Review of: "Evaluation of Antioxidant Activity and α-Amylase Inhibitory Potential of Melilotus indicus Ethanolic Extract: An In Vitro and In Silico Study"

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Potential competing interests: No potential competing interests to declare.

Constructive Feedback on the Research Article

Strengths

Comprehensive Introduction (Section 1: Introduction)

- The introduction does an excellent job of setting the stage by highlighting the medicinal properties of*Melilotus indicus*. It effectively establishes the rationale for the study, citing the plant's antioxidant and anti-diabetic potential.
- The historical and phytochemical background strengthens the relevance of the research, providing a clear justification for the investigation.

Detailed Methodology (Section 2: Materials and Methods)

- The extraction process and determination of flavonoid content are well-explained. The use of specific methods like the aluminum chloride method for flavonoid determination adds credibility and reproducibility to the study.
- The molecular docking section is well-structured, demonstrating thoughtful experimental design and modern techniques like in-silico modeling (Section 2.5.3: Docking and Building Complexes).

Data Presentation (Section 3: Results and Discussion)

- The quantitative presentation of flavonoid content (0.49 mg/g extract ± 3.818) and IC50 value for antioxidant activity is clear and adds substantial value to the research.
- The docking study (Section 3.4) effectively compares the binding scores of different compounds with the standard drug acarbose, presenting the data in a meaningful way to show the potential of *Melilotus indicus* phenolic compounds.

Areas for Improvement

Abstract: Lack of Detail

- The abstract provides a good overview but could benefit from more precise details regarding the methods and the statistical significance of the results. Including the actual binding energies or key findings in this section would give

readers a better understanding of the research outcomes upfront.

Clarity in Results Interpretation (Section 3: Results and Discussion)

- While the results are clearly presented, the interpretation could be more nuanced. For instance, the difference in IC50 values between *Melilotus indicus* extract and vitamin C (Section 3.3: Antioxidant Activity) is noted but not explained in depth. Explaining why the extract has a significantly higher IC50 compared to vitamin C would improve the discussion of its antioxidant potential. This might include a comparison with other plant-based antioxidants or a discussion of possible synergies between the extract's compounds.

Statistical Rigor (Section 2.4.2: Statistical Analysis)

- The statistical analysis of the DPPH scavenging assay (p-value = 0.188) suggests that the results are not statistically significant. This raises concerns about the robustness of the findings. To address this, the authors could conduct additional replicates or explore alternative statistical methods that might reveal more significant trends. Reporting confidence intervals could also provide a clearer picture of the data's reliability.

Docking Study (Section 3.4: Docking Study)

- The docking scores are well-presented, but more discussion around their biological relevance is needed. Although the binding score for chlorogenic acid was favorable, it did not outperform the drug acarbose. The paper would benefit from a discussion on how the docking results might translate to in vivo efficacy and whether modifications to the compounds could improve their inhibitory potential.

Lack of In Vivo Correlation

- The study is solely in vitro and in silico, which limits its application. Suggest adding or planning in vivo studies to validate the findings, especially since molecular docking alone does not guarantee therapeutic efficacy.

Figures and Visuals (Section 2: Materials and Methods)

- While the inclusion of figures showing the plant’s distribution (Figure 2) is helpful, additional visuals such as chromatograms or docking visualization results would enhance the clarity of the experimental outcomes, especially for readers less familiar with molecular docking.

Practical Suggestions

Refine the Discussion of Statistical Results

- Since the antioxidant activity comparison between the extract and vitamin C did not yield statistically significant results, it would be prudent to reanalyze the data or add further experimental replicates. This could provide a more robust statistical comparison, which would strengthen the conclusions.

Expand on the Molecular Docking Discussion

- Consider a more in-depth analysis of the docking interactions, possibly discussing specific residues involved in the binding and how these interactions compare with other natural α-amylase inhibitors. Additionally, mention whether structural modifications to the phenolic compounds might improve their inhibitory effects.

Future Work Section

- While the conclusion briefly mentions potential future work, a dedicated section outlining the next steps (e.g., in vivo studies, compound modification) would add more depth to the paper and guide subsequent research.

Add Controls for Extraction Efficiency

- The extraction process could include a comparison with other solvents or extraction techniques to show that ethanol is indeed the most efficient method for extracting active compounds. This would strengthen the validity of the flavonoid content and antioxidant activity measurements.

Conclusion

This paper presents a solid investigation into the antioxidant and α-amylase inhibitory activities of*Melilotus indicus*. The strengths lie in the clear methodology and comprehensive use of in vitro and in silico techniques. However, further improvements can be made in statistical rigor, result interpretation, and future work planning. Including more context around the biological relevance of the docking study and adding in vivo validation would significantly bolster the paper's impact.

Here are a few alternative statistical methods that might improve the analysis, along with explanations on how they could be applied to the data:

1. **ANOVA (Analysis of Variance)**

- **When to use:** When comparing more than two groups or treatment conditions (e.g., different concentrations of the extract) and analyzing whether there are statistically significant differences between them.
- **Application in the study:** If you test the antioxidant activity (e.g., DPPH scavenging capacity) at multiple concentrations of the *Melilotus indicus* extract, ANOVA could help determine whether the differences between these concentrations are significant.
- **Benefit:** It allows for a more comprehensive comparison across multiple groups, reducing the chance of Type I errors (false positives) that can occur with multiple t-tests.

2. **Post-Hoc Tests (Tukey's HSD, Bonferroni Correction)**

- **When to use:** After performing an ANOVA, if you find significant differences, a post-hoc test helps pinpoint exactly which groups differ from each other.
- **Application in the study:** After finding significant differences in flavonoid content or antioxidant activity, use a post-

hoc test to identify which concentrations or conditions differ. For instance, is there a statistically significant improvement in activity at a specific extract concentration?

- **Benefit:** It helps clarify specific pairwise comparisons between groups, minimizing the risk of false positives due to multiple comparisons.

3. Linear or Nonlinear Regression Analysis

- **When to use:** When investigating relationships between a continuous independent variable (e.g., concentration of extract) and a dependent variable (e.g., antioxidant activity or inhibitory potential).
- **Application in the study:** Regression can be used to analyze the relationship between the concentration of the extract and the % inhibition of DPPH. You could fit a regression model to predict how much antioxidant activity is expected as concentration increases.
- **Benefit:** Provides a more nuanced understanding of dose-response relationships, allowing for predictions and confidence intervals around the results.

4. Confidence Intervals

- **When to use:** To provide a range within which the true value of a parameter (e.g., the IC50 value for antioxidant activity) is likely to fall.
- **Application in the study:** Instead of just reporting the IC50 or p-values, report confidence intervals (e.g., 95%) around your results. This will give a clearer picture of the uncertainty associated with the estimates.
- **Benefit:** Provides a more intuitive understanding of the reliability of the results, rather than just relying on binary significance testing.

5. Mann-Whitney U Test or Wilcoxon Signed-Rank Test

- **When to use:** If the data are not normally distributed or the sample sizes are small, non-parametric tests such as the Mann-Whitney U test (for two independent groups) or the Wilcoxon signed-rank test (for paired data) can be used as alternatives to the t-test.
- **Application in the study:** If the assumption of normality does not hold for the DPPH assay results or flavonoid content data, these tests could be more appropriate for comparing groups (e.g., extract vs. control).
- **Benefit:** Non-parametric tests do not rely on normal distribution assumptions and are more robust with small sample sizes or skewed data.

To validate the **in vitro** and **in silico** findings of *Melilotus indicus* ethanolic extract's antioxidant activity and α-amylase inhibitory potential, **in vivo** studies would be crucial for assessing the extract's efficacy, safety, and mechanism of action in living organisms. Here are some specific **in vivo** studies you could conduct:

1. Antioxidant Activity Assessment in Animal Models

- **Objective:** Validate the in vitro antioxidant activity of the extract in a biological system.
- **Animal Model:** Use rats or mice exposed to oxidative stress-inducing agents such as carbon tetrachloride (CCl_4), hydrogen peroxide (H_2O_2), or lipopolysaccharides (LPS).
- **Study Design:**
  - Divide the animals into groups: control (normal), stress-induced (without treatment), and stress-induced (treated with *Melilotus indicus* extract at different doses).
  - Measure markers of oxidative stress such as lipid peroxidation (malondialdehyde levels), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activity in serum or tissues.
- **Expected Outcome:** If the extract has significant antioxidant effects, animals treated with it should show reduced oxidative stress markers compared to the untreated stress-induced group.

2. Anti-Diabetic Study to Test α-Amylase Inhibition in vivo

- **Objective:** Validate the α-amylase inhibitory potential of the extract in an in vivo setting, supporting its use as an anti-diabetic agent.
- **Animal Model:** Use diabetic rats or mice induced with streptozotocin (STZ) or alloxan, which mimics the type 1 diabetes condition by destroying pancreatic β-cells, or a high-fat diet combined with STZ to mimic type 2 diabetes.
- **Study Design:**
  - Divide the animals into control (non-diabetic), diabetic control, and diabetic treated with*Melilotus indicus* extract (various doses), and an additional group treated with a standard α-amylase inhibitor like acarbose.
  - Monitor fasting blood glucose levels, oral glucose tolerance test (OGTT), and insulin levels.
  - After treatment, collect pancreatic tissue for histopathological analysis and measure serum α-amylase levels to directly assess enzyme inhibition.
- **Expected Outcome:** If the extract is an effective α-amylase inhibitor, treated diabetic animals should show improved glucose tolerance, reduced fasting blood glucose levels, and lower α-amylase activity compared to untreated diabetic controls.
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