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A study of the *in vitro* free radical-scavenging property of *Hedyotis diffusa* using nitric oxide assay

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Abstract
Introduction: *Hedyotis diffusa,* known as Snake Needle Grass, is a herb used in traditional Chinese medicine for the treatment of multiple ailments, especially cancer. It is found to possess anti-proliferative, anti-inflammatory and anti-ageing properties.
Aim: To study the free radical scavenging property of the ethanolic extract of *Hedyotis diffusa* using Nitric oxide assay.
Materials and Methods: A dried sample of *Hedyotis diffusa* was extracted using 85% ethanol. Various concentrations of the extract were mixed with Sodium nitroprusside(SNP) in Phosphate Buffer Saline(PBS). Then Griess reagent was added and the absorbance studied using a spectrophotometer at 546nm. Quercetin solution was used as the standard.
Result: The herb exhibited maximal activity of 72.28% at the concentration of 1000 µg/ml. The IC_{50} value of Quercetin & Herb was found to be 10.24 µg/ml & 104.18 µg/ml respectively.
Conclusion: The ethanolic extract of *Hedyotis diffusa* demonstrated a concentration-dependent free radical scavenging property which can be postulated as the mechanism of action in its anti-cancer, anti-inflammatory and anti-ageing properties.
Keywords: *Hedyotis diffusa*, Free radicals, nitric oxide assay, anti-cancer

1. Introduction

Herbs have been used as medicine for centuries before modern medicine came into being. Nature has bestowed us with millions of plant species and ancient medicinal systems have studied and utilized the properties of different parts of plants and applied them to cure various diseases since time immemorial. Lately, these herbal remedies have re-emerged in significance where modern medicine has failed to find a remedy or in terms of disastrous or troublesome side effects. There has been an explosion of research into the chemical constituents, properties and mechanism of action of these herbal formulations. And the knowledge thus gained can be applied to further the frontiers of modern medicine.

Hedyotis diffusa, also called *Oldenlandia diffusa,* commonly called Snake-Needle Grass, of the plant family Rubiaceae[1], is an annual herb distributed in south-east Asia, including India[2]. It grows on open, sandy and damp soils[1]. All the parts of the herb are used in treatment[3]. It has been in clinical use for several thousand years. It is said to possess diuretic, anti-inflammatory, anti-bacterial, anti-viral and anti-cancer properties[4].

About 15% of the herbal anti-cancer formulas in China contain this herb. It has been used to treat cervical cancer, colorectal cancer, gastric carcinoma, multiple myeloma, leukemia, breast cancer, hepatocellular carcinoma, esophageal carcinoma, etc. Both pre-clinical and clinical studies have established the safety and efficacy of *H. diffusa* in treating the above mentioned cancers. The main chemical constituents that have been studied and postulated to possess these anti-cancer properties are polyphenols, terpenoids, anthraquinones, flavonoids and others[4]. It is also used to treat hepatitis, snake bites, pneumonia, appendicitis, urinary tract infections, etc and as topical application over cellulitis, abscesses, carbuncles, etc. In addition, it is said to enhance vitality, and is used on a daily basis in herbal teas, to prevent diseases[4]. It is also used in combination with other herbs such as *Scutellaria barbata*[5,6], *Siraitia grosvenorii*, etc. The ethanolic extract of *Hedyotis diffusa* (EEHDW) has been shown to produce apoptosis by activating the mitochondrial pathway in human colon carcinoma cell line HT-29[7], by suppressing Sonic hedgehog signaling[8] and inhibiting tumour angiogenesis[9]. *In vivo*, *H. diffusa* has been shown to inhibit colorectal carcinoma by inhibiting STAT3 signaling pathway[10]. *Hedyotis diffusa* extract was also found to diminish the cytotoxic effects of chemotherapeutic drugs in breast cancer patients[11]. These are the postulated mechanisms of its anti-cancer effect. Further study is still under way to elucidate its various actions. The following study was undertaken to determine the free radical scavenging activity of this herb.

Figure 1: The dried sample of *Hedyotis diffusa*

2. Materials and Methods

2.1 Materials

Griess reagent (1% sulphonilamide, 0.1% N 1-naphthylethylenediamine, 2% orthophosphoric acid), Sodium nitroprusside (SNP), Phosphate buffer saline (PBS) and Quercetin were purchased from A to Z Lab Needs, Chennai, India. A dried sample of *Heydotis diffusa* was purchased locally.

2.2 Preparation of the Extracts

Reflux method was used to extract 500g of Hedyotis diffusa with 5000ml of 85% ethanol and then filtered. A rotary evaporator was then used to evaporate the ethanolic extract. This was then brought to a relative density of 1.05 by concentrating it, and a spray dryer was used to produce the dried powder of the ethanolic extract by spraying desiccation. The required concentrations of the extract were then prepared by dissolving the powder in saline(0.6g/mL)[7].

2.3 Principle

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates Nitric oxide which interacts with oxygen to produce Nitrite ions, which is measured at 546nm by spectrophotometer in the presence of Griess reagent[12].

2.4 Procedure

1 ml of SNP (5mM) in PBS was taken in 7 different test tubes and 7 different concentrations (10, 50,100, 200, 400, 800 & 1000µg/ml) of ethanolic extracts of the herb were added to the test-tubes. The test tubes were then incubated at 29°C for 3 hrs. Similar concentrations of quercetin were prepared and incubated in a similar manner which was taken as reference anti-oxidant in the study. For control, a test tube filled with distilled water was taken and conducted in an identical manner.

After 3 hours, one ml of Griess reagent was used to dilute the incubated samples. The absorbance that is formed as a result of diazotization of nitrite with sulphanilamide and consecutive coupling with naphthylethylenediamine dichloride was analyzed on Spectrophotometer at 546 nm[13]. Percentage of Inhibition of NO scavenging activity is given by the formula:

$$\text{Nitric Oxide scavenged (\%)} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

Where, $A_{control}$= Absorbance of control and A_{test}= Absorbance of test sample.

2.5 Statistical Methods: The IC50 values were obtained by Probit analysis using Graphpad Prism 6 software.

3. Results

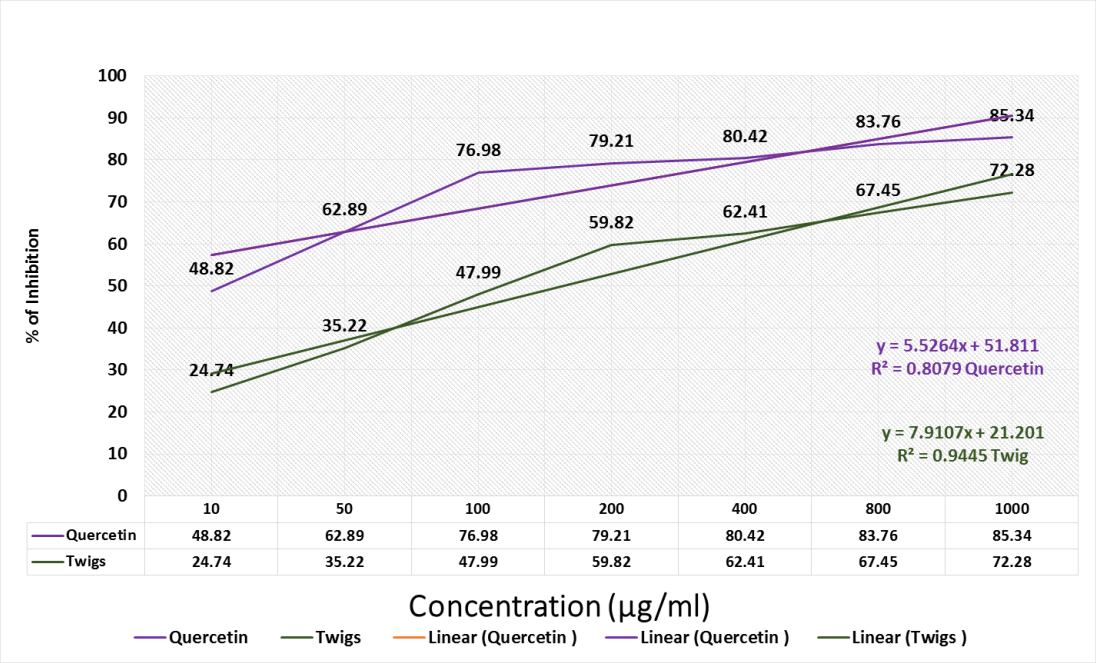
The Nitric oxide scavenging activity of the ethanolic extract of *Hedyotis diffusa* and Quercetin solution which was used as the standard at varying concentrations is represented in Table 1. In the study we found the IC50 value of Quercetin & herb to be 10.24 µg/ml & 104.18 µg/ml respectively. The coefficient of determination, r^2 for H. diffusa was 0.9445, whereas r^2 for Quercetin was 0.8079. This result is statistically significant(>0.8). This is represented in Graph 1.

Table 1. Table showing percentage inhibition of Nitric oxide free radical by *Hedyotis diffusa* and Quercetin at varying concentrations:-

Concentration (µg/ml)	Percentage Inhibition (%)	
	Standard(Quercetin)	*Hedyotis diffusa*
10	48.82±0.72	24.74±0.18
50	62.89±0.84	35.22±0.91
100	76.98±0.54	47.99±1.79
200	79.21±1.14	59.82±1.62
400	80.42±1.06	62.41±0.68
800	83.76±0.98	67.45±0.49
1000*	**85.34±1.02***	**72.28±0.98***

Note: The maximum free radical scavenging activity of the herb Hedyotis diffusa of 72.8±0.98% was seen at a concentration of 1000µg/mL.

Graph 1: Graph showing the coefficient of determination, R^2



4. Discussion

Oxygen, although indispensable to human life, can also create havoc in the human body by creating free radicals. These free radicals are, in turn, highly reactive species with an extra free electron which can react with various biomolecules like DNA, proteins, glycoproteins and lipids and damage them, causing premature ageing, multiple sclerosis, atherosclerosis, cancer, Alzheimer's disease, diabetes, etc[13].

Nitric oxide is one such simple but highly dynamic moiety setting off numerous chain reactions in the body. Its action and effects have been extensively researched after the journal Science crowned it as the Molecule of the Year in the year 1992[14]. It plays an essential role in platelet function, neural transmission and blood pressure regulation, along with anticancer and antimicrobial effects[15]. These functions are best performed at low concentrations of nitric oxide. On the other hand, at higher concentrations, it reacts with oxygen to form intermediary compounds such as NO_2, N_2O_4, N_3O_4 and the stable products nitrate and nitrite. It can also combine with the superoxide radical to form peroxynitrite. These free radicals are implicated in deamination and oxidation of the nitrogenous bases forming the genetic code and also inhibit key enzymes in DNA repair. This can cause mutations and trigger carcinogenesis[16].

Thus, the ability to scavenge excess nitric oxide by *Hedyotis diffusa* can be tapped to prevent and treat cancers, delay ageing and prevent numerous diseases where oxidative stress is the culprit.

From the above study, we have demonstrated that the ethanolic extract of the plant Hedyotis diffusa has significant concentration-dependent Nitric oxide free radical-scavenging property. This property can be implicated in the mechanism of action in the anti-inflammatory, anti-cancer and anti-ageing uses of this herb. This finding may open up new avenues for further research on this herb.

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