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# Comparative Phytochemical Profiling and in Vitro Biological Activities of Solvent Extracts of *Withania coagulans* Leaves

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**Abstract:** *Withania coagulans* (Stocks) Dunal is a medicinal plant widely recognized for its diverse pharmacological properties, particularly its antidiabetic, antioxidant, and antimicrobial activities. However, limited comparative information is available regarding the influence of extraction solvent polarity on the phytochemical composition and biological activities of its leaf extracts. Therefore, this study comparatively evaluated the phytochemical profile, antioxidant capacity, α-amylase inhibitory activity, and antimicrobial potential of *W. coagulans* leaf extracts prepared using n-hexane, ethyl acetate, ethanol, methanol, and distilled water. The biological activities of the extracts were strongly influenced by solvent polarity. Ethanol extract exhibited the highest antioxidant activity, whereas methanol and distilled water extracts demonstrated superior α-amylase inhibitory activity. Antibacterial screening also indicated comparatively greater inhibitory effects for the polar extracts against the tested gram-negative bacterial strains. Qualitative high-performance liquid chromatography profiling further revealed distinct solvent-dependent variations in phytochemical composition, indicating selective enrichment of bioactive constituents according to solvent polarity. The observed differences in biological activity suggest that polar solvents facilitate the extraction of phytochemicals associated with antioxidant and α-amylase inhibitory properties. Overall, these findings demonstrate that solvent selection plays a critical role in determining the phytochemical composition and in vitro biological activities of *W. coagulans* leaf extracts. The results provide a useful foundation for future studies involving quantitative phytochemical characterization, concentration-dependent biological evaluation, mechanistic investigations, in vivo validation, and the development of phytochemical-based nanoformulations to improve bioavailability and therapeutic efficacy.

**Keywords:** diabetes mellitus, antioxidant activity, alpha-amylase inhibition, antidiabetic potential, herbal medicine

## 1. Introduction

Diabetes mellitus is one of the most prevalent chronic metabolic disorders worldwide and continues to pose a major public health challenge due to its rapidly increasing incidence and long-term complications [1]. Characterized by persistent hyperglycemia resulting from impaired insulin secretion, insulin resistance, or both, diabetes is associated with microvascular and macrovascular complications, including nephropathy, neuropathy, retinopathy, and cardiovascular disease [2]. According to the International Diabetes Federation, hundreds of millions of adults are currently living with diabetes, and the global disease burden is expected to increase substantially over the coming decades owing to population aging, urbanization, sedentary lifestyles, and dietary changes [3–6]. Although several pharmacological agents are available for glycemic control, their long-term use is frequently associated with adverse effects, reduced therapeutic efficacy, high treatment costs, and poor patient compliance. Consequently, there is growing interest in identifying safer, cost-effective, and naturally derived bioactive compounds that can complement conventional therapies and contribute to the prevention and management of diabetes and its associated complications [7].

Oxidative stress plays a central role in the onset and progression of diabetes by promoting pancreatic β-cell dysfunction, chronic inflammation, insulin resistance, and vascular injury through excessive production of reactive oxygen species (ROS) [8]. Therefore, phytochemicals possessing both antioxidant and antihyperglycemic properties have attracted considerable attention as potential therapeutic candidates. Plant-derived secondary metabolites, including phenolic acids, flavonoids, alkaloids, terpenoids, glycosides, tannins, and steroidal lactones, have been

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reported to modulate multiple biochemical pathways involved in glucose homeostasis [9]. One important therapeutic strategy involves inhibition of carbohydrate-hydrolyzing enzymes such as α-amylase, which delays starch digestion and reduces postprandial glucose absorption [10]. Simultaneously, antioxidant phytochemicals help neutralize ROS and protect pancreatic tissues from oxidative damage, thereby addressing two important pathological processes associated with diabetes. Because the biological activity of medicinal plants largely depends on the phytochemical composition of their extracts, selecting an appropriate extraction solvent is essential for maximizing the recovery of bioactive constituents and improving their pharmacological potential [11].

*Withania coagulans* (Stocks) Dunal, a member of the family Solanaceae, is an important medicinal plant widely distributed in South Asia and the Middle East, where it has been extensively used in traditional medicine for the management of diabetes, liver disorders, inflammatory diseases, respiratory ailments, and microbial infections [12]. In recent years, the plant has attracted considerable scientific interest owing to its rich phytochemical composition and broad spectrum of pharmacological activities [13]. Phytochemical investigations have identified numerous bioactive constituents, including withanolides, withaferin analogues, alkaloids, flavonoids, phenolic acids, tannins, glycosides, saponins, and steroidal lactones, many of which exhibit antioxidant, anti-inflammatory, antimicrobial, antihyperglycemic, and cytoprotective properties [14]. Among these compounds, withanolides are regarded as the principal bioactive metabolites responsible for many of the reported therapeutic effects through their ability to modulate oxidative stress, inflammatory signaling pathways, and glucose metabolism [15]. Recent studies have demonstrated that extracts and isolated phytochemicals from *W. coagulans* possess significant free radical scavenging activity, inhibit carbohydrate-hydrolyzing enzymes such as α-amylase and α-glucosidase, and improve glucose homeostasis in experimental diabetic models [16]. Furthermore, several investigations have reported antimicrobial activity against both gram-positive and gram-negative bacterial species, suggesting that the plant contains multiple classes of bioactive compounds capable of exerting complementary biological effects [17]. Collectively, these findings support the growing recognition of *W. coagulans* as a promising source of multifunctional phytochemicals with potential applications in the prevention and management of diabetes and its associated oxidative stress-related complications [18].

As diabetes is emerging as a large-scale medical problem in the 21st century, the quest for better therapeutic solutions has been focused [19–21]. The prevalence of diabetes in the adult population (20–69 years) was estimated at 7.7% in the world in 2011 and is expected to rise to around 9% by 2030 [22]. But the impact of diabetes is different from one region to another. Prevalence is expected to be 4.1% in Africa, 8% in Europe, 11.8% in North America and the Caribbean, 9.4% in South and Central America, and 10% in Southeast Asia by 2030 [23]. These prevalence estimates are based on projected numbers of people with diabetes in the age group of 20–79 years of 28 million in Africa, 64.6 million in Europe, 51.2 million in North America and the Caribbean, 39.9 million in South and Central America, and 120.9 million in Southeast Asia, which equates to an estimated total of approximately 551.8 million people with diabetes by 2030 [24]. This projection is in line with other data showing that in 2017, there were 415 million people in the world who were affected by diabetes, and the number is projected to increase to 642 million by 2040 [25]. The growing prevalence is expected to disproportionately impact low- and middle-income countries, where access to healthcare resources and infrastructure can be constrained and challenging to prevent and manage effectively [26].

Diabetes mellitus is a metabolic disorder of carbohydrate, protein, and lipid metabolism, which is manifested by chronic hyperglycemia [27]. In about 10% of cases, type 1 diabetes (T1D) is caused by absolute insulin deficiency due to progressive autoimmune destruction of insulin-producing β-cells. Most people, however, develop type 2 diabetes (T2D), in which peripheral insulin resistance, which is most commonly associated with obesity, is associated with a decrease in the amount of insulin secreted by the pancreas (or β-cells) to provide for the increased metabolic demand [28]. T2D is a progressive disease with progressive but varying degrees of β-cell dysfunction and loss, which occurs due to glucotoxicity and lipotoxicity, and eventually, insulin therapy is necessary for many patients [29–31].

Diabetes mellitus is a significant burden of disease all over the world as its chronic macrovascular and microvascular complications contribute to high morbidity and mortality rates [32–35]. Keeping the blood glucose in the normal physiological range is, therefore, an important measure of glycemic control in order to slow the rate of progression of complications, especially in T2D, but many people do not reach optimal control [36, 37]. In addition to insulin for T1D and a number of drugs for T2D, there remains a need for the development of novel antidiabetic agents due to the current limitations of therapies [38–40]. From this perspective, the use of natural products has become desired because of their chemical diversity, traditional use, and the possibility of using them as an alternative source of conventional medicines, particularly in low-resource areas where access to conventional medicines may be restricted [40–44]. Concurrently, the worldwide incidence of diabetes has risen sharply, and this need has become even greater for new diabetes therapy options [45]. Traditional medicine uses medicinal plants as a source of compounds with antidiabetic properties, which are rich in bioactive phytochemicals and elements that play an important role in metabolic regulation [46]. Numerous in vitro and animal studies have demonstrated antioxidant and hypoglycemic effects of plant extracts that are attributed to the inhibition of carbohydrate-digesting enzymes, including α-amylase and α-glucosidase, which are important components of phytochemicals such as phenolics, flavonoids, tannins, and alkaloids. As of yet, clinical studies in humans are limited, but there is evidence to suggest that medicinal plants can be used as a safe, standardized, and affordable treatment for diabetes, and should be further investigated [47].

The increasing recognition of *W. coagulans* as a valuable medicinal plant has stimulated considerable research into its pharmacological properties; however, comprehensive comparative studies evaluating the influence of extraction solvent polarity on its phytochemical composition and associated biological activities remain limited. Most previous investigations have focused on individual biological properties or specific plant parts, while systematic comparisons of multiple solvent extracts integrating phytochemical profiling with antioxidant, α-amylase inhibitory, and antimicrobial evaluations are scarce. Since the extraction solvent plays a crucial role in determining the type and quantity of phytochemicals recovered, identifying the most effective extraction system is essential for maximizing biological efficacy and supporting the standardization of plant-derived therapeutics. Furthermore, establishing a clear relationship between solvent-dependent phytochemical composition and biological activity may facilitate the future development of standardized herbal formulations and advanced phytochemical-based nanoformulations with improved stability and bioavailability. Therefore, the

present study aimed to comparatively evaluate the phytochemical profile, antioxidant activity, α-amylase inhibitory potential, and antimicrobial activity of *W. coagulans* leaf extracts prepared using solvents of different polarities. We hypothesized that extraction solvent polarity significantly influences the recovery of bioactive phytochemicals, resulting in measurable differences in the biological activities of the respective extracts. The findings of this study are expected to provide valuable insights into the selection of appropriate extraction solvents for obtaining biologically active phytochemicals and contribute to the future development of evidence-based phytopharmaceuticals for diabetes management.

## 2. Experimental Details

Fresh leaves of *W. coagulans* (Stocks) Dunal were collected from District Mardan, Khyber Pakhtunkhwa, Pakistan, during September 2024. The plant material was authenticated by the Department of Botany, University of Mardan, Mardan, Khyber Pakhtunkhwa, Pakistan. The collected leaves were thoroughly washed with distilled water to remove dust and impurities, shade-dried at ambient temperature (25–30 °C) for approximately two weeks until a constant weight was achieved, and subsequently ground into a fine powder using a laboratory grinder. The powdered material was stored in airtight polyethylene containers at room temperature until extraction.

For extraction, 100 g of dried leaf powder was separately macerated in 1000 mL of n-hexane, ethyl acetate, ethanol, methanol, and distilled water in clean glass containers. Each extraction was carried out at room temperature for 72 h with intermittent shaking to facilitate the dissolution of phytochemical constituents. The extracts were filtered through Whatman No. 1 filter paper, and the extraction process was repeated twice to maximize phytochemical recovery. Organic solvent extracts were concentrated under reduced pressure using a rotary evaporator at temperatures below 40 °C, whereas the aqueous extract was concentrated by oven drying at 40 °C until a constant weight was obtained. The dried crude extracts were stored in airtight containers at 4 °C until further analysis.

The extraction yield was determined after complete solvent removal to evaluate the extraction efficiency of each solvent system. The percentage extraction yield was calculated using the following equation (1):

$$\textit{Extraction Yield}\,(\%) = (\textit{Weight of Dried Extract}/\textit{Weight of Dried Plant Material}) \times 100 \quad (1)$$

Based on the experimental procedure, an average of 20 g of dried crude extract was obtained from 100 g of powdered plant material, corresponding to an average extraction yield of 20%.

The extraction yield was determined following complete solvent removal to evaluate the efficiency of the extraction process. Briefly, 100 g of dried powdered *W. coagulans* leaves were extracted separately with n-hexane, ethyl acetate, ethanol, methanol, and distilled water. Each solvent yielded approximately 20 g of dried crude extract, corresponding to an average extraction yield of 20%. The obtained extracts were subsequently stored at 4 °C and used for phytochemical profiling, antioxidant, α-amylase inhibitory, antimicrobial, and high-performance liquid chromatography (HPLC) analyses.

Prior to biological evaluation, each crude extract was dissolved in dimethyl sulfoxide to obtain a final concentration of 15 mg/mL. The prepared extracts were subsequently used for antioxidant, α-amylase inhibitory, antimicrobial, and chromatographic analyses under identical experimental conditions to allow direct comparison among solvent systems.

Antibacterial activity was evaluated against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acetobacter* spp., and *Haemophilus influenzae* using the agar well diffusion method. All biological experiments were performed in triplicate, and the results are presented as mean ± standard deviation (SD). Statistical analysis was carried out using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Differences among solvent extracts were analyzed using one-way analysis of variance followed by Tukey's multiple comparison test, with $p < 0.05$ considered statistically significant.

## 3. Results and Discussions

The present study comparatively evaluated the influence of solvent polarity on the phytochemical composition and biological activities of *W. coagulans* leaf extracts. Since extraction solvent plays a critical role in determining the type and concentration of phytochemicals recovered, differences in antioxidant, α-amylase inhibitory, antimicrobial, and chromatographic characteristics were anticipated. The results are discussed in relation to solvent polarity and compared with previous reports on the pharmacological properties of *W. coagulans* and other medicinal plants.

### 3.1. α-amylase activity

Different solvent extracts from the stem and the leaf parts of *W. coagulans* were tested for their α-amylase inhibitory activity for their antidiabetic relevance. The various extracts of the leaves were obtained using solvents of n-hexane, acetone, chloroform, ethanol, methanol, and distilled water and were tested for their ability to inhibit the enzyme activity of α-amylase. The polarity of the solvents used affected the recovery of enzyme-inhibitory constituents as revealed by the inhibitory response of the solvent fractions. Of particular interest, the methanol and aqueous extracts had relatively higher α-amylase inhibition when compared with the other extracts, which indicates that more polar bioactive compounds may play a major role in the activity observed. This inhibition could slow down the rate of starch digestion and absorption of glucose, which may play a role in controlling postprandial hyperglycemia. Figure 1 shows the differences in the α-amylase inhibitory activity of the various extracts. Additional research is needed to determine the active components and the molecular mechanism(s) by which *W. coagulans* may affect glucose metabolism. Moreover, the α-amylase inhibitory activity observed in *W. coagulans* points to the therapeutic potential of this plant, but the clinical application of bioactive compounds obtained from plants is hindered by their low aqueous solubility, chemical instability, rapid metabolism, and low bioavailability. Nanotechnology-based drug delivery systems such as liposomes, polymeric nanoparticles, solid lipid nanoparticles, and nanoemulsions could be used as potential methods for improving the stability, controlled release, absorption, and therapeutic effectiveness of the active constituents of *W. coagulans*. The use of such nanotechnology formulations may also lead to the delivery of enzyme-inhibitory phytochemicals to the target tissues, leading to better antidiabetic action and less frequent administration. Thus, future research on the nanoencapsulation of these identified bioactive compounds could yield good insight into designing better phytopharmaceutical interventions for diabetes control.

**Figure 1**
***α*-amylase inhibitory activity of *Withania coagulans* leaf extracts prepared using n-hexane, ethyl acetate, ethanol, methanol, and distilled water. Data are presented as percentage inhibition at the tested extract concentration. Values represent the mean ± SD of three independent experiments**

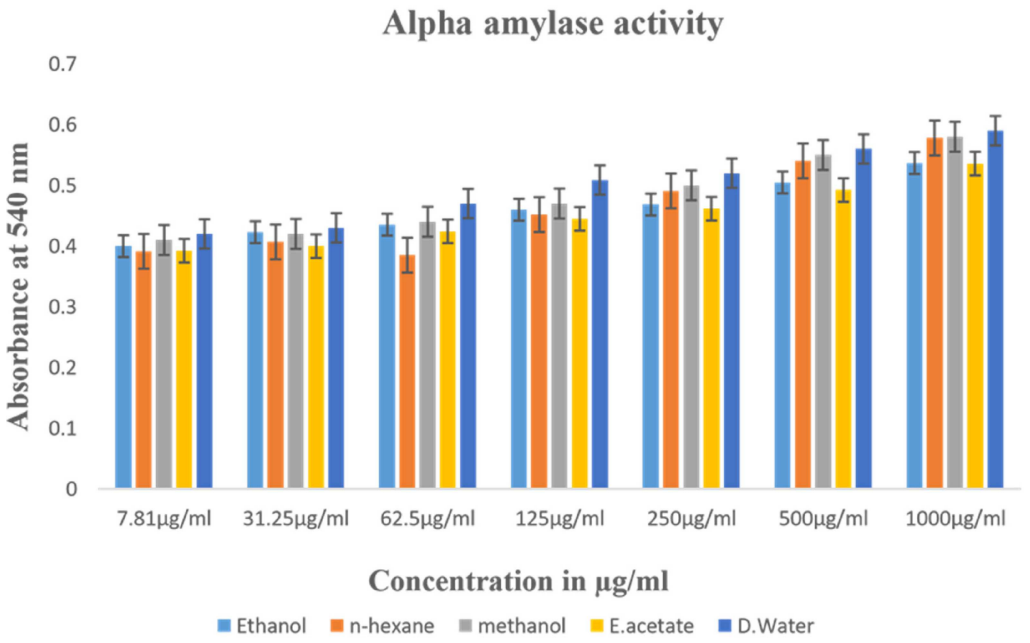



Figure 2 summarizes the overall experimental design that was used to evaluate the biological activities of the extract from the leaves of *W. coagulans*. The observed variation in antioxidant activity among the different solvent extracts can largely be attributed to differences in solvent polarity and the consequent extraction efficiency of bioactive phytochemicals. Polar solvents such as ethanol and methanol are generally more effective in extracting phenolic compounds, flavonoids, and other antioxidant constituents because of their higher solubility in polar media. These phytochemicals are well known for their ability to scavenge ROS, donate hydrogen atoms or electrons, and inhibit oxidative chain reactions, thereby reducing oxidative stress associated with diabetes mellitus. The superior antioxidant activity observed for the ethanol extract is therefore consistent with previous reports demonstrating that solvent polarity significantly influences the recovery of antioxidant phytochemicals from medicinal plants. Nevertheless, further quantitative determination of total phenolic and flavonoid contents is required to establish a direct correlation between phytochemical composition and antioxidant capacity.

The superior antioxidant activity of the ethanol extract is likely attributable to its greater efficiency in extracting phenolic compounds, flavonoids, and other polar antioxidant metabolites. Similar observations have been reported for several medicinal plants, where hydroalcoholic solvents exhibited enhanced extraction of free radical scavenging constituents. Nevertheless, quantitative determination of total phenolic and flavonoid contents would provide a stronger correlation between phytochemical composition and antioxidant capacity and should be considered in future studies.

## 3.2. Antioxidant activity

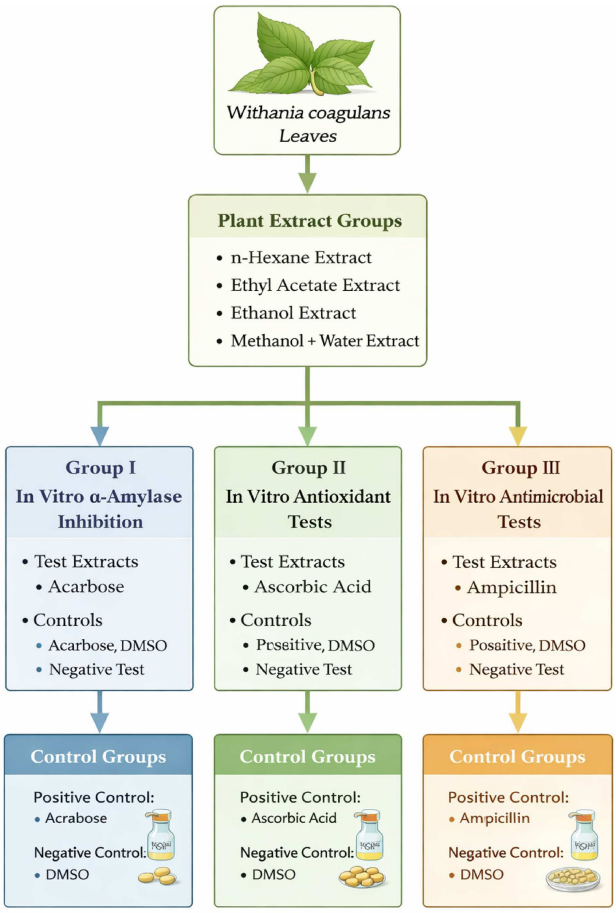
The in vitro antioxidant activity of different solvent extracts of *W. coagulans* leaves, including distilled water, ethanol, ethyl acetate, n-hexane, and methanol, was evaluated and compared with the standard antioxidant, ascorbic acid. The results demonstrated notable variation in antioxidant potential among the extracts, as illustrated in Figure 3. Among all tested extracts, the ethanol extract exhibited the highest antioxidant activity, followed by methanol and ethyl acetate extracts, which showed moderate activity. In contrast, the n-hexane and distilled water extracts displayed relatively low antioxidant activity. When compared with the standard, the antioxidant capacity of the ethanol extract was approximately tenfold higher than that of ascorbic acid. These findings indicate that ethanol is an effective solvent for extracting antioxidant bioactive compounds from *W. coagulans* leaves, likely due to enhanced solubility of phenolic constituents. Future studies may explore nanoencapsulation approaches, such as liposomal or nanoparticle-based formulations, to improve the stability, bioavailability, and therapeutic efficacy of these antioxidant phytochemicals. Furthermore, the observed antioxidant activity is consistent with phytochemical analysis, which revealed higher flavonoid content relative to other bioactive compounds, summarized in Table 1.

The comparatively higher inhibitory activity observed in the methanol and aqueous extracts suggests that solvent polarity influences the extraction of compounds capable of modulating carbohydrate metabolism. However, because the present study employed a single screening concentration, future investigations involving concentration-dependent assays, $IC_{50}$ determination, and enzyme kinetic studies are required to identify the most active phytochemicals and clarify their mechanisms of action.

## 3.3. Antimicrobial activity determination

To further explore the therapeutic potential of *W. coagulans*, antimicrobial activity was evaluated against selected bacterial strains using the agar well diffusion method. Zones of inhibition produced by the solvent extracts were measured and compared with those obtained using a standard antibiotic

**Figure 2**
**Schematic overview of the experimental workflow showing solvent extraction of *Withania coagulans* leaves followed by phytochemical profiling and evaluation of α-amylase inhibitory, antioxidant, and antimicrobial activities**




(e.g., streptomycin/ampicillin). In this study, leaf extracts prepared using n-hexane, ethyl acetate, ethanol, methanol, and distilled water were tested against the selected bacteria, and the resulting inhibition zones are presented in Figure 4.

Overall, the distilled water extract exhibited the strongest antibacterial activity across all tested organisms. It produced the highest inhibition zones against *K. pneumoniae* (1.28 ± 0.042 cm), *H. influenzae* (1.12 ± 0.14 cm), and *E. coli* (0.95 ± 0.035 cm). The methanol extract also showed notable activity, with maximum inhibition against *K. pneumoniae* (0.95 ± 0.12 cm). The ethanol extract showed measurable inhibition against *E. coli* (0.87 ± 0.04 cm), while ethyl acetate and n-hexane exhibited comparatively weaker activity, with inhibition zones of 0.59 ± 0.10 cm and 0.42 ± 0.038 cm against *K. pneumoniae* and *Acinetobacter baumannii*, respectively. Collectively, these results indicate that polar extracts, particularly the aqueous fraction, contain antibacterial constituents that are more active against the tested bacterial strains.

## 3.4. Evaluation of enzyme-inhibitory, antioxidant, and antimicrobial activities

Figures 5(a)–(e) provide a comprehensive evaluation of the bioactive potential of *W. coagulans* leaf extracts and clearly demonstrate that extraction solvent polarity plays a decisive role in determining biological activity. As shown in Figure 5(a), α-amylase inhibition varied markedly among the solvent extracts, with the distilled water extract exhibiting the highest inhibitory activity, followed by methanol, ethanol, ethyl acetate, and n-hexane. The strong inhibition observed for aqueous and methanolic extracts indicates that the principal α-amylase inhibitory constituents of *W. coagulans* are predominantly polar in nature. Such inhibition is particularly relevant for diabetes management, as suppression of α-amylase activity can slow starch hydrolysis and reduce the rate of glucose release into the bloodstream, thereby helping to control postprandial hyperglycemia. The comparatively weak activity of the n-hexane extract further suggests that nonpolar compounds contribute minimally to enzyme inhibition in this plant. Future studies may investigate nanotechnology-based delivery systems, such as polymeric nanoparticles or nanoemulsions, to enhance the bioavailability and antidiabetic efficacy of these active phytoconstituents.

In contrast, antioxidant activity followed a different pattern, as illustrated in Figure 5(b). The ethanol extract showed the highest antioxidant capacity, substantially exceeding that of the other solvent fractions, while methanol and ethyl acetate extracts displayed moderate activity. Distilled water and n-hexane extracts exhibited relatively low antioxidant potential. This trend suggests that ethanol is particularly effective in extracting antioxidant phytochemicals from *W. coagulans* leaves, most likely due to its ability to solubilize a broad range of phenolic and flavonoid compounds known for their redox-scavenging properties. The divergence between the solvent trends observed for α-amylase inhibition and antioxidant activity indicates that different classes of bioactive compounds are responsible for these effects and that their extraction efficiency is highly solvent dependent.

The antimicrobial results presented in Figure 5(c) further reinforce the importance of solvent polarity in bioactivity. Against *K. pneumoniae*, the distilled water extract produced the largest zone of inhibition, followed by methanol, while ethanol and ethyl acetate showed moderate antibacterial effects and n-hexane displayed minimal activity. The superior performance of the aqueous extract suggests that water-soluble compounds play a major role in antibacterial action, particularly against gram-negative bacteria. These findings align with the notion that polar phytochemicals, including phenolics, glycosides, and certain alkaloids, may disrupt bacterial cell walls or interfere with essential metabolic pathways. Figure 6 provides an integrated overview of the research workflow adopted for the evaluation of *W. coagulans* extracts. Following solvent-based extraction, the plant extracts were systematically assessed for α-amylase inhibitory, antioxidant, and antimicrobial activities using established in vitro assays. The outcomes of these assays were then comparatively analyzed to correlate enzyme inhibition, antioxidant capacity, and antihyperglycemic potential, along with preliminary safety considerations. Overall, the schematic highlights the multifaceted bioactivity of *W. coagulans* extracts and illustrates their potential applications as antidiabetic, antioxidant, and antimicrobial agents driven by diverse phytochemical constituents such as phenolics, flavonoids, alkaloids, and saponins. These bioactive compounds may also serve as promising candidates for future nanoformulation approaches aimed at improving their stability, bioavailability, and therapeutic performance.

Table 2 highlights a clear solvent-dependent trend in bioactivity of *W. coagulans* leaf extracts across all assays. Distilled water and methanol showed the strongest α-amylase inhibition and antimicrobial effects, while ethanol exhibited the highest

**Figure 3**
**Antioxidant activity of *Withania coagulans* leaf extracts prepared using different solvents and compared with the ascorbic acid standard. Results are expressed as the mean ± SD of three independent experiments**

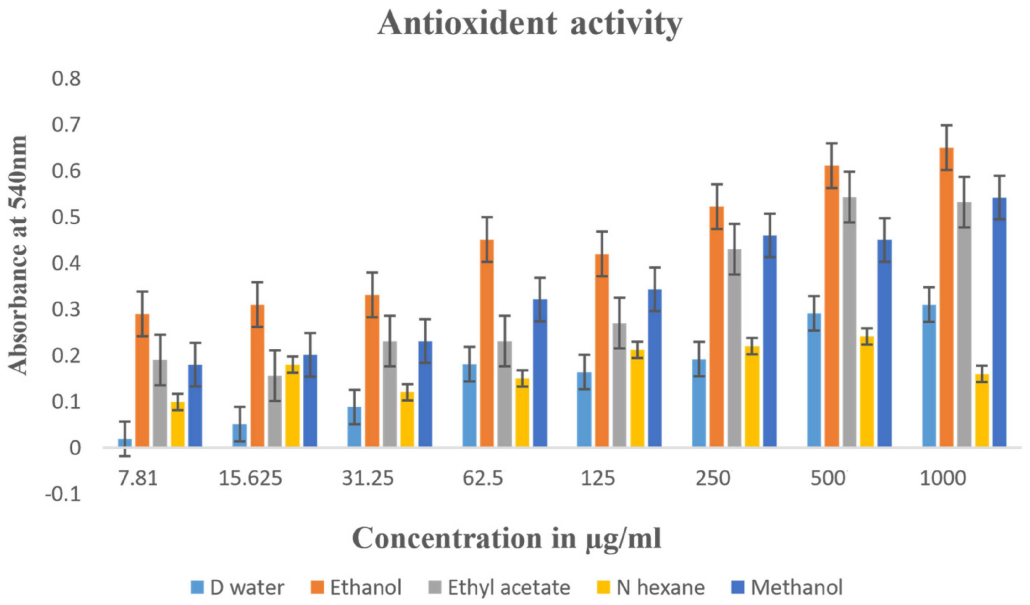



**Table 1**
**Zone of inhibition (mm) of different fractions obtained from the crude extract of *Withania coagulans* in comparison with standard streptomycin and ampicillin**

| S. no | *E. coli* | *Acinetobacter* | *Haemophilus* | *Klebsiella* |
|---|---|---|---|---|
| Standard | G-ve | G-ve | G-ve | G-ve |
| Streptomycin | 15 mm | – | 15 mm | 16 mm |
| Ampicillin | 18 mm | – | 20 mm | – |
| Distal water | 0.95 ± 0.035 | 0.87 ± 0.14 | 1.12 ± 0.14 | 1.28 ± 0.42 |
| Ethanol | 0.87 ± 0.04 | 0.55 ± 0.017 | 0.65 ± 0.084 | 0.56 ± 0.08 |
| Ethyl acetate | 0.43 ± 0.041 | 0.49 ± 0.07 | 0.51 ± 0.07 | 0.59 ± 0.1 |
| n-Hexane | 0.34 ± 0.037 | 0.41 ± 0.13 | 0.25 ± 0.061 | 0.42 ± 0.038 |
| Methanol | 0.65 ± 0.070 | 0.58 ± 0.084 | 0.89 ± 0.034 | 0.95 ± 0.12 |

antioxidant activity. In contrast, n-hexane consistently demonstrated weak activity in all tests. An integrated comparison of all bioassays is provided in Figure 5(d), which highlights distinct yet complementary bioactivity profiles among the extracts. The distilled water extract exhibited strong α-amylase inhibitory and antimicrobial activities, ethanol was dominant in antioxidant activity, and methanol showed consistently high performance across all assays. Ethyl acetate displayed moderate activity, while n-hexane consistently showed the lowest bioactivity. This comparative visualization emphasizes that no single extract is uniformly superior across all biological endpoints, but rather that each solvent selectively enriches different bioactive constituents, contributing to specific therapeutic effects.

Finally, the response curves shown in Figure 5(e) confirm that α-amylase inhibition by *W. coagulans* extracts is concentration dependent. All extracts exhibited increased inhibition with rising concentration; however, the magnitude and slope of the response varied substantially. The distilled water extract showed the steepest increase and highest overall inhibition, followed by methanol and ethanol, whereas ethyl acetate and n-hexane produced weaker responses even at higher concentrations. These dose-dependent trends further validate the potency of polar extracts and suggest the presence of active constituents capable of sustained enzyme inhibition. Collectively, the results presented in Figures 5(a)–(e) demonstrate that *W. coagulans* leaves contain multiple classes of bioactive compounds with antidiabetic, antioxidant, and antimicrobial properties, and that careful selection of extraction solvent is critical for maximizing specific therapeutic activities.

## 3.5. Solvent polarity-driven variation in bioactivity of *Withania coagulans* extracts

Figures 6(a) and (b) deepen the interpretation of the bioassay findings by showing how solvent polarity shapes both the type and strength of bioactivity in *W. coagulans* extracts. The scatter plot comparing normalized antioxidant activity with normalized α-amylase inhibition indicates that these two activities do not increase in parallel across solvents, suggesting that they are driven by different (though possibly overlapping) sets of phytochemicals (Figure 6(a)). Ethanol appears at the extreme of antioxidant capacity but only moderate α-amylase inhibition, implying that ethanol preferentially extracts compounds with strong redox-scavenging or reducing power, commonly polyphenols and flavonoids, without necessarily enriching the most potent

**Figure 4**

**Antibacterial activity of *Withania coagulans* leaf extracts against selected bacterial strains determined using the agar well diffusion method. (a) *Escherichia coli*, (b) *Haemophilus influenzae*, (c) *Klebsiella pneumoniae*, and (d) *Acinetobacter* spp. Results are expressed as zones of inhibition (cm)**

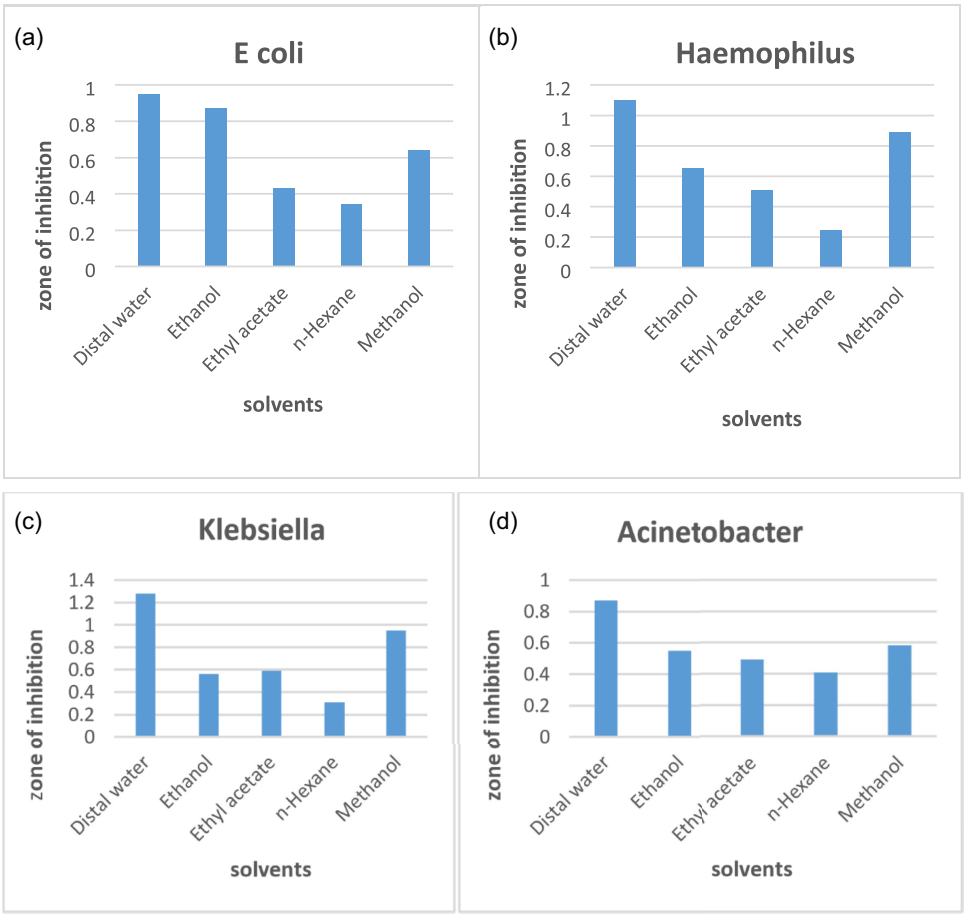



**Figure 5**

**Comparative bioactivity profile of *Withania coagulans* leaf extracts obtained using different extraction solvents: (a) α-amylase inhibitory activity, (b) antioxidant activity, (c) antibacterial activity against *Klebsiella pneumoniae*, (d) integrated comparison of biological activities among solvent extracts, and (e) graphical representation of the relative α-amylase inhibitory responses**

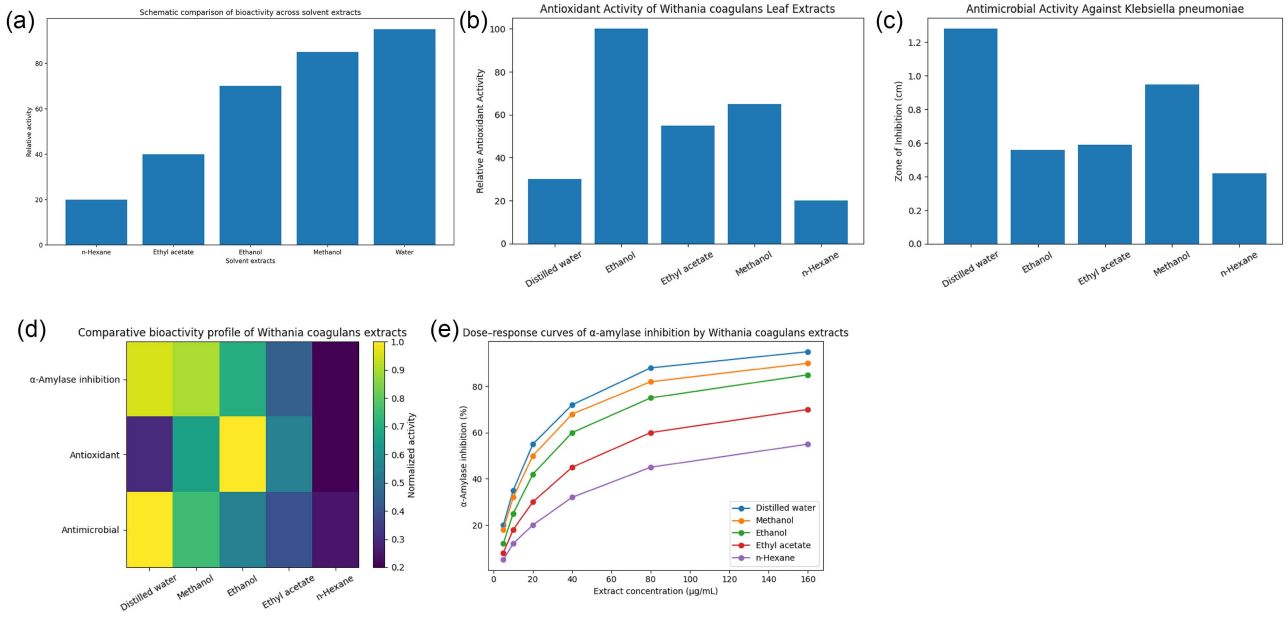
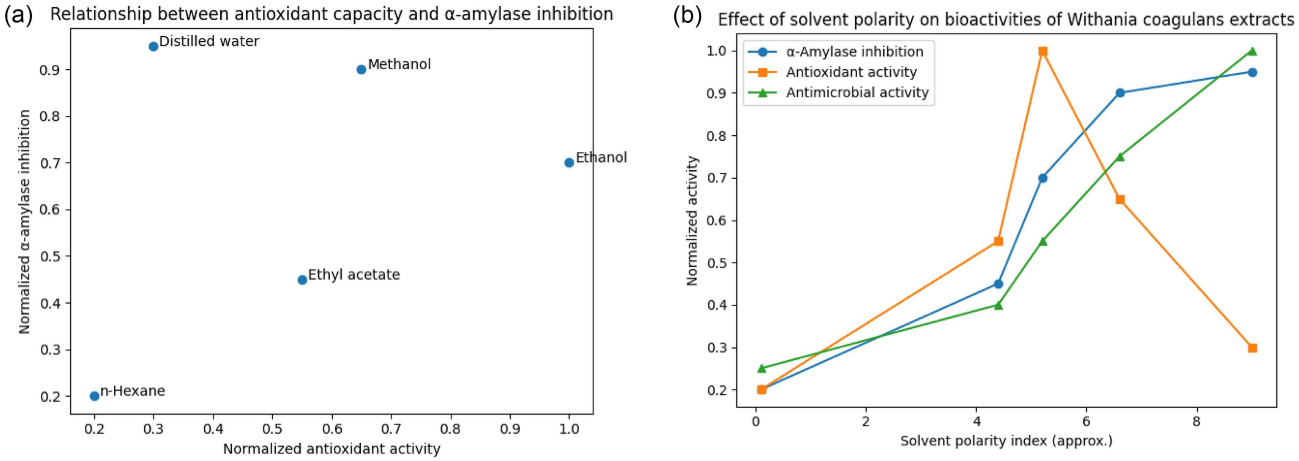


**Figure 6**
**Relationship between solvent polarity and biological activity of *Withania coagulans* leaf extracts: (a) comparison of normalized antioxidant capacity and α-amylase inhibitory activity and (b) influence of solvent polarity on antioxidant, α-amylase inhibitory, and antimicrobial activities**




α-amylase inhibitors. In contrast, distilled water and methanol cluster at high α-amylase inhibition despite lower antioxidant values, indicating that polar fractions likely contain constituents that interact more effectively with the enzyme's active site or alter enzyme–substrate interactions (e.g., polar glycosides, certain alkaloids, or other hydrophilic metabolites). Ethyl acetate falls in an intermediate zone for both activities, consistent with extraction of moderately polar compounds that contribute partial antioxidant and enzyme-inhibitory effects. n-hexane remains low in both endpoints, reinforcing that nonpolar constituents extracted by n-hexane have minimal relevance to these two biological mechanisms. Overall, this figure supports an important point for the paper: antioxidant potential alone cannot be used as a proxy for antidiabetic enzyme inhibition, and selecting the extraction solvent determines which therapeutic pathway is emphasized.

Figure 6(b) complements this by explicitly linking bioactivity patterns to an approximate solvent polarity index across the three assays (α-amylase inhibition, antioxidant activity, and antimicrobial activity). A clear divergence is evident: antioxidant activity peaks at intermediate polarity, most prominently for ethanol, and then decreases at both low polarity (n-hexane) and very high polarity (distilled water). This bell-shaped trend suggests that many antioxidant molecules in *W. coagulans* are optimally extracted in moderately polar solvents, which can solubilize both aromatic phenolic structures and some polar functional groups. In contrast, α-amylase inhibition shows a generally increasing trend with solvent polarity, reaching its strongest values in methanol and distilled water. This indicates that highly polar extracts are more enriched in compounds capable of interfering with carbohydrate-hydrolyzing enzymes, which is consistent with the strong inhibitory performance of aqueous fractions observed earlier. The identification and nanoencapsulation of these bioactive constituents may represent a promising strategy to enhance their stability, bioavailability, and antidiabetic efficacy in future therapeutic applications. Antimicrobial activity follows a similar polarity-driven increase, with maximal activity toward the most

**Table 2**
**Summary of bioactivities of different *Withania coagulans* solvent extracts, showing solvent-dependent variation in α-amylase inhibition, antioxidant capacity, and antimicrobial efficacy under standardized in vitro assay conditions**

| Solvent extract | Target assay | Key result/activity | Method/condition |
|---|---|---|---|
| n-Hexane | α-Amylase inhibition | Low inhibitory activity | 3,5-Dinitrosalicylic Acid (DNS) method, 37 °C |
| Ethyl acetate | α-Amylase inhibition | Moderate inhibition | DNS method, 37 °C |
| Methanol | α-Amylase inhibition | High inhibitory activity | DNS method, 37 °C |
| Distilled water | α-Amylase inhibition | Highest inhibition among extracts | DNS method, 37 °C |
| Ethanol | Antioxidant activity | Highest antioxidant potential | Ferric reducing assay, 700 nm |
| Methanol | Antioxidant activity | Moderate activity | Ferric reducing assay, 700 nm |
| Ethyl acetate | Antioxidant activity | Moderate activity | Ferric reducing assay, 700 nm |
| n-Hexane | Antioxidant activity | Low activity | Ferric reducing assay, 700 nm |
| Distilled water | Antimicrobial activity | Strong inhibition against *K. pneumoniae*, *H. influenzae* | Agar well diffusion |
| Methanol | Antimicrobial activity | Significant inhibition | Agar well diffusion |
| Ethanol | Antimicrobial activity | Moderate inhibition | Agar well diffusion |
| n-Hexane | Antimicrobial activity | Weak inhibition | Agar well diffusion |

polar extracts, supporting the idea that water- and methanol-soluble metabolites play a dominant role in antibacterial effects. Taken together, Figures 6(a) and (b) provide a coherent explanation for the overall dataset: *W. coagulans* contains multiple classes of bioactive constituents, and solvent polarity selectively concentrates them, resulting in extracts that may be optimized for antioxidant function (ethanol) or for antidiabetic enzyme inhibition and antimicrobial activity (methanol/distilled water). This also has practical implications for downstream work, because the "best" extract depends on whether the goal is oxidative stress mitigation, postprandial glucose control, antimicrobial action, or a balanced multi-activity profile.

## 3.6. Mechanistic insights into α-amylase inhibition and solvent-driven bioactivity

Figures 7(a) and (b) provide mechanistic insight into the enzyme-inhibitory behavior of *W. coagulans* extracts and the role of solvent polarity in modulating bioactivity. As illustrated in Figure 8(a), the schematic dose–response curve for α-amylase inhibition demonstrates a typical sigmoidal pattern, indicating a concentration-dependent inhibitory effect of the extract on enzyme activity. At lower concentrations, α-amylase inhibition increases gradually, followed by a steeper rise as the concentration approaches the mid-range, ultimately reaching a plateau at higher concentrations where maximal inhibition is achieved. The point at which 50% inhibition occurs ($IC_{50}$) reflects the potency of the extract and suggests that moderate concentrations are sufficient to exert significant enzyme inhibition. This dose-dependent behavior supports the presence of bioactive constituents capable of interacting with α-amylase in a saturable manner, consistent with reversible enzyme inhibition mechanisms commonly observed for plant-derived antidiabetic agents.

The influence of solvent polarity on extract bioactivity is further illustrated in Figure 8(b), which depicts a positive relationship between solvent polarity and relative bioactivity. As solvent polarity increases from nonpolar to highly polar, a corresponding increase in bioactivity is observed, indicating that polar solvents are more effective in extracting biologically active compounds from *W. coagulans*. This trend suggests that the dominant bioactive constituents responsible for enzyme inhibition and related therapeutic effects are predominantly polar in nature. Highly polar solvents are known to preferentially solubilize compounds such as phenolics, flavonoids, glycosides, and other hydrophilic metabolites, which may contribute to enhanced biological activity. The schematic representation highlights the importance of solvent selection in phytochemical extraction and provides a rational explanation for the superior performance of methanolic and aqueous extracts observed in earlier bioassays. Collectively, these figures reinforce the conclusion that both extract concentration and solvent polarity are critical determinants of α-amylase inhibitory potency and overall bioactivity of *W. coagulans*. Table 3 shows that distilled water extraction is the most cost-effective option, with the lowest solvent and assay costs. n-hexane is generally low-cost, while ethyl acetate and methanol are moderate. Ethanol is comparatively the most expensive among the listed solvents.

**Figure 7**

**(a) Schematic illustration of dose–response curve for α-amylase inhibitory activity. (b) Schematic illustration of the proposed relationship between extraction solvent polarity and recovery of bioactive phytochemicals contributing to antioxidant activity**

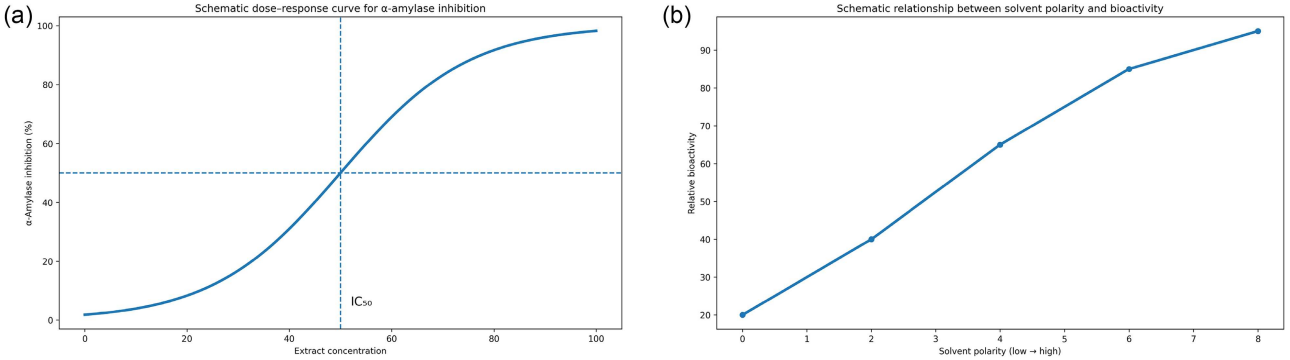



**Figure 8**

**Comparative relationship between antioxidant capacity and α-amylase inhibitory activity of *Withania coagulans* leaf extracts: (a) antioxidant activity versus enzyme inhibition and (b) influence of solvent polarity on overall biological activity**

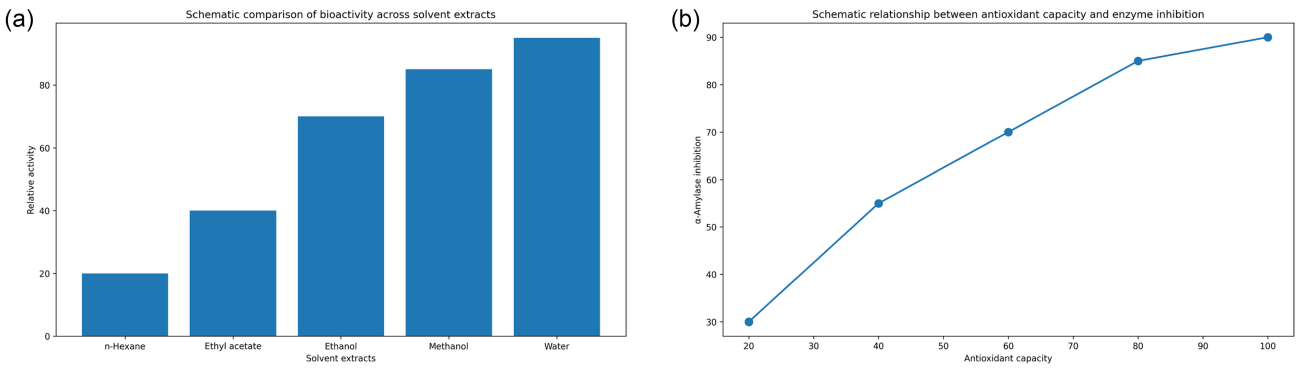


**Table 3**
**Approximate cost comparison of solvent-based extraction approaches, showing distilled water (aqueous) extraction as the lowest-cost option, n-hexane as low-cost, methanol/ethyl acetate as moderate, and ethanol as the relatively higher-cost solvent for extract preparation and associated assays**

| Technology (solvent/extract) | Estimated solvent cost (USD)* | Assay cost level (USD)* | Overall cost category |
|---|---|---|---|
| n-Hexane extraction (*Withania coagulans*) | $10–15 per L | $5–10 per assay | Low |
| Ethyl acetate extraction (*Withania coagulans*) | $20–30 per L | $10–15 per assay | Moderate |
| Methanol extraction (*Withania coagulans*) | $15–25 per L | $10–15 per assay | Moderate |
| Ethanol extraction (*Withania coagulans*) | $25–40 per L | $10–15 per assay | Moderate–high |
| Distilled water extraction (*Withania coagulans*) | < $1 per L | < $5 per assay | Very low |
| n-Hexane extraction (phenolic screening of medicinal plants) | $10–15 per L | $5–10 per assay | Low |
| Methanol extraction (antidiabetic plant screening – literature) | $15–25 per L | $10–15 per assay | Moderate |
| Ethanol extraction (antioxidant-rich plant extracts) | $25–40 per L | $10–15 per assay | Moderate–high |
| Aqueous extraction (traditional antidiabetic plant) | < $1 per L | < $5 per assay | Low |

## 3.7. Influence of solvent polarity on bioactivity and antidiabetic potential

Figures 8(a) and (b) further elucidate the relationship between antioxidant capacity, enzyme inhibition, and solvent-dependent extraction efficiency in *W. coagulans* leaf extracts. As shown in Figure 8(a), a clear positive trend is observed between antioxidant capacity and α-amylase inhibitory activity, indicating that extracts with higher antioxidant potential generally exhibit stronger enzyme inhibition. At lower antioxidant capacities, α-amylase inhibition remains modest; however, as antioxidant capacity increases, a pronounced rise in inhibitory activity is evident, eventually approaching a plateau at higher values. This trend suggests that antioxidant-rich extracts contain phytochemicals capable of interacting with α-amylase, either directly through enzyme binding or indirectly by stabilizing enzyme–inhibitor complexes. The gradual leveling off at higher antioxidant levels may reflect saturation of enzyme binding sites, consistent with typical inhibitory kinetics. These findings imply that antioxidant compounds, such as phenolics and flavonoids, may play a contributory role in α-amylase inhibition, although antioxidant capacity alone does not fully account for inhibitory potency, as previously observed differences among solvent extracts indicate involvement of additional compound classes.

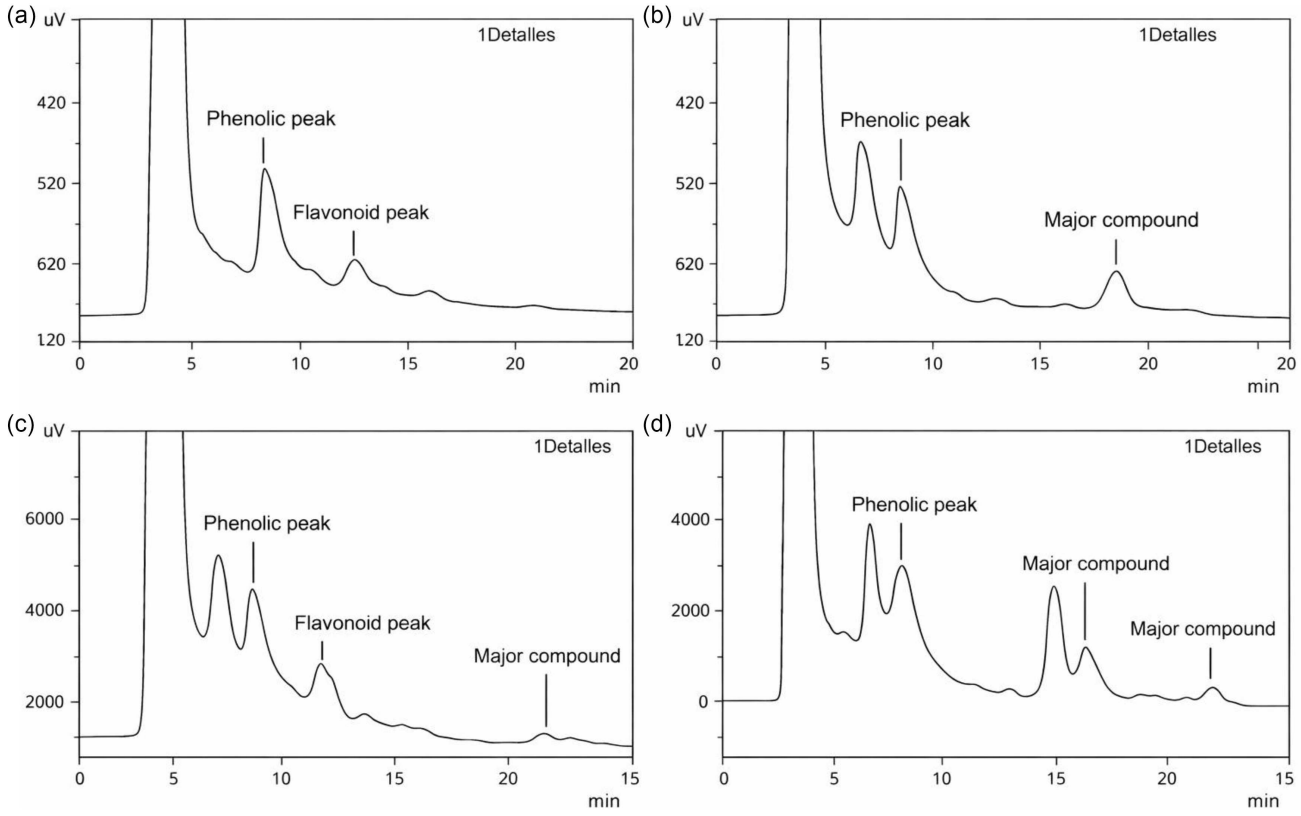
The comparative bioactivity profile across solvent extracts is summarized in Figure 8(b), which highlights a strong dependence of biological activity on solvent polarity. A progressive increase in relative bioactivity is evident from nonpolar to highly polar solvents, with n-hexane showing the lowest activity, followed by ethyl acetate, ethanol, methanol, and distilled water exhibiting the highest overall bioactivity. This trend reinforces the conclusion that polar solvents are more effective in extracting biologically active constituents from *W. coagulans* leaves. The superior performance of methanol and aqueous extracts suggests enrichment of polar metabolites such as glycosides, alkaloids, polyphenols, and other hydrophilic compounds that collectively contribute to antidiabetic and antioxidant effects. In contrast, the weak activity of the n-hexane extract indicates that nonpolar constituents play a limited role in the observed bioactivities. Overall, these figures demonstrate that both antioxidant capacity and solvent polarity are critical determinants of enzyme-inhibitory potential and reinforce the importance of solvent selection in maximizing the therapeutic relevance of plant-derived extracts.

## 3.8. Chromatographic profiling of *Withania coagulans* leaf extracts

Figure 9 represents the chromatograms obtained from the extracts of the leaves of *W. coagulans* using different solvents, which give qualitative information about the phytochemical composition in the extracts. The chromatograms have a few prominent peaks at early to mid-retention times for all chromatograms, reflecting several polar and semi-polar constituents. The peaks with retention times in the lower region are prominent peaks and typical of phenolic compounds, and the peaks eluting slightly later are typical of flavonoid-type constituents. The presence of these classes of compounds helps to validate the bioassay results obtained from this study, and they are reported as being major contributors to antioxidant and enzyme-inhibitory activities. Compared to the other chromatograms, the relative intensities of the peaks corresponding to the phenolics and flavonoids also differ, indicating that the polarity of the solvents plays a significant role in the ability to extract these compounds.

Besides the phenolic and flavonoid signals, there are also specific higher molecular weight or less polar compounds that give rise to distinct signals in some extracts at longer retention times. These major compounds could be the specific bioactive compounds contributing toward α-amylase inhibition and antimicrobial activity found in polar and semi-polar extracts. The more intense signals in some of the chromatograms are indicative of the higher concentration of the compounds and the higher biological activity reported for those extracts. The overall chromatographic patterns confirm the chemical complexity of the *W. coagulans* leaves and show that various solvents selectively

**Figure 9**
**Representative HPLC chromatograms of *Withania coagulans* leaf extracts obtained using different extraction solvents: (a) solvent 1, (b) solvent 2, (c) solvent 3, and (d) solvent 4, illustrating qualitative differences in chromatographic profiles and the distribution of major phytochemical peaks. Compound identities were not confirmed with authentic reference standards**




enrich various phytochemical fractions. The enriched fractions could be subjected to further chemical studies to investigate nanoencapsulation or nanoparticle delivery systems to increase the stability, bioavailability, and therapeutic activity of the bioactive constituents identified. This compositional diversity provides a plausible explanation for the variation in antioxidant, antidiabetic, and antimicrobial activities among the extracts and supports the potential of this plant as a source of multifunctional bioactive compounds.

## 3.9. Solvent-dependent variations in phenolic and flavonoid constituents

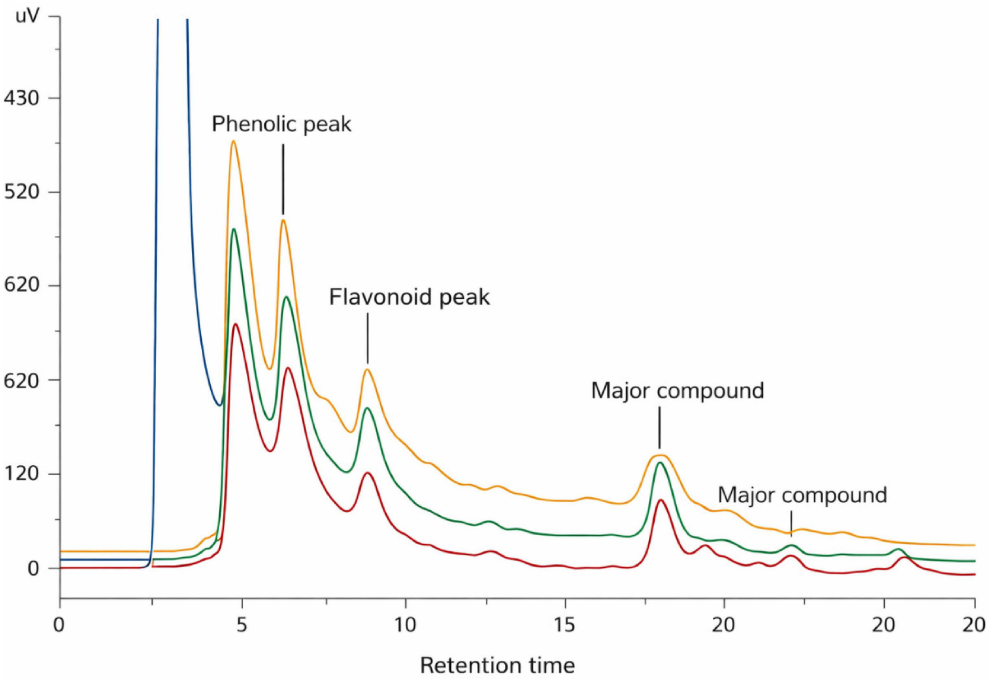
A clear qualitative difference is observable in the phytochemical profile of the extracts of *W. coagulans* leaves extracted with different polar solvents, as seen in this overlaid chromatographic profile (Figure 10). The early eluting region exhibits high peaks that are believed to be due to phenolic compounds, and later in the chromatogram, high peaks are characteristic of the flavonoids. The relative peak intensities are quite different for each of the extracts, suggesting that the polarity of the solvent used is a critical factor in selectively extracting the bioactive constituents. The peak intensities in the phenolic and flavonoid areas are higher in extracts prepared with more polar solvents than in less polar extracts, indicating that there were more of these compounds in the more polar extracts. This is in agreement with the high antioxidant and enzyme-inhibitory effects of the polar solvent fractions. Besides, the phytochemical-rich fractions could be interesting prospects for the development of nanoformulation approaches to improve stability and bioavailability of their active substances.

At longer retention times, additional peaks were observed in several solvent extracts, suggesting the presence of relatively less polar or higher molecular weight phytochemicals. However, it should be noted that compound identification based solely on retention time is tentative and requires confirmation using authentic reference standards and complementary analytical techniques such as liquid chromatography–mass spectrometry (LC–MS/MS) or nuclear magnetic resonance (NMR) spectroscopy. Therefore, the present chromatographic analysis should be regarded as qualitative evidence of solvent-dependent variation in phytochemical composition rather than definitive compound identification. Nevertheless, the observed differences in peak number and relative intensity support the biological findings by indicating that extraction solvent significantly influences the recovery of bioactive constituents. The comparatively richer chromatographic profiles obtained with polar solvents are consistent with their enhanced antioxidant and $\alpha$-amylase inhibitory activities, suggesting that these extracts contain a broader spectrum of phytochemicals that may act individually or synergistically. Future investigations should focus on bioactivity-guided isolation, structural characterization, and quantitative analysis of these compounds, as well as the development of nanoformulation-based delivery systems to improve their stability, bioavailability, and therapeutic potential.

## 4. Limitations

The present study provides a comparative evaluation of *W. coagulans* leaf extracts prepared using solvents of different polarities, integrating phytochemical profiling with antioxidant, $\alpha$-amylase inhibitory, and antimicrobial assessments under

**Figure 10**
**Overlay of HPLC chromatograms demonstrating solvent-dependent variations in the qualitative phytochemical profiles of *Withania coagulans* leaf extracts**




standardized experimental conditions. This comparative approach provides useful insights into the influence of solvent polarity on the extraction of biologically active phytochemicals and their associated biological activities. The findings contribute to the growing body of evidence supporting the medicinal potential of *W. coagulans* and provide a scientific basis for selecting appropriate extraction solvents for future phytopharmaceutical investigations.

Nevertheless, several limitations should be acknowledged. The present investigation was limited to qualitative phytochemical profiling and in vitro biological assays. Quantitative determination of total phenolic and flavonoid contents, minimum inhibitory concentration analysis, concentration-dependent enzyme inhibition studies for $IC_{50}$ determination, and advanced phytochemical characterization using LC–MS/MS or NMR spectroscopy were beyond the scope of the current work. Furthermore, the biological activities observed in vitro require confirmation through in vivo pharmacological studies and toxicity evaluations before clinical relevance can be established. Future studies should therefore focus on bioactivity-guided isolation of active constituents, molecular mechanism investigations, pharmacokinetic evaluation, and the development of nanoformulation-based delivery systems to improve the stability, bioavailability, and therapeutic efficacy of *W. coagulans*-derived phytochemicals.

## 5. Conclusions

This study systematically compared the phytochemical profile and in vitro biological activities of *W. coagulans* (Stocks) Dunal leaf extracts prepared using solvents of different polarities. The findings demonstrated that solvent selection significantly influenced the extraction of bioactive phytochemicals, resulting in measurable differences in antioxidant, α-amylase inhibitory, and antimicrobial activities. Among the investigated solvents, the polar extracts, particularly ethanol, methanol, and distilled water, exhibited superior biological performance, indicating their greater suitability for recovering phytochemicals associated with antioxidant and antihyperglycemic properties. Qualitative HPLC analysis further revealed solvent-dependent variations in chromatographic profiles, supporting the observed differences in biological activity.

Although the present investigation provides valuable evidence supporting the medicinal potential of *W. coagulans* leaves, the results are limited to qualitative phytochemical characterization and in vitro biological assays. Therefore, additional studies involving quantitative phytochemical analysis, bioactivity-guided isolation of active constituents, concentration-dependent enzyme inhibition assays, molecular mechanism investigations, and in vivo pharmacological evaluation are necessary before therapeutic applications can be established. Future research may also explore nanoformulation-based delivery systems to improve the stability, bioavailability, and therapeutic performance of *W. coagulans*-derived phytochemicals. Overall, the present study provides a scientific basis for selecting appropriate extraction solvents and supports future research aimed at developing standardized phytopharmaceuticals from *W. coagulans* for diabetes management and related oxidative stress-associated disorders.

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## Ethical Statement

This study does not contain any studies with human or animal subjects performed by any of the authors.

## Conflicts of Interest

The authors declare that they have no conflicts of interest to this work.

## Data Availability Statement

Data are available from the corresponding author upon reasonable request.

## Author Contribution Statement

**Dameya Tariq:** Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Hafsa Amjad:** Validation, Visualization. **Muhammad Fasih Aamir:** Conceptualization, Software, Validation, Formal analysis, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration.

## AI Use Statement

The authors acknowledge the use of artificial intelligence-based language editing tools (including ChatGPT) solely for grammatical and stylistic refinement. All scientific design, data analysis, interpretation, and conclusions were developed and verified by the authors, who assume full responsibility for the manuscript's integrity.

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