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.

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

Study Overview

This study investigates the antioxidant activity and α-amylase inhibitory potential of*Melilotus indicus* ethanolic extract, analyzing flavonoid content and conducting molecular docking to explore potential interactions with α-amylase. The research aims to validate *Melilotus indicus* as a source of natural antioxidants and inhibitors for α-amylase, which could contribute to anti-diabetic therapies. The study encompasses both in vitro and in silico methodologies, involving flavonoid quantification, DPPH antioxidant assays, and docking studies comparing compounds from *Melilotus indicus* with the drug acarbose.

Strengths of the Study

Combination of In Vitro and In Silico Approaches The dual approach of using both in vitro assays and in silico docking gives a broader context to the findings. The DPPH radical scavenging assay provides quantitative data, while the docking studies allow for an exploration of molecular interactions with α-amylase.

Relevance of Study: The focus on flavonoid content, antioxidant potential, and α-amylase inhibition aligns well with current research trends on natural product-based treatments for diabetes. The exploration of *Melilotus indicus*, a plant known for its medicinal value, offers potential for drug discovery.

Clear Objectives and Methodology: The study's objectives are clearly defined, and the methods for flavonoid quantification, antioxidant assessment, and molecular docking are well-structured. The use of standards such as quercetin and ascorbic acid provides a basis for comparison.

Critical Issues and Areas for Improvement

Lack of Phytochemical Profiling

- While the study claims that flavonoids are the primary phenolic constituents, the analysis remains narrow. A broader

phytochemical profiling using advanced techniques such as high-performance liquid chromatography (HPLC) or mass spectrometry would have provided a more comprehensive understanding of the extract's composition.
- The study does not provide specific data on other relevant phytochemicals like coumarins, saponins, and alkaloids, which are known constituents of *Melilotus indicus*. Without this, the study fails to correlate the biological activities with the full range of active compounds.

Inadequate Controls and Comparative Standards

- The antioxidant activity of the extract is compared only to vitamin C, which is an exceptionally strong antioxidant. This comparison might be misleading, as the extract shows significantly lower activity (IC50 = 1.6 mg/mL vs. vitamin C's IC50 = 0.01 mg/mL). A broader range of reference compounds with varying antioxidant capacities should have been included to provide a more realistic evaluation of the extract's potential.

I recommend focusing on the following**three widely used compounds** for comparison in antioxidant studies:

Ascorbic Acid (Vitamin C):

- A strong antioxidant with well-known properties. It serves as a standard for many antioxidant assays.
- **IC50 Value**: ~0.01 mg/mL in DPPH assays.
- **Rationale**: Since it has a high antioxidant capacity, comparing the**Melilotus indicus** extract to ascorbic acid provides insight into how potent the plant extract is in relation to a known, highly effective antioxidant.

Trolox:

- A water-soluble analog of Vitamin E, commonly used in antioxidant research.
- **IC50 Value**: ~0.02-0.05 mg/mL in DPPH assays.
- **Rationale**: Trolox is often used in assays to compare antioxidant capacity. Including it as a reference helps situate the plant extract's activity against both water- and lipid-soluble antioxidants.

Butylated Hydroxytoluene (BHT):

- A synthetic antioxidant commonly used in food preservation.
- **IC50 Value**: ~0.03-0.06 mg/mL in DPPH assays.
- **Rationale**: As a synthetic antioxidant with lower activity than ascorbic acid, it allows for a comparison to see whether the plant extract is potentially more suitable as a natural alternative to synthetic compounds like BHT.
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Low Flavonoid Content

- The flavonoid content (0.49 mg/g ± 3.818) appears low, which may undermine the significance of the antioxidant activity observed. The study should have discussed whether this low flavonoid content is consistent with other studies on *Melilotus indicus* or whether extraction efficiency was a limiting factor.

Incomplete Validation of Antioxidant Activity

- The study employs only the DPPH assay for antioxidant evaluation. Since DPPH is limited to measuring radical scavenging activity, other assays like ABTS, ferric reducing antioxidant power (FRAP), or ORAC should have been included to confirm the antioxidant potential from different mechanistic perspectives.

Weakness in Molecular Docking Study

- The docking scores for chlorogenic acid and kaempferol (-6.09196091 and -5.45953274 kcal/mol) are higher (less favorable) than that of acarbose (-8.8864727 kcal/mol), indicating weaker binding. Despite this, the study makes optimistic conclusions about the potential of these compounds. This overstates the relevance of the results, as the weaker docking scores suggest they may not be as effective inhibitors as acarbose.
- Moreover, the study does not provide any post-docking validation, such as molecular dynamics simulations or in vitro α-amylase inhibition assays, which would have solidified the docking results. The in silico findings remain theoretical without experimental verification.

Statistical Analysis

- The statistical analysis section is very brief, only mentioning the use of t-tests for DPPH assays. There is no clear discussion of whether the results are statistically significant. Detailed statistical analyses with confidence intervals, standard deviations, and p-values are necessary to validate the findings rigorously.

Recommended Statistical Methods:

ANOVA (Analysis of Variance):

- To compare the antioxidant activities (IC50 values) of**Melilotus indicus** extract and the three reference compounds (Ascorbic Acid, Trolox, and BHT). It will help identify statistically significant differences between them.

Independent T-Test:

- To directly compare the IC50 value of the**Melilotus indicus** extract with **one key reference** (e.g., Ascorbic Acid). This can help assess whether the plant extract's antioxidant activity is significantly different from the gold standard.

Regression Analysis:

- To examine the dose-response relationship for the extract's antioxidant activity (e.g., in DPPH assays). This ensures the consistency and reliability of IC50 determination for your study.

Absence of a Dose-Response Curve for α-Amylase Inhibition

- The study focuses on molecular docking but does not perform in vitro α-amylase inhibition assays to confirm the computational predictions. Without these experimental data, the biological relevance of the docking results is

speculative.

Lack of Mechanistic Insights

- The study does not delve into the mechanisms by which the flavonoids or phenolic acids might exert their antioxidant or α-amylase inhibitory effects. Providing mechanistic insights through detailed biochemical discussions or exploring structure-activity relationships (SAR) could have strengthened the study's impact.

Recommendations for Improvement

Phytochemical Characterization: Use techniques such as HPLC or GC-MS to identify and quantify other important phytochemicals in the extract, providing a more complete profile of the active compounds.

Broaden Antioxidant Assays: Incorporate additional antioxidant assays like ABTS or FRAP to verify the antioxidant potential and better understand the mechanisms involved.

In Vitro α-Amylase Inhibition: Perform actual in vitro α-amylase inhibition assays to confirm the findings from the molecular docking study. The study’s conclusions on antidiabetic potential will remain speculative without these data.

Statistical Rigor: Apply more detailed statistical analysis to all experimental data. Significance testing (with clear p-values) is essential to validate any claims made regarding the activity of the extract.

Mechanistic Studies: Include mechanistic studies or a SAR analysis to understand how specific phytochemicals are contributing to the antioxidant and inhibitory activities.

Suitability for Publication:

While the study has merits, particularly in combining in vitro and in silico approaches, it suffers from several weaknesses. The lack of comprehensive phytochemical analysis, reliance on limited assays for antioxidant activity, and the absence of experimental validation for docking results are significant flaws. Without addressing these issues, the study's conclusions are not fully supported by the data. **In its current state, the study is not suitable for publication**, although it may be acceptable after substantial revision and additional experimental work.