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REVIEWER'S REPORT

Manuscript No.: IJAR- 59138

Title: ECO-FRIENDLY PAINT INCORPORATING A COMBINATION OF TEA TREE OIL AND CLOVE ESSENTIAL OIL AS A NATURAL ANTIFUNGAL AGENT AGAINST THE GROWTH OF ASPERGILLUS NIGER.

Recommendation:
Accept as it is .....
Accept after Major revision.....
Accept after Minor revision
Do not accept (*Reasons below*)

Rating	Excel.	Good	Fair	Poor
Originality		✓		
Techn. Quality		✓		
Clarity			✓	
Significance			✓	

Reviewer Name: Dr. ANAPANA GOPAL

Reviewer's Comment for Publication.

General Comments

The manuscript presents an experimental evaluation of tea tree oil and clove essential oil incorporated into a water-based paint formulation for inhibition of *Aspergillus niger*. The study compares an unamended control with 2% tea tree oil, 2% clove essential oil, and a 1% + 1% combination, using four replicates per treatment. Mycelial coverage was quantified using ImageJ and analysed using ANOVA, Tukey's HSD, Fisher's exact test and additional non-parametric approaches.

The manuscript addresses a relevant topic at the intersection of **natural antifungal agents, sustainable materials and fungal control in water-based paints**. The experimental results indicate substantial suppression of *A. niger* growth by all essential-oil treatments, while the combination treatment produced zero measurable mycelial coverage in all four replicates.

The manuscript also demonstrates commendable awareness of several of its own limitations, particularly the absence of a Tween-80 vehicle control, small number of replicates, absence of a concentration series, lack of formal synergy testing, uncontrolled incubation conditions, use of a single fungal species and short observation period.

However, **Minor revision is required**. The most important concerns involve experimental controls, replication and experimental-unit structure, the interpretation of synergy and fungistatic activity, biosafety/experimental conditions, physical paint-quality assessment, and the strength of the conclusions relative to the available data.

ISSN: 2320-5407
International Journal of Advanced Research
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www.journalijar.com

REVIEWER'S REPORT

1. Originality and Scientific Contribution

The manuscript identifies a potentially useful research gap: most previous essential-oil antifungal assays have examined oils in free form, whereas this study investigates their activity after incorporation into a dried polymer paint film.

This is a potentially interesting contribution.

However, the authors should strengthen the novelty statement by clearly explaining:

- what previous studies have tested essential oils specifically in paint films;
- whether tea tree oil and clove oil have previously been incorporated individually or together into architectural paints;
- what is novel about the 1% + 1% formulation;
- whether the novelty is the formulation, the test system, the combination, or the comparative experimental design.

The claim that such combinations **"have, to our knowledge, not been examined at all"** should be supported by a more systematic literature search or should be expressed more cautiously.

2. Minor Concern: Absence of a Vehicle Control

This is the most important experimental-design issue.

The control P0 contained **no active agent and no Tween 80**, whereas P1, P2 and P3 each contained **2% Tween 80** in addition to the essential oils.

Thus, the observed difference between P0 and the amended treatments cannot be attributed exclusively to tea tree oil and/or clove oil.

Tween 80 itself may influence microbial growth or membrane properties. The manuscript correctly acknowledges this limitation.

The authors should therefore:

- explicitly identify P0 versus amended formulations as a **confounded comparison**;
- avoid stating that the essential oils alone account for the entire observed effect;
- include a paint + 2% Tween 80 vehicle control in future experimental work;
- preferably discuss how this missing control affects interpretation of the present findings.

This issue should also be emphasized in the Abstract and Conclusion rather than appearing only in the Limitations section.

ISSN: 2320-5407
International Journal of Advanced Research
Publisher's Name: Jana Publication and Research LLP
www.journalijar.com

REVIEWER'S REPORT

3. Experimental Unit and Blocking Structure

The manuscript describes a completely randomized design with four treatments and four replicates. However, each of the four plastic boxes contained **one coupon from every treatment**, meaning that the box is naturally a blocking factor.

The authors subsequently performed an analysis incorporating box as a blocking factor and reported a non-significant box effect and a significant treatment effect.

This is useful, but the statistical description should be revised.

The authors should clarify:

- whether the primary analysis is a completely randomized design or randomized complete block design;
- how randomization was performed within each box;
- whether box effects were specified before analysis or introduced post hoc;
- whether the four boxes should be considered biological/experimental replicates;
- whether individual coupons within boxes can legitimately be considered independent observations.

Because one coupon from every treatment occurs within each box, the **paired/block structure is central to the design**, not merely an optional sensitivity analysis.

4. Small Number of Replicates

Only **four replicates per treatment** were used.

The manuscript appropriately recognizes that this produces limited statistical power, particularly for detecting differences among the amended formulations.

Nevertheless, the manuscript occasionally makes relatively strong claims from this small experiment.

The authors should ensure that:

- treatment-vs-control effects are clearly distinguished from comparisons among P1, P2 and P3;
- non-significant comparisons are not interpreted as evidence of equivalence;
- conclusions regarding the superiority or practical advantage of P3 remain appropriately qualified.

The statement that the combination's advantage is **"very likely real"** should be reconsidered because the mean-based comparison did not statistically distinguish P3 from P1 or P2.

ISSN: 2320-5407
International Journal of Advanced Research
Publisher's Name: Jana Publication and Research LLP
www.journalijar.com

REVIEWER'S REPORT

5. Interpretation of the P3 Combination

The most striking result is that P3, containing **1% tea tree oil + 1% clove oil**, produced 0% measurable mycelial coverage in all four replicates, compared with 12.45% for P1 and 9.58% for P2.

This is an interesting observation.

However, the manuscript must distinguish among:

- complete inhibition in the present experiment;
- additive activity;
- synergistic activity;
- statistical superiority.

The authors themselves correctly state that formal synergy cannot be established without concentration-response data and a suitable combination assay.

The wording **"the interaction is at least additive"** should nevertheless be reconsidered. Demonstrating additivity itself requires an appropriate reference model and dose-response information. The current experiment shows that the combination produced greater observed inhibition than either tested single formulation, but it does not formally establish an additive interaction.

A safer interpretation would be:

"The combination produced complete inhibition under the tested conditions, despite containing only 1% of each oil; however, additive or synergistic interaction cannot be formally distinguished from the present experimental design."

6. Lack of a 1% Single-Oil Treatment

The study compares:

- P1: 2% tea tree oil;
- P2: 2% clove oil;
- P3: 1% tea tree + 1% clove.

There is no 1% tea tree-only or 1% clove-only treatment.

This is important because the authors discuss whether the combination produces an effect greater than expected from the individual components.

Without testing the individual oils at 1%, it is difficult to determine the dose-response behaviour of either oil at the same component concentration used in P3.

The authors appropriately recognize this limitation, but it should be given greater prominence because it directly affects the interpretation of the combination treatment.

ISSN: 2320-5407
International Journal of Advanced Research
Publisher's Name: Jana Publication and Research LLP
www.journalijar.com

REVIEWER'S REPORT

7. Concentration–Response Relationship

Only one concentration level for each single oil and one combination ratio was examined.

Consequently, the study cannot determine:

- minimum inhibitory concentration;
- optimum concentration;
- concentration-dependent inhibition;
- dose-response relationships;
- whether lower concentrations could provide adequate protection;
- whether higher concentrations adversely affect the paint film.

The authors acknowledge this limitation.

A concentration-response experiment should therefore be recommended as an essential follow-up study.

8. Fungistatic Versus Fungicidal Interpretation

The manuscript concludes that the P3 treatment is **fungistatic rather than fungicidal** because spores were observed on the surface while no measurable mycelium developed.

This conclusion is too strong based solely on the reported observations.

The presence of spores without visible mycelial growth does not by itself establish that the spores remain viable or that the formulation merely inhibited germination rather than killed the fungal propagules.

The manuscript itself proposes that a subsequent viability/recovery experiment is required.

Therefore, throughout the manuscript, the authors should use wording such as:

"The observed effect is consistent with a fungistatic effect, but fungistatic activity was not experimentally confirmed."

This distinction is scientifically important.

9. ImageJ Measurement and Observer Bias

Mycelial coverage was measured digitally using ImageJ.

The manuscript acknowledges that segmentation thresholds were manually selected and therefore could introduce observer bias.

The authors should provide more methodological information regarding:

ISSN: 2320-5407
International Journal of Advanced Research
Publisher's Name: Jana Publication and Research LLP
www.journalijar.com

- image acquisition conditions;
- camera/device used;
- illumination;
- distance and orientation;
- image resolution;
- thresholding procedure;
- whether the same threshold was applied to all samples;
- whether measurements were performed blind to treatment.

Ideally, measurements should be independently assessed by more than one observer, with agreement reported.

10. Incubation Environment and Experimental Control

The assay was conducted in **non-airtight plastic boxes in an outdoor fifth-floor reading area**, because the laboratory lacked a laminar-flow cabinet. Temperature and humidity were monitored at approximately 27–30°C and 80% relative humidity.

This introduces potentially important environmental variability.

The authors themselves identify possible differences in:

- airflow;
- volatile-oil loss;
- temperature;
- humidity;
- film thickness;
- emulsion stability.

The control treatment itself varied substantially among boxes, from 39.94% to 98.18% coverage.

Therefore, the environmental conditions should be described more precisely, and the conclusions should acknowledge that this was a **proof-of-concept laboratory assay under monitored but not fully controlled environmental conditions**.

11. Biosafety and Laboratory Conditions

A potentially significant issue is that *A. niger* culture development and the antifungal assay were conducted in an **open fifth-floor reading area** rather than in a dedicated laboratory environment.

Because the study involves fungal culture and aerosolizable spores, the authors should provide a clear statement concerning:

- institutional biosafety approval or authorization, if applicable;
- containment and handling procedures;
- disposal/decontamination procedures;

ISSN: 2320-5407
International Journal of Advanced Research
Publisher's Name: Jana Publication and Research LLP
www.journalijar.com

- measures used to prevent environmental contamination and exposure.

This is particularly important because the manuscript describes an educational/school laboratory setting. The study should demonstrate that appropriate biosafety procedures were followed.

12. Paint Film Preparation Requires Better Standardisation

The paint was applied manually to 2 × 2 cm wooden coupons and allowed to dry for **10 minutes at room temperature** before exposure to the inoculated medium.

The manuscript later acknowledges that manual brushing could result in differences in film thickness and active-agent loading.

This is an important source of experimental variability.

The authors should report, if available:

- amount of paint applied per coupon;
- wet-film thickness;
- dry-film thickness;
- number of coats;
- drying conditions;
- mixing duration;
- time between formulation and application.

Future studies should use a controlled coating method to reduce variability.

13. Paint Formulation Stability

The manuscript states that the oils were mixed with Tween 80 and stirred until homogeneous before storage.

However, no information is provided about:

- duration of storage before application;
- emulsion stability;
- phase separation;
- oil distribution;
- pH;
- viscosity;
- whether formulations remained homogeneous throughout application.

The authors themselves suggest that phase separation could explain some experimental variability.

This should be discussed more prominently in the Methods and Limitations.

ISSN: 2320-5407
International Journal of Advanced Research
Publisher's Name: Jana Publication and Research LLP
www.journalijar.com

REVIEWER'S REPORT

14. Physical Quality of Paint Film

The manuscript reports no cracking, peeling or visually observable colour change and reports similar scratch-test observations among treatments.

This is useful as an initial observation, but it does not establish that the physical properties of the paint are unaffected.

The authors appropriately acknowledge that standard tests such as adhesion, opacity, drying time, hardness and colorimetry were not conducted.

Therefore, statements such as **"without visible damage to the film"** are acceptable, but stronger claims concerning unchanged paint quality should be avoided.

The conclusion should explicitly state that **only qualitative physical assessment was performed**.

15. Statistical Analysis

The manuscript provides considerably more statistical information than is common in small experimental studies, which is a strength.

The reported ANOVA gives:

- treatment df = 3;
- error df = 12;
- F = 16.15;
- p = 0.0002;
- $\eta^2 = 0.80$;
- $\omega^2 = 0.74$.

The authors also report Levene's test and Shapiro-Wilk results and perform non-parametric cross-checks.

However, the statistical analysis should be presented more carefully because:

1. the sample size is very small;
2. the data are bounded percentages;
3. P3 has zero variance;
4. the observations have a block structure;
5. P0 differs from the other groups in Tween-80 content.

The authors should clarify which analysis is considered the **primary analysis** and which are sensitivity analyses.

16. Tukey's HSD and Interpretation of Non-Significant Differences

Tukey's HSD separated P0 from P1, P2 and P3, but did not separate P1, P2 and P3.

ISSN: 2320-5407
International Journal of Advanced Research
Publisher's Name: Jana Publication and Research LLP
www.journalijar.com

REVIEWER'S REPORT

This is an important result and should be emphasized more clearly.

In particular, the manuscript correctly states that the present study **cannot demonstrate that clove oil outperforms tea tree oil**.

The same caution should be applied to P3.

Although P3 achieved zero growth in all four replicates, the mean comparison did not statistically distinguish P3 from P1 or P2.

Therefore, claims that P3 is definitively superior should be avoided.

17. Fisher's Exact Test

The binary growth-incidence analysis is a useful supplementary analysis.

The study reports:

- P0: 4/4 growth;
- P1: 4/4 growth;
- P2: 3/4 growth;
- P3: 0/4 growth.

P3 differed significantly from P0 and P1 but not from P2.

This result actually provides a more nuanced interpretation than the manuscript's broader claims.

The authors should clearly state that the evidence for P3's complete suppression is strongest against P0 and P1 in the Fisher analysis, whereas the difference from P2 was not statistically significant.

18. Relative Inhibition Analysis

Relative inhibition was calculated separately within each box. P1, P2 and P3 showed mean relative inhibition of 81.03%, 85.85% and 100%, respectively.

This is useful descriptively.

However, because the control itself varied considerably among boxes, the authors should explain whether this calculation was intended only as a descriptive normalization or whether inferential conclusions were based on it.

The equation and values should be checked carefully for rounding and reproducibility.

19. Box 2 Anomaly

The discussion of the Box 2 anomaly is thoughtful and transparent.

ISSN: 2320-5407
International Journal of Advanced Research
Publisher's Name: Jana Publication and Research LLP
www.journalijar.com

REVIEWER'S REPORT

The authors identify several possible sources:

- manual coating thickness;
- airflow and volatile loss;
- emulsion stability;
- handling differences.

This is a strength of the manuscript.

However, these explanations remain hypotheses because they were not experimentally tested.

The language should therefore use:

"possible explanations include…"

rather than implying that sample preparation or handling was definitively responsible.

20. Mechanistic Interpretation

The manuscript attributes tea tree oil activity mainly to terpinen-4-ol and α-terpineol and clove activity mainly to eugenol, with proposed membrane-related mechanisms.

These mechanisms provide useful biological context.

However, the current study did **not measure**:

- terpinen-4-ol;
- eugenol;
- membrane damage;
- metabolic disruption;
- compound diffusion from the paint film.

Therefore, the mechanistic explanation should be presented as **literature-supported interpretation**, not as a mechanism demonstrated by the current experiment.

In particular, the statement that the compounds "remain able to diffuse or volatilise out of the paint layer" is plausible but was not directly measured.

21. Claims Regarding Replacement of Synthetic Biocides

The manuscript describes the essential oils as a plausible replacement for synthetic biocides and compares them with silver nanoparticles.

The current experiment, however, does not directly compare the formulation against:

- a conventional commercial antifungal paint;
- silver nanoparticles;
- another synthetic biocide;

ISSN: 2320-5407
International Journal of Advanced Research
Publisher's Name: Jana Publication and Research LLP
www.journalijar.com

- a standard commercial preservative.

Therefore, the study demonstrates **antifungal potential**, but it does not establish equivalence or superiority to existing commercial biocides.

The conclusion should use terms such as:

"potential natural antifungal additive"

or

"promising proof-of-concept formulation"

rather than implying that replacement has already been demonstrated.

22. Long-Term Durability

The manuscript correctly recognizes that the two-week assay does not represent the expected service life of a wall coating.

This is a Minor limitation because essential oils are volatile compounds.

The authors should emphasize that the current experiment demonstrates **short-term antifungal activity under the tested conditions**, not long-term protection.

Aging, repeated wetting/drying, UV exposure and extended storage/use conditions would be relevant in future validation studies.

23. Use of a Single Fungal Species

Only *A. niger* was tested.

The authors acknowledge that real building surfaces contain diverse fungal communities, including *Cladosporium*, *Penicillium* and *Aureobasidium*.

Therefore, the conclusions should be restricted specifically to *A. niger*.

The phrase **"antifungal agent"** may be acceptable in the title, but the broader discussion should avoid implying broad-spectrum activity until additional fungal species have been evaluated.

24. Introduction Requires Some Refinement

The Introduction provides a reasonable background concerning water-based paints, fungal colonisation and essential oils.

However, some statements are broader than necessary.

ISSN: 2320-5407
International Journal of Advanced Research
Publisher's Name: Jana Publication and Research LLP
www.journalijar.com

For example, the discussion of *A. niger*-associated disease and the long list of affected organs and mycotoxins may distract from the central material-science question.

The Introduction would be stronger if it focused more directly on:

- fungal colonisation of painted surfaces;
- limitations of conventional and eco-friendly paint formulations;
- antifungal properties of tea tree and clove oils;
- challenges of incorporating volatile compounds into polymer films;
- the specific research gap addressed by the experiment.

25. References and Citations

The reference list includes relevant sources concerning:

- eco-friendly paints;
- silver nanoparticles;
- *A. niger*;
- tea tree oil;
- clove oil;
- essential-oil mechanisms;
- synergy.

However, the authors should carefully check that each citation directly supports the associated claim.

In particular, the manuscript should distinguish evidence obtained from:

- studies on *A. niger*;
- studies on other fungal species;
- free essential oils;
- essential oils incorporated into materials;
- direct application versus vapour exposure.

For example, evidence from dermatophytic fungi should not automatically be presented as direct evidence for the behaviour of clove oil against *A. niger*.

The reference formatting should also be standardized according to the target journal.

26. AI Declaration

The manuscript states that artificial intelligence was used for **reference searching and background reading**, specifically mentioning Bohrium and OPSI 2026 guidelines.

The authors should ensure that this declaration follows the exact AI-disclosure requirements of the target journal.

If the journal has a prescribed wording or policy, that wording should be used.

ISSN: 2320-5407
International Journal of Advanced Research
Publisher's Name: Jana Publication and Research LLP
www.journalijar.com

REVIEWER'S REPORT

27. Language and Editorial Quality

The manuscript is generally readable and has a clear scientific structure, but substantial language and formatting editing is recommended.

Examples include:

- title written entirely in capital letters;
- "Keywords: -" formatting;
- "Introduction:-", "Materials and Methods:-", etc.;
- inconsistent use of abbreviations;
- lengthy sentences in the Discussion;
- occasional repetitive statements;
- inconsistent terminology such as "essential oil," "oil," and "active agent";
- excessive use of quotation marks around terms;
- some conclusions are stronger than the statistical evidence permits.

The manuscript would benefit from professional English editing and formatting according to the target journal's style.

28. Figures and Tables

The manuscript includes several useful tables and graphical presentations.

Table 1

The replicate-level mycelial coverage values are valuable because they allow readers to see the substantial variability among experimental units.

Table 2

The descriptive statistics clearly demonstrate the reduction in coverage and the high variability of P1 and P2.

Table 3

Relative inhibition by box is useful, particularly because the control coverage differs considerably among boxes.

Figures 1–3

The graphical presentation is generally clear. Figure 1 on page 5 appropriately indicates the statistical groupings, while Figure 2 displays replicate-level variability and Figure 3 illustrates relative inhibition.

However, figure labels, fonts, statistical notation and treatment abbreviations should be checked against journal requirements.

ISSN: 2320-5407
International Journal of Advanced Research
Publisher's Name: Jana Publication and Research LLP
www.journalijar.com

REVIEWER'S REPORT

Strengths of the Manuscript

The manuscript has several important strengths:

- Relevant and potentially novel research question.
- Clear comparison among control, individual essential oils and combination treatment.
- Direct testing of essential oils within a paint matrix rather than only in free form.
- Replicate-level data are provided.
- Mycelial coverage is quantitatively assessed using ImageJ.
- Appropriate attention is given to percentage-data transformation.
- ANOVA, post-hoc and non-parametric analyses are reported.
- Effect sizes are reported.
- Box-level variability is explicitly examined.
- The authors are appropriately cautious in stating that synergy has not been formally demonstrated.
- The manuscript explicitly acknowledges the absence of a vehicle control.
- Physical paint-film observations are included.
- Limitations are unusually transparent and comprehensive for a short experimental study.

Overall Recommendation

The manuscript contains a **potentially useful proof-of-concept experimental study** demonstrating substantial inhibition of *A. niger* growth on a water-based paint film containing tea tree oil and clove essential oil. The zero measurable mycelial coverage observed for the 1% + 1% formulation in all four replicates is an interesting finding.

The study is strengthened by transparent reporting of replicate-level results, statistical analyses and limitations. However, the lack of a Tween-80 vehicle control, very small replication, blocked experimental structure, uncontrolled environmental conditions and absence of formal synergy and fungicidal/fungistatic testing substantially limit the strength of the current conclusions.

The manuscript should therefore be revised to ensure that the conclusions reflect **antifungal activity observed under the specific experimental conditions**, rather than established synergy, fungicidal action, long-term protection, or replacement of commercial biocides.

Final Decision: Minor Revision