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# In Vitro Anticancer Activity of *Kokilaksha* (*Hygrophila spinosa* T Ander.) in Osteosarcoma Cell Lines

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## ABSTRACT

**Introduction:** The drug Kokilaksha (Hygrophila spinosa T Ander.) found growing as a weed in the paddy fields is one of the commonly used drugs to treat Vatarakta (Gouty arthritis) as per classical texts of Ayurveda. A thorough literary search on the disease Vatarakta reveals that in Gambhira awastha (chronic stage) the disease involves deeper dhatus like Sandhi, Asthi, Majja; and Arbuda is mentioned as one of the upadravas (complications) of Gambhira Vatarakta according to Acharya Sushruta. Both malignant as well as benign tumours are considered under the term Arbuda. The research profile of Kokilaksha reveals its potent anti-tumour activity and efficacy in the Erythropoietic system. The current study is intended to evaluate the in vitro anticancer activity of Kashaya and hydroalcoholic extract of the drug Kokilaksha (Hygrophila spinosa T Ander.) in osteosarcoma cell lines.
**Materials and Methods:** The in vitro study consisting of two osteosarcoma cell lines namely MG-63 and SAOS-2 were subjected to the hydro alcoholic extract and kashaya of Kokilaksha (Hygrophila spinosa T Ander.). The anticancer activity was carried out using three assays namely MTT assay, Nitric oxide scavenging activity and LDH assay.
**Results:** The in vitro study showed both the hydro alcoholic extract and kashaya of Kokilaksha (Hygrophila spinosa T Ander.) exhibited anticancer activity in a time and concentration dependent manner with respect to all the three assays in both MG-63 and SAOS-2 osteosarcoma cell lines.

***KEYWORDS:*** *Kokilaksha* (*Hygrophila spinosa* T Ander.), osteosarcoma cell lines, MTT assay, Nitric oxide scavenging activity, LDH assay, hydro alcoholic extract, *Kashaya*

## INTRODUCTION

Cancer one of the most dreaded diseases of the 20th century is spreading further with continuance and increasing incidence in the 21st century. The Global Cancer burden is estimated to have risen to 19.3 million new cases and 10 million deaths as per WHO statistics September 2021 and it is expected to be 28.4 million cases by 2040 [1,2]. Multidisciplinary scientific investigations are making best possible efforts to counter the disease especially considering a greater emphasis being given towards the researches in complementary and alternative medicine dealing with cancer management. In terms of Primary bone cancer 28% are osteosarcoma cases. Less than 0.2% of all cancers are primary bone cancers, however

it is much more common for bones to be the sight of metastasis from other cancers. Hence there is an utmost need to invest in a drug which is effective in this direction [2,3].

The drug *Kokilaksha* which is found growing as a weed in the paddy fields is one of the drugs of choice to treat *Vatarakta* (Gouty arthritis) in Ayurveda texts. A thorough literary search on the disease *Vatarakta* reveals that in chronic stages the disease involves deeper tissues like bone and bone marrow ; and *Arbuda* is mentioned as one of the *upadravas* (complications) of *Gambhira Vatarakta* [4,5]. The research profile of the drug *Kokilaksha* reveals its potent anti-tumour activity [6,7]. Action of the drug has also been proven in the erythropoietic system .Therefore it was felt to look for the action and effectiveness of the drug in malignant conditions of bone tissue. Hence present study was aimed at evaluating the anti-cancer property of *Kokilaksha* (*Hygrophila spinosa* T Ander) in osteosarcoma cell lines.

## MATERIALS AND METHODS

### Phase 1

Collection of plant material : Fresh whole plants of *Kokilaksha* ( *Hygrophila spinosa* T Ander. ) were collected during the month of October from in and around the campus of Sri Sri College of Ayurvedic Science and Research , Bengaluru. The plant parts were washed thoroughly with tap water and air dried in shade at room temperature. They were then mechanically powdered and sieved.

Identification and authentication of drug : The collected drug sample was botanically identified and authenticated by the taxonomist, Department of Dravyaguna at Sri Sri College of Ayurvedic Science and Research, Bengaluru.

### Phase 2

Preparation of hydro-alcoholic extract and *kashaya* of *Kokilaksha:* Hydro alcoholic extract was prepared using 20 gms of powdered plant material ; ethanol and distilled water as solvent in the ratio of 70:30. This was then heated under reflux at a temperature between 80-85℃ for 3-4 hours followed by filtration. Filtrate was evaporated using water bath at the temperature between 80-85℃ and dried. Obtained dry extract was preserved in air tight bottles at $5^0$C until further use [8].

*Kokilaksha kashaya* was prepared as per general *Kashaya paaka Vidhi* of Sharangadhara Samhita [9].

### Phase 3

Cell lines: MG-63 andSAOS-2 osteosarcoma cell lines were obtained from National Centre for Cell Science (NCCS), Pune, India.

Chemicals for Cell culture: DMEM with phenol red indicator GIBCO [Cat#: 11965-084] , MEM GIBCO [Cat#:11095-072] ,FBS GIBCO [Cat#:10270106] ,PenStrep GIBCO [Cat#15140122] (100X-Concentration), Dulbecco's Phosphate Buffered Saline (PBS) Millipore [Cat#: BSS2010-B] , Trypsin - EDTA (0.25%) GIBCO [Cat#: 25200-072] , DMSO (Hybri-MaxTM) SIGMA [Cat#: D-4540] , DMSO (Dimethyl sulphoxide) Extra pure, 99% SRL [Cat#: 43404]

Cell Culture: MG -63 cells were maintained in the logarithmic phase of growth in MEM supplemented with 20%FBS, 500µl MEM Non-Essential acids, IX PenStrep solution in 100mm culture plates at $37^0$C in a humidified (92.7%RH) incubator operating at $37^0$C and 5% carbon dioxide . SAOS-2 osteosarcoma cell lines were maintained in the logarithmic phase of growth in DMEM supplemented with 15%FBS and IX PenStrep solution in 100mmcultureplates at $37^0$C in a humidified (92.7%RH) incubator operating at $37^0$C and 5% carbon dioxide.

Treatment: Exponentially growing cells were treated with serial dilutions of hydro alcoholic extract (1mg/ml ,0.5mg/ml, 0.25mg/ml, 0.125 mg/ml,0.06 mg/ml ,0.03 mg/ml ,0.015 mg/ml ,0.078 mg/ml) and *kashaya* (50 %, 25 %, 12.5 %,6.25 %,3.13 %,1.56 %,0.78 %) of *Kokilaksha* for 24h, 48h and 72h.

**1.Cytotoxicity Assay [10]**

**MTT Assay**

The MTT [3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide] colorimetric assay was used to screen for cytotoxic activity. Briefly, MG-63 and SAOS-2 cells were seeded at a density of $5\times10^3$ cells/well and $8\times10^3$ cells/well in 96-well plates .Cells and test compounds were prepared in 96-well plates containing a final volume of 100 µl/ well and incubated for the desired period of exposure (24h, 48h and 72h). Post the incubation the culture supernatant was aspirated out. The cells were again incubated with 100 µl MTT Solution per well to achieve a final concentration of 0.45 mg/ml for 4 hours at 37°C and then with 150 µl Solubilization solution DMSO. The plates were read at 570 nm on a scanning multi-well spectrophotometer. Data was represented as the mean values for three independent experiments . Percentage cell viability was calculated using the equation:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of cells (treated)}}{\text{Absorbance of cells (untreated)}} \times 100$$

The concentrations of the drug at which increased cytotoxic activity was exhibited through MTT assay was taken for the Nitric oxide scavenging activity and LDH assay .

**2. Nitric Oxide Scavenging Activity [11,12]**

The nitric oxide scavenging activity was measured according to the method of Marcocci et al., 1994

**3. Estimation of Serum LDH (LDH Assay)**

1000µl of reagent was mixed to 25 µl of test sample in Eppendorf tube. This mixture was aspirated and absorbance was directly recorded at 405nm by using auto analyzers BTS -350

## STATISTICAL ANALYSIS

The collected data was represented in the form of tables. The difference among data was statistically analyzed using One way ANOVA test followed by Dunnetts test of significance. Graph Pad Prism 5 Software version was used for statistical analysis

## RESULTS

The results of MTT assay for Hydroalcoholic extract and *Kashaya* of *Kokilaksha* (*Hygrophila spinosa* T Ander) on MG-63 and SAOS-2 osteosarcoma cell lines were represented in the Tables (1-10)

**Table 1: Effect of hydroalcoholic extract *of Kokilaksha* [*Hygrophila spinosa* T Ander] on MTT Assay in MG -63 osteosarcoma cell lines at 24 , 48 and 72 hours**

| Groups | Drug treatment | MTT (% viability) at 24 hours | MTT (% viability) at 48 hours | MTT (% viability) at 72 hours |
|---|---|---|---|---|
| I | Control (Cancer cell) | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| II | HEK (1mg/ml) | 92.14± 8.74 $^{ns}$ | 87.01 ± 9.16 $^{ns}$ | 81.12 ± 7.05$^{**}$ |
| III | HEK (0.5mg/ml) | 91.81 ± 4.72 $^{ns}$ | 83.57 ± 10.51 $^{ns}$ | 84.00 ± 5.68$^{**}$ |
| IV | HEK (0.25mg/ml) | 71.11 ± 16.69$^{*}$ | 83.16 ± 13.31 $^{ns}$ | 86.02 ± 7.11$^{*}$ |
| V | HEK (0.125 mg/ml) | 85.52 ± 15.77$^{ns}$ | 81.60 ± 4.23 $^{ns}$ | 81.35 ± 2.19$^{**}$ |
| VI | HEK (0.06 mg/ml) | 92.74± 9.16 $^{ns}$ | 78.88 ±8.03 $^{ns}$ | 83.73 ± 5.40$^{**}$ |
| VII | HEK (0.03 mg/ml) | 86.02 ± 8.80 $^{ns}$ | 86.94 ± 1.44 $^{ns}$ | 90.82 ± 3.37 $^{ns}$ |
| VIII | HEK (0.015 mg/ml) | 90.37± 6.43 $^{ns}$ | 97.80 ± 16.47 $^{ns}$ | 96.21 ±3.29 $^{ns}$ |
| IX | HEK (0.078 mg/ml) | 82.62 ± 10.79 $^{ns}$ | 88.20 ± 4.47 $^{ns}$ | 93.94 ± 11.56 $^{ns}$ |

All the values were expressed in Mean ± SD (n=9). *P* values calculated by one way ANOVA analysis followed by Dunnetts 't' test of significance in comparison with control group.**P < 0.01, *= P < 0.05, ns – non significant as compared to control. HEK-hydroalcoholic extract of *Kokilaksha*

The cell viability after 24 hours of incubation with hydroalcoholic extract of *Kokilaksha* was found to significantly (P<0.05) decrease compared to control on treatment with 0.25 mg/ml concentration of the drug .Post 48hours of incubation the cell viability was found to be non-significant at all the different concentrations of the drug . After 72 hours of incubation cell viability was found to significantly (P<0.01) decrease to 81.12 ± 7.05%,84.00 ± 5.68 % ,81.35 ± 2.19% and 83.73 ± 5.40 % compared to control on treatment with 1 mg/ml, 0.5

mg/ml, 0.125 mg/ml and 0.06 mg/ml concentrations of the drug (Table 1 )

**Table 2: Effect of *Kashaya* of *Kokilaksha* [*Hygrophila spinosa* T Ander] on MTT Assay in MG-63 osteosarcoma cell lines at 24 , 48 and 72 hours**

| Groups | Drug treatment | MTT (% viability) at 24 hours | MTT (% viability) at 48 hours | MTT (% viability) at 72 hours |
|---|---|---|---|---|
| I | Control (Cancer cell) | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| II | KS (50 %) | 9.30 ±0.76$^{**}$ | 6.18 ± 0.75$^{**}$ | 4.79 ± 0.50$^{**}$ |
| III | KS (25 %) | 77.53 ±7.78$^{*}$ | 5.55 ± 0.96$^{**}$ | 3.97 ± 0.20$^{**}$ |
| IV | KS (12.5 %) | 84.84 ±4.72$^{ns}$ | 84.32 ± 10.98$^{ns}$ | 4.51 ± 0.80$^{**}$ |
| V | KS (6.25 %) | 86.73 ±11.50$^{ns}$ | 90.73 ± 8.66$^{ns}$ | 78.84 ±10.24$^{**}$ |
| VI | KS (3.13 %) | 94.76 ±10.35$^{ns}$ | 85.76 ± 3.52$^{ns}$ | 95.56 ± 9.69$^{ns}$ |
| VII | KS (1.56 %) | 88.98 ±10.67$^{ns}$ | 91.40 ± 10.017$^{ns}$ | 91.40 ± 4.72$^{ns}$ |
| VIII | KS (0.78 %) | 92.41 ±9.28$^{ns}$ | 93.16 ± 9.32$^{ns}$ | 97.67 ± 7.00$^{ns}$ |

All the values were expressed in Mean ± SD (n=8). *P* values calculated by one way ANOVA analysis followed by Dunnetts 't' test of significance in comparison with control group.**=P < 0.01,*= P < 0.05,ns – non significant, as compared to control. KS-*Kashaya* of *Kokilaksha*

The cell viability after 24 hours of incubation with *Kashaya* of *Kokilaksha* was found to significantly (P<0.01) decrease to 9.30 ±0.76 % compared to control on treatment with 50 % concentration of the drug. On treatment with 25 % concentration of drug the cell viability was found to significantly (P < 0.05) decrease to 77.53 ±7.78% compared to control (Fig 4).After 48 hours of incubation cell viability was found to significantly (P<0.01) decrease to 6.18 ± 0.75 % and 5.55 ± 0.96 % compared to control on treatment with 50 % and 25 % concentrations of the drug respectively (Fig 5). Post 72 hours of incubation cell viability was found to significantly (P<0.01) decrease to 4.79 ± 0.50 % , 3.97 ± 0.20% , 4.51 ± 0.80 % and 78.84 ±10.24 % compared to control on treatment with 50 % , 25 %, 12.5 % and 6.25 % concentrations of the drug respectively (Table 2)

**Table 3: Effect of hydroalcoholic extract of *Kokilaksha* [*Hygrophila spinosa* T Ander] on MTT Assay in SAOS-2 osteosarcoma cell lines at 24 , 48 and 72 hours**

| Groups | Drug treatment | MTT (% viability) at 24 hours | MTT (% viability) at 48 hours | MTT (% viability) at 72 hours |
|---|---|---|---|---|
| I | Control (Cancer cell) | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| II | HEK (1mg/ml) | 98.75 ± 2.00$^{ns}$ | 48.18 ± 4.52$^{**}$ | 31.40 ± 1.36$^{**}$ |
| III | HEK (0.5mg/ml) | 109.88 ±7.64$^{ns}$ | 81.63 ± 7.05$^{**}$ | 72.68 ± 20.85$^{ns}$ |
| IV | HEK (0.25mg/ml) | 112.26 ± 7.53$^{ns}$ | 89.09 ± 0.49$^{*}$ | 85.40 ± 16.81$^{ns}$ |
| V | HEK (0.125 mg/ml) | 101.07 ± 8.70$^{ns}$ | 91.33 ± 2.05$^{ns}$ | 97.11 ± 24.27$^{ns}$ |
| VI | HEK (0.06 mg/ml) | 98.74 ±5.59$^{ns}$ | 97.93 ± 4.23$^{ns}$ | 84.20 ±11.07$^{ns}$ |
| VII | HEK (0.03 mg/ml) | 101.31 ± 8.30$^{ns}$ | 100.18 ± 4.9$^{ns}$ | 99.62 ± 11.08$^{ns}$ |
| VIII | HEK (0.015 mg/ml) | 102.10± 10.25$^{ns}$ | 105.71 ± 5.29$^{ns}$ | 103.15 ± 13.84$^{ns}$ |
| IX | HEK (0.078 mg/ml) | 98.71± 7.92$^{ns}$ | 102.69 ± 5.38$^{ns}$ | 111.83 ± 10.20$^{ns}$ |

All the values were expressed in Mean ± SD (n=9). *P* values calculated by one way ANOVA analysis followed by Dunnetts 't' test of significance in comparison with control group . **P < 0.01, *= P < 0.05, ns – non significant as compared to control. HEK-hydroalcoholic extract of *Kokilaksha*

The cell viability after 24 hours of incubation with hydroalcoholic extract of *Kokilaksha* was found to non-significant at all the concentrations of drug in SAOS-2 cell lines. The cell viability after 48 hours of incubation with hydroalcoholic extract of *Kokilaksha* was found to significantly (P<0.01) decrease to 48.18 ± 4.52% and 81.63 ± 7.05 % , compared to control on treatment with 1 mg/ml and 0.5 mg/ml concentrations of the drug respectively. On treatment with 0.25mg/ml concentration of drug the cell viability was found to significantly (P<0.05) decrease to 89.09 ± 0.49 % compared to control. After 72 hours of incubation with hydroalcoholic extract of

*Kokilaksha* cell viability was found to significantly (P<0.01) decrease to 31.40 ± 1.36 %, compared to control on treatment with 1 mg/ml concentration of the drug. (Table 3)

**Table 4: Effect of *Kashaya* of *Kokilaksha* [*Hygrophila spinosa* T Ander] on MTT Assay in SAOS-2 osteosarcoma cell lines at 24 , 48 and 72 hours**

| Groups | Drug treatment | MTT (% viability) at 24 hours | MTT (% viability) at 48 hours | MTT (% viability) at 72 hours |
|---|---|---|---|---|
| I | Control (Cancer cell) | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| II | KS (50 %) | 32.78 ± 14.65** | 15.30 ± 8.08** | 8.74 ± 2.30** |
| III | KS (25 %) | 85.43 ± 29.67ns | 20.57 ±7.89** | 15.79 ± 1.77** |
| IV | KS (12.5 %) | 99.22 ± 10.14 ns | 84.00 ±25.85ns | 61.01 ± 24.52ns |
| V | KS (6.25 %) | 100.82 ±5.75 ns | 92.69 ± 24.67 ns | 93.20± 20.72 ns |
| VI | KS (3.13 %) | 105.81 ± 3.27 ns | 86.18± 16.88 ns | 92.48 ± 22.63 ns |
| VII | KS (1.56 %) | 104.92 ± 3.15 ns | 87.15 ± 26.52 ns | 91.04 ± 21.08 ns |
| VIII | KS (0.78 %) | 94.33 ± 2.50 ns | 79.17 ± 32.71 ns | 75.89 ± 15.21 ns |

All the values were expressed in Mean ± SD (n=8). *P* values calculated by one way ANOVA analysis followed by Dunnetts 't' test of significance in comparison with control group. **=P < 0.01, ns – non significant, as compared to control. KS-*Kashaya* of *Kokilaksha*

The cell viability after 24 hours of incubation with *Kashaya* of *Kokilaksha* was found to significantly (P<0.01) decrease to 32.78 ± 14.65 % compared to control on treatment with 50 %, concentration of the drug. After 48 hours of incubation with *Kokilaksha Kashaya* the cell viability was found to significantly (P<0.01) decrease to 15.30 ± 8.08 % and 20.57 ±7.89 % compared to control on treatment with 50 % and 25 % concentrations of the drug respectively . The cell viability after 72 hours of incubation with *Kokilaksha Kashaya* was found to significantly (P<0.01) decrease to 8.74 ± 2.30 % and 15.79 ± 1.77 % compared to control on treatment with 50 % and 25 % concentrations of the drug respectively. (Table 4)

**Table 5: Effect of hydroalcoholic extract of *Kokilaksha* [*Hygrophila spinosa* T Ander] on Nitric oxide inhibitory ratio in MG- 63 cell lines at 24 , 48 and 72 hours**

| Groups | Drug treatment | Nitric oxide Inhibitory ratio at 24 hours | Nitric oxide Inhibitory ratio at 48 hours | Nitric oxide Inhibitory ratio at 72 hours |
|---|---|---|---|---|
| I | Control Cancer cell) | - | - | - |
| II | HEK (1 mg/ml) | 3.09 ± 0.01 | 15.33 ± 0.17 | 28.42 ± 0.11 |
| III | HEK (0.5mg/ml) | 4.80 ± 0.77 | 14.36 ± 0.89 | 23.54 ± 0.04 |
| IV | HEK (0.25 mg/ml) | 5.35 ± 0.79 | 0.33 ± 0.08 | 16.45 ± 0.50 |

All the values were expressed in Mean ± SD (n=3). HEK- hydroalcoholic extract of *Kokilaksha*

The Nitric oxide inhibitory ratio in the group treated with 1mg/ml was found to be 3.09±0.01 post 24 hours of incubation with hydroalcoholic extract of *Kokilaksha* .On treatment with successively decreasing concentration of the extract at 0.5 mg/ml and 0.25 mg/ml the ratio was found to be increasing to 4.80±0.77 and 5.35±0.79 respectively. (Table 5)

The Nitric oxide inhibitory ratio in the group treated with 1mg/ml was found to be 15.33±0.17 post 48 hours of incubation with hydroalcoholic extract of *Kokilaksha* .On treatment with successively decreasing concentration of the extract at 0.5 mg/ml and 0.25 mg/ml the ratio was found to slightly decrease to 14.36± 0.89 and 0.33±0.08 respectively. (Table 5)

The Nitric oxide inhibitory ratio in the group treated with 1mg/ml was found to be 28.42±0.11 post 72 hours of incubation with hydroalcoholic extract of *Kokilaksha* .On treatment with decreasing concentration of the extract at 0.5 mg/ml and 0.25 mg/ml the nitric oxide inhibitory ratio was found to be decrease to 23.54±0.04 and 16.45±0.50 respectively. (Table 5)

**Table 6: Effect of *Kashaya* of *Kokilaksha* [*Hygrophila spinosa* T Ander] on Nitric oxide inhibitory ratio in MG- 63 cell lines at 24, 48 and 72 hours**

| Groups | Drug treatment | Nitric oxide Inhibitory ratio at 24 hours | Nitric oxide Inhibitory ratio at 48 hours | Nitric oxide Inhibitory ratio at 72 hours |
|---|---|---|---|---|
| I | Control (Cancer cell) | - | - | - |
| II | KS (50 %) | 22.35 ± 0.49 | 47.33 ± 0.24 | 59.64 ± 1.54 |
| III | KS (25 %) | 14.43 ± 1.07 | 39.23 ± 0.26 | 45.90 ± 1.11 |
| IV | KS (12.5 %) | 2.30 ± 0.10 | 6.52 ± 0.17 | 37.80 ± 0.70 |

All the values were expressed in Mean ± SD (n=3). KS- *Kashaya* of *Kokilaksha*

The Nitric oxide inhibitory ratio in the group treated with 50 % drug concentration was found to be the maximum at 22.35±0.49 post 24 hours of incubation with *Kashaya* of *Kokilaksha* .On treatment with decreasing concentration of the *kashaya* at 25 % and 12.5 % the nitric oxide inhibitory ratio was found to gradually decrease to 14.43±1.07 and 2.30±0.10 respectively. The Nitric oxide inhibitory ratio post 48 hours of incubation with *Kokilaksha Kashaya* in the group treated with 50 % concentration of the drug was found to be the maximum at 47.33±0.24 .On treatment with decreasing concentration of the *Kashaya* at 25 % and 12.5 % the nitric oxide inhibitory ratio was found to decrease from 39.23±0.26 to 6.5±0.17 respectively. The highest inhibition was shown at the maximum concentration of 50% *Kashaya*. The Nitric oxide inhibitory ratio in the group treated with 50 % concentration of the drug was found to be the highest at 59.64 ±1.54 post 72 hours of incubation with *Kashaya* of *Kokilaksha* .On treatment with decreasing concentration of the *Kashaya* at 25 % and 12.5 % the nitric oxide inhibitory ratio was found to slightly decrease to 45.90±1.11 and 37.80±0.70 respectively. The highest inhibition was shown at the maximum concentration of *Kashaya* here. (Table 6)

**Table 7: Effect of hydroalcoholic extract of *Kokilaksha* [*Hygrophila spinosa* T Ander] on LDH in MG- 63 cell lines at 24 ,48 and 72 hours**

| Groups | Drug treatment | LDH (U/L) at 24 hours | LDH (U/L) at 48 hours | LDH (U/L) at 72 hours |
|---|---|---|---|---|
| I | Control (Cancer cell) | 1321.00 ± 5.56 | 1460.67 ± 4.04 | 974.67 ± 4.36 |
| II | HEK (1 mg/ml) | 1278.83 ± 3.54 ** | 1405.00 ± 4.58** | 623.53 ± 5.12** |
| III | HEK (0.5 mg/ml) | 1307.66 ± 4.16* | 1434.33 ± 4.04* | 646.50 ± 5.47** |
| IV | HEK (0.25 mg/ml) | 1314.43 ± 5.13 ns | 1450.90 ± 1.77ns | 748.37 ± 3.57* |

All the values were expressed in Mean ± SD (n=3). *P* values calculated by one way ANOVA analysis followed by Dunnetts 't' test of significance in comparison with control group. **P < 0.01, *= P < 0.05, ns – non significant as compared to control. HEK- hydroalcoholic extract of *Kokilaksha*

The LDH concentration in control was found to be 1321.00±5.56 U/L after 24 hours of incubation with hydroalcoholic extract of *Kokilaksha*. On treatment with 1 mg/ml concentration of drug the LDH was found significant (P<0.01) decrease to 1278.83±3.54 U/L and LDH was found significant (P<0.05) decrease to 1307.66±4.16 in 0.5 mg/ml compared to control. Post 48 hours incubation with hydroalcoholic extract of *Kokilaksha* the LDH concentration in the control was found to be 1460.67±4.04 U/L . On treatment with 1 mg/ml concentration of drug the LDH concentration was found to significantly (P<0.01) decrease to 1405.00±4.58 U/L and LDH was found to significantly (P<0.05) decrease to 1434.33±4.04 U/L in 0.5 mg/ml compared to control. The LDH concentration in the control was 974.67±4.36 U/L after 72 hours of incubation with hydroalcoholic extract of *Kokilaksha*. On treatment with 1 mg/ml and 0.5 mg/ml concentration of drug the LDH concentration was found to significantly (P<0.01) decrease to 623.53±5.12 U/L and 646.50±5.47 U/L and respectively. LDH was found to significantly (P<0.05) decrease

to 748.37±3.57 U/L in 0.25 mg/ml compared to control. (Table 7)

**Table 8 : Effect of *Kashaya* of *Kokilaksha* [*Hygrophila spinosa* T Ander] on LDH in MG- 63 cell lines at 24, 48 and 72 hours**

| Groups | Drug treatment | LDH (U/L) at 24 hours | LDH (U/L) at 48 hours | LDH (U/L) at 72 hours |
|---|---|---|---|---|
| I | Control (Cancer cell) | 1117.26 ±3.16 | 763.73 ± 3.71 | 1220.33 ± 2.51 |
| II | KS (50 %) | 579.50 ±2.19** | 100.17 ± 2.06** | 103.67 ± 2.14** |
| III | KS (25 %) | 702.73 ±2.70** | 329.40 ± 1.90** | 593.20 ± 2.75** |
| IV | KS (12.5 %) | 1084.70 ±4.09** | 600.43 ± 2.58** | 627.37 ± 3.80** |

All the values were expressed in Mean ± SD (n=3). *P* values calculated by one way ANOVA analysis followed by Dunnetts 't' test of significance in comparison with control group. **=P < 0.01, as compared to control. KS- *Kashaya* of *Kokilaksha*

After 24 hours of incubation with *Kashaya* of *Kokilaksha* LDH concentration in the control was found to be 1117.26±3.16 U/L. On treatment with 50 %, 25 % and 12.5 % concentration of drug the LDH concentration was found to significantly (P<0.01) decrease to 579.50±2.19 U/L, 702.73±2.70 U/L and 1084.70±4.09 U/L respectively compared to control. LDH concentration in the control was found to be 763.73±3.71 U/L after 48 hours of incubation with *Kokilaksha Kashaya* . On treatment with 50 %, 25 % and 12.5 % concentration of drug the LDH concentration was found to significantly (P<0.01) decrease to 100.17±2.06 U/L, 329.40±1.90 U/L and 600.43±2.58 U/L respectively compared to control. The LDH concentration in the control was found to be 1220.33±2.51 U/L after 72 hours of incubation with *Kokilaksha Kashaya*. On treatment with 50 %, 25 % and 12.5 % concentration of drug the LDH concentration was found to significantly (P<0.01) decrease to 103.67±2.14 U/L, 593.20±2.75 U/L and 627.37±3.80 U/L respectively compared to control. (Table 8)

**Table 9 : Effect of hydroalcoholic extract of *Kokilaksha* [*Hygrophila spinosa* T Ander] on LDH in SAOS-2 cell lines at 24 ,48 and 72 hours**

| Groups | Drug treatment | LDH (U/L) at 24 hours | LDH (U/L) at 48 hours | LDH (U/L) at 72hours |
|---|---|---|---|---|
| I | Control (Cancer cell) | 105.40 ± 4.56 | 1495.67 ± 4.51 | 464.80 ± 3.97 |
| II | HEK (1 mg/ml) | 89.60 ± 4.00** | 1343.97 ± 4.22** | 253.33 ± 3.06 ** |
| III | HEK (0.5 mg/ml) | 100.27 ± 2.64 ns | 1454.67 ± 4.51* | 329.00 ± 2.82** |
| IV | HEK (0.25 mg/ml) | 104.13 ± 2.65ns | 1495.33 ± 5.03ns | 419.13 ± 3.45** |

All the values were expressed in Mean ± SD (n=3). *P* values calculated by one way ANOVA analysis followed by Dunnetts 't' test of significance in comparison with control group. **=P < 0.01, *= P < 0.05, ns – non significant as compared to control. HEK- hydroalcoholic extract of *Kokilaksha*

The LDH concentration in the control group was found to be 105.40±4.56 U/L after 24 hours of incubation with hydroalcoholic extract of *Kokilaksha*. On treatment with 1 mg/ml concentration of drug the LDH concentration was found to significantly (P<0.01) decrease to 89.60±4.00 U/L compared to control. After 48 hours of incubation with hydroalcoholic extract of *Kokilaksha* the LDH concentration in the control group was found to be 1495.67±4.51 U/L. On treatment with 1 mg/ml concentration of drug the concentration was found to significantly (P<0.01) decrease to 1343.97±4.22 U/L. LDH was found to significantly (P<0.05) decrease to 1454.67±4.51 U/L in 0.5 mg/ml compared to control. The LDH concentration in the control group was found to be 464.80 ± 3.97 U/L after 72 hours of incubation with hydroalcoholic extract of *Kokilaksha*. On treatment with 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml concentration of drug the LDH concentration was found to significantly (P<0.01) decrease to 253.33±3.06 U/L, 329.00±2.82 U/L and 419.13±3.45 U/L respectively compared to control. (Table 9)

**Table 10 : Effect of *Kashaya* of *Kokilaksha* [*Hygrophila spinosa* T Ander] on LDH in SAOS-2 cell lines at 24, 48 and 72 hours**

| Groups | Drug treatment | LDH (U/L) at 24 hours | LDH (U/L) at 48 hours | LDH (U/L) at 72 hours |
|---|---|---|---|---|
| I | Control (Cancer cell) | 466.37 ± 3.32 | 1216.00 ± 4.58 | 1680.73 ± 3.61 |
| II | KS (50 %) | 183.33 ± 2.15** | 108.73 ± 3.30** | 216.83 ± 3.31** |
| III | KS (25 %) | 327.00 ± 2.65** | 141.73 ± 3.25** | 461.23 ± 2.93** |
| IV | KS (12.5 %) | 434.67 ± 5.69** | 462.90 ± 3.85** | 463.57 ± 2.40** |

All the values were expressed in Mean ± SD (n=3). *P* values calculated by one way ANOVA analysis followed by Dunnetts 't' test of significance in comparison with control group . **=P < 0.01, ns – non significant as compared to control. KS- *Kashaya* of *Kokilaksha*

The LDH concentration in the control was found to be 466.37±3.32 U/L after 24 hours of incubation with *Kokilaksha kashaya* . On treatment with 50 %, 25 % and 12.5 % concentration of drug the LDH concentration was found to significantly (P<0.01) decrease to 183.33±2.15 U/L , 327.00±2.65 U/L and 434.6 ±5.69 U/L respectively compared to control .After 48 hours of incubation with *Kokilaksha kashaya* the LDH concentration in the control was found to be 1216.00±4.58 U/L. On treatment with 50 %, 25 % and 12.5 % concentration of drug the LDH concentration was found to significantly (P<0.01) decrease to 108.73±3.30 U/L , 141.73±3.25 U/L and 462.90±3.85 U/L respectively compared to control. Post 72 hours of incubation with *Kokilaksha kashaya* LDH concentration in the control was found to be 1680.73±3.61 U/L. On treatment with 50 %, 25 % and 12.5 % concentration of drug the LDH concentration was found to significantly (P<0.01) decrease to 216.83±3.31 U/L , 461.23±2.93 U/L and 463.57 ±2.40 U/L respectively compared to control (Table 10)

## DISCUSSION [13,14,15]

Any anti-cancerous activity cannot be approached through one particular assay. Hence some general assays like cytotoxic activity, free radical scavenging activity etc. along with the disease specific assays have been resorted to in the present study. Among the general activities looked for in the anticancer activities; MTT assay and nitric oxide scavenging activity was chosen and LDH assay as a disease specific assay was chosen for the current study.

MTT assay is one of the preliminary assays to assess the cell viability and cytotoxicity. Hence owing to its feasibility and it being the most common assay to test the cytotoxic activity this particular assay was chosen. The Nitric oxide scavenging activity was selected to test the action of drug on free radical scavenging. The raised LDH levels being a favourable prognostic factor for osteosarcoma disease; hence the LDH assay as a disease specific was chosen as a disease specific assay for the current study.MG-63 and SAOS-2 osteosarcoma cell lines were used owing to their easy availability during the period of study.

The results of the study are indicative that the drug *Kokilaksha* in the form of *Kashaya* and hydroalcoholic extract exhibits a concentration and time dependent activity with respect to all the three assays in both the osteosarcoma cell lines used in the anticancer activity evaluation in the present in vitro study. The result of the three assays carried out and the result obtained infers the drug *Kokilaksha* may be more effective in SAOS-2 cell lines. The anticancer activity of the drug *Kokilaksha* elicited above with the three assays may be attributed to the presence of alkaloids, flavonoids, saponins, carbohydrates and phenols.

### Probable Mode of Action

[16,17,18,19,20,21,22,23,24]

The promising anticancer activity of alkaloids has been reported by restraining the enzyme topoisomerase associated with replication of DNA replication , instigate apoptosis, autophagy and necroptosis, suppression of angiogenesis and modulation of various other intracellular targets and signalling pathways owing to their diverse chemical structure. The presence of 40 % of alkaloid found during the quantification

suggests it be quite significant to perform cytotoxic activity.

The quantification of 32.64 % flavonoids as witnessed in the current study supports its role of in the anticancer activity. [24]

Flavonoids are the largest group of naturally occurring phenols which have been reported to show effective anti-oxidant and free radical scavenging activities. Their anticancer activity is related to their modulation of signal transduction pathways within cancer cells. By means of inhibiting cell proliferation, cell cycle progression, oxidative stress, angiogenesis and metastasis while promoting apoptosis. They have been found to inhibit cell growth and trigger cell death in different cancers by inducing excessive and sustained autophagy or impairing autophagy flux suggesting promising effectiveness in apoptosis resistant cancer. Flavonoid apigenin which is one of the flavonoids reported in *Kokilaksha* has been proven to have anti-inflammatory, antioxidant, antitumor and anticancer activity. It is found to possess cytotoxic action against various cancer cell lines. Apigenin was also found to inhibit the expression of Wnt/β-catenin pathway in Osteosarcoma cells along with disruption in the mitochondrial pathway. Another flavonoid reported in the drug is luteolin is known to induce apoptosis and inhibit cell proliferation, metastasis and angiogenesis. It is also found to sensitize cancer cells to the therapeutic-induced cytotoxicity along with suppressing cell survival pathways like tumour suppressor p53 pathway, downregulation of BCL-2 and Caspase 3 expression and upregulation of BAX protein . The anti-oxidant property of flavonoids has also been shown to act against the lipid peroxidation that acts as a natural promoter of decay of osteosarcoma cells. The flavonoids are attributed with the antioxidant activity which helps in scavenging of free radicals thereby preventing the disastrous effect of peroxynitrates on cells [20]

The presence of 20.24 % of saponins in the drug may suggest it to also play a significant role in anticancer activity. Saponins have been reported in general to bring about down regulation of Wnt/β-catenin pathway, VEGF, and Myc gene expression and up regulation of p53 pathway which play an important role in Osteosarcoma pathogenesis [25,26].

Quantification of the drug *Kokilaksha* revealed presence of 10.15 % carbohydrates [24]. *Kokilaksha* has been documented to contain polysaccharides [27] which have been documented in preclinical models to reduce tumor growth and prolong survival by immune modulation mechanism, apoptosis, anti-angiogenesis and cell cycle arrest. Phenols present in *Kokilaksha* also may be responsible for anticancer activity due to their complementary and overlapping mechanisms of action including antioxidant activity and scavenging free radicals; modulation of carcinogen metabolism; regulation of gene expression on oncogenes and tumor suppressor genes in cell proliferation and differentiation. They bring about inhibition of signal transduction pathways including nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB), modulation of enzyme activities in detoxification, oxidation, and reduction; anti-inflammatory properties by stimulation of the immune system and suppression of angiogenesis.

As per classical texts of *Ayurveda Kokilaksha* being a popularly appreciated single drug in the treatment of *Vatarakta* as per the classics; should be effective to treat the complications related to the disease as well. *Arbuda* being an *upadrava* in *Vatarakta* wherein *medas ,asthi, majja* are the deeper *dhatus* that are involved and affected; the drug *Kokilaksha* might support these *dhatu*s preventing them from undergoing destruction as is evident in *Vatarakta* as per the classical texts .This may be attributed to *Kokilaksha* being *madhura rasa, Madura vipaka* and *sheeta veerya* in nature.

## CONCLUSION

The current in vitro anticancer study revealed both the hydro alcoholic extract as well as *kashaya* of *Kokilaksha* have exhibited anticancer activity in a time and concentration dependent manner with respect to MTT assay, Nitric oxide scavenging activity and LDH assay in MG-63 and SAOS- 2 cell lines. The higher percentage of alkaloids, flavonoids and saponins along with the carbohydrates & phenols reported in the drug sample may be attributed to the anticancer activity of *Kokilaksha* .

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**Conflict of Interest:** None

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