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Research Article

IN VITRO HIV-1 REVERSE TRANSCRIPTASE INHIBITION BY ALKALOIDS ISOLATED FROM LEAVES OF *ECLIPTA ALBA*

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ABSTRACT

The high price of the highly active anti-retroviral therapy (HAART) programme has encumbered its distribution to over 90% of the HIV/AIDS inhabitants in the world. This reality has instantly called for the essential to progress low-cost alternate anti-HIV/AIDS treatment. The objective of this study is to isolate alkaloid from *E. alba* leaves for their activities against HIV-1 reverse transcriptase. Collected leaves of *E. alba* were extracted with altered solvents and the purity of isolated alkaloids was checked by TLC and qualitative phytochemical analysis were done. Peripheral Blood Mononuclear Cells (PBMCs) isolated from healthy donors by ficoll–hypaque density gradient centrifugation method. Cell viability test was achieved on isolated phytochemicals by MTT assay against PBMC and HIV-1 RT inhibition activity was determined by HIV-1 Reverse Transcriptase (p66) Capture ELISA. In the HIV-1 reverse transcriptase assay, the isolated alkaloid showed 89% of HIV-1 reverse transcription with IC_{50} of 5 µg/ml. MTT assay revealed that, the alkaloid isolated from *E. alba* had no cytotoxic activity (IC_{50} values higher than 100 µg/ml). Characterization of important biologically-active alkaloid from *E. alba* plant will certainly be supportive in defending and handling various viral diseases in human beings. The results of the present study sustenance the medicinal usage of the alkaloid isolated from the leaves of *E. alba* can be used as antiviral agents and can be subjected to characterize the therapeutic drugs and undergo further pharmacological screening that can be used as sources for new drugs.

Keywords: *Eclipta alba,* alkaloid, HIV-1 reverse transcriptase, MTT assay, PBMCs, cytotoxic activity

INTRODUCTION

In the modern healing system, individuals have in progress observing at the ancient remedial systems like Ayurveda, Siddha and Unani due to the opposing effects associated with synthetic drugs. Mostly in developing countries, herbal remedies play significant role in health care series. The unusual extensive definition for therapeutic plants was merged by the ancient Indian literature and it measured all plant parts to be prospective sources of remedial components[1]. A diverse array of chemicals is synthesized by curative plants. In hepatic and spleen enlargement *Eclipta alba* is used as a stimulant and diuretic and it is also used for catarrhal jaundice and for skin diseases.[2, 3] Plant-derived traditional medications can be used to protect different diseases as they cover a diversity of secondary metabolites to which the bacterial species may not be resistant.

The plant *Eclipta alba* (L.) is commonly known as false daisy which is annual herbaceous and belonging to Asteraceae family. The leaves of this plant are opposite, lanceolate and sessile. The plant is an erect, roughly hairy, annual, much branched and rooting at the nodes. It is also known as Bhringaraj and Karisilakanni, which is found a common weed throughout India ascending up to 6000 f. *E. alba* has been used in various parts of tropical and sub-tropical regions like south America, Asia, Africa. The plant is generally used in hair oil all over India for healthy black and long hair[4]. This plant is well recognised and several *in vitro* and *in vivo* studies describe its anti-ageing agent and anti-hepatotoxic properties[5]. Coumestans like wedelol acetone, desmethyl wedelolactone[6], furano-coumarins, ecalbatin[7], eleanane & taraxastane glycosides[8,9], are the consisting main active principles in *E. alba.* The safety, efficacy and quality of some of bioactive principles have not been scientifically validated and antibacterial activity was also not studied for isolated phytochemicals present in it. Therefore, the objective of this present study is to isolate alkaloid from *E. alba* leaves for their activities against HIV-1 reverse transcriptase.

MATERIALS AND METHODS

Plant Material and Alkaloids Extraction

Leaves of *E. alba* were collected in Kakatiya University Campus in July 2016. The plant was botanically authenticated by Prof. V.S. Raju, Senior Taxonomist at Department of Botany, Kakatiya University, India. The collected sample was grinded, and this ground sample (500 g) was made alkaline with 30% ammonia and extracted with chloroform at room temperature for a total period of 24 h. The extract was partitioned between chloroform and 5% HCl. Finally, chloroform was totally evaporated from the organic phase to form the alkaloid powder (5.2 g) .

Thin Layer Chromatography (TLC)

To determine the purity and relative to front (R_f) of isolated compound, a thin layer chromatography was carried out as per conventional one-dimensional ascending method using silica gel 60F254, 7x6 cm (Merck) were cut with ordinary household scissors. Plate marking were made with soft pencil. Glass capillaries were used to spot the sample for TLC. Applied 1 µl of extract by using capillary at distance of 1 cm. After pre-saturation with mobile phase (chloroform: methanol- 0.5:9.5) for 20 minutes the plates were dried and the spot which appeared were developed

with iodine vapour. The movement of sample was expressed by its retention factor (Rf) and values were calculated as:

R_f = Distance travelled by the solute/Distance travelled by solvent front TLC plates.

Qualitative Determination of Phytochemicals

Qualitative phytochemical analyses of the isolated alkaloid were performed by following the protocol of J.B. Harborne (1973)[9].

Tannins: 200 mg of plant material was boiled in 10 ml distilled water and few drops of $FeCl_3$ were added to the filtrate; a blue-black precipitate indicated the presence of Tannins.

Alkaloids: 200 mg plant material was boiled in 10 ml methanol and filtered. 1% HCl was added than 6 drops of Dragen doff reagent was added, and brownish-red precipitate was the indication for the presence of alkaloids.

Saponins (Frothing test): 5 ml distilled water was added to 200 mg plant material. 0.5 ml filtrate was diluted to 5 ml with distilled water and shaken vigorously for 2 minutes. Formation of stable foam indicates the presence of saponins.

Cardiac Glycosides (Keller-Kiliani test): 2 ml filtrate was treated with 1 ml glacial acetic acid containing few drops of $FeCl_3$. Conc. H_2SO_4 was added to the above mixture giving green-blue colour depicting the positive results for presence of cardiac glycosides.

Steroids (Liebermann-Burchard reaction): To 10 ml chloroform 200 mg plant material was added. In the ratio of 1:1, acetic anhydride was added which resulted into the formation of blue-green ring pointing towards the presence of steroids.

Terpenoids (Salkowski test): 2 ml of chloroform ($CHCl_3$) and 3 ml\of concentrated sulphuric acid (H_2SO_4) were carefully added to 200 mg plant material. A reddish-brown colouration signified the presence of terpenoids.

Flavonoids: To the aqueous filtrate 5 ml of dilute ammonia solution was added, followed by concentrated H_2SO_4. A yellow colouration indicated the presence of flavonoids.

Anthraquinones: 500 mg of dried plant leaves were boiled in 10% HCl for 5 mins and filtrate was allowed to cool. Few drops of 10% NH_3, equal volume of $CHCl_3$ was added to 2 ml filtrate. The formation of rose-pink colour implies the presence of Anthraquinones.

Reducing Sugars: To the 10 ml of aqueous extract a few drops of Fehling's solution A and B were added; an orange red precipitate suggests the presence of reducing sugars.

Quantitative Determination of Alkaloids

The content of alkaloids was restrained by following the protocol described by Harborne (1984)[10]. To prepare the suspension, dispersing 5 g of the dried leaves in 10% acetic acid solution in ethanol and kept at 28^0C for 4 hrs which was further filtered through Whatman Number 42. Alkaloid was precipitated by concentrating the filtrate to one quarter of its original volume and then drops of conc. aqueous NH_4OH were added. Finally, the precipitate was washed with 1% ammonia solution and dried at 80^0C in the oven. The alkaloid content alkaloid was calculated and expressed as mg/g of sample.

Anti-HIV Activity of Isolated Alkaloid Compound

The HIV reverse transcriptase enzyme inhibition due to purified compound was determined using HIV-1 Reverse Transcriptase (p66) Capture ELISA test (Immuno Diagnostics, Inc, USA). 100 µl of different concentrations of crude extract sample and standard drug-AZT were added to respective wells of microplate. 10-fold serial dilutions were made (1 mg/ml to 31.5 µg/ml). 100 µl of HIV-1 Reverse Transcriptase (p66) added to all wells. Without compound was used as blank and AZT was used as standard. The mixture of samples and HIV-1 RT (p66) were incubated at room temperature for 1 hour. Washed the plate three times with wash buffer. 100 µl of Anti-p66 Monoclonal Antibody (detector reagent) was added to all wells and incubated the plate in dark for 1 hour. After one hour of incubation in the dark, the plate washed three times with washing buffer. 100 µl of TMB substrate was added to each well and incubated the plate for 30 minutes up to formation of blue colour. Added 100 µl of stop solution to all wells and read the absorbance at 450 nm with using microplate Elisa reader. The HIV-1 RT inhibition efficiency was calculated with compared to the blank control and calculate the percentage of inhibition by using following equation:

% of HIV-1 RT inhibition = 100 – Sample OD / Control OD x 100

Cytotoxicity Screening by MTT Assay

Cell viability was evaluated by the MTT 3-(4, 5 dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) test method. MTT (5 mg/ml) was dissolved in PBS. The solution was filtered through a 0.2 m filter and stored at 2 - 8°C. PBMC cells (10^3 cells/well) were seeded in 100 µl RPMI-1640 medium in 96-well culture plates prior to the treatment with isolated compound and standard drug AZT and were incubated in a humidified atmosphere with 5% CO_2, 37°C, for 24 h (overnight) or until total adhesion to surface. For MTT assay the medium from the wells was removed carefully after incubation. Each well was washed with MEM (w/o) FCS for 2-3 times. After that, 100 µl fresh medium containing serially dilutions of isolated compound of *E. alba* leaves or AZT were added to each well, and incubated for another 48 hrs. Diluted isolated compound or AZT solutions were freshly prepared in DMSO prior to each experiment. AZT treated PBMC and DMSO treated PBMC were used as positive and negative controls respectively. For MTT assay, 10 µl MTT (5 mg/ml) was added into each well to generate formazan, and then cells were incubated in humidified atmosphere with 5% CO_2 at 37°C for 4 h. After removing the supernatant, 100 µl DMSO was added to dissolve the purple crystal resulting from MTT reduction. The extent of MTT reduction to formazan within cells was measured by absorbance at 595 nm by a microplate reader (Robonik, Mumbai). High optical density readings corresponded to a high intensity of dye colour that is to a high number of viable cells able to metabolize MTT salts.

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death. Each experiment was performed in triplicates for three times. The percentage of proliferation was calculated by the following formula:

% Cell viability = (OD sample – OD control) / OD control × 100

Determination of IC_{50}

The effect of alkaloid compound was expressed by IC_{50} values. IC_{50}, the concentration of compound required to inhibit 50 % cell growth, was determined by plotting a graph (dose-response curves) of Log (concentration of Extract) vs % cell viability. The IC_{50} value was determined from the plotted curve.

Statistical Analysis

Results were expressed as the mean ± SD of values obtained in triplicate from three independent experiments. Statistical analysis was done using EXCEL (DATA ANALYSIS) for determining ANOVA. A p value of < 0.01 was considered statistically highly significant, p <0.05 in significant.

RESULTS

Anti-HIV Activity of Isolated Alkaloid Compound from *E. alba*

The anti-HIV activity was measured using HIV-1 Reverse Transcriptase (p66) Capture ELISA test and the IC_{50} values were determined. The results of the screening are shown in Figure-1. At the beginning of the study it was hypothesized that compounds present in the leaves of the plant could be active against HIV-1 RT. At 125 ug/ml concentration the isolated compound of *E. alba* showed more potent HIV-1 RT inhibition while standard drug shows at 250 µg/ml. The activity of HIV-1 RT inhibition is improved up to 125 µg/ml from 31.2 µg/ml and after that the inhibition activity was reducing. The standard drug inhibition activity also growing up to 250 µg/ml concentration, but after up to 1000 µg/ml the activity was slightly declining. These results conclude that the alkaloid compound from *E. alba* shows, 93% of HIV-1 RT inhibition at 125 µg/ml with IC_{50} of 775 µg/ml while the standard drug shows 94% of HIV-1 RT inhibition at 250 µg/ml with IC_{50} of <31.2 µg/ml. Therefore, the alkaloid compound shows anti-HIV activity nearly similar to used standard drug (AZT) showed in figure.

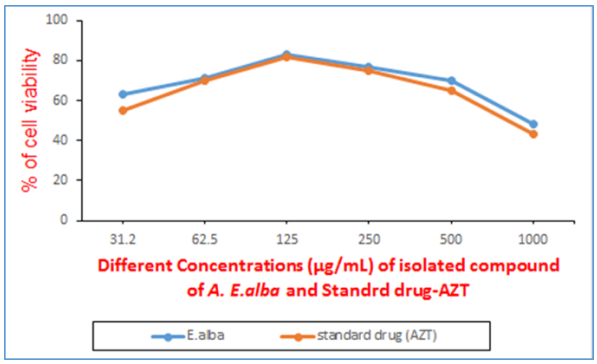

Figure 1: The HIV-1 RT inhibition of isolated alkaloid compound from *E. alba* using HIV-1 Reverse Transcriptase (p66) Capture ELISA

By isolating and identifying these bioactive compounds new drugs can be formulated to treat various diseases and disorders. The main limitation in the use of traditional remedies is the lack of standardization of raw material, manufacturing progress and the final product[10]. A biomarker on the other hand is a group of chemical compounds which are not unique for that plant material but also correlates with biological efficacy. So, the need arises to lay standards by which the right material could be selected and incorporated into the formulation[11]. Medicinal plants are reported to possess a wide range of properties including anti-carcinogenic, anti-inflammatory, antioxidant, antitumor, antimicrobial, immune modulatory, anti-helminthic, antiviral, and antibiotic activities[12, 13]. Medicinal plants is a table food with unbelievable medicinal properties. In the previous studies.

Many scientists such as[14-19], have shown that methanolic extracts from medicinal plants are inhibitory than the aqueous extracts in screening for anti-HIV activity. However, in this study the butanol extracts of *E. alba* showed the highest anti-HIV activity. This is in keeping with studies of[20]; and[21] WHO reported that phytochemicals extracted with butanol and water are more commonly effective inhibitors of HIV virus. The results suggest that the separation of polar and non-polar compounds can increase the chance of finding highly active anti-viral compounds with low cytotoxicity[19]. The alkaloid compound from *E. alba* showed strong anti-HIV activity with IC_{50} of 775 µg/ml and this plant is used traditionally as a treatment of viral diseases and the different compounds are alkaloid, but there is no literature of its anti-HIV activity. Therefore, it may be the first report of this isolated alkaloid compound which showed HIV-1 RT inhibition.

Isolation of PBMC Cells

Aseptically 2.5ml of HiSep media transferred in to a 15ml heparin coated test tubes and overlay with 7.5ml diluted blood (blood sample from healthy volunteers were collected by venepuncture from Kakatiya University health centre and blood sample were diluted at 1:1 ratio with PBS). Centrifuged at 1,000 x g for 30 min. During the centrifugation the PBMC's moved from the plasma and were suspended in the density gradient, isolating them from erythrocytes and granulocytes. The PBMC's layer was removed and then washed twice with PBS and centrifuged at 400 x g. The supernatant was then removed and the PBMC's were re suspended in RPMI 1640 medium.

Cytotoxicity of Active Compound on PBMC Cells

In vitro assay of cytotoxic activity of isolated compound of *E. alba* aerial parts against PBMC cells at different concentrations was evaluated by MTT assay. MTT assay is based on the metabolic reduction of MTT into formazan crystals on treatment with PBMC cells. The inhibitory activities of isolated compound were compared with the standard drug AZT. Cell proliferation of compound was determined by an inhibitory concentration at 50% growth (IC_{50}). The PBMC cell viability percentage were found to be at different concentration of isolated compound (Table-

1). Cytotoxic activity at the different concentrations of 15.6 µg, 31.25 µg, 62.5 µg, 125 µg, 250 µg, 500 µg and 1000 µg/ml showed effective inhibition against PBMC cells. Therefore, data indicate that cytotoxicity of alkaloid compound was influenced by their concentration. Increased percentage of inhibition by suppressing viability was observed from Figure-2 that a gradually increase in percentage in all the treatments. However, at 250 µg/ml of tested drug AZT shows 49.20 ± 1.00 cell viability against PBMC cells was observed whereas isolated compound only crossed 50% inhibition at 250 µg/ml. Therefore, the percentage of 50% inhibition concentration (IC_{50}) is 250 µg/ml.

Table 1: Cell proliferative effect of isolated active compound on PBMC cells

Concentration (µg/ml)	% of Cell viability	
	Isolated alkaloid Compound	Standard Drug (AZT)
15.6	97.45 ± 0.23***	92.18 + 1.18
31.25	92.0 ± 0.37**	89.15 ± 0.98
62.5	83.05 ± 0.96**	78.19 ± 1.13
125	69.82 ± 1.16*	67.6 ± 1.98
250	52.6 ± 0.87*	49.20 ± 1.00
500	33.7 ± 0.96**	37.5 ± 0.95
1000	20.6 ± 0.67*	25.9 ± 0.32
IC_{50}	250 (µg/ml) *	255 (µg/ml)

* P< 0.05, ** p < 0.01, ***p < 0.001 value are considered statistically significant

This study revealed that, at 250 µg/ml the isolated alkaloid showed highest HIV-1 RT inhibition and at 15.6 µg/ml the cell viability percentage is highest. The 50% cell viability showed less toxicity of alkaloid compound. The precise statement must be that the MTT bioassay is used as a parameter for mitochondrial or glycolytic metabolic activity of living cells and if that activity of experimental and control cells is the same, it could be theoretical that any reduction of metabolic activity in experimental wells could suggest a reduced cell viability or inhibition of cell proliferation. Cytotoxicity screening models provide important preliminary data to help selecting plant extracts with potential antineoplastic properties for future work[22,23]. Extracts of *E .alba* reduce the risk of cancer due to the presence of flavonoids[24]. In this study the alkaloid compound from *E. alba* showed less toxicity at lowest concentration and also showed HIV-1 RT inhibition.

Phytomedicines have been used since ancient times to treat various infections but clinical studies are limited[25]. Safety is a major requirement for an antiviral agent and in the search for new drugs it is important to consider possible secondary effects. The minimal cytotoxicity observed in the extracts explored may be due to the presence of cytoprotective components. These cytoprotective (chemical compounds) components of plants have been reported in other studies on plant extracts [26].

CONCLUSION

The isolated alkaloid compound from *E. alba* appeared to have the potential to act as a basis of useful drugs and also to progress the health status of the consumers as a result of the occurrence of active compound that are vital for decent health. The single compound from leaves of this plant can provide lead molecules which could be useful substrate for the synthesis of new broad spectrum for anti-HIV and the treatment of infections caused by the pathogenic organisms. Therefore, it can be concluded that this concentration of active compound could be perhaps used securely against HIV. Based on these results, the alkaloid compound of *Eclipta alba* potentially to be developed as herbal medicine can replace the standard antiretroviral (AZT) drugs against PBMC cells.

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