

Safety Evaluation of *Syzygium jambolanum* on the Development of Zebrafish Embryos

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ABSTRACT

Background: Gestational diabetes mellitus, a hyperglycaemic condition that develops at the time of pregnancy, causes approximately 7% of complications during pregnancies world wide. *Syzygium jambolanum* has been proved clinically for the management of diabetes mellitus and is being prescribed for the management of diabetes in pregnant women. **Objectives:** This study has been designed to evaluate the safety of *Syzygium jambolanum* mother tincture on the development of zebrafish embryos. **Materials and Methods:** Zebrafish embryos were exposed to 1%, 5% and 10% of *Syzygium jambolanum* mother tincture with the same concentration of alcohol. Observations like mortality, hatching at 24, 48 and 72 hr post fertilization and yolk sac edema, length, eye width, pericardial edema and heart rate were recorded at 72 hr post fertilization. The locomotor activity was recorded at 7 day post fertilization. **Results:** Results showed that mortality in zebrafish embryos with the treatment of *Syzygium jambolanum* mother tincture at 10% could be due to alcohol as similar results were observed with 10% alcohol also. Moreover, changes observed in heart beat in embryos with the treatment of alcohol at different concentration were reduced by the *Syzygium jambolanum* indicating its possible protective role in cardiac parameters. **Conclusion:** Results suggest the safety of *Syzygium jambolanum* mother tincture at low concentrations (1% and 5%) on development of zebrafish embryos. The embryo toxicity induced by 10% concentration of *Syzygium jambolanum* may be attributed to the ethanol. Further developmental toxicity studies on animals need to be carried out to validate the results obtained followed by clinical validation.

Key words: *Danio rerio*, Development toxicity, Embryo toxicity, *Syzygium jambolanum*, Zebrafish.

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INTRODUCTION

Syzygium jambolanum (L.) Alston (*Syzygium cumini*; *Eugenia jambolanum* etc.) is commonly known as rose apple, Jamun, or black plum and it belongs to Myrtaceae family. It is mostly found in Southeast Asia and is widely distributed in India mainly in the north-eastern region. Due to the medicinal properties of *S. jambolanum*, each part of the plant has been used for the treatment of several disorders in the natural medicinal systems.¹ The seed of syzygium has always remained to be a keen area of interest among scientific communities as it has an enormous

source of phenolic acids,² flavonoids like quercetin, rutin, kaempferol myricetin, etc.³ Many experimental studies have reported that it exhibited antioxidant,^{1,4} anti-inflammatory,⁵ antibacterial,⁶ antifungal,⁷ anti-diabetic,⁸ anti-HIV,⁹ analgesic and antipyretic activities¹⁰ and genoprotective activities.¹¹

In homeopathy, *Syzygium jambolanum* mother tincture (S.J. ϕ) i.e. ethanolic seed tincture is used as a remedy in the management of diabetes mellitus and gestational diabetes. S.J. ϕ was reported to have active chemical



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constituents like Jamboline glycoside, ellagic acid, tannin and Gallic acid.¹²

S.J. ϕ has been found to show a protective effect on carbohydrate and lipid metabolic disorders in experimental diabetic rats.¹³ S.J. ϕ and 6C, 30C potencies were found to exhibit anti-diabetic effects by activating insulin signalling molecules in the skeletal muscle of diabetic rats.¹⁴ Furthermore, *in vitro* studies of S.J. ϕ have reported antiglycation and cell protection ability¹⁵ confirming its anti-diabetic activity. According to data, gestational diabetes mellitus is one of the major concern and it is necessary to check the safe effect of anti-diabetic remedies on embryo when it consume during pregnancy.¹⁶⁻²⁴

Zebrafish (*Danio rerio*), is widely applied as an alternative animal model for high throughput screening of drugs. *Danio rerio* is small in size, easy to breed, cost-effective, having less generation interval and genetic homology to humans. The most striking feature of this model is transparent embryos provide a great opportunity to observe morphological developmental processes and a high level of structural conservation^{25,26} as well as very readily assessment of developmental abnormalities in zebrafish embryos like a disturbance in length, retina size, heart rate, edema in pericardial and yolk sac.²⁷ These features of Zebrafish and its embryos provide advantage over other models and led this model to be used in the determination of cytotoxicity of number of solutions, chemical agents and natural drugs/plant extracts.^{28,29} Clinically S.J. ϕ is also used to manage gestational diabetes but reports on the developmental effects of S.J. ϕ on developing embryos are not available. Hence, this study was planned to investigate the developmental effects and safety of S.J. ϕ on zebrafish embryos compared to ethanol at same concentrations.

MATERIALS AND METHODS

Drug Preparation

Homoeopathic mother tincture (ϕ) of *Syzygium jambolanum* was prepared and standardised in the laboratory as mentioned in Homoeopathic Pharmacopoeia of India (HPI). Briefly, 100g material was weighed and poured in a specified concentration of alcohol. The material was kept for a given period of time. After specified time, the tincture was separated from material and store in amber colour glass bottle. Alcohol and S.J. ϕ were diluted to 1, 5 and 10% concentrations using medium used for embryo development (embryo medium; 5 mmol/L NaCl, 0.33 mmol/L CaCl₂, 0.33 mmol/L MgSO₄·7H₂O, 0.17 mmol/L KCl).³⁰

Zebrafish Husbandry and Management

Zebrafish were housed in 7 different groups having 10 Zebrafish/ 5L tank in clean poly-sulphone filled with filtered, charcoal and UV treated water. The environment of the zebrafish facility was kept controlled with a temperature at 27-29°C, light/dark cycle of ~14/10 hrs and pH- 6.8-7.5. The fishes were provided constant filtration and aeration.³⁰ Zebrafish was fed with dry brine shrimp tetrabits diet manufactured by Tetra GmbH, Germany, thrice a day.

Zebrafish breeding and collection of eggs

On the day before the test, male and female zebrafish (1:2) were transferred to the breeding tank a few hrs before the onset of darkness. Spawn traps were placed into the tanks to collect the eggs.^{31,32} The light was switched on the next morning. The process of mating, spawning and fertilisation approximately completed within 45 min. The fish were returned to their home tank and the spawn traps with the collected eggs were carefully removed and the eggs were placed in the Petri dishes containing embryo medium.

Egg differentiation

At 26°C, fertilised eggs were collected after 15 min post-fertilization. At this stage, fertilised eggs were identified and selected for the study by using an inverted microscope (RTC-7, Radical scientific equipments Pvt. Ltd., Ambala, India).

Grouping

Fertilised eggs were divided randomly into seven groups ($n=10$) namely; Group I; Water (Control), Group II-IV; Alcohol (ethyl alcohol) (Alc 1%,5% and 10%) and Group V-VII; S.J. ϕ (S. J. 1%,5% and 10%) in 24 well plates for further experiment. In the initial experiments carried out at our facility, alcohol at more than 10% was found to be lethal to embryos. Therefore to minimize the toxic effect of vehicle, the concentration of alcohol and test drug was kept 10% or less for this study.³³

Exposure of drugs

Each group of embryos was exposed to its corresponding concentration of drug for a period of one hr after 3 hr post fertilisation (hpf) of embryos. In zebra fish embryo development, Blastula period represents a key phase which occurs from 2 hpf to 5 hpf approximately. During 3 hpf-4 hpf blastula period, embryo acquires distinctive features like acquiring a spherical shape, formation of yolk sac nuclei with meta synchronous division. Hence, in the present study effects of one hour exposure of S.J. ϕ during the initial phases of embryonic development i.e. 3-4 hpf was

investigated.²⁶ In parallel, control (embryo medium) and alcohol groups in the same concentration were also exposed.

Brief Procedure

The procedure for the zebrafish embryo toxicity test (FET) was adopted from several standard methods.^{34,35} Zebrafish were approved by the Institutional Animal Ethics Committee (IAEC), DDPRCRIH (DDPR-CRIH/Pharmacology/CPCSEA/IAEC/2018/005). The test was initiated immediately after fertilisation of the eggs and terminated after 7 days post fertilisation (dpf) or 168 hpf. Fertilized randomized viable eggs were transferred to 24-well plates. The plates were pre-conditioned for 24 hr with solutions and refilled with 2 ml/well freshly prepared test solutions. The exposure time of embryos with the test drug was one hour. After one hour, test solutions were replaced by an embryo medium for the entire duration of the study. The embryos were incubated at 26°C with the replacement of embryo medium on daily basis.

Observations

Observations were recorded on each tested embryo group under RTC-7 inverted microscope to confirm the morphological characteristics and changes. The following parameters were observed by blinded observers periodically: mortality, hatching, coagulation, on 24, 48 and 72 hpf and heartbeat, pericardial edema, yolk sac edema, length and eye-width was recorded on 72 hpf. Morphometric image analysis was done using Image-J analysis software. Heartbeat was recorded for one minute on camera and after that counted by a blinded observer. On the 7th day after fertilization (168 hpf), the parameters of locomotor activity: total distance traveled and average speed. Briefly, the well plate was divided into the quadrant and the locomotor activity was recorded by making protocol with the help of video tracking software (ANY maze software, 5.21).

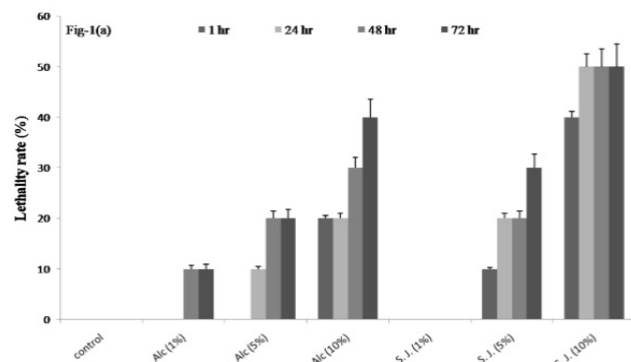


Figure 1(a): Effects of one-hour exposure of S.J. φ (1, 5 and 10%) on % of mortality rate.

Statistical Analysis

The mean \pm standard error (SEM) of different values from each sample was calculated. One-way analysis of variance (ANOVA) was used for statistical analysis of data followed by Tukey's *post hoc* test to monitor significance among test groups with control and its corresponding alcohol group. The comparison between the groups was considered significant having $^a p < 0.05$.

RESULTS

Effect of S.J. φ (1, 5 and 10%) on the mortality of zebrafish embryos

The effect of different concentrations of S.J. φ (1, 5 and 10%) on the mortality rate is illustrated in Figure 1(a). Alcohol and S.J. (φ) at 10% showed mortality which may be due to alcohol.

Effect of S.J. φ (1, 5 and 10%) on hatching rate of zebrafish embryos

The effect of S.J. φ (1, 5 and 10%) on the hatching rate is depicted in Figure 1(b). The result showed that S.J. φ 1% at 48 hpf whereas 5% at 48 and 72 hpf delayed hatching as compared to control and alcohol groups.

Effect of S.J. φ (1, 5 and 10%) on morphological changes in zebrafish embryos

After fertilization, the embryos were exposed with S. J. φ at 1, 5 and 10%; and the images at 72 hpf, Figure 2(a) shows significant morphological changes; yolk sac edema 2(b), pericardial edema 2(c), length 2(d) and eye width 2(e) in larvae. Results of Figure 2b revealed that yolk sac edema was not found in S. J.; treated groups whereas alcohol at 10% showed significant edema ($^a p \leq 0.05$) as compared to the control group.

Pericardial edema in zebrafish embryo (Figure 2c) had significantly occurred with S.J. φ 5% ($^a p \leq 0.05$) as compared to the control group.

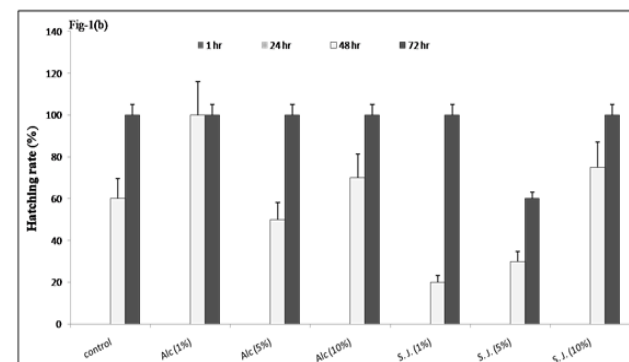


Figure 1(b): Effects of one-hour exposure of S.J. φ (1, 5 and 10%) on % of hatching rate of survived zebrafish embryos.

As seen in Figure 2(d), the length of the zebrafish larvae was not changed in S.J. ϕ treated groups whereas a significant increase ($^ap \leq 0.05$) in length was observed in the alcohol 10% group as compared to the control group.

No significant ($^ap \geq 0.05$) effect was observed with one hr exposure of S.J. ϕ on eye width as compared to the control group whereas S.J. ϕ at 1% significantly decreased ($^bp \leq 0.05$) the eye width as compared to alcohol 1% group as depicted in Figure 2(e).

Effect of S.J. ϕ (1, 5 and 10%) on heart rate

The effect of S.J. ϕ on cardiac function was observed by recording the heartbeats at 72 hpf (Figure 3). At 72 hpf, the heart rate was significantly increased ($^ap \leq 0.05$) with all concentrations of alcohol as compared to control. S.J. ϕ at 1 and 5% significantly decreased the increased effect of alcohol 1 and 5% concentration that is equivalent to the control group. There was a significant increase in a heartbeat ($^ap \leq 0.05$) in 10% of the S.J. ϕ group which may be the effect of alcohol as the same effect observed with alcohol 10%.

Effect of S.J. ϕ (1, 5 and 10%) on behavior parameters

The effect of different concentrations of S.J. ϕ on the behavior of zebrafish embryos in terms of locomotor activity at 7 dpf is demonstrated in Figure 4. *Post hoc* analysis revealed that there was no alteration found in total distance traveled (Figure 4a) and average speed

(Figure 4b) of the larvae at 7 dpf in treated groups ($^ap \geq 0.05$) when compared to control.

DISCUSSION

Safety evaluation of Homeopathic medicines mainly those are prescribed in Mother tincture form and of plant-derived is pre-clinically unexplored despite having good pharmacological effect. Among them, several homeopathic medicines are prescribed in pregnancy but their effects on developing embryos are not investigated by scientific methods. Zebrafish embryos have recently emerged as a reliable method in assessing the effects of herbal medicines as well as synthetic compounds on embryonic development.³⁶⁻³⁸ From the literature survey it has been found that S.J. ϕ is widely prescribed for managing diabetes as well as for gestational diabetes.

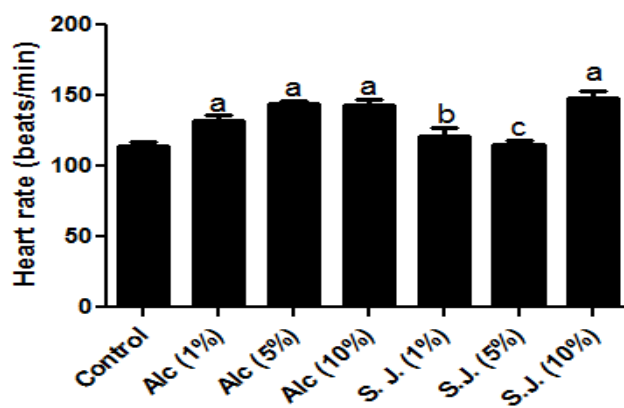


Figure 3: Effects of one-hour exposure of S.J. ϕ (1, 5 and 10%) on the heart rate of embryos at 72 hpf. All values are Mean \pm SEM (n=6), $^ap \leq 0.05$ when compared to control group, $^bp \leq 0.05$ compared to alcohol-1% and $^cp \leq 0.05$ as compared to Alc-5% (One-way ANOVA subsequently Tukey's *post hoc* test).

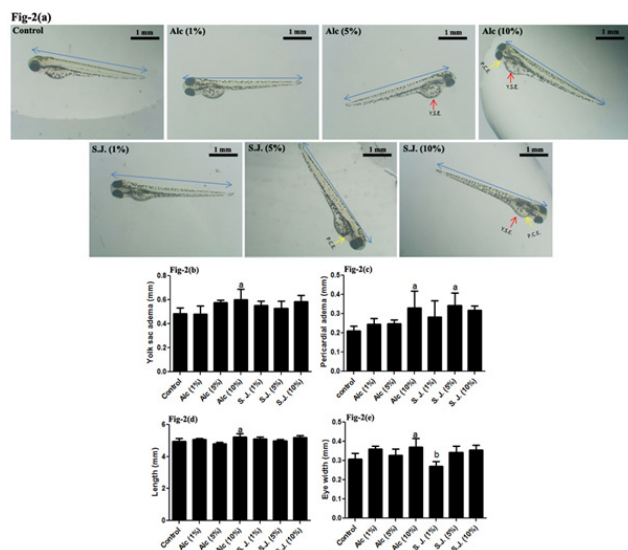


Figure 2: Larvae images at 72 hpf (a), effects of one-hour exposure of S.J. ϕ (1, 5 and 10%) on yolk sac edema (b), pericardial edema (c), length (d) and eye width (e) of the zebrafish embryo. Different values from each group are Mean \pm SEM (n=6), $^ap \leq 0.05$ as compared to control, $^bp \leq 0.05$ as compared to Alc-1% (One-way ANOVA subsequently Tukey's *post hoc* test).

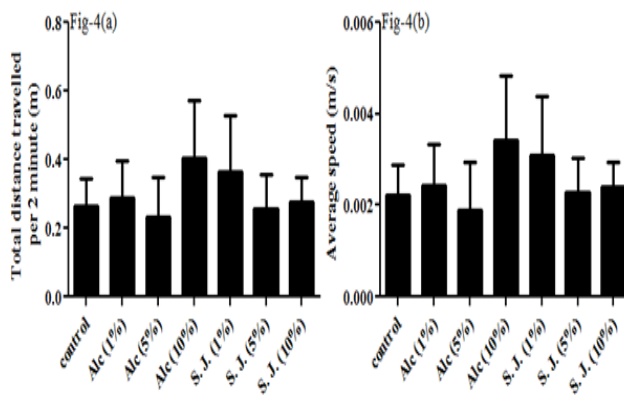


Figure 4: Effects of one-hour exposure of S.J. ϕ (1, 5 and 10%) on the behavior of zebrafish larvae at 7 dpf in terms of locomotor activity (total distance traveled; a and average speed; b). All values are calculated as Mean \pm SEM (n=6), the analysis was done using one-way ANOVA followed by *post hoc* (Tukey's) test.

To explore its safety aspects, the present study was designed to find out the developmental effects of S.J. ϕ on zebrafish embryos.

For the assessment of the safety of the embryos, primary observational parameters are an effect on mortality and hatching. In addition to this, following observational parameters like effect on the heart (lack in heartbeat, pericardial adema), yolk sac deformities, the effect on length, eye width and behavior were selected as these are the target sites of any drug that may cause toxic effect at a very initial stage. The selected observations were similar in earlier embryonic and teratogenic studies carried out on different chemicals reflecting the predictive power of zebrafish embryo bioassay for embryonic development in mammals.^{36,39,40} From the previous data reported³³ which was further verified at our lab also in initial experiments of alcohol for 1 hr showed significant mortality when concentration of alcohol was more than 10%. Some mortality was observed at 10% concentration, however it was non-significant. Hence 10% concentration was taken as the highest concentration along with 5% as middle and 1% as the lowest dose for further experiment.

Coagulation of embryo and lack of heartbeat were considered as indicators of mortality during embryogenesis. In this study, S.J. ϕ at 10% concentration showed mortality [Figure 1(a)] in zebrafish embryo comparable to the control group (alcohol at 10%) that indicates the embryotoxic effect of S.J. ϕ may be due to alcohol which needs further in-depth investigation.

For the transformation from embryo to larva hatching is an important process^{41,42} and normally, hatching occurs between 48 to 72 hpf.⁴³ In the current study, 1 and 5% concentration of S.J. ϕ showed a little delay in the hatching of survived embryos [Figure 1(b)]. This change may be due to alteration in hatching enzyme activity and the movement of embryos.⁴²

Morphological developments play an important role in the evaluation of early-stage developmental effects of many compounds. The yolk sac provides nutrients to the embryo inside the chorion before 4 dpf.^{44,45} No significant changes were observed in yolk sac edema [Figure 2(b)] as well as in length [Figure 2(d)] after exposure of S.J. ϕ groups as compared to control group. Whereas alcohol 10% showed significant yolk sac edema which was not observed in S.J. ϕ 10% which may be the indication of the masking effect of drug upon alcohol effect. The eye width of zebrafish larvae was not affected [Figure 2(e)] after the exposure of S.J. ϕ .

In the present study, pericardial edema and changes in the heartbeat of zebrafish larvae were noted as the sign of heart malformation. S.J. ϕ 5% showed a significant

increase in pericardial edema [Figure 2c] compared to control. Besides, at 72 hpf, S.J. ϕ was ameliorated alcohol-induced increased heartbeat at 1% and 5% concentration [Figure 3]. This effect on heartbeat may be due to the diminishing effect of S.J. ϕ on alcohol in the early life stages of development which needs to be further studied.

The swimming pattern is one of the important parts of behavioural research when the model used is an aquatic organism. In the present study, S.J. ϕ did not affect the behavioural parameters (total distance travelled, average speed) of larvae indicating that S.J. ϕ 1 hr exposure may not have significant CNS effects [Figure 4(a) and 4(b)].

CONCLUSION

From the present study it may be concluded that safety of *Syzygium jambolanum* ϕ at low concentrations (1% and 5%) on development of zebrafish embryos. The embryo toxicity induced by 10% concentration of *Syzygium jambolanum* may be attributed to the ethanol. In addition, *Syzygium jambolanum* ϕ was found to be effective against the alcohol-induced heart malformation. This result suggests the possibility of the effective role of S.J. ϕ in diabetic patients with cardiac complications. Further developmental toxicity studies on animals need to be carried out to validate the results obtained followed by clinical validation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

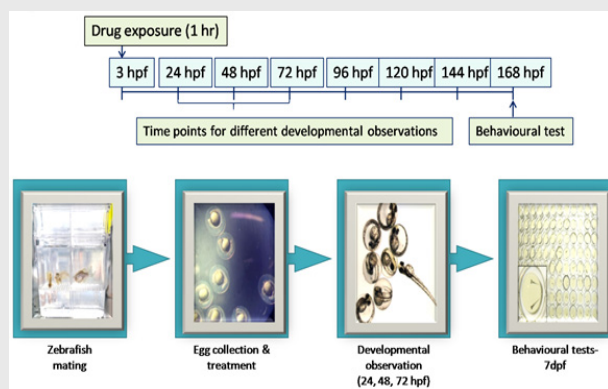
S.J.: *Syzygium jambolanum*; **HIV:** Human immunodeficiency virus; **ϕ :** Mother tincture; **C:** Centesimal; **GDM:** Gestational diabetes mellitus; **HPI:** Homoeopathic Pharmacopoeia of India; **g:** gram; **%:** Percentage; **ltr:** Litre; **UV:** Ultraviolet; **°C:** Degree centigrade; **hrs:** Hours; **min:** Minute; **Alc:** Alcohol; **hpf:** hours post fertilisation; **FET:** Fetal embryo toxicity test; **IAEC:** Institutional Animal Ethics Committee; **dpf:** days post fertilisation; **ml:** millilitre; **SEM:** Standard

error of mean; **CNS**: Central nervous system; **ANOVA**: Analysis of variance.

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PICTORIAL ABSTRACT



SUMMARY

Based on results, it may be summarized that *Syzygium jambolanum* ϕ at a higher concentration (10%) showed mortality which might be alcohol-induced also. Despite mortality at higher concentrations, *Syzygium jambolanum* ϕ at different concentrations found to be beneficial against the alcohol-induced heart malformation suggested the possibility of the effective role of S.J. ϕ in diabetic patients with cardiac complications. Therefore, it can be concluded that *Syzygium jambolanum* ϕ at low concentration was found to be safe in the early life stage of zebrafish embryos. However, detailed developmental toxicity assessment should be carried out at employing higher animal models for better clinical correlation.

About Authors



Mahima Sharma, working as Research Associate (Pharmacology) at Department of Pharmacology at Dr. D P Rastogi, Central Research Institute for Homeopathy, Noida. She has expertise in pre-clinical rodents as well as zebrafish animal studies. She established and developed Zebrafish and Microbiology lab for research work. She has presented her research work at number of national & international platform and got many awards. She published many articles in peer reviewed journal.



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Dr. Arun Kumar, did Ph.D life Sciences from Defence R&D Organization (DRDO), Delhi and have expertise in pre-clinical studies of drug discovery including toxicity, efficacy, and molecular mechanism of drugs in both *in-vitro* and *in-vivo* models. He has many publications, awards and professional memberships in his credits. He served as molecular biologist at CRIH, Noida.



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