RESEARCH PAPER

# Free Radical Scavenging Activity and Antioxidants of hydrocotyle vulgaris L. (Pennywort): Baseline Study inSkin cancer cell line

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**ABSTRACT**

Structural diversity of medicinal herbs makes them a valuable source of novel lead compounds against therapeutic targets that are newly discovered by genomics, proteomics, and high-throughput screening Oxidative stress become the most common health issue in the 21$^{st}$century. Nowadays the usage of Medicinal plants has been increased due to the high anti oxidant properties of Herbal plants. The current study focus on the anti oxidant and anti cancerproperties of the leaf extract of Hydrocotylevulgaris.Results revealed the presence of flavanoidsalkaloids in the Hydrocotyle leaf extract. Good trends for Anti oxidant and free radical scavenging activity.Flavonoids possess a number of medicinal benefits, including anticancer, antioxidant, anti-inflammatory, and antiviral properties they also have neuroprotective and cardio-protective effects. It also has the ability toinhibit the skin cancer cells in human body due to high anti-oxidantproperty.It has a very good IC 50 Value against skin cancer cell line.

Keywords: Hydro cotyle vulgaris, Anti-oxidant, DNA Fragmentation

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## INTRODUCTION

H.Vulgaris.(L)(Popularly known as Acaricoba, Water pennywort, Marshpennywort), is a perennial creeping herb, grown widely in the Americas and mainly native to Brazil . It is a small creeping wet plant native to North America. Nowadays it iscommonlyused in southern states of India as an edible leaf. It grows as a perennial herbaceous plant and only reaches stature heights of 5 to 20 centimeters.Karuppusamy S, Ali MA, Rajasekaran K, Lee J et al.With a slight smell of carrot. It is a crawling aquatic plant this plant is grown for ground cover in ponds. Traditionally, H. Vulgaris. has been used as anti-inflammatory, memory stimulant and anxiolytic herbal medicine (P.G. Pietta et al).The main secondary metabolites previously identified in the plant were volatile constituents, flavonoids,sterols), and saponins . The plant also has high relevance in phytotherapy and in the Ayurvedicmedicine (Indian) because of its potential anxiolytic and memory stimulanteffects. This plant becomesa best option to prevent skin cancer at the early stage...It is a small creeping wet plant native to North America. Nowadays it iscommonlyused in southern states of India as an edible leaf. It grows as a perennial herbaceous plant and only reaches stature heights of 5 to 20 centimeters.(Conforti F, Sosa S, Marrelli M et al) With a slight smell of carrot.

Nowadays the cancer patients are increasing. Cancers in young adults are more common in 21$^{st}$century especially skin cancers. The most common type of skin cancer is Basal cell carcinoma.BCC frequently develops in people who have fair skin. People who have skin of color also get this skin cancer BCCs often look like a flesh-colored round growth, pearl-like bump, or a pinkish patch of skin. The epidermal layer manifests into skin cancer, and based on the involvement of cell type, skin cancer is categorized in two major groups, namely melanoma and non-melanoma skin cancers (NMSCs). NMSCs are further classified into two broad categories: basal cell carcinoma (BCC) and squamous cell carcinoma (SCC)(B.Poljšak, R. Dahmane et al). The current study revel that the leaf extract of Hydro cotyle vulgaris has very good IC 50 value against the skin cancer cell line. It also has good trend for anti oxidant and free radical scavenging activity.Flavanoicd content in very high in hydocotylevulgaris.Flavonoids can protect the brain from oxidative stress and have anticonvulsive effectsIn a DPPH assay, flavonoids interact with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical to provide information on their reactivity.(N.P. Das,

T.A. Pereira et al) Skin is protection for our body from external components. So, protecting the skin in very important task.

The effect of treatment on photosynthesis and antioxidant defense system including superoxide dismutase, peroxidase and catalase in plant roots and leaves were evaluated.Non-steroidal anti-inflammatory drugs (NSAIDS) are commonly used for treatment of pain and many inflammatory disorders.(R. Amarowicz et al)Because of the side effects they produce, like ulcer and renal disorder, many people rely on the use of herbal medicine as a source of bioactive metabolites with fewer side effects.Hydrocotylevulgaris for Skin allergies, ulcers and wound healing.(An introduction to free radical Biochemistry ).It also used for rheumatism, headaches, dizziness, bloody stools. - Leaves used to dress burns or applied to skin diseases. - In Malaya, traditionally used for treating wounds and as a diuretics.It also has anxiolytic (anti-anxiety) and antidepressant-like effects for improving mood and reducing symptoms of stress and anxiety. Cardiovascular health: Hydro cotyle asiatica consists of vasodilator (blood vessel-widening) effects, which help in lowering blood pressure and improving blood circulation.

## MATERIALS AND METHODS

### Plant material preparations

Plant material Hydrocotyle vulgaris L. (Pennywort) was harvested. The plant's leaves and stems werecleaned, and placed on a screen for air drying (depending on the condition of the plant and the temperature). After the air drying process, the plant material was powdered using mechanical Grinding. From this, 2 kilograms was set aside as sample for standardized laboratory testing ofHydrocotyle vulgaris L. (Pennywort).Soxhlet assisted extraction the powders of samples were extracted exhaustively in Soxhletusing water as solvent.

### Phytochemical constituents

From the initial two (2) kilogram-powdered samples of the Hydrocotyle vulgaris L. (Pennywort)that was extracted (aqueous) via soxhlet the presence of various phytochemical constituents wasalso examined particularly the flavonoid content of the plant material.

### ETBr AO staining

A cover slip was placed into the 24 well tissue culture plate and 0.5 x 10 6 cells/ml/well of 431 (human skin cancer) cells were seeded in a DMEM growth medium. After overnight incubation, the cells were washed with PBS and treated with sample 107.9 ug/ml in a serum freeDMEM medium and incubated at 37 o C in 5% Co2 incubator for 24 hours. After incubation, 10 µlof 1 mg/ml acridine orange and ethidium bromide were added to the wells and mixed gently. Samples in the coverslip were evaluated immediately within an hour. At least 100 cells were examined with a fluorescence microscope using a fluorescent filter.

### DAPI STAINING

To determine the number of nuclei and to asses gross cell morphology. Similarly, thecells were seeded on a glass cover slip in a 24-well plate and treated for 24 hours with thecompound. The fixed cells were permeabilized with 0.2 percent triton X-100 (50µl) for 10minutes at room temperature before being incubated for 3 minutes with 10 µl of DAPI using cover slip to ensure uniform stain distribution. The cells were examined using a fluorescent microscope.

### Evaluation of anti oxidant property

DPPH radical scavenging activity

Various concentrations of the sample (4.0 ml) were mixed with 1.0 ml of solutioncontaining DPPH radicals, resulting in the final concentration of DPPH being 0.2 mM.Themixture were shaken vigorously and left to stand for 30 min, and the absorbance was measuredat 517 nm. Ascorbic acid was used as control.(Shimada, K., Fujikawa, K et al)

### Hydroxyl radical scavenging assay

A reaction mixture of 3.0 ml volume contained, 1.0 ml of 1.5 mM FeSO4, 0.7 ml of 6 mMhydrogenperoxide, 0.3 ml of 20 mM sodium salicylate and 1.0 ml of different concentrations (5-100 µg / ml) of sample. After incubation for an hour at 37 o C, the absorbance of the hydroxylatedsalicylate complex was measured at 562 nm. Vitamin E was used as positive control.( Rajeshwar, Y., Senthilkumar et al).

### Superoxide radical scavenging activity

The assay tubes containing various concentration of sample 0.2 ml of EDTA, 0.1 ml ofnitroblue tetrazolium, 0.05 ml of riboflavin and 2.25 ml of phosphate buffer and control tubes were set up without the sample theactivity of the standard antioxidants was also carriedout.Theinitial optical uniformly with a fluorescent lamp for 30 min. A 560 nm was measured again and difference in optical density was taken as the quantum of superoxide production. The percentage inhibition was calculated by comparing with the optical densify the control tubes.( Liu F al)

### Evaluation of anti cancer property

### MTT Assay

The skin cancer cell line (A431) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS).The cells were maintained at 37 0 C, 5% CO2, 95% air and 100% relative humidity.

Maintenancecultures were passage weekly, and the culture medium was changed twice a week. Mosmann, T., 1983.

**Apoptosis activity**

**MMP Assay**

The A 431cells ($1x10^5$ cells/well) were plated to a 24 well plate in a DMEM growth medium forovernight at37°C. The wells were washed with sterile PBS and treated with sampleJ at the concentration of 107.9µg/ml in a serum free DMEM. The treated cells and control cells were incubated at 37°C in a humidified 5% CO2 incubator for 24 h. The measurement of mitochondrial membrane potential for the treated and control cells was carried out according to the manufacturer's instruction.Breifly,the cells were incubated with 100 µl/well of JC-10 dye loading solution and plate was protected from light. The plate was incubated for 30–60 minutes in a 5% CO2 at 37 °C. After incubation, 100 µl of buffer B was added to each sample/well. Finally, the plate was centrifuged at 800 rpm for 2 minutes and the fluorescence was observed at 490/525 and 540/590 ratio.

**DNA fragmentation assay**

Briefly, A 431 cells were seeded in a six-well plate at a density of 1 x $10^6$ cells/well and incubated for 24 h at 37°C in a humidified 5% CO2 incubator. The wells were washed with sterile PBS and treated with 107.9 µg/ml of plant extract sample in a serum free DMEM medium and incubated for 24 h at 37°C in a humidified 5% CO2 incubator. The Hep G2 cells were harvested by trypsinization in a 1.5 ml tube and centrifuged at 10,000 rpm for 10 min. After centrifugation the supernatant was discarded and 500 µL of lysis buffer was added to the cell pellet, incubated in room temperature for 1 hr. Then 700 µL of phenol-chloroform-isoamyl alcohol was added and mixed by inversion, and then centrifuged at 10000 rpm for 5 minutes. The aquatic (upper) phase was transferred into a new Eppendorf tubes. An equal volume of cold isopropanol was added into tubes, and mixed gently by inversion. The tubes were then centrifuged at 10000 rpm for 5 minutes and discarded the supernatant followed by the pellet was air-dried for 30 minutes. Then, the dried DNA was dissolved in 50 µL distilled water. Furthermore, the extracted DNA was quantified by UV spectrophotometer using optical density (OD) at 260 nm and 280 nm.

**RESULTS AND DISCUSSION**

**Phytochemical constituents**

The qualitative analysis of the leaf extract of Hydrocotyle vulgaris showed the presence of saponins flavanoids and tanins.Presence of high flavanoids indicate the anti cancer and anti oxidant properties of the extract.

**Anti oxidant activity**

**DPPH SCAVENGING ASSAY**

The extract of Hydrocotyle vulgaris showd the IC 50 value of 425.18 mg/dl.The compund Flavanoid are mainly resposible for the radical scavenging activity of the plant.Hydocotyle vulgaris showd high content of Flavanoid and that lead to high radical scavenging activity.from the graph it is understood that the percetage of inhibition of the plant extract increases when the concentrationof the extract increases.

**TABLE 1**

| S. No. | | % inhibition | | | | |
|---|---|---|---|---|---|---|
| | | 100 (µg/ml) | 200 (µg/ml) | 300 (µg/ml) | 400 (µg/ml) | 500 (µg/ml) |
| 1 | Plant extract | 11.55 ± 0.45 | 27.27 ± 0.50 | 39.52 ± 0.59 | 47.62 ± 0.30 | 58.27 ± 0.25 |
| 2 | Standard (BHT) | 13.38 ± 0.55 | 35.28 ± 0.30 | 46.17 ± 0.28 | 58.02 ± 0.50 | 67.26 ± 0.25 |

**HYDROXY RADICAL SCAVENGING ACTIVITY**

The compounds such as flavonoids, which contain hydroxyls, are responsible for the radical scavenging effect in the plants[3], [17]. The high contents (+++) of these phytochemicals inHydrocotylevulgarisLexplain its high radical scavenging activity that led to high antioxidant activity. The compounds such as flavonoids, which contain hydroxyls, are responsible for the radical scavenging effect in the plants[3], [17]. The high contents (+++) of these phytochemicals inHydrocotylevulgarisL. can explain its high radical scavenging activity that led to high antioxidant activity

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**TABLE 2: HYDROXY RADICAL SCAVENGING ACTIVITY**

| S. No. | | % inhibition | | | | |
|---|---|---|---|---|---|---|
| | | 100 (µg/ml) | 200 (µg/ml) | 300 (µg/ml) | 400 (µg/ml) | 500 (µg/ml) |
| 1 | Plant extract | 10.16 ± 0.45 | 24.28 ± 0.10 | 37.30 ± 0.30 | 51.17 ± 0.45 | 67.39 ± 0.50 |
| 2 | Standard (Vitamin E) | 15.30 ± 0.12 | 31.40 ± 0.45 | 45.53 ± 0.45 | 63.10 ± 0.55 | 72.10 ± 0.25 |

The standard used against the extract of Hydro cotyle vulgaris was found to be Vitamin E. The Standard showed more inhibition than plant extract. The IC50 Value of the standard was found to be 325.23 and the IC 50 value of the plant extract was found to be 392.19. From the above results it is understood that the extract of Hydro cotyle vulgaris has the ability to inhibit the activity of hydroxy radicals as well as DPPH.

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**Apoptosis activity**

**MMP activity**

The aqueous extract of hydrocotyle vulgaris hasthe ability to inhibit the apoptosis by increasing the mitochondrial membrane potential.Apoptosis removes cells in the upper epidermis that may have genetic alterations that couldlead to cancer

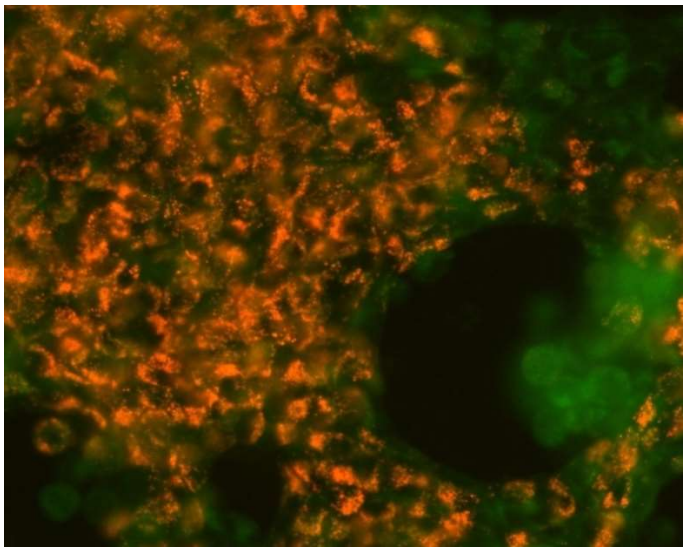


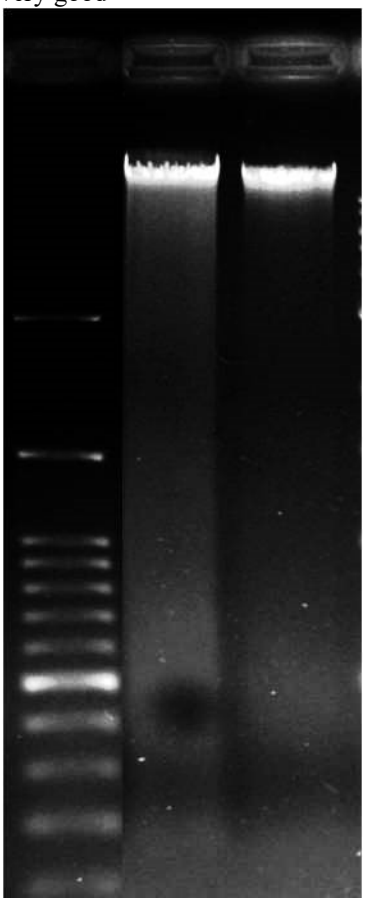
**FIGURE 1**

**DNA FRAGMENTATION**

DNA fragmentation is a hallmark of apoptosis, or programmed cell death. When the DNA fragmentation factor(DFF) is inhibited or lost, cells exposed to ionizing radiation are more likely to survive. This can lead to increased gene mutations, gene amplifications, and chromosomal instability. DNA fragmentation plays a role in skin cancer by helping to remove cells that have DNA damage, The high dna fragmentation ability of hydrocotyle vulgaris is due to the presence of high amount flavanoids in it.Extracted DNA was

quantified by UV spectrophotometer using optical density (OD) at 260 nm and 280 nm. Then the equal amount of each DNA samples was run at 0.8 % of agarose gel electrophoresis along with 100 bp ladderThe A431 Skin cancer cell line get a very good od value of 1.83 during dna quantification.it indicates that after the fragmented DNA was not contaminated with protein or RNA.

**Agarose gel electrophoresis (0.8%)**



**(A)** DNA ladder
**(B)** 107.9 µg/ml of plant extract
**(C)** Control

**DAPI STAINING**

The dye, 4',6-diamidino-2-phenylindole (DAPI), binds to DNA in the nucleus of cells.Since DAPI binds to DNA, it can be used to determine the relative amount of DNA in cells for cell cycle analysis.

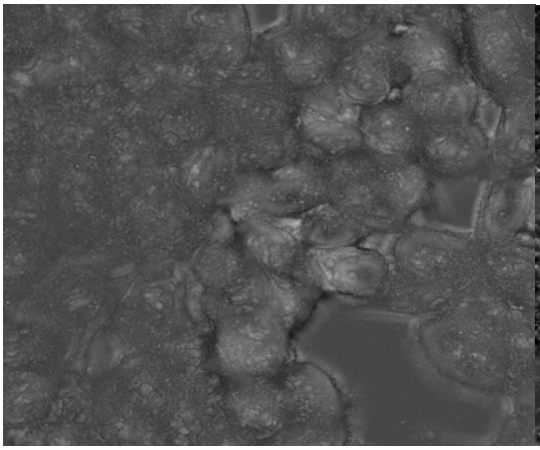


Figure 1

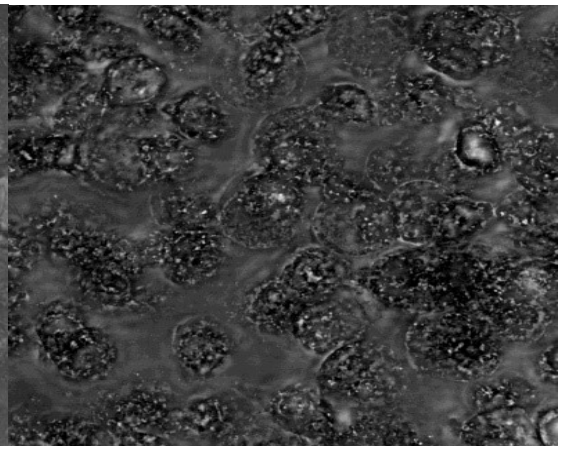


Figure 2

Control Treated sample

**ETBR AO STAINING**

AO/EB staining can be used to study apoptosis in cells, such as breast cancer cells, osteosarcoma cells, and plant cells. Early apoptotic cells Appear bright green. In the current study the control gets dark green colour in ETBR AO Staining. Whereas the plat extract get the combined colour of both red and dark green

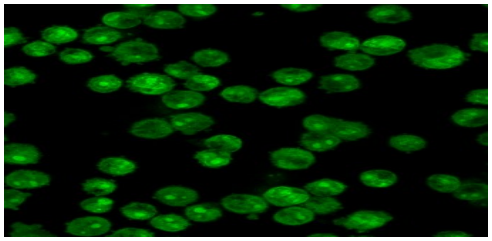


Figure 1 Control

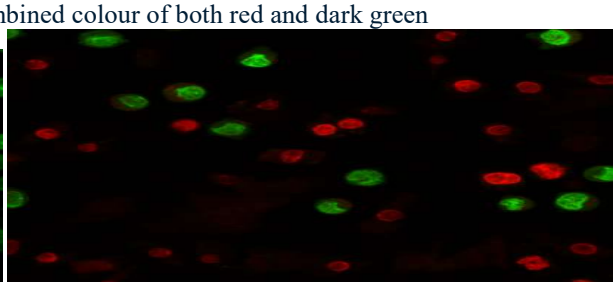
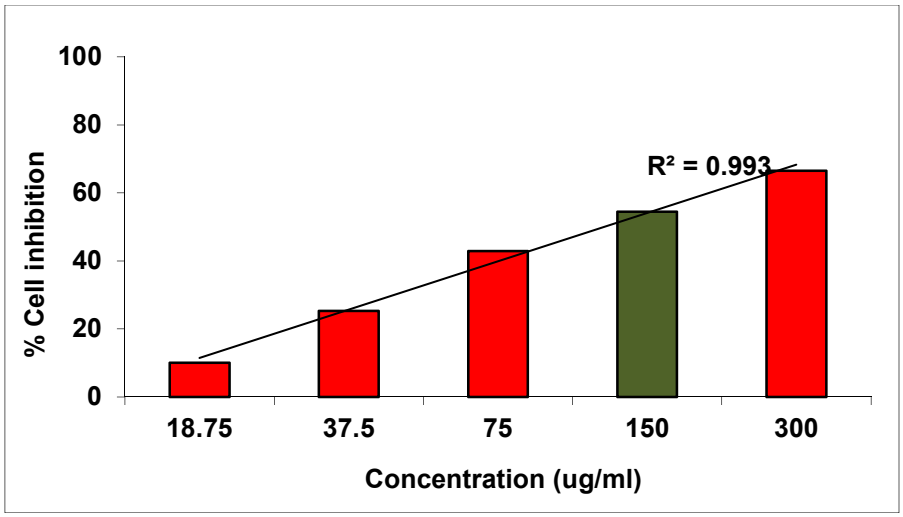


Figure 2 Sample of plant extract

**Anti cancer activity**

Skin cancer cell line




The above graph showed that the plant extract of hydro cotyle vulgaris has the ability to inhibit skin cancer cell. The IC 50 value of the extract against skin cancer cell line was found to 107.92. It indicates that the aqueous extract of hydro cotyle vulgaris will inhibit the cancer cells at the skin.

**DISCUSSION**

Results revealed the presence of flavonoids alkaloids in the Hydrocotyle leaf extract.Good trends for Anti-oxidant and free radical scavenging activity.Flavonoids possess a number ofmedicinal benefits, including anticancer, antioxidant, anti-inflammatory, and antiviral properties they alsohave neuroprotective and cardio-protective effects. It also has the ability to inhibit the skin cancercells in human body due to high anti-oxidantproperty.Similar studies was carried out in Other plants like Hydrocotyleumbelletta.H. umbellata L. (popularly known as Acaricoba, Water pennywort, Marshpennywort) , is a perennial creeping herb, grown widely in the Americasand mainly native to Brazil (Conforti F, Sosa S, Marrelli M et al).• Traditionally, H. umbellata L. has been used as anti-inflammatory, memorystimulant and anxiolytic herbal medicine.The main secondary metabolites previously identified in the plant were volatile constituents (Sherif A Hamdy [a,*], Esther T Menze [b], Hala M El Hefnawy et al), flavonoids,sterolsand saponins.The plant also has high relevance in phytotherapy and in the Ayurvedicmedicine (Indian) because of its potential anxiolytic and memory stimulant effects (Ayyadurai et al., 2026;Lakshmikanthan et al., 2026;Muthu et al., 2024).

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