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COMPARATIVE *IN-VITRO* EVALUATION OF *VITEX LEUCOXYLON* LINN. BARK FOR ANTIOXIDANT ACTIVITY

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ABSTRACT:

Cellular damage arising from free radical is one of the fundamental mechanism underlying a number of human neurodegenerative disorder like diabetes, inflammation, Alzheimer's disease, autoimmune pathologic and digestive system disorder. Thus antioxidant plays an important role in the treatment of such disease. The present study aims at a comparative evaluation of ethyl acetate, hexane and methanol extract of *Vitex leucoxylon* Linn. bark for antioxidant activity. *Vitex leucoxylon* Linn. a medicinal plant of the verbenaceae family, used in traditional medicine for relieving headache and catarrh. HIME was 1.8964 µg/ml after 48 h of incubation. In this study, it was observed that HIME induces a concentration dependent inhibition of HT29 cells, with an IC_{50} value of 1.8964 µg/ml after 48 h of incubation.

INTRODUCTION:

Vitex leucoxylon Linn. (Verbenaceae) commonly known as Songarbhi (Marathi) an excellent herbal crude drug in the nature which has composition of the entire essential constituent that are required for normal and good health of human. It is small to large tree with a sort thick trunk and a spreading crown and almost throughout the Deccan peninsula of India up to an altitude 900 metres, it extends northwards up to Jhansi and part of Bihar. The trees are generally found on the river bank, stream & ponds. The root and the bark are astringent and roots are used as a febrifuge. The leaves are smoked for reliving headache and catarrh and are also used for medicinal baths in fever and anaemia [1].

General pharmacological studies revealed anti-psychotic, anti-depressant, analgesic, antiinflammatory, anti-parkinsonian and anti-microbial activities of aqueous and ethanolic extracts of leaves of *V. Leucoxylon* [2]**.** Sarma *et al* [3] have studied the anti-inflammatory and wound healing properties of the crude alcoholic extract of the leaves in acute inflammation model [3]. The roots and bark are astringent and the roots are reported to be used as a febrifuge. β-Sitosterol, dimethyl terphthalate, vitexin, isovitexin, agnuside and aucubin were isolated from the leaves or barks of *V. Leucoxylon*[4]. Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals[5]. Free radicals are fundamental to any biochemical process and antirepresent an essential part of aerobic life and metabolism [6]. The most common reactive oxygen species (ROS) include superoxide (O_2) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO) radicals and reactive hydroxyl (OH) radicals. The nitrogen derived free radicals are nitric oxide (NO) and peroxynitrite anion. ROS have been implicated in over a hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome [7].

In treatment of these diseases, antioxidant therapy has gained an immense importance. Current research is now directed towards finding naturally occurring antioxidants of plant origin. Antioxidants have been reported to prevent oxidative damage by free radical and ROS, and may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating, and catalytic metals and also by acting as oxygen scavengers [8-9]. Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability [10]. Flavonoids and phenolic compounds

widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti inflammatory, anticarcinogenic etc[11]. They were also suggested to be a potential iron chelator [12-13]. Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties. In view of this and the present understanding about ROS-induced multiple diseases, we have selected one of such ayurvedic herb *Vitex leucoxylon* Linn. The objective of this investigation was to ascertain the scientific basis for the use of this plant in the treatment of antioxidant, using different antioxidant models.

MATERIALS AND METHODS:

Vitex leucoxylon Linn. (Verbenaceae) were collected in flowering stage during late September from the natural population of Jhansi (U.P.) and authenticated by Dr. P.B Singh, Head of regional Research Institute Jhansi, shade dried and powdered then passed from 40# mesh size.

PREPARATION OF VARIOUS EXTRACTS OF *VITEX LEUCOXYLON*:

Powdered material (750 g) of *V. leucoxylon* bark, was extracted with hexane (2 L), ethyl acetate (1.75 L) and methanol (1.75 L) using a Soxhlet apparatus and the spent material was then successively extracted with aqueous methanol (80%, 2 L) and water (2 L). The extract was concentrated in a rotary flash evaporator and dried in desiccators.

Hydroxyl Radical Scavenging Activity: The scavenging capacity for hydroxyl radical was determined according to the modified method [14]. The assay was performed by adding 0.1 ml of EDTA, 0.01 ml of ferric chloride, 0.1 ml of hydrogen peroxide, 0.36 ml of deoxyribose, 1.0 ml of test solutions (5-100 µg/ml) in distilled water, 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1 ml of ascorbic acid were dissolved in sequence. The mixture was then incubated at 37°C for 1 hr and 1.0 ml portion of the incubated mixture was mixed with 10% TCA and 1.0 ml of 0.5% TBA to develop the pink chromogen and measured at 532 nm.

DPPH Radical Scavenging Activity:

The free radical scavenging activity was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH [14]. A 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of control i.e. standard butylated hydroxyl toluene (BHT) at different concentration (25-100 µg/ml) and test solutions at different concentrations (5-100µg/ml) in different test tubes. Thirty minutes later, the absorbances were measured at 517 nm.

Nitric Oxide Scavenging Activity:

Nitric oxide scavenging activity was measured by the spectrophotometric

method [15]. Sodium nitroprusside (5 mM) in phosphate-buffer saline was mixed with a control without the test compound, but with an equivalent amount of methanol. Test solutions at different concentrations (5-100 µg/ml) were dissolved in methanol and incubated at 25°C for 30 min. After 30 min, to 1.5 ml of the incubated solution was diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylenediamine dichloride). The absorbance of the chromophore formed during the diazotization of the nitrile with sulphanilamide and the subsequent coupling with naphthyethylene diamine dihydrochloride was measured at 546 nm.

Superoxide Scavenging:

Superoxide scavenging was carried out using the alkaline dimethyl sulfoxide (DMSO) method [16]. Solid potassium superoxide was allowed to stand in contact with dry DMSO for at least 24 hrs and the solution was filtered immediately before use; the filtrate (200 µl) was added to 2.8 ml of an aquous solution containing nitroblue tetrazolium (56 µM), EDTA (10 µM) and potassium phosphate buffer (10 µM, pH 7.4). Test solutions at different concentrations (5-100 µg/ml) were added and absorbances were recorded at 560 nm against the control.

Statistical Analysis:

The results are presented as mean ± SEM. All parameters were analysed using Student's *t*-test. $P<0.05$ was considered as significant.

RESULTS:

Inhibition of DPPH Radical:

The potential decrease in the concentration of DPPH radial due to scavenging property of ethyl acetate extract of *Vitex leucoxylon* Linn and BHT showed significant free radical scavenging activity viz. 88.52 and 86.73 %, respectively at 100 µg/ml, whereas Hexane and Methanol extract of *Vitex leucoxylon* Linn. did not show any significant activity (Table 1).

Nitric Oxide Scavenging Activity:

The scavenging of nitric oxide by ethyl acetate extract of *Vitex leucoxylon* Linn and BHT was concentration dependent. There was a moderate inhibition of nitric oxide formation with the maximum inhibition being 74.00 and 82.24% respectively at 100µg/ml ethyl acetate extract of *Vitex leucoxylon* Linn and BHT. Similar results were not found in case of Hexane and Methanol extract of *Vitex leucoxylon* Linn (Table 1).

Table 1: Free radical scavenging activity of various extracts of *Vitex leucoxylon* Linn.

Drug	Concentration (µg/ml)	DPPH radical inhibition (%)	Nitric oxide
Ethyl acetate extract of *Vitex leucoxylon* Linn. (ECVL)	5	10.60±0.2698	42.70±0.5411
	10	17.24±1.396	51.67±0.5457*
	25	54.21±2.191**	62.29±1.0380**
	50	84.29±0.1402***	71.00±0.9290***
	100	88.52±0.3861***	74.00±1.7698***
Hexane extract of *Vitex leucoxylon* Linn. (HEVL)	5	09.53±0.5543	37.57±0.6910
	10	10.02±1.029	41.28±0.5382
	25	17.74±0.4495	44.18±0.4970
	50	20.99±0.5698	47.24±0.6458*
	100	26.74±1.6920	49.11±0.2250*
Methanol extract of *Vitex leucoxylon* Linn. (MEVL)	5	06.24±0.109	02.54±0.103
	10	12.43±0.122	06.88±0.142
	25	20.26±0.002	13.99±0.005
	50	22.26±0.009	26.28±0.008
	100	25.59±0.004	33.81±0.029
Butylated hydroxyl toluene (BHT)	25	86.73±0.3915	77.13±0.6458
	50	88.47±0.1520	79.23±1.7770
	100	91.45±0.1782	82.24±0.4976

Values are mean± SEM, 6 independent analysis, P<0.05*, P<0.01, P<0.001*** as compared to standard (Student's *t*-test).**

Superoxide Radical Scavenging: The ethyl acetate extract of Vitex leucoxylon Linn and BHT showed a moderate inhibition of the superoxide radical 74.22 and 81.76% respectively at 100 µg/ml. There was no significant inhibition of superoxide radical by Hexane and Methanol extract of *Vitex leucoxylon* Linn. (Table 2). **Hydroxyl Radical Activity:** The effect of ethyl acetate extract of *Vitex leucoxylon* Linn and BHT on hydroxyl radical and iron (II)-dependent deoxyribose damage was protected significantly at all concentrations; the percentage of inhibition of hydroxyl radical being 79.04 and 73.03 % respectively at 100 µg/ml. No significant inhibition of superoxide radical by Hexane and Methanol extract of *Vitex leucoxylon* Linn. (Table 2)

DISCUSSION:

The antioxidative system protects the organism against ROS-induced oxidative damage. There are restrictions on the use of synthetic antioxidants such as BHT, as they are suspected to be carcinogenic . Natural antioxidants therefore have gained importance. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to form a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. .The ethyl acetate extract of *Vitex leucoxylon* Linn has potent antioxidant and free radical scavenging effects in different *in-vitro* systems, but Hexane and Methanol extract of *Vitex leucoxylon* Linn. showed no significant effects as compared to standard BHT.

Table 2: Free radical scavenging activity of various extracts of *Vitex leucoxylon* Linn.

Drug	Conc. (µg/ml)	Superoxide inhibition (%)	Hydroxyl radical inhibition (%)
Ethyl acetate extract of *Vitex leucoxylon* Linn. (ECVL)	5	35.65±0.9198*	46.99±0.7081*
	10	57.05±1.2561***	52.37±0.5575**
	25	68.70±0.7579***	61.71±0.3296***
	50	71.50±0.8742***	67.15±0.6439***
	100	74.22±0.5889***	79.04±0.6439***
Hexane extract of *Vitex leucoxylon* Linn. (HEVL)	5	28.56±1.6000	42.83±0.6519
	10	39.61±1.8190	49.36±0.8242*
	25	38.40±1.7762	52.81±0.6751*
	50	38.49±1.8220*	62.83±0.4191*
	100	43.46±1.6551**	67.77±0.3100
Methanol extract of *Vitex leucoxylon* Linn. (MEVL)	5	05.12±0.748	04.11±0.529
	10	08.50±0.539	05.66±0.549
	25	23.28±0.649	19.91±0.639
	50	28.26±0.674	32.35±0.458
	100	35.26±0.229	43.88±0.367
Butylated hydroxyl toluene (BHT)	25	74.82±0.8156	57.77±0.3100
	50	77.06±0.8905	70.58±0.7873
	100	81.76±1.6011	73.03±0.3610

Values are mean± SEM, 6 independent analysis, P<0.05*, P<0.01, P<0.001*** as compared to standard (Student's *t*-test)**

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