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Research Article

Investigation on Photodecomposition of Standardised Ethyl Acetate Fraction of *Katha*

Pankaj Sharma*

ABSTRACT

Objective: The present study delves into photodecomposition of a standardised ethyl acetate fraction of *Katha* and manifest how the antioxidant potential degrades exponentially with time. **Methods:** Coarsely dried heart wood of *Acacia catechu* (L.f.) Willd. was treated with 10 % hydro-alcoholic solution to obtain *Katha* as final product. The powdered *Katha* was standardised *via* pharmacognostic parameters and further subjected to soxhlet extraction using ether, ethyl acetate and ethanol to obtain different extracts/fractions. Phytochemical investigations were carried out to screen polyphenols (tannins) of interest which later were confirmed by thin layer chromatography (TLC). Photodecomposition of ethyl acetate fraction with respect to its *in-vitro* antioxidant potential using DPPH and nitric oxide scavenging assay was determined at 0 h, 4 h, 8 h, 12 h, 16 h, 20 h and 24 h of 250, 500, 750 and 1000 µg/ml concentrations respectively. Ethyl acetate fraction conceded the highest amount of polyphenols. **Results:** The extract was found to be unstable at room temperature and displayed a quick change in colour from light orange to deep dark orange within few hours, which also abide by its decreasing antioxidant activity exponentially with time. Antioxidant potential was observed in the following order in dose dependant manner: 0 h > 4 h > 8 h > 12 h > 16 h > 20 h > 24 h. C**onclusion**: Moreover, published literature should have not mentioned the age of extract they have used for their study, which may explain the wide differences in the observations reported with this plant.

Key words: *Acacia catechu*, DPPH, Antioxidant, Nitric oxide scavenging, Free radical, *Katha*.

Pankaj Sharma*

Department of Pharmacy, Institute of Pharmacy and Research, S.J.J.T.U, Vidyanagari, Jhunjuhunu, Rajasthan, INDIA.

Correspondence

Mr. Pankaj Sharma

Research Scholar, Department of Pharmacy, Institute of Pharmacy and Research, S.J.J.T University, Jhunjhunu, Rajasthan - 333001, INDIA.

Phone no: +918626976382

E mail: pankajsharmadrugs@gmail.com

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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.[1] *Acacia catechu* (L.f.) Willd. known by the name '*khadira*' in Ayurveda belongs to Leguminosae family.[2] The plant is a small moderate sized tree about 10-13 meter height extensively scattered throughout India.[3] The medicinal significance of this plant is reflected by the use of leaves, bark and heartwood in several Ayurvedic formulations for thousand years.[4] Heartwood of *A. catechu* has more potent medicinal activity in comparison to its leaves and bark.[5] The heartwood extract of *A. catechu* has been reported to have various pharmacological effects like antioxidant,[6] anti-inflammatory,[7] antimicrobial,[8] immunomodulatory,[9] antipyretic, hypoglycaemic,[10] anti-diarrhoeal and hepatoprotective[11] etc. The plant contains polyphenols like tannins, flavonoids alongwith carbohydrates and proteins.[12] The major constituents of the plant includes catechin (-), epicatechin, epigallocatechin, epicatechingallate and epigallocatechin gallate. Other constituents include rocatechin, phloroglucin, protocatechuic acid, quercetin, poriferasterol glucosides, lupenone, lupeol, procyanidin AC, kaempferol, dihydrokaemferol, toxifolin, (+)-afzelchin gum and minerals.[13] The heartwood of this plant is known by the name '*Katha*' which is well known for its diverse pharmacological properties.[14,15] In the present study the stability of standardised ethyl acetate extract and changes in antioxidant potential was investigated through *in-vitro* studies as illustrated in Figure 1. Storage conditions were already reported to exert marked influence over chemical composition of crude drugs.[16,17] The aim of this study was to determine antioxidant capacity of ethyl acetate fraction of *Katha* obtained from the heart wood of plant *A. catechu* and to access its photodecomposition based on temperature and storage conditions.

MATERIALS AND METHODS

Plant material

The heartwood of the plant was collected in the month of November 2019 from Hamirpur 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 plant extract (Katha)

The heartwood of *A. catechu* was dried at room temperature (25 ± 2°C) for four consecutive weeks and pulverised. *Katha* was obtained from the heartwood of *A. catechu* by boiling the chips of heartwood with 10% hydro-alcoholic solution.[18]

Standardisation of katha

The specimen was processed for pharmacognostic standardisation *viz* morphological studies, powder

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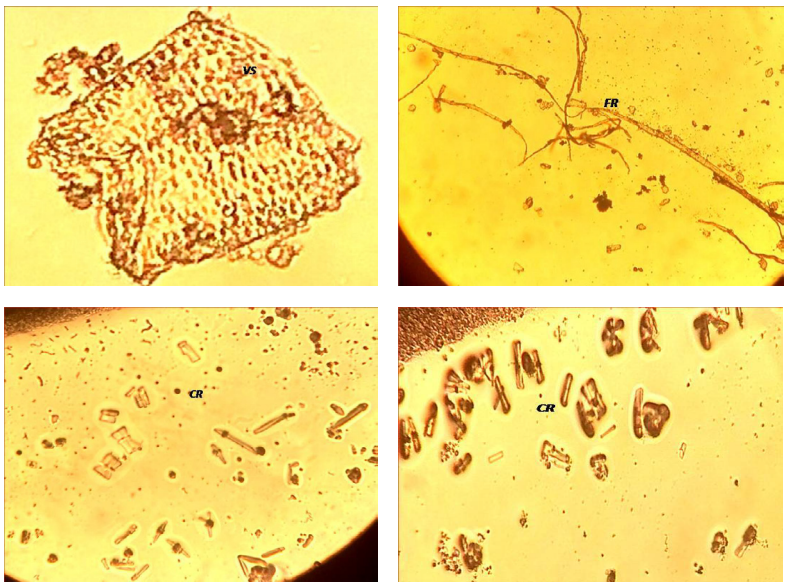

Figure 1: Microscopic characteristics of *Katha*.VS: vessels, CR: crystals, FR: fibres.

microscopy, ash value determination, florescence analysis and loss on drying. Each extract was then screened for secondary metabolites. Presence of active antioxidant compound was confirmed with the help of thin layer chromatography by using desired mobile and stationary phases. These parameters are considered essential in quality control of the crude drugs.[19]

Soxhlation of katha

Katha chips obtained from heartwood of *A. catechu* is powdered and extracted by successive solvent extraction technique using soxhlet apparatus. 100 g of coarsely powdered drug was allowed to get extracted with different solvents in order of increasing polarity *viz*. ether, ethyl acetate and ethanol.

Quantitative phytochemical screening

Quantitative phytochemical screenings of ethyl acetate fraction of Katha were performed as per standard protocols to detect the amount of total phenols and total flavonoids in ethyl acetate fraction.

Determination of total phenolic content

Folin-Ciocalteu reagent was used to evaluate the total phenolic content of the extract using gallic acid as standard.[20] Standard curve of Gallic acid was prepared by taking 500, 250, 125, 62.5, 31.25 and 15.625 µg/ml concentrations. Procedure for determining absorption of various concentrations is same as follows for extract fractions. All the samples were subjected to temperature of 60°C on water bath for 1h 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 525nm using UV Spectrophotometer. All the readings were taken in triplicate. Total phenolic content was expressed in mg Gallic acid equivalent (GAE) per gram of extracts, using calibration curve.

Estimation of flavonoid content

Most commonly used method to determine total flavonoids contents by taking Quercetin as standard.[21] Different concentration of extract and standard was prepared as above and 100, 50, 25, 12.5 µg/ml extract and standard was added to the test tube containing 75 µL of 5% NaNO2 solution. Mixture was allowed to stand for 10 minute. 150 µL of a 10% AlCl3.6H2O solution was then added to every sample and were allowed to stand for 5 minutes. Further 0.5 mL NaOH (1 M) and 2.5 mL of distilled water was 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 mg Querctine equivalent (QE)/g by using the linear regression equation obtained for Quercetin.

Antioxidant activity

In the present study, two commonly used antioxidant evaluation methods such as DPPH radical scavenging activity and Nitric oxide radical scavenging assay were selected to determine the antioxidant potential of ethyl acetate fraction of *Katha*.

Determination of Free Radical Scavenging Activity by DPPH Method

DPPH radical scavenging activity of ethyl acetate fraction of *Katha* was determined according to the standard method with slight alteration. The reaction mixture containing 500, 1000 µg/ml of extract concentration and 2 ml of DPPH (0.1 Mm in methanol) was allowed to stand for 15 minutes in dark at room temperature. Absorbance was measured using double beam UV-VIS spectrophotometer (shimadzu-1601) and tested against the blank. The scavenging potential was calculated by using the following equation.[22]

$$\%\ Inhibition = \frac{B^{o} - B^{1}}{B^{o}} X100$$

Where B^{o} is the absorbance of negative control

B^{1} is the absorbance of the reaction mixture

Determination of Free Radical Scavenging Activity by Nitric Oxide Radical Method

Griess reagent was prepared by mixing same amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid. Ethyl acetate extract

was added with similar volume of freshly prepared sample. Nitric oxide radical so generated was found to be inhibited by ethyl acetate extract at different concentrations. Decolouration due to reaction by polyphenols in ethyl acetate fraction with the nitric oxide free radical was then measured spectrophotometrically. The percentage inhibition of ethyl acetate extract and standard was recorded. All the experiments related to antioxidant activity were performed in triplicate.[23]

RESULTS

Pharmacognostic data

Katha occurs in pieces of variable sizes of 4 to 4.5 cm in length and 3.5 to 4.5 cm in breath, yellowish brown in colour, fracture is hard, characteristic odour with astringent taste. Powder microscopy of *Katha* revealed the presence of acicular type crystals, fibers and pitted vessels as shown in Figure 1. Successive extractive values with ether, ethyl acetate and ethanol were found as 1.2 % w/w, 4.5% w/w and 3.5 % w/w respectively Table 1**.** 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. Moisture content of the drug was found to be between 9-13% w/w. On exposure to UV light *Katha* showed dark brown fluorescence.

Percentage yield of katha

Different extracts were obtained upon successively treated the sample (*Katha*) with different solvents as mentioned in Table 1.

Phytochemical Studies

Preliminary phytochemical screening of ether, ethyl acetate and ethanolic extract of *Katha* showed the presence of tannins, flavonoids, saponins and triterpenes as shown in Table 2 but the main allure of screening was presence of polyphenols (tannins, flavonoids) in ethyl acetate extract. Tannins reported in this plant have already been documented to exhibit pharmacological as well as physiological properties (6-11). Furthermore, ethyl acetate extract of *Katha* is having pink colour with no odour and obtained as fine powder as shown in Figure 2a. Ethyl acetate extract is highly soluble in methanol, ethyl acetate, slightly soluble in benzene, petroleum ether and insoluble in water. Melting point of the extract was found to be between 150-210°C and in UV light extract showed magenta colour fluorescence.

TLC profile

TLC of ethyl acetate extract on precoated TLC plates Merck GF-254 using Chloroform: Methanol: Water (8:2:0.5) as mobile phase showed four different spots at Rf 0.2, 0.4, 0.5, 0.6 after detection with vanillin sulphuric acid reagent and in UV-366 nm as shown in Figure 2b which were later identified as epicatechin/catechin, epigallocatechin, querecetin and procyanidin AC respectively.

Quantitative phytochemical screening

The standard curve of Gallic acid obtained at different concentration (µg/ml) 500, 250, 125, 62.5, 31.25 & 15.625 respectively. Total phenolic content of the extract fractions was calculated in terms of mg GAE per

Table 1: Extractive yield (%).

Extract	% Extractive value (w/w)
Katha (Sovent-10% hydro alcoholic solution)	11.87
Ether	1.2
Ethyl acetate	4.5
Ethanol	3.5

Table 2: Preliminary phytochemical screening of extract.

Metabolite	Test	EE	ETE	EHT
Tannins	Gelatin	+	+++	++
Flavonoids	Lead acetate, Alkaline reagent	++	+++	+
Triterpenes	Liebermann burchard	+	+	+
Glycosides	Modified borntagers	-	-	+
Saponins	Froth formation	+		++
Steroids	Salkowski	-	-	-
Alkaloids	Mayers and wagner reagent	-	-	-

+++ active constituent is highly present, ++ active constituent is moderately present, + active constituent is slightly present, - active constituent absent, EE: Ether extract, ETC: Ethyl acetate extract, EHT: Ethanol extract.

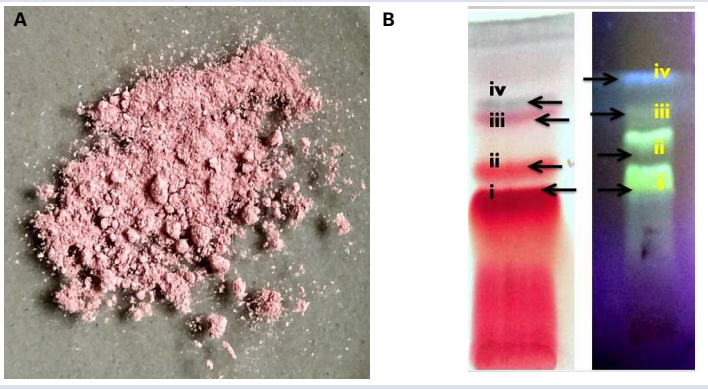

Figure 2: A. Ethyl acetate extract of *Katha*. **B**. TLC of ethyl acetate extract of *Katha*.

gram of extracts. Total phenolic contents were calculated using linear regression equation y=0.003X + 0.033 at R^2=0.998. Total phenolic content in Ethyl acetate extract were calculated to be 96.4 mg GAE/ gram of extract fractions respectively. In another side estimation of total flavonoids content, the standard curve of Querctine was plotted at different concentration (µg/ml) 100, 50, 25, 12.5 & 6.75 respectively. Linear regression was applied to obtained curve. Total flavonoid content were then calculated from equation y=0.009x -0.014 at R^2=0.999 in terms of mg QE/g of extract fraction. Total flavanoid content in Ethyl acetate extract were calculated to be 46.43 mg QE/g of extract fractions.

Determination of free radical scavenging activity by DPPH method

DPPH radical showed a strong absorption maximum at 517 nm (purple). In presence of antioxidant the colour reaction takes place. DPPH free radical scavenging activity of ethyl acetate extract of *Katha* at various concentrations 500 µg/ml and 1000 µg/ml was determined by taking sample with different storage conditions as shown in Figure 3 and Table 3. The first sample selected was kept at light under ordinary room temperature and the other sample was stored in dark under controlled environment.

Determination of free radical scavenging activity by nitric oxide radical method

Percentage free radical scavenging was plotted against concentration of the extracts as shown in Figure 4. The Nitric oxide free radical scavenging activity of ethyl acetate extract of *Katha* at various concentrations *viz* 500 µg/ml and 1000 µg/ml was determined by taking sample with different storage conditions Table 4. The first sample was kept at light under ordinary room temperature and the second sample was stored in dark under controlled conditions. It was observed that the sample placed in dark and in controlled conditions does not show any change in the scavenging potential whereas the sample placed in light, under ordinary temperature undergoes deterioration exponentially with time.

Table 3: Photo-decomposition of the ethyl acetate extract of *Katha* by DPPH.

Time (h)	% AA	% BB	% CC	% DD
0	11.56 %	11.56 %	21.68 %	21.67 %
4	11.16 %	11.53 %	19.93 %	21.66 %
8	10.64 %	11.57 %	18.02 %	21.65 %
12	10.01 %	11.54 %	16.35 %	21.68 %
16	9.36 %	11.56 %	15.98 %	21.64 %
20	8.96 %	11.58 %	14.69 %	21.66 %
24	7.74 %	11.59 %	12.92 %	21.65 %

% AA: 500 µg/ml in presence of light, % BB: 500 µg/ml in dark, % CC: 1000 µg/ml in presence of light, % DD: 1000 µg/ml in dark.

Table 4: Photo-decomposition of ethyl acetate extract of *Katha* by Nitric oxide scavenging method.

Time (h)	% AA	% BB	% CC	% DD
0	17.56 %	17.56 %	28.93 %	28.94 %
4	16.25 %	17.53 %	26.43 %	28.95 %
8	15.69 %	17.54 %	25.45 %	28.92 %
12	14.68 %	17.55 %	22.35 %	28.93 %
16	12.08 %	17.54 %	19.98 %	28.94 %
20	11.35 %	17.55 %	18.69 %	28.96 %
24	10.97 %	17.56 %	16.92 %	28.95 %

% AA: 500 µg/ml in presence of light, % BB: 500 µg/ml in dark, % CC: 1000 µg/ml in presence of light, % DD: 1000 µg/ml in dark.

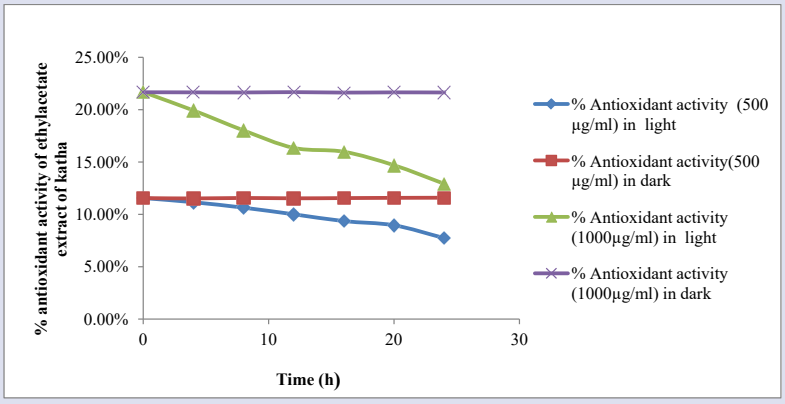

Figure 3: Percentage deterioration in antioxidant activity in DPPH free radical scavenging assay.

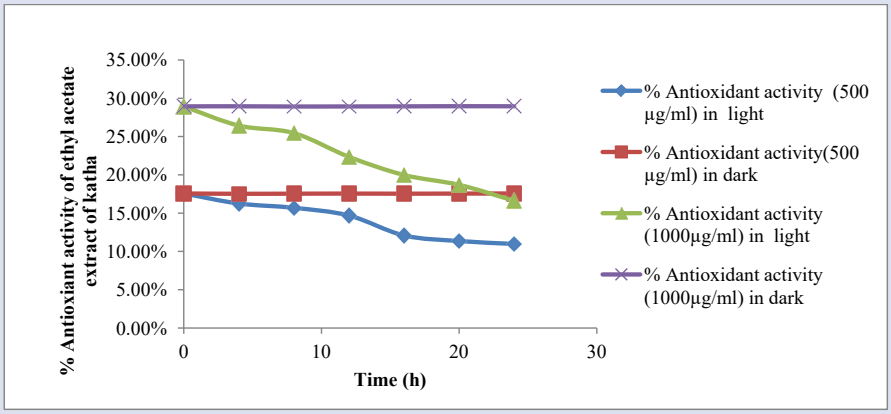

Figure 4: Percentage deterioration in antioxidant activity in nitric oxide scavenging assay.

DISCUSSION

Katha obtained by boiling the heartwood of *A. catechu* with 10 % hydro-alcoholic solution resulted in increase in the percentage yield value by 10 % w/w. In chromatographic studies four active substances were identified on the basis of their respective Rf values. But the major concern of this study was to determine the photo-decomposition of ethyl acetate extract at different storage conditions. The extract is highly unstable in its solution form and its antioxidant potential degrades exponentially with time. For evaluating its antioxidant potential two methods were used (DPPH free radical scavenging and nitric oxide free radical scavenging). Results showed that antioxidant potential degrades in dose dependant manner: 0 h > 4 h > 8 h > 12 h > 16 h > 20 h > 24 h, respectively. As the duration of storage of extract at room temperature under ordinary conditions increased, the antioxidant potential showed a marked decline. It is pertinent here to mention that the sample (ethyl acetate extract) placed in dark under controlled conditions does not show any change in the scavenging potential whereas the sample placed in light, under ordinary temperature conditions undergoes deterioration augmented with time.

CONCLUSION

Standardised ethyl acetate extract of *Katha* was found to be highly unstable in its solution form and showed exponential decline in antioxidant potential with time. Therefore, for the experimental purpose, it should be preserved properly to avoid photodecomposition and to alleviate chances of deviation. The overall outcome of this study can be considered as very promising in establishing stability profile of plant extract with respect to storage conditions and time. Hence, from this study it might be postulated that plant extracts containing polyphenols are highly sensitive and undergoes photo-degradation exponentially with time so special care should be given while handling these plant extracts which otherwise might causes deviation in the therapeutic profile of the drugs like in our case antioxidant activity.

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ETHICS APPROVAL AND CONCENT TO PARTICIPATE

No applicable.

HUMAN AND ANIMAL RIGHTS

No animals/Humans were used for studies that are base of this research.

CONCENT FOR PUBLICATION

Not applicable.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest, financial or otherwise.

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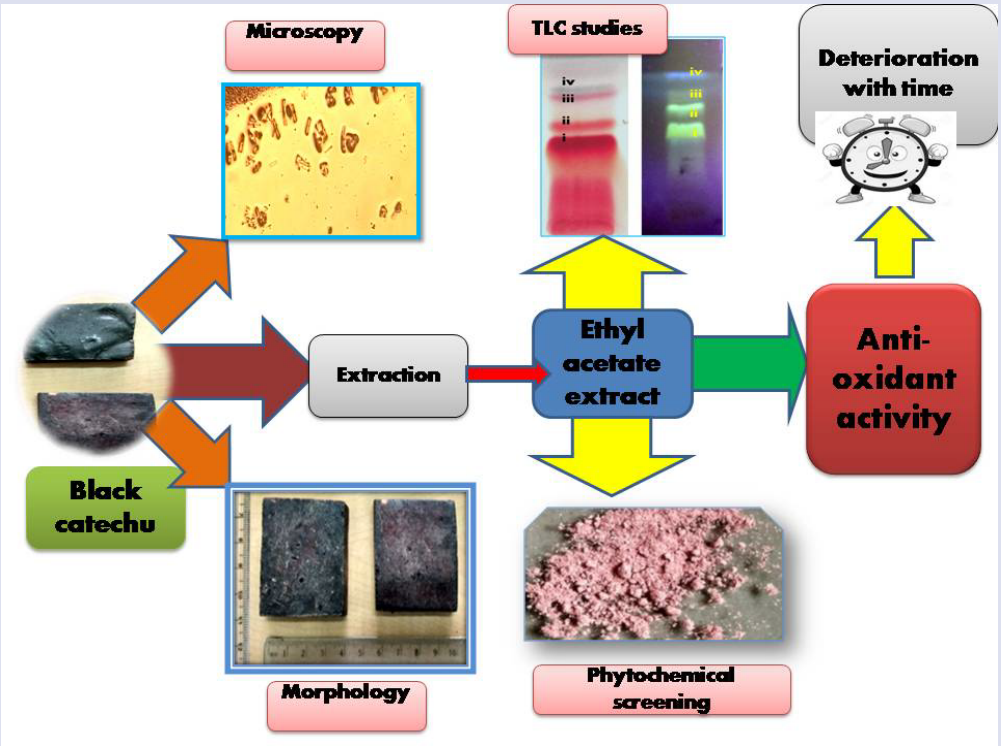
GRAPHICAL ABSTRACT



ABOUT AUTHORS

Mr. Pankaj Sharma is a PhD Research Scholar in SJJT University, Rajasthan (India). His current area of Research is design and development of Phytosomes of herbal drug containing polyphenolic & flavonoids.

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