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Research Article

COMPARATIVE EVALUATION OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF *KAEMPFERIA GALANGA* FOR NATURAL AND MICROPROPAGATED PLANT

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ABSTRACT

Shoots and roots were induced from axillary buds of *Kaempferia galanga* when cultured on Murashige and Skoog (MS) medium supplemented with NAA + BAP (0.1mg + 1.0 mg/l). Liquid detergent (2%) + Alcohol (70%) + Mercuric chloride (0.1%) used for surface sterilization of explants. Rhizomes were developed after four months of transferring in to earthen pots. The methanolic extracts of both micropropagated plant rhizome and natural plant rhizome were screened for antimicrobial activities against two gram -ve and two gram +ve pathogenic bacteria i.e. *Escherichia coli, Salmonella typhi, Bacillus subtilis* and *Staphylococcus aureus*. The methanolic extract of micropropagated plant was exhibited significant inhibition activity compared to natural plant extract. The antioxidant activity of methanolic extract of the rhizomes of micropropagated plant has not showed significant activity than natural plant rhizome extract.

Keywords: *Kaempferia galanga*, Micropropagation, Murashige and Skoog medium

INTRODUCTION

Kaempferia galanga Linn (*Zingiberaceae*), is an acaulescent perennial growing in Southern China, Indochina, Malaysia, India and Thailand[1]. This species is annual and 2-4 plants can be obtained in a year from one rhizome[2]. For rapid multiplication of slow propagated species tissue culture propagation is ideal solution[2]. Currently, efforts are on *in vitro* propagation using rhizome a storage organ inducing for efficient acclimatization and to minimize injury during Transportation[2]. It has reported to have Expectorant, Carminative, Diuretic, Coughs, Pectorial infections and inflammatory tumors[3], Antioxidants[4], and Antimicrobial[5]. The methanolic Extract of the rhizome contains ethyl p-methoxy trans-cinnamate which is highly cytotoxic to HELA cells[6]. The rhizome extract has been potentially active against bacterial infections[7].

In this paper attempt has been made to investigate comparative Antimicrobial and Antioxidant effectiveness from the rhizome of both natural and micropropagated plant.

MATERIALS AND METHODS

Plant material

Plants of *Kaempferia galanga* Linn were collected from Foundation for Revitalization of Local Health Tradition (FRLHT), Bangalore, in the month of March. It was identified and authenticated by Dr. Jawahar C Raveendran (FRLHT), Bangalore. The Identified and authenticated plant was used for tissue culture studies.

Axillary buds (1-2cm) were dissected out and washed thoroughly under running tap water for half an hour. The explants were immersed in 2% soap solution for 15minutes before sterilization in a solution of 70% ethyl alcohol for 5min and 0.1% (w/v) of mercuric chloride for 8-10minutes. After thorough rinsing in sterile distilled water, the explants were transplanted onto the culture medium.

Culture media and conditions

The sterilized axillary bud explants were inoculated on MS medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar and 0.01% myo-inositol and different concentrations of growth regulators such as NAA, BAP, BA and Kinetin. The pH of the medium was adjusted to 5.7 prior to autoclaving for 15min at 121^{0}C. Cultures were incubated at 25±1^{0}C under 16 hour Photoperiod.

In vitro plantlet production

Initiation of growth occurred with in ten days of inoculation on MS medium supplemented with different hormonal combinations. However, the highest number of shoots and roots were induced simultaneously on MS medium supplemented with NAA + BAP (0.1mg + 1.0mg/l) after 60 days. All the regenerated shoots were deep green and healthy in appearance.

Hardening and transplantation

The rooted plantlets isolated from clusters were transferred to garden pots containing sand: soil (1: 1) and kept in the greenhouse. With in 20-30 days in soil the potted plants begin to form new leaves and resumed new growth. Watering with tap water during early morning and late evening were done for a period of 3 months with constant monitoring. The rhizomes were developed with in 4 months after transferring into the garden pots.

Thin layer chromatography

Sample preparation

500mg of rhizomes from natural and *in vitro* regenerated plants were dried, coarsely powdered and extracted with 50 ml of methanol for 1 hour using reflux condenser. The extract was filtered and evaporated to dryness. 2 mg of each dried extracts were dissolved in 1 ml of methanol and these were subjected to TLC analysis.

Preparation of standard: 1 mg of ethyl-p-methoxy cinnamate was dissolved in 0.5 ml of methanol.

Thin layer chromatography was carried out by using methanolic extracts of both natural and micropropagated plant rhizome. Ethyl-p-methoxy cinnamate was used as reference standard using n-hexane: ethyl acetate (9:1) as solvent system and silica gel GF_{254} as stationary phase. . It is detected in UV 254nm, UV 366nm and then sprayed with vanillin-sulphuric acid reagent and dried at 110^{0}C for 10 minutes. The Rf value of methanolic extract was comparable with the Rf value of standard

Antimicrobial screening

Screening of antibacterial activity of both natural and micropropagated plantlet was done by using agar diffusion method. Four organisms two Gram negative *i.e Escherichia coli* (ATCC 1536), *Salmonella typhi* (ATCC 14028) and two gram *positive i.e. Bacillus Subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 29737) were used in the present study to determine the antibacterial activity of the crude extracts. In disc diffusion method, nutrient media was used as a culture media and the cavities were made aseptically over the bacterial culture on nutrient agar plates using borer and filled with Standards Ciprofloxacin (100µg/disc) and Amoxicillin (100µg/disc) as positive control, natural plant rhizome extract

(1mg/disc), micropropagated plant rhizome extract (1mg/disc) in Dimethyl formamide (DMF) and only solvent (DMF) as negative control and incubated at 37^{0}C for 24 hours. After incubation for 24hours, the zone of inhibition around the discs was measured by millimeter scale. The experiment was replicated two times to confirm the reproducible results. The sensitivity of the microorganism species to the plant extracts was determined by measuring the size of inhibitory zones (including the diameter of disc) on the agar surface around the disks. All experiments were performed in duplicate.

Determination of minimum inhibitory concentrations (MIC)

A 16h culture was diluted with a sterile physiological saline solution (0.85% w/v sodium chloride) to achieve an inoculums size of approximately 10^6 colony forming unit ml^{-1}. A serial dilution was carried out to give final concentrations between 0.1 to 1.0mg /0.1ml. The tubes were inoculated with 20µl of the bacterial suspension per ml nutrient broth, homogenized and incubated at 37°C. The MIC value was determined as the lowest concentration of the crude extract in the broth medium that inhibited the visible growth of the test microorganism.

Screening for antioxidant activity

Screening of the both natural and *in vitro* regenerated plant rhizome extracts for antioxidant activity can be done by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay reported by Chan *et al.*, 2008[8] was adopted with modifications. This method depends upon the reduction of purple DPPH to a yellow colored diphenyl picrylhydrazyl. The determination of the disappearance for the free radicals was done using spectrophotometer. The remaining DPPH which showed maximum absorption at 516nm was measured. Different dilutions of the extract (0.1 to 0.6mg/ml) were prepared in methanol. One ml of a 0.3mM DPPH in methanol solution was added to 2.5ml of sample solution of different concentrations of both the extracts (Test solutions). One ml of methanol was added to 2.5ml the sample solutions of different concentrations (blank solutions). One ml of DPPH solution plus 2.5ml of methanol was used as negative control. The blank for this solution is methanol. As DPPH is sensitive to light, it is exposed to the minimum possible light. These solutions were allowed to react at room temperature for 30minutes. The absorbance values were measured at 516nm and converted into the percentage antioxidant activity using the following equation:

Scavenging capacity (%) = 100-absorbance of sample x 100/absorbance of control

RESULTS AND DISCUSSION

Initiation of shoots and roots from in *Kaempferia galanga* appeared to be rather difficult initially due to heavy fungal and bacterial contamination. The method followed viz., immersion in 2% soap solution for 15minutes before treating with solution of 70% ethyl alcohol for 5min and 0.1% (w/v) of mercuric chloride for 8-10minutes helped to overcome this problem (90%).

After 30 days, the average number o shoots and roots were more in the medium supplemented with NAA + BAP (0.1mg + 1.0mg/l) (Table 1). After 45days the grown plantlet were transferred to earthen pot containing sand: cocopeat: compost (1:1:1) and development of plant was observed. The rhizome developed after 2-4 months of transferring into earthen pots containing sand and soil.

The constituents of the rhizomes of *K.galanga* reported, have included cineol, borneol, 3-carene, camphene, kaempferide, cinnamaldhyde, p-methoxycinnamic acid, ethyl cinnamate and ethyl-p-methoxy cinnamate. Ethyl-p-cinnamate was reported to inhibit monoamino oxidase. The methanolic extract of *K. galanga*, which identifies as ethyl cinnamate, ethyl-p-methoxy cinnamate and p-methoxy cinnamic acid showed larvicidal activity against the second stage larva of dog roundworm.

The results are reported here are the average values of two experiments. The methanolic extract of the micropropagated plant rhizome (1000µg/0.1ml) was found to have significant zone of inhibition compared to that of natural plant against two gram negative (*Escherichia coli, Salmonella typhi*) and two gram positive organism (*Bacillus subtilis, Staphylococcus aureus*) compared with positive control Ciprofloxacin (100µg/0.1ml) and Amoxicillin (100µg/0.1ml). Dimethyl formamide was used as negative control. The MIC value of methanolic extracts against two gram positive and two gram negative organisms were found to be 400µg/0.1ml in micropropagated plant extract and 500µg in natural plant extract.

Kaempferol, a natural flavonoid isolated from K. galanga has been shown to reduce the risk of heart disease, pancreatic and lung cancer[9].

DPPH is relatively stable nitrogen centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH reacts with suitable reducing agents as a result of which the electrons become paired off forming the corresponding hydrazine. The solution therefore loses colour stoichometrically depending on the number of electrons taken up. From the results it may be postulated that Natural plant rhizome extract have more hydrogen donors thus scavenging of free radical DPPH is significant compared to Micropropagated plant rhizome extracts.

Table 1: Effect of growth regulators on shoot and root initiation from axillary buds of *K. galanga* on MS medium (observations after 60days)

S.No.	Hormonal combination mg/l	Shoots*	Roots*	Leaves*
1	4.0 BA	1.7±0.82	9.5±2.99	4.7±1.2
2	2.2 BA	1.4±0.51	5.2±1.8	3.6±0.84
3	2.2 BA + 1.07 NAA	2.6±0.96	13.10±1.52	6.3±1.33
4	2.2 BA + 4.6 Kinetin	1.8±0.78	4.4±0.96	4.8±.78
5	0.5 NAA + 1.0 BA	4.4±0.84	10.80±1.68	5.9±0.78
6	2.0 NAA + 5.0 BAP	2.6±.84	10.40±3.82	5.6±1.26
7	1.0 TDZ	1.3±0.48	0.80±0.78	1.8±0.78
8	0.1 NAA + 1.0 BAP	11.10±1.59	30.60±2.87	18.80±1.39

*Data represent an average of 10 explants / treatment. Figures in parenthesis represent standard Deviation.

Table 2: Antibacterial activity of methanolic extracts of the rhizome of *Kaempferia galanga*

S. No.	Name of the drug	Conc. µg/disc	*E.coli* mm	*S.aureus* Mm	*S. typhi* mm	*B. subtilis* mm
1	Ciprofloxacin	100	26.00±0.81	32.50±1.29	30.75±0.95	40.25±1.25
2	Amoxicillin	100	25.75±0.95	21.25±0.95	19.75±0.95	19.75±0.47
3	Natural plant rhizome extract	1000	12.00±0.81	11.25±0.50	11.00±0.81	11.25±0.95
4	Micro propagated plant rhizome extract	1000	14.75±0.50	14.25±0.50	12.25±0.95	14.00±0.81

*The values (average of duplicates in 2 directions) of diameter of zone of inhibition at 100µg for Standards and 1000µg for extracts.

Table 3: DPPH free radical scavenging activity of methanolic extracts of the rhizome of *Kaempferia galanga*

S.No.	Concentration(µg/ml)	Natural Plant Extract	*In vitro* Plant Extract
1	100	43.23±0.32	41.83±0.58
2	200	51.49±0.65	47.23±0.76
3	300	74.52±0.73	72.38±0.37
4	400	82.43±0.44	82.22±0.82
5	500	87.17±0.42	86.37±0.56
6	600	94.68±0.60	92.25±0.87

*Data represent an average of four measurements. Figures in parenthesis represent standard Deviation.

Fig. 1: *In vitro* plantlet formation in *Kaempferia galanga*: (A). Initiation of growth (after 20days); (B). High frequency shoot multiplication and rooting after 40days of subculture; (C). Regenerated *Kaempferia galanga*; (D). Rhizome formation in *in vitro* plantlets transferred to the garden pot containing sand and soil.

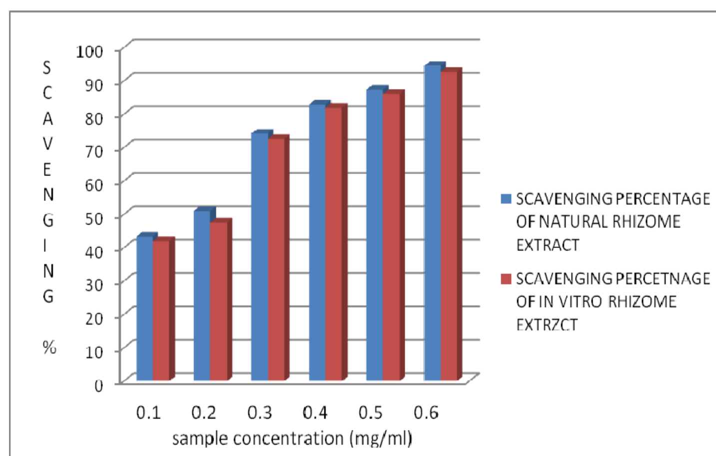

Fig. 2: Radical Scavenging activity of methanolic extracts of both natural and *in vitro* regenerated plant rhizome extract using 1,1-Diphenyl-2-picrylhydrazyl (DPPH).

CONCLUSION

Acclimatization of plantlets is the crucial phase where plantlets are in transition from *in vitro* phase to *in vivo* phase. Generally, higher sophistication (with controlled high humidity, temperature and accurate potting mixture) is required for higher plantlet survival. Simultaneous shoots and roots were observed on MS media supplemented with NAA + BAP (0.1mg + 1.0 mg/l). In the present case, acclimazation of Kaempferia plantlets has been achieved easily with 80-90% survival when transferred to a pot containing sand: soil (1:1) and rhizome development observed after 2-4 months of transferred into the pot. The entire procedure could be completed without callus formation. This was helpful in ex-situ conservation and large scale multiplication of these plants. The ethyl-p-methoxy cinnamate in both natural and in vitro regenerated plant rhizome was identified by using TLC in presence of reference standards. Here Rf value was found to be same as like Rf value of reference standards.

This is the first report which describes the comparison of antimicrobial activity and antioxidant activity between methanolic extracts of the rhizome of natural and *in vitro* regenerated plant. The antimicrobial activity was found to be significant in micropropagated plant compared to natural plant and antioxidant activity was found to be less in rhizome extract of micropropagated plant compared to natural plant.

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