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DEVELOPMENT OF AN AUXOTROPHIC *IN VITRO* SYSTEM FOR *KAEMPFERIA PARVIFLORA* WALL. EX. BAKER USING SUPER ABSORBANT HYDROGEL WITH ITS PHYTOCHEMICAL STUDY

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ABSTRACT

Kaempferia parviflora (black ginger) is a medicinal plant belongs to family zingiberaceae which is used for its property like antioxidant, anti-allergenic, anti-cancer, anti-microbial, anti-inflammatory, and anti-obesity etc. Due to its different properties black ginger shows significant value in pharmaceutical field and used as alternative medicine for treating various types of diseases like Fungal disease, Gastrointestinal disorders, and allergies.

In this study use of super absorbent hydrogel is done for development of auxotrophic *in vitro* system for *Kaempferia parviflora* in which crystals of potassium polyacrylate is gelatinised by water along with plant growth hormones and *in vitro* system is prepared for *Kaempferia parviflora* leaves and also study of phytochemical present in rhizome of black ginger.

Moreover, study for secondary metabolites is also carried by performing HPTLC technique on extraction of rhizome which is prepared in methanol and water as a solvent and study for total phenolic content present in rhizome is also carried out by using gallic acid as a standard solution.

Key words: *Kaempferia parviflora*, hydrogel, potassium polyacrylate, phytochemical analysis

INTRODUCTION

Kaempferia parviflora Wall. Ex Baker (Krachai dum) commonly known as black ginger is native species of Thailand. It is herbaceous plant with purple rhizomes with lanceolate shape leaves which is 6 to 8 cm long and it produce purple and white flower [2].

It belongs to Zingiberaceae family in which many members are used in culinary field to enhance taste and aroma of food. In this family many members are used as medicinal plant like that *Kaempferia parviflora* is used as medicinal plant due to its antioxidant, anti-allergenic, anti-cancer, anti-microbial, anti-inflammatory, and anti-obesity properties and shows significant value in pharmaceutical field [1]. It is used as alternative medicine for treating various types of diseases like Fungal disease, Gastrointestinal disorders, and allergies. It is also known for enhance response to sexual erotic stimuli in men [3].

Various uses of *K. parviflora* increase its mass production as a raw materials therefore easy and quick method is required for its large production [1]. In this study production of *K. parviflora* is done in *in vitro* condition by using super absorbent hydrogel technique in which potassium polyacrylate is used as media for regeneration of *K. parviflora* leaves.

As rapid growth of population it increases the demand for agricultural product which also increase a use of water consumption in irrigation purpose. So, for better use of rain water in some continent use of super absorbing hydrogel is used in agricultural field for its water holding capacity [16]. Some study shows the application of potassium polyacrylate hydrogel is useful in sandy soil than organic soil which have higher content of clay as it does not affect the water needs of soil [17].

For hydrogel, in general there are two groups of polymers are used in agricultural field: water insoluble and water-soluble polymers. Water soluble polymer have linear chain while water insoluble polymer contains cross linked structure [18]. Water insoluble polymers can absorb water up to 100 to 1000 times of their weight like sodium polyacrylate but in agriculture field polyacrylate is used because of its degradation capacity in soil [12].

In different studies it shows that polyacrylate is used to reduce the use of water because it has the ability to absorb large quantity of water at a time and release it in controlled and gradual manner and it maximise the use of rain water to the field. To fulfil this purpose potassium polyacrylate is used instead of sodium polyacrylate because sodium polyacrylate plays important role in increase in pH of soil in field.

In this study potassium polyacrylate is used in in vitro condition for micropropagation growth of *Kaempferia parviflora [4]*. For these leaves of *Kaempferia parviflora* is used along with potassium polyacrylate for micropropagation system. Potassium polyacrylate is white crystalline structure which shows gel formation structure with addition of water and gelatinized.

In this *in vitro* condition crystals of potassium polyacrylate is gelatinized with water and preparation for plant regeneration is done by using plant hormones which is indole-3- butyric acid and 6-benzykaminopurine in this gelatinized medium which contain potassium polyacrylate.

In this condition medium containing water with potassium polyacrylate along with plant hormone is used for regeneration of *Kaempferia parviflora* specifically leaves of *Kaempferia parviflora* is used for micropropagation at pH 5.8 with concentration of plant hormones for indole-3-butyric acid (IBA) 0.4 mg/litre and for 6-Benzylaminopurine (BAP) 2mg/litre.

In this study the goals are (1) use of super absorbent hydrogel of potassium polyacrylate in micropropagation system of *Kaempferia parviflora* leaves. And (2) study of phytochemical screening of extract of rhizome in different solvent system like water and methanol.

Phytochemical screening is carried out with view to determine the constituents of protein, carbohydrates, tannins, alkaloids, flavonoids, terpenoids, and glycosides [5]. For determination of constituents different qualitative test is carried out in laboratory of Parul university, Limda, Vadodara. Test like xantho-proteic test, Molisch's test, $Fecl_3$ reagent, Wagner's test, reaction with concentrated hydrochloric acid (HCL) and lead acetate, Salkowski test, Legal's test respectively for preliminary phytochemical screening determination.

In this study primary and secondary metabolites are taken into concern. For primary metabolites phytochemical screening is done in which presence of different functional group in chemical present in rhizome is checked by performing different kind of chemical test.

For secondary metabolite analysis and separation of chemical analysis is achieved by performing the technique of HPTLC (High performance thin layer chromatography) [22]. Recently HPTLC method is under focus because of its minimum solvent consumption, offline technique, zero waiting time for the instrument set up. it makes the method more suitable for the routine analysis in a crop improvement plan [23].

HPTLC technique is performed on both extracts containing water and methanol. For HPTLC analysis stock solution which is 1-gram dried powder in 10 ml methanol and in water respectively is spotted on Merck, HPTLC Silica gel 60 F_{254} which is stationary phase in range of 5- 10 µL. For HPTLC process toluene: ethyl acetate (6:2 v/v) used as a mobile phase for separation of a secondary metabolites present in *Kaempferia parviflora* rhizome and it shows the more separation rather than another ratio of mobile phase. Due to absence of standard chemical solution, it is not possible to identify all chemicals present in rhizome extraction but after comparing the result with other research it is possible to identify some chemical present in rhizome by its R_F value.

In *Kaempferia parviflora* flavonoids and phenolic compounds are main composition which is present in different chemical form [26]. So quantitative study of phenol or total phenol/polyphenol content is also carried out for rhizome extract by using Folin-Ciocalteu reagent in which folin-Ciocalteu reagent not only measure phenol content but also all reducing substances present in sample solutions [27].

In Folin-Ciocalteu assay reduction of FC reagent in presence of phenolic compound result in formation of molybdenum-tungsten blue that is measured spectrophotometrically at known wavelength. Usually, this test shows the linear relation between concentration and absorbance.

For this study in *Kaempferia parviflora* extract gallic acid is taken as standard solution in which different concentration of gallic acid behaves as standard and graph is plotted for plant sample solution for total phenol content.

MATERIALS AND METHOD

Collection of plant material

Kaempferia parviflora is native species of Thailand. This study is carried out in Vadodara, India. so, the material for study is collected in form of rhizomes by ordering them online from seeds village brand and grow them till mature leaves grow it take about 3 weeks for mature growth of rhizomes of *Kaempferia parviflora*.

Preparation of leaves of *Kaempferia parviflora* for micropropagation

Leaves are collected from grown plant of *Kaempferia parviflora*. Washed thoroughly with running tap water to remove dirt and soil from its surface [2]. Decontamination of this leaves is achieved by using 1% solution of tween-20 detergent which is prepared by adding 1 ml of tween-20 detergent solution in 99 ml of distilled water. For decontamination purpose leaves are washed in 1% solution of tween-20 for 10 minutes. After 10 minutes these leaves are taken out from tween-20 solution and kept in distilled water. At the time of inoculation of leaves in micropropagation media it is trimmed at the edges and petiole by using sterile blade. After trimming these leaves are cut into small pieces for inoculation in sugar tubes by sterile blade.

Preparation of *in vitro* system for regeneration of *Kaempferia parviflora*

For micropropagation system preparation hydrogel of potassium polyacrylate is used along with water and plant hormones. In this study crystals of potassium polyacrylate is used. For media preparation crystals of potassium polyacrylate is gelatinised with water in sugar tubes [18]. Preparation of one sugar tube is done by using 20 ml distilled water and 3-gram potassium polyacrylate along with 2 ml of 2mg/lit working solution of 6-Benzylaminopurine and 0.4 ml of 0.4 mg/lit working solution of indole-3-butyric acid at pH 5.8. This media is prepared by mixing water and plant hormones with crystals of potassium polyacrylate for 5 minutes for complete gelatinization of crystals (figure-3)

Inoculation of *Kaempferia parviflora* leaves in *in vitro* system

Washed leaves are inoculated in hydrogel containing media under sterile condition by using laminar air flow which is sterilized by UV light for 20 minutes. All work is done by using sterile equipment like sterile blade, petri plate and forceps etc.

Preparation of extraction of *Kaempferia parviflora* rhizome

Rhizome of *Kaempferia parviflora* is washed with water and chopped into small pieces then dried under shadow dry condition for 2 weeks and grind to form powder which is used for preparation of extraction [10]. Extraction is prepared into 1:10 in which 2g of dried powder of rhizome is mixed with 20 ml of water and 2g of dried powder of rhizome is mixed with 20 ml of extra pure methanol for preparation of water extraction and methanolic extraction respectively. For extraction preparation this solution which is dried powder with water and methanol are leave under continuously shaking condition for 24 hours and after 24 hours shaking it is filtered by using Whatman filter paper and this filtrate is used for further study of phytochemical analysis as extract [8].

Qualitative test for phytochemical analysis of extraction of rhizome.

For phytochemical screening of extract of *Kaempferia parviflora* rhizome different test is performed at Parul University, Limda, Vadodara. In this study test for Protein, carbohydrates, tannins, alkaloids, flavonoids, terpenoids and glycosides is performed for checking the presence of this phytochemicals.

Xantho-proteic test for protein

In this test 1ml extraction is mixed with 1ml of concentrated HNO_3 and cool for 1 minute then add 2ml of 40% NaOH. Observe the colour change to dark yellow to orange.

Molisch's test for carbohydrates

In 2ml of sample extraction 1 ml of Molisch reagent is added and from the side wall of test tube concentrated H_2SO_4 is added. Positive result shows formation of purple ring.

Test for tannins ($Fecl_3$ reagent)

Presence of tannins can be checked by adding 1ml of distilled water in 1 ml of extract and 2 drops of ferric chloride ($Fecl_3$) solution. Positive result shows the colour change of greenish to black colour.

Wagner's test for Alkaloids

Wagner's test is performed by adding Wagner's reagent directly into extract of sample. Reagent contains Iodine and potassium iodide in distilled water. Positive result shows the formation of reddish to brown precipitation.

Test for flavonoids

A. concentrated hydrochloric acid (Conc. HCL)

Addition of 1ml concentrated HCL in 1ml of extract shows the colour change to red colouration.

B. lead acetate

Presence of flavonoids can be confirmed by adding few drops of lead acetate in 1ml of extract which shows the formation of yellow colour precipitates and with addition of concentrated HCL it converted into colourless solution which indicate the formation of flavonoids.

Salkowski test for terpenoids

For terpenoids test 1ml of extract is treated with 2ml of chloroform and 3 ml of concentrated sulfuric acid (conc. H_2SO_4) presence of terpenoids shows the formation of brown colouration.

Legal's test for glycosides

For legal's test 1ml of extract is treated with pyridine and sodium nitroprusside with the addition of 10% NaOH it shows the pink colouration of solution which indicate the positive result for glycosides.

Secondary metabolite analysis by HPTLC technique:

For HPTLC study some conditions are maintain which include:

Stationary phase: Merck, HPTLC silica gel 60 F_{254}

Mobile phase: Toluene: ethyl acetate (6:2 v/v)

Volume of mobile phase: 16ml

Chamber saturation time: 20 minutes

Temperature: room temperature

Wavelength of detection: 254nm, 366nm

Wavelength range: 200nm to 400nm

Lamp: Deuterium & Tungsten

Scanner type: Spectrum

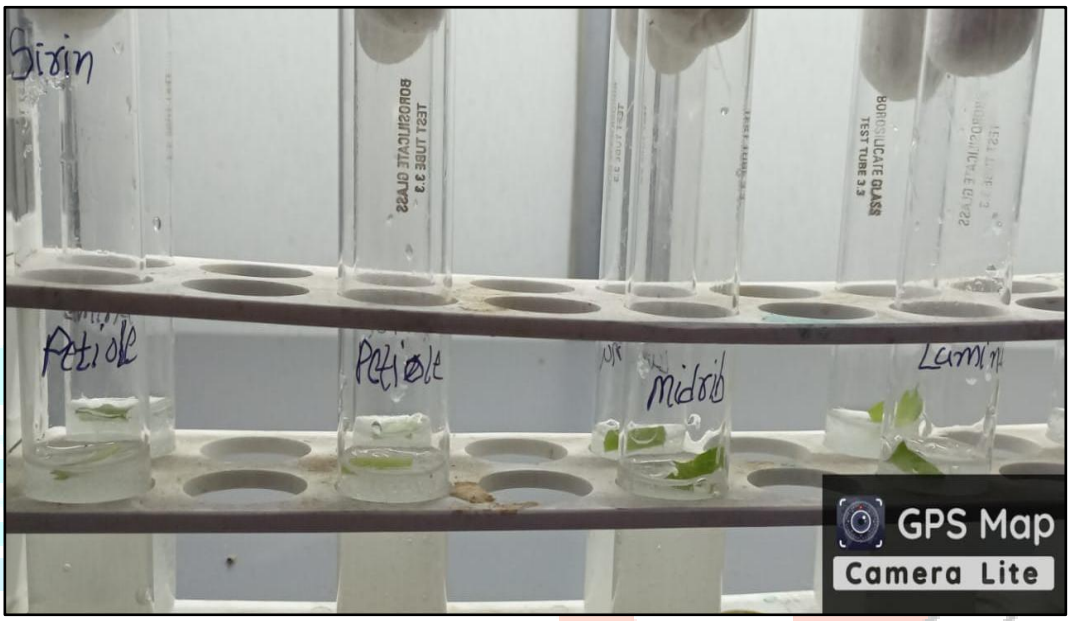
Scanner 4: 271118

In phytochemical analysis quantitative study for phenolic compound in which Folin-Ciocalteu assay is used to determine the total phenolic compound present in rhizome extraction of *Kaempferia parviflora* is also carried out. In this assay concentration was quantified by spectrophotometry method. For chemical reaction mixture contains 1ml of plant extract and 9ml water with 1ml of Folin-Ciocalteu reagent with shaking for 5minutes. After 5min 10ml of 7% Na_2CO_3 solution was treated with mixture. For sample solution total volume became 25ml and for standard solution gallic acid solution at different concentration was prepared for analysis. These prepared solutions are incubated for 90 minutes and then absorbance was taken at 550nm by using visible spectrophotometer.

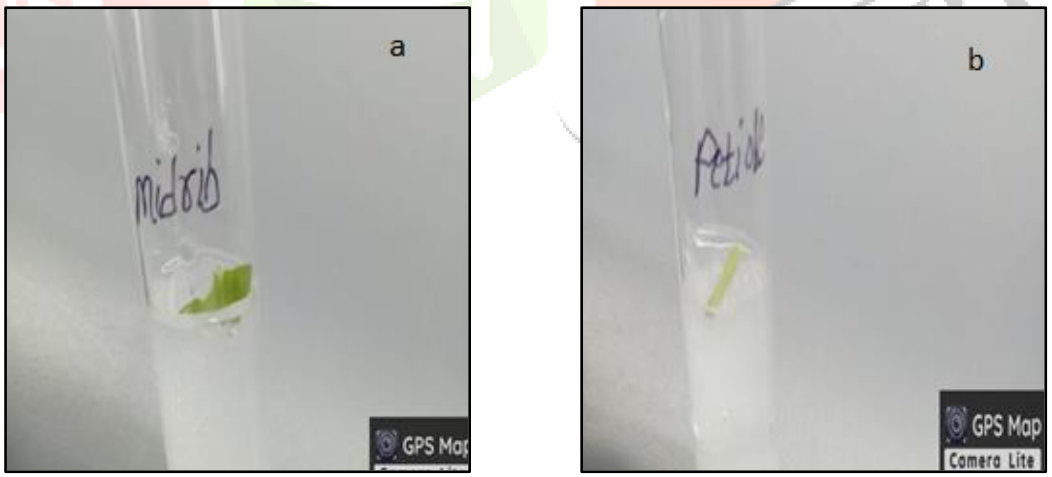
RESULT

Development of an *in vitro* system of *Kaempferia parviflora* by using super absorbent hydrogel is done in which crystals of potassium polyacrylate which is water insoluble hydrogel and already used in agricultural field is taken for preparation of *in vitro* system for regeneration of *Kaempferia parviflora*.

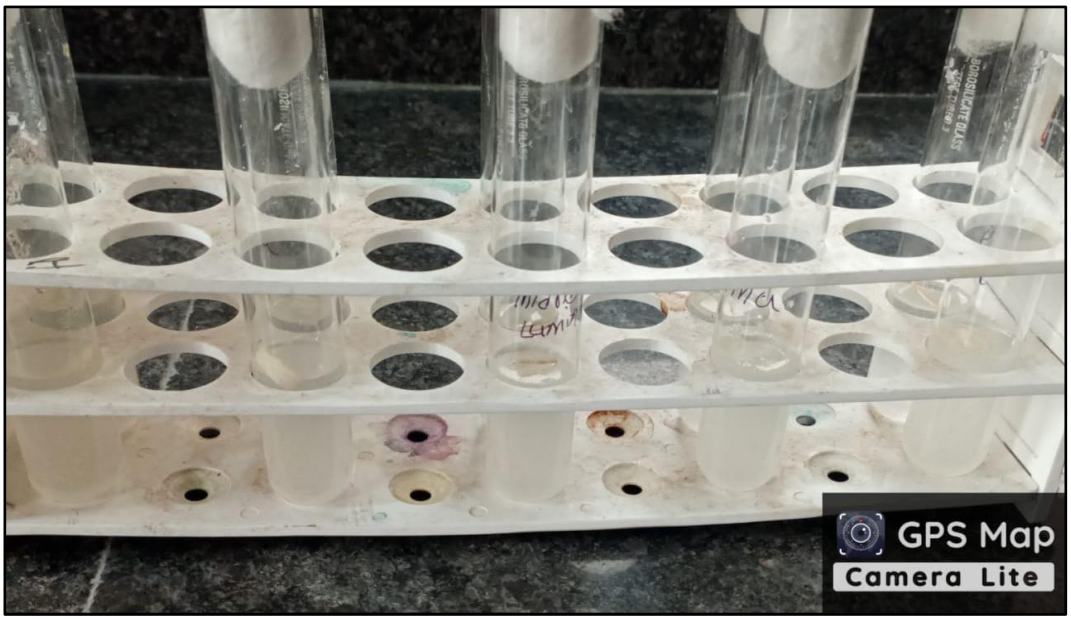
Kaempferia parviflora is a medicinal plant used in pharmaceuticals for its various clinical property and in culinary business for enhancing the taste of food. For its various uses demand of this plant is increase and quick easy method is required for its fast and large production. For this purpose, *in vitro* system in which gelatinized hydro gel along with plant growth hormone is used for better production. In *in vitro* system which is prepared by using hydrogel plant remain healthy for 1st week after inoculation but after one week plant does not show positive response to this system by showing characteristic like yellowing of inoculated plant leaves and after 3rd week it shows complete yellowing of inoculated part.



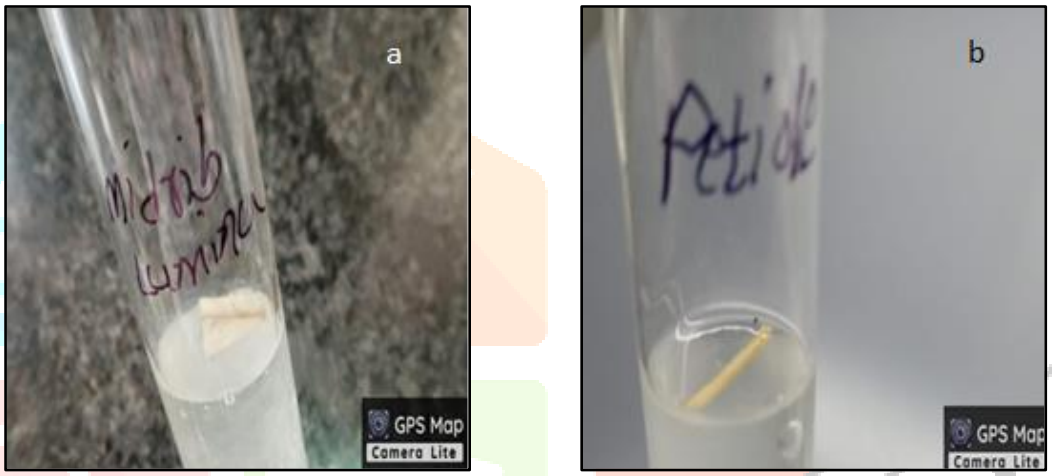
Result of micropropagation system for *Kaempferia parviflora* leaves after 2 weeks of inoculation



Result for midrib of leaves of *Kaempferia parviflora* after 2 weeks. (a), Result for petiole of leaves of Kaempferia parviflora after 2 weeks. (b)



Result of micropropagation system for *Kaempferia parviflora* leaves after 3 weeks of inoculation



Result for midrib of leaves of *Kaempferia parviflora* after 3 weeks. (a), Result for petiole of leaves of *Kaempferia parviflora* after 3 weeks (b)

For further study of *Kaempferia parviflora* phytochemical analysis of extraction of rhizome in two different solvent system which is water and methanol is carried out. For qualitative phytochemical analysis different test is carried out on extraction of rhizome which shows result by mostly changing the colour of solution which indicate the chemical reaction of reagent and chemical present in rhizome extraction.

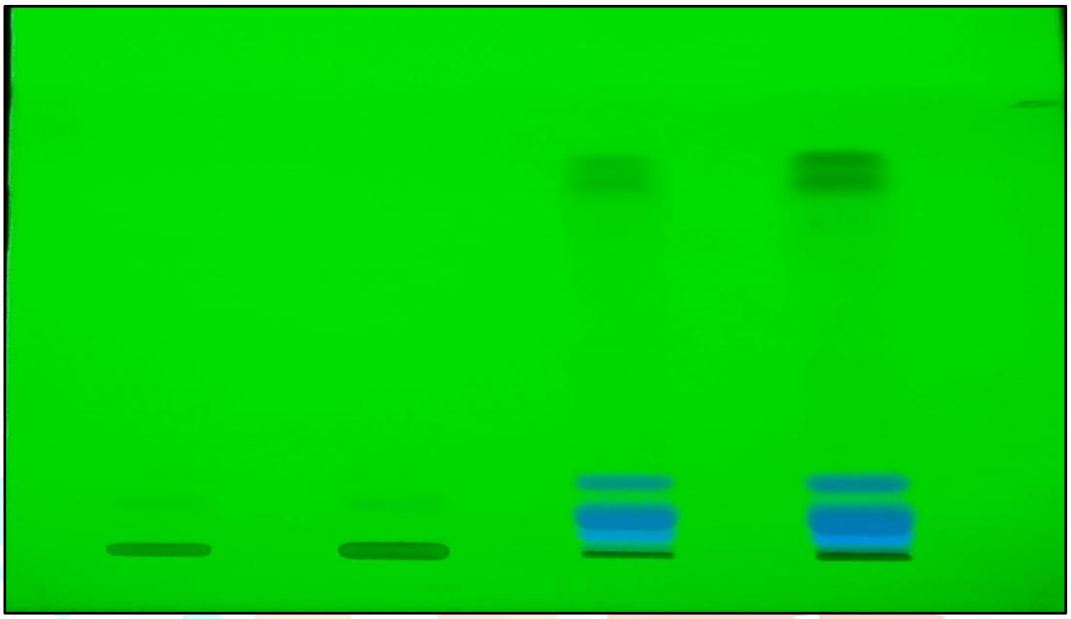
Table.1=result for phytochemical analysis of extraction of rhizome.

SR.NO.	TEST NAME	WATER EXTRACT	METHANOLIC EXTRACT
1.	Xanthoproteic test for protein	+	+
2.	Molisch test for carbohydrate	+	+
3.	$FeCL_3$ reagent: For phenol For tannins	 + +	 + +
4.	Reaction with: a. concentrated HCL b. lead acetate for flavonoids	 + -	 + +
5.	Wagner's test for alkaloids	+	+
6.	Salkowski test for terpenoids	-	+
7.	Legal's test for glycosides	+	+

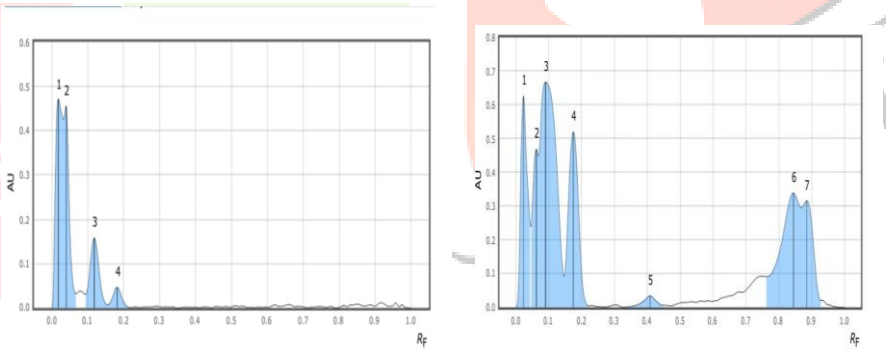
+ = positive - = negative

This table shows the presence of different chemical group present in rhizome extract which can be used in different pharmaceuticals product for better use in curing the different diseases and for further study of medicinal plant.

For secondary metabolite analysis HPTLC shows the separation of different chemicals present in rhizome of *Kaempferia parviflora*. Given below figure (20) and figure (21) of TLC plate shows the separation of chemicals present in rhizome at 254nm and 366nm. For HPTLC technique it shows the different R_F value for different chemical at the wavelength of 254nm and 366nm. But due to absence of standard solution for chemicals it is not possible to identify all chemicals present in extraction.



TLC plate visualization under UV light



Graph:1. Graphical representation of HPTLC for extraction in water(a) and methanolic(b) solvent at 254nm respectively.

Peak	Solvent	R_F Value	Area (%)
1	Water	0.018	41.34
2	Wate	0.040	33.64
3	Water	0.118	19.48
4	Water	0.182	5.55

Table :2. Rf value for water solvent at 254nm

Peak	Solvent	R_F Value	Area (%)
1	Methanol	0.024	12.66
2	Methanol	0.063	7.93
3	Methanol	0.090	31..97
4	Methanol	0.175	15.76
5	Methanol	0.408	1.39
6	Methanol	0.844	19.72
7	Methanol	0.885	10.57

Table:3. Rf value for methanolic solvent at 254nm

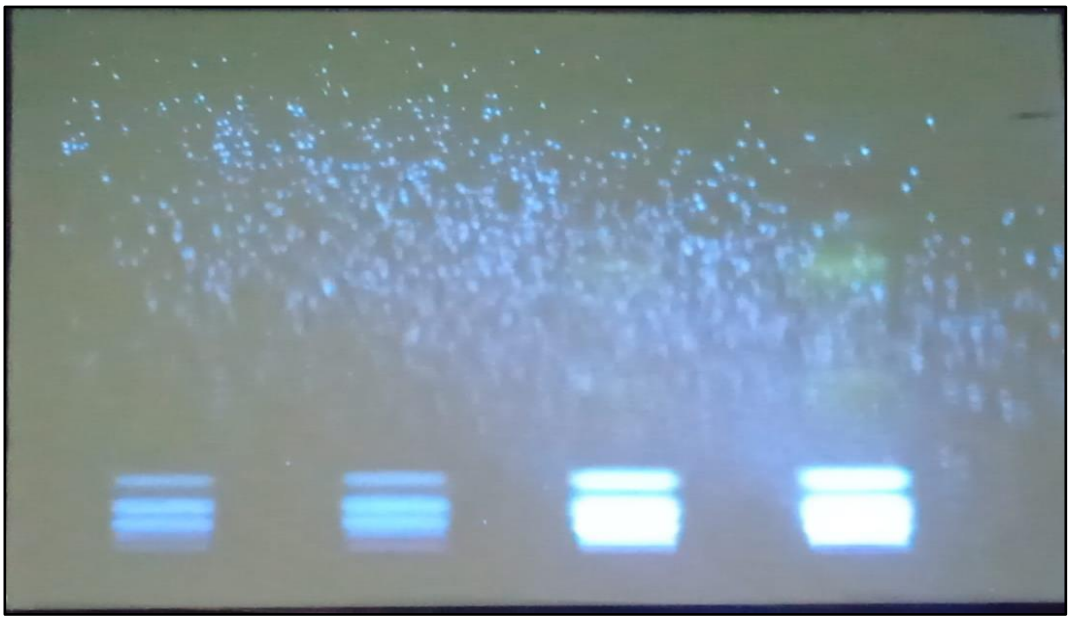
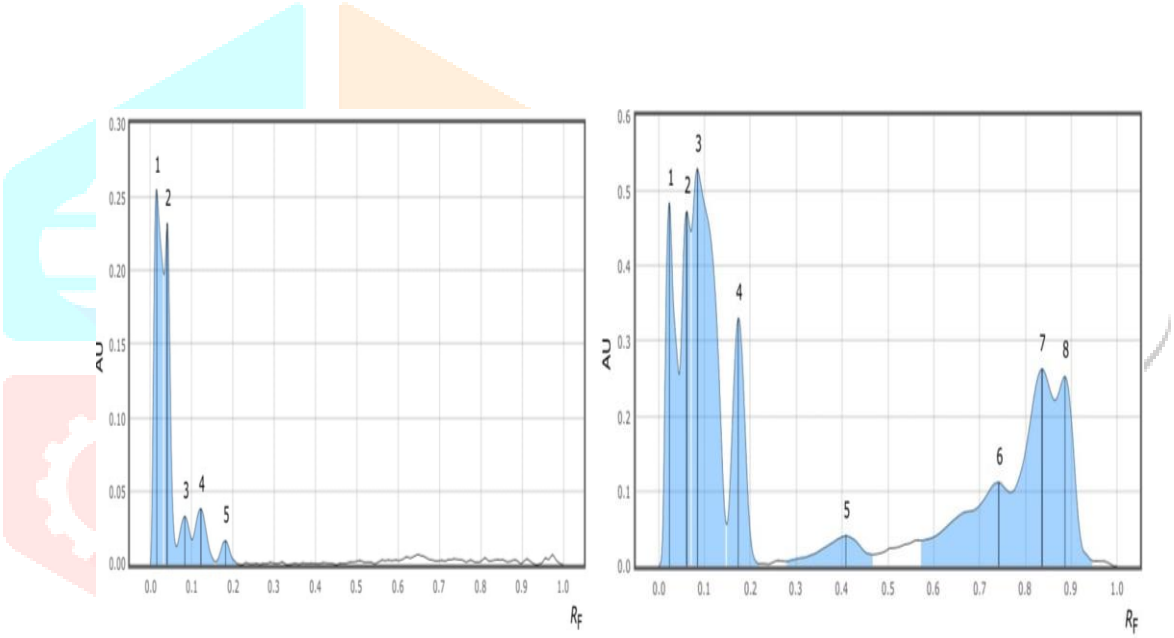


Figure: 21. TLC plate visualization under visible light.



Graph:2. Graphical representation of extraction of rhizome in water(a) and methanolic(b) solvent at 366nm respectively.

Peaks	Solvent	Rf value	Area
1	Water	0.015	48.68
2	Water	0.040	29.80
3	Water	0.083	7.83
4	Water	0.122	10.11
5	Water	0.182	3.58

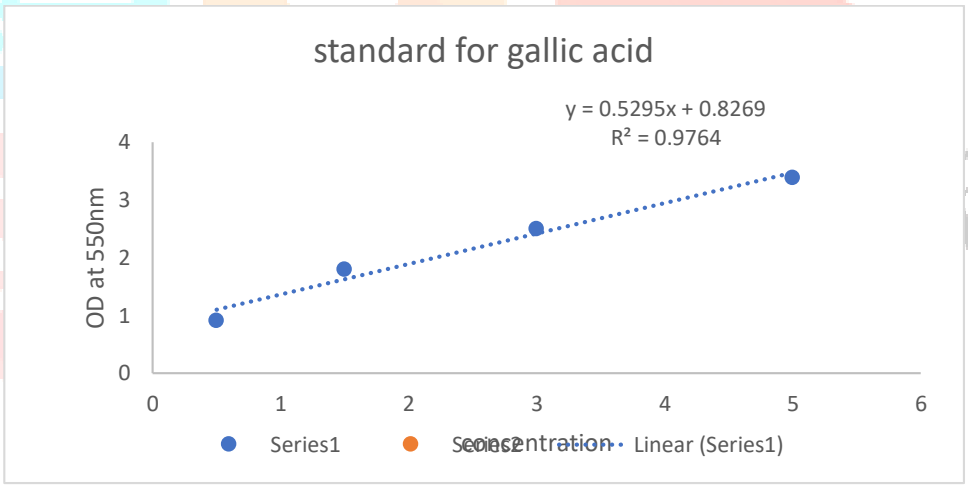
Table:4. Rf value for water solvent at 366nm

Peaks	Solvent	Rf value	Area (%)
1	Methanol	0.024	10.67
2	Methanol	0.061	9.18
3	Methanol	0.085	26.96
4	Methanol	0.174	9.54
5	Methanol	0.408	3.93
6	Methanol	0.742	12.70
7	Methanol	0.836	16.86
8	Methanol	0.886	10.17

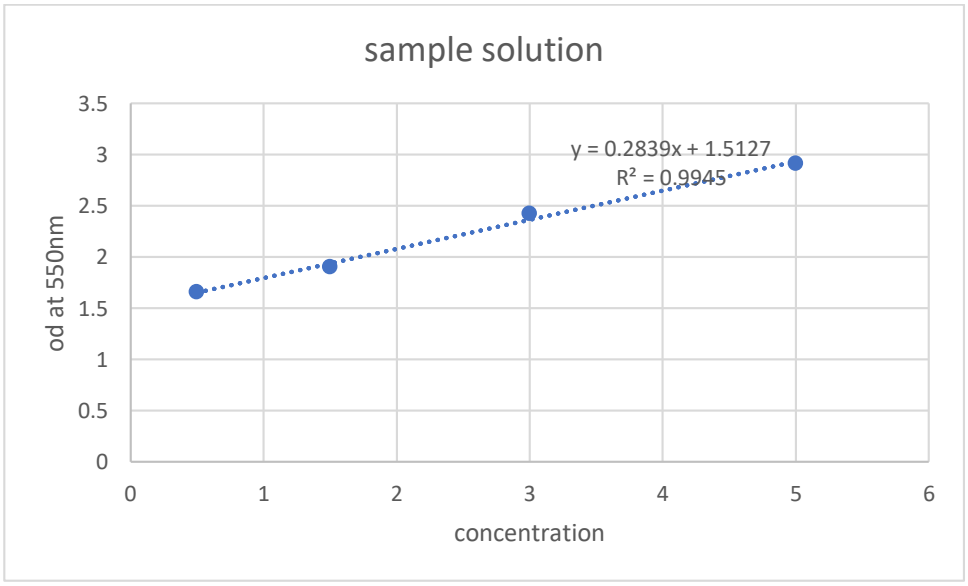
Table:5. Rf value for methanolic solvent at 366nm

Due to absence of standard solution of chemicals present in rhizome of *Kaempferia parviflora* it is not possible to identify all chemicals that are separated by HPTLC method but by comparing the results with previous study it can be concluded that in water extract not many chemicals are separed but in methanolic extract 6-gingerol and 6-shogaol is identify.

For quantification of phenolic compound different absorbance value was obtained by visible spectrophotometry method.



Graph:3. Graphical representation of standard solution of gallic acid for phenolic content at 550nm.



Graph:4. Graphical representation of sample solution of extraction of rhizome in methanol of kaempferia parviflora for total phenolic content at 550nm.

Total content of phenolic compound in standard solution of gallic acid can be calculated by:

Y = mx+c

Y = 0.5295+ 0.8269

1.663 = 0.5295+ 0.8269

1.663 – 0.8269 = 0.5295x

0.8361 = 0.5295x

X = 0.8361/0.5295

X = 1.5790 mg/L

Concentration of phenolic content in standard of gallic acid is 1.5790mg/litre at 550 wavelength.

For concentration of phenolic content in rhizome of *Kaempferia parviflora*:

Y = mx+c

Y = 0.2839x + c

Y = 0.2839x + 1.5127

1.66 = 0.2839x + 1.5127

1.66 -1.5127 = 0.2839x

0.1473 = 0.2839x

X = 0.1473/ 0.2839

X = 0.5188 mg/L

Total phenolic content in rhizome of *Kaempferia parviflora* at 550nm wavelength is 0.5188mg/L.

CONCLUSION

In this study auxotrophic *in vitro* system is prepared for *Kaempferia parviflora* by using hydrogel of potassium polyacrylate which shows positive response at agricultural field for better growth of agricultural product because of its high water holding capacity along with the presence of plant growth hormones.

Due to some unknown reasons *Kaempferia parviflora* does not show positive response to this system but in future if there is further study carried out for hydrogel there is a possibility to use hydrogel in *in vitro* condition for plant regeneration and in tissue culture field.

Along with *in vitro* system some phytochemical study is also carried out in which phytochemical screening is done by performing chemical test which shows result by colour change and confirm the presence of functional group

For secondary metabolites HPTLC method was performed in which different secondary metabolites are separed in which 6-gingerol and 6-shogaol is identified but due to absence of standard solution it is not possible to identify all separeted chemical but if further study done on secondary metabolites it can be used in other pharmaceutical products.

For quantitative analysis Folin-Ciocalteu assay was performed and total amount of phenolic compound is calculated by using gallic acid as standard solution.