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Antioxidant and Anti-inflammatory activities of *Hydrocotyle verticillata* methanol extract

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Abstract

The main aim of this study is to evaluate the antioxidant, anti-inflammatory properties and FT-IR analysis of *Hydrocotyle verticillata* methanol extract. The antioxidant activity like DPPH free radical scavenging activity, Hydroxyl radical scavenging assay, superoxide anion radical scavenging assay and nitric oxide radical scavenging assay was undergone for the extracts of *H. verticillata*. The methanol extract was studied for *in vitro* anti-inflammatory activity in the morphological changes of RAW 264.7 cells were visualized. Anti-inflammatory activity Inhibition of cyclooxygenase enzyme activity of maximum inhibition of (36.36%), lipoxygenase activity (33.46%), Myeloperoxidase (0.001353 U/ml), Inducible Nitric Oxide Synthase (48.50%) and Estimation of cellular nitrite levels (932.085 µg). FTIR analysis was undergone and the major peak attained for *H. verticillata* methanol extract in peak 2924.40 cm^{-1} functional groups Alkanes (-CH_2-).

Keywords: Antioxidant activity, Anti-inflammatory, FT-IR

Introduction

Medicinal plants are regularly used to treat ailments because of minimal side effect and cost effectiveness. The potential for developing antimicrobial, high toxicity property from higher plants appears rewarding as it may lead to the development of phytomedicine. Hence, there is a worldwide interest to isolate biologically active compounds from higher plant species that are possible sources of medicine. The bioactive components of these plants are great sources for new therapeutic agents. These therapeutic agents are of great importance because these pose new hope for disease prevention (Nathalie and Lydia, 2013). Herbal medicines have been known to man for centuries. Therapeutic efficacy of many indigenous plants for several disorders has been described by practitioners of traditional medicine (Abi *et al*., 2005).

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The medicinal plants of secondary metabolites are an excellent natural source of therapeutics and are subsequently used for the treatment of innumerable disease and infections (Gurning *et al*., 2021). Human infection which is a major source of distress is frequently caused by pathogens such as bacteria and fungi (Cavicchioli *et al.,* 2019).

A free radical is a chemical species, capable of independent existence possessing one or more unpaired electron. The free radicals are less stable than non-radicals and are capable of reacting indiscriminately with molecules. Once radicals are formed, they can either react with another radical or with another non-radical molecule by various interactions. When two radicals collide with their unpaired electron, forms a covalent bond. The most molecules found *in vivo* are non radicals. Radical donates its unpaired electron to the other molecules, or takes one electron from it, thus transforming its radical character. At the same time, a new radical is formed (Halliwell and Gutteridge 1984).

These natural antioxidants that are consumed via our diet have beneficial effects on human health, including inhibition of mutagenesis and carcinogenesis (Thaipong *et al*., 2006). Hence there is growing interest in natural polyphenolic antioxidants, present in medicinal and dietary plants that help assuage oxidative damage (Luqman *et al*., 2012).

Inflammation is associated with the characteristics like pain, swelling, redness, loss of function in the affected area and heat accumulation in the inflamed area. A significant role in human health is being played by natural products with respect to preventing and treating inflammatory conditions. Besides various synthetic anti-inflammatory agents (non-steroidal anti-inflammatory drugs) available, herbal medicine still plays a major role to cure various health conditions as large number of medicinal plants possess secondary compounds that retard the key steps of the inflammation pathway (Sonam and Sanjay, 2020). In the present study that the *Hydrocotyle verticillata* extract was allowed for *in vitro* antioxidant activity and anti-inflammatory.

Materials and Methods

Plant secondary metabolic extraction

Hydrocotyle verticillata aerial parts were shade dried for 7 days in order to remove chlorophyll content and finely ground by the mechanical blender. The powdered material was stored in a container for further use. After the setup of Soxhlet apparatus using clamps and mounts. The round bottom flask is filled with methanol solvent around 250 ml (Pandi and Johanna, 2015).

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Anti oxidant activity

DPPH free radical scavenging activity

The free radical scavenging activity of the fractions was measured in vitro by 2,2′-diphenyl-1-picrylhydrazyl (DPPH) assay according to the standard method (Williams et al., 1995). The stock solution was prepared by dissolving 24 mg DPPH with 100 ml of ethanol stored at 20°C until required. The working solution was obtained by diluting DPPH solution with ethanol and 3 ml aliquot of this solution was mixed with 1 ml of sample at various concentrations (100, 200 and 300 μg/ml). The reaction mixture was shaken well and incubated in the dark for 15 min at room temperature. Then the absorbance was taken at 517 nm. The control was prepared without any sample and scavenging activity was estimated based on the percentage of DPPH radical scavenging as the following equation.

Percentage of inhibition = [(control OD−sample OD) / (control OD)] ×100.

Hydroxyl radical scavenging assay

The reaction mixture contained 0.8 mL of phosphate buffer solution (50 mmol L-1, pH 7.4), 0.2 mL of a sample of different concentrations (100, 200 and 300 μg/ml), 0.2 mL of EDTA (1.04 mmol L-1), 0.2 mL of FeCl3(1 mmol L-1), and 0.2 mL of 2-deoxyribose (60 mmol L-1). The mixtures were kept in a water bath at 37 °C and the reaction was started by adding 0.2 mL of ascorbic acid (2 mmol L-1) and 0.2 mL of H2O2 (10 mmol L-1). After incubation at 37 °C for 1 h, 2 mL of cold thiobarbituric acid (10 g L-1) was added to the reaction mixture followed by 2 mL of HCl (25%). The mixture was heated at 100 °C for 15 min and then cooled down with water. The absorbance of solution was measured at 532 nm with a spectrophotometer. The hydroxyl radical scavenging capacity was evaluated with the inhibition percentage of 2-deoxyribose oxidation on hydroxyl radicals (Halliwell and Arnoma, 1987).

The scavenging percentage was calculated according to the following formula:

Scavenging effect (%) = [(control OD−sample OD)/ (control OD)] ×100

Superoxide anion radical scavenging assay

The assay for superoxide anion radical scavenging activity was supported by riboflavin-light-NBT system (Beauchamp and Fridovich, 1971). 1 ml of extracts was taken at different concentrations (20, 40, 60, 80 and 100 μg/ml) and mixed with 0.1 ml of Riboflavin solution (20 μg), 0.2 ml of EDTA solution (12 mM), 0.2 ml of methanol and 0. 1 ml of Nitro-blue tetrazoliumn (0.5 mM) were mixed in test tube and reaction mixture was diluted up to 3 ml

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with phosphate buffer (50 mM). After 20 min of incubation at room temperature, the absorbance was measured at 560 nm. Ascorbic acid was used as standard. The scavenging ability of the plant extract was determined by the following equation:

Scavenging effect (%) = [(control OD−sample OD)/(control OD)]×100

Nitric oxide radical scavenging assay

Nitric oxide radical scavenging activity was determined by Griess Ilosvay reaction using sodium nitroprusside. In a typical experiment, the reaction mixture containing 2 mL of sodium nitroprusside (10 mM) and 0.5 mL of phosphate buffer (pH-7.4) was mixed with 0.5 mL of sample or vitamin-C and incubated for 150 min at 25 °C. After the incubation period was over, 0.5 mL of nitrite was pipetted out and 1mL of sulfanilic acid reagent (0.33% of sulfanilic acid in 2% glacial acetic acid) was added to it and kept for 5 min. Then, 1 mL of 1% naphthyl ethylene diamine dihydrochloride (NEDD) was added and allowed to stand for 30 min at 25 °C. The absorbance of pink colour of the solution was read at 540 nm (Badusha *et al*., 2020). The percentage of nitric oxide inhibition was calculated using the following equation:

Percentage (%) of nitric oxide radical scavenging assay = [(A0-A1)/A0] × 100.

where A0 was the absorbance of control, and A1 was the absorbance of the treated sample.

Anti-inflammatory activity

RAW 264.7 cells was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's modified Eagles medium, DMEM (Sigma aldrich, USA).

Cyclooxygenase (COX) activity

The COX activity was assayed by the method of Walker and Gierse. 100µl cell lysate was incubated with Tris-HCl buffer (pH 8), glutathione 5 mM/L, and hemoglobin 5 mM/L for 1 minute at 25°C. The reaction was initiated by the addition of arachidonic acid 200 mM/L and terminated after 20 minutes incubation at 37°C, by the addition 200µL of 10% trichloroacetic acid in 1 N hydrochloric acid. After centrifugation and addition of 200µL of 1% thiobarbituric acid the tubes were boiled for 20 minutes (Jager et al., 1996). After cooling, the tubes were centrifuged for three minutes. COX activity was determined by reading absorbance at 632 nm and percentage inhibition of COX activity was determined as per the following formula

Percentage inhibition of the enzyme was calculated as,

% inhibition = ((Absorbance of control-Absorbance of test)/Absorbance of control) × 100

Lipoxygenase (LOX) activity

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The determination of LOX activity was done as per methods of Axelrod *et al*. Briefly, the reaction mixture (2 mL final volume) contained Tris-HCl buffer (pH 7.4), 50 µL of cell lysate, and sodium linoleate (200 µL). The LOX activity was monitored as an increase of absorbance at 234 nm (Agilent Cary 60), which reflects the formation of 5-hydroxyeicosatetraenoic acid (Yawer et al., 2007).

Percentage inhibition of the enzyme was calculated as,

% inhibition = ((Absorbance of control-Absorbance of test)/Absorbance of control) × 100

Myeloperoxidase (MPO) activity

Cell lysate was homogenized in 50 mM potassium phosphate buffer and 0.57% hexadecyltrimethyl ammonium bromide (HTAB). The samples were centrifuged at 2000 g for 30 minutes at 4°C, and supernatant was assayed for MPO activity. MPO in the sample was activated by the addition of 50 mM phosphate buffer (pH 6) containing 1.67 mg/mL guaiacol and 0.0005% H_2O_2. The change in absorbance at 460 nm was measured. MPO activity was presented as units per mL of cell lysate. One unit of MPO activity was defined as that degrading 1 µM of peroxide per minute at 25°C (Franck *et al*., 2006).

$U = (\Delta OD \cdot 4 \cdot Vt \cdot \text{dilution factor}) / (L \cdot £470 \cdot \Delta t \cdot Vs)$

ΔOD = density change

Vt = total volume (mL) (1.1 mL)

L=light path (1 cm)

£470 = extinction coefficient for tetraguaiacol (26.6 $mM^{-1} \cdot cm^{-1}$,)

Inducible Nitric Oxide Synthase

Nitric oxide synthase was determined by the method described by Salter et al., (1997). Cell lysate was homogenized in 2ml of HEPES buffer. The assay system contained 0.1ml - 2µmol/L L-Arginine, 0.1ml- 4µmol/L manganese chloride, 0.1ml-10mmol/L 30µg dithiothreitol (DTT), 0.1ml- 1mmol/L NADPH, 0.1ml- 4µmol/L tetrahydropterin, 0.1 ml 10µmol/L oxygenated haemoglobin and 0.1ml cell lysate. Increase in absorbance was recorded at 401nm and enzyme activity was determined as per the following equation.

Percentage inhibition of the enzyme was calculated as,

% inhibition = ((Absorbance of control-Absorbance of test)/Absorbance of control) × 100

Estimation of Cellular Nitrite Levels

The level of nitrites was estimated by the method of Lepoivre et al. (Lepoivre et. al. 1990) To 0.5 mL of cell lysate, 0.1 mL of 3% sulphosalicylic acid was added and vortexed well

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for 30 minutes. The samples were then centrifuged at 5,000 rpm for 15 minutes. The protein-free supernatant was used for the estimation of nitrite levels. To 200 µL of the supernatant, 30 µL of 10% NaOH was added, followed by 300 µL of Tris-HCl buffer and mixed well. To this, 530 µL of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diaminedihydrochloride) was added and incubated in the dark for 10–15 minutes, and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained.

FT-IR analysis of *H. verticillata* extract

FT-IR is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in compounds. The wavelength of light absorbed discharacteristic of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined (Ashokkumar and Ramaswamy, 2014). FT-IR spectral investigation of the *H. verticillata* extract sample accomplished using FT-IR Shimadzu IR Prestige-21 (FT-IR 84005). FT-IR spectrum provides the compositional and functional information of *H. verticillata* extract. Dried powder of different solvent extracts used for FTIR analysis. 10 mg of the dried extract powder was encapsulated in 100mg of KBr pellet, in order to prepare translucent sample discs. The different extract of *H. verticillata* was loaded in FTIR spectroscope (Shimadzu, IRAffinity1, Japan), with a Scan range from 400 to 4000 cm^{-1} with a resolution of 4 $cm^{-1.}$

3. Results

In vitro antioxidant activity

The *in vitro* antioxidant activity of *H. verticillata* extracts was present in total DPPH, Hydroxyl radical scavenging assay, Superoxide anion radical scavenging assay and Nitric oxide radical scavenging assay showed in table 1.

Table 1. Antioxidant activity of *H. verticillata* methanol extracts

Antioxidant activity	Sample concentration			IC_{50} value
	100 µg/ml	200 µg/ml	300 µg/ml	
DPPH	60.36 ± 0.003	71.48 ± 0.07	89.217± 0.05	35.794
Hydroxyl Radical Scavenging assay	42.121 ± 0.215	48.077 ± 0.012	52.591 ± 0.163	245.91

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Superoxide anion radical assay	57.465 ± 0.004	66.170 ± 0.021	75.492 ± 0.045	18.3207
Nitric oxide radical assay	50.205 ± 0.056	54.017 ± 0.012	57.067 ± 0.005	90.3235
Standard (Ascorbic acid)	55.469 ± 0.005	60.381 ± 0.005	66.750 ± 0.006	7.34568

Anti-inflammatory activity of *H. verticillata*

Cyclooxygenase (COX) activity

Inhibition of cyclooxygenase enzyme activity by the *H. verticillata* methanol extract was recorded as percentage inhibition of prostaglandin biosynthesis showed in table 2. A maximum inhibition of 100 µl/ml (36.36%) is required for plant extracts to be considered active.

Table 2. Cyclooxygenase activity of *H. verticillata* methanol extract

Volume (µl/ml)	OD at 632nm	Percentage inhibition
H. verticillata methanol extract		
LPS	0.1246	0.00
25	0.109	12.52
50	0.0983	21.11
100	0.0793	36.36

Lipoxygenase (LOX) activity

The effect of *H. verticillata* methanol extract on lipoxygenase activity is shown in table 3. The LOX activity (12-arachidonate LOX purified from rat lung cytosol fraction) was monitored as an increase in the absorbance at 234 nm, which reflects the formation of hydroperoxylinoleic acid. The highest inhibitory effect was obtained for *H. verticillata* methanol extract (100 µl/ml = 33.46%).

Table 3. Lipoxygenase inhibitory activity of *H. verticillata* methanol extract

Volume (µl/ml)	OD at 234nm	Percentage inhibition
H. verticillata methanol extract		
LPS	0.1883	0.00
25	0.156	17.15

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50	0.1418	24.69
100	0.1253	33.46

Myeloperoxidase (MPO) activity

Myeloperoxidase inhibition values of different containing *H. verticillata* methanol extract are given in table 4. As observed from the results 100 µl/ml (0.001353 U/ml) displayed the highest MPO inhibitory effects when compared to other concentration.

Table 4. Myeloperoxidase inhibitory activity of *H. verticillata* methanol extract

Volume (µl/ml)	**ΔOD**	**Enzyme Activity (U/ml)**
H. verticillata methanol extract		
LPS	0.0156	0.005148
25	0.01	0.0033
50	0.0065	0.002145
100	0.0041	0.001353

Inducible Nitric Oxide Synthase

The inhibitory activity of NO production in the LPS stimulated RAW 264.7 cells was inhibitors from *H. verticillata* methanol extract. As observed from the results 100 µl/ml (48.50%) displayed the highest Inducible Nitric Oxide Synthase inhibitory effects showed in table 5.

Table 5. Inducible Nitric Oxide Synthase activity of *H. verticillata* methanol extract

Volume (µl/ml)	**Δ OD**	**Percentage of Inhibition**
H. verticillata methanol extract		
LPS	0.0233	0.00
25	0.0192	17.60
50	0.0167	28.33
100	0.012	48.50

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Estimation of Cellular Nitrite Levels

The nitrite levels of *H. verticillata* methanol extract treated RAW264.7 cell showed in table 6. The minimum cellular nitrite levels of (689.04 µg) and a maximal level of (932.085 µg) *H. verticillata* extracts respectively.

Table 6. Estimation of cellular nitrite levels

Volume (µl/ml)	OD	Concentration of Nitite (µg)
H. verticillata methanol extract		
LPS	0.2552	1263.24
25	0.1883	932.085
50	0.1686	834.57
100	0.1392	689.04

FT-IR analysis of *H. verticillata* extract

FTIR analysis was evaluated that there are multiple spectrum was displayed such as methanol extract of *H. verticillata* 1444.07 cm^{-1} Misc (S=O sulfate), 1739.25 cm^{-1} Esters (RCOOR`) and 2924.40 cm^{-1} Alkanes ($-CH_2-$) showed in fig. 1 and table 7.

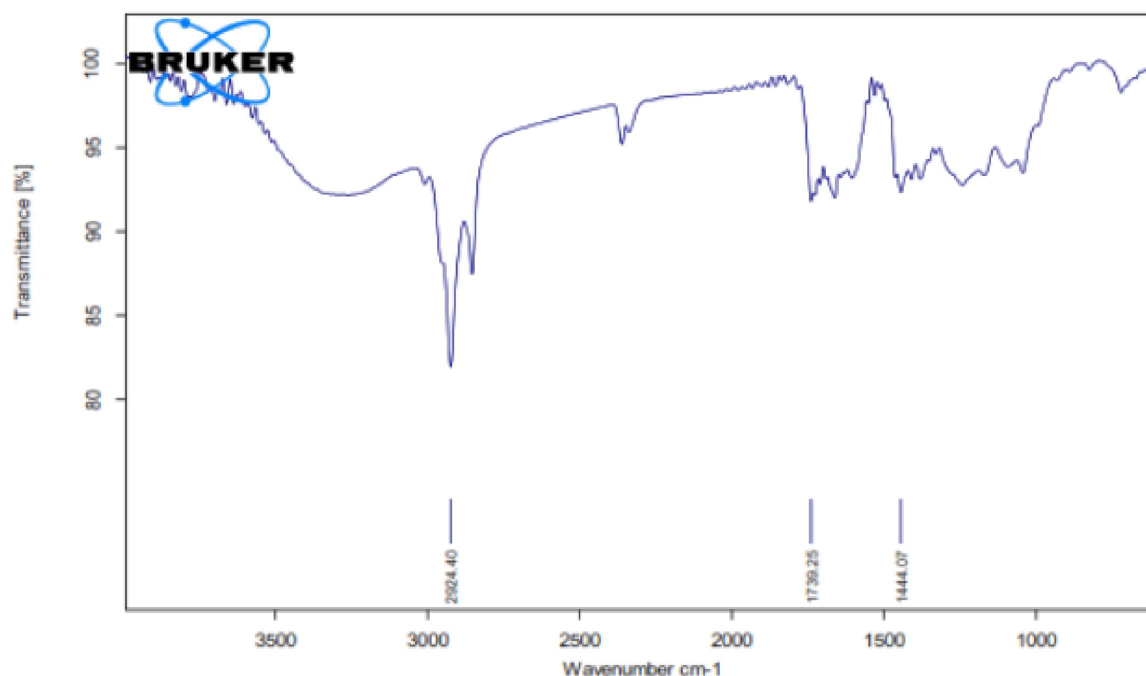

Figure 1. FTIR spectrum of methanol extract of *H. verticillata*

Table 7. Structural feature of the methanol extract of *H. verticillata* by FTIR spectrum

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Wavelength	Functional groups	Structure
1444.07	Misc	S=O sulphate
1739.25	Esters	RCOOR`
2924.40	Alkanes	$-CH_2-$

Discussion

In this contemporary study of *H. verticillata* the various concentration of plant extracts were insisted by the DDPH assay. The maximum DPPH scavenging activity for the Chloroform (IC_{50} value 332.98) and standard ascorbic acid (IC_{50} value 425.04). Cerretani and Bendini, (2010) reported that the DPPH assay is considered to be more selective because aromatic acid with a single hydroxyl group does not react with DPPH radicals. Sharma and Bhat, (2009) reported that the DPPH radical scavenging activity is a very common and an easy *in vitro* method to evaluate the antioxidant capacity of different plant extracts. In this method scavenging of the free stable DPPH radical (2,2'-diphenyl-1-picrylhydrazyl radical) by some antioxidant substances present in plant extract can be adjudged. The colour of DPPH solution is deep purple (absorption at 517 nm) which turns into yellow after being exposed with the proton donating compounds *ie.* antioxidants present in the extract solution.

In present study, the hydroxyl scavenging assay effect of *H. verticillata* extracts were studied and compared with the standard ascorbic acid. Similarly, Lipinski, (2011) reported that the hydroxyl radical scavenging activity was also used to evaluate the antioxidant potential of the 15 plant samples. Hydroxyl radicals are able to reduce disulfide bonds specifically in fibrinogen, resulting in abnormal spatial configurations and this reaction is found to be responsible for the occurrence of many diseases such as cancer, atherosclerosis and neurological disorders. This type of adverse effects of the reaction of hydroxyl radicals can be prevented by non-reducing substances obtained from natural sources.

In present study, the superoxide anion radical scavenging assay effect of *H. verticillata* extracts were studied and compared with the standard ascorbic acid. Attarde *et al.* (2011) reported that the living cells when oxygen is taken up by the cell superoxide anion radicals are immediately produced inside cells. Superoxide anion radicals are highly reactive free radicals produced endogenously by xanthine oxidase which converts hypoxanthine to uric acid. Harmful effects of these radicals on various cellular components leading to numerous diseases and are also involved in lipid peroxidation.

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In this extant study the nitric oxide radical scavenging assay effect of *H. verticillata* extracts were studied and compared with the standard ascorbic acid. Likewise, Nathan, (1992) reported that the nitric oxide is a free radical produced in mammalian cells involved in regulation of various physiological processes. However, excess production of nitric oxide is associated with several inflammatory diseases.

In the present study, the COX activity was assayed by the method of Walker and Gierse. Inhibition of cyclooxygenase enzyme maximum inhibition of 100 µl/ml (36.36%) and minimum 25 µl/ml (12.52%). Similarly, Eldeen and Van, (2008) reported that the inhibition of cyclooxygenase (COX-1 and COX-2) enzyme activity by the plant extracts was recorded as percentage inhibition of prostaglandin biosynthesis. A minimum inhibition of 50% is required for plant extracts to be considered active. Cronstein, (2002) reported that the inhibition of COX-1, as mentioned earlier, may lead to adverse effects on the gastrointestinal mucosa which may cause gastric ulceration and increase the risk of adverse cardiovascular events. However, some authors also reported that COX-2 is constitutively expressed in some tissues. The prostaglandins produced by COX-2 play an important role in the biological and/or physiological functions of such tissues.

In the present work, the effect of *H. verticillata* extracts on lipoxygenase activity in highest inhibitory effect was obtained for 100 µl/ml (33.46%). Yamamoto, (1992) reported that the lipoxygenase are the family of the key enzyme in the biosynthesis of leukotrienes that are postulated to play an important role in the pathophysiology of several inflammatory disseases. Trouillas *et al*. (2003) reported that the LOXs have been postulated to play an important role in the pathophysiology of several inflammatory and allergic diseases. Reactive oxygen radicals are well known to be produced during the inflammatory process. ROS have been implicated in the process of inflammation.

In the present work, anti-inflammatory activity of *H. verticillata* extracts through the inhibition of myeloperoxidase. Rainatou *et al.* (2015) reported that the Myeloperoxidase (MPO) represents the most abundant proinflammatory enzyme whose release may be associated in the pathogenesis of several diseases. MPO was used as marker of stimulated neutrophil degranulation. The *n*-butanolic subfraction has a better inhibitory effect on the release of MPO compared to that of the ethyl acetate subfraction and gallic acid.

In the present study, vasodilation and hypotension seen in septic shock and inflammation are known to be caused by nitric oxide (NO), which is produced in significant

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levels by inducible nitric oxide synthase (iNOS). Aktan *et al*. (2003) reported that the inhibition of iNOS (inducible nitric oxide synthase) may be beneficial for the treatment of inflammatory disease. Murakami and Ohigashi, (2007) reported that the NO is the main product, which formationis regulated by the NOSs. The NOSs include iNOS (inducible nitric oxide synthases), eNOS (endothelial nitric oxide synthases) and nNOS (neuronal nitric oxide synthases). Most significantly, iNOS is highly expressed in macrophages and its activation leads to organ destruction in some inflammatory and autoimmune diseases.

In the present study, anti-inflammatory activity of *H. verticillata* extracts through the cellular nitrite levels were (689.04 µg) and (932.085 µg) in the absence and presence of 0.2% PBS, respectively. Similarly, Poonsit *et al*. (2016) reported that the nitrite levels of *T. diversifolia* aqueous extract-treated RAW264.7 cell showed a minimum level of (0.01 ± 0.10) mmol/L and a maximal level of (0.37 ± 0.09) mmol/L at 0.94 and 15 mg/mL of *T. diversifolia* aqueous extract, respectively. In addition, *T. diversifolia* aqueous extract exhibited LPS-induced NO suppression in a concentration-dependent manner.

In the present study, FTIR was performed to identify the functional groups present in the different solvent extract of *H. verticillata*. Similarly, Pandi *et al*. (2019) reported that the *Hydrilla verticillata* extract FTIR spectral analysis confirmed the different functional groups such as amides, alcohol, phenol, phosphorus and halogen compounds in the extract. Likewise, Lohithasu and Ramana, (2016) explained that the Drug-excipient compatibilities studies were carried out by using FTIR spectroscopy. FTIR Spectra of drug (diclofenac sodium) and optimized formulation F-4 (drug, excipients and gum mixture) were analysed.

Conclusion

From the obtained results, it was concluded that the methanolic leaf extracts of *Hydrocotyle verticillata* leaves have appreciable antioxidant and anti-inflammatory capacity.

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