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IN VITRO ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF *HYDROCOTYLE JAVANICA* THUNB (APIACEAE)

Krithika N.*[1] and Arumugasamy K.[2]

[1]Assistant Professor of Botany, PG and Research Department of Plant Biology and Plant Biotechnology, Quaid-E-Millath Government College for Women (Autonomous), Chennai-600 002, TamilNadu, India.

[2]Associate Professor of Botany, PG and Research Department of Botany, Kongunadu Arts and Science College (Autonomous), Coimbatore – 641 029, Tamil Nadu, India.

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***Corresponding Author**
Krithika N.
Assistant Professor of Botany, PG and Research Department of Plant Biology and Plant Biotechnology, Quaid-E-Millath Government College for Women (Autonomous), Chennai-600 002, TamilNadu, India.

ABSTRACT

The antioxidant and radical scavenging activities of whole plant methanolic extract of *Hydrocotyle javanica* Thunb. were evaluated by DPPH, hydroxyl radical scavenging, ABTS and SOD assays. Formalin induced paw edema (Hemamalini *et al*, 2010*)* on Wistar albino rats was followed to evaluate the anti-inflammatory activity of *H. javanica.* The animals were divided into five groups (Group I- Control, Group II, III & IV plant extract, Group V- standard drug Indomethacin). The paw oedema diameter of the rats were measured by using vernier calliper. The methanol extract evinced higher antioxidant activities in all except DPPH assay with IC50 values of 38.26 in SOD, 38.16 in ABTS and 37.92 in hydroxyl radical scavenging assays.. A dose dependent decrease in edema with progression in time was observed. The percent inhibition after 180 min was highest (85.26) in the Group IV plant extract of 450 mg kg^{-1} treated rats as compared to the Group-V indomethacin treated rats (84.68). Similarly the paw oedema volume decreased significantly in the 450 mg kg^{-1} (20.84±1.18) of plant extract treated rats whereas the 150 mg kg^{-1} and 300 mg kg^{-1} of plant extract treated rats showed a higher paw oedema volume of 32.26±1.15 and 23.15±1.26 respectively as compared to the standard drug effect (21.65±1.34) at 180 min. The selected plant *H. javanica* is an ethnomedicinal herb with powerful antioxidant activities that may aid in various biological and pharmacological functions. The methanol extract evinced remarkable antioxidant and radical scavenging

activities. Hence it can be proposed as a natural and potent antioxidant. The properties can be harnessed for anti-inflammatory drug development.

KEYWORDS: Antioxidant, anti-inflammatory, *Hydrocotyle javanica*, wistar, formalin, drug.

INTRODUCTION

Hydrocotyle javanica Thunb. commonly called as „ java pennywort" is a naturally growing perennial herb distributed globally in tropical Asia and Oceania. It is a procumbent herb growing in moist shaded places at high altitudinal regions in Nilgiris district, Tamilnadu, India. Traditionally, the fresh plant parts of *H. javanica* are crushed and ingested orally to cure sores of throats and lungs, as an aperient, against fever, and applied as a paste in skin diseases. The juice of its leaf had been used in the form of eye drops to cure eye infection; leaf paste as a dressing of wounds to reduce swelling and juice of shoots could treat gastritis and constipation (Warrier *et al*., 2010).

Antioxidants are molecules or ions or a relatively stable radicals that slow down or prevent the oxidation of other molecules (Halliwell and Gutteridge, 2007). The involvement of free radicals in the pathogenesis of a given disease could be measured by the antioxidative potency or capacity. Inflammation is an integrated response of various defense mechanisms by the body to the invasion of a foreign matter. Clinical medicine normally employs non-steroidal anti-inflammatory or steroidal drugs, but these are known to produce a number of side effects such as gastric irritation, stomach ulcers. Therefore there is a demand for herbal medicines that are known to have no side effects in alleviating inflammation. Hence the present study was undertaken to determine the anti-inflammatory activity of *H. javanica.*

MATERIALS AND METHODS

Plant material – Collection and processing

The whole plant of *H. javanica was* washed and air dried under shade for few days. It was then powdered in a pulveriser and passed through a 100 mesh sieve to get a fine powder. 250g of the powder was defatted in petroleum ether and later subjected to Soxhlet extraction using 750 mL of methanol. The extract was reduced to dry residue under high pressure at low temperatures and stored in aseptic conditions in the refrigerator for further studies.

***In vitro* antioxidant activity**

The whole plant methanol extract of *H. javanica* was assessed for its antioxidant activities by employing different *in vitro* antioxidant methods such as DPPH, Hydroxyl, ABTS radical scavenging and SOD. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula

$$\% \text{ radical scavenging activity} = (A_0\text{-}A_1) / A_0 \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance of the sample.

DPPH· radial scavenging activity (Blios, 1958)

H. javanica methanol extract was taken in various concentrations (100 to 600 µg dL^{-1}) and added to 5 mL of 0.1 mM methanolic solution of DPPH· and allowed to stand for 20 min at 27ºC. The absorbance of the samples was measured at 517 nm against the standard ascorbic acid. The results were expressed as percent inhibition.

Hydroxyl radical scavenging activity (Halliwell, 2000)

Hydroxyl radicals were generated from $FeCl_3$ and hydrogen peroxide. The degradation of deoxyribose by $^{\bullet}OH$ formed a reactive species malon-di-aldehyde, which formed an adduct with thiobarbituric acid (TBA). The adduct, MDA–TBA (MDA=Malondialdehyde), was measured for its absorption at 532 nm spectrophotometrically. The hydroxyl radical scavenging activity of the extract was reported as percent inhibition of deoxyribose degradation against the standard ascorbic acid.

Antioxidant activity by radical cation ($ABTS^{\bullet+}$) (Re *et al*., 1999).

$ABTS^{\bullet+}$ was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium per sulphate in the dark for 12 - 16 h at room temperature. Prior to assay, this solution was diluted in ethanol and equilibrated at 30 ºC to give an absorbance of 0.70 ±0.02 at 734 nm. The stock solution of the *H. javanica* extract was diluted such that after introduction of 10 µL aliquots into the assay, they produced between 20-80 % inhibitions of the blank absorbance. After the addition of 1 mL of diluted ABTS•+ solution to 10 µL of sample or Ascorbic acid standards in ethanol, absorbance was measured at 734 nm, at 30 ºC exactly 30 min after the initial mixing. Triplicate determinations were made at each dilution of the standard, and the percentage inhibition was calculated.

Super Oxide radical scavenging activity (SOD) (Beauchamp and Fridovich, 1999)

The one mL reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (7 µM), NBT (50 µM), PMS (15 µM) in various concentrations (100 to 600 µg dL^{-1}) of *H. javanica* methanol extract. At ambient temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of Formazan generated. All tests were performed in triplicates. Ascorbic acid was used as a standard.

Anti-inflammatory study

Formalin induced paw oedema (Hemamalini *et al.*, 2010)

Anti-inflammatory activity was evaluated by formalin induced paw oedema method. Albino rats were divided into five groups of six animals in each group. All the group animals were injected with 0.1 mL of 1 % formalin in 0.9 % normal saline, under the plantar aponeurosis of the right hind paw 30 min after respective treatments. The paw oedema diameter of the rats were measured by using the vernier calliper just before and every hour up to 4 h after formalin injection and the paw oedema was expressed in centimetres.

Experimental Design

Group I: Paw edema induced rats that served as control were given 0.9 % normal saline orally by using an intragastric catheter tube (IGC)

Group II: Paw edema induced rats were given *H. javanica* extract (150 mg kg^{-1} body weight) orally by using IGC.

Group III: Paw edema induced rats were given *H. javanica* extract (300 mg kg^{-1} b.w.) orally by using IGC.

Group IV: Paw edema induced rats were given *H. javanica* extract (450 mg kg^{-1} b. w.) orally by using IGC.

Group V: Paw edema induced rats were given Indomethacin (10 mg kg^{-1} b. w.) orally by using IGC.

RESULTS AND DISCUSSION

In the DPPH assay the methanol extract had an IC50 value of 31.15 which was comparable to that of the standard ascorbic acid (31.16) (Table 1). ABTS assay is better to assess the antiradical capacity of both hydrophilic and lipophilic antioxidant because it can be used in both organic and aqueous solvent system as compared to other antioxidant assay. In all the assays there was a concentration dependent antioxidant activity by the *H.javanica* methanol extracts. The antioxidant activities might offer some lead clues that would explain the

pathologic mechanism of abnormal free radical metabolism. Plants are endowed with free radical scavenging molecules, such as vitamins, terpenoids, phenolic acids, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains and other metabolites, which are rich in antioxidant activities (Aiyegoro *et al*., 2010). A linear relationship between the antioxidant activities and phenolic compounds in the methanolic extracts of *Halenia elliptica* have been reported (Huang *et al*. 2010). Phenolic compounds have redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. The redox potential of phenolic compounds played an important role in determining the antioxidant potential (Boulanouar *et al*., 2017; Labiad *et al*., 2017)

Table 1: *In vitro* antioxidant activity of *H. javanica* whole plant methanol extract.

Antioxidant assays		Concentration of *H. javanica* extract (µg dL^{-1})					IC 50 (µg mL^{-1})
		100	200	400	800	1600	
DPPH	Standard Ascorbic acid	22.93 ±0.22	36.13 ±0.44	52.16 ±0.77	73.26 ±0.92	133.46 ±0.67	31.16
	H.javanica methanol extract	31.16 ±0.56	41.22 ±0.31	69.26 ±0.24	88.16 ±0.17	141.63 ±0.24	31.13
OH	Standard Ascorbic acid	21.93 ±0.21	37.84 ±0.18	60.22 ±0.84	76.93 ±0.88	129.26 ±0.84	32.11
	H.javanica methanol extract	24.22 ±0.16	39.22 ±0.74	64.36 ±0.13	81.22 ±0.76	143.16 ±0.37	37.92
ABTS	Standard Ascorbic acid	23.16 ±0.28	39.15 ±0.92	56.88 ±0.16	71.92 ±0.27	118.33 ±0.16	29.84
	H.javanica methanol extract	36.16 ±0.22	53.84 ±0.13	69.13 ±0.22	91.65 ±0.13	152.16 ±0.22	38.16
SOD	Standard Ascorbic acid	26.81 ±0.16	34.13 ±0.22	66.82 ±0.96	81.31 ±0.72	104.26 ±0.33	28.16
	H.javanica methanol extract	31.36 ±0.26	43.16 ±0.84	69.33 ±0.16	91.16. ±0.28	127.16 ±0.91	32.92

Each value is expressed as percentage of activity mean ± standard deviation (n=3)

Anti-inflammatory activity

The *H. javanica* methanol extracts significantly reduced the formalin induced paw oedema in rats at 120 and 180 min in a dose dependent manner with a maximum effect at 450 mg kg^{-1} dosage level (Table 2). The percent inhibition showed an increasing value with an increase in plant extract concentrations. The percent inhibition after 180 min was highest (85.26) in the Group IV plant extract of 450 mg kg^{-1} treated rats as compared to the Group-V indomethacin treated rats (84.68). The other two plant extract treated groups showed a lower value (77.17, 83.62) in comparison to the standard drug treated rats. Similarly the paw oedema volume

decreased significantly in the 450 mg kg-1 (20.84±1.18) of plant extract treated rats whereas the 150 mg kg-1 and 300 mg kg-1 of plant extract treated rats showed a higher paw oedema volume of 32.26±1.15 and 23.15±1.26 respectively as compared to the standard drug effect (21.65±1.34) at 180 min.

Table 2: Effect of *H. javanica* methanol extract on the percentage inhibition of formalin induced paw oedema in albino rats.

	Dose	Oedema volume (mL)				Percent Inhibition after 180 min
		0 Min	60 min	120 min	180 min	
Group I - Control Normal saline	0.9 %	31.10± 1.08	73.1± 1.24	113.24 ±2.84	141.35 ±3.16	-
Group II – *H. javanica* methanol extract	150 mg kg^{-1}	32.65± 1.16	49.2± 1.31*	41.36± 1.36***	32.26± 1.15***	77.17
Group III – *H. javanica* methanol extract	300 mg kg^{-1}	29.55± 1.24	42.8± 1.26**	28.24± 1.35***	23.15± 1.26***	83.62
Group IV – *H. javanica* methanol extract	450 mg kg^{-1}	28.12± 0.84	40.16± 1.21***	26.22± 1.18***	20.84± 1.18***	85.26
Group V – Indomethacin	10 mg kg^{-1}	29.85 ±0.98	41.3± 1.36***	27.16± 1.34	21.65± 1.34***	84.68

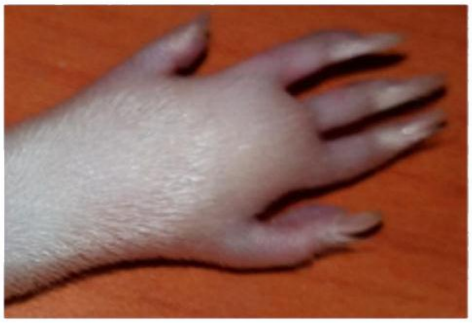
Each Value is SEM ± 6 individual observations *P < 0.05; **P<0.01; *** P<0.001, compared paw oedema induced control vs drug treated rats

The data obtained in this study evince notable antioxidant activities exhibited by different solvent extracts of the ethnomedicinal plant *H. javanica* in various assays. The methanol extract of *H. javanica* proved to be a capable free radical scavenger. In an earlier study the presence of various phytocompounds have been reported (Krithika and Arumugasamy, 2016).

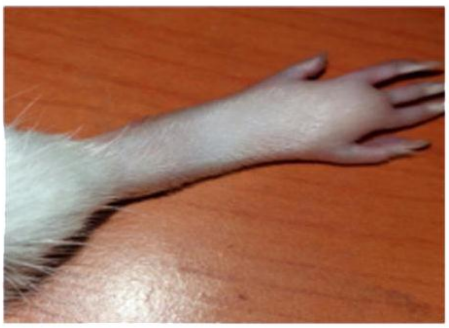
In the present study *H. javanica* methanol extract reduced the formalin induced paw edema in a dose dependent manner with progression in time. Since rapid oedema developed in the rats after formalin injection, this method was reliable for a localized inflammatory response in the late phase. The formalin test normally produces distinct biphasic response, the early and late phase. Centrally acting drugs such as opiods exhibit inhibitory effects in both phases. However, Indomethacin exhibits inhibitory effects only in the late phase. The inflammatory pain could have been characterized by the sensitization of the dorsal horn neurons (Il-Ok Lee

and You-Seong, 2002). The inflammatory process involves a complex interplay between cells of the blood, the blood vessels themselves and the cells of the involved tissue. This process can be seen as a coordinated response of a large number of cells to an initial stimulus (Amritpal *et al.*, 2008).) These effects have been suggested to be associated with the inhibition of pro-inflammatory mediators, including TNF-α and Interleukin-6 levels, brain cyclooxygenase-2 expression and PGE2 production, as well as liver myeloperoxidase activity, which might be mediated by the up-regulation of liver heme oxygenase-1 (Wan *et al.*, 2013).

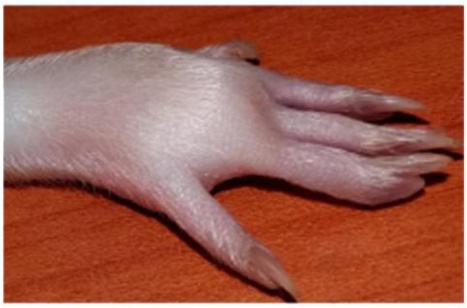
Macrophotography showing the effect of H. javanica extract on formalin induced rat paw oedema



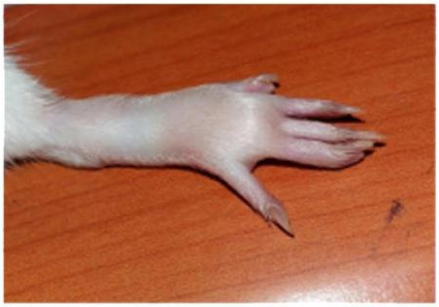
Paw oedema induced Control



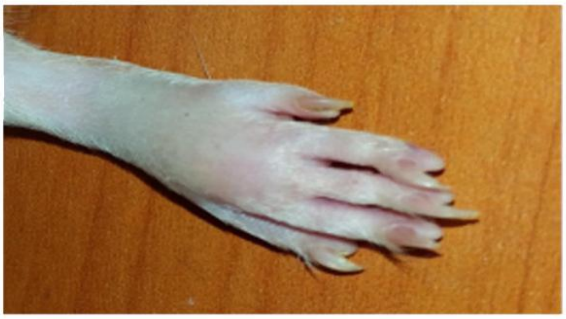
300 mg kg-1 H. javanica extract treated



150 mg kg-1 H. javanica extract treated



450 mg kg-1 H H. javanica extract treated



Standard drug Indomethacin treated

CONCLUSION

The selected plant *H. javanica* is an ethno medicinal herb with powerful antioxidant activities that may aid in various biological and pharmacological functions. The methanol extract

evinced remarkable antioxidant and radical scavenging activities. Hence it can be proposed as a natural and potent antioxidant. These findings explicitly suggested that the plant *H. javanica* alleviated the pathological effects pertaining to inflammation. Thus *H. javanica* could be suggested as a natural anti-inflammatory herbal medicine.

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