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RESEARCH ARTICLE

Phytochemical Characterization of *Aloe Vera* Extracts and Their Inhibitory Activities against Key Cancer-related Enzymes

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ABSTRACT

This study presents the first comparative investigation of the inhibitory effects of *Aloe vera* leaf gel (aqueous) and leaf skin (aqueous, methanolic, and ethyl acetate) extracts and the main components of the plant (aloin, aloe-emodin, and acemannan) on some cancer-related enzymes, and also the phytochemical characterization of the extracts. According to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, quinic acid (ranging from 42.55 to 54.48 mg/g) was the most abundant compound in all extracts except for the ethyl acetate. High-performance LC analysis indicated that aloin was present in higher quantities than aloe-emodin across all extracts. The highest concentration of aloin was observed in the ethyl acetate extract (36.33 ± 0.60 μg/mg), as well as that of aloe-emodin in the methanolic extract (0.91 ± 0.07 μg/mg). Aqueous (gel), methanolic, and ethyl acetate extracts showed strong inhibitory activity against thioredoxin reductase (TrxR), while all extracts demonstrated low inhibition against histone deacetylase (HDAC) and myeloperoxidase (MPO). Among the tested components, aloe-emodin exhibited the most potent TrxR inhibition (IC_{50} = 1.46 ± 0.14 μg/mL). Additionally, acemannan showed the highest inhibitory activity on HDAC (IC_{50} = 38.57 ± 2.82 μg/mL), and aloin demonstrated the strongest inhibition against MPO (IC_{50} = 28.40 ± 0.13 μg/mL). These findings suggest that *A. vera* extracts and the tested components possess promising anticancer potential through their ability to inhibit key enzymes involved in cancer. This provides novel insights into *A. vera*'s anticancer potential. Further studies are also warranted to elucidate the underlying mechanisms and evaluate the in vivo efficacy of the extracts /components.

1 | Introduction

Enzyme inhibitors play a crucial role in drug discovery and development, with numerous naturally derived anticancer drugs exerting their therapeutic effects through enzyme inhibition [1]. Investigating the inhibitory effects of plant extracts and natural compounds on various enzymes is a promising strategy for discovering novel anticancer agents. Thioredoxin reductase (TrxR), a pivotal component of the thioredoxin (Trx) system (comprising Trx, TrxR, and nicotinamide adenine dinucleotide

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phosphate [NADPH]), is essential for regulating DNA and protein synthesis/repair, and maintaining intracellular redox balance [2]. Its involvement in various cellular processes, including cell growth, p53 activity, and protection against oxidative stress, highlights its importance. The strong association of the Trx system with aggressive tumor growth makes TrxR an attractive target for cancer therapy, with many existing clinical anticancer drugs already demonstrating inhibitory effects on this enzyme [3].

Histone deacetylases (HDACs) constitute a family of enzymes that remove acetyl groups from histones, thereby regulating the expression of tumor suppressor genes [4]. Overexpression of HDACs is a hallmark in various cancer types, including pancreatic, colorectal, breast, lung, colon, and liver cancers, as well as leukemias and lymphomas. HDAC inhibitors interfere with HDAC activity to regulate key biological events in cancer cells, such as cell cycle progression, differentiation, and apoptosis. Several HDAC inhibitors have received FDA approval for cancer treatment, and many more are in advanced clinical development for both hematological malignancies and solid tumors, underscoring their therapeutic relevance [4, 5].

Myeloperoxidase (MPO), a heme peroxidase enzyme predominantly found in neutrophils, is vital for the immune system. It combats microbes by generating potent oxidizing products, such as hypochlorous acid (HOCl). While MPO inhibition reduces antimicrobial efficacy, it preserves the phagocytic activity crucial for innate immunity [6]. Elevated HOCl concentrations in tumor cells can induce DNA damage, thereby promoting malignant cell transformation [7]. MPO can support tumor initiation, progression, and metastasis, yet it can also enhance the innate immune response during tumor elimination. Elevated MPO levels have been observed in various cancer types, including lung, gynecological, and colon cancers. The −463G polymorphism in the *MPO* gene is linked to higher MPO expression and an increased risk for numerous cancers. Furthermore, MPO contributes to tumor progression by protecting cancer cells from caspase-3-mediated apoptosis [6]. Although MPO inhibitors are not yet used in the clinic, studies with drugs like AZD3241 (verdiperstat) have shown promising results in mouse models for treatment-resistant melanoma [8]. Therefore, modulating MPO activity presents a promising strategy for novel anticancer interventions.

Aloe vera (L.) Burm. f. (Xanthorrhoeaceae, formerly Liliaceae), a succulent plant indigenous to tropical regions such as North and South Africa, Madagascar, the Canary Islands, the Arabian Peninsula, and the Mediterranean region. For centuries, it has been utilized in medicinal, cosmetic, and nutritional applications [9, 10]. Numerous studies highlight *A. vera*'s diverse therapeutic potential, revealing antioxidant, antiageing, antiinflammatory, antiviral, antimicrobial, antibacterial, and anticancer activities [11–13]. These documented medicinal qualities are broadly attributed to the plant's rich composition of bioactive compounds [14]. Studies report that its anticancer effects are demonstrated through its antiproliferative and immunomodulatory mechanisms, which are also attributed to its rich bioactive compounds [1]. The rich composition of *A. vera* includes key phytoconstituents such as anthraquinones, naphthalenones, saponins, polysaccharides, proteins, enzymes, along with specific compounds like mannose-6-phosphate, salicylic acid, lignin, and various organic acids [1, 15].

The *A. vera* leaf is composed of three distinct layers, each with unique characteristics and compounds. The outermost layer is the green rind, also referred to as the skin [12]. The middle layer of the leaf produces a yellowish liquid, known as latex, which contains major secondary metabolites like anthraquinones (e.g., aloins and aloe-emodin), glycosylated chromones (e.g., aloesin), and polyphenols [1]. The innermost part of the leaf is the clear, mucilaginous gel, which consists of up to 98% water and various compounds, predominantly polysaccharides (e.g., acemannan), as well as sterols, polyphenols, and alkaloids [14, 16].

Aloin has been widely confirmed by studies to possess diverse pharmacological activities, including antioxidant, antitumor, antiinflammatory, antiosteoporotic, organ protective, antiviral, antimicrobial, antiparasitic, and laxative, as well as roles in blood lipid and glucose regulation [17, 18]. Numerous studies have reported that aloin exerts its anticancer effects through diverse mechanisms, including antiproliferative potential (reducing tumor growth, inducing cell cycle arrest, and downregulating cell cycle proteins), proapoptotic activities (modulating mitochondrial pathways, activating caspases, and affecting survival proteins), and the regulation of key molecular pathways such as PI3K/AKT/mTOR, and MAPK-related cascades. Furthermore, aloin demonstrates antimetastatic and antiangiogenic properties [17].

Aloe-emodin is one of the most studied components of *A. vera* leaf due to its cytotoxic, antitumor, antimicrobial, and antiinflammatory properties [19, 20]. Some studies propose aloe-emodin as a novel anticancer drug [21, 22]. It has been reported that aloe-emodin may be a potential antineoplastic agent due to its ability to selectively inhibit the growth of various cancer cells, including colon, lung, hepatic, and breast cancers. Its anticancer mechanisms include inhibiting proliferation, inducing apoptosis, inhibiting metastasis, potentially reversing multidrug resistance, and even demonstrating potential as a photosensitizer in photodynamic therapy for skin cancer [16].

Acemannan, an acetylated β-(1,4) soluble polymannose, is the main bioactive polysaccharide of *A. vera* gel. It possesses diverse activities, including immunoregulation, anticancer, antioxidation, wound healing, bone proliferation promotion, neuroprotection, and intestinal health promotion. Acemannan exerts its anticancer effect through multiple mechanisms, including immunomodulation and antiproliferative effects [23]. The effect is thought to be due to the activation of multipotent effector cells in the immune system, mainly macrophages, and the stimulation of cytokine production [24].

Given this background, this study aimed to investigate the inhibitory effects of *A. vera* leaf gel (aqueous) and leaf skin (aqueous, methanolic, and ethyl acetate) extracts and the purchased main components of the plant (aloin, aloe-emodin, and acemannan) on cancer-related enzymes (TrxR, HDAC, and MPO). Furthermore, phytochemical characterization of the extracts was conducted by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and high-performance LC (HPLC) analysis. To the best of our knowledge, this is the first comprehensive study evaluating the inhibitory effects of *A. vera* extracts and the components on the mentioned specific enzymes.

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TABLE 1 | Compounds detected *in A. vera* extracts by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (mg analyte/g extract).

		Leaf gel	Leaf skin		
	Analyte	**Aqueous**	**Aqueous**	**Methanolic**	**Ethyl acetate**
1	Quinic acid	42.55	54.48	51.26	0.04
2	Nicotiflorin	N.D.	0.64	0.91	0.83
3	p-Coumaric acid	N.D.	0.18	N.D.	0.37
4	Chlorogenic acid	0.04	0.16	0.25	0.09
5	Astragalin	N.D.	N.D.	0.08	0.11
6	Gentisic acid	0.01	0.06	N.D.	0.02
7	Caffeic acid	N.D.	0.01	0.03	0.02
8	Isoquercitrin	N.D.	N.D.	0.03	0.04

N.D. Not determined.

2 | Results and Discussion

2.1 | Phytochemical Content of the Extracts

LC-MS/MS analysis of *A. vera* extracts revealed the presence of eight compounds among the 56 investigated. The corresponding LC-MS/MS chromatograms are presented in the supporting information (Figure S1). Quinic acid was the most abundant compound in *A. vera* leaf gel aqueous, leaf skin aqueous, and leaf skin methanolic extracts, with the leaf skin aqueous extract demonstrating the highest concentration (Table 1). Conversely, *A. vera* leaf skin ethyl acetate extract was rich in nicotiflorin. The remaining six compounds (*p*-coumaric acid, chlorogenic acid, astragalin, gentisic acid, caffeic acid, and isoquercitrin) were found in generally low amounts in extracts where they were detected, while being absent in others. Our study demonstrates the significant effect of the extraction solvent on the yield and composition of compounds obtained from *A. vera*.

Quinic acid is well-documented in the literature for possessing numerous biological properties, including anticancer effects. These anticancer effects have been demonstrated to occur through various mechanisms, such as promoting apoptosis and reducing matrix metallopeptidase 9 expression [25]. A detailed prior study reported that quinic acid was present in *A. vera* leaf gel fillet (11.63 mg/100 g in fresh gel and 689 mg/100 g in dry powder) [26]. In line with our observations, Anzano et al. [27] also investigated *A. vera* leaf phytochemical composition using LC-high-resolution MS/MS, reporting high levels of quinic acid in the extracts that varied with the plants' geographical origin. A study reported that the phenolic compound profiles from different parts of *A. vera*, including the peel, flower, gel, and root, were characterized using ultra-HPLC-photo diode array-Quadrupole time-of-flight/MS (UHPLC-PDA-QOT/MS) methods. It was identified 25 phenolic compounds, including various groups such as cinnamic acids and their derivatives (e.g., caffeic acid and chlorogenic acid), chromones, anthracene compounds and their derivatives, and flavonoids (e.g., isoquercitrin). Their findings indicated that the peel and flowers were richest in these compounds, while the gel contained the least [28]. Another study revealed that *A. vera* green rind extracts were rich in alkaloids, tannins, flavonoids, sterols, triterpenes, and mucilages, while coumarins and saponins were notably absent [29].

The following compounds were not detected in any of the extracts: 1,5-dicaffeoylquinic acid, 4-hydroxybenzoic acid, acacetin, aconitic acid, amentoflavone, apigenin, catechin, chrysin, cosmosiin, coumarin, cyranoside, daidzein, daidzin, ellagic acid, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, ferulic acid, ferulic acid-d3-IS, fisetin, fumaric acid, genistein, genistin, gallic acid, hesperetin, hesperidin, kaempferol, luteolin, miquelianin, naringenin, o-coumaric acid, piceid, protocatechuic acid, protocatechuic aldehyde, quercetin, quercetin-d3-IS, quercitrin, rosmarinic acid, rutin, rutin-d3-IS, salicylic acid, sinapic acid, syringic acid, syringic aldehyde, tannic acid, vanilic acid, and vanillin.

When the aloin and aloe-emodin content of the *A. vera* extracts was measured by HPLC analysis, it was revealed that aloin is the major compound in all extracts, with the highest concentration observed in the leaf skin ethyl acetate extract. However, aloe-emodin, while present in lower amounts in all extracts, showed its highest concentration in the leaf skin methanolic extract (Table 2). These results indicate that the extraction solvent significantly influences the yield of these compounds. The corresponding HPLC chromatograms of standard aloin and aloe-emodin solutions, as well as various *A. vera* leaf extracts, are presented in supporting information (Figure S2).

Arsene et al. [30], using HPLC-MS/MS, reported that *A. vera* leaf aqua extract had a different profile. They found that aloe-emodin and its derivatives were the main components (approximately 75.74%), with aloesin being a significant constituent within this group (30.22%), alongside detected aloins (approximately 2.45%). Another study by Quispe et al. [28] reported, through their UHPLC-PDA-QOT/MS analysis, that aloin was present in all parts of *A. vera* plant (peel, flower, gel, and root). They also noted that aloe-emodin-diglucoside was detected in the peel, flower, and root, while aloe-emodin-8-O-glucoside was exclusively found in the flower; this corroborates our results regarding the higher amounts of aloin in the extracts.

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TABLE 2 | Aloin and aloe-emodin concentrations of *A. vera* extracts determined by high-performance liquid chromatography (HPLC).

	Aloin (μg/mg extract)	Aloe-emodin (μg/mg extract)
***A. vera* leaf gel aqueous extract**	7.97 ± 0.21	0.29 ± 0.06
***A. vera* leaf skin aqueous extract**	14.93 ± 0.29	0.20 ± 0.02
***A. vera* leaf skin methanolic extract**	15.60 ± 1.27	0.91 ± 0.07
***A. vera* leaf skin ethyl acetate extract**	36.33 ± 0.60	0.23 ± 0.02

Results are given as mean ± S.D.

2.2 | TrxR Inhibitory Activity

TrxR inhibitory activities of *A. vera* extracts and the tested components are shown in Table 3. Among the extracts, *A. vera* leaf gel aqueous and leaf skin ethyl acetate extracts exhibited significantly higher ($p < 0.05$) TrxR inhibitory activity compared to curcumin, as evidenced by their lower IC_{50} values. Similarly, among the tested components, aloe-emodin demonstrated the strongest TrxR inhibitory activity, followed by aloin, both significantly surpassing the inhibitory activity of curcumin ($p < 0.05$).

Given the potent TrxR inhibitory activities of aloe-emodin and aloin, along with their quantified presence in the extracts (Table 2), these compounds are likely key contributors to the observed TrxR inhibition of the extracts. Specifically, the high aloin content in the *A. vera* leaf skin ethyl acetate extract corresponds with its strong TrxR inhibitory activity. Similarly, the notable TrxR inhibition of the methanolic extract may be partially attributed to its higher aloe-emodin concentration.

While no studies on TrxR inhibitory activity of aloin or aloe-emodin exist in the literature, numerous reports indicate their anticancer effects through various mechanisms, including their potential to induce apoptosis in cancer cells, antiproliferative effects, and antimetastatic and antiangiogenic properties [19, 21, 22, 31]. An in vitro study showed that aloin treatment suppressed the angiogenic response of vascular endothelial growth factor (VEGF)-induced human endothelial cells by inhibiting their proliferation and migration [32]. Nićiforović et al. [31] showed that aloin induces apoptosis and causes cell cycle arrest in cancer cells, accompanied by significant changes in antioxidant enzyme activities (e.g., inhibition of CuZnSOD), leading to H_2O_2 accumulation responsible for its cytotoxic effects. Furthermore, aloin was reported that aloin inhibit tumor angiogenesis and growth by blocking STAT3 activation [33]. Another study reported that aloin had chemopreventive effects against 1,2-dimethylhydrazine-induced precancerous colon lesions in Wistar rats [34].

Studies have reported that aloe-emodin exhibits antiproliferative effects in various types of cancer, including lung, squamous, glioma, and neuroectodermal cancer cells [19, 35, 36]. It has also been shown that aloe-emodin inhibits the activity and gene expression of N-acetyltransferase in human malignant melanoma cells [37]. Furthermore, it has been reported that aloe-emodin induces apoptosis in diverse cancer cells, including T24 bladder cancer cells [38], HepG2 hepatoma cells [39], human gastric carcinoma cells (AGS and NCI-N87) [40], and leukemia/lymphoma (K562 and P3HR-1) cells [41]. The apoptotic mechanism, as observed in these studies, often involved mitochondrial factor release, caspase-3 and caspase-9 activations [40, 41], and can occur through oxidative stress [39]. A study has demonstrated that aloe-emodin suppresses casein kinase II activity in gastric carcinoma cells [40]. Furthermore, Luo et al. [42] showed that aloe-emodin, in combination with radiation, significantly suppresses the proliferation of HeLa cells by inducing apoptosis. A study reported that aloe-emodin's cytotoxic effect on B16F10 mouse melanoma cells is via necrosis [43]. Ur Rehman et al. [44] demonstrated that aloe-emodin showed cytotoxic activity on MDA-MB-231 cells and strongly inhibited the human carbonic anhydrase II (hCA-II). Similarly, Khan et al. [45] demonstrated that aloe-emodin showed significant antiproliferative activity on prostate cancer cells and also strongly inhibited hCA-II.

2.3 | HDAC Inhibitory Activity

Table 3 summarizes the HDAC inhibitory activities of *A. vera* extracts and the tested components, expressed as IC_{50} values, and compares them to the potent HDAC inhibitor, Trichostatin A. The results indicate that acemannan exhibited the most significantly potent HDAC inhibitory activity among all tested components as well as extracts ($p < 0.05$). While the leaf gel aqueous extract also demonstrated significant HDAC inhibitory activity, all other *A. vera* extracts and tested components exhibited considerably lower activity compared to Trichostatin A ($p < 0.05$). Given that acemannan is the well-known main polysaccharide of *A. vera* gel [23], the high HDAC inhibitory activity observed in *A. vera* gel aqueous extract may be attributed to the gel's high acemannan content. While acemannan's antitumor properties have been primarily attributed to its immunostimulatory activity [23, 24], this novel finding of its HDAC inhibitory activity is significant to its mechanisms in anticancer effects.

While no studies on acemannan's HDAC inhibition have been reported, other research in the literature investigating its anticancer effects does exist. A study reported that acemannan enhances antigen recognition by inducing tumor cell cytotoxicity in murine peritoneal macrophage cells [46]. Tong et al. [47] showed that it inhibits tumor growth in colon cancer models by inducing apoptosis through mechanisms that alter mitochondrial membrane permeability, promote Bax translocation, and trigger the caspase cascade. Additionally, it has been reported that polymannose-rich *Aloe* gel inhibited tumor growth in colon cancer models by modulating key signaling pathways like NF-κB,

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TABLE 3 | The enzyme inhibitory activities (IC_{50} values) of *A. vera* extracts and the compounds.

	TrxR (μg/mL)	HDAC (μg/mL)	MPO (μg/mL)
***A. vera* leaf gel aqueous extract**	16.14 ± 0.33[a]	239.78 ± 4.13[a]	274.94 ± 8.02[a]
***A. vera* leaf skin aqueous extract**	134.92 ± 7.26[b]	1523.76 ± 11.10[b]	152.94 ± 3.82[b]
***A. vera* leaf skin methanolic extract**	18.00 ± 1.60[a, g]	2345.14 ± 136.25[c]	147.17 ± 4.04[b,c]
***A. vera* leaf skin ethyl acetate extract**	9.95 ± 2.39[c]	798.30 ± 38.57[d]	N.D.
Aloin	7.35 ± 1.76[c,d]	2036.46 ± 138.82[c]	28.40 ± 0.13[d]
Aloe-emodin	1.46 ± 0.14[e]	2763.46 ± 10.42[e]	No inhibition
Acemannan	1004.17 ± 36.59[f]	38.57 ± 2.82[f]	483.94 ± 52.75[e]
Standard inhibitor*	20.48 ± 0.94[g]	0.0060 ± 0.0002[g]	0.158 ± 0.005[f]

Results are given as mean ± S.D. Different superscript letters (a, b, c, d, e, f, g) in the same column indicate a significant difference ($p < 0.05$). TrxR: Thioredoxin reductase, HDAC: Histone deacetylase; MPO: Myeloperoxidase. N.D.: The inhibition could not be determined because of the poor solubility of the ethyl acetate extract. IC_{50}: The inhibitory concentration of the extract to inhibit the activity of the enzyme by 50%.
*Standard inhibitors used in each assay were: curcumin for TrxR, trichostatin A for HDAC, and Sigma 475944 for MPO.

STAT3, and cell cycle-related factors (e.g., CDK4, cyclin D1, ERK1/2) [48]. Another study reported that *A. vera* leaf gel extract, a rich source of acemannan, exhibited a tumor preventive effect in mice against Ehrlich ascites tumors [49].

2.4 | MPO Inhibitory Activity

MPO inhibitory activities of *A. vera* extracts and the tested components are shown in Table 3. Aloin demonstrated the significantly highest MPO inhibitory activity among the tested extracts and components ($p < 0.05$). While the leaf skin aqueous and methanolic extracts displayed similar MPO inhibitory activity, the inhibitory activity of the leaf skin ethyl acetate extract could not be measured under experimental conditions due to its low solubility. Furthermore, aloe-emodin exhibited no significant MPO inhibitory activity at the tested concentrations. Given the high MPO inhibitory activity of aloin and its considerable levels in the *A. vera* extracts (Table 2), it is plausible that aloin serves as a contributor to the observed MPO inhibitory effects of these extracts.

Considering MPO's numerous roles in tumor progression, such as promoting oxidative damage and protecting cancer cells from apoptosis [6], it is thought that the MPO inhibitory activities of aloin and *A. vera* may also contribute to their anticancer effects through this inhibitory mechanism. Unlike our findings, Pasala et al. [50] demonstrated that aloe-emodin nanoparticles significantly reduced MPO levels in stroke rats, suggesting a direct inhibitory effect on this inflammatory enzyme. Also, Park et al. [51] showed that aloesin supplementation (0.1% and 0.5%) significantly reduced both MPO activity and messenger RNA expression of inflammatory markers tumor necrosis factor-α and interleukin-1β in a rat model of ulcerative colitis.

3 | Conclusion

This study offers novel information regarding the enzyme inhibitory activities of the various *A. vera* leaf extracts and the main components of the plant (aloin, aloe-emodin, and acemannan) on some cancer-related enzymes (TrxR, HDAC, and MPO) and phytochemical characterization of these extracts.

LC-MS/MS analysis revealed that quinic acid was the most abundant compound found in *A. vera* extracts, while according to HPLC analysis, the aloin content of all extracts was higher than the aloe-emodin content. Also, our results demonstrated that aloe-emodin, aloin, and *A. vera* extracts (gel aqueous and skin ethyl acetate) had strong TrxR inhibitory activity (IC_{50} ranging from 1.46 to 16.14 μg/mL) compared to the standard inhibitor curcumin (IC_{50} = 20.48 μg/mL). Furthermore, acemannan (IC_{50} = 38.57 μg/mL) emerged as the most potent HDAC inhibitor, as well as aloin (IC_{50} = 28.40 μg/mL) demonstrated the highest MPO inhibitory activity among the tested components. These observed inhibitory activities of *A. vera* extracts may be due to aloin, aloe-emodin, and acemannan content, which are the main components of the plant.

While existing literature has shown the anticancer effects of *A. vera* and its components through various other mechanisms,

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the identification of inhibitory effects on TrxR, HDAC, and MPO in this study will contribute to a more comprehensive understanding of the plant's underlying anticancer potential. However, it is important to note that this research represents a preliminary exploration, and further studies are warranted to evaluate the in vivo efficacy of these compounds in preclinical models of cancer.

4 | Experimental

4.1 | The Chemicals

β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), aloe-emodin, aloin, curcumin, HDAC assay kit, MPO, o-dianisidine dihydrochloride, MPO inhibitor, and TrxR assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acemannan was purchased from Clearsynth (Mumbai, India). All other chemicals were analytical grade.

4.2 | The Plant

A. vera leaves were collected from the plant, which was cultivated in the greenhouse of Istanbul University Alfred Heilbronn Botanical Garden (Herbarium of Istanbul University Faculty of Pharmacy ISTE 65118).

4.3 | Preparation of the Extracts

Fresh leaves were harvested, washed thoroughly with deionized water, weighed, and cut longitudinally. The gel was carefully collected. The remaining leaf skins were cut into small pieces. The extraction method previously described by Candoken et al. [43] was applied with some modifications.

A. vera leaf gel aqueous extract: The collected gel was homogenized with ice-cold phosphate-buffered saline (PBS) using a blender (Waring Commercial, USA). The homogenate was filtered through a layer of cheesecloth, and the filtrate was lyophilized (Labconco, USA). The obtained extract was stored at −20°C until further use.

A. vera leaf skin aqueous extract: Leaf skin pieces were homogenized with ice-cold PBS using a blender. The homogenate was filtered through cheesecloth, and the filtrate was centrifuged (Thermo Fisher Scientific, USA) at 3500 rpm. The supernatant was collected and lyophilized, then the obtained extract was stored at −20°C.

A. vera leaf skin methanolic and ethyl acetate extracts: Leaf skin pieces were homogenized with ice-cold solvent (methanol/ethyl acetate) using a blender. The homogenates were mixed on a magnetic stirrer for 45 min at room temperature and left to stand overnight in the dark. Then each homogenates were filtered through cheesecloth and the filtrates were centrifuged at 3500 rpm. The supernatants were collected and evaporated to dryness using a rotary evaporator (Büchi, Switzerland). The dried extracts were stored at −20°C until further analysis.

4.4 | Phytochemical Analysis of the Extracts

4.4.1 | LC-MS/MS Method

Phytochemical content screening of the extracts was performed using an LC-MS/MS method at Dicle University Science and Technology Application and Research Center (DUBTAM). A Shimadzu-Nexera model UHPLC coupled with an MS/MS was used to accomplish quantitative evaluation of 53 phytochemicals. Consequently, the chromatographic separation was performed on a reversed phase Agilent Poroshell 120 EC-C18 model (150 mm × 2,1 mm, 2,7 μm) analytical column. The column temperature was set to 40°C. The elution gradient was composed of eluent A (water+5 mM ammonium formate+0.1% formic acid) and eluent B (methanol+5 mM ammonium formate+0.1% formic acid). The following gradient elution profile was used: 20%–100% B (0–25 min), 100% B (25–35 min), 20% B (35–45 min). Furthermore, the solvent flow rate and injection volume were settled as 0.5 mL/min and 5 μL, respectively.

The MS detection was carried out using a Shimadzu LCMS-8040 model tandem mass spectrometer equipped with an electrospray ionization (ESI) source operating in both negative and positive ionization modes. LC-ESI-MS/MS data were acquired and processed by LabSolutions software (Shimadzu). The MRM multiple reaction monitoring (MRM) mode was used for the quantification of the phytochemicals. The MS operating conditions were applied as: drying gas (N_2) flow, 15 L/min; nebulizing gas (N_2) flow, 3 L/min; DL temperature, 250°C; heat block temperature, 400°C; and interface temperature, 350°C [52]. The analytical method validation parameters for the LC-MS/MS analysis of the extracts are presented in the supporting information (Table S1).

4.4.2 | HPLC Method

Aloin and aloe-emodin contents of *A. vera* extracts were determined by Shimadzu LC20AT HPLC system with UV detection (Shimadzu, Kyoto, Japan). A C18 guard column (10 × 2.1 mm, 3 μm, GL Sciences, Tokyo, Japan) was used. Separation was achieved by a C18 analytical column (Intersil ODS-3, 150 × 4.6 mm, 5 μm, GL Sciences, Tokyo, Japan) at 40°C. The analytes were eluted by a mobile phase consisting of two components as 0.1% aqueous o-phosphoric acid solution (A) and methanol (B), with a gradient program. After stabilization, the system was held at 25% eluent B flow for 10 min, followed by a linear increase to 40% in 10 min and an isocratic hold for 10 min. Then, eluent B flow was increased to 70% in 10 min and kept for 20 min. Finally, eluent B flow was set to its original percentage of 25% in the next 5 min. The flow rate was 1,0 mL/min and the injection volume was set to 20 μL. Aloin was detected at 210 nm, whereas the detection wavelength of aloe-emodin was 254 nm. The analytes were quantified by the external standard method. Calibration curves were plotted in the concentration range of 1.00–50.00 μg/mL ($y = 46418x + 5488.8$; $R^2 = 0.9998$) for aloin and 0.10–2.00 μg/mL ($y = 120559x - 8438.8$; $R^2 = 0.998$) for aloe-emodin ($n = 3$).

For the preparation of samples, 1000 μg/mL solutions of each extract were dissolved in methanol. Solutions were filtered

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through a 0.45 μm syringe tip filter before analysis. All samples were analyzed in triplicate. The analytical figures of merit for aloin and aloe-emodin and the precision and accuracy of the HPLC method are presented in supporting information, Tables S2 and S3, respectively.

4.4.3 | Enzyme Inhibition Assays

Extracts, commercially purchased main components of the plant (aloin, aloe-emodin, and acemannan), and standard enzyme inhibitors (curcumin, Trichostatin A, MPO inhibitor Sigma 475944) were dissolved in dimethyl sulfoxide (DMSO) to prepare their stock solutions. On the day of the experiment, working solutions of each sample were prepared by diluting the stock solutions with DMSO to obtain the desired concentrations.

All enzyme inhibition assays were performed in triplicate. The results of the enzyme inhibitory activities are presented as half-maximal inhibitory concentrations (IC_{50} values). IC_{50} values were determined by nonlinear regression analysis of the dose-response curves generated for each sample. Independent-samples t-tests were performed using Microsoft Excel 365 (Microsoft, USA) to determine significant differences in IC_{50} values between samples.

4.4.4 | Determination of TrxR Inhibitory Activity

The method for determining TrxR inhibitory activity was adapted from the manufacturer's instructions (TrxR assay kit, Sigma-Aldrich, CS0170) [53]. The assay is based on the reduction of DTNB to 5-thio-2-nitrobenzoic acid (TNB) by NADPH, which results in the formation of a strong yellow color measured spectrophotometrically at 412 nm.

For the assay, 20 μL of the sample solution, 150 μL of working buffer (prepared by mixing EDTA-potassium phosphate buffer and NADPH solution according to the manufacturer's instructions), and 10 μL of DTNB solution (60.5 mM) were added to 20 μL of enzyme solution (1 U/mL) in a 96-well plate. The change in absorbance at 412 nm was monitored for 20 min using a microplate reader (BioTek, USA). A control reaction was performed in parallel, replacing the sample with an equal volume of the appropriate solvent. Curcumin was used as a standard inhibitor. TrxR inhibitory activity was calculated as a percentage based on the following formula:

$$\text{TrxR inhibition \%} = \left(1 - \frac{\text{Absorbance change of sample at 412 nm}}{\text{Absorbance change of control at 412 nm}}\right) \times 100$$

4.4.5 | Determination of HDAC Inhibitory Activity

HDAC inhibitory activity was assessed using an HDAC assay kit (Sigma-Aldrich, CS1010) following the manufacturer's protocol. The assay is based on the enzymatic deacetylation of a fluorogenic substrate. Specifically, HDAC activity results in the removal of acetyl groups from lysine residues within the substrate. Subsequently, the addition of a developer solution induces fluorescence emission upon interaction with the deacetylated lysine residues.

For the assay, 30 μL of assay buffer, 15 μL of HeLa nuclear extract (containing HDAC), and 50 μL of the fluorogenic substrate solution were added to 5 μL of the sample solution in a 96-well plate. The reaction mixture was incubated at 30°C for 30 min. Subsequently, 10 μL of the developer solution was added, and the plate was incubated for an additional 10 min at room temperature. Fluorescence intensity was measured using a fluorescence microplate reader (BioTek FLx800) with excitation and emission wavelengths set at 350-380 nm and 440-480 nm, respectively. A control reaction was performed in parallel, replacing the sample with an equal volume of the appropriate solvent. Trichostatin A was used as a standard inhibitor. The HDAC inhibitory activity was calculated as a percentage according to the formula below:

$$\text{HDAC inhibition \%} = \left[\left(1 - \frac{\text{Fluorescence value of sample}}{\text{Fluorescence value of control}}\right) \times 100\right]$$

4.4.6 | Determination of MPO Inhibitory Activity

The method for determining MPO inhibitory activity was adapted from the protocol described by Nastasijević et al. [54]. This assay is based on the measurement of the colored product formed during the oxidation of o-dianisidine dihydrochloride by reactive oxygen species generated by MPO. Specifically, MPO catalyzes the conversion of hydrogen peroxide to HOCl, which then oxidizes o-dianisidine dihydrochloride to a colored product.

For the assay, 10 μL of the sample solution, 15 μL of potassium phosphate buffer, 200 μL of o-dianisidine dihydrochloride solution (1.5 mM), and 25 μL of MPO solution (0.8 U/mL) were mixed in a suitable microplate well. The increase in absorbance at 460 nm was monitored for 10 min at 25°C using a microplate reader. A control reaction was performed in parallel, replacing the sample with an equal volume of the appropriate solvent. An inhibitor compound (Sigma-Aldrich 475944) was used as a standard inhibitor. MPO inhibitory activity was calculated as a percentage according to the following formula:

$$\text{MPO inhibition \%} = \left(1 - \frac{\text{Absorbance change of the sample at 460 nm}}{\text{Absorbance change of the control at 460 nm}}\right) \times 100$$

Author Contributions

Ege Artun: Methodology, Formal analysis. **Eda Dagsuyu**: Methodology, Investigation, Formal analysis, Data curation. **Gozde Hasbal-Celikok**: Methodology, Investigation, Formal analysis, Data curation, Writing-original draft, Writing- review & editing. **Ozlem Kurt-Sirin**: Methodology, Formal analysis. **Pelin Koseoglu-Yilmaz**: Methodology, Investigation, Formal analysis, Data curation. **Mustafa Abdullah Yilmaz**: Methodology, Formal analysis, Data curation. **Tugba Yilmaz-Ozden**: Methodology, Investigation, Formal analysis, Data curation, Writing-original draft, Writing- review & editing. **Refiye Yanardag**: Conceptualization, Project administration, Supervision, Writing-original draft. **Nuriye Akev**: Conceptualization, Project administration, Supervision, Writing-original draft, Writing- review & editing

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting file 1: Figure S1 LC-MSMS Chromatographic Analysis.pdf **Supporting file 2**: Figure S2 HPLC chromatograms.pdf **Supporting file 3**: SUPPLEMENTARY TABLES 1-3.docx

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