Evaluation of antimitotic activity of *Rotula aquatica* (Lour): A traditional herb used in treatment of cancer


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*Rotula aquatica* was extensively used by vaidyas (Ayurvedic practitioners) in holistic treatment of cancer. In the present study, an attempt has been made to evaluate the antimitotic activity of *R. aquatica*. Preliminary antimitotic screening was done using *Allium cepa* root tip assay. The mitotic index of the root tips markedly decreased with increasing concentration of the aqueous extract. The different fractions obtained by successive extraction of *R. aquatica* using solvents of increasing polarity were also evaluated for their antimitotic activity. Tannins were isolated which showed a better activity than the non-tannin fraction. Experiments were also carried out with incorporation of folic acid in the aqueous extract. Folic acid inhibited the antimitotic activity of aqueous extract of *R. aquatica* in a dose dependent manner. The results obtained were compared with methotrexate—a known drug available in market as anti-cancer agent. The studies were extended to human cells using 3 pancreatic cancer cell lines, viz: HPAF-II, BxPC-3, and CAPAN-2. Extract of *R. aquatica* was found to be extremely effective in the prevention of cell proliferation of the pancreatic cancer cell lines. The phytochemical evaluation revealed presence of polyphenols (tannins) and sterols. A HPTLC fingerprinting was developed and studied. Two compounds were isolated and subjected to spectral studies like UV, IR and mass spectrums. The empirical formula was derived by considering this data with elemental analysis of the compounds.

**Keywords:** *Rotula aquatica*, *Allium cepa*, MTT assay, Antimitotic herb.

*Rotula aquatica* Lour. is a small shrub, branched, often submerged in water and found in sandy and sappy beds of central, western and southern India. A lecction of the roots (pashanbedh) is greatly valued in Ayurvedic medicine and is used as a diuretic and laxative, for treating piles, stones in bladder and genereal diseases. The diuretic action of the roots is attributed to the presence of allantoin. A sterol named habdiol is also extracted from the roots.

Cancer is essentially a problem of abnormal cell growth. Under the influence of chemicals, viruses, and free radicals, normal cells are converted to umour masses that divide in an uncontrolled manner.

The cytotoxic effect of plant polyphenols is shown to be mediated through apoptosis. Considering the ability of these natural polyphenols especially the tannins to absorb proteins and metal ions, there is a possibility that they can elicit apoptosis signals through various receptors or proteins. Apart from this, they are excellent antioxidants and they thus prevent the free radical attack on DNA by acting as scavengers of these free radicals. A number of polyphenols are topo-II poisons inhibiting topo I / II isomerases thus enhancing the DNA cleavage.

Another possible mechanism of action reported for anticancer drugs is inhibition of DNA synthesis and thus prevention of cell division. Folic acid supplied from the diet is essential for the production of tetrahydrofolic acid (THF). The conversion of folic acid to THF is carried out by an enzyme folate reductase. Anticancer drugs compete with folic acid for this enzyme thus restricting the production of THF required for synthesis of DNA and consequently for cell replication. Cells which do not have adequate production of THF eventually die.

The antimitotic activity was screened using *Allium cepa* root meristematic cells which have been used extensively in screening of drugs with antimitotic activity. The roots of all plants have distinguished regions, one of them being the region of cell division that lies beyond the root cap and extends a few mm after that. Cells of this region undergo repeated divisions. The rate of cell division is higher in this
region compared to that of the other tissues. This region is called the meristematic region (meristos: divided). This division is similar to the above mentioned cancer division in humans. Hence, these meristematic cells can be used for preliminary screening of drugs with anticancer activity. Even though doubts can be raised about extrapolation of results from plant tissue to animals and finally to humans, Khilman has noted that plant cells are 1000 times more resistant to colchicine which is a potent anti-carcinogenic and which acts by inhibiting the microtubule formation. Thus, it is possible that chemicals that affect plant chromosomes will also affect animals.

We may thus hypothesize that if R. aquatica extract is effective against A. cepa root cells, it will also have antimitotic effect against animal and human cells. To evaluate this hypothesis, it was thought worthwhile to evaluate the activity of the extracts of R. aquatica on 3 established human pancreatic cell lines also.

Polypehols have shown anticarcinogenic potential against PC-12 (human prostate cancer cells), HL-60 (human promyelocytic leukemia) by inducing apoptosis. Pancreatic cancer is extremely resistant to the induction of apoptosis by chemotherapy. Agents that regulate apoptotic sensitivity may lead to chemosensitization of pancreatic cancer. Three pancreatic cancer cell lines were used, viz. HPAF-II, BxPC-3 and CAPAN-2, which resemble well, moderately and poorly differentiated pancreatic cancer cells respectively. The growth of pancreatic cancer is driven by multiple factors like mutations in K-ras leading to inappropriate activation of p21

Additional information on chemosensitization of pancreatic cancer was found.

Preparation of plant extracts—The entire herb was air-dried in the laboratory at room temperature. It was then powdered (24 mesh) and was extracted with hot water by boiling for 30 min to get the aqueous extract. The extract obtained was concentrated and dried under controlled temperature (60°C). The dried powder was successively extracted with other solvents viz. a) petroleum ether (60-80°C), b) benzene, c) chloroform, d) acetone, e) ethanol and f) water. Extraction with each solvent was done in a water bath for 60 min with a reflux condenser. Each time, before extracting with the next solvent, the marc was dried in an air oven below 50°C. Each extract was concentrated and evaporated to dry extract. The solvent : drug ratio for extraction was 5:1. Extracts of desired concentrations were prepared for further study using these dried extracts.

Antimitotic activity—This activity was evaluated using A. cepa root meristematic cells. A. cepa bulbs were sprouted in tap water for 48 hr at room temperature. The bulbs that developed uniform roots were used for the experiment. These roots were treated with above prepared extracts of 10 mg/ml concentration. Water was used as a control. Methotrexate was used as a standard control. After 3 hr of treatment, the root tips were fixed in a fixing solution of acetic acid and alcohol. Squash preparations were made by staining with aceticarmine stain. The mitotic index was calculated as:

\[
\text{Mitotic Index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100
\]

The aqueous extract was also subjected to qualitative test which revealed the presence of tannins and steroids. Tannins were isolated from the water extract. The experiment was repeated with tannin and non-tannin fractions. Squash preparations made as above and observed. A similar experiment was undertaken to find out the probable mechanism of action through which the extract acts. Folic acid was added to the aqueous solution of methotrexate and
aqueous extract of *R. aquatica*. Squash preparations made as above from the treated roots were observed.

**Cell proliferation assay**—Three cell lines (BxPC-3, Capan-2 and HPAF-II) were obtained from the American Type Culture Collection and grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS), penicillin G (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO₂ at 37°C. The effect of *R. aquatica* on cell viability and growth was determined using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) colorimetric assay [using a commercially available kit (Boehringer Mannheim, Germany)]. The compounds were dissolved in water as stock solution (1000x) and then diluted with RPMI-1640 for cell culture experiments. All solutions were prepared fresh on the day of testing. Cells (BxPC-3, Capan-2 and HPAF-II) were seeded at a density of 1x10⁴ per well in 96-well plates in RPMI-1640 containing FBS (10%). After 24 hr, fresh medium was added containing aqueous extract of *R. aquatica* at concentrations of 0, 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴ mg/ml. After 4 days of incubation, in medium containing extract of *R. aquatica* cells were further incubated for 4 hr with the metabolic substrate, tetrazolium salt MTT, at a final concentration of 0.5 mg/ml. Living cells metabolized tetrazolium to formazan which was detected spectrophotometrically at 540 nm with multiwell spectrophotometer (ELISA Reader, Biotek Instruments Inc., Burlington, VT).

**HPTLC method for fingerprinting**—HPTLC fingerprinting studies were carried out by mixing 10mg of aqueous extract in 10ml of methanol. This solution was spotted as 20, 40, 60 and 80μl on a HPTLC silica GF₂₅₄ plate. The plate was developed using solvent system chloroform: ammonia: ethanol (4:5:1). The fingerprint patterns of *R. aquatica* were visualised at 254 and 366nm.

**Preparative TLC**—A slurry of silica gel GF₂₅₄ was made in distilled water. This slurry was then applied to glass plates (12.5 x 12.5cm) with the aid of a TLC spreader to obtain preparative silica gel plates having thickness of about 0.5mm. The plates were dried in an oven at 105°C and activated 2 hr before use.

**Sample preparation and application**—Aqueous extract (1mg/ml) was spotted on the plate as a band of length 50mm length. The plates were developed using the solvent system. The plates were visualised at 254nm, the band marked, scrapped, vortexed with methanol and centrifuged. The supernatant was separated, evaporated and residue dried with nitrogen gas. The dried residue was subjected to spectral analysis, viz: UV, IR, Mass and elemental.

**Statistical analysis**—The data was subjected to statistical analysis using analysis of variance followed by appropriate post-hoc tests. P <0.05 was considered as significant.

**Results**

**Effect of aqueous extract of *R. aquatica* on mitotic activity**—Antimitotic activity of the total aqueous extract was comparable to the activity of methotrexate (Table 1). The activity of remaining fractionated extracts was less than that of the total aqueous extracts. A one-way ANOVA showed that there was a significant effect of treatment on mitotic activity (F₈,₁₈ = 580.80, P<0.001). Post-hoc analysis using the Newman-Keuls test showed that the activity of all the different fractions were significant when compared with vehicle control. The aqueous extract showed lowest mitotic index i.e. highest activity (Table 1) amongst all the different fractionated extracts.

Table 2 shows activity of four different concentrations of the total extract and methotrexate. It was observed that the extract produced a dose-dependent decrease in the mitosis of *A. cepa* root tips. The phases were differentiated in each case and it was observed that the number of non-dividing cells increase with an increase in concentration of the extract. A one-way ANOVA of the data showed that the aqueous extract produced a dose-dependent decrease in the mitotic activity of *A. cepa* root tip cells (F₈,₁₈ = 443.83, P<0.001). Post-hoc analysis using Newman-Keuls test showed that the maximal activity of all the different fractionated extracts was less than that of the total aqueous extracts. A one-way ANOVA showed that there was a significant effect of treatment on mitotic activity (F₈,₁₈ = 580.80, P<0.001). Post-hoc analysis using the Newman-Keuls test showed that the activity of all the different fractions were significant when compared with vehicle control. The aqueous extract showed lowest mitotic index i.e. highest activity (Table 1) amongst all the different fractionated extracts.

**Table 1**—Antimitotic activity after treatment of *A. cepa* roots with fractionated extracts of *R. aquatica*

<table>
<thead>
<tr>
<th>Different solutions used for treatment</th>
<th>Concentration (mg/ml)</th>
<th>Mitotic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg</td>
<td>SD</td>
</tr>
<tr>
<td>Plain Tap water</td>
<td>85</td>
<td>0.58</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>10</td>
<td>2.65</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td>74</td>
<td>3.61</td>
</tr>
<tr>
<td>Benzene extract</td>
<td>74</td>
<td>1.15</td>
</tr>
<tr>
<td>Chloroform extract</td>
<td>70</td>
<td>0.00</td>
</tr>
<tr>
<td>Acetone extract</td>
<td>63</td>
<td>2.89</td>
</tr>
<tr>
<td>Ethanol extract</td>
<td>22</td>
<td>2.89</td>
</tr>
<tr>
<td>Remaining aqueous extract</td>
<td>19</td>
<td>1.73</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.10</td>
<td>1.53</td>
</tr>
</tbody>
</table>
inhibition produced by \textit{R. aquatica} extract was not significantly different to that of methotrexate. The cell divisions were differentiated and number of cells in each phase of cell division i.e either prophase, metaphase, anaphase, or telophase were recorded (Table 2). Thus, the number of cells entering prophase decreased with increase in concentration of the extracts. Since the cells do not enter prophase, further stages of cell division also decrease with increase in concentration of the aqueous extract.

\textbf{Effect of tannin and non-tannin fractions on mitotic activity (Table 3)—A one-way ANOVA showed that there was a significant decrease in mitotic index by all 3 fractions viz., tannin, non-tannin and aqueous fraction ($F_{3,8} = 2022.42; P < 0.001$). Post-hoc analysis using Newman-Keuls test showed that the total extract was the most effective. It was observed that the tannin fraction showed better activity than non-tannin fraction.}

\textbf{Effect of folic acid on antimitotic activity of \textit{R. aquatica} and methotrexate (Table 4)—Analysis of the data using a 3-way ANOVA showed that there was a significant effect of the pretreatment with folic acid on the mitotic activity.}

\begin{table}[h]
\centering
\small
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Treatment} & \multicolumn{2}{c|}{\textbf{Conc. (mg/ml)}} & \textbf{Mitotic Index} & \textbf{Avg} & \textbf{SD} & \textbf{SEM} \\
\hline
\textbf{Tannin fraction of aqueous extract of \textit{R. aquatica}} & 10 & 22 & 1.00 & 0.58 \\
\textbf{Non-tannin fraction of aqueous extract of \textit{R. aquatica}} & 10 & 13 & 1.53 & 0.88 \\
\textbf{Aqueous extract of \textit{R. aquatica}} & 10 & 12 & 0.58 & 0.33 \\
\hline
\end{tabular}
\caption{Antimitotic activity after treatment of \textit{A. cepa} roots with tannin and non-tannin fraction of aqueous extract of \textit{R. aquatica}}
\end{table}

\begin{table}[h]
\centering
\small
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{Concentration (mg/ml)} & \textbf{Non-dividing cells} & \textbf{Mitotic Index} & \textbf{Avg} & \textbf{SD} & \textbf{SEM} \\
\hline
\textbf{Aqueous extract of \textit{R. aquatica}} & & & & & & \\
0.3 & 25 & 68 & 3 & 3 & 1 & 75 & 2.08 & 1.20 \\
1 & 35 & 58 & 5 & 1 & 1 & 65 & 1.00 & 0.58 \\
6 & 58 & 39 & 2 & 1 & - & 42 & 0.58 & 0.33 \\
10 & 88 & 9 & 2 & 1 & - & 12 & 1.53 & 0.88 \\
\textbf{Methotrexate} & & & & & & \\
0.004 & 50 & 21 & 11 & 10 & 8 & 50 & 0.58 & 0.33 \\
0.020 & 60 & 15 & 10 & 8 & 7 & 40 & 1.53 & 1.00 \\
0.040 & 70 & 13 & 8 & 5 & 4 & 30 & 1.15 & 0.67 \\
0.10 & 90 & 5 & 3 & 2 & - & 10 & 1.00 & 0.58 \\
\hline
\end{tabular}
\caption{Antimitotic activity after treatment of \textit{A. cepa} roots with aqueous extract of \textit{R. aquatica} and methotrexate}
\end{table}

\begin{table}[h]
\centering
\small
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Treatment} & \textbf{Concentrations (mg/ml)} & \textbf{Mitotic Index} & \textbf{Avg} & \textbf{SD} & \textbf{SEM} \\
\hline
\textbf{Folic acid} & 83 & 1.15 & 0.67 \\
\textbf{Folic acid} & 78 & 0.58 & 0.33 \\
\textbf{Aqueous extract of \textit{R. aquatica} + Folic acid} & 42 & 0.58 & 0.33 \\
\textbf{Aqueous extract of \textit{R. aquatica} + Folic acid} & 52 & 0.58 & 0.33 \\
\textbf{Aqueous extract of \textit{R. aquatica} + Folic acid} & 58 & 0.58 & 0.33 \\
\textbf{Aqueous extract of \textit{R. aquatica} + Folic acid} & 65 & 1.73 & 1.00 \\
\textbf{Aqueous extract of \textit{R. aquatica} + Folic acid} & 68 & 1.53 & 0.88 \\
\textbf{Aqueous extract of \textit{R. aquatica} + Folic acid} & 0.1 & 0.00 & 0.00 \\
\textbf{Aqueous extract of \textit{R. aquatica} + Folic acid} & 43 & 2.08 & 1.20 \\
\textbf{Aqueous extract of \textit{R. aquatica} + Folic acid} & 48 & 0.58 & 0.33 \\
\textbf{Aqueous extract of \textit{R. aquatica} + Folic acid} & 52 & 1.53 & 0.88 \\
\textbf{Aqueous extract of \textit{R. aquatica} + Folic acid} & 52 & 2.31 & 1.33 \\
\textbf{Methotrexate} & 0.1 & 0.1 & 0.00 & 0.00 \\
\textbf{Methotrexate + Folic acid} & 0.1 & 0.78 & 0.03 & 0.02 \\
\textbf{Methotrexate + Folic acid} & 0.83 & 0.02 & 0.01 \\
\textbf{Plain water} & 0.85 & 0.02 & 0.02 \\
\hline
\end{tabular}
\caption{Antimitotic activity after treatment of \textit{A. cepa} roots with addition of folic acid to aqueous extract of \textit{R. aquatica} and methotrexate}
\end{table}
acid on the antimitotic activity of *R. aquatica* and methotrexate ($F_{3,30} = 746.05$). A control with only folic acid did not show any difference in mitotic index when compared to that of plain water. The mitotic index increased when folic acid was added to the total aqueous extract of *R. aquatica* and methotrexate solution which otherwise reduced the mitotic activity in the absence of folic acid. This however, did not increase with increase in folic acid concentration suggesting that the effect was not dose-dependent. Comparing the mitotic index of methotrexate and *R. aquatica* it was observed that incorporation of folic acid increased the mitotic index significantly in case of methotrexate, but not so in case of *R. aquatica*. Post-hoc analysis of the data showed that folic acid inhibited the anti-mitotic activity of methotrexate to a greater extent as compared to *R. aquatica*.

Effect of *R. aquatica* on cell viability of human pancreatic cancer cell lines (Fig. 1)—A one-way analysis of the data of each of the cell lines showed a significant effect of the extract on each of the cell lines ($F_{3,42} = 58.53, 58.53, 131.67$) respectively. Post-hoc analysis showed that the extracts were most effective at a concentration of 1 mg/ml.

*Spectral analysis*—Two spots were observed when the plate was visualised under UV. The Rf was found to be 0.3 and 0.7 for spot 1 and spot 2 respectively. The maximum absorption was recorded at 219.8 and 225.4 nm for spots 1 and 2 respectively. Characteristics bands seen under IR spectrum have been shown in Table 5.

The mass spectrum showed most intense peaks at (m/z)—(A) Spot 1: 388(M+H), 387(base peak), 369,354,335,293,279,256,223,199,149,97,71,57; and (B) Spot 2: 387(M+H), 386(base peak), 383, 354, 311, 309,278,256,213,199,185,149,129,97,83,57. The molecular weight of compound (spot 1) was 388. This was same as that of a steroid. The mass fragmentation pattern was matched. The most intense peaks were found to be same as that of a steroidal alcohol. The molecular formula from elemental analysis was found to be $C_{26}H_{46}NO$. The compound, thus, could be a steroidal alcohol with a side chain containing an amide or nitrogen in the heterocyclic ring and in such case might be a steroidal alkaloid.

The molecular weight of compound isolated (spot 2) was 387. This was also same as that of a sterol. The mass fragmentation pattern was found to be typical of a steroidal alcohol. The molecular peaks were found to be same as that of a steroidal alcohol. The molecular formula from elemental analysis was found to be $C_{25}H_{39}SO$. The compound thus, could be a steroidal alcohol. These compounds can occur as steroidal glycosidic alkaloid and steroidal glycoside in the herb. Only the aglycone has been detected as the...
glycoside gets hydrolysed in the process of extraction. The melting point of compound for spot 1 and spot 2 was found to be 170°C and 190°C respectively. Both the compounds showed positive response to Liebermann - Burchard's test, thus confirming the identity of a steroidal alcohol.

Discussion
The results from our study showed that the aqueous extract of *R. aquatica* had excellent anti-mitotic activity, that was comparable to the activity of methotrexate. The fractionation of the aqueous extract into tannin and non-tannin fractions showed that each of these individual fractions possessed good anti-mitotic activity but were not as potent as the total aqueous extract. The addition of folic acid inhibited the anti-mitotic activity of *R. aquatica* significantly, but not completely. It completely inhibited the activity of methotrexate.

Aqueous extract of *R. aquatica* was also effective in reducing the cell viability of 3 human pancreatic cancer cell lines that may be acting following the same mechanisms as those in the onion cells. By virtue of this, if the extract is administered in humans it may prevent cell proliferation by directly combining with DNA leading to DNA fragmentation, by inhibiting the folic acid pathway or by combining with cell receptors/enzymes and eliciting signals for cell apoptosis.

The phytochemical analysis of *R. aquatica* revealed that the plant was rich in polyphenols (tannins) and steroids. The polyphenols have been shown to be potent anti-mitotic agents in numerous assays. Thus, it was not surprising that the aqueous extract of *R. aquatica* showed good anti-mitotic activity. The tannin and non-tannin fractions showed good anti-mitotic activity, but less than that of the total aqueous extract. It may be, therefore, concluded that the non-tannin fraction contains steroids along with other polar constituents. Though, the probability of steroids extracted in a polar solvent was low, these steroids occurred as aglycones of the saponin glycosides after the glycosides was hydrolyzed. There might be other polar compounds in the total extracts that might be acting synergistically with the tannins and steroids of *R. aquatica*. Steroids have been shown to be useful in treatment of cancer. Hence, the steroids from *R. aquatica* must be contributing to the anticancer potential of the herb.

The aqueous extract of *R. aquatica* seems to prevent prophase stage in cell division where the DNA duplication occurs. Methotrexate is a known anticancer-drug that inhibits DNA synthesis. When folic acid was supplemented to the cells with methotrexate and the total aqueous extract it was seen that mitotic index increased. Thus, it may be concluded that the extract may be acting through this pathway inhibiting tetrahydrofolic acid and hence folic acid required for DNA synthesis thereby arresting cell division. Methotrexate is known to compete with folic acid for the enzyme THF reductase, the total aqueous extract of *R. aquatica* may also be competing with folic acid thus inhibiting the DNA synthesis. Hence, increase in folic acid concentration increases the mitotic index due to more availability of folic acid. However, the mitotic index does not increase significantly in case of *R. aquatica*, as compared to that of methotrexate. This may be because the extract may be mediating its effect through other mechanisms also. The extract may be binding with different cell proteins responsible for cell division. The antioxidant potential of the extract
established in another experiment in our laboratory, the extract shows good antioxidant activity which so may be one of the contributing factors to its anticancer potential.

The spectral analysis of the isolated compounds shows that the compounds may be steroidal glycosidic alkaloid and a steroidal alcohol. The study throws light on potential use of *R. aquatica* in treatment of cancer.

References


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