


Article

Phytochemical Composition and Antioxidant and Anti-Inflammatory Activities of *Humboldtia sanjappae* Sasidh. & Sujanapal, an Endemic Medicinal Plant to the Western Ghats

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Abstract: Ethnomedicinal plants are important sources of drug candidates, and many of these plants, especially in the Western Ghats, are underexplored. *Humboldtia*, a genus within the Fabaceae family, thrives in the biodiversity of the Western Ghats, Kerala, India, and holds significant ethnobotanical importance. However, many *Humboldtia* species remain understudied in terms of their biological efficacy, while some lack scientific validation for their traditional uses. However, *Humboldtia sanjappae*, an underexplored plant, was investigated for the phytochemical composition of the plant, and its antioxidant, enzyme-inhibitory, anti-inflammatory, and antibacterial activities were assessed. The LC-MS analysis indicated the presence of several bioactive substances, such as Naringenin, Luteolin, and Pomiferin. The results revealed that the ethanol extract of *H. sanjappae* exhibited significant in vitro DPPH scavenging activity ($6.53 \pm 1.49 \mu\text{g/mL}$). Additionally, it demonstrated noteworthy FRAP (Ferric Reducing Antioxidant Power) activity ($8.46 \pm 1.38 \mu\text{g/mL}$). Moreover, the ethanol extract of *H. sanjappae* exhibited notable efficacy in inhibiting the activities of α -amylase ($47.60 \pm 0.19 \mu\text{g/mL}$) and β -glucosidase ($32.09 \pm 0.54 \mu\text{g/mL}$). The pre-treatment with the extract decreased the LPS-stimulated release of cytokines in the Raw 264.7 macrophages, demonstrating the anti-inflammatory potential. Further, the antibacterial properties were also evident in both Gram-positive and Gram-negative bacteria. The observed high zone of inhibition in the disc diffusion assay and MIC values were also promising. *H. sanjappae* displays significant anti-inflammatory, antioxidant, antidiabetic, and antibacterial properties, likely attributable to its rich composition of various biological compounds such as Naringenin, Luteolin, Epicatechin, Maritemin, and Pomiferin. Serving as a promising reservoir of these beneficial molecules, the potential of *H. sanjappae* as a valuable source for bioactive ingredients within the realms of nutraceutical and pharmaceutical industries is underscored, showcasing its potential for diverse applications.

Keywords: *Humboldtia sanjappae*; LC-MS analysis; radical scavenging; anti-inflammatory activity; cytokine release



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1. Introduction

Humans have always battled with various infections. In addition to these, recent decades have witnessed a significant increase in the occurrence of various non-communicable diseases. These diseases have been associated with increased mortality globally. The changes in lifestyle comprising dietary changes and reduced physical activity have resulted in a sudden increase in the number of patients. The role of oxidative stress and inflammation is

impeccable in the onset of these diseases. Oxidative stress, the imbalance between the generation of reactive molecules and its scavenging, plays significant roles in non-communicable diseases [1]. Together with this, numerous studies have established that inflammation has a role in the progression of different diseases. The link between inflammatory response and the onset of different cancers such as ovarian cancer and pancreatic cancer has been studied well [2,3]. Recent studies suggest that neuroinflammation in Alzheimer's will escalate the disease progression. Recently, studies on *Gynostemma pentaphyllum* (Thunb.) Makino demonstrated a protective effect against the inflammation [4,5]. New therapies indicate that the anti-inflammatory treatments associated with cardiovascular disease are a promising strategy to bring down the succession of the disease [6]. Inflammatory processes in the host defense system should be highly regulated, and the loss of control is problematic. So, the inflammatory molecules have become the primary target for the prevention of various diseases, in which the main signaling pathways such as the nuclear factor- κ B (NF- κ B) signaling pathway, Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, and mitogen-activated protein kinase (MAPK) signaling pathway might be brought under control to prevent the diseases [7–9]; the JAK/STAT is focused on more because this pathway is associated with the pathogenesis of different inflammatory diseases such as Rheumatoid Arthritis (RA) and Inflammatory Bowel Disease (IBD) [10,11]. Even though inflammation is an evolutionarily conserved mechanism for the organism's survival [12], it is essential to control the prolonged release of anti-inflammatory mediators to prevent the development of various diseases [13]. Different natural products have recently been investigated and have given satisfactory results in this respect [14,15]. Excess inflammation in the body will lead to the development of Reactive Oxygen Species (ROS); if the concentration of the same exceeds a limit, the body will not be able to neutralize the same [16]. Due to this reason, pharmaceutical and food industries have considerably used natural products with antioxidant capabilities, and herbal products that promote health have also become highly popular in recent years [17,18].

It has been discovered that several plant medicines have a variety of pharmacological properties, including antioxidant, anti-inflammatory, anticancer, neuroprotective, and hypolipidemic properties [8,19,20]. These activities are attributed to the non-nutritive chemicals present in the plants [10]. Findings from studies conducted in mice suggest that the leaves of *Hemigraphis alternata* exhibit anti-inflammatory, anti-nociceptive, and anti-diarrheal activities [21]. These kinds of phytochemicals are reported in several plant families such as Lamiaceae, Zingiberaceae, Malvaceae, Acanthaceae, and Apocyanacea [22,23]. Fabaceae is one such family with an abundance of different phytochemicals which are good in curing various diseases [24].

The genus *Humboldtia* belongs to the family Fabaceae. The plants of the particular genus are well known for their traditional uses and pharmacological properties [25]. Research has been conducted on certain species of *Humboldtia*, revealing their rich phytochemical composition. These plants have been found to contain valuable phytochemicals, including phenols, flavonoids, saponins, tannins, terpenoids, cardiac glycosides, apigenin, steroids, phlobatannins, and more [26–28]. In the realm of traditional medicine, the bark of *Humboldtia* species held curative significance, being employed to address conditions such as biliousness, leprosy, ulcers, and epilepsy and acting as an anticonvulsant [29]. The remediation of biliousness, impurities in the blood, ulcers, and epilepsy all involved the preparation of a decoction from the bark powder [30].

Humboldtia brunonis Wall, *Humboldtia unijuga* Bedd., and *Humboldtia vahliana* Wight are well known for their pharmacological efficiency and their antioxidant, anti-inflammatory, anticancer, antimicrobial, and anti-depressant effects [28,29,31–33]. *H. brunonis* fulfilled roles as a stypitic, demulcent, anthelmintic, ulcer remedy, stomachic, astringent, and treatment for menstrual and urinary issues [34]. Furthermore, the local populations residing in Karnataka's Shiradi and Bisle Ghats harnessed the leaves and bark of *H. brunonis* for arthritis and diabetes treatments, a practice detailed by Prasad and Kumar [26]. It was documented that the *H. brunonis* bark and leaves were utilized for addressing wounds,

menstruation disorders, and overgrowth issues [35]. *Humboldtia unijuga*, referred to as 'palakan' by the Kani tribes in Agasthyamala, was employed to treat ailments such as headaches, chickenpox, and snake bites [36]. It has been discovered that the plant possesses Erythrodiol-3-acetate and 2,4-di-tert-butylphenol, which have been demonstrated to exhibit anti-inflammatory and anticancer properties [25].

The plant species *Humboldtia sanjappae*, belonging to the Fabaceae family, and its related species exhibit a diverse range of pharmacological properties [28,29,31–33]. However, the phytochemical constituents and pharmacological effects of this plant remain largely unexplored. Currently, there are no existing reports on the antioxidant and anti-inflammatory activities of this plant, and data regarding its phytochemical composition are also lacking. Consequently, this study represents a pioneering effort to investigate the phytochemical profile of the plant and evaluate its potential antioxidant, enzyme-inhibitory, anti-inflammatory, and antibacterial properties. To identify the bioactive phytochemicals, LC-MS analysis is employed as a key analytical technique.

2. Results and Discussion

2.1. Quantitative and Qualitative Estimation of Phytochemicals in *H. sanjappae*

The *H. sanjappae* extract was analyzed using LC-MS, which indicated the presence of flavonoids, including Naringenin, Luteolin, and Pomiferin, as well as phenols such as Epicatechin and Maritemin (Figure 1, Table 1). Flavonoids and phenolic substances not only have antioxidant properties, but they also work well as anti-inflammatory agents [37]. Various studies provide support for the immune-modulating effects of polyphenols and flavonoids. Seed polyphenols extracted from *Nigella sativa* L. were evaluated for their analgesic and anti-inflammatory properties. The study findings demonstrated that these polyphenols effectively reduced paw edema induced by carrageenan [38]. Concentrated extract derived from *Dendrobium loddigesii* Rolfe, rich in polyphenols, was administered to treat diabetic mice. The outcomes indicated that this extract exhibited the capability to lower blood glucose levels, reduce body weight, decrease levels of low-density lipoprotein cholesterol, and elevate insulin levels within the mice [39]. Flavonoids are part of the category of polyphenolic natural compounds, encompassing over 4000 identified variations. The advantageous biological effects of flavonoids are unquestionably intertwined with their structural composition and properties, rendering them prime contenders for pharmaceutical development. Numerous inflammatory molecules such as TNF- α , IL-1, IL-6, IL-17, and IFN- γ , released via the activation of various signaling pathways, primarily the NF- κ B pathway, have been demonstrated to be inhibited upon administration of flavonoid [40]. Scientists detected that supplementation of Epicatechin potentially contributed to reducing inflammation and enhancing insulin sensitivity in visceral adipose tissue of high-fat fed mice [41]. The presence of phytochemicals such as Epicatechin in the plant may be responsible for its observed antidiabetic activity. However, additional experiments and studies are necessary to validate this hypothesis and confirm the specific compounds and mechanisms involved in the plant's potential benefits for diabetes management. Certainly, the antioxidant properties of the extract can play a crucial role in managing oxidative stress in individuals with diabetes. As diabetes can cause substantial cellular damage, including in the brain, combating oxidative stress with antioxidant compounds becomes important [42]. By reducing oxidative stress, the extract's antioxidant properties may help protect cells, mitigate damage, and contribute to improved overall health in diabetic patients.

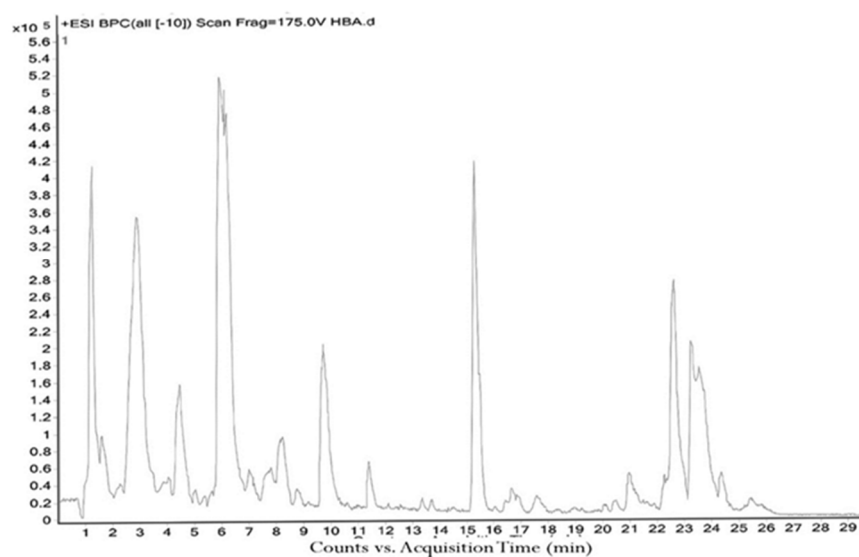
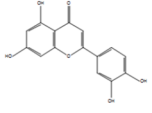
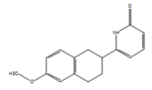
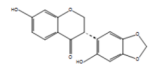
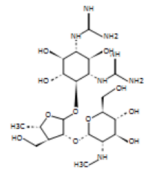
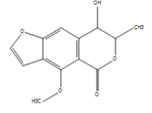
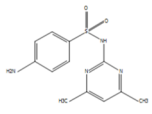
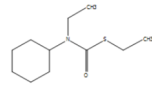
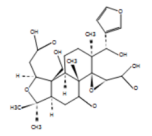
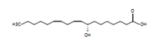
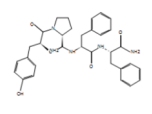
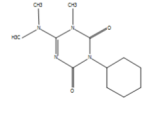
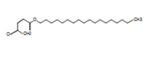


Figure 1. The LC–MS total ion chromatogram of *H. sanjappae* ethanol extract.

Table 1. LC–MS analysis and chemical composition of *H. sanjappae*.

Sl. No.	RT	m/z ^a	m/z ^b	Name of the Compound	Fragments	Mol. Wt.	Chemical Formula	Structure
1	3.575	577.14	577.14	Richotomine	483.13, 315.11, 197.80	532.14	C ₃₀ H ₂₀ N ₄ O ₆	
2	3.773	579.15	579.15	Procyanidin B7	579.14, 443.15, 383.12, 227.17	578.14	C ₃₀ H ₂₆ O ₁₂	
3	4.166	289.07	289.07	(–)-Epicatechin	289.07, 226.07	290.08	C ₁₅ H ₁₄ O ₆	
4	4.633	513.14	513.14	2'',6''-Di-O-acetylononin	513.14, 289.07	514.15	C ₂₆ H ₂₆ O ₁₁	
5	5.908	494.24	494.24	Ryanodine	494.23, 189.07	493.23	C ₂₅ H ₃₅ NO ₉	
6	5.919	465.16	465.16	Pomiferin	421.16, 213.07	420.16	C ₂₅ H ₂₄ O ₆	
7	6.312	549.22	549.22	Cymorcin diglucoside	431.10, 253.03	490.21	C ₂₂ H ₃₄ O ₁₂	
8	8.763	287.05	287.06	Maritimetin	287.05, 283.15, 267.15	286.05	C ₁₅ H ₁₀ O ₆	
9	9.101	285.04	285.04	Luteolin	285.04, 215.09	286.05	C ₁₅ H ₁₀ O ₆	

Table 1. Cont.

Sl. No.	RT	<i>m/z</i> ^a	<i>m/z</i> ^b	Name of the Compound	Fragments	Mol. Wt.	Chemical Formula	Structure
10	9.475	271.06	271.06	Naringenin	271.06, 259.12, 248.97	272.07	C ₁₅ H ₁₂ O ₅	
11	9.681	271.06	271.06	Coriandrone E	251.16, 179.10	248.07	C ₁₃ H ₁₂ O ₅	
12	10.067	271.06	271.06	Morindone	271.05, 267.15, 253.14	270.05	C ₁₅ H ₁₀ O ₅	
13	10.197	301.07	301.07	(+)-Sophorol	271.05, 301.06, 295.18, 277.17	300.06	C ₁₆ H ₁₂ O ₆	
14	10.485	269.08	269.08	Formononetin	258.04, 179.10, 139.15	268.07	C ₁₆ H ₁₂ O ₄	
15	11.846	283.19	283.19	Lactapiperanol C	279.09, 265.17	282.18	C ₁₆ H ₂₆ O ₄	
16	15.229	507.23	507.22	Limonate	507.22, 351.25, 238.12	506.22	C ₂₆ H ₃₄ O ₁₀	
17	16.573	295.23	295.23	17-Hydroxylinolenic acid	295.22, 284.32, 277.21	294.22	C ₁₈ H ₃₀ O ₃	
18	19.676	645.42	645.42	Capsanthin 5,6-epoxide	529.30, 403.26, 238.89	600.42	C ₄₀ H ₅₆ O ₄	
19	21.644	593.27	593.27	Ganoderic acid F	415.35, 227.17, 570.41	570.28	C ₃₂ H ₄₂ O ₉	
20	22.179	471.35	471.35	delta-Maslinic acid	471.85, 311.17, 248.97	472.36	C ₃₀ H ₄₈ O ₄	
21	23.291	413.26	413.27	D8'-Merulinic acid A	391.28, 279.15, 149.02	390.28	C ₂₄ H ₃₈ O ₄	

^a: calculated *m/Z* ratio, ^b: Reference *m/Z* ratio.

Several investigators have noted natural substances' anti-inflammatory properties, including multiple preclinical experiments [43–46]. As a result, we infer that the antioxidant and anti-inflammatory properties exhibited by the ethanol extract of *H. sanjappae* must be due to the presence of various phytochemical components such as polyphenols, flavonoids, isocoumarins, etc., and also that these many different phytochemicals in plants offer a valuable source of antimicrobial compounds with immense therapeutic potential [47].

Antibiotic-resistant bacteria have emerged as a significant global health concern [48,49]. Plants have long been renowned for their antibacterial powers as nature's medicine [50]. Polyphenols and essential oils, among other bioactive substances, have powerful antibacterial properties [30,51–53]. Adopting plant phenomena might open the way for innovative and long-lasting antibacterial treatments because traditional antibiotics are becoming less effective, leading to a pressing need for the development of new and effective antimicrobial agents. In this context, the rich diversity of plant species provides a vast array of bioactive compounds that can be explored for their antimicrobial properties. Polyphenols and flavonoids possess well-documented antimicrobial properties [54,55], exhibiting inhibitory effects against a broad spectrum of bacteria [55,56].

Our research has supplied substantiating proof of the elevated levels of these compounds in the ethanol extract of *H. sanjappae*. Specifically, the total phenolic content measured at 378.77 ± 6.62 μg equivalent per milligram and the total flavonoid content recorded at 204.76 ± 6.10 μg equivalent per milligram both emphasize the concentration within the extract (Table 2). Given its rich content of polyphenols and flavonoids, the extract from *H. sanjappae* shows promise as a potential source for the development of novel antibiotics. Considering its antimicrobial potential, the polyphenol- and flavonoid-rich extract of *H. sanjappae* warrants further exploration in the search for new antibiotic compounds.

Table 2. The total polyphenol and flavonoid contents of *H. sanjappae* ethanol extract.

Assay	μg Equivalent/mg of Extract
Total phenolic content	378.77 ± 6.62
Total flavonoid content	204.76 ± 6.10

2.2. In Vitro Antioxidant Activities of *H. sanjappae* Extract

Species of the Fabaceae family consist of phytochemicals responsible for the plant's antioxidant potential [57]. The different genera of the family are established as having antioxidant potential [58]. In vivo studies of *Tamarindus*, a related genus of *Humboldtia*, showed that it exhibits potent antioxidant activity [59], and the antioxidant potential of species within the genus *Humboldtia* has been previously explored, and their effectiveness has been reported [27,31]. The IC_{50} value of *H. sanjappae* bark extract in the anti-DPPH radical scavenging assay was shown to be 6.53 ± 1.49 $\mu\text{g}/\text{mL}$. Likewise, Table 3 shows other antioxidant activity in Ferric Reducing Antioxidant Power, represented by its value of 8.46 ± 1.38 $\mu\text{g}/\text{mL}$. The antioxidant properties of the plant must be assigned to the different phytochemicals present in the extract; those bioactive compounds identified from LC-MS analysis are listed in the Table 1. For example, previous studies demonstrated that anticancer properties of Epicatechin are linked to its antioxidative potential [60]. Another component present in the extract is Luteolin, which is found in glycosylated forms in a variety of vegetables and fruits and is classified within the flavone subclass of flavonoids. Its documented effects include in vivo anti-inflammatory [61], antioxidative [62–64], antidiabetic [61], antimicrobial [65], and anti-cancer [66,67] activities. The antioxidant properties of Naringenin [68,69], Morindone [70,71], Capsanthin 5,6-epoxide [72,73], and Ganoderic acid F [74] have been previously established. Therefore, these compounds could potentially account for the robust antioxidant activity observed in the extract. Oxidative stress plays a critical role as an independent factor in the development of numerous chronic diseases, including cancer, diabetes, and cardiovascular diseases [75–78]. Therefore, the antioxidant properties found in the plant extract can be beneficial in the management of diseases that are linked to oxidative stress.

Table 3. In vitro antioxidant and antidiabetic activities of *H. sanjappae* expressed as IC₅₀ values (µg/mL).

Activity	IC ₅₀ Value(µg/mL)		
	HSE	Ascorbic Acid	Acarbose
DPPH scavenging	6.53 ± 1.49	2.11 ± 0.25	>200
FRAP value	8.46 ± 1.38	4.15 ± 0.47	>200
α-amylase	47.60 ± 0.19	33.92 ± 2.54	122.18 ± 3.08
α-glucosidase	32.09 ± 0.54	29.85 ± 2.01	103.45 ± 2.68

2.3. Enzyme Inhibitory Properties of *H. sanjappae* Ethanol Extract

The extract was examined for its enzyme-inhibitory properties against key enzymes associated with type 2 diabetes mellitus, namely α-amylase and α-glucosidase. The IC₅₀ value for the inhibition of α-amylase and α-glucosidase by the extract was determined to be 47.60 ± 0.19 µg/mL and for the inhibition of β-glucosidase, 32.09 ± 0.54 µg/mL (Table 3). The standard antidiabetic drug acarbox exhibited an IC₅₀ value of 122.18 ± 3.08 and 103.45 ± 2.68 µg/mL against α-amylase and α-glucosidase enzymes, respectively. Hence, the plant extract contains stronger antidiabetic compounds compared to the acarbose. The α-amylase and α-glucosidase are enzymes that play crucial roles in carbohydrate metabolism and are frequently targeted by antidiabetic medications [79]. Indeed, the inhibition of α-amylase and α-glucosidase by the *H. sanjappae* extract may contribute to its potent antidiabetic activity. By inhibiting these enzymes involved in carbohydrate metabolism, the extract can potentially help regulate blood glucose levels and manage diabetes effectively. The enzyme-inhibitory properties are well corroborated by the major bioactive substances observed in the plant using LC-MS analysis. Epicatechin, Luteolin, and Naringenin were reported to inhibit the α-amylase and α-glucosidase in in vitro and animal model studies [80,81]. In addition, the reports clearly indicated the antidiabetic properties of these bioactive compounds in independent studies.

2.4. Anti-Inflammatory Activity of *H. sanjappae*

The anti-inflammatory activity of the extract was evaluated using Raw 264.7 macrophages as the model. Raw 264.7 cells stimulated with lipopolysaccharide (LPS) are a widely utilized cellular model of inflammation [82]. The lipopolysaccharide is the cell wall component of most of the Gram-negative bacteria; the LPS stimulates the macrophage in a toll-like-receptor-dependent manner [83]. In the present study, the normal macrophage was estimated for the level of IL-1β, and it was estimated to be 64.6 ± 1.9 pg/mg protein. However, there was observed a significant elevation in the IL-1β levels upon stimulation with the lipopolysaccharide to 573.4 ± 4.5 pg/mg protein. The increased level of IL-1β is an indicator of inflammation in the cellular conditions [84–86]. However, the pre-treatment of macrophages with the different doses of HSE resulted in a significant reduction in IL-1β levels (Table 4). The pre-treatment of Raw 264.7 cells with 5 µg/mL of HSE resulted in cellular IL-1β levels of 403.7 ± 6.2 pg/mg protein ($p < 0.01$). Similarly, the pre-treatment with 10 µg/mL (298.5 ± 8.4 pg/mg protein) and 20 µg/mL of HSE (156.2 ± 3.4 pg/mg protein) resulted in lower IL-1β levels ($p < 0.001$). The reduction in IL-1β levels is indicative of the anti-inflammatory potential of the HSE at the respective treatment doses.

Together with IL-1β, IL-6 was also found to significantly influence the inflammation in macrophages [87,88]. IL-6 has a major role in the innate immune defense systems [89]; however, the same molecule is associated with the progression of various diseases [90,91]. The level of IL-6 in the untreated macrophages without LPS stimulation was estimated to be 133.4 ± 5.8 pg/mg protein; however, the exposure of LPS elevated the cellular IL-6 levels to 628.5 ± 8.2 pg/mg protein. On the contrary, the level was brought down by the treatment with 5 and 10 µg/mL of *H. sanjappae* extract, which reduced the cellular IL-6 levels to 507.1 ± 8.1 ($p < 0.05$) and 388.4 ± 2.8 pg/mg protein ($p < 0.001$). In the highest concentration of *H. sanjappae* extract treatment, the IL-6 was estimated to be 291.3 ± 6.6 pg/mg protein ($p < 0.001$).

Table 4. Anti-inflammatory activity of *H. sanjappae* extract against lipopolysaccharide-induced activation of Raw 264.7 cells and comparison with standard aspirin (1 mM).

	IL-1 β (pg/mg Protein)	IL-6 (pg/mg Protein)	TNF- α (pg/mg Protein)	NO (μ M/mg Protein)
Untreated	64.6 \pm 1.9	133.4 \pm 5.8	115.2 \pm 3.1	10.7 \pm 0.64
LPS Control	573.4 \pm 4.5	628.5 \pm 8.2	856.0 \pm 11.2	75.2 \pm 2.1
Aspirin (1 mM)	147.5 \pm 5.1 ***	209.5 \pm 9.1 ***	247.5 \pm 6.3 ***	22.7 \pm 1.7 ***
HSE 5 μ g/mL	403.7 \pm 6.2 **	507.1 \pm 8.1 *	715.2 \pm 8.8 *	58.8 \pm 3.4 *
HSE 10 μ g/mL	298.5 \pm 8.4 ***	388.4 \pm 2.8 ***	602.8 \pm 5.2 ***	42.3 \pm 1.9 ***
HSE 20 μ g/mL	156.2 \pm 3.4 ***	291.3 \pm 6.6 ***	493.7 \pm 6.4 ***	30.7 \pm 2.5 ***

* indicates significant variation with respect to LPS control ($p < 0.05$); ** indicates higher significant variation with respect to that of LPS control ($p < 0.01$), and *** indicates highest significant variation with respect to that of LPS control ($p < 0.001$). All the results are indicated as mean \pm standard deviation of six independent experiments.

The TNF- α levels are crucial for the survival and proliferation of various cancer cells [92]. The cytokine is also important in the progression events of cancers including metastasis and stemness [93]. The level of TNF- α in the untreated and unstimulated Raw 264.7 macrophages was estimated to be 115.2 \pm 3.1 pg/mg protein. However, the level was elevated to 856.0 \pm 11.2 pg/mg protein upon stimulation by the LPS. This clearly indicated the induction of acute inflammation in the experimental condition. In 5 μ g/mL *H. sanjappae* treated macrophages, the level of TNF- α was reduced to 715.2 \pm 8.8 pg/mg protein. A similar decrease in the TNF- α level was also noted in the 10 and 20 μ g/mL *H. sanjappae* treatment, which brought down the TNF- α level to 602.8 \pm 5.2 and 493.7 \pm 6.4 pg/mg protein.

The nitric oxide level is also an important inflammatory indicator in cells; the inducible nitric oxide synthase is an enzyme responsible for the overwhelming load of nitric oxide in the body [94]. Despite its physiological and immunological importance, nitric oxide is often associated with chronic inflammation and is thereby involved in many of the degenerative diseases [95]. The level of nitric oxide in the untreated macrophage cells was estimated to be 10.7 \pm 0.64 μ M/mg protein. The level was increased to 75.2 \pm 2.1 μ M/mg protein in the macrophages exposed to LPS. Interestingly, the level was brought down by the pre-treatment with the 5 μ g/mL of HSE (58.8 \pm 3.4 μ M/mg protein). In the 10 μ g/mL of *H. sanjappae* extract treatment, the NO level was estimated to be 42.3 \pm 1.9 μ M/mg protein, and, in the 20 μ g/mL HSE treatment, it was further reduced to 30.7 \pm 2.5 μ M/mg protein. Hence, it is clearly indicated that the pre-treatment with different doses of HSE dose-dependently reduced the inflammatory insults in cultured macrophages.

Pathogen-associated molecular pattern molecules (PAMPs) or damage-associated molecular pattern molecules (DAMPs) are the two types of molecules that trigger the production and release of IL-1 β . LPS is the main outer surface membrane component and is a highly potent PAMP which stimulates innate or natural immunity in various eukaryotic cells [96]. LPS-induced inflammatory responses are linked to the production of ROS in cells [97]. *H. sanjappae* extract was found to possess anti-inflammatory potential in a dose-dependent manner (Table 4). In vitro analysis revealed that it inhibits nitric oxide (NO) radicals. The inflammatory insults caused by LPS are prevented in cells pre-treated with *H. sanjappae* extract, and cytokine level is also reduced as a result. Interleukin-1 β (IL-1 β) is a potent proinflammatory cytokine vital in the host cell defense reaction to infection [98]. After LPS stimulation, macrophages were shown to have a considerably higher level of IL-1 β ; however, pre-treatment with *H. sanjappae* extract at various dosages dramatically reduced the IL-1 β levels in the macrophages. Like IL- β , interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) are significant mediators of innate immunity [99]. LPS also elevated the level of these cytokines. Application of the *H. sanjappae* extract reduced the elevated levels of TNF- α and IL-6.

The bioactive flavonoids present in the *H. sanjappae* extract such as Epicatechin, Luteolin, and Naringenin might play an important role in the anti-inflammatory potential

of the plant. Independent studies have reported the anti-inflammatory potential of these bioactive flavonoid molecules in cultured macrophages and animals [37].

To further explain the mechanism of anti-inflammatory activity, the expression of genes NF-KB and COX2 was assessed. Compared to the untreated LPS control, the *H. sanjappae* extract treatment significantly brought down the expression of NF-KB and COX2. The expression of NF-KB is a crucial event in inflammation, and it is associated with various diseases including cancers. Likewise, the COX2 is a well-known inflammatory enzyme associated with the production of prostaglandins. The expression of COX2 is also evident in different forms of cancers (Figure 2).

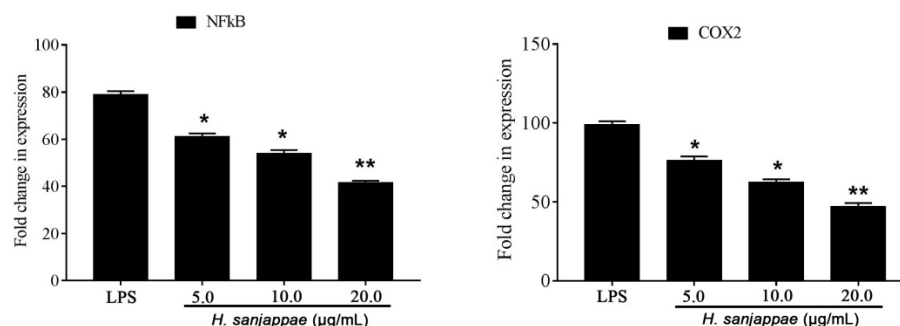


Figure 2. The expression of NF-KB and COX2 genes in the Raw 264.7 macrophages treated with LPS and different doses of *H. sanjappae* leaf methanol extract. * indicates significant variation with respect to LPS control ($p < 0.05$); ** indicates higher significant variation with respect to that of LPS control ($p < 0.01$).

2.5. Antibacterial Activity of *H. sanjappae*

The antibacterial activity of *H. sanjappae* is being documented for the first time, while antimicrobial properties of *H. brunonis* have previously been reported [100]. Previous studies have investigated the antimicrobial potential of different extracts from *H. brunonis*, where the methanolic extract of the leaves [101] and aqueous extract of stem and leaf [33] exhibited significant antibacterial activity. It is worth noting that many species within the genus have not been extensively studied in this regard. Therefore, there remains a considerable knowledge gap regarding the antimicrobial potential of the majority of species within the genus. The present study observed a significant antibacterial activity of the plant against different pathogenic microbes (Table 5). The highest activity was observed against *Pseudomonas aeruginosa* (24.1 ± 0.3 mm) followed by *Salmonella enterica* (22.1 ± 0.1 mm). The lowest activity was observed against *E. coli* (18.5 ± 0.2 mm). The standard antibiotic gentamicin (20 µg) had growth inhibition zones of 21.7 ± 0.5 , 22.1 ± 0.1 , 19.7 ± 0.2 , and 20.5 ± 0.2 mm against *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. enterica*, respectively. Likewise, the minimum inhibitory concentration was found to be highly effective against *P. aeruginosa* (0.625 ± 0.02 mg/mL) followed by *Salmonella enterica* (1.00 ± 0.01 mg/mL). The lowest activity was observed against *E. coli* (1.50 ± 0.01 mg/mL). The MIC values of gentamicin were found to range between 0.325 and 0.625 mg/mL (Table 5).

Table 5. Efficacy of *H. sanjappae* (HSE) as an antimicrobial agent estimated using disc diffusion method and minimum inhibitory concentrations and comparison with gentamicin (GM).

Bacteria	Zone of Inhibition (mm)		MIC Concentration (mg/mL)	
	HSE	GM (20 µg)	HSE	GM
<i>Escherichia coli</i>	18.5 ± 0.2	21.7 ± 0.5	1.50 ± 0.01	0.325
<i>Pseudomonas aeruginosa</i>	24.1 ± 0.3	22.1 ± 0.1	0.625 ± 0.02	0.325
<i>Staphylococcus aureus</i>	20.6 ± 0.3	19.7 ± 0.2	1.25 ± 0.04	0.625
<i>Salmonella enterica</i>	22.1 ± 0.1	20.5 ± 0.2	1.00 ± 0.01	0.625

All the results are indicated as mean \pm standard deviation of six independent experiments.

3. Materials and Methods

3.1. *Humboldtia sanjappae* Collection and Extraction

The *Humboldtia sanjappae* plant samples were collected on 12 December 2022 from Ernakulam District, Western Ghats of Kerala (10.04829° N, 76.8399° E). The mature leaves and the bark of the plants collected were carefully cleaned of all kinds of dust via washing. These materials were dried under shade for 21 days and powdered using a mixer grinder; the powder was extracted with 100% ethanol. About 5 g of each material was subjected to 6 h of extraction. All the extracts were evaporated to dryness using a rotary evaporator, and extract yield was calculated for the same.

3.2. Phytochemical Analysis of *Humboldtia sanjappae*

A preliminary phytochemical analysis of all samples was performed to determine the presence of biologically important secondary metabolites such as alkaloids, flavonoids, terpenoids, steroids, carbohydrates, saponins, etc. All tests were conducted following the conventional procedures described by Yadav, Agarwala, and Harborne [102,103]. The total phenolic content (TPC) and the total flavonoid content (TFC) were determined spectrophotometrically. TPC was found using the Folin–Ciocalteu reagent assay [104]. A standard curve was constructed using Gallic Acid standards. TPC was measured in Gallic Acid Equivalents (GAE). TFC was determined using an aluminium chloride colorimetric assay [105]. The flavonoid content was estimated using the standard quercetin calibration curve. TFC was measured in terms of quercetin equivalents.

The LC-MS analysis was carried out according to the previous methods of House et al. [106]. Briefly, the HR-LCMS-Q-TOF analysis was carried out using Agilent 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA). Accurately, 10 µL of the extract was injected into the system, and the run was carried out using water (0.1% formic acid *v/v*) (A) and methanol (B) as solvents. The gradient elution mode was used as follows: 1–10 min 95% A, 10–20 min 75% A, 20–25 min 50% A, 25–30 min 30% A, 30–40 min 95% B. The flow rate was set at 0.3 mL/min, and pressure was 1200 bar.

3.3. Analysis of the Antioxidant Activity of *H. sanjappae* Ethanol Extract

The antioxidant activities were assessed by evaluating the scavenging potentials of various radicals, such as diphenyl picryl hydrazyl (DPPH) and FRAP (Ferric Reducing Antioxidant Power). These methods allow for the measurement of the ability of the tested samples to neutralize or reduce these radicals, providing insights into their antioxidant properties [96,97]. A solution of DPPH was prepared by dissolving it in methanol at a concentration of 0.1 mM. Varying concentrations of the extract were mixed with the DPPH solution. The resulting mixture was then incubated in a dark environment at a temperature of 30 degrees Celsius for 20 min. The change in absorbance of the solution was measured and used to estimate the percentage inhibition [96]. The stock solutions consisted of a 300 mM acetate buffer (prepared by dissolving 3.1 g of sodium acetate and 16 mL of acetic acid) with a pH of 3.6, a 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution (3.12 mg/mL) in 40 mM HCl, and a 20 mM FeCl₃ solution (3.25 mg/mL). To prepare the fresh working solution, 25 mL of acetate buffer, 2.5 mL of TPTZ solution, and 2.5 mL of FeCl₃ solution were mixed together. The resulting solution was warmed at 37 °C before use. For the FRAP assay, a test sample of varying concentrations was mixed with 2.80 mL of the prepared FRAP solution. The mixture was allowed to react for 30 min under dark conditions. After the incubation period, the absorbance of the colored product, known as the ferrous tripyridyltriazine complex, was measured at a wavelength of 593 nm [98]. The control in the experiment refers to the reaction mixture in which the test sample was not added.

3.4. Analysis of the *H. sanjappae* Ethanol Extract on Activities of Enzymes

To assess the enzyme-inhibitory properties of the test samples, specific enzymes related to diabetes and secondary diabetic complications were targeted. The inhibitory effects on α -amylase [45] and α -glucosidase [46] were examined using standard procedures.

3.5. Effect of *H. sanjappae* Ethanol Extract on Lipopolysaccharide-Induced Anti-Inflammatory Activity in Macrophages

The murine Raw 264.7 cells were seeded at 1×10^7 cells/mL in a 24-well plate containing complete growth media. The *H. sanjappae* extract was diluted in RPMI-1640 media at different concentrations (5, 10, and 20 μ g/mL). A standard anti-inflammatory compound, aspirin, was also used as positive control at a concentration of 1 mM. The cells were then treated with lipopolysaccharide (LPS) at a concentration of 1 μ g/mL for 24 h. The levels of inflammatory cytokines such as interleukin-1 β , interleukin-6, and tumor necrosis factor- α were measured using PeproTech ELISA kits. The production of nitric oxide in the media by the macrophages was quantified biochemically using the Griess method [106]. The gene expression of NF-KB and Cyclooxygenase 2 was determined by real-time PCR according to the $\Delta\Delta$ CT method. Briefly, the total RNA was isolated, and cDNA was synthesized using standard kits from Takara (Bangalore, India). The real-time PCR analysis was carried out using the temperature cycle of 95 °C (melting temperature) for 15 s, 60 °C (annealing temperature) for 45 s, and 73 °C (extension temperature) for 30 s. The cycle was repeated 40 times, and CT values were calculated using the Applied Biosystems 7300 software. The primer sequences used in the study are listed in Supplementary Table S5.

3.6. Antibacterial Activity of *H. sanjappae* Ethanol Extract

The antibacterial activity of *H. sanjappae* was estimated in terms of the disc diffusion method according to the methods of Webber et al. [107]. The extracts were placed in circular discs and kept in the bacterial culture plate at 80 mm distance to one another. The growth inhibition zone in each of the bacterial cultures was determined and expressed as zone of inhibition in mm. The MIC value was determined according to the previous methods of Morgan et al. [108]. The gentamicin was used as a standard antibacterial agent at a concentration of 20 μ g.

3.7. Statistical Analysis

The data were represented as mean of three independent experiments with triplicate analysis. The statistical operations were carried out using GraphPad Prism 7.0.

4. Conclusions

Most of the species in the genus *Humboldtia* have not been evaluated for their pharmacological potential despite their relevance in ethnomedicine. The present study for the first time reports the phytochemical composition and antioxidant, antimicrobial, and anti-inflammatory activities of *H. sanjappae*, a native of the Western Ghats of India. The study concludes that the plant has strong antioxidant properties in terms of radical scavenging and reducing potentials, and it is also effective as an antibacterial agent. Further, the extract inhibited the cytokine levels in Raw 264.7 macrophages, which is indicative of its anti-inflammatory properties. Enzymes such as α -amylase and α -glucosidase are important in controlling how our bodies absorb carbs and are frequently targeted by diabetic drugs [79]. Indeed, the potent antidiabetic actions of HSE may be connected to its capacity to inhibit α -amylase and α -glucosidase. The extract may help regulate blood sugar levels and successfully manage diabetes by inhibiting certain carbohydrate-processing enzymes.

By carefully studying and testing, we have clearly shown that bark extract of *H. sanjappae* made with ethanol is really good at reducing inflammation, controlling diabetes, fighting bacteria, and acting as an antioxidant. These different benefits not only highlight how valuable *H. sanjappae* is, but also remind us that using plants for medicine has always been a great way to create a variety of medicines. There are many examples from

history where medicinal plants have led to big changes in medicine. For example, aspirin, which comes from the bark of the willow tree (*Salix alba* L.), changed how we manage pain and helped develop other drugs such as NSAIDs (non-steroidal anti-inflammatory drugs) [109]. Additionally, the Madagascar periwinkle plant (*Catharanthus roseus* L.) gave us vinblastine and vincristine, powerful compounds that have really changed how we treat cancer [110].

Significantly, more than half of the drugs utilized worldwide in modern pharmaceuticals have their origins in natural sources [111,112]. The worldwide commercial success of established and effective pharmaceuticals taken from many plant kinds demonstrates the importance of medicinal plants as potential drug reservoirs. Quinine, an anti-malarial alkaloid derived from the bark of *Cinchona officinalis* L., is one example. Furthermore, chloroquine, derived from quinine, not only modulates inflammatory autoimmune responses but has recently shown promise in anticancer therapy [112,113].

The polypharmacological potential of *H. sanjappae*, as evidenced by its diverse array of beneficial properties, aligns perfectly with this lineage of discovery. Its capacity to simultaneously tackle a range of health factors—spanning from inflammation and diabetes to bacterial infections and oxidative stress—resonates with the holistic approach of medicinal plants. These qualities offer the potential for more complete and refined therapeutic treatments, acknowledging the complexities of human health.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules28196875/s1>. Table S1: Qualitative analysis of phytochemicals present in different extracts of *Humboldtia sanjappae*; Table S2: Percentage yield of different extracts of *H. sanjappae*; Table S3: Antioxidant activity of different extracts of *H. sanjappae*; Table S4: Total phenol and total flavonoid contents of different extracts of *H. sanjappae*; Table S5: The forward and reverse primer sequences of different genes used for real-time PCR analysis.

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Sample Availability: Samples of the compounds are available from the authors.

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