

Earthworm, an in Vivo System to Screen Proliferative and Antimitotic Compounds

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Abstract— Amputated earthworm, *Eudrilus eugeniae*, possessing clitellum region has regenerating ability and develop into complete worms. Earthworm regeneration assay was developed based on this phenomenon and utilized to identify the presence of antimitotic compounds from the rhizomes of medicinal plant *Acorus calamus*. In continuation of that study, rhizomes of *A. calamus* was extracted with water and crude residue was obtained after evaporation. Subsequently, to separate the compounds from crude residue it was extracted with five different organic solvents individually such as benzene, chloroform, ethyl acetate, ethanol and methanol. All five different fractions were examined on earthworm regeneration assay to identify its antimitotic potential. Fractions extracted with benzene and chloroform did not inhibit regeneration as blastema was observed. In contrast fractions extracted with methanol and ethyl acetate inhibited the development of blastema. It suggests that methanol and ethyl acetate fractions might possess antimitotic compounds. TLC analysis with ethyl acetate fraction revealed two distinct bands. Compounds eluted from TLC plates decreased mitotic index of *Allium cepa* root tips and supporting the presence of antimitotic compounds. Surprisingly, fractions extracted with ethanol increased the regeneration capacity of the worms. Our results demonstrate that rhizomes of *Acorus calamus* possess both antimitotic and proliferative compounds. Hence we suggest that earthworms could be used as an in vivo system to screen both proliferative and antimitotic compounds from unknown sources.

Keywords- *Eudrilus eugeniae*, *Acorus calamus*, earthworm, antimitotic, proliferative, aqueous extract

I. INTRODUCTION

Medicinal plants are one of the major sources of active ingredients in pharmaceutical products throughout world [1]. Pharmacologically active plant-derived natural products provide leads to the development of new drugs [2]. Angiosperms are known for their scope in providing clinically valuable compounds to treat cancerous growth [3], [4]. Plants have also been source of antimitotic compounds,

Revised Manuscript Received on December 15, 2019.

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which have great potential to treat cancerous growth [5]. Screening of anticancerous or antimitotic compounds from natural resources is necessary to find new leads or effective drug molecules [6].

Many plants, cell culture or animal-based methods have been in practice to screen or validate the active compounds from natural products [7], [8], [9]. Cell proliferation assays such as dye exclusion methods, ATP assay, clonogenic cell survival assay and other methods using cell cultures are in use [10]. Well established in vivo systems to study compounds that induce proliferation are rare.

Many animals have the ability to regenerate new cells or tissues through cell proliferation [11]. However, earthworms have been recently suggested as one of the simple model systems to study regeneration of new tissues [12]. Earthworm species *Eudrilus eugeniae*, *Eisenia fetida*, *Lumbricus terrestris* and *Allolobophora chlorotica* have been reported to regenerate brains as well [13]. *E. eugeniae* is a segmented worm and has good regeneration ability when it possesses clitellum region. We have recently reported that regeneration ability of this worm can be used to validate antimitotic compounds from plant extracts [9]. Single medicinal plant may possess many different pharmacologically significant compounds. *Acorus calamus* plant is reported to have various compounds and is useful to treat cancer, arthritis, inflammatory diseases, diarrhea, dyspepsia, skin disorders, sinusitis, respiratory problems, fevers, bronchitis and other disorders [14]. Bioactive compounds can be separated either by sequential or differential extraction methods either with organic solvents or water. In this study we have used differential extraction method to separate compounds with antimitotic activity from proliferative activity in *A. calamus*. Different fractions partitioned from aqueous extract of *A. calamus* rhizomes have been tested on amputated earthworms. We report here that earthworm regeneration could be used to identify not only compounds with antimitotic property but also with potential in cell proliferation by using the medicinal plant.

II. MATERIALS AND METHOD

A. Plant materials & Chemicals

The rhizomes of *A. calamus* L. and bulbs of *Allium cepa* L. were purchased and authenticated as described earlier [9]. All chemicals and solvents were purchased in analytical grade from HiMedia or Merck Ltd., India.

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B. Preparation of rhizome extract

Rhizomes of *A. calamus* were washed thoroughly, air dried, and powdered. Powdered sample [1g] was extracted in 10 mL of sterile distilled water and kept overnight in a shaker at 30°C. Aqueous extracted samples were filtered by using Whatman No. 1 filter paper and followed by 0.2- μ m filter. Water was evaporated to dryness using a rotary evaporator at room temperature. The resultant residue stored in an air-tight container at 4°C until further use. The crude aqueous extract residue was differentially partitioned with five different solvents such as benzene, chloroform, ethyl acetate, methanol and ethanol. One ml of organic solvent was used for every 0.1g of crude residue obtained from aqueous extract. Subsequently, organic solvents were removed in fume hood. The residues obtained were again dissolved in sterile distilled water and tested with earthworm regeneration assay.

C. Culture and maintenance of earthworm

Adult earthworms, *E. eugeniae*, were collected from the Agricultural Support Centre, Tamil Nadu Agricultural University, Tirunelveli, Tamil Nadu, India. It was maintained in a plastic tub containing soil, cow dung, and leaf litter in appropriate moisture conditions.

D. Earthworm regeneration assay

Earthworm regeneration assay with amputated worms were followed as described earlier [9]. It was used to check the antimitotic potential of a known or unknown compound. Briefly, healthy adult worms were selected and amputated with a sterile scalpel blade on the 30th segment from the mouth. Amputated worms retain the anterior parts of the body including the clitellum region were kept in separate beds and monitored every 24 h, for the growth of the blastema. Earthworms were injected between the clitellum and the amputation region with 10 μ L of distilled water or other extract. Injections were performed at 24-h intervals for 8 consecutive days. Five worms were used for each treatment and all experiments were performed at least 3 times.

E. Thin Layer Chromatography

Crude residue obtained from aqueous extract of rhizomes was extracted with ethyl acetate. For every 1 g of dried sample, 10 ml ethyl acetate was used for extraction. Aliquot of 10 μ l was loaded in precoated TLC plate and the sample was resolved by the solvent, petroleum ether. TLC plate was stained with iodine vapors and Rf values were calculated.

F. Allium cepa root tip assay

Onion bulbs with newly germinated roots were incubated for 16 hours with distilled water or compounds eluted from TLC plates. Onion bulbs treated with distilled water were used as the control. Following the incubation period, root tips were fixed with ethanol and acetic acid and subsequently treated with 1 N hydrochloric acid. Root tips were washed and stained with 0.5% safranin stain (W/V) before observing under a light microscope. Chromosome morphology and mitotic stages were observed to calculate mitotic index. The mitotic index was calculated as indicated earlier [9].

G. Statistical analysis

Statistical analysis was performed (mean, standard deviation, and standard error) with unpaired Student's t-test

in the software tool GraphPad Prism 6.0. $P < 0.05$ was considered statistically significant.

III. RESULTS AND DISCUSSION

Phytochemical analysis of *A. calamus* has been mostly done with organic solvents and many compounds reported are hydrophobic (Table 1). Asarones are major compounds identified in rhizomes and leaves of this plant. Phytochemical studies showed that asarones of *A. calamus* are lipophilic compounds [15]. Asarones have been shown to possess activity to treat pain, inflammation and tumor [16]. We are interested in identifying hydrophilic compounds from this rhizome and hence water was initially used to extract the compounds. We have recently shown that aqueous extract of *A. calamus* possesses antimitotic activity by using earthworm regeneration ability [9]. Aqueous extract of rhizomes of *A. calamus* would possess many compounds. The dried powder obtained from aqueous extract was differentially fractionated with five different solvents such as benzene, chloroform, ethyl acetate, methanol and ethanol. Organic solvents were evaporated following the extraction and dissolved again in water before injection into worms.

Fractions partitioned from aqueous extract were examined on regeneration of tissues in amputated earthworms as described earlier [9]. Samples of sterile distilled water (control) or fractions extracted were injected once every 24 hours for a period of 8 days and monitored for development of blastema. Wounds in amputated region of all the worms were healed within 24 hours. Initiation of blastema from the amputated region was observed from 3rd day in control worms and developed well after 8 days (Fig 1A). Development of blastema was found in worms injected with fractions partitioned from benzene, ethanol and chloroform (Fig 1C, D and E). It suggests that these three fractions may not have compounds with antimitotic property. Regeneration from amputated region was not detected in worms injected with fractions partitioned from methanol and ethyl acetate, until 8 days from amputation (Fig 1B and F). It suggests that these two fractions may possess the compounds that arrest cell division. Surprisingly, the fraction isolated using ethanol induced the regeneration from wounded region much better than controls. It indicates that ethanol fraction may have compounds that promote cell division or other factors that are necessary for growth of new cells.

Although fractions partitioned from ethyl acetate and methanol inhibited blastema development, lethality of worms were approximately 50% when methanol extracted samples were tested. Methanol fractions might have had compounds that were cytotoxic and hence it was not analyzed further. Ethyl acetate and ethanol fractions were injected into

Table-I: Phytochemicals in *A. calamus*

S.no	Compound Name	Solvent	Extracted with solvent
1	Benzene,1,2-dimethoxy-4-(2-propenyl)	Organic	Ethanol
2	Shyobunone	Organic	Ethanol
3	Octadecadienoicacid	Organic	Ethanol
4	β Asarone	Organic	Ethanol, Propanol
5	Tetracyclo(6.2.1.0(3.8)0(3.9))undecanol4,4,11,11-tetramethyl	Organic	Ethanol
6	Trimethoxyamphetamine,2,3,5	Organic	Ethanol
7	Pyrimidin-2-one,4-(N-methylureido)-1-(4-methylaminocarbonyloxymethyl)	Organic	Ethanol
8	4a,7-Methano-4Ah-naphth(1,8a-b)oxirene,octahydro-4,4,8,8-tetramethyl	Organic	Ethanol
9	n-Hexadecanoic acid	Organic	Ethanol
10	Preisocalamendiol	Organic	Propanol
11	B-ocimene	Organic	Propanol
12	Methyleugenol	Organic	Propanol
13	Asarylaldehyde	Organic	Chloroform
14	Acoramone	Organic	Chloroform
15	s-cadinol	Organic	Ethanol

worms and the development of blastema was observed very closely until 8th day of amputation. Worms injected with sterile distilled water had blastema development from 3rd day (Fig 2A) grown gradually on 5th and 8th day (Fig 2 B and C). Aqueous extract of rhizomes was used as a positive control as it has already been shown to have antimetabolic compounds [9]. No growth from amputated region was observed on 3rd, 5th or 8th day from aqueous extract of rhizomes (Fig 2 D, E and F). It corresponds well as we reported earlier [9]. Similarly, ethyl acetate fraction injected worms did not develop any blastema on 3rd, 5th or 8th day (Fig 2 G, H, I). It suggests that compounds that were originally responsible for antimetabolic activity in the aqueous extract were partitioned well with ethyl acetate. Generation of blastema was marginally better on 3rd day in worms injected with fractions from ethanol compared to controls (Fig 2 J). Growth of blastema was better after 5th day (Fig 2 K) and become distinct after 8 days (Fig 2L). It shows that ethanol fraction did not have significant amount of antimetabolic compounds. Conversely, this fraction possesses compounds that play significant role in enhancement of cell division, growth or regeneration. The activity of compounds responsible for proliferation might have been suppressed by the presence of antimetabolic compounds in aqueous extract. Further analysis on ethanol fraction would reveal the properties of compounds responsible for proliferation. These results clearly suggest that earthworm's regeneration system could be utilized to examine various fractions and narrow down to fewer compounds from the crude extract.

Extraction of *A. calamus* rhizome powder directly with absolute methanol or ethanol resulted in getting α or β asarone compounds [15, 17]. Many therapeutic properties of this medicinal plant, including anticancer, is attributed toward these two asarones [18, 16]. Extraction with ethanol and subsequent partition with petroleum ether showed the presence of α -asarone and had insecticidal activity [19]. These data suggest that asarones are highly hydrophobic and

getting extracted with organic solvents. Interestingly, ethanol extracts partitioned subsequently with ethyl acetate, which was devoid of β -asarone, enhanced adipocyte differentiation in 3T3-L1 cells and the identified compound was named as 22-((6-deoxy- α -L-rhamnopyranosyl)oxy)-3,23- [20]. It indicates that compound which has activity towards differentiation of adipose tissue act better when they are devoid of compound like β -asarone. It corresponds well with our data where aqueous fraction inhibited blastema development, however, the ethanol fraction partitioned from aqueous extract induced cell proliferation. It suggests that compounds that induce proliferation are suppressed by compounds that inhibit cell division. It also depends on the concentration of both compounds available in the extract.

However, when the extract was done with 50% ethanol, amelioration of nephrotoxicity was found in experiments with mouse [21]. In another report, 50% ethanol extract showed anti-inflammatory and anti-oxidative response in mouse [22]. These results suggest that rhizome possess many compounds and partitioned differently based on the extraction procedure. Aqueous extract of leaves of *A. calamus* has been shown to have anti-inflammatory activity by using HaCaT cells [23]. Aqueous extract of rhizomes has also been shown to have wound healing and anti-inflammatory activity *in vivo* and *in vitro* respectively [24]. These data strongly suggest that aqueous extract possess wound-healing compounds. Proliferation of cells in amputated worms in ethanol partitioned fractions could be due to the presence of wound healing, anti-inflammatory or anti-oxidative compounds. To identify the compounds which inhibited blastema development, fractions partitioned with ethyl acetate from crude residue obtained from aqueous extract was analyzed in TLC plates with petroleum ether as mobile phase. Two spots having Rf value of 0.03 and 0.06 were found after staining with iodine (Fig 3). Compounds from both spots were scratched off from the Preparative TLC plates and examined for their antimetabolic potential using *Allium cepa* root tips. Many numbers of cells in the root tips were going through different mitotic phases in the samples treated with water. However, the number of mitotic phases were less in samples treated with ethyl acetate fraction. Mitotic index was calculated and found that it significantly reduced in *A. cepa* root tips that were treated with compounds isolated from TLC compared to control samples [Fig 4]. Extraction of rhizomes and leaves of *A. calamus* with organic solvents and subsequent analysis with TLC has shown to have α -asarone and β -asarone [25].

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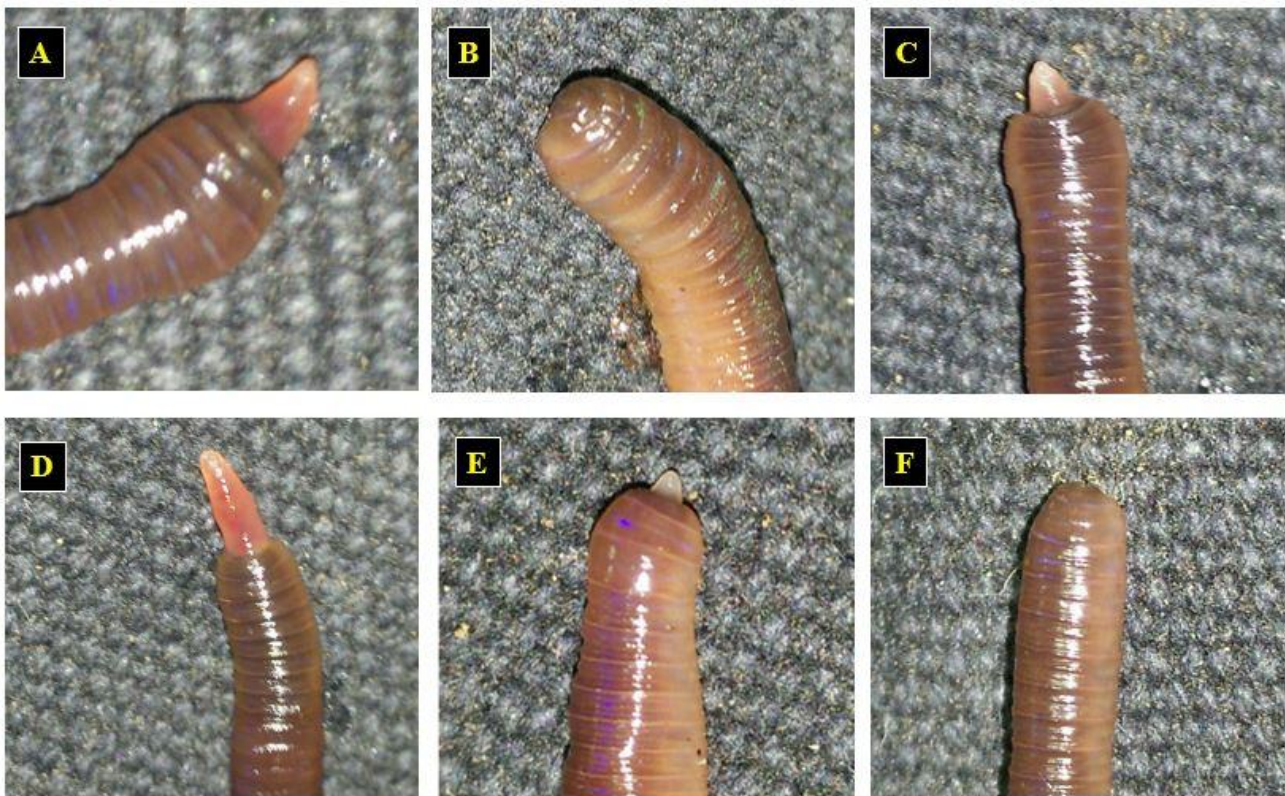


Fig.1 Evaluation of blastema development in *E. eugeniae* with differential extraction of *A. calamus*.

The worms were injected once in 24 hrs for a period of 8 days with sterile water [A] or the aqueous extract of rhizome partitioned with methanol [B], benzene [C], ethanol [D], chloroform [E] or ethyl acetate [F]. All photos were taken on 8th day. Blastema development was not seen in worms injected with aqueous extract partitioned with methanol [B] and ethyl acetate [F]. The regeneration of new tissues was found better from extract partitioned with ethanol [D] compared to control [A], benzene [C] or chloroform [E].

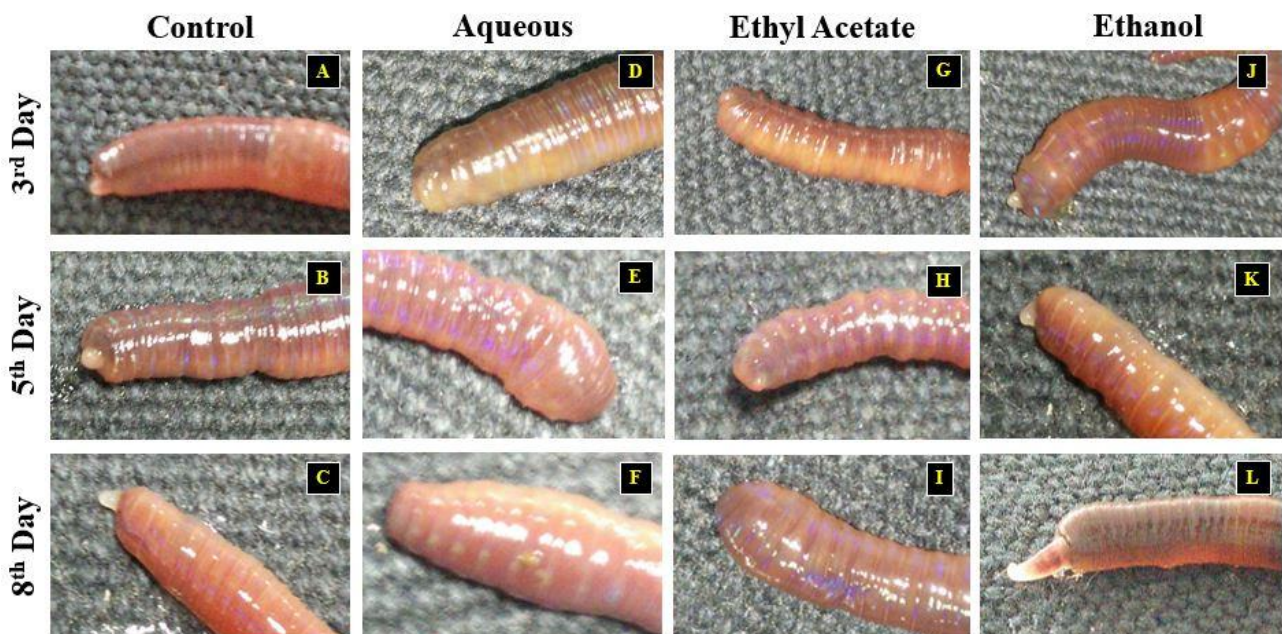


Fig 2. Proliferative and antimitotic compounds from aqueous extract of *A. calamus*.

The control worms were injected with distilled water once in 24 hrs for a period of 8 days and the development of blastema was photographed after 3rd [A], 5th [B] and 8th day [C]. Aqueous extract of *A. calamus* rhizomes was injected similarly for 8 consecutive days and blastema development was not found after 3rd [D], 5th [E] and 8th day [F]. Ethyl acetate extract partitioned from aqueous extract injected worms did not show any regeneration after 3rd [G], 5th [H] and 8th day [I]. Ethanol extract partitioned from aqueous extract injected worms showed better proliferation from amputated region after 3rd [J], 5th [K] and 8th day [L].

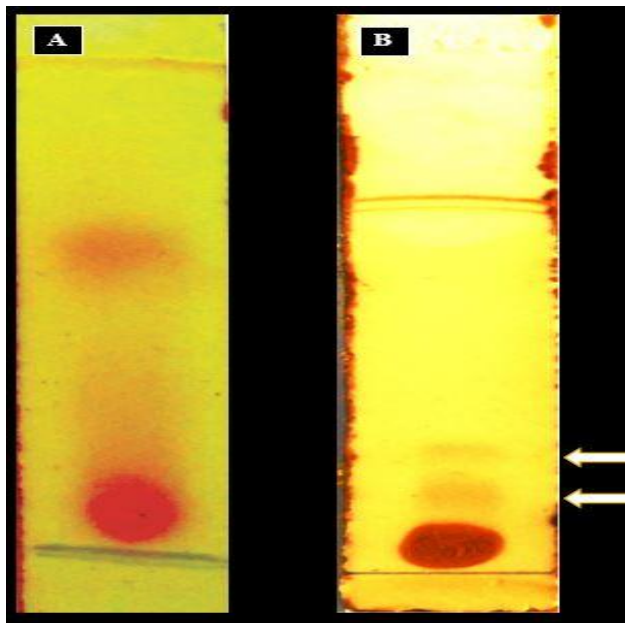


Fig 3. TLC analysis of *A. calamus* extracts.

Aqueous extract of rhizomes [A] or ethyl acetate fraction from aqueous extract [B] were loaded on precoated silica gel plates and mobilized with petroleum ether. Photographs were taken after staining in iodine chamber. Arrows indicate two bands found in sample partitioned with ethyl acetate.

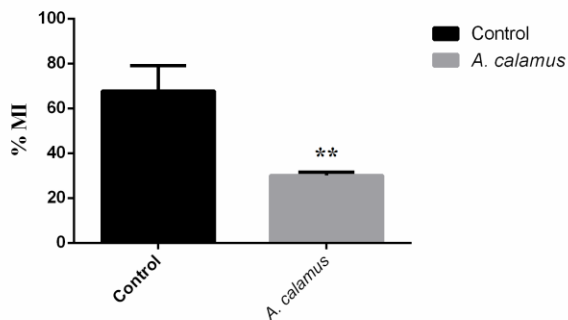


Fig 4. Inhibition of cell division in *A. cepa* root tips by ethyl acetate fraction.

The graph shows percentage of cells observed in root tips with four phases of mitotic cell division in water-treated control samples or ethyl acetate fraction partitioned from aqueous extract of *A. calamus*. Mean SEM bar is shown and ** indicates the significant difference.

Hence we extracted the rhizomes initially with sterile water, lipophilic compounds like asarones would have not been extracted. Our results with TLC suggest that fractions partitioned with ethyl acetate possess antimutagenic compounds. Further experiments are required to provide more knowledge on these compounds. Interestingly, earthworms' regeneration ability has been utilized recently to study the role of TCTP protein in development of blastema from amputated worms [26]. Taken together, our data strongly suggest that earthworms could be used as a simple *in vivo* system to identify both antimutagenic and proliferative compounds by utilizing their regeneration ability. Similar compounds from any medicinal plant or other sources could be tested by utilizing earthworms.

ACKNOWLEDGMENT

This research was supported by the Kalasalingam Academy of Research and Education (KARE), deemed to be university, Krishnankoil, Tamil Nadu, India. Mr. C. Rajesh and Mr. R. Prakash are thankful to KARE for receiving the research fellowship.

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