

Phytochemical Characterisation and In Vitro Cyclooxygenase Inhibitory Activity of Gbeful Super Bitters: Scientific Validation of its Claimed use in Pain Management

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ABSTRACT

Gbeful Super Bitters is a polyherbal alcoholic bitter drink claimed to possess several medicinal properties, including analgesic effects, but scientific evidence for its bioactive constituents and these properties has not been documented. This study aimed to characterise the phytochemical constituents of Gbeful Super Bitters and evaluate its in vitro analgesic potential. Qualitative and quantitative phytochemical analyses were carried out using standard procedures. Gas Chromatography–Mass Spectrometry (GC-MS) analysis was used to identify bioactive compounds, with identification based on the NIST mass spectral library. In vitro analgesic potential was measured by a cyclooxygenase (COX) inhibition assay at 25, 50, 100, 200 and 400 µg/mL, with diclofenac sodium as the reference standard. Data are presented as mean ± SD, and IC₅₀ values were determined by nonlinear regression. Data were presented as mean ± standard deviation, and IC₅₀ values were established by nonlinear regression analysis. Phytochemical screening detected flavonoids, phenols, alkaloids, tannins, saponins, steroids, glycosides and terpenoids. Total phenolic (85.62 ± 2.14 mg GAE/mL), terpenoid (63.74 ± 1.89 mg LAE/mL), and flavonoid (47.38 ± 1.56 mg QE/mL) contents were highest. GC-MS identified phytol (11.94%), β-caryophyllene (10.82%), eugenol (7.21%), limonene (4.67%) and α-pinene (3.82%) as major compounds. The extract inhibited COX activity in a concentration-dependent manner (18.64–78.91% inhibition; IC₅₀ = 112.64 µg/mL), less potent than diclofenac sodium (IC₅₀ = 38.27 µg/mL) but with substantial COX-inhibitory activity Gbeful Super Bitters contains a lot of bioactive phytochemicals and shows in vitro analgesic activity via COX inhibition, supporting its traditional use for back and waist pain and warranting further pharmacological evaluation and standardisation.

Keywords: Gbeful Super Bitters; Phytochemicals; Phytochemical Characterisation; Gas Chromatography–Mass Spectrometry; Cyclooxygenase Inhibition; Analgesic Activity; Polyherbal Formulation; Bioactive Compounds; Pain Management; NIST Mass Spectral Library.

1.0. Introduction

Long before now, natural products have played important roles in the discovery and development of medicines. A crucial fact is that in many developing nations, including Nigeria, traditional herbal medicines have been adopted as part of routine healthcare because they are affordable, accessible and generally accepted among local communities [1,2]. These traditional herbal medicines are obtained from medicinal plants, which are a rich source of bioactive compounds such as flavonoids, alkaloids, tannins, saponins, terpenoids, phenols and glycosides. These bioactive compounds influence several physiological processes and promote individual health [3,4]. For instance, they have been known for their analgesic [5], anti-inflammatory [6], antioxidant [7], antimicrobial [2] and immune-modulating effects [8]. The continued focus on medicinal plants shows that they are reliable and potent in preventing and treating a wide range of diseases [2, 9, 10].

Pain is among the most prevalent reasons prompting individuals to seek medical attention, thereby underscoring the ongoing need for safer and more efficacious pain relief medications. While traditional painkillers are often used, long-term use can lead to side effects that reduce their effectiveness in real-world clinical settings [11, 12]. As a result, plant-derived compounds have become a promising alternative because they can modulate pain pathways in different and complementary ways. These mechanisms include inhibiting enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX) and reducing the activity of pro-inflammatory mediators such as tumour necrosis factor-alpha (TNF-α), interleukin-1β and interleukin-6 [13-15], controlling pathways like NF-κB and MAPK [11],

interacting with opioid receptors, removing harmful reactive oxygen species and adjusting transient receptor potential (TRP) ion channels that are involved in sensing pain. In polyherbal mixtures, the various phytochemicals can synergistically interact to enhance their overall effectiveness, leading to better treatment outcomes [16, 17].

Gbefun Super Bitters is a commercially available product made from a combination of several herbs. It is commonly promoted for its ability to relieve pain and offer other health benefits. Although its rate of consumption has been growing, little or no information regarding its phytoconstituents and efficacy in reducing pain. There remains a deficiency in comprehensive studies concerning the specific phytochemicals it contains and its analgesic mechanisms. Having this information is crucial for confirming its traditional uses, ensuring consistent quality and building a more solid scientific basis for its medical applications.

1.1. Study Aim and Objectives

Based on the aforementioned knowledge gaps, this study seeks to examine the phytochemical constituents of Gbefun Super Bitters using Gas chromatography–mass spectrometry (GC–MS) and assess its in vitro analgesic activity by cyclooxygenase inhibition. The study had four specific objectives, which are to:

- 1) Identify the qualitative and quantitative phytochemical composition of Gbefun Super Bitters.
- 2) Analyse and characterise the major bioactive constituents of Gbefun Super Bitters using gas chromatography–mass spectrometry (GC–MS).
- 3) Ascertain the in vitro analgesic activity of Gbefun Super Bitters through cyclooxygenase (COX) inhibition.
- 4) To connect the identified phytochemicals and GC–MS-detected compounds to the pain-relieving properties of the product.

2.0. Materials and Methods

2.1. Collection and Preparation of Sample

Commercially packaged Gbefun Super Bitters was obtained from an authorised distributor in Port Harcourt, Rivers State, Nigeria. The samples were taken to the laboratory under suitable storage conditions before phytochemical, GC–MS and in vitro analyses. Five hundred millilitres (500 mL) of Gbefun Super Bitters was measured and divided into portions for subsequent phytochemical, GC–MS and in vitro analgesic activity assay.



Figure 1. Commercially packaged Gbefun Super Bitters alcoholic drink.

2.2. Phytochemical Screening

2.2.1. Qualitative Phytochemical Screening

Qualitative phytochemical analysis was conducted following the procedures outlined by Harborne [18] and Trease and Evans [19], with some slight changes.

2.2.1.1. Test for Alkaloids

Alkaloids were identified using Mayer's reagent. A 5 mL portion of Gbeful Super Bitters was treated with dilute hydrochloric acid and warmed gently in a water bath. The mixture was then filtered and the filtrate was split into two parts. A few drops of Mayer's reagent were added. The formation of a creamy-coloured precipitate indicated the presence of alkaloids.

2.2.1.2. Test for Flavonoids

Flavonoids were tested using the Shinoda test. In this method, 5 mL of Gbeful Super Bitters was mixed with small pieces of magnesium ribbon, accompanied by the addition of a few drops of concentrated hydrochloric acid. The formation of a pinkish-red colouration confirmed the presence of flavonoids.

2.2.1.3. Test for Phenols

The ferric chloride test was used to observe the presence of phenols. Exactly 2 mL of Gbeful Super Bitters was treated with a few drops of 5% ferric chloride solution. The appearance of a violet colouration confirmed the presence of phenols.

2.2.1.4. Test for Tannins

Tannins were identified using the ferric chloride test. Here, 2 mL of Gbeful Super Bitters was mixed with a few drops of 5% ferric chloride solution. The formation of a green colouration indicated the presence of tannins.

2.2.1.5. Test for Saponins

The frothing test was used to confirm if saponins were present. About 5 mL of Gbeful Super Bitters was added to 10 mL of distilled water in a test tube and shaken vigorously for about 5 min. The presence of froth that persisted for several minutes, measuring at least 1 cm in height, was considered evidence of saponins.

2.2.1.6. Test for Terpenoids

The Salkowski reaction test was adopted to confirm the presence of terpenoids. A total of 2 mL of Gbeful Super Bitters was combined with 2 mL of chloroform. Then, 2 mL of concentrated tetraoxosulphate (VI) acid was added carefully along the side of the test tube. The appearance of a reddish-brown ring at the interface confirmed the presence of terpenoids.

2.2.1.7. Test for Steroids

The Liebermann-Burchard reaction test was used to identify steroids. Approximately 2 mL of Gbeful Super Bitters was mixed with 2 mL of chloroform, followed by 2 mL of acetic acid and a few drops of concentrated tetraoxosulphate (VI) acid. A blue-green colouration was considered an indicator of steroids.

2.2.1.8. Test for Glycosides

The Keller–Killiani test was employed to ascertain if cardiac glycosides were present. Approximately 2 mL of Gbfun Super Bitters was treated with glacial acetic acid containing a small amount of ferric chloride solution. Then, a few drops of concentrated tetraoxosulphate (VI) acid were added carefully along the wall of the test tube. The formation of a brown ring at the junction of the two layers confirmed the presence of cardiac glycosides.

2.2.2. Quantitative Phytochemical Analysis

Quantitative phytochemical analysis was carried out using standard spectrophotometric methods as described by Harborne [18] and other established techniques.

2.2.2.1. Determination of Total Phenolic Content

Total phenolic content was determined by the Folin–Ciocalteu method described by Singleton et al. [20]. Gbfun Super Bitters (1 mL) was mixed with diluted Folin–Ciocalteu reagent (5 mL), shaken, and left to stand for 5 minutes. Sodium carbonate solution (7.5%, 4 mL) was then added and the mixture was incubated in the dark at room temperature for 30 minutes. Absorbance was read at 765 nm using a UV–visible spectrophotometer (Model UV-1800, Shimadzu Corporation, Kyoto, Japan). A calibration curve was prepared using gallic acid at varying concentrations, and total phenolic content was calculated from this curve and expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE/g).

2.2.2.2. Determination of Total Flavonoid Content

Total flavonoid content was determined using the aluminium chloride colourimetric method of Zhishen et al. [21], as modified by Owo [6]. To 1 mL of extract, sodium nitrite (5%), aluminium chloride (10%) and sodium hydroxide (1 M) were added sequentially at fixed time intervals, and the mixture was then diluted with distilled water. Absorbance was measured at 510 nm, and flavonoid content was calculated from a quercetin calibration curve and expressed as milligrams of quercetin equivalents per gram of sample (mg QE/g).

2.2.2.3. Determination of Total Tannin Content

Total tannin content was measured using the Folin–Ciocalteu method, with slight modifications as described by Broadhurst and Jones [22]. About 1 mL of Gbfun Super Bitters was mixed with Folin–Ciocalteu reagent and sodium carbonate solution. The mixture was allowed to incubate and absorbance was measured at 725 nm using a spectrophotometer. Tannic acid was used as the reference standard for the calibration curve. The tannin content was calculated and expressed as milligrams of tannic acid equivalents per gram of sample (mg TAE/g).

2.2.2.4. Determination of Total Alkaloid Content

Total alkaloid content was measured using a gravimetric technique as described by Harborne [18] and modified by Owo [6]. About 1 mL of Gbfun Super Bitters was mixed with 1 mL of diluted hydrochloric acid and left to settle before being filtered. The liquid that passed through the filter was then treated with ammonium hydroxide solution to cause the alkaloids to form a solid precipitate. This precipitate was gathered, rinsed, dried until it reached a stable weight and then precisely weighed. The percentage of alkaloids present was calculated using the formula provided:

Alkaloid content (%) = Weight of alkaloid precipitate/Weight of sample $\times$ 100

2.2.2.5. Determination of Total Saponin Content

The total saponin content was determined using the gravimetric method outlined by Obadoni and Ochuko [23]. Specifically, 1 mL of Gbefun super bitters extract was subjected to solvent extraction and the resulting solution was concentrated to promote saponin precipitation. The precipitated saponins were then filtered out, dried until they reached a constant weight and weighed. The percentage of saponin content was calculated using the provided formula:

Saponin content (%) = (Weight of dried saponin extract $\times$ 100)/Weight of sample

All quantitative analyses were performed in triplicate and results were expressed as mean $\pm$ standard deviation.

2.2.2.6. Determination of Total Terpenoid Content

Total terpenoid content was determined by the spectrophotometric method of Ferguson [24], with slight modification. Gbefun Super Bitters (1 mL) was extracted with petroleum ether (5 mL) by vigorous shaking for 10 minutes. After phase separation, the petroleum ether layer was collected, evaporated to dryness, and the residue reconstituted in a known volume of petroleum ether. For colour development, 1 mL of the reconstituted extract was mixed with 1 mL of chloroform, followed by 1 mL of concentrated sulphuric acid, and the mixture was left to stand at room temperature for 30 minutes. Absorbance was read at 538 nm using a UV–visible spectrophotometer. A calibration curve was prepared using linalool as reference standard, and total terpenoid content was calculated from the curve and expressed as milligrams of linalool equivalents per millilitre of sample (mg LLE/mL), using the formula:

Total terpenoid content (mg LLE/mL)

= Concentration obtained from linalool calibration curve $\times$ dilution factor

All analyses were carried out in triplicate and results were expressed as mean $\pm$ standard deviation.

2.3. Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

Gas chromatography–mass spectrometry (GC–MS) analysis was carried out to identify the volatile and semi-volatile bioactive compounds present in Gbefun Super Bitters. The concentrated extract was filtered through a 0.22 μ m membrane filter before analysis and 1.0 μ L of the filtrate was injected into the GC–MS system, comprising an Agilent 7890B gas chromatograph coupled to an Agilent 5977A mass selective detector (Agilent Technologies, Santa Clara, CA, USA).

Separation was achieved on an HP-5MS fused silica capillary column (30 m $\times$ 0.25 mm i.d., 0.25 μ m film thickness; 5% phenyl methylpolysiloxane stationary phase), with helium (99.999% purity) as carrier gas at a constant flow rate of 1.0 mL/min. The injector operated in split mode (10:1 split ratio) at 250°C. The oven temperature was held at 60°C for 2 minutes, ramped to 200°C at 10°C/min, then to 300°C at 5°C/min, and held at 300°C for 10 minutes, giving a total run time of 52 minutes. The mass spectrometer operated in electron ionisation (EI) mode at 70 eV, with ion source, quadrupole and transfer line temperatures set at 230°C, 150°C and 280°C, respectively. Data were acquired in full-scan mode over an m/z range of 40–650, with a solvent delay of 3 minutes.

Compounds were identified from their retention times, peak areas and mass spectral profiles, and their relative abundance was expressed as a percentage of the total ion chromatogram peak area.

2.4. Compound Identification Using the NIST Mass Spectral Library

The compounds, found using GC–MS analysis, were identified by comparing their mass spectra to reference spectra stored in the National Institute of Standards and Technology (NIST) Mass Spectral Library (NIST/EPA/NIH Mass Spectral Library, Version 2.2). The identification of each compound was based on analysing fragmentation patterns, molecular ion peaks, retention times, molecular weights and similarity scores against known reference compounds in the NIST database. Only those compounds that had acceptable matching scores were considered tentatively identified. For each compound that was identified, details such as retention time (RT), molecular formula, molecular weight (MW), percentage peak area and the compound name were noted down. The relative amount of each compound was calculated based on its peak area in the total ion chromatogram, with the sum of all peak areas taken as 100%. The identified compounds were then reviewed in scientific literature to find out what biological and pharmacological effects they are known to have, especially focusing on their potential analgesic properties. The detected plant compounds were then linked to the observed in vitro analgesic effect of Gbfun super bitters to explore possible connections between the chemical makeup and the biological effects.

2.5. In Vitro Evaluation of Analgesic Activity by Cyclooxygenase (COX) Inhibition Assay

The in vitro analgesic properties of Gbfun Super Bitters were studied by testing how effectively they can block the activity of cyclooxygenase (COX) enzymes. This was done using a Cyclooxygenase Inhibitor Screening Assay Kit from Cayman Chemical, Ann Arbor, MI, USA, following the instructions provided by the manufacturer. The method used to measure this involved a colourimetric method to find out the production of prostaglandins, which are substances created by COX enzymes. Since COX enzymes convert arachidonic acid into prostaglandins, which play a major role in causing pain and inflammation, reducing their activity was seen as a sign that Gbfun super bitters might have analgesic effects.

The herbal preparation was diluted step by step with the assay buffer to reach final concentrations of 25, 50, 100, 200 and 400 µg/mL. Diclofenac sodium was used as the positive control, while the assay buffer with the enzyme and solvent without the herbal preparation was used as the negative control.

For each test, 10 µL of the sample or standard solution was combined with 150 µL of assay buffer that included haematin and COX-1 enzyme. This mixture was incubated at 37°C for 10 minutes. After that, 20 µL of arachidonic acid was added to start the reaction. The mixture was then incubated at 37°C for 2 minutes. The reaction was stopped by adding 30 µL of stop reagent. To allow the colour to develop, the chromogenic reagent from the kit was added and the absorbance was determined at 590 nm using a microplate reader.

The percentage inhibition of cyclooxygenase activity was calculated using the equation:

$$\% \text{ COX inhibition} = \frac{(A_c - A_s)}{A_c} \times 100$$

where: (Ac) = absorbance of the negative control; and (As) = absorbance of the sample or standard.

The IC₅₀ value, representing the concentration of Gbeful super bitters needed to inhibit 50% of cyclooxygenase activity, was determined by plotting the percentage inhibition against the corresponding extract concentrations and analysing the resulting dose–response curve using nonlinear regression. All measurements were performed in triplicate and the results were expressed as mean ± standard deviation to indicate data variability.

2.6. Statistical Analysis

All experiments were performed in triplicate, and results are expressed as mean ± SD. Data were analysed by one-way analysis of variance (ANOVA) using SPSS version 27.0 (IBM Corp., Armonk, NY, USA), with Tukey's post hoc test applied for multiple comparisons where appropriate; $p < 0.05$ was considered statistically significant. Graphs were plotted and IC₅₀ values for the COX inhibition assay were determined using GraphPad Prism version 9.0 (GraphPad Software Inc., San Diego, CA, USA).

3.0. Results and Discussion

3.1. Results

3.1.1. Qualitative Phytochemical Profile of Gbeful super bitters

The qualitative phytochemical screening of Gbeful super bitters revealed the presence of alkaloids, flavonoids, phenols, tannins, saponins, terpenoids, steroids and glycosides as presented in Table 1.

Among the detected phytochemicals, flavonoids, phenols and terpenoids showed high abundance, while alkaloids, tannins, saponins and glycosides were moderately present. Steroids were slightly present.

Table 1. Qualitative phytochemical profile of Gbeful super bitters.

Phytochemical Constituent	Relative Abundance
Alkaloids	++
Flavonoids	+++
Phenols	+++
Tannins	++
Saponins	++
Terpenoids	+++
Steroids	+
Glycosides	++

Key: +++ = Highly abundant; ++ = Moderately abundant; + = Slightly present; – = Absent.

3.1.2. Quantitative Phytochemical Composition of Gbeful super bitters

The quantitative phytochemical composition of Gbeful super bitters is presented in Table 2. The results revealed varying concentrations of bioactive constituents. Total phenolic content was the most abundant phytochemical constituent (85.62 ± 2.14 mg GAE/mL), followed by total terpenoid content (63.74 ± 1.89 mg LEE/mL) and total flavonoid content (47.38 ± 1.56 mg QE/mL). Other detected constituents included total tannins (29.81 ± 1.03 mg

TAE/mL), total alkaloids (18.47 ± 0.84 mg/mL) and total saponins (14.26 ± 0.72 mg/mL). The high abundance of phenols, terpenoids and flavonoids suggests that these phytochemical classes represent the major bioactive constituents of Gbeful super bitters.

Table 2. Quantitative Phytochemical Profile of Gbeful Super Bitters.

Phytochemical Constituent	Concentration (Mean $\pm$ SD)
Total phenolic content (mg GAE/mL)	85.62 ± 2.14
Total flavonoid content (mg QE/mL)	47.38 ± 1.56
Total terpenoid content (mg LEE/mL)	63.74 ± 1.89
Total tannin content (mg TAE/mL)	29.81 ± 1.03
Total alkaloid content (mg/mL)	18.47 ± 0.84
Total saponin content (mg/mL)	14.26 ± 0.72

Values are presented as mean $\pm$ standard deviation (SD) of triplicate determinations.

3.1.3. Bioactive Compounds Detected in Gbeful Super Bitters by GC–MS Analysis

The GC–MS analysis of Gbeful Super Bitters showed the presence of a range of bioactive compounds from different phytochemical groups, including terpenoids, phenols and flavonoids (Table 3). The detected compounds were characterised using their retention times, molecular formulae, molecular weights and relative peak abundances, which provided information on their identity and distribution within Gbeful Super Bitters.

Phytol was the most abundant compound detected, with a peak area of 11.94%. This was followed by β -caryophyllene (10.82%) and eugenol (7.21%). Other compounds detected in appreciable amounts included limonene (4.67%) and α -pinene (3.82%). The detection of these bioactive compounds indicates the complex phytochemical nature of Gbeful Super Bitters and provides insight into the possible compounds that may contribute to its reported biological activities.

Flavonoid-related compounds detected after derivatisation included quercetin trimethylsilyl ether (2.84%), kaempferol trimethylsilyl ether (2.31%), catechin TMS derivative (2.09%), naringenin TMS ether (1.93%) and rutin derivative (1.76%).

Table 3. Bioactive Compounds Identified in Gbeful super bitters by GC–MS Analysis.

Peak No.	RT (min)	Compound	Class	Molecular Formula	MW (g/mol)	PA (%)	Reported Pharmacological Activity
1	6.21	α -Pinene	Terpenoid	C ₁₀ H ₁₆	136.24	3.82	Anti-inflammatory, analgesic
2	10.32	Limonene	Terpenoid	C ₁₀ H ₁₆	136.24	4.67	Antioxidant, analgesic
3	16.75	Eugenol	Phenolic compound	C ₁₀ H ₁₂ O ₂	164.20	7.21	Analgesic, anti-inflammatory

4	22.68	β -Caryophyllene	Sesquiterpene noid	$C_{15}H_{24}$	204.35	10.82	CB ₂ receptor-mediated analgesic activity
5	26.18	Phytol	Diterpenoid	$C_{20}H_{40}O$	296.53	11.94	Antioxidant, analgesic
6	28.42	quercetin trimethylsilyl ether	Flavonoid	$C_{18}H_{20}O_7Si_3$	452.70	2.84	Antioxidant, anti-inflammatory, analgesic
7	30.15	kaempferol trimethylsilyl ether	Flavonoid	$C_{21}H_{28}O_6Si_3$	472.71	2.31	Anti-inflammatory, antioxidant
8	31.26	Rutin derivative	Flavonoid glycoside	$C_{33}H_{44}O_{16}Si_5$	868.00	1.76	Antioxidant, anti-inflammatory
9	32.18	catechin TMS derivative	Flavonoid	$C_{24}H_{36}O_6Si_3$	516.80	2.09	Antioxidant, analgesic
10	33.47	Naringenin TMS ether	Flavonoid	$C_{27}H_{46}O_6Si_3$	554.86	1.93	Anti-inflammatory, analgesic

RT = Retention time; MW = Molecular weight.

3.1.4. In Vitro Cyclooxygenase (COX) Inhibitory Activity of Gbefun Super Bitters Extract

The in vitro inhibitory effect of Gbefun Super Bitters extract on cyclooxygenase (COX) activity is presented in Table 4. The extract demonstrated a dose-dependent suppression of COX activity, as indicated by the progressive increase in percentage inhibition with increasing extract concentrations. The COX inhibitory activity of Gbefun super bitters extract increased from $18.64 \pm 1.25\%$ at 25 $\mu\text{g/mL}$ to $78.91 \pm 3.21\%$ at 400 $\mu\text{g/mL}$.

Diclofenac sodium, which served as the reference standard, showed greater inhibitory activity at all tested concentrations, with inhibition increasing from $42.56 \pm 2.01\%$ at 25 $\mu\text{g/mL}$ to $92.45 \pm 3.52\%$ at 400 $\mu\text{g/mL}$. Nonlinear regression analysis estimated the IC₅₀ value of Gbefun Super Bitters extract at 112.64 $\mu\text{g/mL}$, whereas diclofenac sodium exhibited a lower IC₅₀ value of 38.27 $\mu\text{g/mL}$, indicating stronger COX inhibitory activity.

Table 4. Effect of Gbefun super bitters Extract on Cyclooxygenase (COX) Inhibition Activity and IC₅₀ Values.

Treatment	Concentration ($\mu\text{g/mL}$)	COX Inhibition (%)	IC ₅₀ ($\mu\text{g/mL}$)
Gbefun super bitters extract	25	18.64 ± 1.25	112.64
	50	31.42 ± 1.76	
	100	46.83 ± 2.14	
	200	62.75 ± 2.83	
	400	78.91 ± 3.21	
Diclofenac sodium	25	42.56 ± 2.01	38.27
	50	56.78 ± 2.45	
	100	71.34 ± 2.67	
	200	84.62 ± 3.14	
	400	92.45 ± 3.52	

Values are presented as mean $\pm$ standard deviation (SD) of triplicate determinations. IC₅₀ represents the concentration required to inhibit 50% of cyclooxygenase activity, calculated by nonlinear regression analysis of concentration–response curves.

3.2. Discussion

This study aimed to examine the phytochemical makeup of Gbfun Super Bitters, identify its active components using gas chromatography–mass spectrometry (GC–MS) and evaluate its potential pain-relieving properties through an *in vitro* cyclooxygenase (COX) inhibition test.

The results of this study offer a better understanding of the phytochemical content and possible analgesic effects of Gbfun Super Bitters. This was done by combining qualitative and quantitative phytochemical tests, identifying bioactive compounds through GC–MS and assessing its ability to block COX activity in a laboratory setting. The qualitative screening showed the presence of alkaloids, flavonoids, phenols, tannins, saponins, terpenoids, steroids and glycosides. The quantitative analysis revealed that phenols, terpenoids and flavonoids were the most common phytochemical groups. The high levels of these components suggest that Gbfun Super Bitters contains a variety of secondary metabolites that could be responsible for its different biological effects. The high presence of phenolics, terpenoids and flavonoids found in this study aligns with earlier studies that strongly held that polyherbal formulations are rich in these bioactive substances due to their broad pharmacological benefits [16, 17, 25].

Phenols were the most prevalent phytochemicals in Gbfun Super Bitters, followed by terpenoids and flavonoids. The high concentration of phenols may play a key role in the biological activities of the herbal bitters. Phenols are known for their antioxidant properties, which comprise the ability to neutralise harmful reactive oxygen species and decrease oxidative stress-related damage in cells during inflammation [26, 27]. Moreover, phenols can influence inflammatory pathways by preventing the activation of nuclear factor kappa B (NF- κ B), which in turn reduces the production of pro-inflammatory molecules like tumour necrosis factor-alpha (TNF- α), interleukin-1 β and interleukin-6 [28-30].

Flavonoids, which were also present in considerable amounts, have been associated with analgesic and anti-inflammatory effects through their ability to inhibit key enzymes involved in inflammation, including cyclooxygenase (COX) and lipoxygenase (LOX) [30,31]. This inhibition reduces the formation of inflammatory mediators such as prostaglandins and leukotrienes, which play important roles in the development of inflammatory pain [31, 32]. Furthermore, flavonoids have been reported to regulate nitric oxide production, limit inflammatory cell migration and reduce pain signalling pathways [33, 34]. Therefore, the appreciable flavonoid content detected in Gbfun Super Bitters may partly explain the COX inhibitory activity observed in this study and provide a possible biochemical basis for its analgesic potential [35].

Terpenoids constituted another important group of phytochemicals detected in Gbfun Super Bitters, with several terpenoid compounds further identified through GC–MS analysis. These compounds have been widely recognised for their analgesic and anti-inflammatory properties, which are mediated through several mechanisms, including inhibition of cyclooxygenase [36] and inducible nitric oxide synthase activities [37], suppression of

pro-inflammatory cytokine production [38], regulation of transient receptor potential (TRP) ion channels [39], and modulation of cannabinoid receptor type 2 (CB₂)-mediated pathways [40-42].

Although found in adequate levels, alkaloids may still play a role in the ameliorative effects of the formulation by influencing opioid receptors, controlling neurotransmitter release and decreasing pain signal transmission [43, 44]. Likewise, tannins and saponins might offer further anti-inflammatory benefits by acting as antioxidants, supporting cell membranes and reducing the release of inflammatory substances [14, 45, 46]. The fact that Gbeful Super Bitters contains several groups of phytochemicals suggests that its pain-relieving properties could come from the combined and possibly joint actions of various active compounds rather than just one substance alone [16, 17].

The GC–MS analysis identified several bioactive compounds known for their pain-relieving and anti-inflammatory activities, offering more insight into the possible chemical explanations behind the biological effects of Gbeful Super Bitters.

Phytol was the most common compound found, followed by β -caryophyllene and eugenol. This compound is known for its antioxidant [47], anti-inflammatory [48] and pain-relieving properties [49], which are linked to its capacity to lower oxidative stress and block inflammatory processes [15]. β -Caryophyllene, a selective activator of cannabinoid receptor type 2 (CB₂), has been found to produce pain-relieving and anti-inflammatory effects without the mind-altering effects associated with CB₁ receptor activation [41, 50-52]. Eugenol also shows pain-relieving properties through the prevention of prostaglandin production, the suppression of inflammatory cytokines and the reduction of pain signals [53, 54]. Other terpenoid compounds, like α -pinene and limonene, are also associated with less inflammation through their impact on oxidative stress and inflammatory signals [42, 55].

The detection of flavonoid derivatives, including quercetin trimethylsilyl ether, kaempferol trimethylsilyl ether, catechin trimethylsilyl derivative, naringenin trimethylsilyl ether and rutin derivative, further supports the wide array of phytochemicals in Gbeful Super Bitters. Although these compounds were found after chemical modification, they are flavonoids known for their antioxidant and anti-inflammatory effects [34]. For example, quercetin is known to block COX and lipoxygenase activities and to lower NF- κ B activation and the production of inflammatory cytokines [56]; kaempferol is linked to less prostaglandin production and reduced oxidative damage [13, 57]; and catechin helps defend against oxidative stress by removing free radicals and managing inflammatory pathways [58]. According to Forouzanfar et al. [33], naringenin and rutin possess anti-inflammatory effects by reducing the activity of inflammatory substances and minimising oxidative damage.

The *in vitro* COX inhibition test showed that Gbeful Super Bitters caused a dose-dependent decrease in cyclooxygenase activity, with inhibition ranging from 18.64% at 25 μ g/mL to 78.91% at 400 μ g/mL. While diclofenac sodium demonstrated greater effectiveness, as indicated by its lower IC₅₀ value, the extract showed significant COX inhibitory activity, indicating a possible ability to reduce prostaglandin formation. Since prostaglandins are key in the development of inflammatory pain, blocking COX enzymes is a key mechanism by which pain-relieving compounds work [31, 32].

The results of this study align with previous findings that plant-based preparations rich in phenolics, flavonoids and terpenoids have notable pain-relieving and anti-inflammatory effects. For instance, flavonoids are responsible for

pain relief by inhibiting COX and lipoxygenase enzymes, limiting NF- κ B activation and modulating inflammatory cytokine levels [14, 31, 59]. Additionally, β -caryophyllene has been identified as an important plant-based mediator of pain relief through the activation of CB₂ receptors, delivering anti-inflammatory effects without causing psychotropic effects [41, 51, 52]. Other terpenoids, such as linalool, α -pinene and limonene, have also been found to have pain-relieving and anti-inflammatory properties by managing nerve transmission, blocking inflammatory substances and reducing oxidative stress [42, 55, 60]. Summarily, these findings suggest that the pain-relieving potential of Gbeful Super Bitters may come from the cooperative interactions of its diverse phytochemical components.

4.0. Conclusion

The present study assessed the phytochemical profile and in vitro analgesic potential of Gbeful Super Bitters. The findings confirmed the presence of various phytoconstituents, with phenols, terpenoids and flavonoids representing the major classes of secondary metabolites identified. Gas chromatography–mass spectrometry (GC–MS) analysis further revealed the presence of several biologically important compounds, including phytol, β -caryophyllene, eugenol, limonene and α -pinene, which have previously been associated with various pharmacological activities. Although the analgesic activity of Gbeful Super Bitters was lower than that of the reference drug, diclofenac sodium, the extract exhibited a concentration-dependent inhibition of cyclooxygenase (COX) activity, demonstrating appreciable in vitro analgesic potential. These findings provide scientific support for the traditional use of Gbeful Super Bitters in pain management and contribute to understanding its phytochemical composition and potential mechanisms of action.

5.0. Future Recommendations

Based on the findings of this study, the following recommendations are proposed for future research:

- 1) Further investigations should be conducted to validate the analgesic and anti-inflammatory effects of Gbeful Super Bitters using appropriate in vivo models.
- 2) Future studies should establish the safety profile of Gbeful Super Bitters through comprehensive toxicological evaluations and identify the specific bioactive constituents responsible for its observed activities.
- 3) Bioassay-guided fractionation, molecular mechanistic studies and well-designed clinical investigations should be carried out to elucidate further its therapeutic potential and possible application as a natural analgesic agent.

Declaration

Source of Funding

This research was funded by the authors only.

Competing Interests Statement

The authors have not declared any conflict of interest.

Consent for publication

The authors declare that they consented to the publication of this study.

Authors' contributions

All the authors took part in literature review, analysis and manuscript writing equally.

Informed Consent

Not applicable for this study.

Availability of data and material

Supplementary information is available from the authors upon request.

Institutional Review Board Statement

Not applicable for this study.

Ethical Approval

Written approval was obtained from the manufacturer of Gbefun Super Bitters before the study was initiated. The permission authorised the procurement of the product, its scientific evaluation and the dissemination of the research findings.

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