Safety Pharmacological studies of *Kalyanaka ghrita*

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ABSTRACT

Safety pharmacology is a study of unfavorable, pharmacodynamic effects of a drug on physiological functions with therapeutic range by using International Conference on Harmonization (ICH) S7A guidelines. The cardiovascular, central nervous, and respiratory systems are most affected by pharmacological side effects, resulting withdrawal of multiple medications from the market. *Kalyanaka ghrita* (KG) is an *Ayurvedic* formulation with ghee as a major basic component, though a promising candidate in treatment of AD, KG has not been documented for its safety profile, which prompted the study. In this study we evaluated safety pharmacology of KG oral (4, 2, 1g/kg), and nasal (100, 50, 25 μl/rat), in Wistar rats for 28 days subjected to CNS, CVS and the respiratory safety profile was evaluated on day 0, 14 and 28. At the end of the study the nasal turbinate was evaluated histopathologically. In the present study KG did not cause any significant change in CNS profile. However KG treatment had increased the grooming and rearing behaviors, which were not significant compared to vehicle control and did not cause change in CVS and respiratory profile upon treatment with KG for 28 days. The epithelium of nasal turbinate of animals was found intact after 28 days of nasal administration. After sustained dosing, the KG oral and intranasal treated groups showed no harmful events, which illustrates the CNS, CVS and respiratory safety profile of *Kalyanaka ghrita*.

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INTRODUCTION

Nonclinical pharmacological studies are categorized as primary pharmacology, secondary pharmacology, and safety pharmacological studies which a crucial role in the drug development and discovery process. Primary and secondary pharmacological studies deal with the mechanism of action, effects related and unrelated to therapeutic targets. The safety pharmacological studies play an important role in identifying potential undesirable pharmacodynamics effects on physiological functions when exposed in the therapeutic dose or more. In drug development process, safety pharmacological studies are conducted prior to first in human trials (FiH) (Bass *et al.*, 2004). Safety pharmacological studies are performed in the early stages of drug development to quantify and mitigate hazards associated with a therapeutic candidate, identify the lead compound using hazard recognition, and reject the novel chemical entity using safety liabilities.

The origins of the safety pharmacology guidelines were established in the international guidelines that are International conference on harmonization (ICH). S7A safety pharmacology first in human trials denotes the non-clinical safety studies conducted

before human clinical trials for pharmaceuticals. The major organ systems investigated are the cardiovascular, central nervous system, and respiratory systems, adverse effects on which lead to withdrawal of several drugs from the market. The SP studies involved in the finding of toxic effects of the drugs in the FiH trials and these organs prefer as core battery systems and these core battery studies carried through ICH S7 and ICH S7B as per the GLP regulations. The other organ systems such as gastrointestinal and renal systems refer to supplementary studies and resemble follow-up studies as complementary to the core battery systems (Amen *et al.*, 2011).

Alzheimer's disease is the most severe form of dementia and the most common form of age-related cognitive impairment among neurodegenerative disorders (Hamdam and Sethu, 2013; Redfern *et al.*, 2005). Several medications, such as acetyl cholinesterase inhibitors and memantine, provide symptomatic relief but do not slow the course of the disease. One of the causes is that Alzheimer's disease progresses through numerous disease pathways, including cholinergic, amyloid, tau, and inflammatory pathways (Hoymann, 2012; Näslund *et al.*, 2000).

As a result, using Ayurvedic formulations to treat numerous mechanisms involved in Alzheimer's disease would be beneficial due to the presence of many phytoconstituents in them. *Kalyanaka ghrita* is a traditional Ayurvedic ghee-based formulation with references in *Vaghbhat Ashtang Hridaya* and Ayurvedic Pharmacopeia of India (API) is reported to have multiple pharmacological uses to treat anemia, epilepsy, schizophrenia, psychosis, obsessive-compulsive disorder, inflammatory conditions, fever, and memory loss (Natsume *et al.*, 2015).

This herbal ghee comprises phytoactive elements of medicinal plants that are both oil soluble and water soluble. Though herbal formulations are perceived to be safe by majority of the people, there are numerous reports of toxic effects various herbs and herbal formulations. These observations mandate the systemic safety pharmacological evaluation of any herbal formulation and its documentation. Though *Kalyanaka ghrita* has been found to improve memory in our pilot studies, the safety pharmacology is not yet reported.

In this present study, we investigate the safety profile and undesirable effects of the oral and intranasal routes of administration of KG on cardiovascular, central nervous systems, and respiratory organs following ICH guidelines of S7A and S7B safety pharmacological core battery studies. (International Committee for Harmonization).

MATERIALS AND METHODS

Drugs and chemicals

Kalyanaka ghrita was prepared as per the API. Cow ghee (Katraj Dairy Pune), thiopentone sodium (Neon Laboratories Ltd, India), heparin sodium (Akums Drugs and Pharmaceuticals Ltd) were used for the study. All other reagents and chemicals were of analytical grade.

Animals

The experiments were carried out using male Wistar rats weighing 180–220 g were purchased from the National Institute of Biosciences, Pune. The animals were kept under standard conditions of 12 h light: 12 h dark cycles with temperature $25 \pm 1^\circ$C, relative humidity of 45-55 % and feed and water *ad libitum*. All the experiments were carried out in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA). The protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy (CPCSEA Registration Number: 1703/PO/Re/S/01/CPCSEA). The protocol approval number was CPCSEA/PCP/PCL07/2020-2021.

For the CNS safety pharmacological studies, animals were divided into 7 groups of 8 animals each and for cardiovascular, and respiratory safety pharmacological studies, the animals were grouped into 7 groups of 6 animals for each group. The groups were named as vehicle control group and were administered with cow ghee, KG oral 4g/kg, 2g/kg, and 1g/kg respectively. Vehicle intranasal group was administered with cow ghee intranasally, 100μl, 50 μl and 25 μl of KG intranasally per animal daily for 28 days, respectively (Bobade *et al.*, 2015)

KG pharmacological investigations on CNS safety

On day 0, day 14, and day 28, animals were subjected to neurobehavioral characteristics of the central nervous system using a modified Irwin test. This test is used for screening of new chemical entities behaviourally for the central nervous system effects at the different doses to find out the potential therapeutic benefits and to choose for the relevant successive assessment which also named as a functional observational battery (FOB). These behavioral batteries were used to compare the therapeutic index to those of therapeutic doses by comparing it to the standard liabilities of the medication class (Moscardo *et al.*, 2009). With refer-

ence to the ICH, this test was used to investigate a series of 58 behavioral observations related to behavioral (awareness, mood, motor activity), neurological (CNS excitation, Motor coordination, muscle tone, reflexes), and autonomic (writhing, pupil size, palpebral opening, exophthalmos, urination, salivation, piloerection, hypothermia, skin color, and heart rate) changes that occur in animals (Baird et al., 1997; Bertaina-Anglade et al., 2006).

These behavioral observations were carried out through undisturbed home cage observation, handling observations, open field functions and few autonomic observations. These observations were analyzed through a scoring system indicating the level of severity for observations: normal conditions of observations were scored as 4; a score down to 0 indicated as decrease in specific parameter while a score of 8 indicated increase of the specific parameter; intermediate scores were also applied according to the severity. Observations generally absent under normal conditions were given a normal score of 0. If present, such observation was performed and evaluated with an increasing score up to 8. As the level of severity in the case of home cage observations and death was possible to be less, these observations were simply recorded as absent (0): if not observed or present (1): if observed (Creason, 1989).

On the day of the test, the blind observer began by observing the animals for 20 seconds in their home cage, after which the animals were transferred to an open arena (LWH: 707040 cm for rats) to estimate the immediate exploratory behavior for 10 seconds, and then observed for 1 minute for another 14 signs that did not require interaction with the animals and the remaining 29 observations that required interaction with the animals. (Mathiasen and Moser, 2018).

KG pharmacological investigations on CVS safety

The ECG and heart rate were assessed on day 0 and on day 28 by using power lab 8 channel recorder (AD Instruments, Australia). The ECG leads (ML, 138, AD Instruments, Australia) were connected to anaesthetized rats to record heart rate and ECG parameters QRS interval, QT interval, QTc.

The hemodynamic parameters were assessed on day 28 using the PowerLab 8 channel recorder (AD Instruments, Australia). The mean BP and heart rate were measured using an invasive method by inserting polyethylene cannula surgically into the anesthetized rat carotid artery and an arterial catheter was connected to a blood pressure (BP) transducer. The systolic, diastolic and mean arterial blood pressures were recorded (Moss, 1999).

KG pharmacological investigations on respiratory safety

The respiratory parameters were estimated using whole-body plethysmography on day 0, day 14, and day 28 respectively. The noninvasive method for the assessment of lung function in conscious rats by estimating ventilation, airflow, lung volume, and gas exchange which is a well established, widely accepted, reliable method for the measurement of lung functions. The animals were acclimatized in the plethysmography for 3 days before the examination as a training session due to overcome the variations in the results the experimental conditions and records were maintained during the acclimatization period but respiratory parameters were not recorded. The animals were administered with the respective vehicle and *Kalyanaka ghrita* doses and allow for 1hr then the animal was placed in the body plethysmograph on the experiment day considered as day 0. After a one-hour post-dose interval, the parameters were assessed. The parameters of respiratory function Expiratory flow rate ((EF50) (mL/s), Peak inspiratory flow rate ((PIF) (mL/s), and Peak expiratory flow rate ((PEF) (mL/s) were all measured at 15-minute intervals throughout a 1.5-hour period. At each time point, respiratory data was recorded for 5 minutes. The flow signal was utilized to calculate RR, TV, and EF50 for each breath, which were then averaged at 10-second intervals. On days 0, 14, and 28, the relevant parameters were assessed, and responses were recorded and preserved using whole-body plethysmography (DeLorme and Moss, 2002; Coggins et al., 1981).

The effects of KG on the nasal turbinates histologically

On day 28, the animals were killed humanely, and the nasal turbinates were collected and fixed in 10% neutral buffer formalin before being stored at room temperature for 72 hours. The samples were subsequently embedded in paraffin molds and sliced into 5μm thickness using a microtome for histopathological tests. Deparaffinized sections were rehydrated in a succession of gradient alcohols before being stained with hematoxylin and eosin (H&E) (Sinha, 1972).

Statistical analysis

CNS safety pharmacological studies

For home cage observations, death, and other observations, the chi-square test was used in the nonparametric Mann Whitney U test in the GraphPad Prsim 5 Demo software in the United States. The rats showed a 50% change in behavior, which was the

smallest difference between the vehicle control and treated groups, indicating that the neurobehavioral change was pharmacologically meaningful (Creason, 1989).

Pharmacological research on CVS safety

The data was analyzed using one-way ANOVA and Dunnet's multiple comparison test (GraphPad Prsim 5 Demo software, USA), with a significance level of 0.05.

Respiratory safety pharmacological studies

Each respiratory parameter data was averaged over a 5-minute period and analyzed using one-way ANOVA followed by Dunnet's multiple comparison test considering $p < 0.05$ as statistically significant.

RESULTS

Effect of *Kalyanaka ghrita* on CNS safety pharmacological parameters

The home cage observations revealed no significant changes in the sensory-motor responses like a corneal response, pinna response, pupil response, tail pinch response, startle and righting reflex, approach response after administration of KG oral (1g/kg, 2g/kg, and 4g/kg) and intranasal 25μl/rat, 50μl/rat, & 100μl/rat) compared to the vehicle control group.

There was an decrease in the grooming behavior on treatment with KG oral and intranasal treated groups compared to vehicle control group (Table 1). However, this decrease was not statistically significant. Furthermore, there was an increase in the rearing response observed with KG oral and intranasal treated groups compared to vehicle control group (Table 1).

There was also no significant change in the autonomic nervous system functions such as salivation, lacrimation, piloerection, excessive urination, diarrhea and respiration in the KG oral and nasal groups compared to vehicle control group.

There was no change in defecation, arousal, vocalization, handling reactivity and stereotypy on treatment with KG when compared to the vehicle control group.

Effect of *Kalyanaka ghrita* on CVS safety pharmacological parameters

Estimation of *Kalyanaka ghrita* on Heart rate (BPM)

There was no significant change in the heart rate on treatment with KG in all tested groups when compared with vehicle control group on both the observed days 0 and 28 (Figure 1).

Effect of *Kalyanaka ghrita* on ECG Parameters

There was no significant change in the QRS complex (s), QT interval (s) and QTc (s) on treatment with KG in all tested groups when compared with vehicle control group on both the observed days 0 and 28 (Figure 1).

Effect of KG on *Kalyanaka ghrita* on arterial blood pressure (mm Hg)

The systolic, diastolic and mean arterial blood pressures were analysed using invasive method on day 28. There was no significant change in the systolic, diastolic and mean arterial blood pressures on treatment with KG oral (1g/kg, 2g/kg, and 4g/kg) and intranasal (25ul/rat, 50ul/rat, & 100ul/rat) as compared to the vehicle control group (Figure 2). These observed effects on heart rate, ECG and blood pressure confirms the cardiological safety pharmacology of *Kalyanaka ghrita*.

Effect of *Kalyanaka ghrita* respiratory safety pharmacological parameters

The basal readings of the respiratory parameters such as RR, TV, MV, EF50, PIF, and PEF were recorded on day 0 and day 28. There was no significant change in the respiratory rate, tidal volume and minute volume upon treatment with KG oral and intranasal when compared to vehicle control group (Figure 3).

There was also no significant difference in expiratory flow, peak inspiratory flow and peak expiratory flow rates on treatment with KG (oral and intranasal) when compared to vehicle control group. These observed effects on respiratory rate, respiratory volume and flow rates confirmed the respiratory safety pharmacology of *Kalyanaka ghrita.*

Effect of *Kalyanaka ghrita* on the histology of nasal turbinates

The epithelium of the nasal turbinates of animals administered with intra nasal *Kalyanaka ghrita* (25,50 and 100 μl/rat) did not show any alterations in the structure when compared to vehicle control group (Figure 4). There were no inflammation or damage observed, which confirmed the safety of *Kalyanaka ghrita* on nasal administration.

DISCUSSION

The importance of safety pharmacological investigations in the drug research and development process cannot be overstated. These investigations are carried out in the drug development system to determine the safety of new chemical entities and formulations in FiH trials, which are carried out in the early stages of the drug discovery process accord-

Table 1: Effect of KG in Open arena on Grooming and Rearing

Day	Parameter	VC	KGL (1g/kg)	KGM (2g/kg)	KGH (4g/kg)	KGIL (25μl)	KGIM (50μl)	KGIH (100μl)
0		4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00	3.89 ± 0.11	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
14	Spontaneous Locomotory activity	3.80 ± 0.17	4.00 ± 0.00	4.00 ± 0.00	3.91 ± 0.08	4.00 ± 0.00	4.00 ± 0.00	3.89 ± 0.11
28		4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00	3.56 ± 0.43
0		1.32 ± 0.24	1.20 ± 0.20	0.67 ± 0.28	0.33 ± 0.22	0.68 ± 0.22	0.33 ± 0.22	1.67 ± 0.28
14	Grooming	1.62 ± 0.25	1.16 ± 0.16	1.60 ± 0.22	1.62 ± 0.22	1.63 ± 0.22	1.57 ± 0.17	1.57 ± 0.28
28		1.64 ± 0.26	1.63 ± 0.22	1.67 ± 0.37	1.68 ± 0.22	1.60 ± 0.22	1.68 ± 0.22	1.67 ± 0.52
0		3.05 ± 0.25	2.67 ± 0.45	3.75 ± 0.25	3.00 ± 0.65	3.50 ± 0.32	3.75 ± 0.45	3.50 ± 0.36
14	Rearing	3.80 ± 0.50	3.80 ± 0.65	3.70 ± 0.65	3.70 ± 0.36	3.75 ± 0.45	3.17 ± 0.16	3.50 ± 0.36
28		3.82 ± 0.15	3.50 ± 0.35	3.75 ± 0.45	3.50 ± 0.36	3.75 ± 0.25	3.77 ± 0.16	3.75 ± 0.45

The data was represented as mean ± SEM(n=8), Data was analyzed by Mann Whitney U test. VC: Vehicle control; KG *Kalyanaka ghrita.*

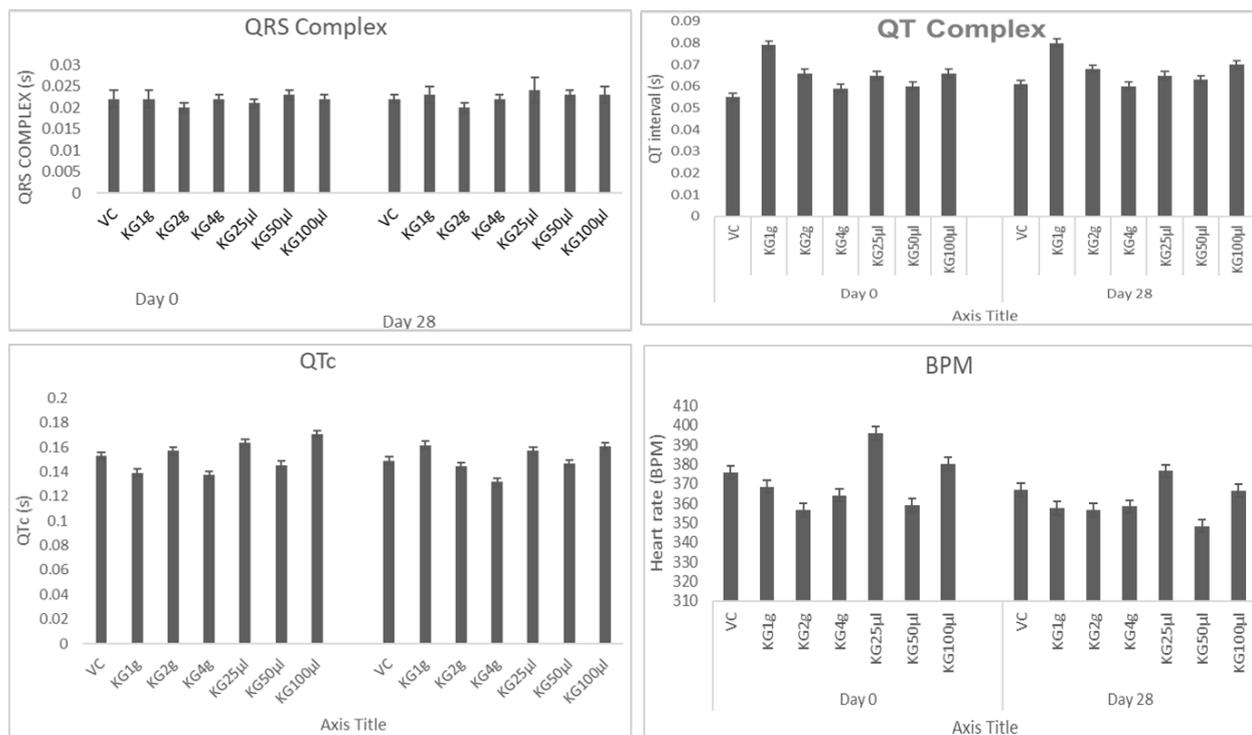**Effect of *KG* on QRS complex (s),QT interval(s), QTc & Heartrate**

Figure 1: For QRS Complex, QT interval, QTc and Heart rate. The data was represented as mean ± SEM (n=6), Data was analyzed by two way ANOVA followed by Bonferroni post test. VC:Vehicle control; KG: *Kalyanaka ghrita*.
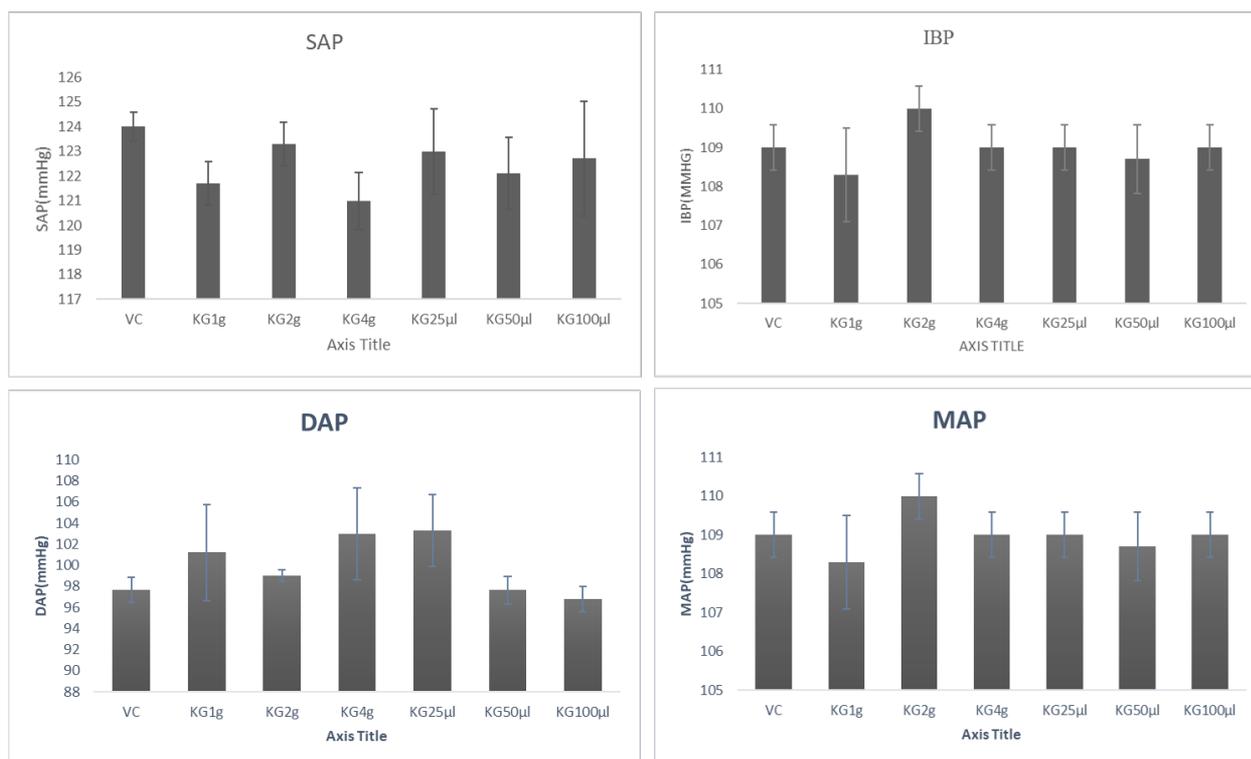
Effect of *KG* on SAP, DAP, IBP &MAP

Figure 2: For cvs SAP, DAP, IBP and MAP. The data was represented as mean $\pm$ SEM (n=6), Data was analyzed by one way ANOVA followed by Dunnett's post hoc test. VC: Vehicle control; KG: *Kalyanaka ghrita.*

ing to the ICH S7 and ICHS7B criteria (Goineau *et al.*, 2013). The neurophysiological observations such as changes in the behavior, motor activity, sensory-motor reflex responses, coordination, may be affected by NCE or herbals which were estimated with Functional observational battery (FOB) or Modified Irwin's test is recommended by ICH.

In the current investigation, animals were subjected to the ICH S7A guidelines in the presence of a blind observer. As noted in the findings section, a total of 58 observations were divided into three categories: neuromuscular, sensory-motor behavioral, and autonomic nervous system observations (Ramos *et al.*, 1997) and were analyzed using the comparison between the vehicle control group and KG oral and nasal groups.

Kalyanaka ghrita has caused no significant changes in ANS related functions and neuromuscular functions in the animals as observed in the KG oral (1 g/kg, 2g/kg, 4g/kg) and KG nasal (25μl/rat, 50μl/rat, and 100μl/rat) treated groups when compared to a vehicle control group. There were nonsignificant decrease in grooming and increase in rearing behaviours observed. The grooming and rearing are strongly linked with agonistic effect

on D_1 receptor and D_2 receptor, respectively. D_1 receptors govern the grooming while D_2 receptors are involved in the process of rearing. Drugs like apomorphine decreased the grooming behavior and increased the rearing behavior. Locomotion, on the other hand, is a key trait connected to dopamine receptor agonism that was not affected by KG treatment. In summary, KG showed no significant action in behavioral parameters, validating Kalyanaka ghrita's CNS safety pharmacology.

Several drugs have minor or major unwanted side effects. Early detection of these drawbacks was performed by safety pharmacology studies which majorly impact cardiovascular system (Rajput *et al.*, 2010). The cardiovascular system is examined for alterations in ventricular depolarization and repolarization, as well as the QT interval, which is defined as the time between the commencement of the QRS complex and the end of the electrocardiogram's T wave (ECG) (Redfern *et al.*, 2003) and (Malik and Camm, 2001). It may prolong due to the effect of the new chemical entity or marketed drug, and hence, this ventricular repolarization may result in ventricular tachyarrhythmia including torsade de pointers (Gras *et al.*, 1996).
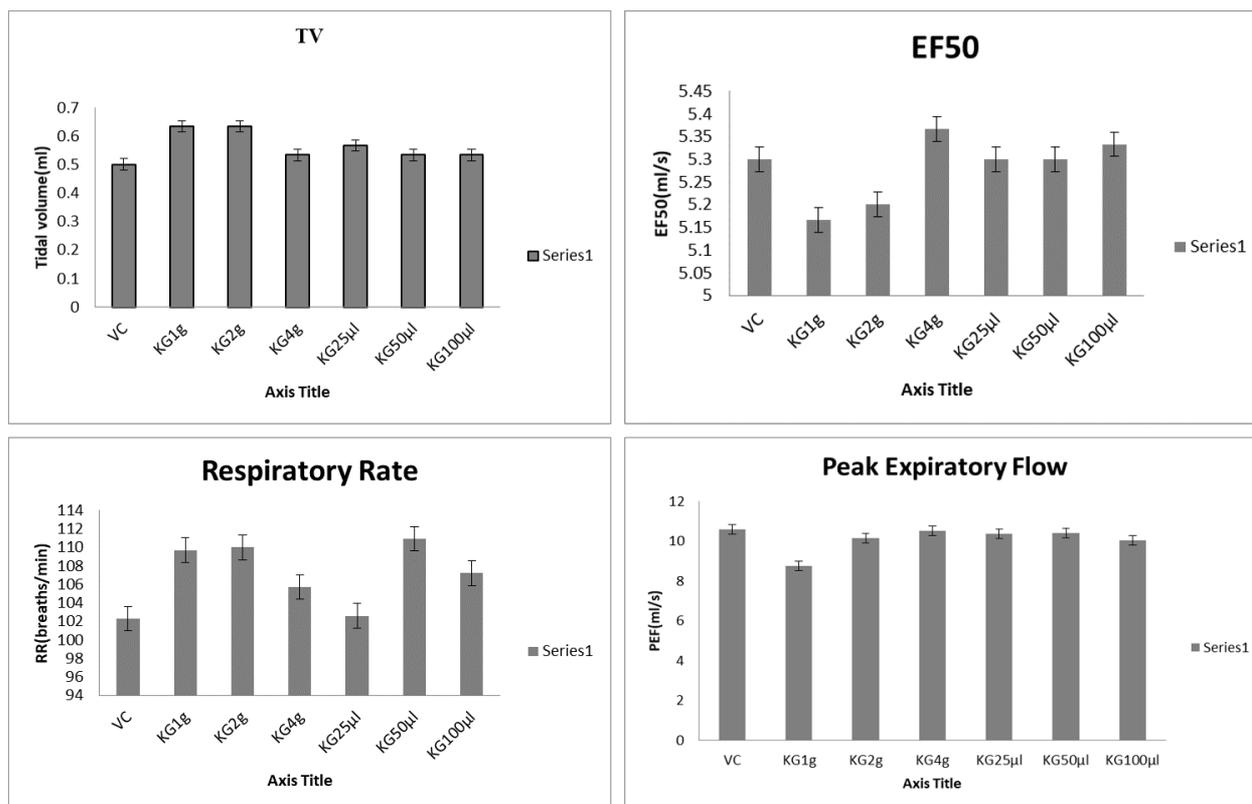
Effect of KG on TV, RR, PEF &EF$_{50,}$ MV & PIF

Figure 3: Effect of KG on TV, RR, PEF &EF$_{50,}$ MV& PIF. The data was represented as mean $\pm$ SEM (n=6), Data was analyzed by one way ANOVA followed by Dunnett's post hoc test. VC: Vehicle control; KG: *Kalyanaka ghrita*.

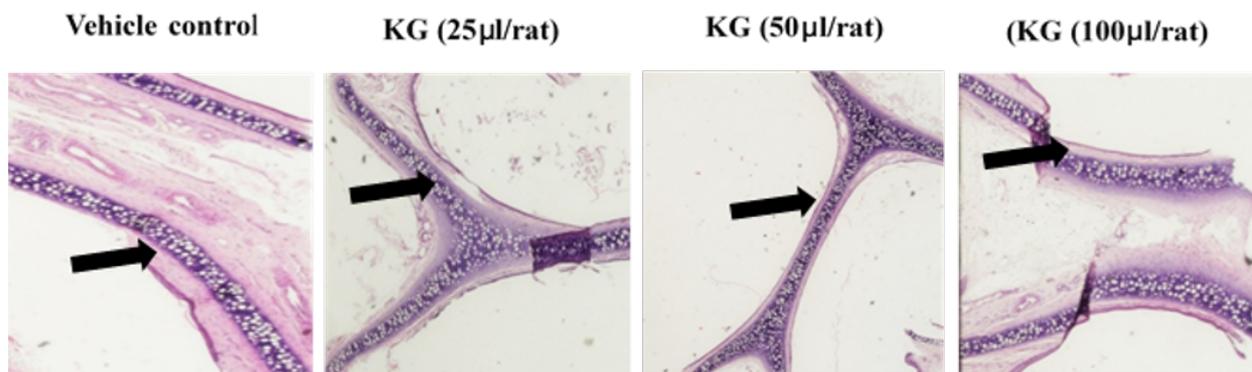**Figure 4: Effect of KG on epithelium of nasal turbinate of the animals. Arrows indicate epithelium of nasal turbinate of the animals. VC: Vehicle control; KG:** *Kalyanaka ghrita*. **Photomicrographs of nasal turbinate from rats stained with Hematoxyline and Eosin stain (H & E Stain), (10x)**

The ICH established the guidelines (ICH S7B) for the detection of potential proarrhythmic effects of pharmaceuticals using safety pharmacological studies. The QTc prolongation also leads to torsade depointes. In the present study KG (oral and intranasal) did not not cause any significant changes on QT and QTc as compared to the vehicle control group. There were no significant change in the heart rate of the KG treatment groups as compared to the vehicle control group. Further, there was no change in the systolic, diastolic and mean arterial blood pressure in *Kalayanak ghrita* trearted groups which confirm KG has not altered the hemodynamic parameters as compared to the vehicle control group. This illustrates that *Kalyanak ghrita* in both the routes of administration has not shown any deleterious effect on cardiovascular functions when compared to the vehicle control group.

The technique of noninvasive whole-body plethysmography is used to assess changes in respiratory physiology and breathing cycle parameters. (Reinmann *et al.*, 2001; Bargeton and Barres, 1969). Plethysmography techniques have been used in numerous research. Whole-body plethysmography permits repeat measurements on the same unrestrained anesthetized animal while forced oscillation needs the termination of animals. As a result, whole-body plethysmography was used in this study (Dolhnikoff *et al.*, 1999; McHugh *et al.*, 2006). Following ICH S7A safety guidelines, the respiratory safety pharmacology of Kalyanaka ghrita intranasal and oral doses were evaluated on days 0, 14, and 28 of administration. The total breathing cycle time of *Kalyanak ghrita* treated groups were not statistically significant when compared to vehicle control group during entire duration of the study period. The inspiratory flow rate and expiratory flow rates were found to be normal in KG treated groups as compared to the vehicle control group.

According to the findings, there were no significant differences in the respiratory parameters between the vehicle control group and the KG oral and intranasal groups in this study. The studies confirmed the respiratory safety profile of *Kalyanaka ghrita*.

The present study confirms the CNS, cardiovascular and respiratory safety profile of *Kalyanaka ghrita* which is of scientific significance and clinical relevance.

CONCLUSION

The potential and safety of the *Kalyanaka ghrita* is established using core battery tests of preclinical CNS safety pharmacology. The cardiovascular and hemodynamic studies confirmed the safety profile of *Kalyanaka ghrita* and it was found to be devoid proarrhythmic risk. The respiratory safety profile of Kalyanaka ghrita was validated in respiratory parameters using whole-body plethysmography. According to the findings of this study, *Kalayanaka ghrita* is a safe medicine that has no CNS, cardiovascular, or respiratory side effects after 28 days of dosage.

Conflict of Interest

The authors declare that they have no conflict of interest.

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