PHYSICAL QUALITY CHARACTERIZATION AND TOTAL FLAVONOID CONTENT OF NEEM LEAF KOMBUCHA (AZADIRACHTA INDICA A. JUSS) AS A NUTRACEUTICAL CANDIDATE

Tiara Ajeng Listyani[1*], Bagas Ardiyantoro[2], Anna Fitriawati[3], Jihannaz Firdamaya[4]
Pharmacy Departement, Health Science Faculty, Universitas Duta Bangsa Surakarta, Indonesia[1234]
*Correspondence Email : tiara_ajenglistyani@udb.ac.id

ABSTRACT

Neem leaves (*Azadirachta indica* A. Juss) contain secondary metabolites with potential health benefits. Fermentation using a SCOBY may modify the phytochemical profile and enhance their potential as functional beverages; however, the strong bitterness of neem leaves remains a challenge for nutraceutical development. This study aimed to characterize the physical quality and initial total flavonoid content (TFC) of neem leaf kombucha at various concentrations and fermentation times. Four formulas (0.3%, 1%, 3%, and 1% with clay pretreatment) were evaluated on days 8, 12, and 14. Physical quality was assessed by measuring pH and alcohol content (refractometry), while TFC was determined using UV-Vis spectrophotometry. On day 14, pH ranged from 2.54 to 3.21 in undiluted samples and from 2.97 to 3.44 after 21-fold dilution, with alcohol contents of 4.3–6.3% and 0.3–0.5%, respectively. TFC values in undiluted samples were 0.02862, 0.03388, 0.03695, and 0.03016 mg QE/mL for the 0.3%, 1%, 3%, and clay-pretreated 1% formulas, respectively, while diluted samples contained 0.01416, 0.02094, 0.02440, and 0.01990 mg QE/mL. Fermentation for 14 days produced the highest flavonoid response, with the 3% concentration showing the highest TFC. The clay-pretreated 1% kombucha retained a substantial flavonoid content and may improve palatability.

KEYWORDS

Azadirachta indica; kombucha; total flavonoid content; fermentation; nutraceutical.

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INTRODUCTION

The utilization of natural materials as sources of functional foods and nutraceutical candidates continues to expand, driven by growing interest in plant based products containing bioactive compounds. One plant with such potential is neem (*Azadirachta indica* A. Juss), a member of the Meliaceae family that has long been used in various traditional medicine systems. Neem leaves are known to contain a diverse range of secondary metabolites, including flavonoids, phenolic compounds, alkaloids, terpenoids,

and various limonoids. Recent studies indicate that neem leaves are an attractive source of phytochemicals for the development of food and pharmaceutical products, although the standardization of their composition, safety, and sensory acceptability still requires further investigation. Flavonoids are an important group of compounds because they are associated with a wide range of biological activities and can serve as one of the chemical parameters for evaluating the quality of natural materials (Andersa *et al*., 2024; Boukeloua *et al*., 2024).

The use of neem leaves as a beverage ingredient is mainly limited by their strong, bitter taste. In addition to biologically valuable components, neem leaves contain various compounds that may influence the sensory characteristics of the product. Andersa et al. (2024) emphasized that sensory acceptability is one of the challenges in developing neem as a food ingredient, and that de-bittering or bitterness-masking approaches are required to improve product palatability. This issue is particularly relevant to the development of beverage formulations, because a product with a high bioactive content may not be accepted if its organoleptic properties do not match consumer preferences. Therefore, the development of neem-based products should consider a balance among the preservation of bioactive compounds, physicochemical characteristics, and product acceptability.

One approach that can be used to develop plant materials into functional beverages is fermentation using a kombucha culture. Kombucha is essentially produced through the fermentation of a sugar-containing substrate using a Symbiotic Culture of Bacteria and Yeast (SCOBY). The activity of yeasts and bacteria during fermentation generates various metabolites, including organic acids, ethanol in varying amounts, and changes in the phenolic components derived from the substrate. In current food technology, the choice of substrate is no longer limited to black or green tea. A variety of plants, fruits, flowers, and herbal materials have been evaluated as alternative substrates for producing kombucha-analog fermented beverages with distinct chemical and sensory characteristics. Barros et al. (2024) showed that the use of alternative herbal substrates holds considerable technological promise; however, research gaps remain, particularly regarding chemical composition, physicochemical quality, sensory characteristics, and product standardization.

Kombucha fermentation not only alters taste and acidity but can also modify the bioactive compounds in plant materials. Liang et al. (2024) reported that fermentation can increase the contents of total polyphenols, total flavonoids, quercetin, kaempferol, and several catechins compared with the unfermented material. These increases are associated with biotransformation processes involving microbial activity, enzymes, organic acids, and the fermentation environment. In that study, total flavonoids increased by approximately 69.23% after fermentation, although the changes in individual compounds did not always follow the same direction. These findings indicate that fermentation time is an important variable, because fermentation can cause the release, transformation, or, under certain conditions, degradation of phenolic components. Therefore, total flavonoid content should be monitored together with physical quality parameters so that the product profile during fermentation can be evaluated more comprehensively.

The pH is an important indicator for monitoring kombucha fermentation. During fermentation, yeasts metabolize sugars and produce intermediate metabolites, while acetic acid bacteria contribute to the formation of organic acids, so the pH of the product gradually decreases. Salinas-Ruiz et al. (2026) showed that increasing fermentation time and temperature led to a progressive decrease in pH, accompanied by increases in acetic acid and lactic acid. These results indicate that pH can serve as a practical parameter for describing the progress of fermentation, although it cannot be used alone to explain all of the chemical changes in the product. Herbal fermentation lasting approximately 7-14 days

is also widely used in the development of kombucha-analog beverages; therefore, observations at multiple time points are needed to determine changes in quality throughout the process (Barros et al., 2024; Salinas-Ruiz et al., 2026).

In addition to pH, alcohol formation is part of the metabolic dynamics of kombucha. Yeasts produce ethanol during sugar fermentation, and part of this ethanol can subsequently be oxidized by acetic acid bacteria to acetic acid. Thus, alcohol content is not static and may change depending on fermentation time, SCOBY composition, sugar content, temperature, and substrate type. Dartora et al. (2023), in a 14-day kombucha fermentation study, showed that fermentation time simultaneously affected pH, ethanol, acetic acid, polyphenol components, and sensory characteristics. This reinforces the importance of monitoring physicochemical parameters during the development of fermented beverages, particularly when the product is intended as a nutraceutical beverage candidate that requires consistent quality. A recent review also emphasized that variations in substrate and fermentation conditions are among the main causes of heterogeneity in kombucha composition and are the reason why standardization of the production process is needed (Pereira, 2026).

From a pharmaceutical perspective, the standardization of raw materials and fermented products is necessary to ensure that changes during processing do not eliminate the chemical characteristics that underlie the functional potential of the material. Total flavonoid content (TFC) can be used as one parameter to quantitatively describe the flavonoid content of a plant matrix or fermented product. However, TFC values are influenced by the raw material, extraction method, fermentation conditions, and analytical method used. Therefore, TFC characterization should not be separated from the quality parameters of the starting material and the process conditions. Studies on neem have also shown that flavonoid content can differ markedly depending on the type of extract and the preparation technique, so control of raw materials and processes is an important part of producing a consistent product (Andersa et al., 2024).

Although research on kombucha and the use of herbal substrates is developing rapidly, information on the use of *A. indica* leaves as a kombucha fermentation substrate remains limited, particularly studies that integrate the initial quality of the simplicia, changes in physicochemical characteristics during fermentation, and total flavonoid content. In addition, the bitterness of neem poses a distinct challenge for product development, so a formulation approach is needed that considers not only the amount of active constituents but also the potential for product acceptance. Based on these considerations, this study was conducted to characterize the physical quality and evaluate the total flavonoid content of neem leaf kombucha at several concentrations and fermentation times, including a material pretreatment approach to reduce bitterness. This study is expected to provide preliminary data on the standardization of neem leaf kombucha and to serve as a basis for developing the formulation as a nutraceutical candidate. The term "candidate" is used because further development still requires analytical validation, safety evaluation, sensory testing, and verification of biological activity in follow-up studies.

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RESEARCH METHOD

Study Design, Time, and Place

This study was conducted as a laboratory experimental study with a descriptive-comparative approach to characterize the physical quality and preliminary total flavonoid content of neem leaf kombucha (*Azadirachta indica* A. Juss) at different neem concentrations and fermentation periods. The observations included neem simplicia quality, pH, apparent alcohol level, and total flavonoid response. Measurements were performed during fermentation on days 8, 12, and 14. The experimental work was conducted in June - August 2026 at the Natural Products Laboratory, Universitas Duta Bangsa Surakarta, Central Java, Indonesia.

Neem leaves used as the raw material were obtained from the Tawangmangu area, Karanganyar Regency, Central Java, Indonesia. The plant material had been botanically authenticated through a determination process and was identified as *Azadirachta indica* A. Juss (family Meliaceae). Standardization of the herbal starting material was incorporated to ensure the consistency of the raw material before fermentation. This approach is consistent with previous work involving Listyani et al., in which organoleptic properties, moisture-related parameters, ash content, and phytochemical characteristics were used as important components of physicochemical standardization of herbal materials.

Materials and Equipment

The main material was dried neem leaf simplicia (*Azadirachta indica* A. Juss). Other materials used for kombucha preparation included sucrose, water, kombucha starter, and a symbiotic culture of bacteria and yeast (SCOBY). Clay was used as a pretreatment material in one of the 1% neem formulations to reduce the characteristic bitterness of neem leaves. Materials used for total flavonoid analysis included ethanol, quercetin as the reference flavonoid, aluminium chloride ($AlCl_3$) 10%, sodium acetate 1 M, methanol, and distilled water. The major instruments included an analytical balance, oven, moisture balance, furnace, calibrated pH meter, refractometer, glassware, micropipettes, cuvettes, and a UV-Visible spectrophotometer. The use of quercetin, $AlCl_3$, sodium acetate, and UV-Visible spectrophotometry was supported by Siregar, Hidayat, and Listyani (2026), who quantified total flavonoids through flavonoid–$AlCl_3$ complex formation and expressed the results as quercetin equivalents.

Preparation and Quality Characterization of Neem Simplicia

Fresh neem leaves were sorted, washed under running water, cut into smaller pieces, dried, and subsequently processed into dried simplicia. The quality of the simplicia was characterized before its use as the fermentation substrate. The evaluated parameters included organoleptic properties, simplicia yield, loss on drying, moisture content, and total ash content. Organoleptic examination included the appearance, color, odor, and taste of the dried leaves. Moisture-related parameters were determined to evaluate the stability of the raw material, whereas ash determination was used as an indicator of the inorganic residue present in the simplicia.

This physicochemical standardization step was intended to minimize variation originating from the herbal starting material before fermentation and follows the general pharmaceutical standardization framework applied by Arinda et al. (2026), which included organoleptic examination, moisture determination, ash content, and phytochemical characterization.

Preparation of Neem Leaf Kombucha

Neem leaf kombucha was prepared by infusing dried neem material in hot water at approximately 80–90°C. Sucrose was added as the carbon source for microbial fermentation. The infusion was subsequently allowed to cool to approximately 22°C before the addition of kombucha starter and SCOBY to avoid thermal damage to the microbial culture.

Four experimental formulations were prepared according to the nominal neem leaf concentration:

1. 0.3% neem leaf kombucha;
2. 1% neem leaf kombucha;
3. 3% neem leaf kombucha; and
4. 1% neem leaf kombucha with clay pretreatment.

For the clay-treated formulation, the neem material underwent a clay-assisted boiling pretreatment prior to kombucha fermentation. This formulation was specifically developed to investigate a practical strategy for reducing the characteristic bitterness of neem while monitoring whether the treatment substantially altered the measured flavonoid response. Fermentation was carried out in clean glass containers under semi-aerobic conditions. The vessels were covered with clean breathable material to permit gas exchange while reducing environmental contamination. Fermentation was maintained under dark conditions, and samples were collected on days 8, 12, and 14.

The use of SCOBY-based fermentation for the development of plant-derived functional beverages is also supported by the work of Listyani, Listyorini, and Fitriawati (2026), who applied kombucha fermentation technology in the development of a plantbased functional beverage.

Preparation of Diluted Kombucha

In addition to the undiluted kombucha, a ready to consume diluted preparation was evaluated to explore the feasibility of developing neem kombucha into a beverage concept comparable to diluted apple cider vinegar. For each formulation, 10 mL of kombucha was mixed with 200 mL of water, resulting in a final volume of approximately 210 mL and a nominal dilution factor of 21.

Both the undiluted and diluted preparations were evaluated for pH, alcohol level, and flavonoid response. For the primary analysis, the total flavonoid values of the diluted samples were reported as the concentration measured in the diluted beverage itself and were not multiplied by 21 to reconstruct the concentration of the original kombucha. This approach was selected to represent the flavonoid concentration potentially consumed in the diluted beverage.

Determination of pH

The pH of each kombucha formulation was measured directly using a calibrated digital pH meter. Before measurement, the instrument was calibrated using standard buffer solutions at pH 4.0 and pH 7.0. The electrode was immersed directly in the sample until a stable reading was obtained.

Measurements were conducted for both undiluted and 21-fold diluted kombucha on fermentation days 8, 12, and 14. Changes in pH were used as a physical quality indicator of fermentation progression because the formation of organic acids during SCOBY fermentation is expected to increase beverage acidity.

Determination of Apparent Alcohol Level

Alcohol related readings were obtained using a refractometric method during fermentation. Measurements were performed on both undiluted and diluted samples on days 8, 12, and 14. Because the refractive index of fermented beverages can also be influenced by residual sugars, organic acids, and other dissolved solids, the values obtained in this study were treated as or preliminary alcohol estimates, rather than confirmatory ethanol concentrations. This distinction was maintained throughout data interpretation, and confirmatory ethanol determination using a selective analytical method is proposed for subsequent research.

Determination of Total Flavonoid Content

The total flavonoid response was determined using an aluminium chloride colorimetric method followed by UV-Visible spectrophotometric measurement. The analytical principle was based on the formation of a colored complex between flavonoids and aluminium ions.

For sample preparation, 10 mL of neem kombucha was mixed with 10 mL of ethanol. An aliquot of the prepared sample was subsequently reacted with $AlCl_3$ 10% and sodium acetate 1 M and incubated before spectrophotometric measurement. Absorbance was measured at approximately 425 nm, which had been selected as the working wavelength for the flavonoid $AlCl_3$ complex.

The underlying analytical approach is consistent with the method reported by Siregar, Hidayat, and Listyani (2026), in which total flavonoid content was determined by UV-Visible spectrophotometry after complex formation with $AlCl_3$ and quantified relative to quercetin. Their study further demonstrated the application of quercetin equivalent reporting for pharmaceutical analysis of flavonoid-containing natural products.

Data Analysis

Data were analyzed descriptively by comparing physical quality and estimated total flavonoid values among the four formulations and across fermentation days 8, 12, and 14. The analysis focused on trends in pH, apparent alcohol level, total flavonoid response, and differences between undiluted and diluted preparations.

RESULT AND DISCUSSION

Quality Characteristics of Neem Leaf Simplicia

The quality of the herbal starting material is an important determinant of the reproducibility of plant-based fermented products. The neem leaf simplicia used in this study produced a yield of 12.17%. Its average loss on drying, moisture content, and total ash content were 6.35 ± 1.74%, 6.83 ± 1.95%, and 9.62 ± 1.83%, respectively, as presented in Table 1. The simplicia was characterized as dried brown leaves with a characteristic odor and distinctly bitter taste.

The relatively low moisture content is important for reducing deterioration of the dried material during storage and for minimizing variability before extraction and fermentation. The ash content reflects the total inorganic residue remaining after incineration and may originate from the intrinsic mineral content of the plant as well as extraneous inorganic material. Therefore, these parameters are relevant not only as descriptive characteristics but also as preliminary indicators of raw-material consistency.

This standardization approach is in agreement with the physicochemical characterization framework reported by Arinda et al. (2026), including **Tiara Ajeng**

Listyani, in which organoleptic characteristics, moisture-related parameters, ash content, and phytochemical screening were used as important quality-control parameters for herbal materials.

Table 1. Physicochemical characteristics of neem leaf simplicia

Parameter	Result
Simplicia yield	12.17%
Loss on drying	6.35 ± 1.74%
Moisture content	6.83 ± 1.95%
Total ash content	9.62 ± 1.83%
Appearance	Dried leaves
Color	Brown
Odor	Characteristic
Taste	Bitter

Qualitative phytochemical screening further demonstrated the presence of flavonoids in the neem simplicia. Flavonoids, tannins, selected alkaloids, and steroids were detected in the starting material. Following kombucha fermentation, flavonoids remained detectable, together with terpenoids and alkaloids.

The persistence of a positive flavonoid reaction after fermentation is relevant because it indicates that fermentation did not completely eliminate the flavonoid-related constituents of neem leaves. However, changes in the qualitative phytochemical pattern before and after fermentation should not be interpreted as direct evidence of formation or degradation of specific compounds. They may reflect differences in extraction, solubility, microbial biotransformation, or the acidic environment generated during fermentation.

Changes in pH and Alcohol Level During Fermentation

A progressive decrease in pH was observed in all neem kombucha formulations from day 8 to day 14, as shown in Table 2.

Table 2. Changes in pH and apparent alcohol readings of undiluted neem kombucha during fermentation.

Formulation	pH Day 8	pH Day 12	pH Day 14	Apparent alcohol Day 8 (%)	Day 12 (%)	Day 14 (%)
Neem 0.3%	2.81	2.64	**2.54**	4.6	4.8	**4.6**
Neem 1%	2.94	2.77	**2.65**	6.4	6.8	**6.3**
Neem 3%	3.36	3.22	**3.21**	4.5	4.2	**4.3**
Neem 1% + clay	3.22	3.01	**2.88**	5.1	4.7	**4.9**

The most pronounced acidification was observed in the 0.3% formulation, in which pH decreased from 2.81 to 2.54. In contrast, the 3% formulation remained at the highest pH on day 14, at 3.21. These differences suggest that neem concentration influenced the physicochemical environment during fermentation. The decrease in pH is consistent with the metabolic activity of the kombucha microbial consortium. Yeasts metabolize fermentable sugars, while acetic acid bacteria subsequently produce organic acids, particularly acetic and gluconic acids. Consequently, fermentation is generally accompanied by progressive acidification. Dartora et al. (2023) similarly reported that fermentation time significantly affected pH, ethanol, acetic acid, and polyphenolic composition during 14 days of green tea kombucha fermentation.

Salinas-Ruiz et al. (2026) also demonstrated that fermentation time is a major determinant of the physicochemical profile of kombucha, including changes in pH and fermentation metabolites. Interestingly, the 3% neem formulation maintained a higher pH than the lower-concentration formulations. This may indicate a greater buffering contribution from plant-derived solids or a different interaction between substrate concentration and microbial metabolism. Nevertheless, this explanation remains a hypothesis because titratable acidity and microbial population dynamics were not quantified in the present study.

The apparent alcohol readings showed a different pattern from pH and did not change monotonically. For example, the 1% formulation increased from 6.4% on day 8 to 6.8% on day 12 and subsequently decreased to 6.3% on day 14. Such variation is biologically plausible because ethanol is simultaneously formed by yeast and consumed by acetic acid bacteria.

However, these values should be interpreted as refractometric alcohol estimates rather than confirmed ethanol concentrations. Recent studies have emphasized that refractometric measurements in kombucha are influenced not only by sugars but also by ethanol, glycerol, organic acids, and other dissolved metabolites. Therefore, refractometric readings are useful for comparative process monitoring but cannot be regarded as selective ethanol quantification.

This analytical limitation does not invalidate the observed fermentation trend, but it limits conclusions regarding actual alcohol concentration. Confirmatory ethanol analysis using a selective method such as HPLC or gas chromatography is therefore appropriate for subsequent studies.

Total Flavonoid Content During Fermentation

The preliminary total flavonoid content showed an overall increasing trend between days 8 and 14 (Table 3). The values are expressed as working estimates in mg quercetin equivalent per milliliter (mg QE/mL) using the same conversion approach throughout the study.

Table 3. Preliminary total flavonoid content of neem leaf kombucha during fermentation

Formulation	Day 8 (mg QE/mL)	Day 12 (mg QE/mL)	Day 14 (mg QE/mL)	Change Day 8–14
Neem 0.3%	0.02243	0.02250	**0.02862**	**+27.60%**
Neem 1%	0.02966	0.03086	**0.03388**	**+14.23%**
Neem 3%	0.02892	0.03105	**0.03695**	**+27.77%**
Neem 1% + clay	0.02345	0.02374	**0.03016**	**+28.61%**

The main scientific finding is that fermentation duration, rather than neem concentration alone, appeared to determine the measured flavonoid response. On day 8, the 1% formulation showed the highest estimated TFC (0.02966 mg QE/mL), slightly exceeding the 3% formulation (0.02892 mg QE/mL). By day 14, however, the 3% formulation produced the highest value of 0.03695 mg QE/mL.

Therefore, increasing the amount of neem did not immediately produce a proportional increase in detectable flavonoids. Instead, the effect of a higher substrate concentration became more apparent as fermentation progressed. This phenomenon may be explained by microbial biotransformation. During kombucha fermentation, organic acids, microbial enzymes, and fermentation-derived solvents can facilitate the release or

conversion of plant phenolics and flavonoid glycosides into more readily detectable compounds.

Liang et al. (2024) reported that kombucha fermentation increased total flavonoids by 69.23%, while quercetin and kaempferol increased by 89.11% and 70.32%, respectively. The authors proposed a synergistic mechanism involving organic acids, microbial enzymes such as tannase and polygalacturonidase, and fermentation-derived alcohols and esters. Related study published in *Food Bioscience* also reported increases in both phenolic and flavonoid contents following kombucha fermentation, supporting the concept that microbial metabolism can modify the phytochemical profile of the original plant substrate.

The analytical approach used in the present study is also consistent with the pharmaceutical quantification of flavonoids reported by Siregar, Hidayat, and Listyani (2026), who used UV-Visible spectrophotometry based on $AlCl_3$ complex formation, quercetin calibration, and quercetin-equivalent expression to determine total flavonoids in *Psidium guajava* fractions. The present results therefore support the hypothesis that fermentation modifies the measurable flavonoid profile of neem kombucha.

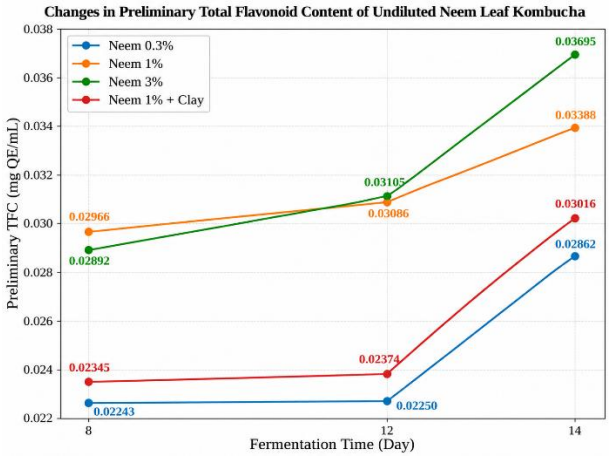


Figure 1. Changes in preliminary total flavonoid content of neem leaf kombucha during 14 days of fermentation.

Effect of Clay Pretreatment

The clay-treated formulation was included because the natural bitterness of neem may limit consumer acceptance. On day 14, the untreated 1% formulation contained an estimated 0.03388 mg QE/mL, whereas the 1% clay-treated formulation contained 0.03016mg QE/mL, representing a difference of approximately 10.98%. The lower value observed after clay treatment is scientifically plausible because clay minerals may interact with polyphenolic compounds. Recent molecular research demonstrated that quercetin can adsorb strongly onto kaolinite surfaces through hydrogen bonding and other surface interactions. Therefore, adsorption onto clay may partly explain the lower flavonoid response observed in the treated formulation. Heating during the pretreatment step may also contribute to differences in extractable compounds. However, the present study did not identify the specific clay mineral or directly quantify adsorption; consequently, these mechanisms should be considered possible explanations rather than confirmed causes. Importantly, the flavonoid response of the clay-treated sample continued to increase during fermentation, from 0.02345 mg QE/mL on day 8 to 0.03016 mg QE/mL on day 14. Thus,

clay pretreatment did not prevent the fermentation-associated increase in measurable flavonoid content. This represents an important formulation finding because bitterness reduction may potentially be achieved without eliminating most of the flavonoid response. Nevertheless, sensory acceptability must be evaluated directly in future studies before any claim regarding improved palatability can be established.

Characteristics of the Diluted Beverage

To explore a beverage concept comparable to diluted apple cider vinegar, the fermented kombucha was diluted by mixing 10 mL of kombucha with 200 mL of water. The day-14 characteristics are summarized in Table 4.

Table 4. Day-14 characteristics of diluted neem kombucha

Formulation	pH	Apparent alcohol (%)	Preliminary TFC (mg QE/mL)
Neem 0.3%	2.97	0.3	**0.01416**
Neem 1%	3.25	0.4	**0.02094**
Neem 3%	3.44	0.3	**0.02440**
Neem 1% + clay	3.24	0.5	**0.01990**

The 3% formulation retained the highest measurable flavonoid response after dilution at 0.02440 mg QE/mL, followed by the 1% formulation at 0.02094 mg QE/mL. A particularly relevant result was observed between the two 1% formulations. The diluted clay-treated formulation contained 0.01990 mg QE/mL compared with 0.02094 mg QE/mL in the untreated formulation. The difference was only approximately 4,97%. Thus, the clay treated product showed approximately 95% of the measured flavonoid response of the untreated 1% product under the diluted beverage condition.

This observation supports the potential use of clay treatment as a formulation strategy, because its impact on the measured flavonoid response became relatively small after dilution. Nevertheless, the measured values after dilution should not be interpreted as true chemical recovery or flavonoid mass retention. A 21-fold aqueous dilution would theoretically produce a substantially greater reduction in concentration if the analytical response were governed by dilution alone. The higher-than-expected measured response indicates that sample matrix effects, color, turbidity, or assay conditions may have influenced spectrophotometric measurement. For this reason, the results are most appropriately described as relative measured flavonoid responses in the ready-to-consume diluted matrix, rather than as absolute flavonoid recovery.

Relationship Between Fermentation Time, pH, and Flavonoid Response

When pH and TFC are considered together, an inverse descriptive pattern becomes evident. From day 8 to day 14, pH decreased in all formulations, while estimated TFC generally increased. For example, in the 3% formulation, the pH decreased from 3.36 to 3.21, whereas the TFC increased from 0.02892 to 0.03695 mg QE/mL. This concurrent pattern does not demonstrate a causal relationship between acidity and flavonoid formation. Instead, both changes are likely consequences of ongoing microbial metabolism. Organic acid accumulation lowers pH, while microbial enzymes and changes in solvent conditions may simultaneously release or transform phenolic compounds. Liang et al. (2024) similarly reported that organic acids and microbial enzymes contributed to the transformation of flavonoid compounds during kombucha fermentation.

Scientific Implications for Nutraceutical Development

Three principal scientific findings were obtained from this study.

First, neem leaf kombucha underwent measurable physicochemical changes during fermentation, particularly progressive acidification, confirming active fermentation of the herbal substrate.

Second, the measurable flavonoid response increased with fermentation time. The 3% formulation produced the highest estimated TFC on day 14, suggesting that a higher neem substrate concentration can provide a greater flavonoid response when sufficient fermentation time is allowed.

Third, clay pretreatment reduced the flavonoid response only modestly relative to untreated 1% kombucha, particularly after dilution. This finding is relevant to product development because it suggests a possible balance between phytochemical content and strategies intended to reduce the characteristic bitterness of neem.

Collectively, these findings support the potential of neem leaf kombucha as a nutraceutical candidate, rather than establishing it as a finished nutraceutical product.

CONCLUSION

This study showed that neem leaf kombucha (*Azadirachta indica* A. Juss) had acceptable physicochemical characteristics and a measurable flavonoid response throughout fermentation. On day 14, the undiluted formulations had pH values ranging from 2.54 to 3.21, while the 3% neem formulation showed the highest preliminary total flavonoid content (TFC) at 0.03695 mg QE/mL. The 1% clay-treated formulation also maintained a substantial TFC of 0.03016 mg QE/mL, and after dilution its TFC reached 0.01990 mg QE/mL, close to the untreated 1% formulation at 0.02094 mg QE/mL. These findings indicate that neem concentration, fermentation time, and clay pretreatment influence the physicochemical and phytochemical characteristics of neem kombucha, supporting its potential for further development as a plant-based nutraceutical beverage candidate.

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REFERENCES

Andersa, K. N., Tamiru, M., Teka, T. A., Ali, I. M., Chane, K. T., Regasa, T. K., & Ahmed, E. H. (2024). Proximate composition, some phytochemical constituents, potential uses, and safety of neem leaf flour: A review. *Food Science & Nutrition, 12*(10), 6929–6937. https://doi.org/10.1002/fsn3.4336 PubMed

Arinda, D., Hidayat, R., Wardani, T. S., & Listyani, T. A. (2026). Karakterisasi fisikokimia dan analisis FTIR (*Fourier transform infrared spectroscopy*) ekstrak etanol 70% daun pelawan (*Tristaniopsis merguensis* (Griff.)). *Jurnal Kesehatan Tambusai, 7*(1), 4001–4011. https://doi.org/10.31004/jkt.v7i1.55666 Journal Universitas Pahlawan

Barros, V. C., Botelho, V. A., & Chisté, R. C. (2024). Alternative substrates for the development of fermented beverages analogous to kombucha: An integrative review. *Foods, 13*(11), 1768. https://doi.org/10.3390/foods13111768 PubMed Central (PMC)

Boukeloua, A., Soudani, L., Bendif, H., Mustafa, A. M., Boga, M., Serralheiro, M. L., Yilmaz, M. A., Yener, I., & Garzoli, S. (2024). A comprehensive study on chemical and biological profiles of Algerian *Azadirachta indica* (A. Juss). *Phytochemistry Letters, 61*, 89–96. https://doi.org/10.1016/j.phytol.2024.03.017 ScienceDirect

Dartora, B., Hickert, L. R., Fabricio, M. F., Ayub, M. A. Z., Furlan, J. M., Wagner, R., Perez, K. J., & Sant'Anna, V. (2023). Understanding the effect of fermentation time on physicochemical characteristics, sensory attributes, and volatile compounds in green tea kombucha. *Food Research International, 174*, 113569. https://doi.org/10.1016/j.foodres.2023.113569 ScienceDirect

Liang, W., Wang, X., Zhang, L., Jiao, S., Song, H., Sun, J., & Wang, D. (2024). Changes and biotransformation mechanism of main functional compounds during kombucha fermentation by the pure cultured tea fungus. *Food Chemistry, 458*, 140242. https://doi.org/10.1016/j.foodchem.2024.140242 PubMed

Listyani, T. A., Listyorini, P. I., & Fitriawati, A. (2026). Transfer ilmu pengetahuan dan teknologi produksi kombucha bunga kupu-kupu antidiabetes bagi Karang Taruna Katajanom di Kelurahan Jayengan Surakarta. *Jurnal Pengabdian Ilmu Kesehatan, 6*(2), 186–197. https://doi.org/10.55606/jpikes.v6i2.7832 Journalshub

Pereira, A. G. (2026). Kombucha as a bioactive functional beverage: Current evidence, production challenges, and future perspectives. *International Journal of Molecular Sciences, 27*(6), 2739. https://doi.org/10.3390/ijms27062739 PubMed Central (PMC)

Salinas-Ruiz, J. P., Guevara García, J., Rios Tovar, D., Cruz Cabrera, R. P., & Torrico, D. D. (2026). Effects of fermentation time and temperature on the physicochemical quality of kombucha. *Foods, 15*(7), 1226. https://doi.org/10.3390/foods15071226 MDPI

Siregar, S. S., Hidayat, R., & Listyani, T. A. (2026). Penetapan total flavonoid fraksi jambu kristal (*Psidium guajava* L.). *Jurnal Kesehatan Tambusai, 7*(1), 630–641. https://doi.org/10.31004/jkt.v7i1.55914