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Effect of *Puta* on *in vitro* anticancer activity of *Shataputi Abhrak Bhasma* on lung, leukemia and prostate cancer cell lines

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ABSTRACT

Background: *Rasashastra* needs to be upgraded using the technological advances, with regards to drug processing, development and therapeutics. The potential of *Rasaaushadhis* need to be explored by subjecting them against newer life threatening diseases like cancer where contemporary medicine has limitations. *Abhrak Bhasma*, one of the drugs of *Rasashastra*, has some peculiar attributes. According to classical *Rasashastra* texts, *Shataputi Abhrak Bhasma* is regarded as a *Rasayan*, whose efficacy is in direct proportion to the number of *Putas*. Thus increasing number of *Putas* not only has a significant effect on the physical, analytical aspects but also the therapeutic effect of the *Abhrak Bhasma*.
Objectives: To screen *in vitro* anticancer activity of *Abhrak Bhasma* at various stages of *Putas* (20, 50, 100). To evaluate and thus validate the principle from classical *Rasashastra* texts, which explains direct relation of number of *Putas* with therapeutic efficacy.
Materials and methods: *Shataputi Abhrak Bhasma*, at various stages of its preparation was subjected to *in vitro* anticancer activity on three different cancer cell lines (LungHOP62, LeukemiaU937, ProstateDU145) at Tata Memorial Centre- Advanced Centre for Treatment, Research Education in Cancer, Navi Mumbai. SRB assay was followed to evaluate the anti-proliferative activity.
Results: It was found that *Abhrak Bhasma* shows concentration dependent positive *in vitro* anticancer activity on all three cell lines with highly significant activity on prostate cancer cell lines. Anticancer activity of *Abhrak Bhasma* is in the order 100 *Puti* > 50 *Puti* > 20 *Puti*. *Shataputi Abhrak Bhasma* had maximum activity on prostate cancer cell lines almost equivalent to positive control drug adriamycin.
Conclusion: The *in vitro* anticancer activity of *Shataputi Abhrak Bhasma* increases with increasing number of *Putas*, thus revalidating the direct relation between number of *Putas* and efficacy of the drug.

1. Introduction

Today, the world is reeling on the verge of an epidemic. The magnitude of mortality or morbidity of this epidemic on humans seems to be unclear and unpredictable. This epidemic is termed as 'CANCER'.

The latest surveys carried out by the WHO, worldwide, establish and reveal some alarming facts about the incidences of cancer and compels the medical fraternity to take immediate steps to curb this growing menace the mankind is facing. Some of the key facts as stated by WHO, updated in February 2015, are as follows [1].

Cancers figure among the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.8 million cancer related deaths in 2012. The number of new cases is expected to rise by about 70% over the next 2 decades. Among men, the 5 most common sites of cancer diagnosed in 2012 were lung, prostate, colo-rectum, stomach, and liver cancer. Among women the 5 most common sites diagnosed were breast, colo-rectum, lung, cervix, and stomach cancer. It is expected that annual cancer cases will rise from 14 million in 2012 to 22 within the next 2 decades.

In the Indian System of Health, earliest and foremost record of cancer could be traced in *Atharvaveda,* of which *Ayurveda* is an *Upaveda*, where the disease was given the nomenclature as '*Apacit*'. Swellings or lumps situated in the deep structures or as chronic ulcers have been categorized under the heading of "*ARBUDA*", where as non-healing ulcer as "*ASADHYA VRANA*".

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As a parallel can be drawn between the various types of cancers and the similar noted references in the *Ayurvedic* classical texts, there is a lot of potential to unravel the various treatment modalities mentioned in *Ayurveda*, which have stood the test of time. With the advent of newer and potentially life threatening disorders, it is the need of the hour to unearth and research various ways to tackle the ailments of the 21st century, keeping in view the principles of *Ayurveda*, which were propagated for maintaining the health of healthy and curing the diseased.

Rasashastra, one of the branches of *Ayurveda*, dealing with the pharmaceutical aspects, has a number of formulations having some unique attributes attached to them. It involves harnessing the therapeutic potential of herbs, metals and minerals by subjecting them to various procedures converting them into bio-assimilable form. The concepts of *Rasashastra*, need to be validated using the scientific and technological advances of today's world, which shall open up new avenues for drug processing and development in *Ayurveda*. This shall pave the way for exploring the *Siddhantas* (principles) given in the form of *Sutras* (verse) and re-establish the efficacies of the *Rasa aushadhis* which have been claimed to have been the saviour of human race from various ailments in the medieval and pre-medieval period.

Abhrak (Mica) is one such entity mentioned in classical *Rasashastra* texts having unique attributes. *Shataputi* and *Sahastraputi Abhrak Bhasma* are indeed unique attributes of *Abhrak*. *Abhrak Bhasma* is repeatedly subjected to *Puta* (incineration in a closed earthen vessel). Also process like *Lohitikaran* where *Shataputi Abhrak Bhasma* is tritutated with some distinct herbs before incinerating and process of *Amrutikaran* where *Abhrak Bhasma* is fried in *Goghruta* (cow ghee) and *Triphala kwath*, have a role in its therapeutic efficacy which needs evaluation. As many as 100 incinerations are mentioned for the preparation of *Shataputi Abhrak Bhasma* which underlines the amount of *Agni Sanskaar* (heat processing) *Abhrak Bhasma* is subjected to before being used therapeutically. *Abhrak Bhasma* is a *Rasayan* (which promotes life) [2] and its therapeutic properties undergo changes with incinerations. This is evident from the *Siddhant* mentioned in *Rasendra Saar Sangraha* [3]. Repeated incinerations lead to fineness in particle size. Recent studies show nanoparticle size range of *Bhasmas* [4]. Thus *Bhasmas* have emerged as *Ayurveda's* Nanomedicine. Properties of *Bhasmas* like *Yogavahitwa* (which promotes passage and movement of a formulation or drug) and *Shighra vyapti* (fast spreading) [5] further underline their similarities with nanoparticles in nanomedicine, having unique properties like fast action and target specific drug delivery. Nanomedicine is emerging as an important field for cancer drug research. The property of *Rasayan*, as mentioned in the classical text *Charak Samhita* can be correlated with the concept of Immunomodulation as is seen from the recent researches [6]. As also, the recent researches have shown that a number of *Rasayan dravyas* mentioned in *Ayurveda* classics have proven anti-cancer properties [7].

The need to screen more drugs of *Rasa Shastra* to find an innovative drug therapy for cancer management was one of the reasons of screening *Shataputi Abhrak Bhasma* on various cancer cell lines. A survey among the various renowned *Vaidyas*, established the fact that *Abhrak Bhasma* is widely used in combination with other dravyas in the management of various types of cancers in various stages. Some references of *Abhrak Bhasma* indicate its use in diseases like *Shopha* [8], *Granthi* [9], *Gulma* [10], *Vrana* [9], *Pandu* [10] which can be considered analogous with some form of benign or malignant tumors. Clinically, *Abhrak Bhasma* works predominantly on *Pranavaha, Raktavaha* and *Mutravaha strotasas* (channels of circulation). Hence, analogous, lung, leukemia and prostate cancer cells lines were selected for screening. Recently a lot of research has been done and is in progress regarding the role of Nanomedicines in cancer management, due to their unique properties like target specificity through drug delivery [11] and minimal dosages thus reducing the ill effects of the chemotherapeutic agents [12]. *Shataputi Abhrak Bhasma* being a *Rasayan,* with reduced particle size, this study was an attempt to screen its anti-cancer activity through preclinical assays and also evaluate the importance of *Putas* in pharmaceutics of *Abhrak Bhasma.*

The aim and objectives of the present study was to screen *in vitro* activity of *Krushnavajra Abhrak Bhasma* on lung (*Pranavaha strotas*), leukemia (*Raktavaha strotas*) and prostate cancer (*Mutravaha strotas*) cell lines and to study the effect of *Puta* on the *in vitro* anticancer activity of *Krushnavajra Abhrak Bhasma*. Also, to validate the *Siddhanta* given in *Rasashastra* texts which says 'As the number of *Putas* is increased the qualities of the drug increases manifold (thousand times)' [3].

2. Materials and methods

2.1. Material

Shataputi Abhrak Bhasma was prepared at the D.Y. Patil School of *Ayurveda* pharmacy after authentication of the raw drug to be *Krushnavajra Abhrak* from University of Pune. Standard Operative Procedure which was standardized by Dr. Deepali Korde and Prof. Dr. Kulwant Singh at ICPT&R, Jamnagar in 2003 was adopted for preparation. The prepared Abhrak Bhasma showed positive results when screened for classical parameters like Nischandratva, Varitaratwa, Rekhapurnatva, Apsumajjanam. It was also subjected to advanced analytical tests like XRD, ICP-AES, SEM-EDS, FEG-SEM, and TGA-DTA. During the process of preparation of the drug, samples of the *Abhrak Bhasma* were collected after 20 *Putas* (AB20), 50 *Putas* (AB50), 100 *Putas* (AB100), after *Lohitikaran* (ABL) and after *Amrutikaran* (ABA). These samples were subjected to *in vitro* anticancer activity.

In vitro anticancer activity of *Shataputi Abhrak Bhasma* was performed at Tata Memorial Centre- Advanced Centre for Treatment, Research Education in Cancer, Navi Mumbai. For the study 3 cells lines were selected ie. lung (HOP62), leukemia (U937), prostate (DU145). Instrument and materials used were SRB calorimeter, 96 well microtitre plates, dimethyl sulfoxide, 10%TCA, sulphorhodamine B dye, 1% acetic acid and plate reader.

2.2. Method

2.2.1. Cancer cell line culture

The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment [13–15], cells were inoculated into 96 well microtiter plates in 100 μl at plating densities as shown in the study details above, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs.

2.2.2. Preparation of solution of test drug

Experimental drugs were initially solubilized in dimethyl sulfoxide at 100 mg/ml and diluted to 1 mg/ml using water and stored frozen prior to use [13]. At the time of drug addition, an aliquot of frozen concentrate (1 mg/ml) was thawed and diluted to 100 μg/ml, 200 μg/ml, 400 μg/ml and 800 μg/ml with complete medium containing test article. Aliquots of 10 μl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 μl of medium, resulting in the required final drug concentrations i.e.10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml.

2.2.3. Plate preparation and drug addition

After compound addition, plates were incubated at standard conditions for 48 h and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 μl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant was discarded; the plates were washed five times with tap water and air dried [13,16].

2.2.4. SRB assay

Sulforhodamine B (SRB) solution (50 μl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature [16,17]. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

2.2.5. End point measurement

Percentage growth was calculated on a plate by plate basis for test wells relative to control wells [13,17]. Percentage growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells × 100.

Using the six absorbance measurements [time zero (Tz), control growth (C) and test growth in the presence of drug at the four concentration levels (Ti)]; the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as [(Ti−Tz)/(C−Tz)] 100 for concentrations for which Ti>/ = Tz (Ti−Tz) positive or zero [(Ti−Tz)/Tz] × 100 for concentrations for which Ti.

The dose response parameters were calculated for each test article. Growth inhibition of 50% (GI_{50}) was calculated from [(Ti−Tz)/(C−Tz)] × 100 = 50, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from Ti = Tz. The LC50 (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from [(Ti−Tz)/Tz] × 100 = −50.

Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested. The summary of the parameters is as follows.

GI_{50} Growth inhibition of 50% (GI_{50}) calculated from [(Ti−Tz)/(C−Tz)] × 100 = 50, drug concentration resulting in a 50% reduction in the net protein increase.

TGI Drug concentration resulting in total growth inhibition (TGI) will calculated from Ti = Tz.

LC50 Concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning, indicating a net loss of cells following treatment is calculated from [(Ti−Tz)/Tz] × 100 = 7−50.

Values obtained were analysed, their graphs were plotted and the final results were expressed in terms of LC50, TGI and GI50 values in comparison to standard or positive control drug taken as adriamycin.

3. Results

There are very few *Rasa dravyas* mentioned in Classical texts of *Rasashastra* where *Acharyas* have mentioned to give 100 *Putas*. *Abhrak* is one among them. Various *Siddhantas* like the one given in *Rasendra Chudamani* which says that when subjected to many *Putas* the dravya becomes finer in particle size and acquires newer unpredictable properties *(Vichitra gunadipti)* [12]. The effect of *Puta* was analysed by screening *Abhrak Bhasma* of 20, 50, 100 *Putas* and after process of *Lohitikaran* and *Amrutikaran*.

Before establishing the activity of the drug clinically, it has to be subjected to test preclinically. In the present study *Abhrak Bhasma* was prepared in D.Y.Patil School of *Ayurveda,* Navi Mumbai and was screened for *in vitro* anticancer activity at Tata Memorial Centre-Advanced Centre for Treatment, Research Education in Cancer, Navi Mumbai.

SRB assay [17] was selected as a preclinical assay for *in vitro* anticancer screening. This anti-proliferative assay was performed to assess growth inhibition. All 5 samples were subjected to screening at 4 different concentrations, viz. 10,20,40,80 μg/ml for 3 times and the average readings of the same were noted. All the samples were tested on 3 different cell lines, namely, lung (HOP62), leukemia (U937), prostate (DU145). The findings were represented in the form of graphs and final conclusions were drawn (Figs. 1–3).

3.1. 20 Puti Abhrak Bhasma (AB20)

From Figures 1–3 it can be seen that *Abhrak Bhasma* after 20 *Putas* shows anti-proliferative/growth inhibitory activity on all the three cell lines.

3.1.1. Lung cancer cell line (HOP62)

On lung cancer cell line AB20 inhibitory average results are 93.5, 91.3, 89.1 and 80.3 as against adriamycin results −23.2, −32.2, −44.4 and −58.1 for 10, 20, 40, 80 μg/ml concentration respectively. Thus AB20 shows little or negligible growth inhibitory activity in concentration dependent manner as against adriamycin.

3.1.2. Leukemia cell line (U937)

On Leukemia cell line AB20 inhibitory average results are 91.5, 88.1, 82.9 and 67.4 as against adriamycin results −13.8, −17.4, −21.7 and −28.6 for 10, 20, 40, 80 μg/ml concentration respectively. Thus AB20 shows little or negligible growth inhibitory activity in concentration dependent manner as against adriamycin.

3.1.3. Prostate cancer cell line (DU145)

On prostate cancer cell line AB20 inhibitory average results are 87.0, 70.4, −2.2 and −24.7 as against adriamycin results −69.6, −70.5, −72.7 and −75.1 for 10, 20, 40, 80 μg/ml concentration respectively. Thus AB20 shows mild to moderate growth inhibitory activity in concentration dependent manner as against adriamycin.

3.2. 50 Puti Abhrak Bhasma (AB50)

From Figures 1–3 it can be seen that *Abhrak Bhasma* after 50 *Putas* shows anti-proliferative/growth inhibitory activity on all the three cell lines.

3.2.1. Lung cancer cell line (HOP62)

On lung cancer cell line AB50 inhibitory average results are 86.2, 77.4, 59.7 and 19.4 as against adriamycin results −23.2, −32.2, −44.4 and −58.1 for 10, 20, 40, 80 μg/ml concentration respectively. Thus AB50 shows mild to moderate growth inhibitory activity in concentration dependent manner as against adriamycin.

3.2.2. Leukemia cell line (U937)

On Leukemia cell line AB50 inhibitory average results are 85.5, 75.0, 50.3 and 18.4 as against adriamycin results −13.8, −17.4, −21.7 and −28.6 for 10, 20, 40, 80 μg/ml concentration respectively. Thus AB50 shows mild to moderate growth inhibitory activity in concentration dependent manner as against adriamycin.

3.2.3. Prostate cancer cell line (DU145)

On prostate cancer cell line AB50 inhibitory average results are −7.5, −18.7, −37.8, and −52.5 as against adriamycin results −69.6, −70.5, −72.7 and −75.1 for 10, 20, 40, 80 μg/ml concentration respectively. Thus AB50 shows significant growth inhibitory activity in concentration dependent manner as against adriamycin.

3.3. 100 puti Abhrak Bhasma (AB100)

From Figures 1–3 it can be seen that *Abhrak Bhasma* after 100 *Putas* shows anti-proliferative/growth inhibitory activity on all the three cell lines.

3.3.1. Lung cancer cell line (HOP62)

On lung cancer cell line AB100 inhibitory average results are 74.0, 67.1, 32.7 and −16.9 as against adriamycin results −23.2, −32.2, −44.4 and −58.1 for 10, 20, 40, 80 μg/ml concentration respectively. Thus AB100 shows mild to moderate growth inhibitory activity in concentration dependent manner as against adriamycin.

3.3.2. Leukemia cell line (U937)

On Leukemia cell line AB100 inhibitory average results are 81.4, 65.3, 28.2 and 10.7 as against adriamycin results −13.8, −17.4, −21.7 and −28.6 for 10, 20, 40, 80 μg/ml concentration respectively. Thus AB100 shows mild to moderate growth inhibitory activity in concentration dependent manner as against adriamycin.

3.3.3. Prostate cancer cell line (DU145)

On prostate cancer cell line AB100 inhibitory average results are −14.2, −29.9, −49.3, and −63.8 as against adriamycin results −69.6, −70.5, −72.7 and −75.1 for 10, 20, 40, 80 μg/ml concentration respectively. Thus AB100 shows highly significant growth inhibitory activity in concentration dependent manner as against adriamycin.

3.4. Abhrak Bhasma after Lohitikaran (ABL)

From Figures 1–3 it can be seen that *Abhrak Bhasma* after Lohitikaran shows anti-proliferative/growth inhibitory activity on all the three cell lines.

3.4.1. Lung cancer cell line (HOP62)

On lung cancer cell line ABL inhibitory average results are 87.4, 78.2, 45.3 and −3.7 as against adriamycin results −23.2, −32.2, −44.4 and −58.1 for 10, 20, 40, 80 μg/ml concentration respectively. Thus ABL shows mild to moderate growth inhibitory activity in concentration dependent manner as against adriamycin.

3.4.2. Leukemia cell line (U937)

On leukemia cell line ABL inhibitory average results are 82.6, 65.4, 36.2 and 16.0 as against adriamycin results −13.8, −17.4, −21.7 and −28.6 for 10, 20, 40, 80 μg/ml concentration respectively. Thus ABL shows mild to moderate growth inhibitory activity in concentration dependent manner as against adriamycin.

3.4.3. Prostate cancer cell line (DU145)

On prostate cancer cell line ABL inhibitory average results are −12.8, −23.7, −44.9, and −58.8 as against adriamycin results −69.6, −70.5, −72.7 and −75.1 for 10, 20, 40, 80 μg/ml concentration respectively. Thus ABL shows highly significant growth inhibitory activity in concentration dependent manner as against adriamycin.

3.5. Abhrak Bhasma after Amrutikaran (ABA)

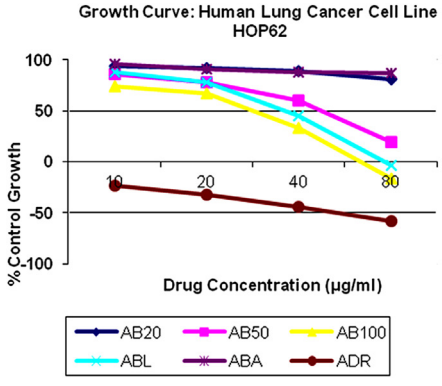
From Figures 1–3 it can be seen that Abhrak Bhasma after Amrutikaran shows negligible anti-proliferative/growth inhibitory activity on lung and leukemia cell lines and no activity on prostate cell line.

3.5.1. Lung cancer cell line (HOP62)

On lung cancer cell line ABA inhibitory average results are 95.8, 90.4, 88.0 and 86.3 as against adriamycin results −23.2, −32.2, −44.4 and −58.1 for 10, 20, 40, 80 μg/ml concentration respectively. Thus ABA shows negligible inhibitory activity in concentration dependent manner as against adriamycin.

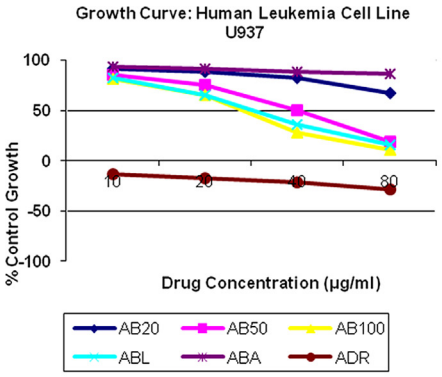
3.5.2. Leukemia cell line (U937)

On Leukemia cell line ABA inhibitory average results are 93.4, 91.2, 88.5 and 86.8 as against adriamycin results −13.8, −17.4, −21.7 and −28.6 for 10, 20, 40, 80 μg/ml concentration respectively. Thus



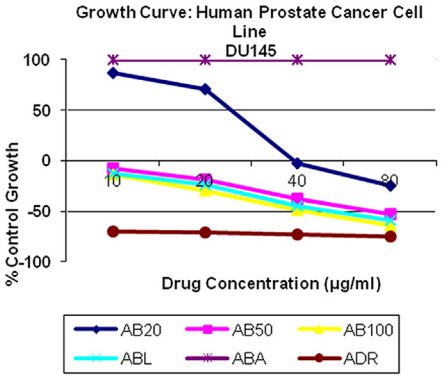
HOP62	LC50	TGI	GI50*
AB20	>80	>80	>80
AB50	>80	>80	49.9
AB100	>80	67.2	32.0
ABL	>80	77.7	39.7
ABA	>80	>80	>80
ADR	58.6	22.7	<10

Fig. 1. Drug concentration (μg/ml) calculated from graph.



U937	LC50	TGI	GI50*
AB20	>80	>80	>80
AB50	>80	>80	46.7
AB100	>80	>80	37.4
ABL	>80	>80	40.7
ABA	>80	>80	>80
ADR	>80	34.5	<10

Fig. 2. Drug concentration (µg/ml) calculated from graph.



DU145	LC50	TGI	GI50*
AB20	>80	58.5	28.7
AB50	64.0	28.7	<10
AB100	56.3	23.5	<10
ABL	59.5	25.5	<10
ABA	>80	>80	>80
ADR	40.2	<10	<10

Fig. 3. Drug concentration (µg/ml) calculated from graph.

ABA shows negligible inhibitory activity in concentration dependent manner as against adriamycin.

3.5.3. *Prostate cancer cell line (DU145)*

On prostate cancer cell line ABA inhibitory average result is 100 as against adriamycin results −69.6, −70.5, −72.7 and −75.1 for 10, 20, 40, 80 µg/ml concentration respectively. Thus ABA shows no growth inhibitory activity as against adriamycin.

4. Discussion

The above findings are an indicator that *Abhrak Bhasma* at different stages of preparation when put to SRB test showed antiproliferative, growth inhibitory activity on all the 3 cell lines selected. The mechanism of *in-vitro* growth inhibition achieved by *Abhrak Bhasma* needs to be evaluated with further studies. *Shataputi Abhrak Bhasma* AB100 showed highest activity among all 5 samples on prostate cancer cell lines. The activity was almost equivalent to the activity of control group standard chemotherapeutic agent adriamycin. This finding needs further evaluation in invivo studies for its mechanism of action.

Putas or incinerations done repeatedly lead to increase in fineness of the *bhasma*, brings about changes in the structure, composition, arrangement of molecules and thus formation of newer compounds. Hence, the activity varied with number of *Putas*. The more the incinerations with *Agni* the better is the activity (antiproliferative in this case).

However, *in vitro* studies have to be supported with invivo studies to further substantiate the findings. Another limitation of the study is decrease in solubility of Abhrak Bhasma after Amrutikaran thus hampering its anti-proliferative activity.

5. Conclusion

The present study signifies the importance of various pharmaceutical processes of *Rasashastra* like *Puta* and the effect they have on the efficacy of the drug. It validates the *Siddhanta* that the activity of *bhasma* increases with increase in *Putas*. Thus, the number of Putas is directly proportional to the efficacy of the drug and inversely proportional to the particle size and the dosage required. Hence, it may be concluded that *Shataputi Abhrak Bhasma*, highly regarded as a *Rasayan* showed positive *in vitro* anticancer activity on prostate cancer cell lines, thus leading to scope for exploring the mechanism of its activity through animal and cclinical studies.

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None.

Conflicts of interest

None.

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