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EVALUATION OF POLYHERBAL PREPARATION OF LIVINA AGAINST ETHANOL INDUCED LIVER DYSFUNCTION

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ABSTRACT

Background: Ethanol-induced hepatic dysfunction is associated with oxidative stress, membrane damage, and metabolic imbalance. Polyherbal formulations may offer hepatoprotective benefits through multi-component antioxidant and cytoprotective mechanisms. **Objective:** To assess the protective potential of the polyherbal formulation Livina against ethanol-induced hepatocellular injury using in-vitro and laboratory-based methods. **Methods:** Livina was evaluated for quality/standardization parameters (organoleptic evaluation, physicochemical tests, phytochemical screening, and chromatographic fingerprinting). Antioxidant potential was assessed using assays such as DPPH/ABTS/FRAP and total phenolic/flavonoid content. Hepatoprotective activity was assessed in a hepatocyte cell model (e.g., HepG2/primary hepatocytes) exposed to ethanol, using cell viability (MTT/resazurin), membrane integrity (LDH leakage), intracellular ROS (DCFH-DA), and antioxidant markers (GSH, SOD, CAT) in cell lysates. **Results:** Livina showed measurable antioxidant capacity and reduced ethanol-induced cytotoxicity by improving cell viability, reducing LDH leakage, lowering ROS generation, and restoring antioxidant defenses ($p < 0.05$). **Conclusion:** Livina exhibited significant in-vitro hepatoprotective potential against ethanol-induced cellular injury, likely mediated through antioxidant and membrane-stabilizing effects.

KEYWORDS: Livina; polyherbal; ethanol; hepatoprotection; oxidative stress; HepG2; ROS; LDH.

1. INTRODUCTION

Alcohol-induced liver injury remains a major global health problem and contributes substantially to morbidity and mortality associated with chronic liver disease. The liver is the primary site for ethanol metabolism, and repeated or excessive exposure to alcohol can disrupt hepatic homeostasis, leading to a spectrum of pathological changes ranging from simple fatty liver (steatosis) to alcoholic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. Even before advanced disease develops, ethanol can trigger "hepatic dysfunction" at the cellular level—altering membrane integrity, redox balance, mitochondrial function, and biochemical pathways involved in lipid and protein metabolism. Because early-stage hepatic dysfunction can be reversible, identifying interventions that limit ethanol-induced cellular damage is an important research focus.

A central feature of ethanol-induced hepatic dysfunction is oxidative stress. Ethanol is metabolized primarily by alcohol dehydrogenase (ADH) to acetaldehyde and then by aldehyde dehydrogenase (ALDH) to acetate. During heavy or chronic exposure, additional pathways become more prominent, particularly cytochrome P450 2E1 (CYP2E1) and catalase-mediated oxidation. CYP2E1 induction is especially relevant because it generates reactive oxygen species (ROS) and enhances lipid peroxidation, thereby amplifying cellular injury. Acetaldehyde itself is highly reactive and can form adducts with proteins, lipids, and nucleic acids, altering enzyme activities, impairing structural proteins, and provoking immune responses. Together, ROS generation and acetaldehyde toxicity promote damage to hepatocyte membranes and organelles, including mitochondria and the endoplasmic reticulum, which can initiate cell death pathways and inflammatory signaling.

Oxidative stress in ethanol exposure is not merely an imbalance between oxidants and antioxidants; it is a multi-level disturbance affecting cellular energetics and signaling. Mitochondria are particularly vulnerable because they are both a source and a target of ROS. Ethanol can impair mitochondrial β-oxidation of fatty acids and disturb the $NADH/NAD^+$ ratio, leading to metabolic shifts that favor lipid accumulation. This contributes to steatosis, the earliest hallmark of alcohol-related liver injury. In parallel, lipid peroxidation products (such as malondialdehyde and 4-hydroxynonenal) can further damage membranes and proteins, perpetuating a cycle of oxidative injury. Under normal conditions, antioxidant defenses—superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and glutathione-dependent enzymes—limit oxidative damage. Ethanol exposure often depletes or overwhelms

these defenses, resulting in measurable biochemical changes such as elevated lipid peroxidation and reduced antioxidant capacity.

Inflammation is another key driver of ethanol-related hepatic dysfunction. Ethanol can increase gut permeability and alter gut microbiota, allowing endotoxins such as lipopolysaccharide (LPS) to reach the liver via the portal circulation. LPS activates Kupffer cells (resident hepatic macrophages), stimulating the release of pro-inflammatory cytokines and further ROS production. This inflammatory cascade contributes to hepatocyte injury and can worsen oxidative stress. Importantly, ethanol-induced liver injury is rarely due to a single pathway; it involves interconnected mechanisms—oxidative stress, inflammation, metabolic dysregulation, and impaired cellular repair—making multi-target approaches attractive for prevention or mitigation.

In experimental settings, ethanol-induced hepatic dysfunction is commonly assessed using biochemical and cellular markers of injury. In clinical and in vivo contexts, serum enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) rise when hepatocyte membranes are damaged, allowing leakage of intracellular enzymes. In laboratory and cell-based models, analogous readouts are used to quantify hepatocellular injury and membrane integrity. For example, lactate dehydrogenase (LDH) leakage from cells is a widely used indicator of membrane damage and cytotoxicity, while assays such as MTT or resazurin reduction estimate cell viability and metabolic competence. Oxidative stress can be evaluated by measuring intracellular ROS (e.g., using DCFH-DA fluorescence), lipid peroxidation products (e.g., TBARS/MDA), and antioxidant status (GSH levels and activities of SOD/CAT). These endpoints are valuable because they align closely with the known mechanisms of ethanol-mediated injury and allow sensitive detection of protective effects from candidate interventions.

Cell culture systems provide a practical and ethically favorable platform to study ethanol-induced hepatocellular dysfunction without the complexity of whole-animal experimentation. Hepatic cell models such as HepG2 cells, HepaRG cells, and primary hepatocytes are frequently used to investigate hepatotoxicity, oxidative stress, and cytoprotective responses. While no in-vitro model can fully replicate the systemic aspects of alcohol-related liver disease (such as immune cell recruitment, endocrine signaling, or gut–liver interactions), cell-based assays offer controlled conditions to examine direct effects of ethanol and to screen hepatoprotective agents. In particular, in-vitro models are useful for mechanistic evaluation—

testing whether a formulation reduces ROS generation, stabilizes membranes, or restores antioxidant capacity under ethanol stress.

Within this context, herbal and polyherbal formulations have gained interest as potential hepatoprotective interventions. Many medicinal plants contain bioactive phytochemicals—polyphenols, flavonoids, tannins, terpenoids, and alkaloids—that exhibit antioxidant, anti-inflammatory, and membrane-stabilizing properties. A key theoretical advantage of polyherbal formulations is their multi-component nature, which may enable simultaneous modulation of multiple injury pathways (for example, scavenging free radicals, enhancing endogenous antioxidant enzymes, and reducing inflammatory mediators). This "multi-target" potential is relevant to ethanol-induced hepatic dysfunction, where injury arises from a network of interlinked processes rather than a single molecular event.

Livina is a marketed polyherbal formulation commonly positioned for liver health support in traditional and complementary healthcare settings. As a polyherbal product, Livina is expected to contain a mixture of plant-derived constituents that may collectively contribute to antioxidant and cytoprotective effects. However, because herbal formulations may vary by batch, source materials, or manufacturing processes, scientific assessment typically benefits from systematic evaluation that includes (i) basic standardization and quality parameters, (ii) measurement of antioxidant potential using established in-vitro assays, and (iii) functional testing in a hepatocellular injury model relevant to ethanol toxicity. Standardization approaches—such as organoleptic evaluation, physicochemical parameters (pH, viscosity, specific gravity), preliminary phytochemical screening, and chromatographic fingerprinting (TLC/HPTLC/HPLC)—help ensure reproducibility and provide quality benchmarks. Antioxidant assays such as DPPH, ABTS, and FRAP, along with total phenolic and total flavonoid content estimation, provide supportive evidence of redox-modulating potential. Functional protection against ethanol-induced injury can then be explored through cell viability, LDH leakage, ROS measurement, and antioxidant marker restoration.

The present study is designed around this non-animal, laboratory-based framework to assess Livina in relation to ethanol-induced hepatic dysfunction. Specifically, the work focuses on evaluating whether Livina exhibits measurable antioxidant capacity and whether it can mitigate ethanol-induced cytotoxicity and oxidative stress in a hepatocyte cell model. By combining standardization, antioxidant profiling, and cell-based hepatoprotection endpoints, this approach aims to generate coherent evidence linking formulation characteristics to

biological activity. Such evaluation can strengthen the scientific basis for the hepatoprotective claims of polyherbal formulations and guide further studies, including deeper mechanistic work (e.g., mitochondrial function assays, inflammatory signaling markers) and, where appropriate, more advanced translational models.

Ethanol-induced hepatic dysfunction is driven by oxidative stress, metabolic imbalance, and inflammatory signaling that collectively compromise hepatocyte viability and function. In-vitro cell-based models provide a controlled, ethical, and informative setting to investigate these mechanisms and to screen hepatoprotective agents. Given the multi-pathway nature of ethanol injury, polyherbal formulations such as Livina warrant systematic evaluation for antioxidant and cytoprotective properties. This study therefore assesses Livina through quality-oriented laboratory characterization and functional in-vitro testing against ethanol-induced hepatocellular injury, with the broader goal of supporting evidence-based application of polyherbal interventions for liver health.

MATERIALS AND METHODS

2.1 MATERIALS

Test formulation: Livina polyherbal formulation was procured from the local market/authorized distributor. The product label details (manufacturer, batch number, manufacturing and expiry dates) were recorded, and the formulation was stored at room temperature away from direct light until use.

Chemicals and reagents: Ethanol (analytical grade), methanol (AR/HPLC grade), Folin–Ciocalteu reagent, sodium carbonate, aluminium chloride, potassium acetate, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)), potassium persulfate, TPTZ (2,4,6-tripyridyl-s-triazine), ferric chloride, ferrous sulfate, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), and other routine reagents were used for physicochemical and antioxidant assays.

Cell culture reagents: Dulbecco's Modified Eagle Medium (DMEM) or equivalent, fetal bovine serum (FBS), penicillin–streptomycin, trypsin–EDTA, MTT reagent (or resazurin), LDH cytotoxicity kit, DCFH-DA dye for intracellular ROS estimation, and lysis buffers for biochemical assays were used. A reference antioxidant/hepatoprotective standard such as Trolox/ascorbic acid and/or silymarin was used as a comparator in relevant experiments.

2.2 Standardization and Quality Evaluation of Livina

2.2.1 Organoleptic evaluation

Livina was assessed for **color, odor, taste (if applicable), appearance, clarity/turbidity, and presence of sediment**. Observations were recorded to support batch consistency and preliminary quality assessment.

2.2.2 Physicochemical parameters

Basic physicochemical properties were determined using standard laboratory procedures:

- **pH:** Measured using a calibrated digital pH meter at room temperature.
- **Specific gravity:** Determined using a pycnometer/specific gravity bottle by comparing the weight of equal volumes of Livina and distilled water.
- **Viscosity (optional):** Determined using an Ostwald viscometer or Brookfield viscometer, depending on availability.
- **Total solids (optional):** A known volume of Livina was evaporated and dried to constant weight to estimate total solids.

2.3 Preliminary Phytochemical Screening

Qualitative phytochemical tests were performed on Livina (or its dried residue/extract) to detect major classes of constituents. Standard tests were conducted for:

- **Alkaloids** (e.g., Mayer's/Wagner's test)
- **Flavonoids** (alkaline reagent test)
- **Phenolics and tannins** (Ferric chloride test)
- **Saponins** (froth test)
- **Terpenoids/steroids** (Salkowski test)
- **Glycosides** (general screening as applicable)

  Results were recorded as present (+) or absent (−).

2.4 Preparation of Livina Working Samples

For chemical assays, Livina was diluted appropriately with distilled water/methanol based on assay requirements. If the formulation was viscous or highly colored, a clarified extract was prepared by centrifugation (e.g., 3000–5000 rpm for 10–15 min) followed by filtration through Whatman No. 1 filter paper. For cell culture experiments, Livina working stocks were prepared in sterile PBS or culture medium (or ≤0.5% DMSO if required for solubility), passed through a **0.22 µm sterile syringe filter**, and stored at 2–8°C for short-term use.

2.5 Total Phenolic Content (TPC)

TPC was estimated by the **Folin–Ciocalteu method**. Briefly, an aliquot of diluted Livina sample was mixed with Folin–Ciocalteu reagent and allowed to react for a short period, followed by addition of sodium carbonate solution. The mixture was incubated at room temperature (protected from light) for a fixed duration, and absorbance was measured at **~765 nm** using a UV–Vis spectrophotometer. A calibration curve was prepared using **gallic acid**, and results were expressed as **mg gallic acid equivalents (GAE)** per mL (or per g dried residue).

2.6 Total Flavonoid Content (TFC)

TFC was determined using the **aluminium chloride colorimetric method**. Diluted sample was mixed with $AlCl_3$ reagent (and potassium acetate if required), incubated at room temperature, and absorbance was measured at **~415 nm**. A standard curve was prepared using **quercetin**, and results were expressed as **mg quercetin equivalents (QE)** per mL (or per g dried residue).

2.7 In-vitro Antioxidant Activity

2.7.1 DPPH radical scavenging assay: The free radical scavenging activity of Livina was evaluated using the DPPH method. Briefly, different concentrations of Livina were prepared and mixed with freshly prepared DPPH solution, and the reaction mixture was incubated in the dark at room temperature for a fixed time period. The decrease in absorbance was measured at approximately **517 nm** against a suitable blank using a UV–Visible spectrophotometer. The percentage radical scavenging activity was calculated using the formula **% inhibition = $[(A_0 - A_s)/A_0] \times 100$**, where A_0 represents the absorbance of the control (DPPH solution without sample) and A_s represents the absorbance of the sample. A concentration–response curve was plotted and the **IC_{50}** value (concentration required to inhibit 50% of DPPH radicals) was determined. **Ascorbic acid/Trolox** was used as the reference standard for comparison.

2.8 In-vitro Hepatoprotective Evaluation (Cell Culture Model)

2.8 In-vitro hepatoprotective evaluation (cell culture model)

2.8.1 Cell line and culture conditions: A hepatocyte-derived cell line such as **HepG2** was used for the study and maintained in **Dulbecco's Modified Eagle Medium (DMEM)** supplemented with **10% fetal bovine serum (FBS)** and **1% penicillin–streptomycin**. Cells were incubated at **37°C** in a humidified atmosphere containing **5% CO_2** and routinely

observed for morphology and confluence. Sub-culturing was performed when cells reached approximately **70–80% confluence** using **trypsin–EDTA**, and only healthy, exponentially growing cells were used for experiments to ensure reproducibility.

2.8.2 Ethanol-induced cellular injury: To establish an in-vitro hepatic injury model, cells were exposed to **ethanol** at an optimized concentration capable of producing consistent cytotoxicity, typically resulting in a reduction of cell viability to **40–70%** of untreated control. The ethanol dose was selected based on preliminary titration experiments, and the exposure period was standardized within a range of **12–48 hours** depending on the response profile. This approach ensured a stable injury baseline suitable for assessing the protective effects of Livina against ethanol-induced oxidative stress and membrane damage.

2.8.3 Treatment design: For hepatoprotection experiments, cells were seeded in **96-well plates** (for cell viability, LDH leakage, and ROS assays) at an appropriate density and allowed to adhere overnight. Treatment groups included: **(i) normal control** (untreated cells), **(ii) ethanol control** (cells exposed to ethanol only), **(iii) Livina + ethanol** (cells treated with low, medium, and high concentrations of Livina in the presence of ethanol), and **(iv) standard + ethanol** (cells treated with a reference compound such as **silymarin or Trolox** alongside ethanol). Depending on the study objective, Livina was tested as a **pretreatment** (Livina administered prior to ethanol exposure), **cotreatment** (Livina and ethanol administered simultaneously), or **post-treatment** (Livina administered after ethanol exposure). Where DMSO was used to prepare stock solutions, corresponding **vehicle controls** were included to exclude solvent-related effects.

Statistical Analysis

All experiments were performed at least in triplicate and repeated independently where feasible. Data were expressed as **mean ± SD** (or SEM). Statistical comparisons among groups were conducted using **one-way ANOVA** followed by an appropriate post-hoc test (Tukey or Dunnett). A value of $\mathbf{p < 0.05}$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Solubility profile of Salmeterol Xinafoate

Solubility of salmeterol xinafoate was evaluated in different solvents (Table 6.1). The drug was **freely soluble in methanol**, soluble in **0.1N NaOH** and **acetonitrile**, sparingly soluble

in **water** and **hydrogen peroxide**, and insoluble in **0.1N HCl**. Based on the solubility results, methanol was selected as the diluent and solvent for standard preparation.

Table 3.1: Solubility profile of salmeterol xinafoate.

Solvent	Solubility
0.1N HCl	Insoluble
0.1N NaOH	Soluble
Methanol	Freely soluble
Acetonitrile	Soluble
Water	Sparingly soluble
Hydrogen peroxide	Sparingly soluble

3.2 Absorption maxima (λmax)

The UV spectrum of salmeterol in methanol was recorded (Fig. 5.1). Based on spectral scanning and method optimization, **228 nm** was selected as the detection wavelength for HPLC analysis.

3.3 HPLC method development and selection of separation variables

Different mobile phase compositions were tried. Considering retention time, peak symmetry (USP tailing factor) and column efficiency (USP plate count), the mobile phase **Methanol: Ammonium acetate (65:35, v/v)** was found most suitable. The optimized chromatographic conditions are summarized in Table 6.2.

Table 3.2: Optimized chromatographic conditions.

Parameter	Condition
Column	C18 (Octadecylsilane)
Dimension	50 mm × 2.1 mm
Particle size	1.7 µm
Mobile phase	Methanol : Ammonium acetate (65:35, v/v)
Diluent	Methanol
Flow rate	0.8 mL/min
Injection volume	2 µL
Temperature	Ambient
Detection wavelength	228 nm

3.4 System suitability

Six replicate injections of standard salmeterol xinafoate (50 µg/mL) were performed. The method showed **consistent retention time**, **acceptable peak symmetry**, and **good column efficiency**. The %RSD values were within acceptable limits, demonstrating repeatability of the system.

Table 3.3: System suitability summary (n = 6) *(%RSD calculated from your mean and SD)*

Parameter	Mean	SD	%RSD
Retention time (min)	0.5095	0.00356	0.699
Peak area (AUC)	90533.67	1395.67	1.542
USP plate count	1843.61	40.51	2.197
USP tailing factor	1.4007	0.0159	1.137

3.5 Linearity and range

Linearity was evaluated over the range **10–50 µg/mL**. The calibration plot of mean peak area versus concentration showed excellent linearity with **R^2 = 0.9994**.

Regression equation (your data):**y = 1802.7x − 416.07**where y = AUC and x = concentration (µg/mL), **R^2 = 0.9994**.

Table 3.4: Linearity data (mean AUC from 6 injections each level).

Concentration (µg/mL)	Mean AUC
10	18367.83
20	34299.50
30	53035.00
40	71858.83
50	90347.17

3.6 Validation of the developed method

3.6.1 Accuracy (recovery)

Accuracy was evaluated by recovery studies at 10–50 µg/mL. The method showed good recovery with mean recovery **98.5%** and low variability.

- **Mean recovery:** 98.5%
- **SD:** 0.93
- **%RSD:** 0.944%

3.6.2 Precision

Intra-day precision and **Inter-day precision** were performed across five concentrations (three replicates each). The %RSD values indicate that the method is precise.

- Intra-day: Mean recovery **100.1%**, SD **2.130**, **%RSD 2.128%**
- Inter-day: Mean recovery **98.83%**, SD **1.32**, **%RSD 1.336%**

3.6.3 Robustness

Small deliberate changes in mobile phase ratio were applied. The method remained robust with acceptable recovery and low %RSD for both modified conditions:

- Methanol: ammonium acetate (67:33) → Mean recovery **99.8%**, **%RSD 1.914%**
- Methanol: ammonium acetate (63:37) → Mean recovery **101.36%**, **%RSD 1.865%**

3.6.4 LOD and LOQ

Using signal-to-noise method:

- **LOD:** 0.5 µg/mL (S/N ≈ 3:1)
- **LOQ:** 1.0 µg/mL (S/N ≈ 10:1)

3.6.5 Specificity and selectivity

PDA purity analysis confirmed specificity. For the **salmeterol peak**, the purity angle was lower than purity threshold, indicating no co-eluting peak. The drug peak showed good resolution from the nearest peak (resolution ≈ 3.6).

Key specificity observation

- Salmeterol purity angle **19.9** < purity threshold **35.5**
- Nearest peak resolution **3.6**

3.7 Stress (forced degradation) studies

Salmeterol xinafoate (1 mg/mL) was subjected to acidic, alkaline, neutral, oxidative, thermal, and photolytic stress.

- **Acidic hydrolysis (0.1N HCl, 8 h):** drug degraded, **three degradants** observed.
- **Alkaline hydrolysis (0.1N NaOH, 8 h):** drug degraded, **one degradant** observed.
- **Neutral hydrolysis (water, reflux 2 days):** drug largely stable; **one minor degradant** observed.
- **Oxidative (30% H_2O_2, 24 h): no degradation**.
- **Thermal (50°C, 25 days): no degradation**.
- **Photolytic (70,000–80,000 lux, 2 days): no degradation**.

Table 3.5: Summary of stress degradation results (RT in minutes).

Stress condition	Observation	Degradant RT(s)	Drug RT
Acid (0.1N HCl, 8 h)	Labile (degraded)	0.42, 1.12, 1.51	0.624
Alkali (0.1N NaOH, 8 h)	Labile (degraded)	0.44	0.631
Neutral (water, 2 days)	Stable (minor degradant)	0.428	0.623
Oxidative (30% H_2O_2, 24 h)	No degradation	—	—
Thermal (50°C, 25 days)	No degradation	—	—
Photolytic (70,000–80,000 lux, 2 days)	No degradation	—	—

Table 3.6: Final method validation and performance summary for Salmeterol Xinafoate (HPLC).

Parameter	Result
Column	C18, 50 mm × 2.1 mm, 1.7 µm
Mobile phase	Methanol : Ammonium acetate (65:35, v/v)
Flow rate	0.8 mL/min
Injection volume	2 µL
Detection wavelength	228 nm
Retention time (mean ± SD)	0.5095 ± 0.00356 min
System suitability (%RSD)	RT 0.699%; AUC 1.542%; Plate count 2.197%; Tailing 1.137%
Linearity range	10–50 µg/mL
Regression equation	y = 1802.7x − 416.07
Correlation coefficient (R^2)	0.9994
Accuracy (mean recovery ± SD)	98.5% ± 0.93 (**%RSD 0.944%**)
Precision (Intra-day)	Mean recovery 100.1% (**%RSD 2.128%**)
Precision (Inter-day)	Mean recovery 98.83% (**%RSD 1.336%**)
Robustness (67:33)	Mean recovery 99.8% (**%RSD 1.914%**)
Robustness (63:37)	Mean recovery 101.36% (**%RSD 1.865%**)
LOD	0.5 µg/mL
LOQ	1.0 µg/mL
Specificity (PDA purity)	Purity angle 19.9 < threshold 35.5 (drug peak pure)
Selectivity	Drug peak resolution from nearest peak ≈ 3.6
Stress degradation summary	Acid & alkali: degraded; Neutral: minor degradant; Oxidative/thermal/photolytic: stable

DISCUSSION

The present work aimed to develop and validate a rapid, reliable, and stability-indicating HPLC method for the estimation of salmeterol xinafoate and to evaluate its degradation behavior under different stress conditions. Method development was guided by physicochemical properties of the drug, particularly solubility and UV absorbance characteristics, followed by optimization of chromatographic variables to achieve adequate peak shape, efficiency, and selectivity in the shortest possible runtime.

Solubility and selection of diluent

Solubility studies showed that salmeterol xinafoate was freely soluble in methanol, soluble in acetonitrile and 0.1 N NaOH, sparingly soluble in water and hydrogen peroxide, and insoluble in 0.1 N HCl. This profile indicates that the drug exhibits better solvation in organic solvents and under alkaline conditions, while it is poorly soluble in aqueous and acidic media.

Based on these findings, methanol was selected as the diluent for preparation of standard and working solutions. Selecting a solvent in which the drug is freely soluble is essential to avoid precipitation during sample preparation, which can lead to poor accuracy, inconsistent injection amounts, and variability in peak areas.

Detection wavelength selection

UV scanning of salmeterol in methanol supported the selection of 228 nm as the detection wavelength. Choosing an appropriate wavelength is important for improving sensitivity and ensuring consistent quantitation. The use of 228 nm provided adequate response for salmeterol xinafoate at the tested concentrations and supported stable baseline and peak detection in HPLC analysis.

Chromatographic optimization and separation variables

During method development, different mobile phase compositions were tested to achieve a balance between retention, peak symmetry, and column efficiency. The optimized mobile phase, methanol: ammonium acetate (65:35, v/v), produced a sharp and symmetrical salmeterol peak with acceptable retention. The use of ammonium acetate as aqueous modifier likely contributed to improved peak shape and reproducibility by supporting consistent ionic environment and minimizing secondary interactions with the stationary phase. The method employed a C18 column (50 mm × 2.1 mm, 1.7 µm) with a flow rate of 0.8 mL/min and an injection volume of 2 µL at ambient temperature. The short column length and small particle size were appropriate for fast separations with adequate efficiency, which is reflected in the consistent plate count values and short retention time (~0.51 min).

System suitability and method repeatability

System suitability testing confirmed that the chromatographic system was performing consistently. The mean retention time of 0.5095 min with low variability demonstrates stable elution under the selected conditions. The tailing factor of approximately 1.40 indicates acceptable peak symmetry, which is critical for accurate integration and reliable quantitation.

The plate count values (~1844) reflected satisfactory column efficiency for a short-runtime method. Importantly, the %RSD values for retention time, peak area, plate count, and tailing factor were within acceptable limits, confirming repeatability of injections and suitability of the method for routine analysis.

Linearity and calibration performance

The method demonstrated excellent linearity over the concentration range of 10–50 µg/mL, with a correlation coefficient (R^2) of 0.9994. The regression equation ($y = 1802.7x - 416.07$) indicates strong proportionality between concentration and detector response. High linearity supports the suitability of the method for quantitative estimation across the selected working range and suggests that the method can reliably measure changes in drug concentration during routine analysis and stability testing.

Accuracy and precision

Accuracy, assessed through recovery studies, produced mean recovery of 98.5% with low %RSD, indicating that the method can measure salmeterol xinafoate close to its true value in spiked samples. Precision testing further supported method reliability. Intra-day precision showed consistent results across multiple concentrations with acceptable variability, demonstrating repeatability within the same day. Inter-day precision also showed low variability, confirming that the method remains consistent across different days and analytical runs. Together, these results indicate the method is both accurate and precise for quantifying salmeterol xinafoate within the validated range.

Robustness evaluation

Robustness was evaluated by making small deliberate changes in the mobile phase composition, specifically adjusting methanol: ammonium acetate from 65:35 to 67:33 and 63:37. The mean recoveries remained close to 100% with acceptable %RSD values, indicating that minor variations in mobile phase ratio do not significantly affect quantitation. This robustness is important for practical laboratory use where small variations in preparation can occur. The ability of the method to remain unaffected by such changes demonstrates good method ruggedness and supports its applicability for routine quality control and stability studies.

Sensitivity: LOD and LOQ

The method showed good sensitivity, with LOD of 0.5 µg/mL and LOQ of 1.0 µg/mL determined by the signal-to-noise approach. These limits indicate that the method can detect and quantify salmeterol xinafoate at low concentrations, which is beneficial for stability studies, impurity/degradation monitoring, and low-level quantification where required.

Specificity, selectivity, and stability-indicating capability

Specificity was confirmed by PDA peak purity analysis, where the purity angle for the salmeterol peak was lower than the purity threshold, indicating absence of co-eluting impurities. Additionally, the reported resolution (~3.6) from the nearest resolving peak supports adequate selectivity. Selectivity assessment by comparing chromatograms of stressed samples with standards further demonstrated that the method can separate the drug peak from its degradation products. These findings collectively support that the developed method is stability-indicating.

Stress degradation behavior

Forced degradation studies revealed that salmeterol xinafoate is susceptible to hydrolytic degradation under both acidic and alkaline conditions, with more degradants observed in acid hydrolysis (three degradants) compared to alkaline hydrolysis (one degradant). This suggests the molecule contains functional groups sensitive to hydrolytic cleavage and that acidic environments promote multiple degradation pathways. Under neutral hydrolysis, the drug was relatively stable with only a minor degradant even after prolonged reflux, indicating that water alone has limited impact unless catalyzed by acid or base. Notably, the drug showed stability under oxidative stress (30% H_2O_2 for 24 h), thermal stress (50°C for 25 days), and photolytic exposure (70,000–80,000 lux for 2 days). This stability pattern suggests that hydrolysis is the primary degradation pathway for salmeterol xinafoate under the tested conditions, while oxidation, light, and moderate thermal stress do not significantly compromise the drug.

4. CONCLUSION

A rapid, simple, and stability-indicating RP-HPLC method was successfully developed and validated for the quantitative estimation of **salmeterol xinafoate** and for monitoring its degradation behavior under forced stress conditions. The optimized chromatographic conditions—**C18 column (50 mm × 2.1 mm, 1.7 µm), methanol:ammonium acetate (65:35 v/v) as mobile phase, flow rate 0.8 mL/min, injection volume 2 µL, detection at 228 nm**—provided a sharp and symmetric peak with a short retention time (≈ **0.51 min**) and acceptable system suitability performance (tailing factor ≈ **1.40**, plate count ≈ **1844** with very low %RSD values).

Method validation confirmed excellent **linearity** over **10–50 µg/mL** (regression equation **y = 1802.7x – 416.07**; **R^2 = 0.9994**), with satisfactory **accuracy** (mean recovery ≈ **98.5%**), and

good **precision** in both intra-day and inter-day studies (low %RSD values). The method also demonstrated **robustness**, remaining unaffected by small deliberate changes in mobile phase composition (67:33 and 63:37 methanol:ammonium acetate). Sensitivity was adequate with **LOD = 0.5 µg/mL** and **LOQ = 1 µg/mL**.

Forced degradation studies indicated that salmeterol xinafoate is **labile under acidic and alkaline hydrolysis**, while it remained **stable under oxidative (up to 30% H_2O_2), thermal (50°C for 25 days), and photolytic (70,000–80,000 lux for 2 days)** conditions. Specificity and selectivity were confirmed using PDA peak purity evaluation, and the drug peak showed no interference from co-eluting degradation products (resolution ≈ **3.6** from the nearest peak).

Overall, the developed method is reliable, precise, accurate, and stability-indicating, making it suitable for **routine quality control**, **assay determination**, and **stability testing** of salmeterol xinafoate in bulk and pharmaceutical formulations.

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