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Bio-enhancement effect of *Bos primigenius indicus* urine isolates on Curcumin anticancer activity using different human cell line models of A549, Hep-G2, MCF-7, Jurkat and K562

Hemanth Kumar Manikyam¹, C.Ramesh², Krishna Mohan Poluri³, Haranath Reddy KASIREDDY⁴, Charitha Devi Mekala⁵

¹Department of chemistry, North East Frontier Technical University, Arunachal Pradesh, India; ²School of pharmaceutical Management, IIHMR University, Jaipur, and Rajasthan India; ³Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India; ⁴Sri Venkateswara Gosamrakshana Shala, Tirumala Tirupati Devasthanams, Andhra Pradesh, India; ⁵Department of Virology, Sri Venkateswara University, Tirupati, Andhra Pradesh, India.

ABSTRACT

The study is conducted to explore possible utilization of cow urine and understanding complete biochemical compounds involved in possible therapeutic applications of cow urine therapy which was used from ancient times. In the present experiment inductively coupled plasma mass spectrometry (ICP-MS) and Gas Chromatography-Mass Spectrometry (GC-MS) methods was used to analyze the complete mineral and compound detection. The test conducted using MTT method using human cancer A549, Hep-G2, MCF-7, Jurkat and K562 cell lines as part of the in vitro preclinical characterization of Cow urine extracted Curcumin and pure Curcumin compounds and compared against positive control Sodium lauryl Sulphate (SLS). More than 100% increment in cell killing at a concentration of 1.25 mg/ml recorded in the cell line for the cow urine extracted Curcumin and less than 70% for the pure Curcumin. Positive control SLS exhibited nearly 100% of killing cells.

Keywords: Cow urine, Curcumin, Anticancer, Ayurveda, cell lines, Bio-enhancement

*Correspondence to Author:

Hemanth Kumar Manikyam
Department of chemistry, North
East Frontier Technical University,
Arunachal Pradesh, India

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INTRODUCTION

Cows were regarded as wealth and were the backbone of the economy of ancient Indians. Wars were fought for acquiring cows. Cattle were one of the most frequently used animals described in Vedas. Cows were regarded as mother ("Gau-mata") and referred to as Aghnya. Prayers were offered to Agni (God of Fire) to kill with his flame all those evil dwellers, who stole milk of cows. Voluminous treatises are also available on cows, e.g., Gau Ayurveda'. During Pauranic period, cow (Kamadhenu) emerging out of Samudra manthan, was considered so valuable that devatas fought with demons and acquired them. Cattle husbandry was well developed during the Rigvedic period (1500– 1000 BC) and the cow (Kamadhenu) was adored and considered the 'best wealth' of mankind. Aryans laid great emphasis on protection of cows. Atharvaveda provides interesting information about ailments of animals, herbal medicines, and cure of diseases. Urine was also considered as an antidote to poisons (Sushruta Samhita).

From the ancient period, cow's urine has been used as a medicine. In Veda, cow's urine was compared to the nectar. In Sushruta, several medicinal properties of cow's urine have been mentioned and cow urine was known to cause weight loss and to cure leprosy, cardiac and kidney problems, indigestion, stomach ache, edema, etc (1). This kind of alternative treatment, termed as 'panchgavya therapy' or 'cowpathy', has been reported to be beneficial even for dreaded diseases like cancer, AIDS and diabetes. Practitioners of Ayurvedic medicine from India routinely use cow urine as a remedy and the medicines made from it are used to cure several diseases. Cow urine has shown anticancer and anti-diabetic activity.

The cow urine has been given two US patents for its bio-enhancer properties especially for early cure of tuberculosis and cancer in human beings. United States Patent and Trade mark Office had granted Patents No 6410059 and

No. 6896907 to an "Indian innovation which has proved that cow urine can make antibiotics, anti-fungal agents more effective" (2,3). Other studies also revealed the immunomodulatory properties of cow urine. Besides, cow urine is said to be a very effective insect repellent when mixed with certain herbs (4).

Curcuma longa, a perennial herb and member of the *Zingiberaceae* (ginger) family, grows to a height of three to five feet and is cultivated extensively in Asia, India, China, and other countries with a tropical climate. Turmeric is used extensively in foods for its flavor and color, as well as having a long tradition of use in the Chinese and Ayurvedic systems of medicine, particularly as an anti-inflammatory and for the treatment of flatulence, jaundice, menstrual difficulties, hematuria, hemorrhage, and colic.

Turmeric can also be applied topically in poultices to relieve pain and inflammation (5). Cancer is a hyperproliferative disorder marked by metastasis into the vital organs of the body through invasion and angiogenesis. Curcumin blocks the transformation, proliferation, and invasion of tumor cells. The biochemical pathways involved in the carcinogenesis process have been investigated extensively over the last four decades. Numerous studies over the last two decades have demonstrated that Curcumin targets several steps in these biochemical pathways, thus showing immense promise for the treatment of cancers.

The present study is carried out to prove the bio-enhancement effect of cow urine using anticancer activity of Curcumin against different cancer cell line models A549, Hep-G2, MCF-7, Jurkat and K562 cells.

MATERIALS AND METHODS

EXTRACTION OF TOTAL MINERALS FROM COW URINE:

1 liter of cow urine was freshly collected and added in equal volume of 250 ml each in 500 ml beakers. 25 ml Aquaregia (3:1, HCL: Nitric

acid) was added in each beaker and stirred for half an hour using magnetic stirrer. Later 250 ml of diethyl ether was added in equal quantities to each beaker and stirred for an hour. Diethyl ether layer was collected using separating funnel and 5 N NaOH solution was added to diethyl ether solution till we get dark purple color layer at bottom of flask indicating mineral separation. The purple colored solution was collected and dried till we get crystals. Then crystallized powder was analyzed for minerals using ICP-MS method.

TOTAL MINERAL ANALYSIS USING ICP-MS:

Inductively coupled plasma mass spectrometry (ICP-MS) is powerful technique for trace analysis of elements and the latter is preferred for ultra-trace levels due to its higher sensitivity. Mass spectrometry with inductively coupled plasma (ICP-MS) is a multi-element technique for analyzing liquid samples, characterized by high selectivity, sensitivity and detection limits much lower than other multi-element techniques. Open beakers heated on hot plates, digestion tubes in a block digester, and digestion bombs placed in microwave ovens are the most commonly used equipment to digest the cow urine sample. Ultra-pure de-ionized water (18 MΩcm⁻¹) from a Milli-Q analytical reagent grade water purification system (Millipore) and ultra-pure HNO₃ 60% (Lot –No B0157318 Merck) were used. In order to validate the method for determining the concentration of metals, NCS ZC 73006 Certified Reference Material was used. For digestion of this reference material, an acid mixture (3 ml HNO₃ ultrapure 60%, 2 ml HF 40%) was added to 0.1 g of sample in a Teflon receptacle, tightly closed. Six such receptacles were inserted in a device made of six stainless steel cylinders mounted between two flanges, to confer pressure resistance. The whole system was put in an oven at 200°C for 12 hours. A colorless solution resulted and ultra-pure water was added up to 50 ml. Calibration standard solutions and internal standards were

prepared by successive dilution of a high purity ICP-multi-element calibration standard (10µg/l from twenty-nine element ICPMS standard, item N9300233, Matrix: 5% HNO₃, PerkinElmer Life and Analytical Sciences). All plastic lab ware used for the sampling and sample treatment were new or cleaned by soaking 24 h first in 10% HNO₃ then in ultra-pure water.

INSTRUMENT

All the determinations were carried out by inductively coupled plasma quadrupole mass spectrometry (ICP-Q-MS). A PerkinElmer ELAN DRC (e) instrument was used with a Mein hart nebulizer and silica cyclonic spray chamber and continuous nebulization. The operating conditions are: Nebulizer Gas flow rates: 0.86 l/min; Auxiliary Gas Flow: 1.2 l/min; Plasma Gas Flow: 15 l/min; Lens Voltage: 7.25 V; ICP RF Power: 1100 W; CeO/Ce = 0.031; Ba⁺⁺/Ba⁺ = 0.016. Prior to analysis, the ICP-MS, located in a temperature-controlled laboratory (20 ± 2 °C), was allowed a sufficient period of time to stabilize before optimization procedures were carried out.

HPLC – LC/MS-ESI

An Agilent HPLC – LC/MS-ESI system consisted of an auto sampler, two pumps for gradient solvent delivery, a column oven operated at 60°C, and a diverge valve to direct LC effluent to mass spectrometer in the elution window 6–16 min. A 250×4.6 mm, 5 mm, jordi-gel DVB polyamine. Column was used. The elution was accomplished by a water (A)/acetonitrile (B) gradient with the following profile: t=0, 5, 7, 15, 16.5, and 19 min, B%=100, 92, 90, 90, 100, and 100, respectively; t=0, 5.1, 15, 16.5, and 19 min, flow rate=1.5, 1.0, 1.0, 1.5, and 1.5 ml min⁻¹, respectively. The HPLC effluent was directed through the diverge valve to a ESI interface on a Agilent 6400 triple quadrupole mass spectrometer. The capillary heater was set at 250°C. The spray voltage was fixed at 4.5 kV.

The collision gas (argon) pressure was established at 2.5 mTorr; the collision energy (voltage in the collision cell) was 16 V. An electron multiplier voltage of 1600 V was used. The sheath and auxiliary gas settings were 100 psi and 30 cc min⁻¹, respectively. The dwell time was 500 ms. The instrument was operated in the negative mode. The parent ion of Allantoin [M-H]⁻ at m/z 157 (159 for internal standard) was admitted to the first quadrupole (Q1). After the collision induced fragmentation in Q2, the product ion at m/z 114 (116 for internal standard) was monitored in Q3. Unit resolution (at half peak height) was used for both Q1 and Q3. Data processing was carried out using a Finnigan Quan Guide data analysis program. Peak area ratios based on SRM of Allantoin (m/z 157114) and the internal standard (m/z 159116) were utilized for the construction of calibration curve and quantization.

CATECHOLAMINE ISOLATION FROM COW URINE BY CATION RESIN EXCHANGE METHOD

1 liter of fresh cow urine was collected. Cation-Resin was prepared by soaking the resin in HCL solution for overnight. The resin was collected and added to cow urine in equal quantity. After overnight incubation the resin separated from the cow urine by Muslin cloth and washed with water to get clear resin. Later the resin was washed with Ammonium Hydroxide solution. The collected solution was evaporated completely to get gray color powder and analyzed for catecholamine.

CATECHOLAMINE DETECTION USING HPLC

Catecholamines play an important role as neurotransmitter and adrenal gland hormones in the human body. Concentrations of three major Catecholamines (norepinephrine, epinephrine, and dopamine) in urine are significant in diagnosis and treatment of several

diseases. The advantage of this method is not only its simplicity but also high sensitivity and selectivity. Furthermore, this method is quite easy-to-use because cleaning of conductive diamond electrode used as the working electrode can be performed routinely without removing it from the flow cell. Manual polishing of the working electrode is not necessary at all. HPLC Conditions Column: Inertsil ODS-4 (5µm, 250×3.0 mm I.D.) Eluent: A) Acetate-citrate buffer B) CH₃CN A/B = 100/16, v/v (Premix) Flow rate: 0.5 mL/min Col. Temp. : 35 °C Detection: ECD 800 mV vs. Ag/AgCl (ED703, Diamond) Inj. Vol.: 20 µL

ACETATE-CITRATE BUFFER

To 500 mL of water, 0.82 g of sodium acetate (anhydrous), 2.10 g of citric acid (monohydrate), and 0.5 g of sodium 1-octanesulfonate was dissolved.

SHORT CHAIN FATTY ACID DETECTION BY LC-MS

Following the liquid liquid extraction procedure, the extract was subjected to a derivatization reaction using N-(3-dimethyl aminopropyl)-N'-carbodiimide (EDC) and 2, 2, 2-Trifluoroethyl amine (TFEA) in phosphate buffer. The resultant solution was diluted and chromatographed using a valve switching system consisting of two HPLC column. Upon initial separation of derivatized butyrate from phosphate salts on an Aquasil C18 column, the fraction was further eluted with second Aquasil C18 column for enhancing the sensitivity. Butyrate-¹³C₄ and butyrate internal standard (butyrate-d₇) was detected by API 4000 LC/MS system under positive MRM mode.

PREPARATION OF COW URINE WITH AND CURCUMIN

The test items Cow urine extracted Curcumin and pure Curcumin 95% were purchased from

Herbal Creations and stored in ambient conditions for further study.

USA) to obtain the sub confluence of cells (70 % to 90 % Confluent).

PREPARATION OF STOCK SOLUTION

Stock concentration of the Test Items Cow urine extracted Curcumin and pure Curcumin were prepared by dissolving the test item in 100% DMSO shown in **table 2** and final stock solution prepared as shown in **table 3**

PREPARATION OF POSITIVE CONTROL

10 % Sodium Lauryl Sulphate (SLS) (w/v) was used as positive control and different concentrations of SLS were used (10, 5, 2.5, 1.25, 0.625, 0.312 percent solutions).

PREPARATION OF NEGATIVE CONTROL

RPMI medium with 0.5% DMSO was taken as negative control.

DESCRIPTION OF CELL LINES

All the cell lines described in **table 2** were purchased from National Center for Cell Sciences, Pune with seeding density of 2×10^4 cells/well stored in liquid nitrogen for further testing purpose.

PREPARATION OF MTT SOLUTION

Stock concentration of 5mg/ml MTT was prepared in PBS and sterile filtered with 0.22µ filter and was used for the study (14).

TEST SYSTEM PREPARATION

Prior to the assay the test system A549, HepG2, MCF-7, Jurkat and K-562 cells were propagated at $37 \pm 1^\circ\text{C}$ in a gaseous environment of $5 \pm 1\%$ Carbon dioxide, in humid environment in tissue culture flasks containing medium, Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA) supplemented with 10 % fetal bovine serum (Invitrogen, USA) and penicillin (100 units) – streptomycin (100 µg) antibiotics (Invitrogen,

CELL SEEDING FOR CYTOTOXICITY ASSESSMENT

Cell monolayer was rinsed with PBS, aspirated off PBS and cells were trypsinized with 0.25% Trypsin with 0.2g/l EDTA in tissue culture flask at $37 \pm 1^\circ\text{C}$ until the cells detached and floated. DMEM with 10% FBS was added into the flask to flush out the cells and centrifuged at 900 rpm for 5 minutes. Cells were resuspended in DMEM medium and cell suspension was subjected for the cell count and viability to determine cell number per ml. Cell number was adjusted to 2×10^5 cells/ml and 0.1 ml of the adjusted cells were seeded in each well of 96 well cell culture plates. Frequent mixing was done during the seeding, to achieve a uniform cell suspension for plating the cells per well. Plates were designated to indicate its contents and date of experiment. Plates were incubated at $37 \pm 1^\circ\text{C}$ for 24 ± 1 hours in gaseous environment of $5\% \pm 1\%$ Carbon dioxide. After 24 ± 1 hours incubation the cell were exposed to different concentrations of test items

Table 2 by replacing the spent medium with 100µl of different concentrations of the test items solution and incubated for 48 ± 1 hours at $37 \pm 1^\circ\text{C}$ in gaseous environment of $5 \pm 1\%$ carbon dioxide. Positive, negative control and blank were dispensed in the designated wells and incubated for 48 ± 1 hours at $37 \pm 1^\circ\text{C}$ in gaseous environment of $5 \pm 1\%$ carbon dioxide. At the end of the 48 ± 1 hours incubation medium with test item / positive control was removed and cells were incubated for 4 hours with 20µl of MTT 5mg/ml solution. After 4 hours incubation formazan crystals formed by mitochondrial reduction of MTT was solubilized by adding 150µl of DMSO. Absorbance was read at 570nm after 10min incubation with vortexing.

DATA ANALYSIS

A decrease in the number of living cells results in a decrease in the metabolic activity in the sample. This decrease directly correlates to the amount of formazan formed as monitored by optical density at 570nm. Percent Viability will be calculated using the below formula (14).

$$\% \text{ Viability} = 100 \left(\frac{\text{O.D Test Item}}{\text{O.D of Control}} \right)$$

$$\% \text{ Activity} = 100 - \% \text{ Viability}$$

RESULTS

TOTAL MINERAL AND TRACE ELEMENT ASSAY

Although the composition of the cow urine sample is fairly unknown or not explored scientifically; but ancient Indian scriptures showed the presence of some trace elements and minerals in it which have some medicinal properties. To explore the unexplored we did ICP_MS for cow urine sample crystalline solid powder which have distinctive odor of it. ICP-MS had shown the signatures of some trace elements and minerals viz Gold, Silver, Iron, Sulphur, Calcium, Selenium etc in it. (Table 1).

ALLANTOIN QUANTIFICATION

A detailed HPLC experiment showed the 83% abundance of 114MW Allantoin in cow urine sample. Generally it produced broadest peak at 11.234 retention time scale. (Fig 1a and 1b)

QUALITATIVE DETECTION OF CATECHOLAMINE

A qualitative HPLC assay of the cow urine sample showed the presence of Nor epinephrine, Epinephrine, DHBA and Dopamine in it. (Fig 2)

CATECHOLAMINE DETECTION BY LC-MS

Mass finger printing assay of the cow urine sample helped to qualitatively understand the composition of it. It showed the presence of short chain fatty acids in it. (Fig 3)

ANTI-CANCER ACTIVITY OF COW URINE ON DIFFERENT CELL LINES

Test results and the graphical representation of the study are summarized in Table 3 and 4 and represented in Fig 4-6.

ANTICANCER ACTIVITY ON A549 CELLS

Cow urine extracted Curcumin has shown 102% and pure Curcumin has shown 60% of inhibition at the concentration of 1.25 µg/ml. Positive control SLS showed the 100% inhibition activity.

ANTICANCER ACTIVITY ON HEP-G2 CELLS

Cow urine extracted Curcumin has shown 105% and pure Curcumin has shown 87% of inhibition at the concentration of 1.25 µg/ml. Positive control SLS showed the 100% inhibition activity.

ANTICANCER ACTIVITY ON MCF-7 CELLS

Cow urine extracted Curcumin has shown 105% and pure Curcumin has shown 87% of inhibition at the concentration of 1.25 µg/ml. Positive control SLS showed the 99% inhibition activity.

ANTICANCER ACTIVITY ON JURKAT CELLS

Cow urine extracted Curcumin has shown 110% and pure Curcumin has shown 23% of inhibition at the concentration of 1.25 µg/ml. Positive control SLS showed the 100% inhibition activity

ANTICANCER ACTIVITY ON K562 CELLS

Cow urine extracted Curcumin has shown 178% and pure Curcumin has shown 164 % of inhibition at the concentration of 1.25 µg/ml. Positive control SLS showed the 100% inhibition activity.

From the results it's been confirmed that Cow urine extracted Curcumin has more anticancer activity when compared to pure Curcumin on all

TABLE 1: MINERAL ANALYSIS OF COW URINE ISOLATE BY ICP-MS

SI no	Parameters	Results	Protocol
1.	Au	<0.01 ppm	ICPMS-1-02
2.	Ag	<0.01 ppm	ICPMS-1-02
3.	Fe	141.3 ppm	ICPMS-1-02
4.	K	37.74%	ICPMS-1-02
5.	Ni	1.66 ppm	ICPMS-1-02
6.	P	268.5 ppm	ICPMS-1-02
7.	Mg	0.69%	ICPMS-1-02
8.	Mn	7.42 ppm	ICPMS-1-02
9.	S (w/w)	2.37%	API-part Vol VII
10.	Ca	0.96 ppm	ICPMS-1-02
11.	Pb	<0.01 ppm	ICPMS-1-02
12.	Se	0.25 ppm	ICPMS-1-02

TABLE NO. 2. PREPARATION OF INITIAL STOCK CONCENTRATION OF TEST ITEMS

S.No	Test Item Name	Initial Stock Concentration in mg/ml (highest soluble conc. in DMSO)	Volume of DMSO initial stock in ml	Volume of DMEM medium in ml	Final Stock in DMEM in mg/ml (1st concentration)
1	Cow urine extracted Curcumin(GOCU)	250	0.025	4.975	1.25
2	Curcumin(CU)	250	0.025	4.975	1.25

From the final stock different concentrations of the final working stocks five concentrations were prepared in DMEM medium by 4 folds and 3 folds serial dilutions as specified in table below. Diluted stocks were used for the study.

TABLE NO. 3. FINAL STOCK CONCENTRATIONS USED IN THE STUDY

S.No	Test Item Name	Final Stock in DMEM in mg/ml	Final working stock Concentration in RPMI in mg/ml	Volume of Working Stock in ml	Volume of DMEM in ml
1	Cow Urine extracted Curcumin (GOCU)	1.25 (4 folds)	0.313,0.078,0.020,0.005,0.001	1.25 ml	3.75 ml
2	Curcumin(CU)	1.25 (4 folds)	0.313,0.078,0.020,0.005,0.001	1.25 ml	3.75 ml

TABLE NO. 4. ANTICANCER PERCENTAGE ACTIVITY OF PURE CURCUMIN

Concentration in mg/ml	Average Percent Activity Curcumin				
	HEPG2	A549	MCF-7	Jurkat	K562
1.250	87.281	60.572	91.339	23.566	164.271
0.313	73.164	67.627	51.388	77.057	88.809
0.078	97.774	63.016	87.008	104.489	78.747
0.020	43.297	40.120	49.814	47.257	62.320
0.005	6.348	1.291	-22.897	33.915	81.211
0.001	-0.474	-6.756	-39.039	15.337	-8.316

TABLE NO. 5. ANTICANCER PERCENTAGE ACTIVITY OF COW URINE EXTRACTED CURCUMIN

Concentration in mg/ml	Average Percent Activity of Cow Urine extracted Curcumin				
	HEPG2	A549	MCF-7	Jurkat	K562
1.250	105.825	102.553	95.513	110.764	178.393
0.313	97.159	80.778	91.661	112.056	125.323
0.078	92.925	79.957	87.302	111.410	89.155
0.020	7.430	37.988	27.704	47.040	37.682
0.005	-6.066	32.320	-37.693	-5.705	16.185
0.001	-10.641	28.962	-32.995	1.292	-4.734

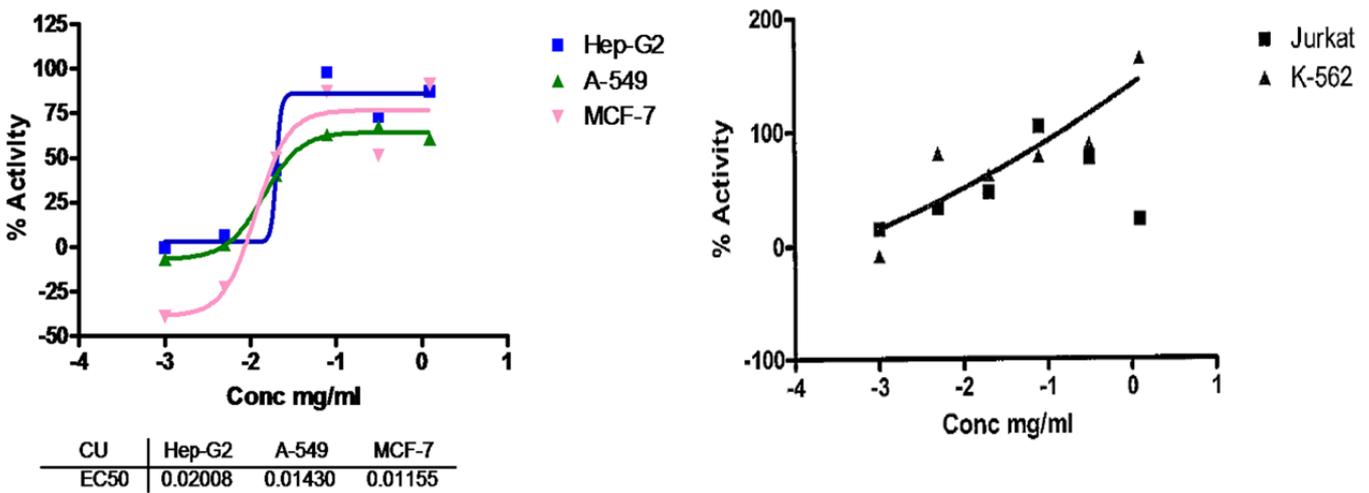


FIGURE 1 percentage activity of pure Curcumin on different cancer cell lines

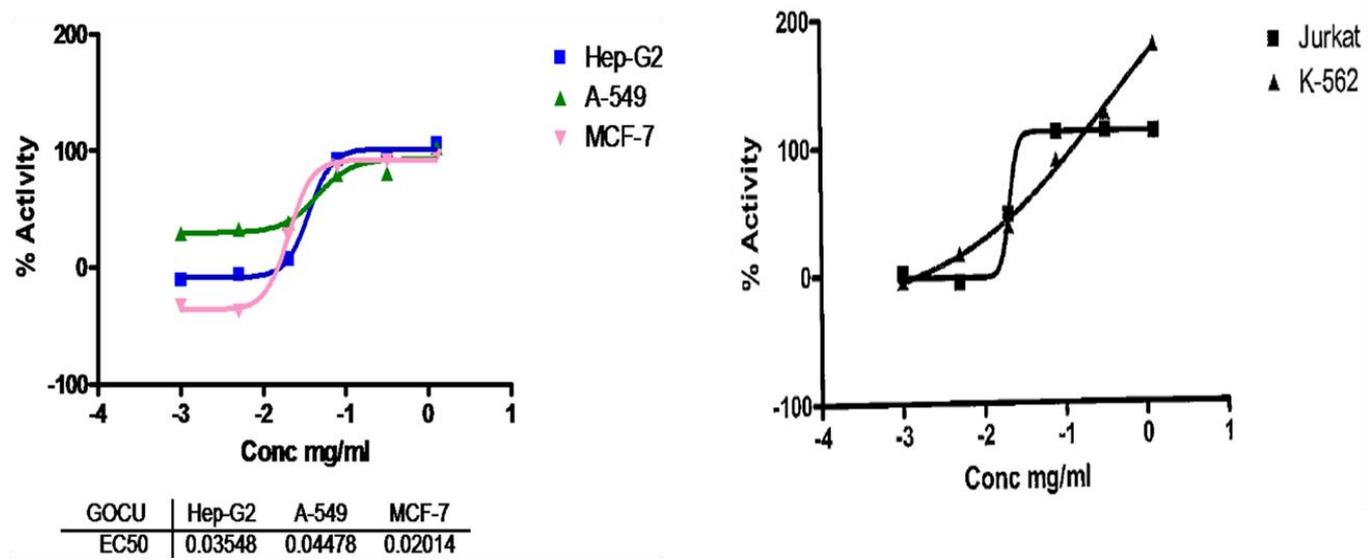


FIGURE 2 percentage activity of cow urine extracted Curcumin on different cancer cell lines

cancer cell line models which proves the Bio-enhancement effect of Cow urine.

DISCUSSION

Cow (*Bos indicus*) urine/Gomutra has been elaborately explained in Ayurveda and described in "Sushruta Samhita", "Ashtanga Sangraha" and other Ayurvedic texts as an effective medicinal substance/secretion of animal origin with innumerable therapeutic properties (6). Bhav Prakash Nighantu describes Gomutra as the best of all types of animal urine (including human) and enumerates its various therapeutic uses (7).

Gomutra is not a toxic waste material. 95% of it is water, 2.5% consists of urea, and the remaining 2.5% is a mixture of minerals, salts, hormones and enzymes (8). Gomutra exhibits the property of Rasayana tattwa responsible for modulating various bodily functions, including immunity. It augments B- and T-lymphocyte blastogenesis; and IgG, IgA and IgM antibody titers in mice. It also increases secretion of interleukin-1 and interleukin-2 (9), phagocytic activity of macrophages, and is thus helpful in the prevention and control of infections. Antimicrobial and germicidal properties of Gomutra are due to the presence of urea (strong effect), creatinine, swarn kshar (aurum hydroxide), carbolic acid, other phenols, calcium and manganese; its anticancer effect is due to uric acid's antioxidant property and Allantoin; immunity is improved by swarnkshar; and wound healing is promoted by Allantoin. Butyrate and some short chain fatty acids had proven to induce apoptosis in human cancer cell lines. (15)

Curcumin is one such agent; derived from turmeric (*Curcumin longa*), it has been used for thousands of years in the Orient as a healing agent for variety of illnesses. Research over the last few decades has shown that Curcumin is a potent anti-inflammatory agent with strong therapeutic potential against a variety of cancers. Curcumin has been shown to

suppress transformation, proliferation, and metastasis of tumors (10).

Bio-enhancing is one of its many properties (11). Cow urine distillate is more effective as a bio-enhancer than cow urine, and increases the effectiveness of antimicrobial, antifungal and anticancer drugs (12). It also increases the activity of gonadotropin releasing hormone conjugate with bovine serum albumin (GnRH-BSA) and zinc (13).

CONCLUSION

Till date the bio-enhancement effect of cow urine distillate has been practically proven only on antibiotics. For the first time in our research we have used different cancer cell line models like A549, Hep-G2, and MCF-7, Jurkat and K562 cells to explain the bio-enhancement effect of cow urine against anticancer activity of Curcumin. From the results in comparison between cow urine extracted Curcumin and pure Curcumin, the cow urine extracted Curcumin had shown more than 100% of inhibition activity against all cancer cell lines where as pure Curcumin has shown nearly 70% activity. This research has opened up an ally of green chemistry for utilization of animal resources in human welfare and medical appliance.

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