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Original Research Article

Prostaglandin Synthesis Inhibitory Activity of *Heliotropium indicum* L. (Boraginaceae) and HPLC-DAD Analysis

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ABSTRACT

Dysmenorrhea a painful gynecological problem reported by women in their reproductive years, is a common condition and is refers to painful menstruation with severe cramping affecting the lower part of the abdomen caused by excessive prostaglandin production within secretory endometrial cell. Already existing drugs have numerous limitations. The objective of the study is to evaluate the cyclooxygenase inhibitory and radical scavenging potential of *Heliotropium indicum* L. (Boraginaceae) in the management of dysmenorrhea. The entire dried *Heliotropium indicum* plant was extracted by successive extraction procedure. Cyclooxygenase assay was carried out using COX-2 Test kit and ABTS assay was also carried out using ABTS Radical cation decoloration assay. HPLC-DAD was used to detect polyphenolic molecules. In cyclooxygenase assay, methanol extract showed highest anti-inflammatory activity with the lowest IC_{50} of 0.553±0.05 mg/mL when compared with Ibuprofen (IC_{50} of 0.065±0.00 mg/mL) at 1-0.03125 mg/mL, n-hexane and ethyl acetate have IC_{50} of 0.782±0.01mg/mL and 0.608±0.01mg/mL, respectively. *Heliotriopium indicum* extract also showed an antioxidative activity when tested with ABTS radical cation decoloration with ethyl acetate having the highest activity (IC_{50} of 0.315±0.00 mg/mL) when compared with Trolox (IC_{50} of 0.034±0.00 mg/mL) at 1-0.03125 mg/mL. Tannic acid, naringenin, garlic acid, quercetin, maleic acid and saponin were identified from HPLC-DAD analysis at various concentration. *Heliotriopium indicum* has the potential to prevent the production of excessive prostaglandin by its ability to inhibit cyclooxygenase-2. Therefore, could be a source of new COX-2 inhibitors.

***Keywords*:** Dysmenorrhoea, *Heliotropium indicum*, Boraginaceae, Antioxidant, Anti-inflammatory, Cyclooxygenase-2, High-Performance Liquid Chromatography-Diode Array Detection

Introduction

Dysmenorrhoea is the medical term for painful menstrual periods caused by uterus contractions, which affects 45 to 95% of menstruating women.[1-2] It can be primary or secondary and is a common complaint among adolescents and women of reproductive age.[3] A study in eastern Nigeria showed that there was a prevalence of 25% of dysmenorrhea, 25% of which were severe enough to prevent them from attending school.[4] Other studies reported a much higher incidence rate of 72.3% and 77.8%, respectively.[5-6]

COX-1 and COX-2 are two closely related forms of cyclooxygenase which transform arachidonate into prostaglandins. The significant differences in their body distribution and role in health and disease lies in the regulation of genes of COX-1 and COX-2.[7-8] COX-1, the predominant constituent form of the enzyme, is expressed throughout the body and provides certain homeostatic functions, such as maintaining normal gastric mucosa, influencing the flow of renal blood, and supporting blood clotting by facilitating platelet aggregation.[9]

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On the contrary, COX-2, the inducible form, is expressed in response to inflammation and other physiological stimuli and growth factors and it's involved in the production of prostaglandins that mediate pain and support the inflammation process.[10]

Efforts have been made to develop drugs that specifically inhibit COX-2 with little or no side effects.[11]The introduction of selective COX-2 inhibitors has the promise of improving the treatment of inflammation processes without the gastrointestinal effects and renal failure associated with existing drugs.[12-13] During menstruation, prostaglandins are released from the endometrium, increasing intrauterine pressure and developing uterine ischemia. Ischemia leads to proinflammatory reaction cascades that lead to the production of reactive oxygen radicals.[14] Oxidative stress has been associated with more than one hundred diseases.[15-16] Increased lipid peroxidation has also been associated with dysmenorrhea, which is an index of oxidative stress.[17] The end products of reactive oxygen species and associated metabolites have become targets in the treatment approach to diseases associated with acute inflammation and endothelial dysfunction.[18] In other cases, if oxidative stress plays a role in the severity of menstrual pain among other factors, antioxidant therapy may be considered as further therapeutic intervention.[19]

Plants contain potential secondary metabolites, such as phenols, triterpenes, flavonoids, and cinnamic acid, which are responsible for their anti-inflammatoryactivity.[20] Harpagoside,an anti-inflammatory component of *Harpagophytum procumbens*, is reported to prevent arachidonic acid metabolism and the biosynthesis of eicosanoid, thereby inhibiting COX-2 and reducing inflammation.[21] *Heliotropium indicum* L. (Boraginaceae) (Figure 1) is traditionally used in various

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folklore systems to treat various diseases in different countries around the world. The decoction of leaves and roots is traditionally used for chicken feces, allergies, blood purification, knee swelling in Bangladesh.[22] In Jamaica, women use flower infusion to treat menorrhagia, and in Senegal and the Philippines, it is used to treat kidney stones.[23] Several pharmacological activities of *H. indicum* have been reported, such as the analgesic effects of the air parts in a mouse model of formalin-induced pain compared to morphine,[24] antithrombotic effects,[25] anti-bacterial and antioxidant activity,[26-27] antidiabetic activity,[28] anti-cataract effect [29] and anti-cancer effects.[30]
In Nigerian ethnomedicine, the sap of the stem of *Heliotropium indicum* is used orally by women to treat dysmenorrhea, but there is insufficient scientific evidence to justify these claims, and there are also limitations in existing drugs that required the search for new prostaglandin synthesis inhibitors. Therefore, the aim of this study is to evaluate the cyclooxygenase inhibitory and radical scavenging potential of *Heliotropium indicum* L. (Boraginaceae) in the management of dysmenorrhea.

Figure 1: *Heliotropium indicum* L.

Materials and Methods

Materials
n-hexane, ethyl acetate and methanol, chloroform, acetonitrile, formic acid, hydrochloric acid (HCl), arachidonic acid, ethylenediaminetetraacetic acid (EDTA), potassium persulphate, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) of Analytical grade, Loba Chemie, India.

Plant collection and preparation:
Whole fresh plant of *Heliotropium indicum* was collected in the month of October, 2021 from Apete area of Ibadan, Oyo state. The plant was identified by a taxonomist Mr. Felix Nwafor of the Department of Pharmacognosy and Environmental Medicine, University of Nigeria Nsukka, where a voucher sample was deposited (PCG/UNN/0416) in the herbarium. The plant was cut into pieces to facilitate drying and air dried for two (2) weeks. The dried samples were grinded using laboratory mill into powdered form and weighed using a weighing balance (M411L, M-Metlar, Nigeria) then stored in an air tight container for further analysis.

Plant extraction technique
Successive extraction was used in increasing polarity of eluent to macerate 346.16 g of powdered plant sample starting with 2L of n-hexane for 72 hours after which it was filtered, the marc was air dried and was further macerated with 2L ethyl acetate and 2L methanol, respectively for another 72 hours. After the extraction process, rotary evaporator (Buchi, R-300) was used to concentrate the filtrate *in vacuo* in other to obtain the various fractions and stored in a refrigerator (Nexus, NX-501) at 4^{o}C until required.

Cyclooxygenase inhibitory assay
The COX 2 inhibitory activities of plant extracts (n-hexane extract of *Heliotropium indicum* (HHI), ethyl acetate extract of *Heliotropium indicum* (EHI), methanol extract of *Heliotropium indicum* (MHI)) were evaluated using COX-2 test kit (Catalogue No. 560131, Cayman Chemical, Ann Arbor, MI, USA); and Ibuprofen (UniCure Ibuten) as positive control.[31] The fractions were dissolved in dimethyl sulfoxide and assayed at a final concentration of 1mg/mL-0.03125 mg/mL. The mixture contained 0.96 mL buffer (0.1 M Tris-HCL, pH-8 containing 5 mM EDTA, and 2 Mm phenol), enzyme (0.01 mL), heme (0.01 mL) and 0.01 mL of each test sample. This was incubated for ten minutes at thirty-seven degree Celsius and arachidonic acid solution (0.01 mL) was added. The reaction was terminated with 0.05 mL of HCl (1.0 M). Finally, reduction of PGH 2 to PGF 2α by stannous chloride (0.1 mL) was measured by EIA. The appearance of bright yellow product was read at 412 nm on a microplate reader. The negative control was prepared with COX-2 inactivated in boiling water for three minutes. The inhibitory percent was calculated by the comparison of compound treated by control incubation. The IC_{50} of each sample was extrapolated from concentration inhibition response curve using regression analysis.

ABTS radical scavenging assay
ABTS radical cation decolorization test was adopted to determine the free radical scavenging activity of plant samples.[32] The ABTS radical cation ($ABTS^{\bullet+}$) was generated by a reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1), stored in darkness at ambient temperature for 12-16 hours before use. The $ABTS^{\bullet+}$ solution was diluted with methanol and obtained 0.700 absorbance at 734 nm. After adding 5 µL plant extract (1-0.03125 mg/mL) to 3.995 mL of diluted ABTS•+ solution, the absorbance was measured for 30 min after initial mixing. A suitable solvent blank was performed in each test. All measurements were made at least three times. The percentage of absorption inhibition at 734 nm was calculated using the formula.
ABTS·+ scavenging effect (%) = ((AB–AA)/ AB)×100 (2), where, AB is absorbance of ABTS radical + methanol; AA is absorbance of ABTS radical + sample extract/standard. Trolox was used as standard substance. All determinations were performed in triplicate (n = 3).

Standards preparation for HPLC-DAD analysis
A mixed standard of 50 µg/L of Caffeic acid, Coumeric acid, Malic acid, Gallic acid, Tannic acid, Rutin, Saponin, Naringenin, Naringin, Quercetin, as well as glutathione was used for calibration based on the equipment-generated standard curve.

HPLC-DAD analysis
The sample (1 g) was dissolved with 1,000 µL of methanol then transferred into a vial for HPLC analysis and the sample ran twice alongside the calibrated standards.

HPLC analysis of various concentrations of the unknown samples was performed using Agilent 1260 Infinity II LC series. Equipped with Diode Array Detector (DAD) WR G71115A (DEAC606992), Column Oven of G7130 (DEAEQ22974), Quat Pump VL G711A (DEAEY01907), Auto-Sampler G7129A (DEAEQ22974) with a Poroshell Column 120 EC-C18 4µm (4.6x150 mm), PN 693970-902 (T), SN USHKB12136, LN B18447. Examination of the various samples was performed with a multi-gradient program by utilization of 0.1% formic acid and Acetonitrile (60:40) as mobile phase, injection volume of 20 µL, oven temperature 28°C with a constant flow rate of 0.700 mL/min, a wavelength of 257 nm with a total running time of 11min.

Formula for calculating the concentration (mg/mL) of phytochemicals is below:

$$Y = \frac{\textbf{Average peak area of sample}}{Average\ peak\ area\ of\ standard} x\ \text{conc of standard}\ x \frac{volume\ of\ diluent}{weight\ of\ sample}$$

Statistical analysis
Graphpad prism 7 was used for the statistical analysis. All data was expressed as mean± S.D. and of triplicate parallel measurements. The statistical analyses were carried out using a single ANOVA, followed by Dunnett's multiple comparison tests at α0.05.

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Results and Discussion

Cyclooxygenase Inhibitory and ABTS radical scavenging activity

Research has shown that women with dysmenorrhea have high levels of prostaglandin hormones that cause inflammation, fever and abdominal pain with cramp. COX-2 inhibitors play an established role in the treatment of inflammatory diseases and have a potential application to the prevention and treatment of other diseases.[33-34] In this study, the inhibitory activity of COX-2 of the entire plant *Heliotropim indicum* was evaluated *in vitro* in order to determine the mechanism by which extracts will show their anti-inflammatory effects using ibuprofen as the standard. All the extracts of *H. indicum* revealed different degree of anti-inflammatory activity with the methanol extract significantly showing the highest activity with the lowest IC_{50} of (0.553±0.05 mg/mL). The IC_{50} of n-hexane extract and ethyl acetate extract was (0.782±0.10 mg/mL) and (0.608±0.01 mg/mL), respectively. The result showed that there is a gradual increase in % inhibition of cyclooxygenase enzyme as concentration increases (Table 1). The extract of *H. indicum* can be verified from the result that it can be used as an anti-inflammatory agent. Kyei *et al*.[35] reported the anti-inflammatory effect of *H. indicum* on lipopolysaccharide-induced uveitis by possibly reducing the production of proinflammatory mediators such as tumor necrosis factor-α (TNF-*α*), prostaglandin E2 (PGE2) and monocyte chemotatic protein-1 (MCP-1). Also, it was reported that *H. indicum* significantly reduced the levels of TNF-α mediates cell survival and pro-inflammatory response.[36] Ren and Torres[37], show that the expression of IL-1and IL-6 was significantly reduced at different doses of *H. indicum*. Srinivas *et al*.[38] reported the anti-inflammatory activity of methanol root extracts from *H. indicum* against carrageenan-induced acute paw inflammation and subacute inflammation models of cotton pellet granuloma.

Table 1: Cyclooxygenases-2 (COX-2) inhibitory activity of fractions of *Heliotropium indicum* whole plant at 1-0.03125 mg/mL

Conc. (mg/mL)	% inhibition of HHI	% inhibition of EHI	% inhibition of MHI	Conc. (mg/mL)	Ibuprofen
1	57.20 ± 5.67	72.30 ± 1.39	67.73 ± 5.12	0.1	67.31 ± 1.66
0.5	40.99 ± 2.21	47.51 ± 0.69	48.34 ± 0.42	0.05	47.23 ± 1.80
0.25	30.06 ± 6.51	34.21 ± 0.69	39.06 ± 1.11	0.025	27.84 ± 1.80
0.125	18.01 ± 6.65	17.45 ± 0.55	31.16 ± 0.97	0.0125	17.31 ± 0.69
0.0625	12.19 ± 1.11	10.94 ± 0.42	31.58 ± 3.60	0.00625	11.63 ± 0.55
0.03125	8.17 ± 3.46	4.02 ± 0.69	16.90 ± 0.28	0.00313	5.26 ± 0.55
IC_{50} (mean ± SD)	0.782 ± 0.01**	0.608 ± 0.01**	0.553 ± 0.05**		0.065 ± 0.00

HHI: n-hexane extract, EHI: Ethyl acetate extract, MHI: Methanol extract
Comparison of extracts with standard was done and level of significance difference represent with asterisk. Extract with no asterisks is not significantly (NS) different from the Standard (Ibuprofen)

Table 2: ABTS inhibitory activity assay of fractions of *Heliotropium indicum* whole plant at 1-0.03125 mg/mL

Conc. (mg/mL)	% inhibition of HHI	% inhibition of EHI	% inhibition of MHI	Conc. (mg/mL)	Trolox
1	47.30 ± 2.70	66.89 ± 0.68	61.42 ± 0.74	0.1	81.76 ± 2.03
0.5	35.81 ± 0.68	62.84 ± 2.03	49.12 ± 0.20	0.05	59.46 ± 2.70
0.25	22.30 ± 0.68	51.35 ± 2.70	35.81 ± 0.00	0.025	52.70 ± 1.35
0.125	16.22 ± 1.35	37.84 ± 1.35	21.62 ± 0.14	0.0125	33.11 ± 0.68
0.0625	10.14 ± 3.38	22.84 ± 1.22	10.54 ± 1.62	0.00625	17.57 ± 1.35
0.03125	5.41 ± 1.35	18.24 ± 2.03	6.95 ± 0.07	0.00313	7.43 ± 0.68
IC_{50} (mean ± SD)	0.987 ± 0.06***	0.315 ± 0.00*	0.676 ± 0.00***		0.034 ± 0.00

HHI: n-hexane extract, EHI: Ethyl acetate extract, MHI: Methanol extract
Comparison of extracts with standard was done and level of significance difference represent with asterisk. Extract with no asterisks is not significantly (NS) different from the Standard (Trolox).

Antioxidants are said to help neutralize free radicals in our bodies and this is thought to promote overall health. Oxidative stress is associated with the pathogenesis of many diseases and can play a significant role in the dysregulation of endothelial cells, including the endothelial cells of the uterus and the severity of dysmenorrhea.[39] Understanding the relationship between oxidative stress and the status of antioxidants in relation to primary dysmenorrhea appears to be crucial to its prevention, diagnosis and treatment.[40] In this study, the ABTS radical scavenging activity of the whole plant of *H. indicum* was evaluated. The result showed ethyl acetate extract has the highest radical scavenging activity of IC_{50} of 0.315±0.00 mg/mL when compared to Trolox (IC_{50} of 0.034±0.00) mg/ml. n-hexane and methanol extract also demonstrated its ability to scavenge ABTS with IC_{50} of 0.987±0.06 and IC_{50} of 0.676±0.00 mg/mL, respectively (Table 2). Phenolic compounds identified are believed to act as primary antioxidants or free radical decanters which have the ability to eliminate inflammatory process which is a complex process resulting to many human diseases.[41-42]

The HPLC separation principle is based on the distribution of the analyte between the mobile and stationary phases. From the analysis, the retention time (min) and Area% for the identified compounds are tannic acid (RT, 2.244, Area %, 0.2647), gallic acid (RT, 2.476, Area%, 3.5270), naringenin/caffeic acid (RT, 2.623, Area%, 4.1235), quercetin (RT, 3.277, Area%, 11.9142), maleic acid (RT, 3.703, Area%, 2.2470) and saponin (RT, 6.204, Area%, 8.6065) (Table 3, Figure 2). The concentrations of phytochemicals detected are as follows: Tannic acid (0.004 mg/mL), naringenin/caffeic acid (0.028 mg/mL), gallic acid (0.038 mg/mL), quercetin (0.067 mg/mL), maleic acid (0.104 mg/mL) and saponin (16.53 mg/mL) (Table 4, Figure 3). These compounds identified by HPLC-DAD analysis are known natural antioxidants, which have broad biological effects, including anti-inflammatory, anti-aging, anti-atherosclerosis and anti-cancer activities which may be responsible for the cyclooxygenase inhibitory and radical scavenging potential of our research plant.[43] For example, it has been reported that naringenin protects cells after damage caused by oxidative stress and inflammatory responses, and this could be attributed to the fact that there are two hydrogen groups in the 5 and 7 positions of the A-ring and a carbonyl group in the 4 positions of the C-ring, which helps naringenin interact with iron and copper ions and thus quenching free radicals.[44-46]

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In addition, naringenin inhibited the release of inflammatory mediators from BV2 microglia stimulated by lipopolysaccharides by inactivating nuclear factor-κB and significantly inhibited the excessive production of nitrogen oxide (NO) and prostaglandin E2 (PGE2)..[47]Gallic acid have been found to show anti-inflammatory activity against the zymosan-induced acute food pad inflammation in mice.[48] Molecular modeling and docking analysis of gallic acid and its structural analogues were considered to be strong COX-2 inhibitors.[49] Experimental evidence also suggests that caffeic acid has a potent antioxidant and anti-inflammatory effect.[50-51] *In silico* study revealed that caffeic acid, a phenolic phytochemical in coffee has therapy potential to inhibit mediator inflammation COX-2[52] and also directly inhibits Fyn kinase activity and UVB-induced COX-2 expression.[53] Quercetin was found to suppress COX-2 expression by inhibiting the p300 signaling and blocking the binding of multiple transactivators to COX-2 promoter.[54] More so, quercetin, a flavonoid found in copious amount from aqueous extract of *Euphorbia heterophylla* was reported to show significant anti-inflammatory activity.[55] In addition, Baharara *et al*.[56] reported that saponins show inhibition of inflammation mediators such as NO, TNF-α and COX-2, and also have significant anti-inflammatory effects on both stages of acute paw swelling induced by carrageenan in mice.[57] Furthermore lonimacranthoide VI, a chlorogenic acid ester saponin was found to inhibit mRNA expression and *in vitro* activity of COX-2 and PGE_2 production in a dose-dependent manner.[58]

Conclusion

Heliotropium indicum has the potential to prevent the excessive production of prostaglandin, which leads to painful menstruation due to its ability to inhibit the cyclooxygenase-2 enzyme. *H. indicum*'s anti-inflammatory and antioxidant activity is due to the abundance of polyphenols identified in them. It is, therefore, recommended that phenolic compounds be isolated with clinical potential in the treatment of dysmenorrhea and other related diseases.

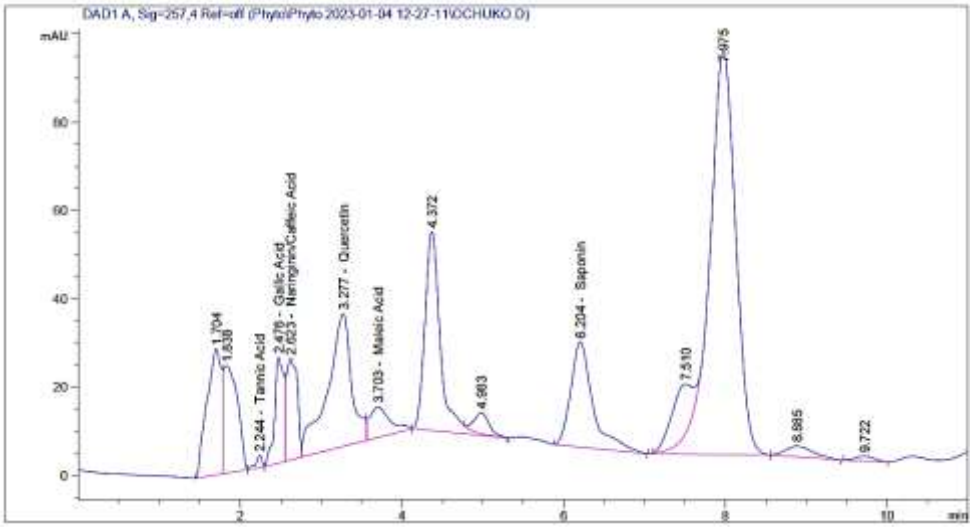

Figure 2: HPLC-DAD chromatogram of ethyl acetate extract of *Heliotropium indicum* whole plant

Table 3: HPLC-DAD analysis data of ethyl acetate extract of *Heliotropium indicum* whole plant

Peak #	RetTime [min]	Width [min]	Area (mAU*s)	Area %	Name of compound
1	1.704	0.1680	352.11612	6.6804	-
2	1.838	0.1657	295.21072	5.6008	-
3	2.244	0.0766	13.95375	0.2647	Tannic Acid
4	2.476	0.1065	185.90089	3.5270	Gallic Acid
5	2.623	0.1242	271.34467	4.1235	Naringinin/Caffeic Acid
6	3.277	0.2954	627.98236	11.9142	Quercetin
7	3.703	0.2453	118.43430	2.2470	Maleic Acid
8	4.372	0.1982	595.40668	11.2962	-
9	4.983	0.1891	61.19262	1.1610	-
10	6.204	0.2760	453.63828	8.6065	Saponin
11	7.510	0.2806	211.01904	4.0035	-
12	7.975	0.3349	2064.00439	39.1588	-
13	8.885	0.3453	57.05576	1.0825	-
14	9.722	0.2631	17.60120	0.3339	-

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Conflict of Interest

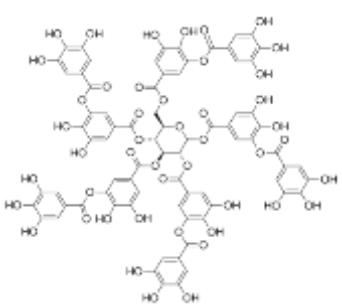
The authors declare no conflict of interest.

Authors' Declaration

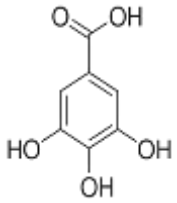
The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 4: Concentration of phytochemicals identified in ethyl acetate extract of *Heliotropium indicum* whole plant using HPLC-DAD

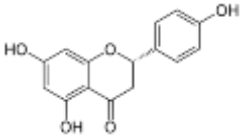
S/N	Phytochemical(s)	Conc. (mg/mL)
1	Tannic acid	0.004
2	Naringenin	0.028
3	Gallic acid	0.038
4	Quercetin	0.067
5	Maleic acid	0.104
6	Saponin	16.53



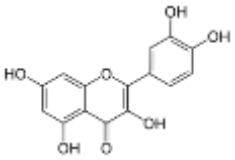
Tannic acid



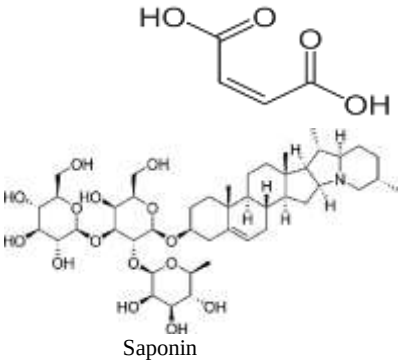
Gallic acid



Naringenin



Quercetin



Saponin

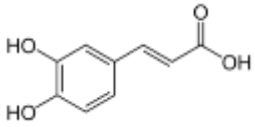


Figure 3: Structures of identified phytochemicals from ethyl acetate extract of *Heliotropium indicum* whole plant using HPLC-DAD

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