Tested at Varying Concentrations Ranging from 100 to 1000 µg/ml

CONCLUSION

The study investigated the antioxidant properties of the individual components of a polyherbal formulation using two established in vitro antioxidant assays. Disaggregating the polyherbal formulation shows that AI components play a lesser role in its antioxidant activity, and that thermal processing exerts both assay- and extract-specific effects. These findings provide an evidence base for standardising extract ratios and processing protocols for this traditional polyherbal formulation.

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