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Regular paper

Isolation, preparation and investigation of leaf extracts of *Aloe barbadensis* for its remedial effects on tumor necrosis factor alpha (TNF-α) and interleukin (IL-6) by *in vivo* and *in silico* approaches in experimental rats

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***Aloe barbadensis* is a stemless plant with a length of 60–100 cm with juicy leaves which is used for its remedial and healing properties in different suburbs of various countries. The present study was conducted to investigate the effect of *A. barbadensis* leaf extract (aqueous and ethanolic) in yeast induced pyrexia and acetic acid induced writhing in rat model to evaluate the antipyretic biomarkers and its phytochemical screening with computational analysis. For analgesic activity model 60 Albino rats (160–200 kg) were divided into four groups. Of the 4 groups, control consisted of 6 rats (Group I) treated with normal saline, standard comprised of 6 rats treated with drug diclofenac (Group I). Experimental groups consisted of 48 rats, treated with *A. barbadensis* ethanolic and aqueous leaf extracts at doses of 50 mg/kg, 100 mg/kg, 200 mg/kg, and 400 mg/kg (Group III. IV). For antipyretic activity group division was same as in analgesic activity. All groups were treated the same as in the analgesic activity except for the second group which was treated with paracetamol. In both antipyretic and analgesic activity at the dose of 400 mg/kg, group III showed significant inhibition. TNF–α and IL-6 showed significant antipyretic activity at a dose of 400 mg/kg. For molecular docking aloe emodin and cholestanol were used as ligand molecules to target proteins Tnf-α and IL-6. Acute oral toxicity study was performed. There was no mortality even at the dose of 2000 mg/kg. Quantitative and qualitative phytochemical screening was performed for the detection of various phytochemicals. Hence, *A. barbadensis* leaf extracts can be used in the form of medicine for the treatment of pain and fever.**

Keywords: induced pyrexia, pain, antipyretic biomarkers, *in-silico, in vivo, Aloe barbadensi*

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Abbreviations: COX-1 and COX-2, Cyclooxygenase 1 and 2; IL-6, interleukin 6; NSAIDs, Nonsteroidal Anti-Inflammatory drugs; TNF-α, tumor necrosis factor alpha

INTRODUCTION

An unpleasant signal that something hurts, which is basically a complex experience that differs greatly from person to person, is called pain. We can also define pain as a somatic sense of severe discomfort, a sign of a disorder and physical injury or even emotional sorrow. The body's defense mechanisms depend largely on pain which also serves as a rapid warning system that instructs the motor neurons in the central nervous system to prevent physical harm. There are two categories of pain: acute and chronic pain (Kumar *et al.,* 2015). NSAIDs reduce pain by blocking the formation of prostaglandins, which is accomplished by decreasing the activity of the enzyme Cyclooxygenase 1 and 2 (COX-1 and COX-2) (PG) (Hayat *et al.,* 2023; Naveed *et al.,* 2023; Waseem *et al.,* 2023; Aqib *et al.,* 2023; Naveed *et al.,* 2022a; Naveed *et al.,* 2022b; Ayesha *et al.,* 2022; Li*et al.,* 2009). Fever is a common component of inflammation in animals, and it amplifies the host's reaction. The hypothalamus generally regulates fever, but certain bacterial or viral illnesses can promote the formation of pyrogens, substances that effectively alter the hypothalamic "thermostat setting" to raise body temperature and cause fever. Endogenous or exogenous pyrogens are both possible (Walter *et al.,* 2016). Infection, tissue injury, inflammation or other disease conditions can cause pyrexia or fever. Among the most common symptoms of these conditions is an increase in the production of cytokines, such as Interleukin-1, Interleukin-6, interferon, and tumor necrosis factor among others. PGE2 synthesis is boosted by the cytokines because they stimulate the arachidonic acid pathway. To elevate body temperature PGE2 activates the hypothalamus, which causes it to increase heat production while minimizing heat loss (Ahmad B *et al.,* 2023; Mathew *et al.,* 2021).

Thousands of people around the world, mostly in developing nations, depend on herbal medication for their health. Traditional and complementary medicine practitioners have extensive experience in the inhibition, diagnosis, and management of a wide range of disorders including infectious diseases, allergies, and hypertension (Hamid *et al.,* 2023; Ammara *et al.,* 2023; Ejaz Ahmad

et al., 2023; Ahmad *et al*., 2023; Rauf *et al.,* 2023; Nayakudya *et al.,* 2020; Sajjad *et al.,* 2014). *Aloe barbadensis* is one of the most popular plant these days and it is attracting a lot of scientific attention. The use of this plant may be traced all the way back to the Babylonian era. It grows in warm areas and is unable to survive in freezing temperatures. According to WHO estimates, traditional medicines are being used by up to 80% of the population. *A. barbadensis* is a stemless plant with a length of 60–100 cm with juicy leaves (Sana *et al.,* 2023; Aziz *et al.,* 2023a; Hena *et al.,* 2023; Wajid *et al.,* 2023; Nureen *et al.,* 2023; Gul *et al.,* 2023). On several plants, *A. barbadensis* bears thick green leaves with white dots on the lower and upper surface of the stem. In Ayurveda, ghritkumari is defined as an *A. barbadensis* being a blood cleanser, anti-inflammatory, uterine tonic, laxative, diuretic, spermatogenic and fever reliever (Saleem *et al.,* 2023; Aziz *et al.,* 2023b; Narjis *et al.,* 2023; Zawar *et al.,* 2023a; Farooq *et al.,* 2022).

Many phytochemicals, vitamins, minerals, and antioxidants are found in the plant. A class of substances known as phytochemicals (derived from the Greek word "phyto," which literally means "plant") are physiologically active molecules found in minute amounts in plants that are not recognized as nutrients, but which appear to protect against degenerative diseases (Zawar *et al.,* 2023b; Tayyaba *et al.,* 2023; Zawar *et al.,* 2023c; Dalia *et al.,* 2017). Molecular docking is a computer simulation technique for predicting the form of a receptor-ligand complex, in which the receptor is a protein, or a nucleic acid molecule (DNA or RNA) and the ligand is a small molecule or another protein; the receptor and ligand are both proteins. Also known as simulation-based prediction, it is a technique for forecasting the location of a ligand in an expected or pre-defined binding site (Ahmad *et al.,* 2023; Bermen *et al.,* 2000).

MATERIALS AND METHODS

Collection of samples and their extracts

A. barbadensis leaves were collected, aqueous and ethanolic extract was prepared by cold maceration method (Singh *et al.,* 2010).

Albino rats

Albino rats (160–200 g) of both sexes were purchased from the animal home for the experiment. Rats were kept in polypropylene cages at the University of Lahore animal house. Rats were fasted before being used in the experiments. After that they were given distilled water and balanced feed.

Groups and treatment schedule

In group I, rats were treated with distilled water 10ml/kg. In analgesic and antipyretic activity in group II rats were treated with diclofenac and paracetamol 100 mg/kg. In group III and IV rats were treated with different quantities of *A. barbadensis* ethanolic and aqueous leaf extracts.

Steps

Fever induced by yeast.

All the groups were injected with yeast below the nape of neck to induce fever after 21 hours, and the highest temperature was 101.58 Fahrenheit**.** Control rats were injected with normal saline, group II, the rats were treated with a dose of paracetamol, while group III and IV rats were treated with the different concentrations of ethanolic and aqueous leaf extracts of *A. barbadensis*. The body temperature (rectum) of rats was measured with digital thermometer after the equal time intervals until fourth hour.

Analgesic activity model

All groups were treated the same as in the analgesic activity except the second group which was treated with diclofenac.

Procedure

Writhing induced by acetic acid

By the injection of acetic acid, the writhing process began in rats. It was injected to determine the potential of leaf extract of *A. barbadensis* in pain process. But 1 hour before the experiment, rats in group I were injected with normal saline (10 ml/kg) intraperitoneally. In group II, the rats were treated with a dose of diclofenac 100 mg/kg. While in groups III and IV the rats were treated with different concentrations of ethanolic and aqueous leaf extracts of *A. barbadensis* at a dose of 50, 100, 200, and 400 mg/kg. For counting the writhes, a stopwatch was used. The rats were placed into different cages during activity.

Acute oral toxicity study

Acute oral toxicity study was carried out using a protocol as described by Srinivasan *et al* (2018).

Qualitative phytochemical analysis of *Aloe barbadensis*

Qualitative and quantitative phytochemical analysis of *A. barbadensis* was done by standard protocol (Usman *et al*., 2020).

In silico antipyretic and analgesic activity of *A. barbadensis*

For *in-silico* investigation, various software of bioinformatics was used to support *A. barbadensis* antipyretic and analgesic activity. Chemskech, chimaera, pymol, pyrx, depth residue, and discovery studio were the computational tools used.

Data analysis

Data were analyzed using PROC GLM in SAS software (version 9.1).

RESULTS

Ethanolic and aqueous extracts of *A. barbadensis*

In this study, the potential of *A. barbadensis* leaf extracts at different doses were observed against rat models. The difference between the groups of rats treated with extracts and the control group was at a significant level. All groups showed a remarkable increase in temperature after being injected with yeast. Group I showed a marked increase in temperature after 1–4 hrs of injection. Meanwhile, experimental groups treated with *A. barbadensis* leaf extracts reduced the temperature from 1 to 4 hours as compared to group I. The improvement was more pronounced at fourth hour in a dose depend-

Table 1. Inhibition (%) of pain by ethanolic and aqueous extracts of *A. barbadensis* and diclofenac on acetic acid induced pain in rats

Treatment	Ethanolic	Aqueous
Control	18.83±0.40	18.83±0.40
Standard	7.36±0.91 (61%)	7.36±0.91 (61%)
Treated at dose 50mg/kg	17.00±0.41 (9%)	17.75±0.95 (-5%)
Treated at dose 100mg/kg	15.25±0.48 (19%)	16.5±0.29 (12%)
Treated at dose 200mg/kg	12.25±0.75 (34%)	13±0.91 (30%)
Treated at dose 400mg/kg	6.50±1.04 (65%)	9.75±0.63 (48%)
p-value	<0.0001	<0.0001

ent manner. Within the experimental groups, *A. barbadensis* leaf ethanolic extract (group III) showed the most pronounced effect as compared to *A. barbadensis* leaf aqueous extracts (group IV). Interleukin-6 and TNF-alpha concentrations in the plasma were increased as a result of yeast injection. In both ethanolic and aqueous extracts TNF-alpha and IL-6 marker values differ considerably between treatment groups. Effect of *A. barbadensis* leaf extracts on IL-6 and TNF-alpha in group III and IV indicated a significant decrease in pyrexia in comparison to control (Fig. 1 and Table 1).

Analgesic activities

In analgesic activity, all groups showed a significant increase in pain which was induced with acetic acid. Group I showed the most evident increase in pain after injection. While group III and IV treated with *A. barbadensis* leaf extracts reduced the temperature as compared to group I.

The acute toxicity results showed that the ethanolic extracts had a high safety profile as neither death nor signs related with toxicity were observed at the highest dose level (2000 mg/kg orally) in the rats. Rats did not show any change in their gross behavior or associated stereotypical symptoms as shown in Table 2.

Qualitative phytochemical screening of aqueous and ethanol gel extracts of *A. barbadensis*

The results of qualitative phytochemical screening of the aqueous and ethanol gel extracts of *A.barbadensis*

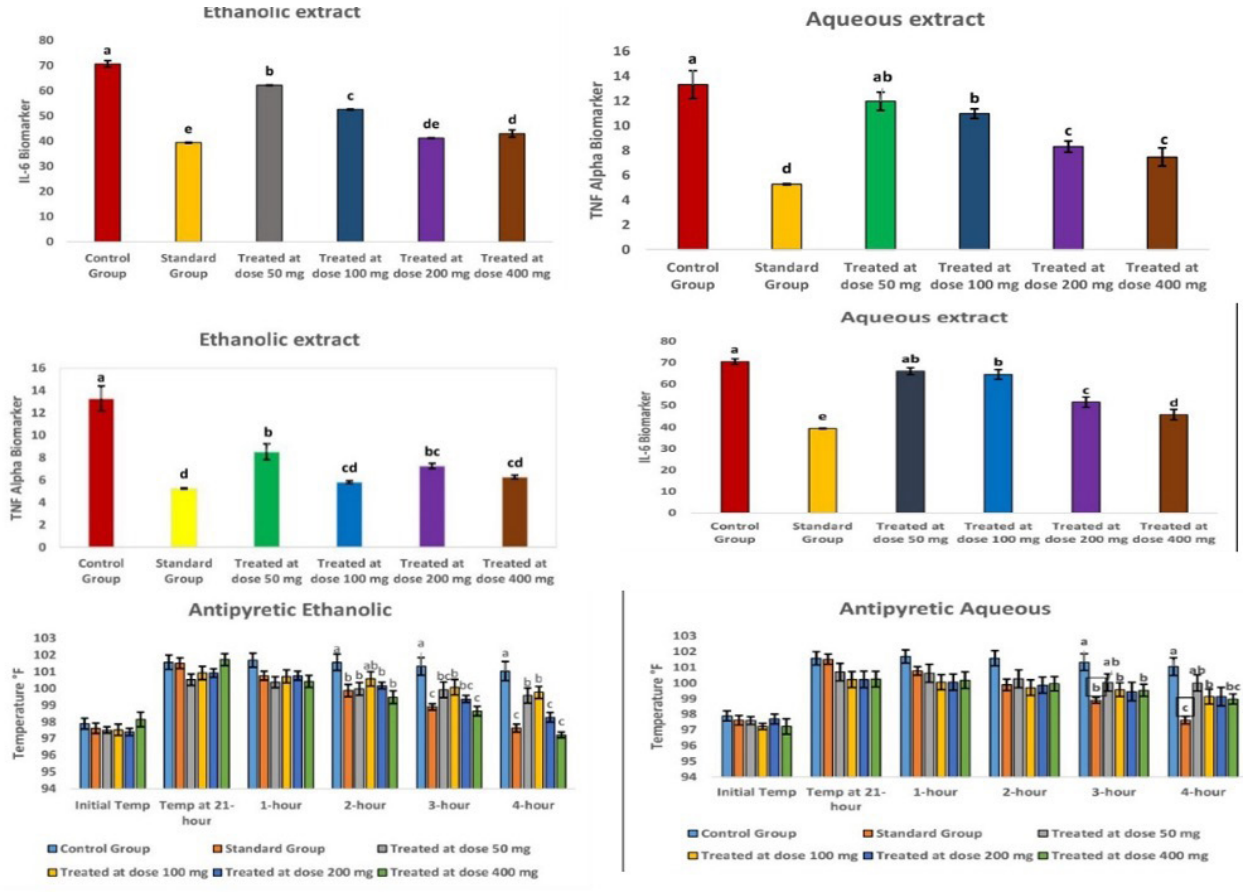

Figure 1. TNF Alpha and IL-6 showing significant upregulation and downregulation and temperature of rats (F°) at 1–4 hours post yeast injection in group I, II, III and IV

Table 2. Effect of acute toxicity by ethanolic extracts of *A. barbadensis* and normal saline in rats

Treatment	Control	Dose A	Dose B	Dose C	*p*-value
AST (µL)	7.40^{c} ±1.21	35.40^{b}±8.78	243.20^{a}±7.74	11.40^{c}±1.36	< 0.0001
ALT(µL)	9.80^{c}±2.08	34.00^{b}±7.09	53.60^{a}±7.59	13.60^{c}±1.57	< 0.0001
Alkp_v	44.60^{b}±7.31	70.80^{b}±10.27	154.40^{a}±9.52	56.40^{b}±8.79	< 0.0001
Bilurobin (mg/dl)	1.68^{a}±0.16	1.50^{a}±0.25	1.88^{a}±0.49	0.36^{b}±0.12	0.0091
Total protein (g/dl)	7.46^{a}±0.27	5.24^{b}±0.63	7.12^{a}±0.43	4.56^{b}±0.70	0.0032
Albumin (pg/dl)	3.20^{ab}±0.29	3.32^{ab}±0.36	4.42^{a}±0.76	2.04^{b}±0.36	0.0247

Superscripts on different means within row differ significantly at $p \leq 0.05$. Dose A=500 mg/kg; Dose B=1000 mg/kg; Dose C=2000 mg/kg

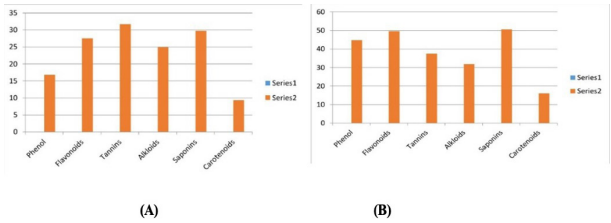

Figure 2. Qualitative phytochemical screening of the aqueous and ethanol gel extracts of *Aloe barbadensis*

Table 3. Qualitative phytochemical screening of the aqueous and ethanol gel extracts of *Aloe barbadensis*

S/N	Phytochemical components	Ethanoic extract	Aqueous extract
1	Tannins	+	+
2	Saponins	+	+
3	Anthraquinones	+	–
4	Glycosides	+	+
5	Alkaloids	–	+
6	Flavonoids	+	+
7	Phenolics	+	+
8	Steroids	–	–
9	Terpenoids	–	–

showed the presence of tannins, saponins, phenolics and flavonoids. Proteins were present in ethanolic extract but absent in aqueous extract while glycosides and alkaloids were present in aqueous extract but absent in ethanolic extract. Terpenoids and steroids were absent in both extracts (Table 3).

Quantitative phytochemical screening showed that ethanolic extract showed a high quantity of phytochemicals as compared to aqueous extract. These bioactive chemicals are important in medicine because they have anti-inflammatory, anti-diabetic, analgesic, anti-oxidant, and antipyretic properties (Fig. 2).

In-silico studies

The objective of the in-silico analysis is to study the potential of phytochemicals to target the cytokine TNF-Alpha and Il-6 protein, hence, showing its antipyretic and analgesic activity. The molecular binding score of the phytochemicals of *A. barbadensis* and the standard drug paracetamol against target TNF-Alpha protein and IL-6 was -4.2 and -5.9 kcal/mol and -6 while the cholestanol showed the highest score amongst the three (Fig. 3 and Fig. 4).

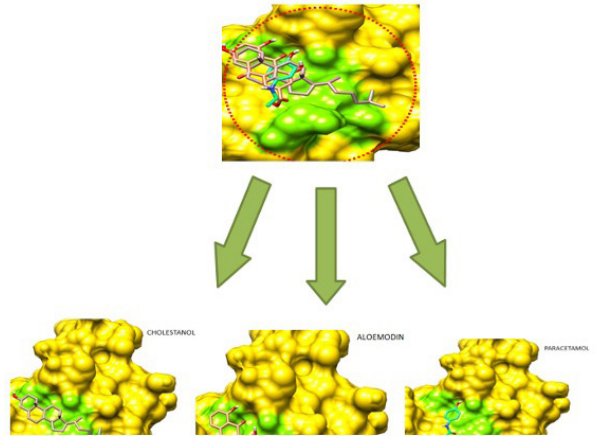

Figure 3. IL-6 and ligand molecules aloe emodin, cholestanol and paracetamol.

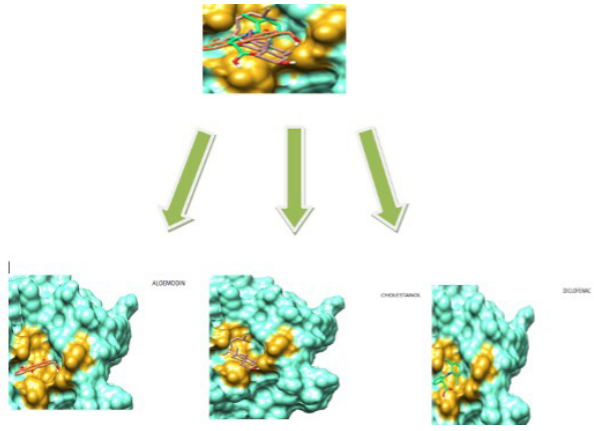

Figure 4. IL-6 and ligand molecules aloe emodin, cholestanol and diclofenac

DISCUSSION

This study was conducted to evaluate the medicinal prospective of *Aloe barbadensis* ethanolic and aqueous leaf extracts against pain and fever. The presence of distinct chemical ingredients was recognized during phytochemical screening of aqueous and ethanolic ex-

tracts of *A. barbadensis*. Previous studies reported that several endogenous pyrogens, including prostaglandin, interleukin-1, interleukin-6, interleukin-8, tumour necrosis factor-alpha, and macrophage protein-1 to induce fever. Tumor necrosis factor and phospholipase A2 may activate prostaglandin production. Brewer's yeast causes pathogenicity by inducing TNF-α and prostaglandin production (Ridtitid *et al.,* 2008).

In the present study, fever was significantly ($p<0.05$) reduced in group III and IV in a dose dependent manner. The higher the dose (400 mg/kg) the greater was inhibition of fever. Brewer's yeast intravenously causes pyrexia by increasing the synthesis of prostaglandins. It is a beneficial test for determining the antipyretic activity of plant materials as well as synthetic medications (Khan *et al.,* 2009). Antipyretic efficacy could be mediated by inhibiting prostaglandin synthesis, similar to how paracetamol works by inhibiting cyclo-oxygenase enzyme activity. Pyrexia is caused by a variety of mediators and inhibiting these mediators has an antipyretic effect. Results of another study were concurrent with our findings (Safari *et al.,* 2016; Okokon *et al.,* 2010; Demoze A *et al.,* 2020; S Alyas *et al.,* 2023; Ghosh *et al.,* 2015; Velázquez-González *et al.,* 2014). Previous studies revealed that antipyretic activity of these extracts could potentially be due to the presence of alkaloids. Prostaglandins, which are involved in pyrexia, are known to be targeted by flavonoids. As a result, the presence of flavonoids in *A. barbadensis* aqueous leaf extract may contribute to its antipyretic activity (Hussain *et al.,* 2022; Safari *et al.,* 2016).

The medicinal ability of ethanolic and aqueous leaf extract of *A.barbadensis* was evaluated against pain. A system of sensory neurons and neural afferent pathways that selectively respond to potentially unpleasant, tissue-damaging stimuli support the sense of pain. The myelinated A delta and unmyelinated C fibres, which are found in skin nerves and in deep somatic and visceral regions, are most responsive to painful stimuli. The primary afferent nociceptors are these pain receptors (Okokon *et al.,* 2010). In this study, fever was remarkably decreased in group III and IV in a dose dependent manner. Endogenous substances, such as bradykinins, serotonin, progesterone, histamine, and substance P are released when acetic acid is exposed to a painful stimulus. The abdominal constriction reaction is thought to be mediated by local peritoneal receptors. Therapy has been linked to levels of PGE2 and PGF2 in peritoneal fluids as well as lipoxygenase products. Previous studies confirmed that the chemo sensitive nociceptors of rats were activated by intraperitoneal dose of acetic acid, resulting in abdominal writhing (Apu *et al.,* 2012; Ara *et al.,* 2010; Zulfikar *et al.,* 2010). The findings of this study are congruent with another study (Ghosh *et al.,* 2015).

The phytocompounds found in the ethanolic and aqueous extracts of *A. barbadensis* may be responsible for the analgesic effects shown in this investigation. The ethanolic extracts may have functioned in a similar way to NSAIDs by inhibiting the COX pathway metabolically. The cyclooxygenase and lipoxygenase pathways, which are important for peripheral nociception, have been revealed to be inhibited by flavonoids (Velázquez-González *et al.,* 2014). Previous studies reported that flavonoids reduce prostaglandin production by reducing prostaglandin synthase's action. Prostaglandin production, which is implicated in pain perception via an opioidergic mechanism, has been shown to be targeted by flavonoids (Lenard *et al.,* 2023; Panda *et al.,* 2009). Flavonoids lower intracellular calcium levels through inhibiting *N*-methyl-D-aspartate (NMDA) receptor activation. The nitric oxide synthase enzyme and phospholipase A2 activity are lowered, leading to a reduction in NO and prostaglandin formation (Ara *et al.,* 2010; Valdes *et al.,* 2023). Previous studies reported that analgesic properties have been attributed to tannins, alkaloids, and steroidal substances. Terpenoids have also been linked to antinociceptive properties via inhibiting thrombocyte aggregation and interfering with pain signaling processes (Ali *et al.,* 2012). Acute toxicity was also performed to evaluate possible adversative effect of repetitive extract's administration to rats at different doses. Our study was correlated with a previous study (Paul *et al.,* 2018; Devaraj *et al.,* 2011).

CONCLUSIONS

Qualitative phytochemical study showed that the crude extract of *A. barbadensis* showed the presence of alkaloid, saponins, flavonoid, terpenoids, tannins, anthraquinones, phenols and steroids. The presence of phytochemicals, such as polyphenols and flavonoids in plants, reduces the risk of chronic diseases and increases the ability of biological systems to trap highly reactive free radical species. Its antipyretic and analgesic action is attributed to the presence of these polyphenols. The results of phytochemical screens were identical to those discovered by other researchers, including alkaloids, saponins, tannins, flavonoids, and steroids.

Declarations

Ethical Approval. The ethical approval for this study was provided Molecular Biology and Biotechnology Bioethical, Biosafety and Biosecurity Committee, The University of Lahore under Ref no: CRiMM/22/Research /146 dated 02/12/2022.

Competing interests. The authors declare no conflict of interest.

Availability of data and materials. All the data has been included in the manuscript.

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