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PLANT SYSTEM AS A TOOL FOR VALIDATING ETHNOBOTANICAL CLAIMS FOR KIDNEY STONE TREATMENT

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ABSTRACT

A renal calculus or kidney stone is one of the most prevalent and widespread conditions in the world, without a guaranteed cure. None of the known and available treatments prevent the recurrence of kidney stone formation. Hence, new and improved treatment methods are constantly being developed. This study claims to use plant systems as tools to provide a scientific basis for ethnobotanical treatments for kidney stones using *Ficus elastica* cystolith and *Colocasia esculenta* raphides as targets and *Tectona grandis* fruit and *Bryophyllum pinnata* leaf extracts as treatments. In addition, the phytochemical analysis of these extracts is also proposed. Observation was performed by photomicrograph of cell cystolith and raphides before and after treatment. The method was specifically used to study dissolution of calcium oxalate crystals and can provide a potential alternative to animal testing. This article emphasizes a method to validate the efficacy of ethnobotanical herbal remedies which show *in vitro* anti-urolithiatic activity. They can be further taken up for *in vivo* studies by treating plant cells containing calcium oxalate crystals as a model to study the effect of plant extracts. An ethical consideration on this alternative method offers a more humane approach to *in vivo* testing for biomedical science.

KEYWORDS: Animal testing, alternative, cystolith, ethnobotany, raphides.

INTRODUCTION

The instinctive behaviour of primitive man helped to associate the beneficial action of plants in the treatment of various ailments. From approximately 11th to 18th centuries, it was assumed that the colour, shape, habitat or other physical characteristics of a plant were

indicative of its medicinal value, for instance, the worm-shaped embryo of *Chenopodium* (worm seed) indicated it to be of value as an anthelmintic, the yellow colour of saffron suggested a possible use in liver disorders and *Rauwolfia serpentina* roots (snake root) should be useful in treating snake bite. However, the use of plants by such inferences was established through trial and error. Later on, a better understanding about the medicinal properties of the plants was gained through rational thought and action.

Herbs and spices have been used since ancient times for their flavouring qualities and also for their preservative and medicinal properties. Approximately two-third of the drugs of the modern medicine system have been developed from natural resources - largely from plants - and are used by people all over the world in the form of folk remedies, traditional or ethnic medicine.[5, 6, 9]

Plants act as an additional lifeline for mankind and in one way or another help various organisms to live and survive. Ethnobotanical studies are often significant in revealing locally important plant species, especially for the discovery of crude drug. Considerable research on pharmacognosy, chemistry, pharmacology and clinical therapeutics has been carried out on native medicinal plans. Traditional knowledge-driven drug development can follow a reverse pharmacology path and thereby reduce time and cost of development. Herbal medicine has gained much popularity because, herbal medicines are effective, and have fewer side effects. Herbal extracts have been used to cure various disorders, spasmodic gastric-intestinal complains, cough, bronchitis, laryngitis, tonsillitis and act as carminative and diuretic agents. Therefore, the demands for these plants are increasing in industrialized and non-industrialized countries. This has lead to an increase in their prices.[5, 6]

Accumulation of calcium to form calcium oxalate is an interesting phenomenon. Cell-mediated crystallization of calcium oxalate in plants includes biomineralization of calcium oxalate crystals in plants. Biomineralization fulfills a variety of crucial functions, including important skeletal and protective roles. In higher plants, calcium oxalate typically develops within intravacuolar membrane chambers of specialized cells. The complex cellular features associated with calcium oxalate crystallization indicate that it constitutes a biologically controlled process, analogous to calcification processes that shape bones, teeth, and shells in animals. Crystals have been observed in members of more than 215 plant families and occur in about 74% of angiosperm families, and are found in almost all organs and tissues of plants. The crystalline form can constitute to about 1% to over 90% of a plant's dry mass.[7, 10] The

formation of calcium oxalate crystals is genetically controlled and the crystals are usually formed in a defined shape and spatial location. Calcium oxalate crystals in higher plants occur in five major forms, namely raphides (acicular crystals that form in bundles), styloids (acicular crystals that form singly), prisms (consisting of simple regular prismatic shapes), druses (a spherical aggregates of crystals) and crystal sand (small tetrahedral crystals that form in clusters). The form, shape and occurrence of calcium oxalate crystals in plants are species- and tissues specific. Calcium oxalate exists in two chemical forms, monohydrate and dihydrate, and both of these occur in plants. The observed morphologies represent elaborations and modifications of basic crystal structure for either the monohydrate or dihydrate form. The monohydrate is more stable and is more commonly found in plants than is the dihydrate. The presence or absence of a particular type of crystal is used as a taxonomic character.[7, 10]

Innovation of new and novel therapeutics is a multi-step process involving drug design, synthesis and its pharmacological screening. Drug development mainly deals with three phases, *viz.* identification of lead compound amongst the million compounds, preclinical studies by *in vitro* and *in vivo* experiments, and clinical studies. Selection of an animal model, cell and tissue culture is one of the most important steps in any of the experimental pharmacological study. A number of other pharmaceutical products, including vaccines, antibiotics, and therapeutic proteins are also made because of them.[8] But this method needs many instruments, chemicals, funds, time, and legal issues are involved. In this paper we are predicting that a plant cell can be used as a dummy or model to study preliminary investigation and even effects efficiency of drugs on a disorder/ diseases. Naturally occurring crystal of calcium oxalate are synthesised by the pathway of oxalate biosynthesis which utilizes ascorbate as the primary precursor. Ascorbate utilized is produced directly within the crystal idioblast itself. Plant crystals are formed from endogenously synthesized oxalic acid, which combines with calcium from the environment. Even in animals the biochemical process involved in calcium oxalate stone formation is super-saturation, nucleation, aggregation, crystal growth, crystal retention and formation of stone granules and finally development of stone.[6]

Many plants with the property of disintegrating and dissolving kidney stones are listed in Ayurveda. In the Indian system of medicine, several herbal remedies have been used for the treatment of kidney failure since the time of Charka and Sushruta. New approaches of using plant extract on plant calcium oxalate crystal will improve and accelerate the discovery of the

right cure. Traditional knowledge serves as a powerful search engine and greatly facilitates intentional, focused and safe natural products research to rediscover the drug discovery process.[9]

Herbal medicines have many phytoconstituents which exert their beneficial effect in kidney stone treatment. Plant extracts contain phytochemicals that inhibit stone formation by inhibiting synthesis and agglomeration of crystals and even dissolve it. Herbal extracts may prevent stone formation because of many reasons like they may have diuretic activity, crystallization inhibiting activity, lithotriptic activity, analgesic and anti-inflammatory activity. For the present study, an ethobotanical survey (data not shown) was conducted in Mahad (Raigad) to identify plants used locally for the treatment of kidney stones. From the plants identified in the survey, *Tectona grandis* and *Bryophyllum pinnata* were selected for the study due to previously published studies that noted their lithotriptic activity.[9] The targets for the extract activity were *Ficus elastica* cystolith and *Colocasia esculenta* raphides.

MATERIALS AND METHOD

Phytochemical screening: *Tectona grandis* fruit and *Bryophyllum pinnata* leaves were air dried till a constant weight was achieved. Preliminary phytochemical screening was performed of *Tectona grandis* fruit and *Bryophyllum pinnata* leaves. The extracts were prepared in chloroform, acetone, 90% methanol and water by sonicating 1 g in 100 mL solvent in an ultrasonic bath for 15 minutes at room temperature. Respective filtrates were used while all phytochemical tests were performed.

Effect of plant extract on calcium oxalate crystals: Extracts of *Tectona grandis* fruit and *Bryophyllum pinnata* leaves were prepared by crushing 1 g of plant material in 10 mL distilled water using a mortar and pestle. The extract was filtered with Whatmann filter paper no. 1. The filtrate was used for further studies. Free hand sections of *Ficus elastica* and *Colocasia esculenta* showing cystoliths and raphides, respectively, were treated with the plant extracts. The sections were incubated in the extract at 25±2 ºC for 20-22 hours. Effects of the extracts on the cystoliths and raphides were observed by comparing photomicrographs taken before and after treatment. Control was maintained by treating cystolith and raphides with normal water. Photomicrographs of *Ficus* cystolith and *Colocasia* raphides were taken at 10x magnification using a Motic Digital Microscope B1 and their size was determined using the Motic Image Plus 2.0 software. The results were derived by observing 30 sections of *Ficus* cystolith and *Colocasia* raphides treated with the extracts.

RESULTS

The phytochemical screening of *Tectona grandis* fruit (Table 1) and *Bryophyllum pinnata* leaf (Table 2) extract was also done. The screening showed while majority of the secondary and primary metabolites were not detected, the presence of alkaloids was detected in all extracts for both plants.

Table 1: Phytochemical screening of *Tectona grandis*

Chemical constituent	Test	Extracts			
		CH	AC	ME	WA
Alkaloids	Dragendroff reagent	+	+	+	+
	Wagner's reagent	+	+	+	+
Anthocyanin	Concentrated HCl and NH_3	+	-	-	-
Antraquinone	Dilute H_2SO_4, benzene and NH_3	+	-	+	-
Carbohydrate	Fehling's test	+	-	-	-
	Benedict's test	-	-	-	-
Cardiac glycosides	Baljet's reagent	-	-	-	+
Emodins	NH_4OH and benzene	-	-	+	+
Flavonoids	Pew's test	-	-	-	-
	Sodium hydroxide test	-	-	-	-
	Ammonium hydroxide test	-	-	-	-
	Alkaline reagent test	-	-	-	-
Glycosides	Kedde's test	+	+	-	-
	Keller-Killani test	+	-	-	-
Polyphenol	Folin-Ciocalteau's test	-	+	+	+
Saponin	Foam test	-	-	-	-
Steroids	Salkowski's test	-	-	-	-
Tannin	Ferric chloride test	-	-	-	-
Terpenoids	Anisaldehyde reagent	-	+	-	-
	Vanillin-sulphuric acid regent	+	-	+	-

(CH: Chloroform; AC: Acetone; ME: 90% Methanol; WA: Water)

Table 2: Phytochemical screening of *Byrophyllum pinnata*

Chemical constituent	Test	Extracts			
		CH	AC	ME	WA
Alkaloids	Dragendroff reagent	+	+	+	+
	Wagner's reagent	+	+	+	+
Anthocyanin	Concentrated HCl and NH_3	-	-	-	-
Antraquinone	Dilute H_2SO_4, benzene and NH_3	-	-	-	-
Carbohydrate	Fehling's test	+	-	-	-
	Benedict's test	-	-	-	-
Cardiac glycosides	Baljet's reagent	-	-	-	-
Emodins	NH_4OH and benzene	-	-	-	-
Flavonoids	Pew's test	-	-	-	-
	Sodium hydroxide test	-	-	-	-
	Ammonium hydroxide test	-	-	-	-

	Alkaline reagent test	-	-	-	-
Glycosides	Kedde's test	+	+	-	-
	Keller-Killani test	+	-	-	-
Polyphenol	Folin-Ciocalteau's test	-	-	+	+
Saponin	Foam test	-	-	-	-
Steroids	Salkowski's test	-	-	-	-
Tannin	Ferric chloride test	-	-	-	-
Terpenoids	Anisaldehyde reagent	-	+	-	-
	Vanillin-sulphuric acid regent	-	-	-	-

(CH: Chloroform; AC: Acetone; ME: 90% Methanol; WA: Water)

Colocasia esculenta petiole sections showing raphides were used for the study. *Tectona grandis* fruit (Figure 2) and *Bryophyllum pinnata* leaves (Figure 3) extracts were used as treatment on raphides. It was seen that the raphides were completely dissolve after 20-22 hours of incubation. The control sections (Figure 1) treated with water did not show any difference on the raphides.

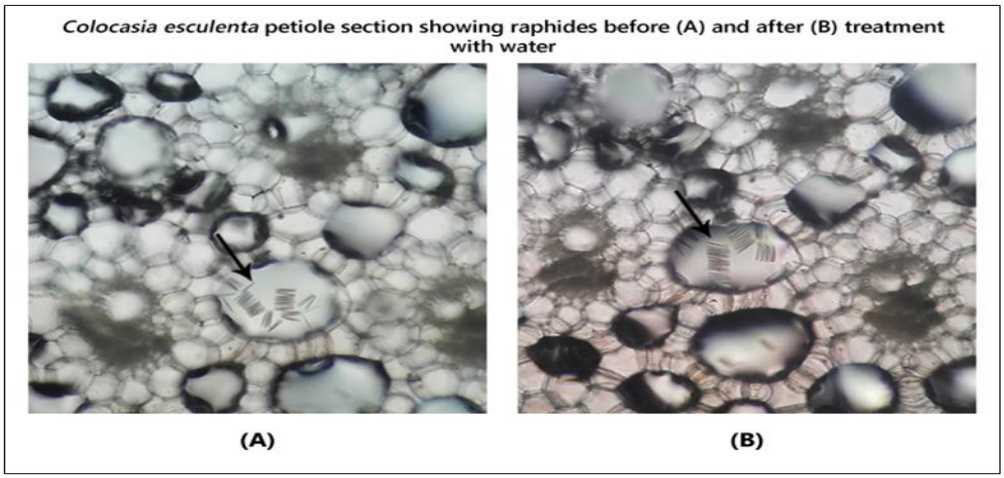

Figure 1: Control for *Colocasia esculenta* raphides

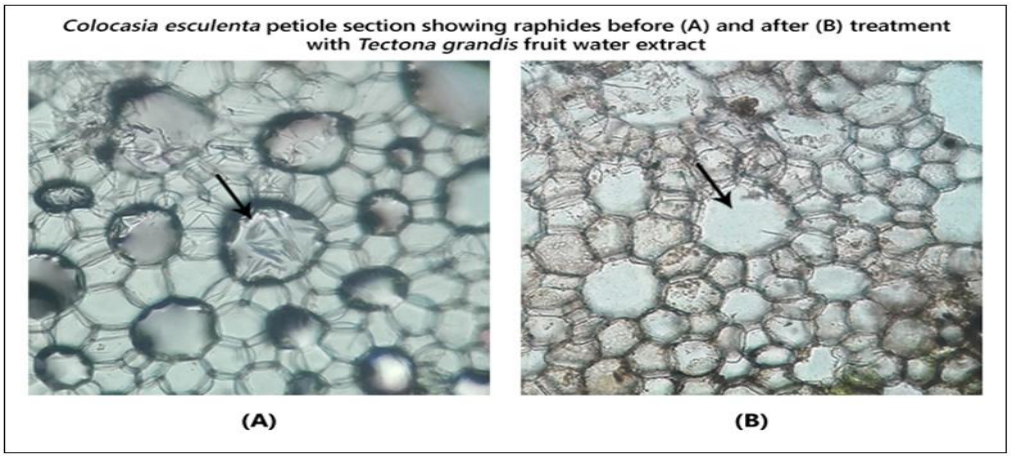

Figure 2: *Colocasia esculenta* raphides treated with *Tectona grandis* fruit extract

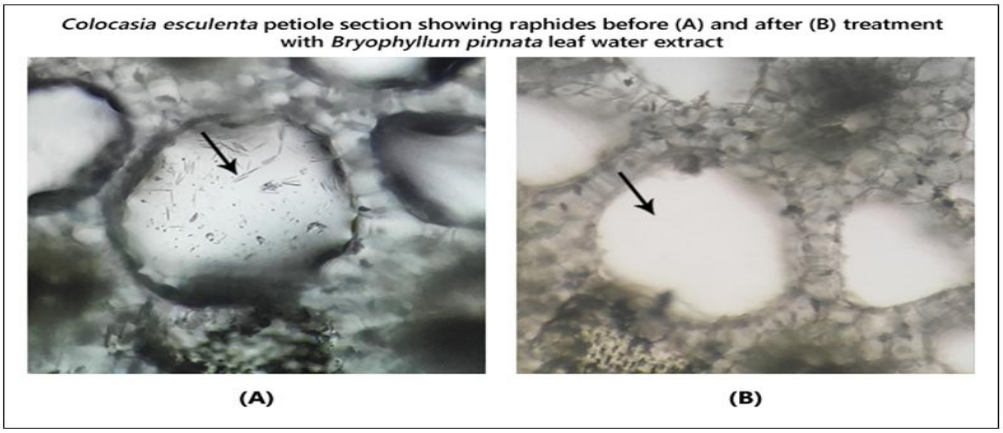

Figure 3: *Colocasia esculenta* raphides treated with *Bryophyllum pinnata* leaf extract

Ficus elastica leaf sections showing cystolith were used for the study. *Tectona grandis* fruit (Figure 5) and *Bryophyllum pinnata* leaf (Figure 6) extracts were used as treatment on cystolith. It was seen that after 20-22 hours the cystolith showed a significant decrease in size. However, the sectioned treated with water (Figure 4) were not affected.

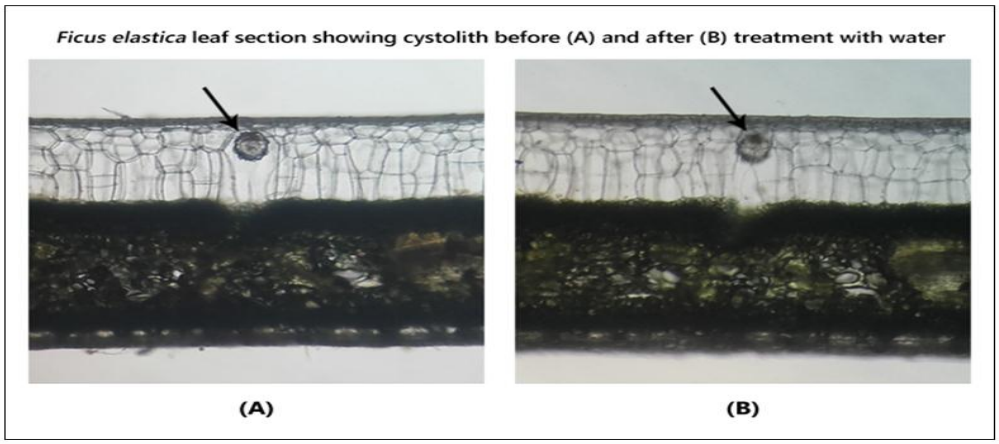

Figure 4: Control for *Ficus elastica* cystolith

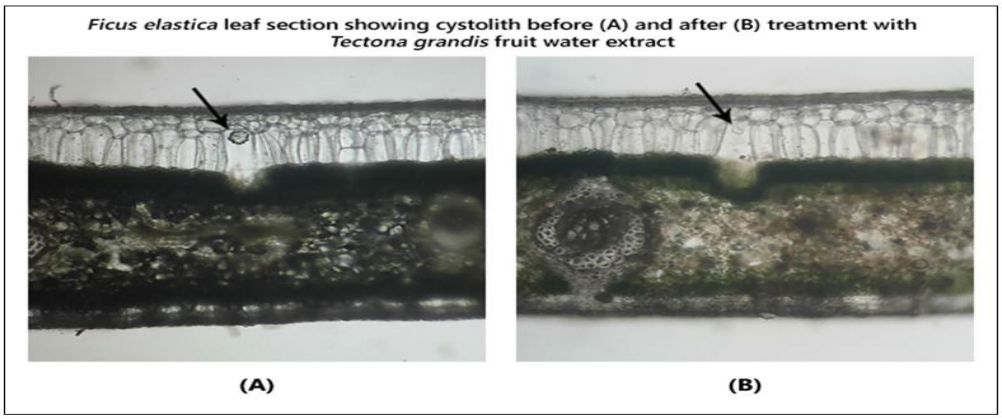

Figure 5: *Ficus elastica* cystolith treated with *Tectona grandis* fruit extract

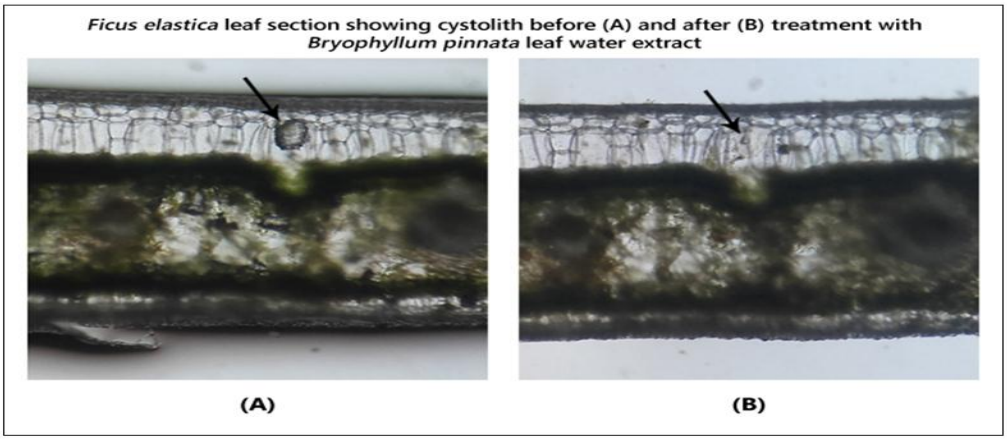

Figure 6: *Ficus elastica* cystolith treated with *Bryophyllum pinnata* leaf extract

Table 3: Effect of plant extracts on the size of calcium oxalate crystals

Calcium oxalate type	Herbal drugs	Before treatment (zero hour)	After treatment (20-22 hours)
Cystolith	*Tectona grandis*	Length: 30.3 µm Breadth: 20.2 µm	Length: 18.5 µm Breadth: 10.0 µm
Cystolith	*Bryophyllum pinnata*	Length: 39.6 µm Breadth: 33.5 µm	Length: 20.0 µm Breadth: 6.5 µm
Cystolith	Control (water)	Length: 40.4 µm Breadth: 29.8 µm	Length: 40.4 µm Breadth: 29.8 µm
Raphides	*Tectona grandis*	Range of lengths for groups for raphides 20-28 µm	Completely dissolved
Raphides	*Bryophyllum pinnata*	Range of lengths for groups for raphides 15-30 µm	Completely dissolved
Raphides	Control (water)	Range of lengths for groups for raphides 21-25 µm	Range of lengths for groups for raphides 21-25 µm

DISCUSSION

In India, 12% and nearly 4-15% of the global population suffer from urinary stone problems; of which 50% may end up with loss of kidney(s) or renal damage. Calcium oxalate stones represent up to 80% of analyzed stones and calcium phosphate accounts for 15-25%, while 10- 15% are mixed stones. The others are struvite 15-30%, cystine 6-10%, and uric acid stones 2-10%. Calcium oxalate stones are of two types, calcium oxalate monohydrate (whewellite) and calcium oxalate dehydrate (weddellite).[3]

Many medicines like Thiazide diuretics (e.g. Hydrochlorothaizide), alkali, (e.g. Potassium citrate), Allopurinol, Sodium cellulose phosphate (SCP), Penicillamine (Cuprimine), Bisphosphonates, Potassium phosphate, *Oxalobacter formigenes* and other probiotics are used to treat the stones formed which act by decreasing the excretion of stone forming agent such as oxalates, calcium, phosphates etc.[2]

Now-a-days, however, herbal medicine has gained much popularity because, herbal medicines are effective, have less side effects and reduce recurrence rate of stone formation, hence search for antilithiatic drugs from natural sources has assumed greater importance and is promising. Herbal medicines have many phytoconstituents which may exert their beneficial effect in kidney stone treatment. Plant extracts contain phytochemicals that inhibit stone formation by inhibiting synthesis and agglomeration of crystals.[1]

Herbal extracts may prevent stone formation because of many reasons like they may have diuretic activity, crystallization inhibiting activity, lithotriptic activity, analgesic and anti-inflammatory activity.[4] However, further research is needed to identify the active principles from medicinal plants to assess their dosage and quality control, and investigate their interactions and adverse effects. Although use of herbal medicine is popular and promising, it is essential to carry out further research to understand the disease, and the mechanism of action of herbal medicines in order to develop efficient and safe lithotriptic agents. But for this, clinical trials on animals are a must. Replacement of animals is what most people think of when you say alternatives to animal testing. The animals are replaced, either by methods that does not involve animals at all (absolute replacement) or by use only the cells or tissues of animals (relative replacement). Our method is absolute replacement as it involves plant cells as a test subject.

As discussed earlier, both animals and plants follow the same process in formation of calcium oxalate crystals. Thus via treating plant cystolith and raphides with well known ethno medicine we have studied the mechanism of dissolution of crystals in plant cells by imaging technique. This will provide an overview of whole treatment mechanism as shown in above images. *Bryophyllum* and *Tectona* extracts seemed to be very effective on calcium oxalate crystals. There is almost complete dissolution of *Ficus* cystolith and *Colocasia* raphides. The sections were also incubated in normal water as treatment control. Medicinal plants comprise of approximately 8000 species and account for about 50% of all the flowering plant species in India. Thus every plant can be used for the study as this method is simple, easy, economical, and precise, with the biggest benefit being that no animal testing is required.

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