Pharmacognostical evaluation and anti-convulsant property of *Annona reticulata* Linn. (Annonaceae) root

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**Abstract**

**Background:** The present investigation was aimed at establishing the pharmacognostical parameters and preliminary phytochemical screening of *Annona reticulata* Linn. (Annonaceae) root. Pharmacognostical studies and phytochemical evaluation of *A. reticulata* root were carried out by following standard procedures to provide information that help to identify the species. The species of *Annonaceae* family are documented to possess anticonvulsant property, while *A. reticulata* and its root are reported to be used as a folklore medicine to treat epilepsy. In this study, anti-convulsant activity of the root extract was further investigated in mouse model with seizures induced by pentylentetrazole (PTZ; 60 mg/kg, i.p.). Diazepam (5 mg/kg) was used as a standard anti-convulsant drug. Prior to induction of seizures, ethanol extract (100, 200, and 400 mg/kg, p.o.,) and 0.1% sodium CMC were administered. Later, the onset, duration of convulsions along with recovery was recorded.

**Results:** *A. reticulata* can be recognized by its crown shape, leaves with lengthy petioles, fleshy petals and heart-shaped fruits. Roots are cylindrical with rootlets and have aromatic odour. Presence of oil globules and rhomboidal calcium oxalate crystals in cortex, sclerenchyma cells in cortex and secondary phloem, starch grains in cortex, secondary phloem and secondary xylem, and uni- to tri-seriate medullary rays are the key diagnostic characters of root. Preliminary phytochemical screening of extracts revealed the presence of phenolic compounds, tannins, alkaloids, carbohydrates, flavonoids, glycosides, saponins, proteins, fixed oil and fats. The total alkaloid content in the root was 0.524% w/w, while total flavonoid and total phenolic content in ethanol extract were 16.65 mg QE/g and 59.54 mg GAE/g, respectively. Based on the values obtained from anti-convulsant activity, it is evident that the ethanol extract offered significant protection against PTZ-induced convulsion in mice.

**Conclusion:** The current pharmacognostical study aids not only in identification of crude drug material but also in establishing the standardization parameters. Further, the findings of this study indicated that the ethanolic extract of *Annona reticulata* Linn root displayed significant anti-convulsant property and this property could be attributed to imperative bioactives like flavonoids, phenolic compounds, alkaloids, in addition to other secondary metabolites.

**Keywords:** *Annona reticulata*, Pharmacognosy, Anti-convulsant, Pentylentetrazole
Background

Epilepsy, a neurological disorder discerned by transient bouts of abnormal excessive and synchronous electrical activity in brain, is associated with various neurobiological, cognitive, psychological signs and symptoms [1]. The management of epilepsy involves usage of diverse category of drugs such as benzodiazepines, barbiturates, gamma aminobutyric acid (GABA) analogs, hydantoins, carbamazepine, etc. [2]. Already existing anti-epileptic drugs were considered to exhibit better efficacy and safety profile. However, these drugs possess one/more inherent adverse effects such as ataxia, dizziness, impaired concentration, sleep disturbance, anorexia, and aggression [3]. Hence, there is immense scope for developing potent and safe drugs for effective treatment of epilepsy.

Herbs with medicinal properties are the oldest accredited health care products used by individuals across the globe. These herbs constitute a major part of the classical formulations prescribed by any of the traditional systems of medicine and/or folk medicines practiced throughout the country [4, 5], and they have been the source of therapeutic agents for years together. Despite the huge advancements brought about in the formulation and development of allopathic medicines, even today 60–80% of population belonging to developing countries still consider herbal medicines to be safe and economic. Moreover, an extensive usage of plant-based remedies as complementary health care has also been documented in developed countries [5]. Ancient knowledge in natural products as source of remedies is being used as an approach in treating various ailments. Awareness and understanding of primordial information on plants led to the exploration of their potential use, and to provide scientific data through research.

The family Annonaceae comprises trees or shrubs often climbing, frequently aromatic and includes various genus, viz. Sagerarea, Uvaria, Cyathocalyx, Artabotrys, Unona, Polyalthia, Popowia, Phaenthus, Goniathalamus, Unona, Polyalthia, Popowia, Phaenthus, Goniathalamus, Mitrephora, Annona, Xylopia, Miliusa, Saccopetalum, Alphonsea and Oropha [6]. Traditionally, the species of this family are used as astringent, analgesic and in various conditions like convulsions, diarrhea, dysentery, rheumatoid pain, neuralgia, etc., [7, 8]. Annona Linn. one among the genus of Annonaceae is reported to possess number of species, out of which A. squamosa Linn. and A. reticulata Linn. are distributed in India [6]. The information on traditional applications of A. reticulata reveals its use in the treatment of epilepsy, constipation, haemorrhage, cardiac problems, dysentery, worm infestation, bacterial infection, dysuria, etc. [9, 10].

This plant contains diverse class of compounds that includes alkaloids, tannins, phenols, flavonoids, glycosides, and steroids. The important constituents of root bark include anonaene, liriiodine, oxoushinsunine, reticuline, while stem bark contains anonaene, oxoushinsunine, reticuline, michelalbine, asimilobine, annomontine, acetogenins, etc. Leaves possess elemol, α-eudesmol, α-cadinol, etc., and seed contains fatty oil and N-fatty acyl tryptamines [10, 11].

Various reports pertinent to this plant suggested its significant antipyretic, anthelmintic, antihyperglycemic, antiulcer, antinociceptive, analgesic, CNS depressant, anti-inflammatory, wound healing, antimarking, antioxidant, antimicrobial activity, etc., [10, 12], while root is reported for antiproliferative [13], cytotoxicity [14], antioxidant, antimicrobial [15], anthelmintic [16], antiplasmodial [17], and larvicidal [18] activity.

Nowadays researches are focused towards herbal therapies to ascertain safe and better remedy for epilepsy, as drugs of natural origin are considered to possess better edge over synthetically derived compounds. The present study was undertaken with an aim to establish the root pharmacognostical parameters and phytochemical profiles that aids in correct identification of the species, besides evaluating its potential anti-convulsant property.

Methods

Collection and authentication of plant material

Root material was collected from Sadahalli village, Devanahalli Taluk, Bangalore, Karnataka, India during July, 2019. Few fresh pieces of root material were stored in 70% alcohol for macro- and microscopical studies. A herbarium was prepared [19] following curatorial practices and deposited along with crude drug material for future reference (UASB-4602).

Pharmacognostical studies

Macroscopical studies were performed on the intact root material collected, while microscopical evaluation was carried out on thin free hand transverse sections. Macerate was prepared by treating small cut pieces of root with 50% HNO₃ along with little quantity of KClO₃ and boiled until brown fumes were observed. Then, it was washed with distilled water thoroughly to obtain acid-free material. The transverse sections, macerate elements and powdered material were treated with various reagents like phloroglucinol:conc. HCl (1:1), safranin, iodine solution and examined under compound binocular microscope. Later, cells, tissues in section and macerate were measured. Histochemical tests were also performed by treating thin transverse sections with various test reagents like chloroglucinol and conc. HCl, millon’s reagent, iodine, sudan III, ferric chloride and ruthenium red solution to identify cell contents [20, 21].
Phytochemical studies
The physico-chemical constants, viz., loss on drying, yield to water and alcohol (95%), ash values (total, acid insoluble, water soluble), were determined. Ultraviolet analysis was performed by treating powder sample with various reagents, and observations were made under visible, 254 nm and 365 nm. Successive solvent extraction was carried out using sequence of solvents with increase in polarity, and the obtained extracts were tested for the presence of various phytochemicals [22–24].

Total alkaloid content
The method outlined by Rinaldi et al.[25] was followed to determine the total alkaloid content. Coarsely powdered root was extracted thrice with 0.1 M phosphoric acid under agitation for 1 h. Then the acid solution was partitioned with hexane and pH adjusted to 9 with 25% ammonium hydroxide. The pH adjusted solution was extracted with chloroform in a separating funnel until the extract showed negative for alkaloids with Dragendorff’s reagent. The chloroform layer was then dried with anhydrous sodium sulphate and concentrated to determine the total alkaloid content.

Total phenolic content
Total phenolic content was calculated using Folin Ciocalteau reagent, and absorbance was measured in UV–visible spectrophotometer (Shimadzu UV-1700). One ml of total ethanol extract and various dilutions of standard gallic acid were separately taken in a 10-ml volumetric flask. To each of the flask, distilled water (5 ml) and Folin Ciocalteu’s reagent (0.5 ml) were added. After 5 min, 20% sodium carbonate solution (1.5 ml) was added, made up to 10 ml with ethanol and incubated for 2 h. The absorbance of the developed intense blue colour was measured at 750 nm and the total phenolics present in the extract was calculated and represented as gallic acid equivalent (GAE) [26].

Total flavonoid content
Total flavonoid content was estimated using aluminium chloride assay. One ml of sample and standard (quercetin) were separately taken in a 10-ml volumetric flask. Distilled water (4 ml) and sodium nitrate (5%, 0.3 ml) were added to each flask. After 5 min, aluminium chloride (0.3 ml; 10%) was added followed by NaOH (2 ml, 1 M) and made up to 10 ml with ethanol. The absorbance of developed orange yellow colour was measured at 510 nm in Shimadzu UV-1700 spectrophotometer. The total flavonoid was calculated and represented as Quercetin equivalent (QE) [26].

Pharmacological activity studies
Chemicals/drugs
Pentylenetetrazole and sodium CMC were obtained from Hi Media, while Diazepam was from Ranbaxy Laboratories Ltd.

Animals
The study protocol was approved by Institutional Animal Ethics Committee and conducted by following CPCSEA guidelines strictly, India. Swiss Albino mice of 10 weeks old were used for establishing the pharmacological activity pertinent to A. reticulata root. The animals of either sex in the weight range of 25–30 g were included in the study, while the animals less than 25 g and more than 30 g, pregnant animals and lactating animals were excluded. The selected animals were kept under quarantine in a clean environment for a period of two weeks in animal house facility (25 °C ± 1 °C) of the faculty and maintained in polypropylene cages under 12 h light/dark cycle. During this period, the animals had water ad libitum and standard pellet diet.

Acute toxicity study
Acute toxicity study of ethanol extract was carried out by following Organization for Economic Co-operation and Development (OECD) 423 guidelines [27]. Initially, animals were fasted overnight prior to dosing with water ad libitum. Extract concentration for administration was prepared in such a way that the volume does not exceed 1 ml/100gm body weight of mice. Three female Swiss albino mice in the weight range of 25–30 g were administered with a dose of 2000 mg/kg suspended in 0.1% Sodium CMC. After administration, each animal was kept under observation for the first 24 h, with subsequent observations once daily were recorded for next consecutive 14 days for the development of abnormality and/or mortality.

Anti-convulsant activity
Pentylenetetrazole (PTZ) induced convulsion
A total of 30 Swiss albino mice of either sex in the weight range of 25–30 g were divided randomly into five groups with six animals in each group (n = 6). Group I—Control group received vehicle (1 ml 0.1% Sodium CMC), Group II—Standard group received diazepam (5 mg/kg, i.p.), Group III—Low-dose-extract-treated group received 100, Group IV—Moderate-dose-extract-treated group received 200, and Group V—High-dose-extract-treated group received 400 mg/kg of ethanol extract. Groups I, III, IV and V were intraperitoneally administered with...
Fig. 1 Macroscopical characters. a Habit, b leaf, c flower, d fruit, e fresh roots, f dry roots
PTZ (60 mg/kg) after one hour of vehicle and extract treatment, while Group II was administered with PTZ (60 mg/kg) after 30 min of standard drug treatment. After PTZ administration, the following parameters such as jerks, straub’s tail and death or survival were monitored and recorded. The latency of onset of convulsion and time taken for recovery were also noted [28, 29]. The data obtained from all the treated groups were compared with control group. After the completion of the study, the animals were euthanized by decapitation method.

Statistical analysis
The results obtained in the study were statistically analysed by one-way analysis of variance (ANOVA) followed by Tukey’s Kramer multiple comparison tests. All the values were expressed as Mean±Standard Error of Mean (n = 6). Statistical analysis was carried out using GraphPad InStat V-3 software.

Results
Pharmacognostical studies
Habit and habitat of A. reticulata
Annona reticulata thrives abundantly well in tropical region under humid climatic conditions with enough water supply and sunlight. Trees are small, with branches being tomentose when young and glabrous as they mature. Leaves are petiolate, oblong-lanceolate, around 10–15 cm long, 3–6 cm wide, with acute/obtuse apex and cuneate/round base. Petiole is around 1–2 cm in length. Flowers are 2–4, on lateral pedicels. Pedicels are around 1–2 cm in length. Fruits are heart shaped or sub-globose with rough outer surface, yellowish-red or yellow inner side as it ripens and contains numerous hard seeds (Fig. 1a–d).

Macroscopical characters
The roots collected are stout, fibrous with numerous secondary rootlets, cylindrical and measure up to 6 cm in diameter. Dried roots are fibrous, hard with thin brown bark and large yellow wood. Root fracture is fibrous and possesses mild sweet taste with aromatic odour (Fig. 1e, f).

Microscopical characters
Transverse section of the root exhibits a circular outline. The outer surface shows rhytidome, followed by a narrow cork, cortex, secondary phloem and a wide secondary xylem region. Cork is made up of 7–10 layers of tangentially elongated tabular parenchyma arranged in radial rows measuring 6.28–15.03×23.18×2.81–5.75–7.56 μm. The cortex is composed of multilayered parenchyma cells measuring 11.78–16.00×22.75×3.59–9.58–13.75 μm. Oil globules (3.30–5.87–8.74 μm), calcium oxalate crystals of rhomboidal shape and occasional groups of scattered lignified stone cells (2.81–4.30–6.56 μm) are observed in cortical region. A narrow secondary phloem next to cortex characterized by thin-walled cells interlaced with groups of lignified sclerenchyma and medullary rays is seen. Next to secondary phloem is a broad secondary xylem composed of medullary rays, vessels and parenchyma. Medullary rays are predominantly uniseriate measuring 6.67–10.99×15.88×3.98–5.59–7.43 μm, while the vessels are oval to spherical in shape and measure 5.89–13.82–39.02×3.57–10.27–45.44 μm. The medullary ray in secondary phloem is broad, while it narrows down in the secondary xylem region. Starch grains of spherical shape measuring 2.05–5.34–10.52 μm were observed in medullary ray parenchyma and xylem parenchyma as well (Fig. 2a1–a7).

Macerate characters
Macerate studies displayed the following elements: Thin-walled parenchyma cells (11.27–15.53–28.03×5.41–7.84–12.58 μm), pitted parenchyma (9.59–26.30–69.69×5.39–10.93–22.80 μm), reticulate parenchyma (15.04–23.71–47.93×3.72–9.23–18.58 μm), vessels with different sizes and shapes, with pitted type of thickenings, sub-terminal openings, drawn out ends measuring 42.84–78.47–178.75×9.27–22.06–72.23 μm and fibres with variable wall thickness, lumen and ends (161.97–348.86–718.97×2.72–9.07–17.05 μm) (Fig. 2a8–a18).

Powder characters
Powder showed fragments of cork cells, groups of lignified sclerenchyma cells, medullary ray cells accompanying xylem elements; spindle-shaped lignified fibres, parenchyma containing starch grains, starch grains of simple and compound type with spherical shape, lignified pitted parenchyma, fragments of vessels with pitted...
Fig. 2 (See legend on previous page.)
thickenings, and rhomboidal calcium oxalate crystals (Fig. 2a19–a31).

**Histochemical studies**

Thin root sections revealed the presence of lignin (pink with phloroglucinol and conc. HCl), starch (blue with iodine solution), protein (red with millon's reagent), lipids (red with sudan III), tannins (bluish black with FeCl₃) and absence of mucilage (no pink with ruthenium red solution).

**Physico-chemical constants**

The physico-chemical constants (loss on drying, ash values, extractive values) determined are reported in Table 1. The colour, consistency and percentage yield of root powder to various solvents are displayed in Table 2.

**Fluorescence analysis**

The characteristic color exhibited by root powder under visible and UV (254 and 365 nm) radiation upon treatment with various solvents/reagents is tabulated in Table 3.

**Preliminary phytochemical screening**

Preliminary phytochemical analysis of extracts obtained through successive solvent extraction revealed the presence of alkaloids, phenolic compounds and tannins, saponins, flavonoids, glycosides, carbohydrates, proteins, fixed oil and fats. Volatile oil from the dried root was found to be 0.2% v/w by hydrodistillation.

**Total alkaloid, total flavonoid and total phenolic content**

The total alkaloid content in root powder was 0.524% w/w, while the total phenolic and total flavonoid content of ethanol extract were found to be 59.54 mg GAE/g and 16.65 mg QE/g, respectively.

**Pharmacological activity studies**

**Acute toxicity study**

Acute toxicity evaluation on experimental animals administered with 2000 mg/kg dose of ethanol extract did not show any signs of toxicity during short-term (24 h) and long-term (14 days) observation period. Therefore, three doses, 1/20th (100 mg/kg, low dose), 1/10th (200 mg/kg, mid dose) and 1/5th (400 mg/kg, high dose)

| Table 1 | physico-chemical constants of *A. reticulata* root powder |
|---------|----------------------------------------------------------|
| Loss on drying (% w/w) | Ash value (% w/w) | Extractive value (% w/w) |
| | Total | Acid insoluble | Water soluble | Water soluble | Alcohol soluble |
| 12.63 | 5.80 | 1.99 | 0.93 | 6.70 | 8.76 |

| Table 2 | Successive solvent extraction of *A. reticulata* root powder |
|---------|---------------------------------------------------------------|
| Solvent | Color | Consistency | Extractive value (% w/w) |
| n-hexane | Dark green | Sticky mass | 4.72 |
| Toluene | Brown | Solid | 2.38 |
| Chloroform | Brown | Solid | 1.22 |
| Ethyl acetate | Brownish green | Solid | 1.37 |
| Ethanol | Reddish brown | Solid | 8.67 |
| Water | Brown | Solid | 4.87 |

| Table 3 | Fluorescence analysis of *A. reticulata* root powder |
|---------|------------------------------------------------------|
| Reagents | Visible light | Ultraviolet light |
| | | Short wave (254 nm) | Long wave (365 nm) |
| Powder as such | Sand stone | Sands of time | Light biscuit-N |
| 50% H₂SO₄ | Coffee | Pine | Maroon |
| 50% HNO₃ | Old brick | Meadow path | Revel |
| 5% KOH | Terracotta | Amazon moss | Milan red |
| Methanol | Autumn gold | Vivid green | Meadow path |
| Ethanol | Vintage walnut | Mehendi-N | Amazon moss |
| In Acetone | Moody maroon | Green gold | Armada |
| 1 N HCL | Nut-brown-N | Pine-N | Autumn gold |
| 1 N Methanolic NaOH | Honey comb | Moghul green | Moghul green |
| 1 N Ethanolic NaOH | Vintage walnut | Dark drama | Nut Brown |
| Powder + dil NH₃ | Moody maroon | Vivid green | Ming red |

All color comparison is based on the “Asian paints” premium gloss enamel card. Asian paints limited, Mumbai
of this tolerated dose (2000 mg/kg) were consequently selected for anticonvulsant activity assessment.

**Anticonvulsant activity study**

PTZ-administered mice exhibited straub’s tail, tonic–clonic convulsion, while pretreatment with diazepam totally eliminated these episodes of seizures. *A. reticulata* root ethanol extract (100, 200 and 400 mg/kg) treatment prolonged the onset of PTZ-induced tonic–clonic convolution, reduced duration of straub’s tail, convulsion and recovery. These effects were found to be dose-dependent when compared to control group animals. An exceptional inhibition of PTZ-induced convulsion was noticed in the animals administered with high dose of 400 mg/kg. Also, a significant reduction in duration of straub’s tail, and recovery compared to mice received PTZ alone was detected in this high-dose-treated animal group (Table 4).

**Discussion**

Macro- and microscopical studies of crude drugs help to evolve diagnostic characters for proper identification [30]. *A. reticulata* (Linn.) can be identified by its characteristic crown shape and aromatic fruits, while root can be recognized by its fibrous fracture, mild sweet taste and aromatic odour. Microscopically, presence of oil content, rhomboidal crystals; and starch grains in medullary ray and xylem parenchyma are considered to be of prime importance. These characters along with various other key elements observed in macerate and powdered drug will aid in establishing the diagnostic characters that facilitate the correct identification of species. Phytochemical studies act as a prerequisite to establish biomarker compounds for the purpose of identification and to determine the quality of plant materials [31]. Moisture, even though an inevitable component in plant materials, should be as low as possible in the crude drugs. Ash values provide knowledge on purity, quality, care taken to prepare the drug, while extractive values signifies the amount of constituents present in the drug material [30]. These physico-chemical parameters reported for the root material may be used to establish standardization parameters. Moreover, fluorescence analysis revealed various colour exhibited by powder under different wavelengths upon treatment with various reagents and these features will enable to, identify the drug in its powder form and differentiate from other powders also.

Acute toxicity study provides adequate information on toxicological properties of the administered substance through a single or short-term exposure in an organism [32]. No toxic signs and/or mortality were observed in acute toxicity study performed at an oral dose of 2000 mg/kg during the observational period of 24 h. As there were no untoward effects with this single dose, it is suggestive of that the medium lethal dose (LD₅₀) is higher than 2000 mg/kg in mice. Considering this, three doses 100 mg/kg (low dose), 200 mg/kg (mid dose) and 400 mg/kg (high dose) were selected for anticonvulsant activity.

The ethanol root extract was evaluated against convulsions induced by PTZ in Swiss Albino mice. PTZ, a potent GABA receptor antagonist inhibits GABA at GABAₐ receptors resulting in continuous stimulation of cortical neurons consecutively causing seizures that resemble Petit mal in humans [33, 34]. Thus, substances

| Groups                        | Onset (s)      | Straub's tail (s) | Convulsion (s) | Recovery (s) |
|-------------------------------|----------------|------------------|----------------|--------------|
| Control (0.1% Sodium CMC)     | 212.16±8.63    | 114.83±8.93      | 55.66±2.53     | 926±49.62    |
| Standard (diazepam (5 mg/kg)+PTZ) | Absent         | —                | —              |              |
| 100 mg/kg+PTZ                 | 207.61±11.05   | 31.5±8.74        | 23±1.42        | 852.5±51.42  |
| 200 mg/kg+PTZ                 | 203.33±5.47    | 15.5±2.59        | 19.16±2.66     | 522.66±28.90 |
| 400 mg/kg+PTZ                 | 208.16±9.81    | 1.5±0.50         | 2.3±0.21       | 377.33±19.88 |

**PTZ pentylentetrazole**

Values are expressed as Mean ± SEM; n = 6. One-way analysis of variance (ANOVA); p value found to be < 0.0001, considered extremely significant. Tukey–Kramer multiple comparisons test

* p < 0.001 in comparison with control
* p < 0.01 in comparison with control
* p < 0.05 in comparison with control
* p < 0.001 in comparison with low dose
* p < 0.01 in comparison with low dose
* p < 0.05 in comparison with low dose
* p < 0.01 in comparison with medium dose
* p < 0.05 in comparison with medium dose
* p < 0.001 in comparison with low dose

Table 4 Effect of ethanol extract of *A. reticulata* root on PTZ induced convolution in mice
Conclusions
The macro-, microscopical studies and preliminary phytochemical analysis of *A. reticulata* root have been carried out, besides anticonvulsant screening of ethanol extract against PTZ-induced convulsion. The diagnostic characters and physico-chemical constants discussed may be of potential application in the identification of the root material, and also to establish standardization parameters. An effective anticonvulsant activity of root extract is further needed to be explored for the accountable bioactive constituents.

Abbreviations
CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; CK: cork; CTX: cortex; SPH: secondary phloem; MR: medulary ray; SXY: secondary xylem; OG: oil globule; SC: sclerenchyma cell; V: vessel; SG: starch grain; CR: crystal.

Acknowledgements
The authors are thankful to Dr. V. Madhavan, HoD, Department of Pharmacognosy, and Dr. J. Anbu, Professor, Department of Pharmacology, Faculty of Pharmacy, M.S.Ra, for their support throughout the work.

Study involving plants
The plant material was authenticated by Dr. A. N. Sriniveswara, Curator, Mahatma Gandhi Botanical Garden, UAS, GKVK, Bangalore.

Authors’ contributions
KSS and MA designed and supervised the work. MKS performed the study and collected the data. Data were interpreted by MKS, KSS and MA. All the authors contributed to the preparation of the manuscript. All authors read and approved the final manuscript.

Funding
No funding was received.

Availability of data and material
Data and material are available upon request.

Declarations

Ethics approval and consent to participate
The animals for this study were obtained from the animal house, Department of Pharmacology, Faculty of Pharmacy, and the study protocol was approved by the Institutional Animal Ethical Committee (IAEC), M.S.Ra, XXII/MSRFPH/COG/M-027.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflicts of interest.

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Received: 15 March 2021   Accepted: 8 August 2021

Published online: 28 August 2021

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