



Pharmacological Study

Anticancer activity of *Arkeshwara Rasa* - A herbo-metallic preparation

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Abstract

Introduction: Though metal based drugs have been prescribed in Ayurveda for centuries to treat various diseases, such as rheumatoid arthritis and cancer, toxicity of these drugs containing heavy metal is a great drawback for practical application. So, proper scientific validation of herbo-metallic drugs like *Arkeshwara Rasa* (AR) have become one of the focused research arena of new drugs against cancers. **Aim:** To investigate the *in vitro* anticancer effects of AR. **Materials and Methods:** Anticancer activity of AR was investigated on two human cancer cell lines, which represent two different tissues (pancreas and skin). Lactate dehydrogenase (LDH) assay for enzyme activity and trypan blue assay for cell morphology were performed for further confirmation. **Results:** AR showed potent activity against pancreatic cancer cells (MIA-PaCa-2). LDH activity confirmed that AR was active against pancreatic cancer cells. Finally, it was observed that AR exhibited significant effects on cancer cells due to synergistic effects of different compounds of AR. **Conclusion:** The study strongly suggests that AR has the potential to be an anticancer drug against pancreatic cancer.

Key words: Anticancer, *Arkeshwara Rasa*, natural products

Introduction

Ayurveda is considered as the most ancient form of traditional medicine in the Indian sub-continent, dating back to more than 3500 years. The basic distinction between Ayurveda and other traditional medicinal systems are availability of several therapeutic approaches.^[1] Regarding that, Ayurveda had its origin in *Atharva Veda*, which consist of various important topics including human health, engineering, and astrology.^[2] In China, the Chinese traditional medicinal system is advancing through promoting its therapies through research and evidence-based approaches.^[3] Similarly, it is essential to study the stage-wise transformations that take place during the preparation of Ayurvedic medicines along with the characterization of final product(s). This will assist to define the chemical nature of Ayurvedic medicines apart from understanding the role of various constituents added during various preparation steps.^[4,5] This traditional form of medicine relies on medicinal plants for

treatment; however, use of other materials like metals in the form of metallic salts, herbo-metallic complexes, and ashes (*"Bhasma"* or calcined form of the metal) have also been reported. A particular category of Ayurvedic herbo-metallic formulation known as *"Rasa"* is particularly noted for using metals and even heavy metals in these formulations. *Rasas* are used for treatment of various complex disorders, such as diabetes, rheumatoid arthritis, cancer, etc., For instance, *Tarakeshwara Rasa* is used for treatment of diabetes. *Tarakeshwara Rasa* contains calcined forms of tin, biotite and iron, as well as mercury in the form of mercuric sulfide or black sulfide of mercury along with other plant products.^[6] Although scientists have expressed concerns on the use of heavy metals in the formulations and possible resultant toxicity, Ayurvedic practitioners insist that calcinations

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or use of metals such as mercury in particularly their sulfide form abolishes metal toxicity.^[7]

Among the hundreds of *Rasas* used by Ayurvedic practitioners, *Arkeshwara Rasa* (AR), consisting of a number of plant parts together with mercuric sulfide, is used for treatment of rheumatoid arthritis. Various plant parts used in AR include dried and powdered fruit rind of *Terminalia belerica* Roxb. (Combretaceae), *Terminalia chebula* Retz. (Combretaceae), and *Phyllanthus emblica* L (Euphorbiaceae). These fruit rinds are used along with whole plants of *Plumbago zeylanica* L. (Plumbaginaceae) and latex of *Calotropis procera* (Aiton) W.T. Aiton (Apocynaceae). The preparation of AR is a complex process.^[8] Ayurvedic practitioners insist that although the formulation is administered orally, there are no adverse side-effects.

It is interesting that the plants or plant parts used in AR have reported anticancer properties. *Triphala* composed of three fruits show potent anticancer activity and also individual fruits show that they have antitumor activity *in vitro* and *in vivo*, respectively.^[9,10] Mercuric sulfide (HgS) is a component of traditional mineral medicine, which has been used as a memory-enhancing drug for more than thousands of years.^[11] Though mercury is known to be toxic in living tissues, salts form of mercury have been traditionally considered nontoxic. Researchers reported that mercury sulfide in traditional medicine has 5000 times less mercury than organic methyl mercury.^[5] The efficacy and safety aspects of mercurial preparations have been assessed.^[12] Traditional medicinal books of Ayurveda and Chinese traditional medicine insist that mercury, minerals and plants or animal products are very useful in cancers. It was therefore of interest to study the anticancer effectiveness of AR against various cancer cell lines and evaluate any mercuric sulfide (an ingredient of AR)-induced toxicity in the same cell lines.

Materials and Methods

Sample preparation

Mercury and sulfur were obtained from Sigma Chemical Co., USA. Plant materials were obtained from Bangladesh after proper identification and authentication by the Medicinal Plant Collection Wing of the University of Development Alternative. *Arkeshwara Rasa* was prepared exactly as previously described by Bhagvatacharya in a classical Ayurvedic text *Rasa Ratna Samucchoy*.^[8] In this process, one part mercury was thoroughly mixed with two parts sulfur in an earthen vessel. The vessel was covered with a circular copper lid, and leakages were sealed with clay. The whole vessel was then covered with live coals for 6–7 h. The lid was then taken off and mercuric sulfide deposited on the lower part of the lid scraped off. Collected mercuric sulfide was then powdered and mixed with latex of *C. procera* and decoction of *Triphala* (fruits of *P. emblica*, *T. belerica* and *T. chebula*) with *P. zeylanica* whole plants. The mixture was boiled for a few hours to dryness and then boiled again with fresh latex of *C. procera* and decoction of *Triphala* with *P. zeylanica* whole plants to dryness. This was done a total of 10 times and final product was obtained as powder.^[8]

In vitro cell cytotoxicity

Human pancreatic cancer cells MIA-PaCa-2 and epithelial carcinoma KB cells were used to determine the cell viability of AR. MIA-PaCa-2 and KB cells were collected from Korea Cell Bank (Seoul, Korea). Both cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ in FA-deficient medium RPMI 1640 with 10% fetal bovine serum. The cells (5 × 10⁶ cells/mL) were grown as a monolayer and harvested with 0.25% trypsin in 0.03% ethylene di-amine tetra acetic acid solution, followed by the seeding of 200 μL of each cell type in 96-well plates and preincubation for 24 h. To investigate the selectivity and cytotoxicity of AR, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed on both cells.^[13] Cells were incubated at 37°C for 24 h; the control was incubated without any drug. Three different concentrations (10, 100, and 500 μg/mL) of each compound were tested. MTT assay is based on the reduction of a yellow tetrazolium component to an insoluble purple-colored formazan produced by the mitochondria of viable cells. After incubation for 24 h, 100 μL of medium containing 20 μL of MTT solution (MTT, USA) were added to each well. The plate was then incubated for an additional 4 h, followed by the addition of 180 μL of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous iso-propanol (Sigma, Milwaukee, USA) to each well. The solution was gently mixed to dissolve MTT formazan crystals. The absorbance of each well was measured with a micro plate reader at 570 nm.

Trypan Blue dye exclusion test

Cell viability of AR was determined by cell morphology using Trypan Blue dye exclusion test.^[14] Following treatments, cell cultures were *in situ* stained with 0.2% Trypan Blue solution (Sigma, USA) at room temperature for 10 min and then fixed with Trypan Blue dye at 4°C. The fixed cultures were rinsed with phosphate-buffered saline and examined under light microscopy at ×10 magnification. Cultures from all treatment conditions were photographed using Zeis microscope.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) test-kit (CytoTox 96® NonRadioactive Cytotoxicity Assay, Promega Co.) was used to investigate the cell membrane integrity as per the procedure described in the kit.^[15] The absorbance at 490 nm was recorded on a Microplate Reader (Varioskan Flash). The LDH leakage (% of positive control) is expressed as the percentage of (test-blank)/(positive-blank), where test is the cells exposed to the AR, positive is the optical density of the positive control cells and blank is the optical density of the wells without cells.

Statistical analysis

Statistical differences were performed using a one-way ANOVA (Origin Pro 8.0). Data were presented as mean ± SEM. *P* < 0.05 were considered as statistically significant.

Results

In vitro anticancer activity

A herbo-metallic Ayurvedic drug (AR) was investigated to evaluate its potential therapeutic efficacy against cancer cells. *In vitro* cytotoxicity of the drug was examined by MTT

cell viability against different human cancer cell lines like, human epidermal carcinoma (KB) cells and human pancreatic cancer (MIA-PaCa2) cells with medium used as control. Cells were treated with different concentrations (10, 100, 500 $\mu\text{g/mL}$) of AR. After treatment with drugs, MIA-PaCa2 cells showed lower cell viability of about 20% compared with the control where medium was used as control (IC_{50} values about 333.33 $\mu\text{g/mL}$). Cell viability decreased with increasing AR concentrations and reached almost 20% at higher concentration of drug (500 $\mu\text{g/mL}$). In contrast, the other cancer cell, KB cells did not show any significant effects (55% cell viability at 500 $\mu\text{g/mL}$ concentration) like pancreatic cells [Figure 1]. It is possible that AR may contain one or more component(s), to which pancreatic cancer cells are more sensitive.

To examine cell viability, *in situ* Trypan Blue dye exclusion test to detect live cells and dead cells was performed. This is a stain that identifies nonviable cells, which absorb dye when observed under a microscope, while viable cells appear unstained. Viable cells have intact cell membranes and so cannot uptake the dye. In contrast, nonviable cells do not have an intact and functional membrane and hence do take up dye from their surroundings. As such, Trypan Blue can easily differentiate between viable and nonviable cells. Cell viability decreased with increasing concentrations of AR. Overall, as evidenced by the morphological data, exposure to 0.5 $\mu\text{g/mL}$ AR for 24 h resulted in more pancreatic cancer cell's death than epithelial carcinoma cells [Figure 2].

Lactate dehydrogenase release activity

LDH is an enzyme which exists in a variety of organisms, including plants and animals and can be measured as a

surrogate for tissue breakdown. For the LDH release assay, dose dependent activity was observed when MIA-PaCa-2 and KB cells were exposed to AR. Indicative cytotoxicity was observed from 10 to 500 $\mu\text{g/mL}$ when both cells were exposed to AR. 10 $\mu\text{g/mL}$ concentration of resulted in 90% of KB cells and 55% of MIA-PaCa-2 cells increase of LDH leakage in comparison to controls, respectively. Increasing the concentration of AR further increased release of LDH. At 500 $\mu\text{g/mL}$, AR exposure resulted in almost 75% release of LDH in MIA-PaCa-2 cells and 95% in KB cells [Figure 3].

Discussion

Herbal medicines have been widely recognized and scientists emphasize the importance and urgency of examining potential drug interactions produced by natural products.^[16] We investigated the mercuric sulfide based herbo-metallic preparation commonly known as AR, which demonstrated potent anticancer activity in a human pancreatic cancer cell line through cell viability and enzymatic activity assays. AR is composed of *Triphala* with *P. zeylanica* and fresh latex of *C. procera* along with mercuric sulfide. Researchers have found that *Triphala* itself as well as the plants individually show cytotoxic activity in cancer cells. One group of researcher reported the differential response of normal and tumor cells to *Triphala in vitro*, and the substantial regression of transplanted tumor in mice fed with *Triphala* points to its potential use as an anticancer drug for clinical treatment.^[10] Another group reported that *Triphala* was more potent on pancreatic cancer *in vivo* and *in vitro*, respectively.^[9] The studies suggest that *Triphala* may

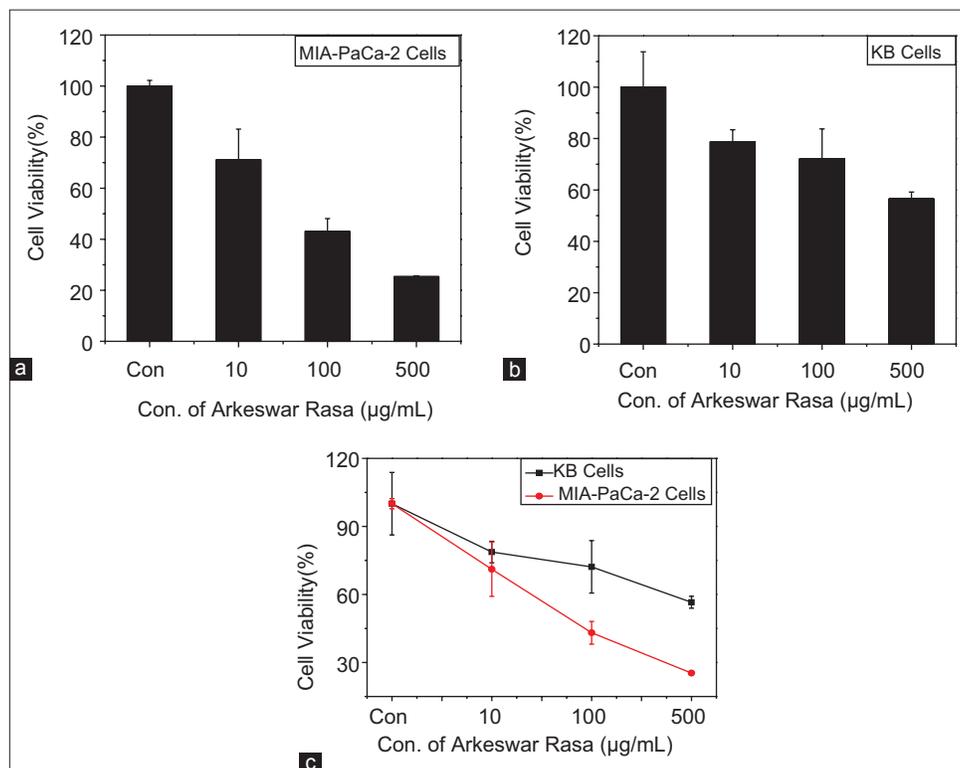


Figure 1: Cell cytotoxicity assay against *Arkeshwara Rasa*. (a) Cell viability assay of human epidermal carcinoma cells about 55% at 500 $\mu\text{g/mL}$ concentration. (b) Cell viability assay of human pancreatic cancer cells about 25% at higher concentration. (c) Comparison between two cells viability of cells against herbal drugs

consist of one or more components, which are more potent on pancreatic cancer cells. A study has shown that extracts of raw fruits of *P. emblica* at 50–100 µg/mL significantly inhibited cell growth of human cancer cell lines.^[17] Extracts of fruits of *T. chebula* demonstrated cytotoxic activity in a number of cell lines like human (MCF-7) and mouse (S115) breast cancer cell line, a human osteosarcoma cell line (HOS-1), a human prostate cancer cell line (PC-3) and a nontumorigenic, immortalized human prostate cell line (PNT1A) by inhibiting the rate of cell proliferation and inducing cell death.^[18] Methanolic extracts of *T. belerica* fruits with others enhancer has been shown to reduce cancer in mice.^[19] Choedon *et al.* reported that it showed potent activity against hepato-carcinogenesis in mice with no side-effects. Methanolic extracts of *C. procera* induced extensive cell death in both Huh-7 and COS-1 cells but not in AML12 cells.^[20] Ethanolic extract of *P. zeylanica* Linn. has been shown to possess significant anticancer activity and also reduced elevated level of lipid peroxidation due to the extract's higher content of terpenoids and flavonoids.^[21,22] Thus the various plant ingredients of AR have been variously reported to

have anticancer activity, and together they might have produced the anticancer effect in the present study. However, individual component(s) of each plant having anticancer effects need to be identified, which is currently under progress in our laboratory.

In Ayurveda, mercuric sulfide is considered nontoxic when prepared as described in the methods section and the process of heating mercury to form mercuric sulfide results in formation of ash. In Ayurveda it is claimed that the process of preparing mercuric sulfide (calcinations) results in abolition of the toxicity of mercury, and enhancing its quality so that it is effectively used by the body. In herbo-metallic preparations, the metal (in this case mercury) is transformed from a heavy, hard and rough structure to a light, soft and smooth powder (*Bhasma*) and the macro-sized particles are reduced to their nano form (usually 10–50 nm) as established by modern microscopic and spectroscopic techniques. It still remains to be firmly established that such herbo-metallic preparations containing mercury have no toxic effects in the body, although our results suggest that mercury in its sulfide form did not produce any toxicity. A recent study has also claimed that herbo-metallic preparations (i.e., preparations containing metals along with herbal components, which together may or may not be in a complex form) as well as *Bhasmas* are safe to use.^[23] Thus, AR can be considered as a potential promising anticancer agent.

Conclusions

The findings indicate that human pancreatic cancer cells are highly sensitive against AR as measured through growth inhibition and LDH release activity. This anticancer activity can be due to the presence of various anticancer phytochemicals present in the various plant ingredients in combination with mercuric sulfide.

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Conflicts of interest

There are no conflicts of interest.

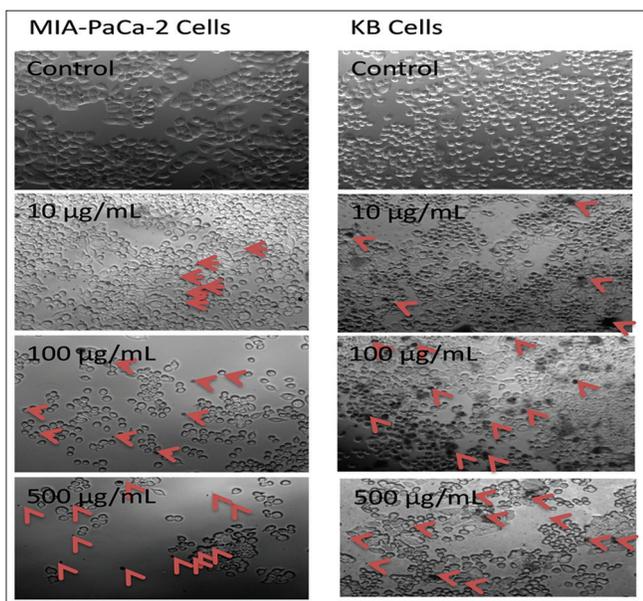


Figure 2: Cell morphology assay against Arkeshwar Rasa with different concentrations of MIA-PaCa-2 cells (left) and KB cells (right)

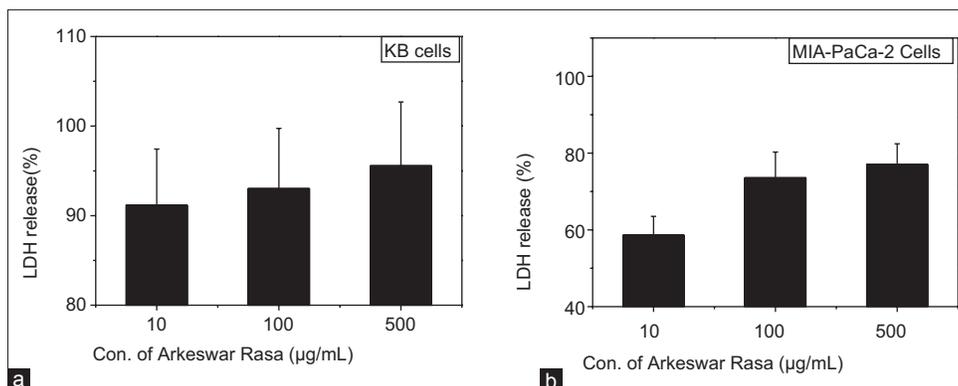


Figure 3: In vitro lactate dehydrogenase release study of (a) KB cells; (b) MIA-PaCa-2 cells

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हिन्दी सारांश

हर्षो मेटालिक योग अर्केश्वर रस का एंटीकैंसर प्रभाव

मो. नफिउज्जाम, मो. नुरुन्नबी, समीर कुमार साह, रौनक जहां, यंग-क्यू ली, मो. रहमतुल्ला

आयुर्वेद में सदियों से भस्मों का प्रयोग विभिन्न रोगों जैसे रुमेटॉइड आर्थाइटिस और कैंसर में किया जा रहा है। आयुर्वेद हर्षो मेटालिक योग अर्केश्वर रस का एंटीकैंसर प्रभाव का परीक्षण करने के लिए मानव के दो विभिन्न ऊतकों (पैन्क्रियाज और त्वचा) कैंसर सेल लास को लिया गया। एन्जाइम क्रियाविधि के लिए लैक्टेट डीहाइड्रोजिनेज और सेल मार्फॉलॉजी के लिए ट्राइपन ब्लू से परीक्षण किया गया। अर्केश्वर रस पैन्क्रियाटिक कैंसर सेल के विरुद्ध प्रभावी रहा और लैक्टेट डीहाइड्रोजिनेज की क्रिया से भी प्रभावी रहा। इससे निष्कर्ष निकला कि अर्केश्वर रस पैन्क्रियाटिक कैंसर के विरुद्ध एक प्रभावी एंटीकैंसर औषधि है।