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HPTLC ANALYSIS AND IN VITRO BIOLOGICAL ACTIVITY OF *DODONAEA VISCOSA*

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ABSTRACT

Dodonaea viscosa is a plant widely distributed in Saudi Arabia. It is traditionally used to treat diabetes, malaria, inflammation angina, arthritis, etc. However, there have been no reports of work done from the plant grown in Saudi Arabia. The current investigation was done to analyze the presence of plant metabolites using HPTLC analysis and to evaluate the in vitro anti-inflammatory and antioxidant activity of the methanolic extract. HPTLC fingerprint analysis of the extract was carried out using CAMAG HPTLC system and was then scanned using CAMAG Scanner 3 at 365 and 254 nm using WinCATS 4 software. The results of the HPTLC investigation of the methanolic extract proved the presence of nine constituents, two of which were identified as kaempferol and rutin. The methanolic and chloroform extracts were analyzed for antioxidant activity by the DPPH method. The methanolic extract showed better results in comparison to the chloroform extract confirming the scavenging capacity of the extract. The inhibition of albumin and proteinase inhibitory activity methods were used to evaluate in vitro anti-inflammatory activity. The percentage of inhibition of protein denaturation of the methanolic extract was within the range of 78.02-85.09%, while the proteinase inhibitory activity was within the range of 31.03-67.24%. It was concluded that the methanolic extract of *Dodonaea viscosa* possesses anti-inflammatory and anti-oxidant properties and this was attributed to the active phytoconstituents.

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Introduction

The phytoconstituents of plants are of importance, as they possess the power to heal various medicinal disorders. [1] Anti-oxidants have the capacity to scavenge free superoxide radicals and protecting the human biological system against the damaging effects of the various oxidative processes. [2, 3] Oxidative stress occurs when there is an excess of ROS and decrease in antioxidant levels and this may bring about tissue damage resulting in inflammation. [4] Researchers have reported that the antioxidants of plant origin could be very important in terms of therapeutic agents in stress-related disorders. [5] Hydroxyl, Superoxide anion, hydrogen peroxide radicals, and peroxynitrite radicals play an important role in the process of inflammation in various tissues. Compounds of natural origin that have scavenging properties toward these radicals are believed to have therapeutic potentials for various inflammatory diseases. The anti-inflammatory drugs mostly available are potential inhibitors of the cyclooxygenase pathway of arachidonic acid metabolism resulting in the production of prostaglandins. Non-steroidal anti-inflammatory opiates and drugs are the classical drugs in the inflammation but on prolonged use may cause some adverse reactions such as renal damage, gastrointestinal disturbances, respiratory depression, and possible dependence. Hence, this has led to an increasing interest in finding new anti-inflammatory and antioxidant natural drugs and medicinal plants with possibly fewer side effects. [6-8] The research on plants with folkloric use as anti-inflammatory agents and pain relievers can be considered a logical research strategy in the search for new drugs with anti-inflammatory and analgesic properties. The chromatographic fingerprinting technique is a major method used for the routine analysis of herbal remedies as it provides better resolution and estimation of active constituents. This method uses a small quantity of mobile phase to simultaneously analyze several samples. [9] *Dodonaea viscosa* belongs to the Sapindaceae

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family and is wildly grown in the Northern Border Province, Hejaz and Asir in Saudi Arabia (2017) [10]. It is called Daidon or Dodanaia in Arabic. Literature review reveals the presence of various constituents including quercetin, rutin, isorhamnetin, dodoneasides A and B, β-pinene, limonene, myrcene, p-cymene, citronellal, ϒ-terpineol, α-spinasterol, geraniol, fraxetin, syringic acid, cleomiscosin A, cleomiscosin C, and β-sitosterol β-D-glucoside. [11-15] Traditionally, this plant was used to treat skin infections, anti-inflammatory, in rheumatism, liver and smooth muscles disorders, as uterine colic, antipruritic in skin rashes, dermatitis, in hemorrhoids, and sore throat. [16-19] This study was designed with the aim of carrying out the HPTLC analysis and evaluating the in vitro antioxidant and anti-inflammatory activity of the plant *Dodonaea viscosa* grown in Rafha, Northern Border Province, Saudi Arabia as there are no reports on investigation of the plant grown here.

Methodology:

Collection and extraction:

Dodonaea viscosa was collected from Rafha, Saudi Arabia. The plant material was cleaned, shade dried, pulverized to a coarse powder and successively extracted with ethyl acetate, chloroform, petroleum ether, methanol and water. Then the extracts were qualitatively analyzed for identification of various phytoconstituents as per the standard procedures.

HPTLC Procedure:

High-Performance Thin-Layer Chromatography was performed on silica gel 60F254 (10 cm× 10 cm; 0.25 mm layer thickness; Merck). The extract was prepared in 10 ml volumetric flask by taking 250 mg of the extract and diluting with ethanol (25 mg/ml) and filtered using a 0.45 syringe filter from which 10 µl was subjected to HPTLC (CAMAG, Switzerland) analysis. The extract and standard samples were spotted on a silica gel 60F254 (Merck, Darmstadt, Germany) TLC plate. The plate was air-dried and then developed using the mobile phase Toluene: Ethyl acetate: Formic acid (5:4:0.2v/v) in a CAMAG Twin Trough glass chamber, which was previously saturated with mobile phase vapor for twenty minutes. After developing, the plate was dried at 65°C for 2 minutes and then scanned using CAMAG Scanner 3 (CAMAG, Switzerland) at 254 and 365 nm using WinCATS 4 software.

Anti-oxidant activity [20, 21]
The antioxidant activity of the methanolic extract was evaluated using 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) method, which is based on the radical-scavenging effect of the DPPH, prepared in methanol and mixed with 1 ml of both the standard and the samples. The mixture absorbance was measured at 517 nm. Methanol with DPPH was used as a blank. The absorbance percentage was calculated using the formula:

$$\% \text{ Absorbance } = \frac{A - B}{A} \times 100$$

A and B are absorbance of the blank and sample, respectively.

Anti-inflammatory activity [22, 23]
The in vitro anti-inflammatory activity was assessed by proteinase inhibitory effect and the inhibition of albumin denaturation.
Inhibition of albumin denaturation: A mixture consisting of different concentrations of the extract and 1% aqueous solution of bovine albumin fraction was prepared. 1N HCl was used to adjust the pH. The samples were heated at 57 ºC for 20 min, cooled, and the turbidity was measured at 660 nm.
Protein denaturation was calculated as the percentage of inhibition by using the following formula.
Percentage of inhibition = (Abs control – Abs sample) × 100/ Abs control
Proteinase inhibitory effect:
The mixture of 0.06mg trypsin, 1ml 20 M HCl buffer, and 1 ml sample of various concentrations was prepared. To the mixture, 1 ml of 0.8% (w/v) casein and 2 ml of 70% perchloric acid were added. The absorbance was recorded at 210 nm. The percentage of inhibition was calculated. The results of the study were expressed as the mean±SD for three replicates.

Results

The plant material was subjected to extraction with petroleum ether, ethyl acetate, chloroform, methanol and water, and the % yield was calculated. The nature and the percentage yield of the extracts are as shown in Table 1.

Table 1: Nature and percentage yield of the extracts:

Sl No	Solvent	Nature	%Yield
1	Petroleum ether (PE)	Light Greenish oily mass	2.1
2	Ethyl acetate (EE)	Dark green mass	3.9

3	Chloroform (CE)	Light brownish-yellow mass	5.2
4	Methanol (ME)	Brown mass	9.8
5.	Water	Dark Brown mass	7.4

Photodocumentation profile of the methanolic extract of *Dodonaea viscosa* under UV 254 nm and UV 366 nm is as shown in figures 1 and 2.

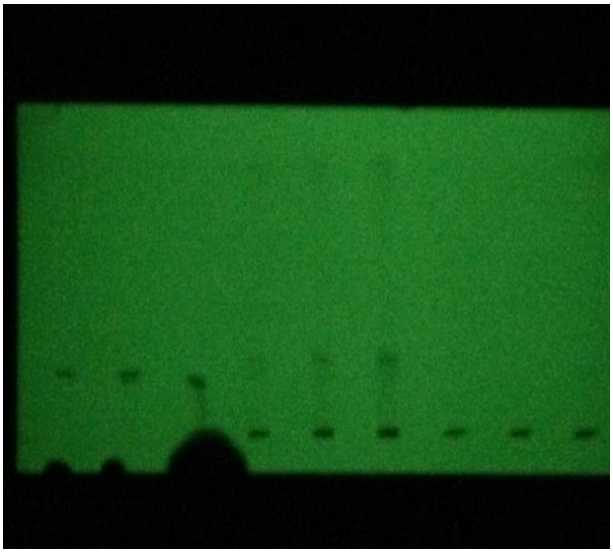

Figure 1: HPTLC chromatoplate of the methanolic extract (254 nm).

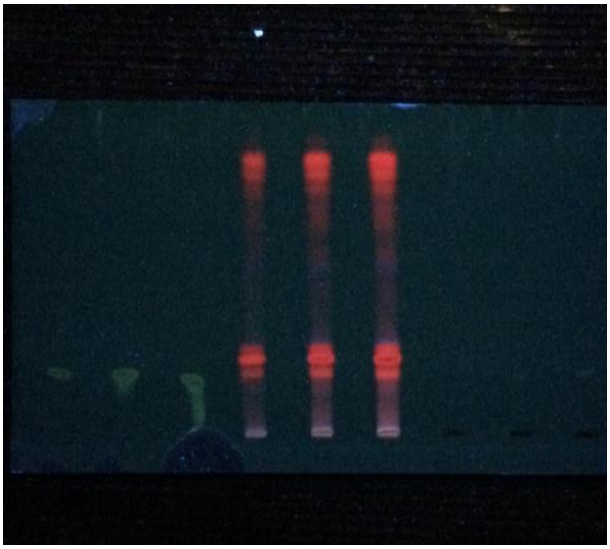

Figure 2: HPTLC chromatoplate of the methanolic extract (366 nm).

As shown in figure 3, the HPTLC chromatogram of the methanolic extract revealed the existence of nine chemical components with Rf values of 0.02, 0.11, 0.14, 0.28, 0.43, 0.62, 0.66, 0.76, and 0.84. Peaks 7 and 9 in the extract were confirmed to be flavonoids kaempferol (Rf=0.66) and rutin (Rf=0.84), respectively as the peaks matched with the peaks present in the chromatogram of the standard kaempferol and rutin.

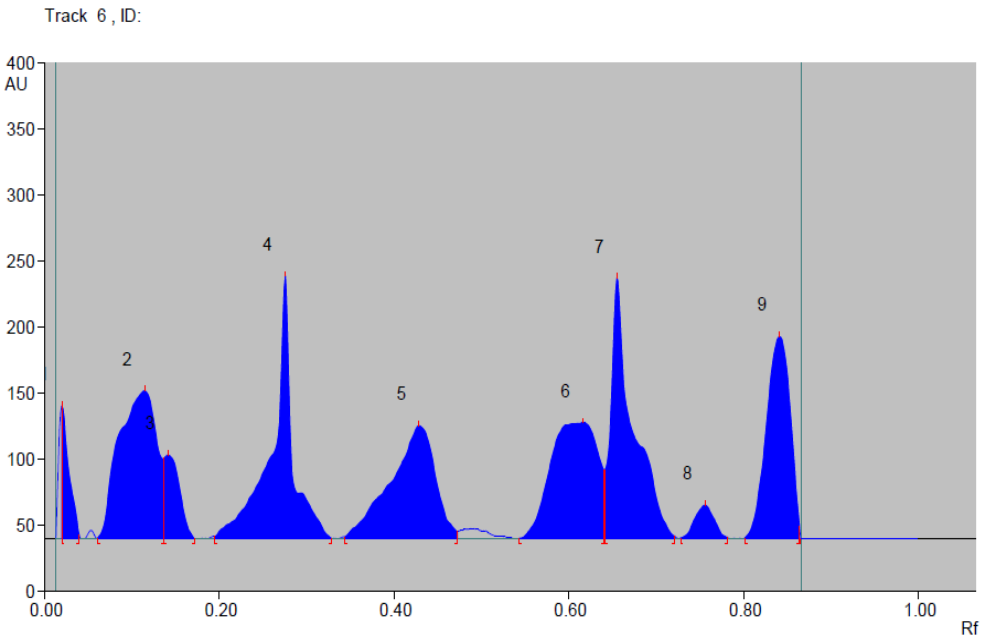

Figure 3: HPTLC Chromatogram of the methanolic extract

Table 2: Rf values of peaks in HPTLC Fingerprint of ME.

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area %
1	0.02 Rf	100.4 AU	0.02 Rf	00.4 AU	9.83 %	0.04 Rf	2.4 AU	2.89 %
2	0.06 Rf	0.4 AU	0.11 Rf	11.9 AU	10.95 %	0.14 Rf	0.2 AU	16.07 %
3	0.14 Rf	60.7 AU	0.14 Rf	63.0 AU	6.17 %	0.17 Rf	0.1 AU	3.67 %
4	0.19 Rf	1.7 AU	0.28 Rf	98.5 AU	19.43 %	0.33 Rf	0.1 AU	15.08 %
5	0.34 Rf	1.2 AU	0.43 Rf	85.3 AU	8.35 %	0.47 Rf	5.4 AU	14.40 %
6	0.54 Rf	0.2 AU	0.62 Rf	87.7 AU	8.58 %	0.64 Rf	1.7 AU	15.82 %
7	0.64 Rf	51.8 AU	0.66 Rf	96.9 AU	19.27 %	0.72 Rf	1.6 AU	16.36 %
8	0.73 Rf	0.3 AU	0.76 Rf	25.2 AU	2.47 %	0.78 Rf	0.9 AU	1.84 %
9	0.80 Rf	0.3 AU	0.84 Rf	52.7 AU	14.95 %	0.87 Rf	9.3 AU	13.86 %

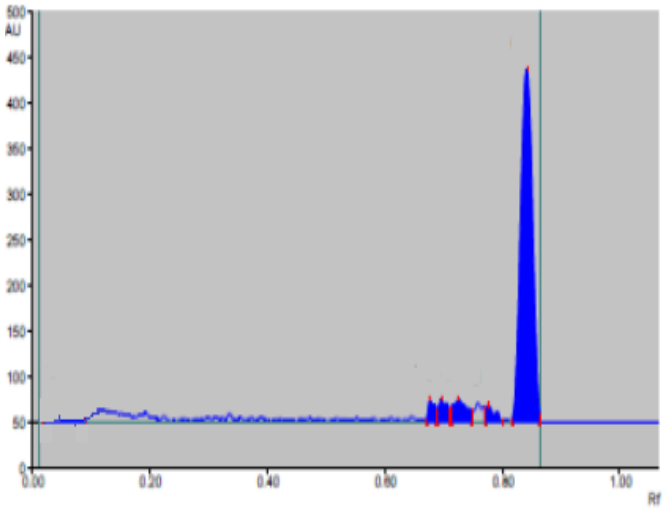

Figure 4: HPTLC chromatogram of the rutin standard

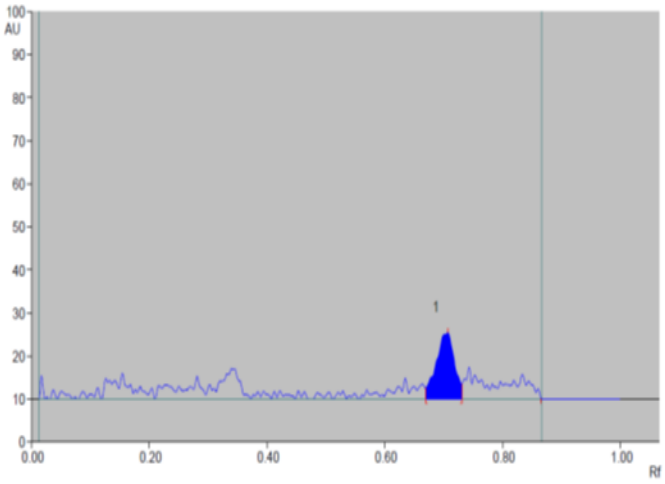

Figure 5: HPTLC chromatogram of the kaempferol standard

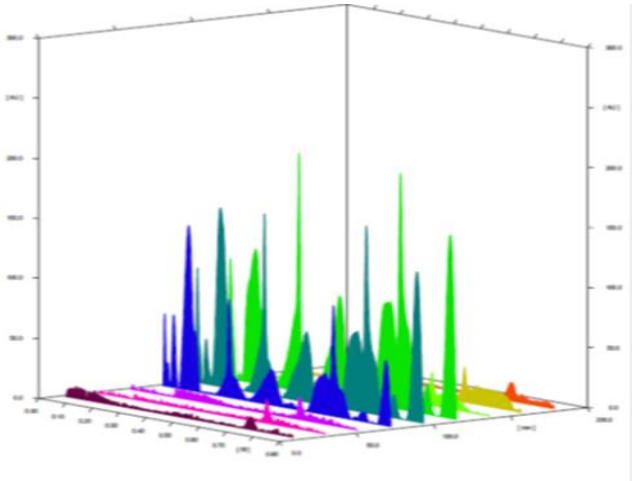

Figure 6: Three-dimensional representation of HPTLC chromatogram measured at 254 nm

Anti-oxidant activity

The results revealed an increase in the scavenging effect with an increase in the concentration of samples. ME exhibited higher DPPH radical scavenging activity when compared to the chloroform extract. At the concentration of 200 µg/ml, methanol extract possessed 93.57% scavenging activity while the chloroform extract at the same concentration showed 84.52%.

Table 3: Anti-oxidant activity of the extract by DPPH method

µg/ml	*Dodonaea viscosa* *Methanolic extract*		*Dodonaea viscosa* *Chloroform extract*	
Extract	Abs	% Inh	Abs	% Inh
20	0.068	83.80	0.122	70.95
40	0.060	85.71	0.115	72.61
80	0.052	87.61	0.109	73.21
100	0.047	88.80	0.097	76.90
120	0.041	90.23	0.091	78.33
140	0.039	90.71	0.084	80.00
160	0.032	92.38	0.076	81.90
180	0.028	93.33	0.079	81.19
200	0.027	93.57	0.065	84.52

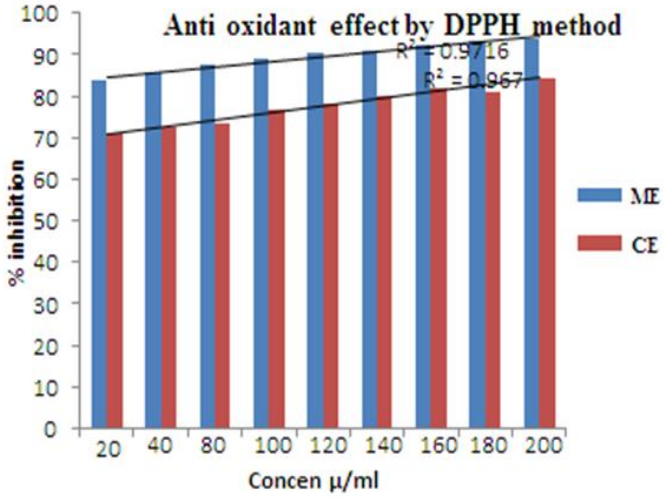

Figure 7: Anti-oxidant activity of the extract by DPPH method

In-vitro anti-inflammatory activity

As shown in Table 4, the percentage of inhibition was highest i.e. 85.09% at the concentration of 500 µg/ml. Diclofenac sodium was used as a standard drug and showed the maximum inhibition of 92.38% at the concentration of 500 µg/ml.

Table 4: Effect of *Dodonaea viscosa* extract on albumin denaturation

Treatment	Concentration (mg)	Absorbance at 660nm	% inhibition
Extract	100	0.007±30.92	78.02
	200 mg	0.011±0.860	79.52
	300 mg	0.005±0.713	83.02
	400 mg	0.005±0.693	83.50
	500 mg	0.014±0.626	85.09
Diclofenac sodium	500 mg	0.009±0.320	92.38

Each of the values represents the mean ± SD. N=3

As shown in Table 5, the methanolic extract exhibited antiproteinase activity at different concentrations with the maximum inhibition of 67.24% at 500 µg/ml. Standard Diclofenac sodium showed the maximum inhibition of 75.86% at 500 µg/ml.

Table 5: Effect of *Dodonaea viscosa* extract on proteinase inhibitor activity

Treatment	Concentration (mg)	Absorbance at 210nm	% inhibition
Extract	100	0.005±0.400	31.03
	200	0.014±0.333	42.58
	300	0.014±0.273	52.93
	400	0.005±0.225	61.20
	500	0.007±0.193	67.24
Diclofenac sodium	500	0.009±0.140	75.86

Each value represents the mean ± SD. N=3

Discussion:

As mentioned in the experimental section, standard methods were followed for the evaluation of phytoconstituents. Chloroform extract gave positive results for the presence of flavonoids, glycosides, sterols, and tannins; ethyl acetate extract demonstrated the presence of phenolic compounds and sterols; while the methanolic extract contained sterols, glycosides, flavonoids, phenolic compounds, saponins, tannins, sugars, and proteins. The methanolic extract was selected for the HPTLC analysis, as maximum classes of phytochemicals were present in it. HPTLC fingerprinting helps in the identification, quality control, and providing basic information used for the identification, isolation, and purification of chemical compounds present in the plant. It is an accurate method to identify plants. This method can also be used for phytochemical profiling and quantification of the plants' components as it is reliable and reproducible. HPTLC can provide sufficient information about the drug and also can be used in the identification, standardization, and quality control. [24-27] As shown in fig 6, the methanolic extract revealed the presence of nine chemical components with Rf values of 0.02, 0.11, 0.14, 0.28, 0.43, 0.62, 0.66, 0.76, and 0.84. Peak 7 and 9 were confirmed to be kaempferol (Rf=0.66) and rutin (Rf=0.84), respectively as the peaks matched with the peaks present in the chromatogram of the standard kaempferol and rutin.

The plant extracts may exert an anti-oxidant activity by various mechanisms including scavenging of reactive species, increasing the antioxidant activity, enzymes suppressing lipid peroxidation recycling, and sometimes by binding pro-oxidant metals. [28] Antioxidant activity can be evaluated using a number of models with different mechanisms such as reducing power, hydrogen atom transfer, metal chelation, single electron transfer, etc. The antioxidant activity was evaluated by DPPH method, which is a common method to investigate the scavenging activity of naturally occurring compounds as it is one of the sensitive, easiest, and rapid, methods. DPPH is a free radical with a maximum absorbance at 517 nm in methanolic solution. The odd electron of the radical is responsible for the appearance of a purple color. When DPPH accepts an electron, which is donated by any antioxidant compound, it is decolorized and can be measured quantitatively from the changes in the absorbance. [21] Table 3 shows the results of in vitro antioxidant activity. The results showed an increase in the scavenging effect along with increasing the concentration of samples. The methanol extract exhibited higher DPPH radical scavenging activity when compared to the chloroform extract. This was due to the presence of various phytoconstituents like flavonoids and other phenolic compounds present in the methanolic extract. Flavonoids possess the chelating property and may act by direct scavenging of free radicals and chelating of transition metal elements. Furthermore, flavonoids can act as intracellular antioxidants by inhibiting enzymes, generating free radicals, like xanthine oxidase, cyclooxygenase, lipoxygenase, etc. [29]

The anti-inflammatory activity of the methanolic extract was evaluated by the inhibition of albumin denaturation assay and proteinase inhibitory effect methods. Any kind of stress can lead to denaturation of proteins leading to the loss of primary and secondary structures. Denaturing of proteins leads to the loss of their biological function and can be one of the causes of inflammation. The albumin denaturation method is a sensitive, reliable, and quick technique to investigate the anti-inflammatory activity of plant extracts. However, the mechanism through which the extract mediates its denaturation effect is not clear. Literature reveals that the interaction of phytochemicals with the aliphatic region around the lysine residue on the albumin protein may be responsible for this activity. It has also been reported that the denaturation of albumin proteins leads to the formation of antigens and these antigens initiate the type III hypersensitive reaction leading to inflammation; hence, inhibition of its denaturation process by any agent indicates its anti-inflammation properties. [30, 31] The plant extract was effective in the inhibition of albumin denaturation induced by heat. Literature reports that leukocyte proteinase is crucial in tissue damage during various inflammatory reactions and a significant protection level was provided by proteinase inhibitors. [31] The maximum inhibition of 500 µg/ml of the extract was 67.24%, while it was 75.86% for the standard Diclofenac sodium at the same concentration.

Conclusion:

HPTLC fingerprinting profile of methanolic extract proved the presence of nine different phytoconstituents, two of which were identified as rutin and kaempferol. The chromatographic profile can be used to identify and evaluate the quality of plants. The results of the in vitro study indicated that the methanolic extract possesses a significant anti-inflammatory and antioxidant activity. This also gives scientific justification for its traditional use. Bioactivity-guided fractionation of the extract may lead to the isolation of active components. However, more research is required for the identification and isolation of the extract components that may be responsible for these activities and the acquired data can provide a basis for further studying of the plant.

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