

INVESTIGATION ON PHARMACOGNOSY OF *KATHA* POWDER AS WELL AS IT'S *IN VITRO* CYTOTOXIC ACTIVITY

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ABSTRACT

Objective: The present study delves into the investigation of quantitative phytochemical in *Katha* powder, and it is *in vitro* cytotoxic activity.

Methods: Coarsely dried chips of *Acacia catechu* heartwood were treated with a 10% hydro-alcoholic solution to obtain *Katha* as the final product. The powdered *Katha* was standardized through pharmacognostic parameters. Phytochemical investigations were carried out to screen polyphenols (tannins and flavonoids) of interest which later were confirmed by thin-layer chromatography. The cytotoxicity effect of *Katha* powder on MCF-7, A431, and HepG2 cells was characterized by the trypan blue dye exclusion and MTT colorimetric assays technique. Control assay was carried out for samples containing only the appropriate volumes of blank solutions and showed no effect on cell growth. Different cells were exposed to *Katha* powder for about 48 h and performed cytotoxicity assays. The effect of *Katha* powder against these cell lines concentration range 10–100 µg/ml showed a decrease in percent cell viability in a dose-dependent manner, as compared with that of the control when examined by the trypan blue exclusion assay technique and MTT colorimetric assays technique.

Results: Quantitative phytochemical investigations were showed that *Katha* is rich in the content of polyphenols (tannins and flavonoids) and having good pharmacological potential. The effect of *Katha* powder against these cell lines concentration range 10–100 µg/ml showed a decrease in percent cell viability in a dose-dependent manner.

Conclusion: So from this investigation it is to be suggested that the *Katha* powder is rich in the phenolic compound and shows a good anticancer effect against MCF-7, A431, and HepG2 cells.

Keywords: *Acacia catechu*, MCF-7, A431, HepG2, Polyphenols, *Katha*.

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INTRODUCTION

The use of herbal medicines has become a global subject with medical and economic ramifications over the past few decades. *Acacia catechu* (L.f.) Willd. is known by the name "*khadira*" in Ayurveda belongs to the Leguminosae family [1]. The plant is a small moderate-sized tree about 10–13 m height extensively scattered throughout India [2]. The medicinal significance of this plant is reflected by the use of leaves, bark, and heartwood in several Ayurvedic formulations for 1000 years [3]. The heartwood of *A. catechu* has more potent medicinal activity in comparison to its leaves and bark. The heartwood extract of *A. catechu* has been reported to have various pharmacological effects such as anti-inflammatory [4], antioxidant [5], antimicrobial, immunomodulatory, antipyretic, hypoglycemic, anti-diarrheal, and hepatoprotective. The plant contains polyphenols such as tannins, and flavonoids along with carbohydrates and proteins [6]. The major constituents of the plant include catechin (-), epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate. Other constituents include rocatechin, phloroglucin, protocatechuic acid, quercetin, poriferasterol glucosides, lupenone, lupeol, procyanidin AC, kaempferol, dihydrokaempferol, taxifolin, (+)-afzelchin gum, and minerals [7]. The heartwood of this plant is known by the name "*Katha*" which is well known for its diverse pharmacological properties [8]. The present study on *Katha* powder delves for investigation of quantitative phytochemical in *Katha*, and its *in vitro* evaluation of cytotoxic activity.

METHODS

Plant material

The heartwood of the plant was collected in November 2019 from Solan district of Himachal Pradesh, India, which further was authenticated

by Raw material herbarium and museum, NISCAIR, New Delhi, India. A voucher specimen of the plant was preserved in the herbarium for reference (NISCAIR/RHHD/Consult/2019/3465-66).

Preparation of *Katha*

The heartwood of *A. catechu* was dried at room temperature (25±2°C) for 4 consecutive weeks and pulverized [9]. *Katha* was obtained from the heartwood of *A. catechu* by boiling the chips of heartwood with a 10% hydro-alcoholic solution. The steps involved in *Katha* processing are to be described in Fig. 1.

Standardization of *Katha*

The specimen was processed for pharmacognostic standardization through morphological studies, powder microscopy, percentage yield of *Katha* after extraction, fluorescence analysis, and loss on drying. The presence of active compounds was confirmed with the help of thin-layer chromatography (TLC) using desired mobile and stationary phases.

Quantitative phytochemical screening

Quantitative phytochemical screenings of *Katha* powder were performed as per standard protocols to detect the number of total phenols, total flavonoids, and total tannin content [10].

Determination of total phenolic content

Folin-Ciocalteu reagent was used to evaluate the total phenolic content of the extract using Gallic acid as standard [11]. The standard curve of Gallic acid was prepared by taking 500, 250, 125, 62.5, 31.25, and 15.625 µg/ml concentrations [12]. The procedure for determining the absorption of various concentrations is the same as follows for *Katha* powder. All the samples were subjected to a temperature of 60°C on

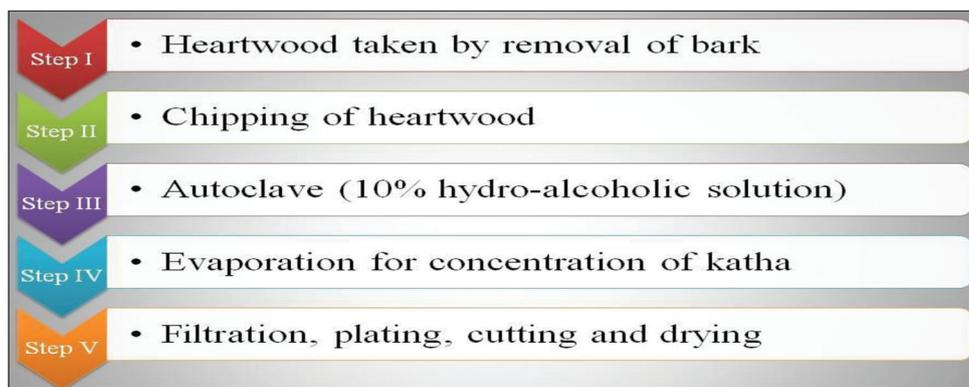


Fig. 1: Preparation of Katha

a water bath for 1 h, followed by cooling to room temperature. 400 μL of this solution was transferred into the test tube containing 1.6 mL of sodium carbonate (7.5% in deionized water) and 2 mL of Folin-Ciocalteu reagent (0.1% in deionized water). Further, all the samples were incubated for 1 h at room temperature. Absorbance was measured at 525 nm using UV-Spectrophotometer. All the readings were taken in triplicate. Total phenolic content was expressed in mg Gallic acid equivalent (GAE) per gram of *Katha* powder, using calibration curve [13].

Estimation of flavonoid content

The most commonly used method to determine total flavonoid contents by taking quercetin as standard [14]. Different concentrations of *Katha* powder and standard were prepared as above and 100, 50, 25, and 12.5 $\mu\text{g}/\text{mL}$ *Katha* powder, and the standard was added to the test tube containing 75 μL of 5% NaNO_2 solution [15]. The mixture was allowed to stand for 10 min. 150 μL of a 10% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ solution were then added to every sample and were allowed to stand for 5 min. Further 0.5 mL NaOH (1 M) and 2.5 mL of distilled water were added to each sample. Absorbance was measured at 510 nm using UV-Spectrophotometer. All the observations were taken in triplicate. Total flavonoid content was calculated as milligram quercetin equivalent (QE)/g using the linear regression equation obtained for quercetin [16].

Detection of tannin content

The total tannin content was determined following the International Standard method with slight alteration using tannic acid as standard [17]. Sample concentrations were taken at 2 mg/mL for each of the *Katha* powder. Initially, 200 μL of the sample were mixed with freshly prepared 200 μL of 0.35% ferric ammonium citrate, followed by 200 μL of 0.8% ammonia solution [18]. Finally, volume was made up to 4 mL by adding 3.4 mL of water. The absorbance of the mixture was measured at 525 nm. The results were expressed as milligram tannic acid equivalent (TAE)/g dry *Katha* powder [19].

In vitro cell lines study of Katha powder

MCF-7, A431, and HepG2 were obtained from the National Center for Cell Science, Pune, and maintained in DMEM supplemented with 10% FBS (Fetal Bovine Serum), 100 $\mu\text{g}/\text{mL}$ streptomycin, and 100 IU/mL penicillin [20]. The cell was inoculated at 37°C in an atmosphere of 5% CO_2 . Human lymphocytes were isolated from peripheral blood as described by cell where suspended in complete RPMI 1640 medium supplemented with 10% FBS, 5 g/mL phytohemagglutinin, and maintained at 37°C in a 5% CO_2 humidified incubator [21]. Stock solutions of extract /compound were prepared in dimethyl sulfoxide (DMSO) and solutions of different concentrations were obtained by diluting with culture medium. The final concentration of DMSO was less than 0.4% in all experiments. For 96 well plates, cells were seeded at approximately 7.5×10^3 – 1.5×10^4 cells per well, and for 24 well plates and seeding density were approximately 1.5×10^4 – 5×10^4 cells per well depending on the doubling time of individual cell lines [22].

Technique used for cell line assay

For the assay study, the two techniques are to be namely used is trypan blue dye exclusion assay and MTT assay technique [23]. The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that lives cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium, whereas dead cells do not trypan blue that is oxidized by living cells to a colorless product and dead cells remain blue colored [24]. Cells were seeded in a 24-well plate and then treated with various concentrations of test samples. The plate was incubated at 37°C and the number of culture cells in the different wells was counted using a hemocytometer after staining with 0.4% trypan blue after 48 h to calculate the percentage viability [25]. Cells were examined and counted under a light microscope at 10 x (Olympus, Japan) [26].

$$\% \text{ Cell viability} = \frac{\text{Number of viable cells (unstained cells)}}{\text{Total number of cells (stained and unstained)}} \times 100 \quad (1)$$

MTT assay is based on the metabolic reduction of MTT by the mitochondrial succinate dehydrogenase of intact cells to purple formazan product. MTT is a mono tetrazolium salt, the reduction of which is one of the most frequently used methods for measuring cell proliferation and cytotoxicity [27]. Cells were incubated in 96-well microtiter plates for 48 h. Following the addition of the test samples, the plates were incubated for an additional 48 h. Controlled well contained medium alone. Three triplicate wells were used in each point in the experiments after 48 h of incubation at 37°C, 50 μL MTT solution (5 mg/mL in phosphate-buffered saline) was added, and incubated for another 4 h at 37°C in a 5% CO_2 incubator. The resulting MTT – Formazan product was dissolved by 100 μL of isopropanol and the plates were gently shaken to solubilize the formed formazan. The amount of formazan was determined by measuring the absorbance (OD) at 570 nm using a Bio-Rad 550 enzymes-linked immunosorbent assay microplate reader [28].

$$\% \text{ Cell viability} = \frac{\text{Number of viable cells (unstained cells)}}{\text{Total number of cells (stained and unstained)}} \times 100 \quad (2)$$

Morphological changes of cells

The analysis of cell morphological changes was evaluated for 48 h in the absence or presence of test samples in 24-well plates. At the end of the incubation period, cells were observed under phase contrast inverted microscopes at $\times 200$ [29].

Hoechst assay

Differential staining with specific fluorochromes can be used to distinguished cells undergoing apoptosis from viable and necrotic cells. The analysis of cell morphology was evaluated Hoechst fluorescence staining [30]. The cells were cultured in 24-well plates to 85% confluence and then treated with the test sample for 48 h,

as described previously. Next, the cell was washed in PBS and fixed with 4% formaldehyde in PBS for 15 min and stained with 10 µg/ml Hoechst 33258 (HO; Sigma, St. Louis, MO, USA) at room temperature for 10 min in PBS. The apoptosis was classified by morphology and color of the cells and qualified. Finally, after the cells were washed with PBS, morphological changes were observed under the fluorescence microscopes [31].

Acridine orange/ethidium bromide (AO/EB) fluorescences staining

The analysis of changes in cell morphology was evaluated using AO/EB fluorescence staining [32]. Briefly, to a suspension of test sample-treated cells were added 4 µl/ml of dye mixture, containing 100 µg/ml acridine orange (Sigma, USA), and 100 µg/ml ethidium visualized immediately under fluorescence microscopes [33].

RESULTS

Pharmacognostic data

Katha occurs in pieces of variable sizes of 4–4.5 cm in length and 3.5–4.5 cm in breadth, yellowish-brown in color, the fracture is hard, characteristic odor with an astringent taste. Powder microscopy of *Katha* revealed the presence of acicular type crystals, fibers, and pitted vessels, as shown in Fig. 2. Total ash and acid insoluble ash value was found to be 11.5% w/w, 0.68% w/w which confirms the presence of inorganic content in the drug. The moisture content of the drug was found to be between 9 and 13% w/w. On exposure to UV light, *Katha* showed dark brown fluorescence.

Percentage yield of *Katha* after extraction

The data about *Katha* content from the heartwood of *A. catechu* are presented in Table 1 showing with maximum *Katha* content of 14.64% w/w was obtained and minimum *Katha* content is 7.95% w/w, respectively.

Phytochemical studies

Preliminary phytochemical screening of *Katha* showed the presence of tannins, flavonoids, saponins, and triterpenes, as shown in Table 2, but the main allure of screening was the presence of polyphenols (tannins and flavonoids) in ethyl acetate extract. The melting point of the extract was found to be between 150 and 210°C and in UV light extract showed magenta color fluorescence.

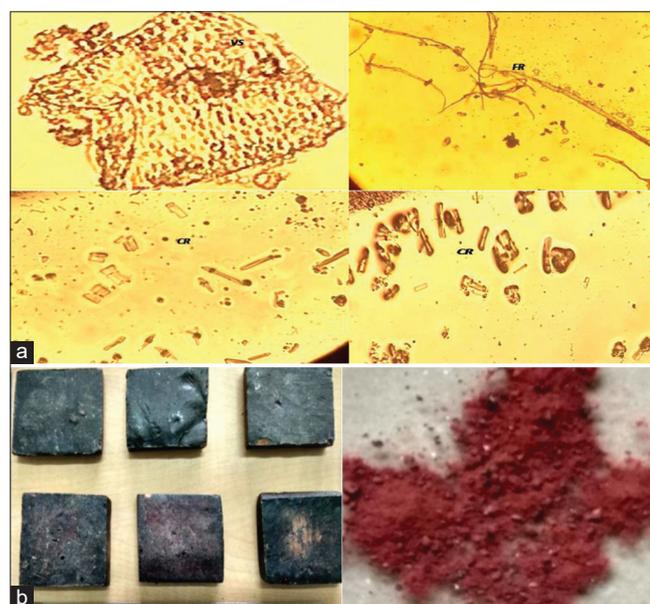


Fig. 2: (a) Microscopic characteristics of *Katha*. (b). Description of *Katha* and its powder. VS: Vessels, CR: Crystals, FR: Fibers

TLC profile

TLC of *Katha* powder on pre-coated TLC plates Merck GF-254 using chloroform:methanol:water (8:2:0.5) as mobile phase showed three different spots at Rf 0.2, 0.4, and 0.5 after detection with vanillin sulfuric acid reagent and in UV-366 nm, as shown in Fig. 3 which were later identified as epicatechin/catechin, epigallocatechin, and quercetin, respectively.

Quantitative phytochemical screening

The standard curve of Gallic acid obtained at different concentration (µg/ml) 500, 250, 125, 62.5, 31.25, and 15.625, respectively. The total phenolic content of the *Katha* powder was calculated in terms of mg GAE per gram of *Katha* powder. Total phenolic contents were calculated using linear regression equation $y=0.003X+0.033$ at $R^2=0.998$. Total phenolic content in *Katha* powder was calculated to be 96.4 mg GAE/Gram of *Katha* powder, respectively. In another side estimation of total flavonoids content, the standard curve of quercetin was plotted at different concentrations (µg/ml) 100, 50, 25, 12.5, and 6.75, respectively. Linear regression was applied to the obtained curve. Total flavonoid content was then calculated from equation $y=0.009x-0.014$ at $R^2=0.999$ in terms of mg QE/g of *Katha* powder. Total flavonoid content in *Katha* powder was calculated to be 46.43 mg QE/g of *Katha* powder. The standard curve of tannic acid obtained at different concentration (µg/ml) 500, 250, 125, 62.5, 31.25, and 15.625, respectively. The total tannin content of the extract fractions was calculated in terms of mg GAE per gram of *Katha* powder. Total tannin contents were calculated using linear regression equation $y=0.016x+0.004$ $R^2=0.999$. The total tannin content in *Katha* powder was calculated to be tannins 96.4 mg TAE/g dry extract of extract fractions, respectively. It is to be represented in Fig. 4.

In vitro cytotoxicity testing of *Katha* powder

In vitro proliferation study

The cytotoxicity effect of *Katha* powder on MCF-7, A431, and HepG2 cells was characterized by the trypan blue dye exclusion method and colorimetric assays using MTT. Control assay was carried out for samples containing only the appropriate volumes of blank solutions and showed no effect on cell growth. Different cells were exposed to *Katha* powder for about 48 h and cytotoxicity assays. The percentage cell viability of different *Katha* extracts on MCF-7, A431, and HepG2 cells was evaluated by the trypan blue dye exclusion method. The

Table 1: The percent yield of *Katha*

Trial	<i>Katha</i> extract	% yield w/w	Average value w/w
Trial I	10% hydro-alcoholic hot distilled water	9.12 %	11.87%
Trial II	10% hydro-alcoholic hot distilled water	11.95 %	
Trial III	10% hydro-alcoholic hot distilled water	14.54 %	

Table 2: Preliminary phytochemical screening of *Katha* powder

S. No.	Metabolite	Test	<i>Katha</i> powder	Inference
1.	Tannins	Gelatin	+++	Present
2.	Flavonoids	Lead acetate, Alkaline reagent	+	Present
3.	Triterpenes	Liebermann-Burchard	+	Present
4.	Glycosides	Modified borntrager's	+	Present
5.	Saponins	Froth formation	++	Present
6.	Steroids	Salkowski	-	Absent
7.	Alkaloids	Mayers and Wagner reagent	-	Absent

+++ Active constituent is highly present, ++ Active constituent is moderately present, + Active constituent is slightly present, - Active constituent absent

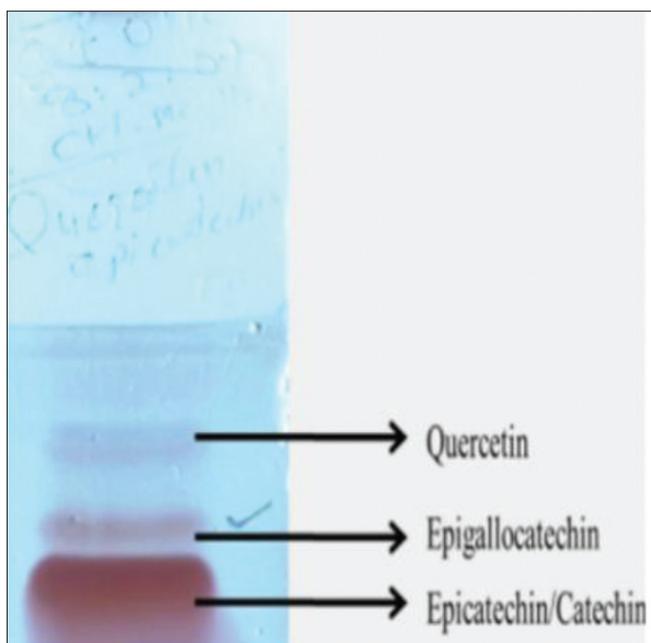


Fig. 3: TLC analysis of Katha powder

results of the trypan blue assay are presented in Table 4, respectively. The effect of Katha powder against these cell lines concentration range 10–100 µg/ml showed a decrease in percent cell viability in a dose-dependent manner, as compared with that of the control when examined by the trypan blue exclusion assay (Fig. 5).

It was found that the percentage growth inhibition increased with increasing concentrations of Katha powder, steadily in the case of the Katha powder against MCF-7, A431, and HepG2 cells. Katha powder showed a good linearity range against MCF-7, A431, and HepG2 cells. Overall, the Katha powder shows the greatest activity against these cell lines.

Effect of Katha powder on human peripheral lymphocytes

The cytotoxic effect of Katha powder on human peripheral lymphocytes was characterized by MTT assay. When the different concentrations of Katha powder (10–100 µg/ml) were added to normal lymphocytes, the Katha powder did not show any toxic effect on lymphocytes. The cell number and cell survival both increased, as the concentration of the Katha powder increased (Fig. 6).

Effect of Katha powder on the morphology of the cells

To investigate the effect of Katha powder on the morphology of the apoptotic cells, Hoechst 33258 (HO) and AO/EB staining were used. Cells stained with HO after treatment with different concentrations

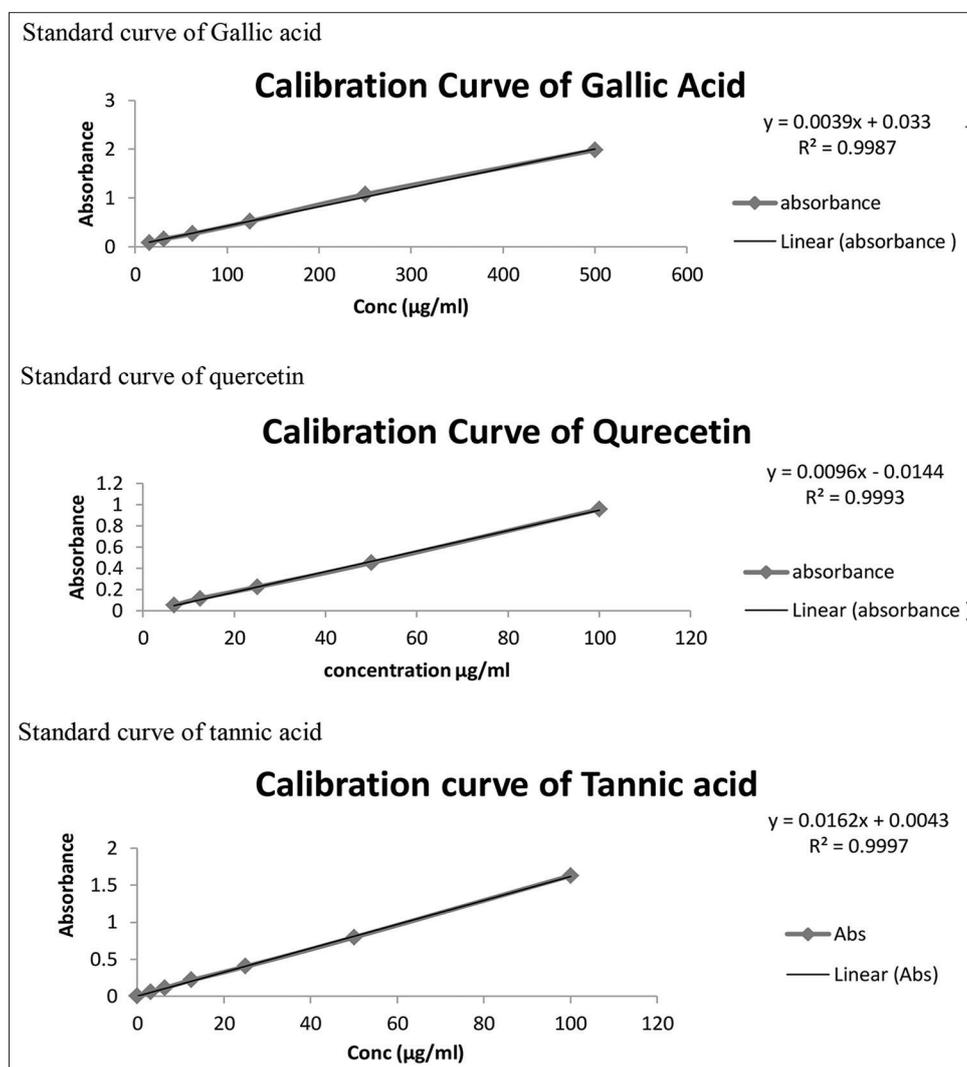


Fig. 4: Standard calibration curve of Gallic acid, quercetin, and tannic acid

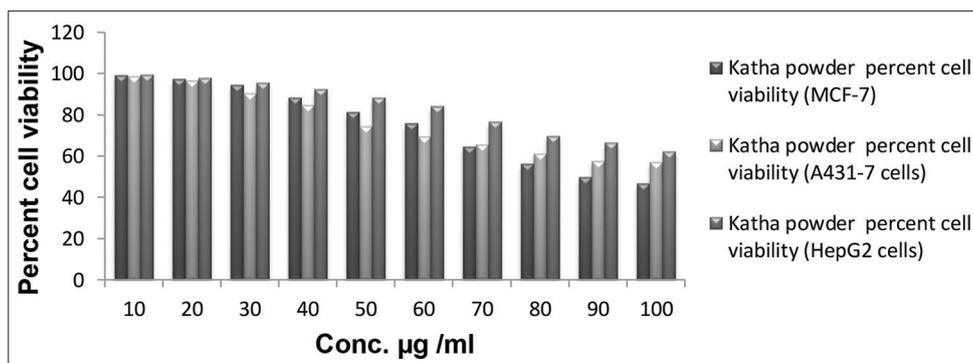


Fig. 5: Percent cells viability of Katha powder (MCF-7, A431-7, and HepG2 cells) using trypan blue exclusion assay

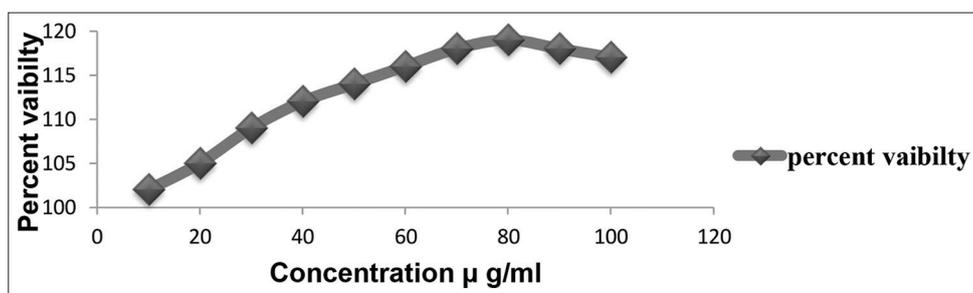


Fig. 6: Effect of Katha powder on the percentage viability of normal lymphocytes as measured by MTT assay

Table 3: Effect of Katha powder on percent cell viability (MCF-7, A431, and HepG2 cells) using trypan blue exclusion assay

Concentration (µg/ml)	Katha powder percent cell viability (MCF-7)	Katha powder percent cell viability (A431-7 cells)	Katha powder percent cell viability (HepG2 cells)
10	99.12	98.73	99.43
20	97.42	96.56	97.83
30	94.57	90.56	95.68
40	88.45	84.75	92.56
50	81.37	74.43	88.36
60	75.89	69.56	84.21
70	64.67	65.72	76.64
80	56.25	61.17	69.73
90	49.78	57.68	66.59
100	46.92	57.21	62.38

Table 4: Effect of Katha powder on human peripheral lymphocytes

S. No.	Concentration in µg/ml	Percent viability			
		Trial 1	Trial 2	Trial 3	Average trial value
1.	10	102	102	102	102
2.	20	105	105	105	105
3.	30	109	109	109	109
4.	40	112	112	112	112
5.	50	114	114	114	114
6.	60	116	116	116	116
7.	70	118	118	118	118
8.	80	119	119	119	119
9.	90	118	118	118	118
10.	10	117	117	117	117

(50 and 100 µg/ml) of Katha powder for 48 h showed marked morphological changes indicative of cell apoptosis were observed, including chromatin condensation and fragmentation in MCF-7 Fig. 7a-f, A431 (Fig. 8a-f), and HepG2 cells (Fig. 9a-f).

As shown in Fig. 7a-f, MCF-7 cells treated with Katha powder exhibited characteristics changes of the apoptosis, for example, cell shrinkage, nuclear condensation, and fragmentation and formation of apoptotic bodies. Cells treated with 50 µg/ml of Katha powder showed the sign of every apoptosis (Fig. 7c and d).

A431 cells treated with Katha powder show characteristics changes of apoptosis, for example, cell shrinkage, nuclear condensation and fragmentation, and formation of apoptotic bodies (Fig. 8).

Cells treated with 50 and 100 µg/ml of Katha powder showed similar features of apoptosis, that is, early apoptosis and chromatin condensation (Fig. 8c and d). It is shown in Fig. 8e and f, the apoptosis nuclei clearly and highly condensed chromatin that was uniformly fluorescent.

The nucleus was shown as bright green and the cell profiles were dark green which suggested that the treatment of HepG2 cells with the Katha powder induce apoptosis. Cells treated with 50 µg/ml of Katha powder shown the sign of early apoptosis (Fig. 9c and d) and, on the other hand, cells treated with 100 µg/ml of the Katha powder similar features of late apoptotic activity with apoptotic bodies formation (Fig. 9e and f).

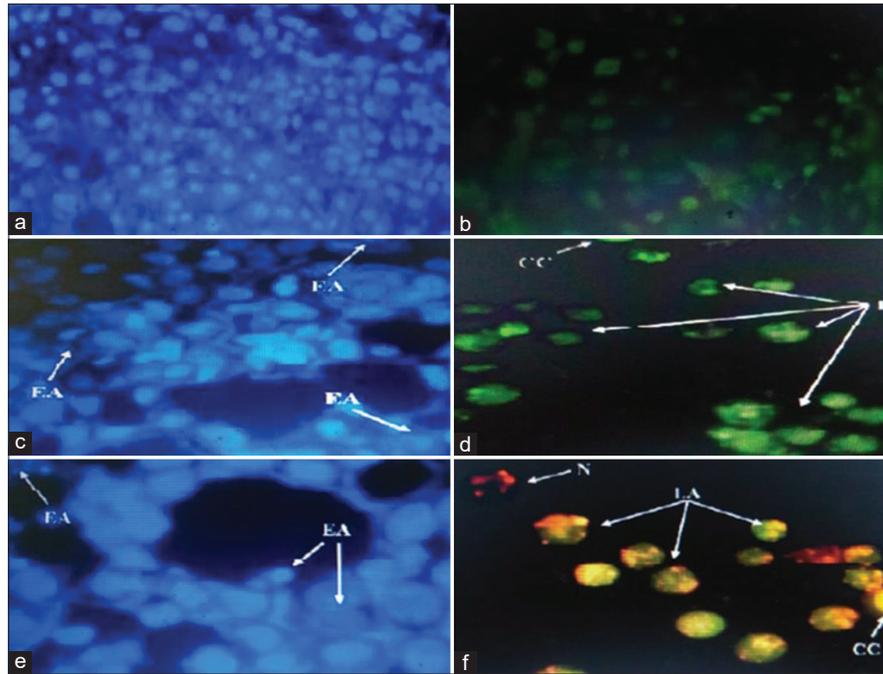


Fig. 7: (a-f) apoptosis assay by Hoechst and acridine orange/ ethidium bromide (MCF-7 cells). (a and b) MCF-7 control cells, (c and d) MCF-7 cells treated with 50 µg/ml of *Katha* powder. (e and f) MCF-7 cells treated with 100 µg/ml of *Katha* powder. Early apoptosis (EA) cells with apoptotic nuclei stained by Hoechst with fluorescence in blue spectrum (arrow); chromatin condensation (CC); late apoptosis (LA); necrosis (N) stained acridine orange and ethidium bromide (AO/EB) shown fluorescence in green and yellow/orange spectrum (arrow). On the other hand, cells treated with 100 µg/ml of *Katha* powder showed a similar feature but with extra features of late apoptotic activity with the formation of the apoptotic bodies (Fig. 7e and f)

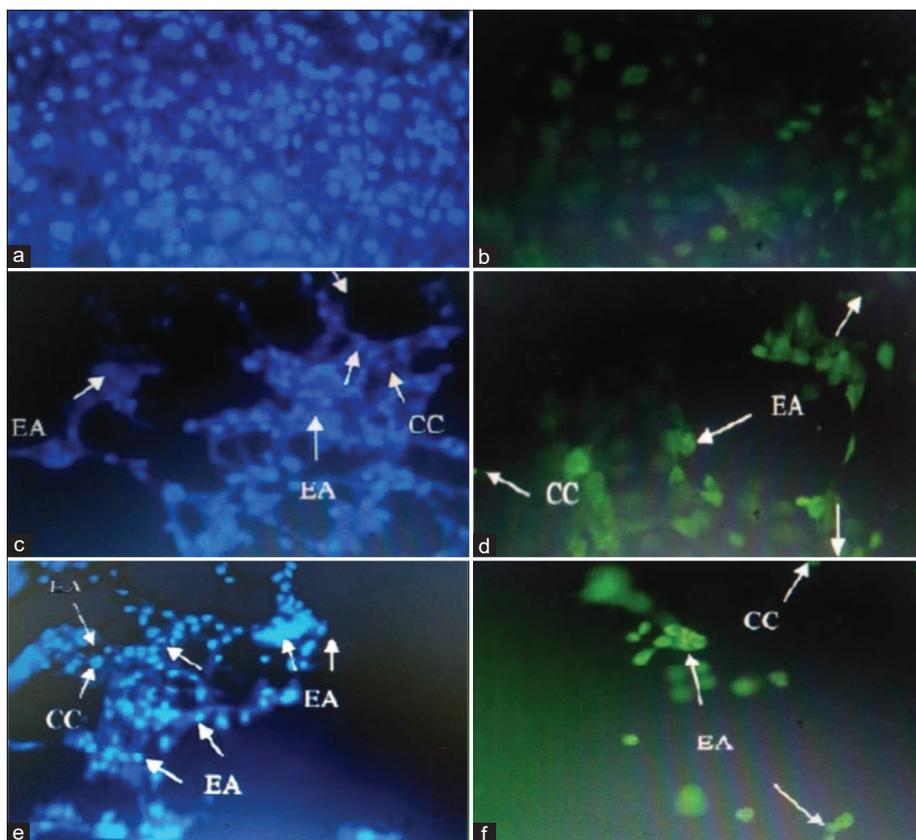


Fig. 8: (a-f) Apoptosis assay by Hoechst and acridine orange/ethidium (AO/EB) bromide (A431 cells). (a and b) A431 control cells (c and d) A431 cells treated with 50 µg/ml of *Katha* powder (e and f) A431 cells treated with 100 µg/ml of *Katha* powder. Early apoptosis (EA); chromatin condensation (CC) cells with apoptotic nuclei stained by Hoechst with fluorescence in blue spectrum (arrow) and cells stained with AO/EB shown fluorescence in the green spectrum (arrow)

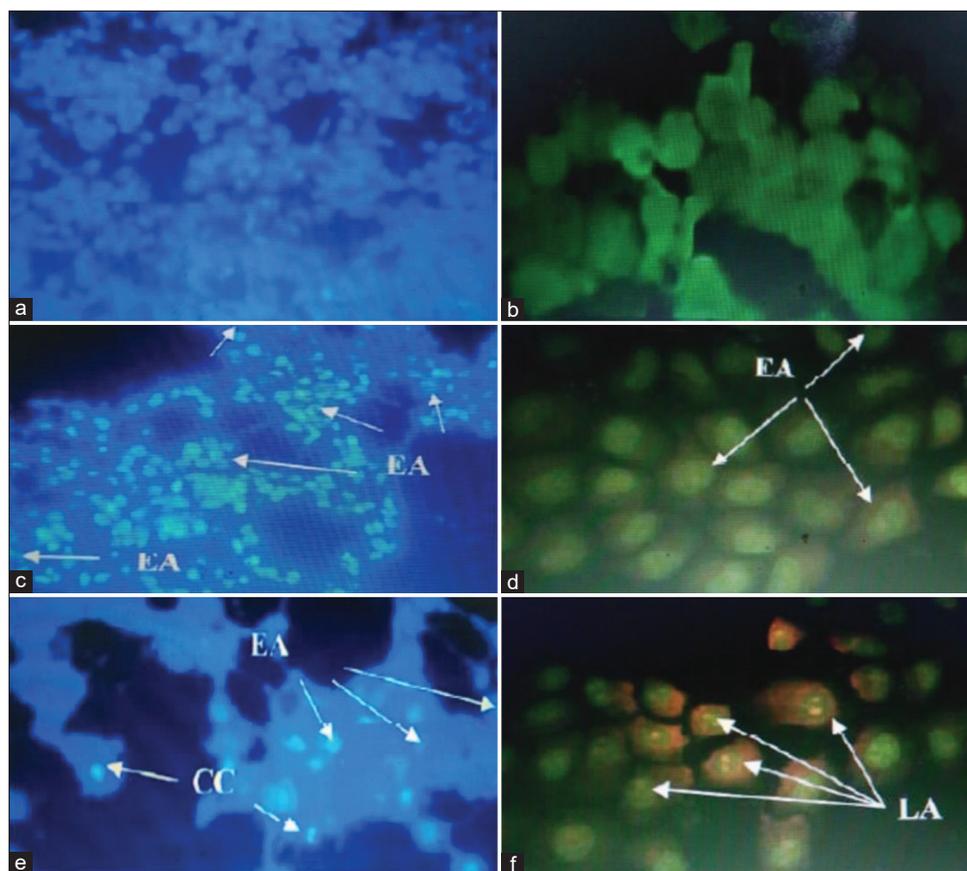


Fig. 9: (a-f) Apoptosis assay by Hoechst and acridine orange/ethidium bromide (HepG2 cells). (a and b) HepG2 control cells. (c and d) HepG2 cells treated with 50 µg/ml of *Katha* powder (e and f) HepG2 cells treated with 100 µg/ml of the *Katha* powder. Early-stage apoptosis (EA); chromatin condensation (CC) cells with apoptotic nuclei stained by Hoechst with fluorescence in blue spectrum (arrow); late apoptosis (LA) stained acridine orange and ethidium bromide (AO/EB) shown fluorescence in green and orange spectrum (arrow).

DISCUSSION

Katha obtained by boiling the heartwood of *A. catechu* with a 10% hydro-alcoholic solution increased the percentage yield value by 10% w/w. In chromatographic studies, four active substances were identified based on their respective Rf values it shows the presence of catechin/epicatechin, epigallocatechin, and quercetin, etc., the cytotoxic effect of the *Katha* powder on MCF-7, A431, and HepG2 cells was characterized by trypan blue dye exclusion method and MTT colorimetric assay. Overall, the *Katha* powder showed the greatest activity against these cancer cell lines and was used to carry further study on human peripheral lymphocytes. For this study, the human peripheral lymphocytes were isolated and the effect of *Katha* powder on the percent viability of normal lymphocytes was calculated. The result showed the *Katha* powder did not show any toxic effect to lymphocytes which concluded that the *Katha* powder has no toxicity to normal cells. So as for as morphological alteration concerned the MCF-7, A431, and HepG2 cell following the treatment with *Katha* powder indicative of cell apoptosis. Besides, marked morphological changes that occurred in the apoptotic cell were also perceived through Hoechst 33258 (HO) and AO/EB staining, and this help in deducing that the cell death observed was not due to necrosis, but due to apoptosis. It was possible to observe a better difference between early and late apoptosis, showing that the *Katha* powder exhibited a longer concentration-response effect for late apoptosis and a smaller concentration effect for early apoptosis, in both of the studied concentration.

CONCLUSION

Katha powder was found rich in the polyphenolic compound, tannin, and flavonoid content. These active compounds potentiate the market

value of *Katha* powder for therapy. Further, if we concern with *in vitro* proliferation of *Katha* powder against these cell lines concentration range 10–100 µg/ml showed a decrease in percent cell viability in a dose-dependent manner, as compared with that of the control when examined by the trypan blue exclusion assay. Overall, the *Katha* powder showed the greatest activity against these cancer cell lines and was used to carry further study on human peripheral lymphocytes. For this study, the human peripheral lymphocytes were isolated and the effect of *Katha* powder on the percent viability of normal lymphocytes was calculated. The result showed that the *Katha* powder did not show any toxic effect to lymphocytes which concluded that the *Katha* powder has no toxicity to normal cells.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest, financial, or otherwise.

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