# Development and Preliminary Evaluation of Herbal-Infused Chocolate with Potential Implications for Cholesterol and Immune Support

Mohini Patel[1*], Falaq Jujara, Nidhi Patel, Jigisha Panchal, Jaswandi Mehetre

[1,2,3,4] *Assistant Professor, School of Pharmacy, ITM SLS Baroda University, Vadodara, Gujarat, India*

[5] *Dean (In-charge), School of Pharmacy, ITM SLS Baroda University, Vadodara, Gujarat, India*

***Corresponding Author: mohinipatel2712@gmail.com**

***Abstract:*** *Lifestyle-related disorders such as hypercholesterolemia and metabolic dysfunction have increased demand for functional foods with added health benefits. This study developed a botanically enriched dark chocolate incorporating flaxseed, ashwagandha, cinnamon, and fennel, all known for cardioprotective, antioxidant, immunomodulatory, and digestive properties. Dark chocolate powder served as the base, while date paste and honey replaced refined sugar. Three formulations with varying sweetener ratios were prepared. Phytochemical screening confirmed the presence of key bioactive compounds, including lignans, withanolides, omega-3 fatty acids, and cinnamaldehyde. Sensory evaluation highlighted differences in taste, aroma, texture, and overall acceptability, with one formulation scoring highest across parameters. Stability testing demonstrated that the preferred formulation maintained desirable appearance, minimal bloom formation, and consistent moisture and hardness during storage. The findings suggest that botanically fortified dark chocolate is a feasible functional food with good sensory appeal, stability, and potential to support cardiovascular health and immune function.*

***Key Word:*** *Herbal-infused chocolate, Cholesterol regulation, Immunomodulatory effects.*

# 1 INTRODUCTION

## 1.1 Cholesterol & Its Health Impact

Cholesterol is essential for maintaining membrane stability, producing steroid hormones, and synthesizing bile acids. However, elevated low-density lipoprotein cholesterol (LDL-C) remains one of the primary contributors to atherosclerosis and cardiovascular diseases (CVDs), including coronary artery disease and stroke—conditions responsible for more than 18 million deaths annually. [1,2] The Global Burden of Disease (GBD) 2019 study reported that high LDL-C accounted for approximately 4.4 million deaths and nearly 98.6 million disability-adjusted life years (DALYs), demonstrating its global health significance. [2]

Despite advancements in diagnostic and therapeutic approaches, hypercholesterolemia affects about 39% of adults worldwide, with many individuals—particularly in low-resource regions—remaining undetected or untreated.[2,4] Modern lifestyle factors such as physical inactivity and diets rich in saturated and trans fats further exacerbate dyslipidaemia and increase the risk of metabolic disorders and CVDs. [5]

### 1.1.1 Risks of High Cholesterol

Persistently elevated LDL-C promotes plaque deposition within arterial walls, reducing blood flow and oxygen supply to vital organs. This significantly increases the likelihood of coronary artery disease, myocardial infarction, stroke, and peripheral artery disease. [5] Dyslipidaemia frequently coexists with obesity, diabetes, and systemic inflammation, amplifying cardiovascular risk and complicating management strategies. Recent evidence also suggests that individuals recovering from COVID-19 may experience post-infection dyslipidaemia characterized by elevated LDL-C and total cholesterol levels, potentially increasing long-term susceptibility to cardiovascular complications. [6]

## 1.2 Immunity

The immune system is a coordinated defense network composed of organs, tissues, and specialized cells that protect the body from harmful microorganisms.

### 1.2.1 Types of Immunity

**1.2.1.1 Innate Immunity:** Innate immunity offers immediate, non-specific protection through physical barriers and cells such as macrophages and natural killer cells, which rapidly respond to invading pathogens.

**1.2.1.2 Adaptive Immunity:** Adaptive immunity develops over time and generates targeted responses through T and B lymphocytes, enabling antibody production and long-term immunological memory.

### 1.2.2 Core Functions of the Immune System

1. Recognition of foreign pathogens
2. Elimination through cellular and molecular mechanisms

3. Development of memory for enhanced future protection

Lifestyle factors—including nutrient-rich diets, adequate sleep, physical activity, and effective stress management—are essential for maintaining strong immune defences. [7] Global challenges such as aging, chronic disease, and infectious threats like COVID-19 have contributed to weakened immune function, disruptions in lipid metabolism, and increased vulnerability to infections and chronic illnesses.[6,8] These issues collectively impose significant public health and economic burdens.[8]

### 1.3 Impact of Hypercholesterolemia on Immune Function

Growing evidence highlights the role of cholesterol in regulating immune responses. Dysregulated cholesterol levels can impair both innate and adaptive immunity.

#### 1.3.1 Cholesterol-Induced Activation of Innate Immunity

Oxidized LDL (oxLDL) activates Toll-like receptors (TLRs), leading to stimulation of the NLRP3 inflammasome and excessive secretion of IL-1β and IL-18. This persistent activation contributes to chronic low-grade inflammation and impaired immune function. [9]

#### 1.3.2 Disrupted Cholesterol Efflux and Immune Imbalance

Dysfunction of cholesterol transporters ABCA1 and ABCG1 causes lipid accumulation in hematopoietic stem and progenitor cells. This shifts differentiation toward inflammatory monocytes and neutrophils, promoting immune imbalance and persistent inflammation. [10]

#### 1.3.3 Oxysterols and Adaptive Immune Regulation:

Cholesterol metabolites such as 25-hydroxycholesterol inhibit SREBP2 activity, suppressing B-cell maturation and antibody production. Oxysterols also disrupt T-cell function and migration, resulting in altered adaptive immunity. [11]

**1.3.4 Hypercholesterolemia and Autoimmunity:** Elevated cholesterol reduces DNase activity, causing extracellular DNA accumulation and formation of autoantibodies such as anti-dsDNA, contributing to autoimmune diseases like systemic lupus erythematosus (SLE). [12]

#### 1.3.5 Clinical Insights into Immune Suppression

Untreated hypercholesterolemia is associated with reduced expression of T-cell, B-cell, and NK-cell activation markers (e.g., CD25, CD69), along with Th1-to-Th2 cytokine shifts. Short-term statin therapy only partially restores these functions, indicating lasting immunological effects. [13]

### 1.4 Synthetic Treatments and Limitations

Pharmacological interventions for dyslipidaemia include statins, fibrates, ezetimibe, and PCSK9 inhibitors. These agents primarily reduce LDL-C levels by inhibiting cholesterol biosynthesis or enhancing hepatic LDL uptake.

**1.4.1 Statins:**

Statins lower intracellular cholesterol and exert anti-inflammatory effects by promoting macrophage polarization from M1 to M2 through JMJD3 activation. They suppress TLR4–MyD88–NF-κB signaling and inhibit NLRP3 inflammasome activity, reducing cytokines such as IL-1β, IL-18, IL-6, and TNF-α. [14] Statins also down regulate MHC II expression and shift cytokine profiles toward Th2 dominance, influencing autoimmune and inflammatory processes. [14, 15] Clinical findings suggest statins may enhance humoral responses to polysaccharide antigens, though their mortality benefit in infections like COVID-19 remains unclear. [15]

**1.4.2 PCSK9 Inhibitors:**

PCSK9 inhibitors—including monoclonal antibodies (e.g., alirocumab) and siRNA-based therapies (e.g., inclisiran)—increase hepatic LDL receptor availability, leading to substantial reductions in circulating LDL-C. Beyond lipid lowering, these agents reduce leukocyte adhesion, suppress pro-inflammatory cytokines, and enhance IL-10 and regulatory T-cell activity. [16] Preclinical research indicates that PCSK9 inhibition enhances antitumor immunity by increasing $CD8^+$ T-cell infiltration and reducing intratumoral Tregs. [17] They also reverse oxLDL-induced dendritic cell activation, promoting immune tolerance. [18]

**1.5 Limitations and Adverse Effects of Synthetic Therapies:** Although statins and PCSK9 inhibitors effectively reduce LDL-C and cardiovascular risk, their long-term use is associated with several drawbacks. Common adverse reactions include muscle-related symptoms such as myalgia and occasional myopathy, elevations in liver enzymes indicating possible hepatic stress, gastrointestinal discomfort, and an increased likelihood of developing type 2 diabetes, particularly at higher statin doses. [19, 20] Some individuals also report cognitive difficulties such as memory impairment and fatigue.

These effects contribute to poor adherence, especially in patients reluctant to commit to lifelong medication. Cost further limits accessibility—PCSK9 inhibitors, as injectable biologics, remain expensive and less feasible in low-resource settings. Concerns regarding infection risk have also emerged; prolonged statin use may produce mild immunosuppressive effects, while PCSK9 inhibitors are linked to nasopharyngitis and flu-like symptoms. [21, 22] The immunomodulatory and muscle-related effects of these drugs can be especially problematic for individuals with pre-existing immune dysfunction. Collectively, these limitations emphasize the need for safer, affordable, and culturally acceptable alternatives, particularly in low- and middle-income countries. [21, 22]

**1.6 Need for Natural Alternatives**

Although statins and other lipid-lowering medications significantly reduce cardiovascular events, their side effects and variable tolerance have led to increased interest in natural, better-tolerated alternatives. Many nutraceuticals—including berberine, red yeast rice, artichoke extract, and bergamot—have demonstrated cholesterol-lowering capacity comparable to low-dose statins. Additionally, a variety of natural agents such as probiotics, curcumin, green tea catechins, and elderberry exhibit

immunomodulatory effects, enhancing innate and adaptive responses without the adverse reactions commonly associated with synthetic drugs. A 2023 network meta-analysis identified several of these substances as among the most effective non-pharmacological interventions for lipid management, particularly for statin-intolerant populations or individuals preferring non-drug approaches. [23]

**1.7 Selected Natural plant-based agents and other contributing substances**

**1.7.1 *Withania somnifera* (Ashwagandha)**

**Figure 1: Ashwagandha**

**Synonyms:** Indian ginseng, Indian winter cherry, poison gooseberry

**Family:** Solanaceae

**1.7.1.1 Geographical Distribution:**

Withania somnifera is widely cultivated across arid tropical regions, including Afghanistan, India, China, several African nations, the Middle East, and Mediterranean territories such as Spain.

**1.7.1.2 Phytochemical Constituents:** Ashwagandha contains a rich phytochemical profile consisting of more than 12 alkaloids, over 40 withanolides, and multiple sitoindosides. Key constituents include alkaloids such as withanine, somniferine, and tropine; steroidal lactones such as withaferin A and withanolides; saponins, tannins, flavonoids (quercetin, kaempferol), phenolic compounds, carbohydrates, β-sitosterol, and other sterols including stigma sterol and cholesterol.

**1.7.1.3 Therapeutic and Pharmacological Activities:** Its diverse composition underlies a broad spectrum of pharmacological effects. Ashwagandha demonstrates anti-inflammatory, antioxidant, antitumour, antidiabetic, antimicrobial, neuroprotective, hepatoprotective, and cardio protective activities. It also supports stress resilience and immune regulation, contributing to both metabolic and immune health. [24]

**1.7.1.4 Traditional and Ethnomedicinal Uses:** In traditional medicine, Ashwagandha roots are used in multiple forms, including milk decoctions for detoxification and pastes for swelling and ulcers. It has been employed for male reproductive health, tumour suppression, respiratory issues, infertility, skin disorders, neurological conditions, rheumatism, digestive problems, and insomnia. It is also believed to enhance immunity and increase white blood cell production. Modern uses include consumption in beverages, such as Ashwagandha-infused cocoa drinks, aimed at boosting immunity and vitality.

**1.7.1.5 Impact on Lipid Profile and Immune Function:**

Ashwagandha has been shown to improve metabolic parameters by reducing blood glucose, LDL-C, and total cholesterol while increasing HDL levels. Its anti-atherogenic properties help prevent plaque formation, and it inhibits lipid peroxidation, promotes lipoprotein lipase activity, and may reduce body weight. It also displays anti-aging effects by enhancing red blood cell levels and hair pigmentation. Immunologically, compounds such as withaferin A and sitoindosides IX and X modulate immune responses, supporting both cellular and humoral immunity. [24]

**1.7.2 *Cinnamomum verum* (Cinnamon)**

**Synonym:** Darchini

**Family:** Lauraceae

**Figure 2: Cinnamon**

**1.7.2.1 Geographical Distribution:** Cinnamon is widely used in culinary and medicinal traditions across Asia, South America, and Australia. Major producers include China, India, Vietnam, Indonesia, Sri Lanka, and Nepal. [25, 26]

**1.7.2.2 Phytochemical Composition:** Its therapeutic properties are attributed primarily to cinnamaldehyde, cinnamic acid, and cinnamate.

**1.7.2.3 Pharmacological Activities:** Cinnamon possesses antioxidant, anti-inflammatory, and antidiabetic actions that support cardiovascular health. It also exhibits antimicrobial, analgesic, anti-arthritic, anticancer, neuroprotective, cytoprotective, and immunomodulatory properties. [26]

**1.7.2.4 Traditional and Ethnomedicinal Uses:** Cinnamon bark has been traditionally used to manage ailments such as menstrual irregularities, arthritis, cardiomyopathy, gastrointestinal disturbances, dizziness, erectile dysfunction, fever, and prostate inflammation. [26]

**1.7.2.5 Impact on Cholesterol Metabolism and Immune Function:**

Cinnamon contributes to lipid regulation through several mechanisms. It inhibits HMG-CoA reductase, a key enzyme in endogenous cholesterol synthesis, thereby lowering LDL-C and elevating HDL-C. Cinnamaldehyde and cinnamic acid enhance lipid oxidation and reduce triglyceride levels. Cinnamon also decreases intestinal absorption of lipids by down regulating NPC1L1 and CD36 transporters in the gut epithelium. Additionally, it suppresses the transcription of genes involved in triglyceride production and ApoB-48 synthesis, further supporting its lipid-lowering and cardio protective effects. [25] Its immunomodulatory actions complement these metabolic benefits, contributing to overall systemic health.

**1.7.3 *Linum usitatissimum* (Flaxseed)**

**Synonyms:** Linseed, Alsi

**Family:** Linaceae

**1.7.3.1 Phytochemical Composition:** Flaxseed contains high levels of α-linolenic acid (ALA), a key omega-3 fatty acid, along with dietary fiber, lignans, and other polyunsaturated fatty acids such as DHA and EPA. These constituents underpin its nutritional and therapeutic significance. [27, 28]

**Figure 3: Flaxseed**

**1.7.3.2 Pharmacological Properties:** The bioactive compounds in flaxseed exhibit diverse

pharmacological effects. Flaxseed has shown protective roles in neurological disorders, cardiovascular disease, atherosclerosis, cancer, osteoporosis, autoimmune conditions, diabetes, arthritis, and hypertension. The anti-inflammatory actions of ALA and other polyunsaturated fatty acids are particularly beneficial in reducing risks of rheumatoid arthritis, asthma, and coronary artery disease. [28]

**1.7.3.3 Traditional and Medicinal Applications:** Traditionally, flaxseed oil, fibers, and lignans have been used to support cardiovascular and metabolic health, reduce inflammation, and aid in preventing cancer, osteoporosis, and neurological dysfunction. Flaxseed proteins also contribute to cardio protective and immune-supportive functions. [27]

**1.7.3.4 Impact on Lipid Profile and Immune Function:**

Flaxseed supplementation has consistently shown lipid-lowering benefits, including reductions in total cholesterol, LDL-C, and triglycerides, alongside increases in HDL-C. Clinical studies confirm its effectiveness in improving lipid metabolism and slowing cardiovascular disease progression. It is well tolerated even in individuals with severe hyperlipidemia, with repeated evidence of improved lipid profiles. [27, 28]

**1.7.4 *Theobroma cacao* (Dark Chocolate)**

**Family:** Malvaceae

**1.7.4.1 Nutritional and Functional Value:**

**Figure 4: Dark Chocolate**

Dark chocolate is considered a superior alternative to milk chocolate due to its significantly higher content of polyphenols and flavonoids. In fact, the concentration of these bioactive compounds in dark chocolate is approximately five times greater than that found in milk or white chocolate. In contrast, milk and white chocolates tend to have higher levels of fat (approximately 30 g per 100 g) and sugar (about 52 g per 100 g), making dark chocolate a more favourable choice for health-conscious consumers.

**1.7.4.2 Phytochemical Composition:** Dark chocolate is a rich source of various phenolic compounds, including catechins**,** epicatechin**,** anthocyanins, and proanthocyanidins, which contribute to its distinct bitterness, astringency, and robust cocoa flavour. Additionally, it contains alkaloids such as theobromine**,** caffeine, and theophylline, which also contribute to its physiological and sensory effects. [29]

**1.7.4.3 Impact on Cholesterol and Immune Function:**

Regular, moderate consumption of dark chocolate may contribute to the maintenance of healthy blood cholesterol levels. The cocoa polyphenols present in dark chocolate have been found to inhibit cholesterol absorption and synthesis by reducing the expression of cholesterol receptors. As a result, consumption may lead to lower total cholesterol and LDL cholesterol levels, although effects on HDL cholesterol are generally minimal. Dark chocolate also plays a role in supporting the immune system by enhancing the production of interferon-gamma (IFN-γ), an important cytokine in immune response regulation. Furthermore, antioxidants in cocoa, such as procyanidins**,** epicatechin, and catechin, can

reduce the oxidation of LDL cholesterol, a critical process involved in the development of atherosclerosis and plaque formation in arteries.

Studies have shown that cocoa products can reduce lipid peroxidation in both the liver and bloodstream, leading to lower levels of malondialdehyde, a marker of oxidative stress. The catechins found in dark chocolate—measured at approximately 12 mg per 100 g—and epicatechin (about 41.5 mg per 100 g) are associated with various physiological benefits. These include increased antioxidant activity in plasma, enhanced dilation of bronchial arteries, improved fat metabolism, and resistance to LDL oxidation, all of which contribute to cardiovascular and metabolic health. [29, 30, 31]

**1.7.5 *Phoenix dactylifera* (Date Palm)**

**Synonym:** Khajoor

**Family:** Arecaceae

**Figure 5: Dates**

**1.7.5.1 Nutritional Importance and Potential as a Natural Sweetener:** Excessive consumption of added sugars in processed foods is a significant contributor to poor dental health and an increased risk of chronic diseases such as obesity, type 2 diabetes, cardiovascular disorders, and metabolic syndrome. In this context, there is growing interest in identifying nutritious, natural alternatives to synthetic sweeteners and refined sugars. One promising candidate is the date fruit, which serves as a nutrient-dense sweetener with a favorable health profile.

**1.7.5.2 Sugar Composition:** Date pulp is composed predominantly of naturally occurring sugars that are easy to digest, including glucose, fructose, mannose, maltose, and sucrose - together accounting for over 80% of its dry weight. These sugars provide energy without the adverse health effects commonly associated with artificial or refined sweeteners. Compared to non-nutritive sweeteners (NNS) such as saccharin, aspartame, and sucralose, or even natural sugar substitutes like stevia and monk fruit, dates offer a balanced nutritional profile with essential macro- and micronutrients.

**1.7.5.3 Nutritional Profile:** Dates are highly nutritious, comprising mainly carbohydrates and dietary fiber, as well as significant levels of potassium, other minerals, vitamins, and various bioactive compounds. These include antioxidant phenolics, carotenoids, and to a lesser extent, lipids and proteins. The rich presence of these nutrients supports their use not only as a food ingredient but also as a functional dietary component with therapeutic potential.

**1.7.5.4 Bioactive Compounds and Health Benefits:** Both date pulp and seeds are abundant in biologically active molecules, with their composition varying across different cultivars. These include polyphenols - mainly flavonoids, as well as carotenoids and phytosterols. Such phytochemicals are responsible for the antioxidant, anti-diabetic, anti-obesity, liver-protective, neuroprotective, and anti-lipidemic effects observed with regular date consumption. Their presence enhances the nutritional and medicinal value of dates.

**1.7.5.5 Applications as a Functional Sweetener:** Dates and their derivatives-such as date syrup, paste, spreads, juices, and liquid sweeteners - not only serve as natural sweetening agents but also supply

vitamins, minerals, and antioxidants, making them vastly superior to refined sugar, which lacks nutritional value ("empty calories"). Moreover, dates offer additional benefits such as functioning as flavouring and colouring agents, further broadening their application in food product formulation. Given their comprehensive nutritional and therapeutic benefits, dates represent a promising natural substitute for refined sugar, offering both sweetness and functional health advantages when included in modern diets. [32]

### 1.7.6 Honey as a Natural Sweetener

**Figure 6: Honey**

Honey is a naturally derived sweet, viscous substance produced by honeybees through the enzymatic conversion of floral nectar. Its sensory qualities—flavour, aroma, and colour—vary according to botanical source and geography, ranging from pale yellow to dark amber, which influences consumer preference. India produces nearly 60,000 tonnes of honey annually, emphasizing its cultural and economic importance. [33] Compositionally, honey consists primarily of fructose and glucose (70–80%), along with water (10–20%) and minor constituents such as organic acids, amino acids, phenolics, vitamins, minerals, enzymes, and proteins. These bioactive compounds contribute to its nutritional value and therapeutic effects. Traditionally used as a natural sweetener, honey is widely incorporated into culinary applications for its pleasant taste and smooth texture. Its increasing use in food and beverage industries is driven by consumer demand for natural, clean-label alternatives. Honey's antioxidant, antimicrobial, and anti-inflammatory properties enhance its functionality in products such as confectionery, baked goods, beverages, spreads, and health-oriented formulations. The presence of polyphenols, peptides, and enzymes contributes to its antioxidant capacity, supporting its role in reducing oxidative stress and improving overall health. [33]

### 1.7.7 *Foeniculum vulgare Mill.* (Fennel)

**Synonym:** Saunf, Variyali

**Family:** Umbelliferae

**Figure 7: Fennel**

**1.7.7.1 Phytochemical Composition:** catechins, tocopherols, and flavonoids. When combined with phenolic antioxidants, organic acids, carotenoids, protein hydrolysates, and tannins Fennel seeds contain essential oils, including trans-anethole (TA), estragole, and fenchone.

1.**7.7.2 Pharmacological Properties:** fennel seeds has a number of pharmacological properties, including antioxidant, anti-inflammatory, antibacterial, antifungal, antiparasitic, antidiabetic, cardio protective, and hepatoprotective, antihyperlipidemic, anticancer, and estrogenic. [29, 30]

**1.7.7.3** ***Foeniculum vulgare***, commonly known as fennel, is cultivated extensively worldwide owing to its rich content of essential oils and its multifaceted applications in traditional medicine and various industries. The seeds are characterized by a naturally sweet and aromatic nature, largely due to the presence of key volatile compounds such as trans-anethole, fenchone, limonene, camphene, and α-pinene. These constituents contribute to fennel's distinctive anise-like scent and flavour, making it a valuable natural additive in a variety of food and beverage products. Historically, fennel has been employed as a flavour enhancer in items such as baked goods, meats, seafood, and alcoholic and non-alcoholic beverages. Recent developments in food technology have explored its use in innovative chocolate formulations, where the herb's unique aromatic compounds enhance the overall sensory profile. In addition to its flavouring potential, fennel essential oil may also provide functional properties due to the presence of bioactive molecules. As a result, fennel is gaining recognition as a functional flavouring agent in the confectionery sector, particularly in the development of value-added chocolate products. [34, 35]

## 2. MATERIALS AND METHODS

### 2.1 Raw Materials

Dark chocolate, Ashwagandha, cinnamon, flaxseed, fennel, honey, dates, and other ingredients used in the formulation were procured from local markets near the School of Pharmacy, ITM SLS Baroda University, Vadodara, and Gujarat, India.

### 2.2 Chemicals and Reagents

Analytical-grade solvents and reagents—including methanol, ethanol, chloroform, n-hexane, Dragendorff's, Hager's, Mayer's, Wagner's reagents, ferric chloride, ammonium solution, concentrated $H_2SO_4$ and HCl, acetic anhydride, gallic acid, Schiff's reagent, Tollen's reagent, bromine water, and potassium permanganate—were used for extraction and phytochemical screening.

### 2.3 Soxhlet Extraction:

Soxhlet extraction was employed to isolate bioactive compounds from plant materials. In this technique, powdered samples placed in a thimble undergo repeated solvent percolation as vapours condense and siphon back into the flask, enabling efficient extraction over 6–48 hours at controlled temperatures (60–100 °C). [36]

**2.3.1 Soxhlet Extraction of cinnamon for Cinnamaldehyde Isolation:** Soxhlet extraction is Cinnamon bark was subjected to Soxhlet extraction using solvents such as ethanol, methanol, acetone, hexane, and petroleum ether. Polar solvents yielded extracts enriched with flavonoids, alkaloids, saponins, tannins, and terpenoids, whereas non-polar solvents produced higher cinnamaldehyde content. For example, extraction of 100 g dried bark with 95% ethanol for six hours produced a 5.2%

yield, with trans-cinnamaldehyde identified as the major constituent. Hexane showed the highest recovery of cinnamaldehyde, reflecting its non-polar characteristics. [26]

**2.3.2 Extraction of *Withania somnifera* (Ashwagandha):** Root and leaf samples of Ashwagandha were initially defatted using n-hexane to remove waxes and lipids. The defatted material was then extracted with 70–90% methanol at approximately 35 °C. The crude extract underwent liquid–liquid partitioning with chloroform, n-butanol, and water to obtain fractions of varying polarity. The aqueous methanol fraction demonstrated the highest concentration of withanolides—the principal bioactive metabolites of Ashwagandha [37]

**2.4 Phytochemical Screening**

Qualitative phytochemical screening was performed to detect the presence of major bioactive compounds in the plant extracts using standard procedures. The methods, visual indicators of positive results, and literature references are summarized below:

**Table 1: Phytochemical Screening of Herbal Plants**

| Phytochemical Group | Test & Procedure | Positive Indication | Reference |
|---|---|---|---|
| **Alkaloids** | **Dragendorff's/Kraut's Test**: A small volume of filtrate was treated with 1–2 mL of Dragendorff's reagent. | Formation of a reddish-brown precipitate indicates alkaloids. | [38] |
| | **Hager's Test**: 1–2 mL of Hager's reagent was added to the filtrate. | A creamy white precipitate confirms alkaloid presence. | [38] |
| | **Mayer's Test**: A few drops of Mayer's reagent were gently added along the test tube wall containing filtrate. | A white or pale yellow precipitate indicates a positive result. | [38] |
| | **Wagner's Test**: The filtrate was mixed with 1–2 drops of Wagner's reagent. | Appearance of a brown or reddish precipitate suggests alkaloids. | [38] |
| **Phenols & Tannins** | **Ferric Chloride Test**: 4 mL of plant extract was combined with 2 mL of 0.2% $FeCl_3$ solution. | Development of green or blue coloration indicates the presence of phenolic compounds and tannins. | [39] |
| **Flavonoids** | **Alkaline Reagent Test**: To 4 mL of extract, 2 mL of ammonium solution and 0.4 mL of $H_2SO_4$ were added sequentially. | Formation of a yellow colour suggests the presence of flavonoids. | [39] |
| | **Shinoda's Test**: The extract was dissolved in 5 mL alcohol, with magnesium ribbon fragments and a few drops of concentrated HCl added. | A pink to crimson coloration indicates flavonoids. | [38] |
| **Saponins** | **Foam Test**: 4 mL of methanolic extract was shaken vigorously for two minutes. | A stable foam persisting for at least 10 minutes confirms the presence of saponins. | [39] |
| **Steroids** | **Salkowski's Test**: Chloroform was added to the extract, followed by concentrated sulphuric acid. | A reddish-brown ring at the interface indicates steroids. | [38] |
| | **Liebermann–Burchard Test:** The extract is treated with acetic anhydride, and concentrated sulphuric acid is added along the test tube wall. | A series of colour changes indicates the presence of sterols or triterpenoids | [38] |

| | | | |
|---|---|---|---|
| **Glycosides** | **Keller–Kiliani Test**: Glacial acetic acid and $FeCl_3$ were added to the extract, followed by sulphuric acid. | Formation of a reddish-brown ring signifies the presence of glycosides. | [38] |
| **Lignans** | **Labat Test**: The extract was treated with gallic acid solution. | An olive green coloration indicates lignans. | [38] |
| | **Furfur aldehyde Test**: The extract was mixed with 2% furfur aldehyde solution. | A red coloration confirms the presence of lignans. | [38] |
| **Aldehyde** | **Schiff's Test:** Add a few drops of Schiff's reagent to the sample and allow it to stand at room temperature. | Magenta or pink coloration indicates the presence of a free aldehyde group (CHO) in cinnamaldehyde | [40] |
| | **Tollen's Test:** Add freshly prepared Tollen's' reagent ($AgNO_3$ + $NH_3$) to the sample and warm in a water bath for 3–5 minutes. | Silver mirror forms on the test tube, confirming the presence of an aldehyde group in cinnamaldehyde. | [40] |
| **Alpha-Linolenic Acid (ALA)** | **Bromine Water Test:** Add bromine water drop wise to the sample in chloroform. Shake gently and observe discoloration. | Brown colour fades due to the reaction of bromine with C=C bonds, confirming the presence of unsaturation in ALA. | [40] |
| | **Baeyer's Test:** Add dilute alkaline potassium permanganate ($KMnO_4$) to the sample. Shake and observe colour change and precipitate formation. | Purple colour disappears, and a brown $MnO_2$ precipitate forms, confirming the presence of unsaturation in ALA | [41] |

## 2.5 LIST OF MATERIALS USED

**Table 2: List of Materials Used**

| Material | Component | Intended Purpose |
|---|---|---|
| **Plant-Derived Components** | Ashwagandha (Root and Leaves) | Incorporated for their therapeutic and pharmacological properties |
| | Cinnamon (Bark) | |
| | Flaxseed (Seed) | |
| **Additional Ingredients** | Dark Chocolate | Served as the base matrix for the herbal chocolate formulation |
| | Date or Honey | Functioned as natural sweeteners to counterbalance the bitterness of dark chocolate |
| | Fennel | Employed to enhance the flavor profile of the final product |
| | Milk | Used as a dispersing agent or solvent in the chocolate preparation process |
| | Coconut Oil & Vitamin E | Serve as natural agents that help preserve product stability and extend shelf life |

### 2.6 Key Bioactive Compounds Contributing to Therapeutic Effects

**Table 3: List of Bioactive Compounds**

| Sr. No. | Ingredient | Principal Bioactive Compound Responsible for Therapeutic Action |
|---|---|---|
| 1 | Dark Chocolate | Flavonoids, primarily Quercetin |
| 2 | Ashwagandha | Withanolide A and Withanolide D |
| 3 | Cinnamon | Cinnamaldehyde |
| 4 | Flax Seed | Alpha-Linolenic Acid (ALA) |

### 2.7 Preparation Process of Herbal Chocolate Batches [42]

**General Flow for Herbal Chocolate Preparation**

↓

Accurate Measurement of Ingredients

↓

Melting or mixing of Dark Chocolate Powder

(50 g chocolate + sufficient milk in double boiler)

↓

Continuous Stirring until Smooth Consistency

↓

Addition of Herbal Components:

Ashwagandha Extract, Cinnamon Extract, Flaxseed Powder,

and a small amount of Fennel Powder

↓

Thorough Mixing for Uniform distribution of ingredients

↓

Addition of Natural Sweetener:

↓

Incorporation of Preservatives:

Vitamin E and Coconut Oil

↓

Pouring into Chocolate Moulds

(Gently tapping to release trapped air)

↓

Allow to cool and solidify for several hours

(Refrigeration for Proper Setting)

↓

De-moulding and Final Packaging

↓

Store in airtight containers for preservation

**Ingredient Composition Used in Different Formulations of Herbal Chocolate**

**Table 4: Ingredient Composition Used in Different Formulations of Herbal Chocolate**

| Sr. No. | Ingredients | Formulation Batch 1 | Formulation Batch 2 | Formulation Batch 3 |
|---|---|---|---|---|
| 1 | Dark Chocolate | 50 g | 50 g | 50 g |
| 2 | Ashwagandha Extract | 2.5 g | 2.5 g | 2.5 g |
| 3 | Cinnamon Extract | 2 g | 2 g | 2 g |
| 4 | Flaxseed Powder | 5 g | 5 g | 5 g |
| 5 | Honey | 15 mL | – | 20 mL |
| 6 | Dates | – | 25 g | 35 g |
| 7 | Fennel Powder | Quantity sufficient (Qs) | Quantity sufficient (Qs) | Quantity sufficient (Qs) |
| 8 | Milk | Quantity sufficient (Qs) | Quantity sufficient (Qs) | Quantity sufficient (Qs) |
| 9 | Coconut Oil & Vitamin E | Quantity sufficient (Qs) | Quantity sufficient (Qs) | Quantity sufficient (Qs) |

**Note:** *"Qs" denotes the quantity sufficient to achieve the desired consistency during formulation.*

**2.8 Medicated Chocolate Formulations: Evaluation and Characteristics**

Medicated chocolate is increasingly being explored as a novel delivery system to improve patient compliance and address specific health concerns. In this study, several quality parameters of medicated chocolate were assessed, including sensory attributes, pH, and blooming behaviour. Additionally, a stability study was performed to monitor changes in the chocolate's physical characteristics over time. [31]

**2.8.1 Sensory Evaluation of Chocolate**

The sensory characteristics of the developed chocolate formulation were assessed based on organoleptic parameters such as colour, aroma, flavour, texture (mouth feel), and visual appeal. These evaluations were conducted using human sensory perception to determine the product's overall acceptability.

**2.8.2 pH Determination:** To measure the pH, 2 grams of the formulated chocolate sample were dissolved in 100 mL of phosphate buffer solution. The pH of this solution was then determined using a digital pH meter equipped with a glass electrode. [31]

**2.8.3 Blooming Assessment**

**Fat Bloom:** Fat bloom was identified by the presence of a dull, whitish coating on the chocolate's surface, resulting from fat crystal migration or recrystallization. This phenomenon typically diminishes the glossiness and alters the texture of the chocolate. Fat bloom can be delayed through storage under stable temperature conditions, which reduces the rate of fat migration or phase transitions.

**Sugar Bloom:** Sugar bloom was recognized as a rough, grainy layer forming on the surface due to condensation. This typically occurs when chocolate is removed from refrigeration, causing surface moisture to dissolve sugars. As the water evaporates, the sugars recrystallize, leading to an uneven and undesirable texture.

**Bloom Testing Protocol:**

Chocolate samples underwent a cycling treatment protocol consisting of:

- **Step 1:** Incubation at 30°C for 11 hours
- **Step 2:** Temperature transition for 1 hour
- **Step 3:** Storage at 18°C for 11 hours
- **Step 4:** Another 1-hour temperature shift

Following the third stage, samples were evaluated for any signs of blooming.

**2.8.4 Stability Studies**

Stability of the chocolate formulation was evaluated under two controlled storage conditions:

- **Condition A:** 25°C with 75% relative humidity (RH)
- **Condition B:** Refrigerated storage at 2–8°C

The samples were stored for a duration of one month, sealed in aluminium foil to maintain quality. Throughout the storage period, organoleptic attributes-such as colour, aroma, taste, mouth feel, and appearance-were periodically monitored to assess the product's physical and sensory stability. [31]

## 3. RESULT AND DISCUSSION

### 3.1 Phytochemical Screening:

**Table 5: Result of Phytochemical screening**

| Test | Ashwagandha | Cinnamon | Flax seed |
|---|---|---|---|
| **Alkaloid** | | | |
| Dragendorff's | + | - | - |
| Wagner | + | - | - |
| Hager | - | - | - |
| Mayer | + | - | - |
| **Saponins** | | | |
| Foam test | + | - | + |
| **Phenolic and Tannins compounds** | | | |
| Ferric Chloride Test | + | + | + |
| **Flavonoids** | | | |
| Shinoda Test | + | + | + |
| Alkaline Reagent Test | + | + | + |
| **Steroids/Triterpenoid** | | | |
| Liebermann–Burchard Test | + | - | + |

| | | | |
|---|---|---|---|
| Salkowski's Test | + | - | + |
| **Glycosides** | | | |
| Keller–Kiliani Test | + | - | - |
| **Lignans** | | | |
| Labat test | - | - | + |
| Furfur aldehyde test | - | - | + |
| **Aldehyde** | | | |
| Schiff's Test | - | + | - |
| Tollen's Test | - | + | - |
| **ALPHA-LINOLENIC ACID (ALA)** | | | |
| Bromine Water Test | - | - | + |
| Baeyer's Test | - | - | + |

### 3.2 Sensory Evaluation and Comparative Analysis of Formulations

**Table 6: Comparative Sensory Characteristics of Chocolate Formulations**

| Parameter | Formulation Batch 1 | Formulation Batch 2 *(Best)* | Formulation Batch 3 |
|---|---|---|---|
| Colour | Brownish | Brownish | Brownish |
| Odour | Mild chocolaty | Chocolaty | Chocolaty |
| Taste | Bitter due to lack of sufficient natural sweetener | Mildly bitter with a pleasant, well-balanced flavour | Sweet, but slightly overpowering and unbalanced flavour |
| Mouth feel | Not smooth; slightly coarse | Smooth texture with a pleasing sensation | Dense and less smooth due to combination of sweeteners |
| Appearance | Slightly dull surface | Shiny and visually attractive surface | Shiny surface but heavier consistency |
| Overall Acceptability | Low (due to bitterness and texture) | High (best result) | Moderate (texture compromised) |

Formulation Batch 2 demonstrated superior sensory properties compared to Batches 1 and 3. The exclusive use of dates (25 g) as a natural sweetener provided a balanced flavour and contributed to a smooth mouth feel without overpowering sweetness. In contrast, Batch 1, which used only honey, failed to adequately mask the bitterness of functional ingredients. Batch 3, combining both dates and honey, did not achieve the desired texture, likely due to moisture imbalance and heavier consistency. Therefore, Batch 2 was identified as the best formulation in terms of overall sensory acceptability. Its balanced taste, smooth mouth feel, and visually appealing appearance make it a promising functional chocolate formulation for consumer use.

### 3.4 pH Analysis of Chocolate Formulations

- **Formulation Batch 1:** pH 5.0
- **Formulation Batch 2:** pH 6.5
- **Formulation Batch 3:** Higher than 6.5 (exact value), indicating increased alkalinity

The ideal pH for chocolate products typically ranges from 6 to 7.5, which balances flavour, texture, and microbial stability. Batch 2, with a pH of 6.5, falls within the optimal range, contributing to better taste, mouth feel, and shelf stability. In contrast, Batch 1, with a more acidic pH of 5.0, may exhibit increased bitterness and less smooth texture, which was consistent with sensory observations. Batch 3, exhibiting a higher pH beyond the ideal range, may face issues related to improper setting, altered flavour profiles, and potential shelf-life concerns.

**3.5 Blooming Test of Chocolate Formulation**

**Table 7: Blooming Test Results of Chocolate Formulations**

| Formulation Batch | Fat Blooming | Sugar Blooming | Additional Observations | Overall Stability |
|---|---|---|---|---|
| Batch 1 | Melting observed (unstable) | Absent | Likely due to honey affecting fat crystallization | Poor stability, melting issue |
| Batch 2 | Absent | Absent | Stable fat matrix, no defects detected | Good stability, no blooming |
| Batch 3 | Absent | Present | Sugar crystallization likely due to moisture content | Moderate stability, sugar bloom present |

The blooming test revealed distinct differences among the three formulations. Batch 1 showed melting during the test, indicating fat instability likely caused by the presence of honey, which adversely affected fat crystallization. Batch 3 exhibited sugar blooming, visible as a whitish surface, likely due to higher moisture from combining honey and dates, which promotes sugar crystallization. Batch 2 demonstrated excellent stability with no signs of either fat or sugar blooming, attributed to the use of dates as sweetener and an optimized formulation that maintained the integrity of the fat matrix preventing crystallization defects. This batch maintained its glossy appearance and smooth texture throughout the test period.

These results indicate that Formulation Batch 2 is superior in terms of physical stability and quality, making it the most promising formulation for shelf-stable functional chocolate products.

**3.6 Stability Evaluation of Chocolate Formulations under Different Storage Conditions**

**Table 8: Stability of Chocolate Formulations under Different Storage Conditions**

| Formulation Batch | Condition A (25°C, 75% RH) | Condition B (2–8°C) | Overall Stability Assessment |
|---|---|---|---|
| **Batch 1** | Melted; | Partially stable; minor texture degradation | **Poor** |

| | | | |
|---|---|---|---|
| | phase separation observed | | Unstable at ambient, weak refrigerated stability |
| **Batch 2** | Stable; retained sensory and physical quality | Stable; no visible or sensory changes | **Good** Most stable under both conditions |
| **Batch 3** | Texture degradation; sugar blooming observed | Surface dullness; slight flavour changes | **Moderate to Poor** Inconsistent and unstable |

The stability study demonstrated that Formulation Batch 2 maintained its organoleptic and physical properties throughout the one-month storage period under both ambient (25°C, 75% RH) and refrigerated (2–8°C) conditions. In contrast, Batch 1 showed significant instability at ambient temperature, melting and undergoing phase separation, while Batch 3**,** which combined both honey and a higher quantity of dates, showed sugar blooming and texture changes under ambient conditions and loss of surface gloss and altered taste under refrigeration. These changes suggest a moisture imbalance and incompatibility of dual sweeteners for long-term stability. These results confirm that Batch 2 is the most suitable formulation in terms of storage stability for functional chocolate products.


Figure 8:
Dark Chocolate Powder used for Herbal Chocolate Formulation

Figure 9:
Packaging Material used for Herbal Chocolate Formulation

Figure 10:
Preparation of Herbal Chocolate Formulation

Figure 11:
Preparation of Herbal Chocolate Formulation

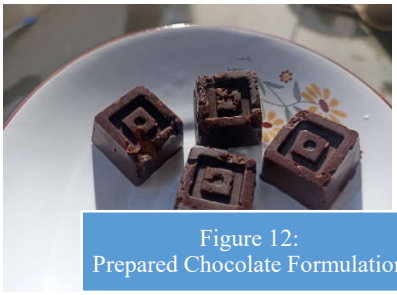

Figure 12:
Prepared Chocolate Formulation

Figure 13:
Prepared Chocolate Formulation

Figure 14:
Best Batch (3) of all Formulated Herbal Chocolate

Figure 15:
Packaging of Prepared Formulated Herbal Chocolate (Batch 3)

Figure No. 16
Final lebeled Product of Formulated Herbal Chocolate (Batch 3)

**CONCLUSION:**

This study successfully developed an herbal-infused dark chocolate formulation targeting cholesterol management and immune enhancement by incorporating bioactive plants-flaxseed, ashwagandha, and cinnamon-along with fennel, milk, and natural sweeteners. Phytochemical screening confirmed the presence of key active compounds in the ingredients: flavonoids (primarily quercetin) in dark chocolate, withanolide A and D in ashwagandha, cinnamaldehyde in cinnamon, and alpha-linolenic acid (ALA) in flaxseed. Three chocolate batches were prepared using honey (Batch 1), dates (Batch 2), and a combination of honey and dates (Batch 3) as sweeteners. Comprehensive sensory evaluation considering colour, odour, taste, mouth feel, and appearance revealed that Batch 2 (date-sweetened) achieved the highest overall acceptability. Physicochemical assessments revealed that Batch 2 maintained optimal pH, showed no fat and sugar blooming, indicative of better product stability. Stability studies under Condition A (25°C, 75% RH) and Condition B (2–8°C) further confirmed that Batch 2 retained its quality attributes more effectively than the other formulations. In conclusion, the date-sweetened herbal dark chocolate offers a promising functional confectionery with enhanced sensory appeal, stability, and potential health benefits related to cholesterol management and immune support. This formulation shows strong potential as a functional, plant-based confectionery product for health-oriented consumers.

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**List of Abbreviations**

- **CVDs** – Cardiovascular Diseases
- **GBD** - Global Burden of Disease
- **DALYs** - Disability-Adjusted Life Years
- **COVID-19** - Coronavirus Disease 2019
- **oxLDL** - Oxidized Low-Density Lipoprotein
- **TLRs** - Toll-Like Receptors
- **NLRP3** - NOD-Like Receptor Family Pyrin Domain Containing 3
- **IL** - Interleukin
- **HSPCs** - Hematopoietic Stem and Progenitor Cells
- **SREBP2** - Sterol Regulatory Element-Binding Protein 2
- **SLE** - Systemic Lupus Erythematosus
- **dsDNA** - Double-Stranded DNA
- **NK Cells** - Natural Killer Cells
- **Th1/Th2** -T Helper 1 / T Helper 2 Cells
- **PCSK9** - Proprotein Convertase Subtilisin/Kexin Type 9
- **siRNA** - Small Interfering Ribonucleic Acid
- **JMJD3** - Jumonji Domain-Containing Protein 3
- **TLR4–MyD88–NF-κB** - Toll-Like Receptor 4–Myeloid Differentiation Primary Response 88–Nuclear Factor Kappa B
- **MHC II** - Major Histocompatibility Complex Class II
- **Tregs** - Regulatory T Cells
- **LDL-C** -Low-Density Lipoprotein Cholesterol
- **HDL-C** - High-Density Lipoprotein Cholesterol
- **ALA** - Alpha-Linolenic Acid
- **DHA** - Docosahexaenoic Acid
- **EPA** - Eicosapentaenoic Acid
- **IFN-γ** - Interferon-Gamma
- **NNS** - Non-Nutritive Sweeteners
- **TA** - Trans-Anethole
- **RH** - Relative Humidity
- **pH** - Potential of Hydrogen
- **Qs** - Quantity Sufficient
- **NPC1L1** - Niemann-Pick C1-Like 1 (cholesterol transporter)
- **ApoB-48** - Apolipoprotein B-48
- **EtOH** – Ethanol, **MeOH** – Methanol, **n-Hex** - n-Hexane

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