

Hager's test

Extract was treated with Hager's reagent (saturated picric acid solution). If a yellow colored precipitate is formed, it indicates the presence of alkaloids.

Test for carbohydrates**Molisch test**

Extract was treated with Molisch reagent (α -naphthol in 95% ethanol) and few drops of sulfuric acid were added through the slide of the test tube. If a violet ring appears at the junction, it indicates the presence of carbohydrates.

Benedict's test

Extract was treated with Benedict's reagent (copper sulfate + sodium citrate + sodium carbonate in water) and heated for 10 min. Red-colored precipitate indicates the presence of sugars.

Fehling's test

Extract was dissolved in 1 ml of distilled water and filter. One milliliter of Fehling's solution A and B was added to the filtrate and heat in a water bath for few minutes. The formation of a brick-red precipitate indicates the presence of reducing sugar.

Iodine test

Extract was treated with 2 ml of iodine solution. A dark purple or black coloration indicates the presence of carbohydrates.

Test for amino acids**Millon's test**

Extract was treated with Millon's reagent (mercuric nitrate in nitric acid). Red color indicates the presence of proteins.

Ninhydrin test

Extract was treated with ninhydrin reagent and ammonia and heated. The violet color indicates the presence of proteins.

Xanthoprotein test

Few drops of nitric acid were added by the sides of the test tube very gently to 1 ml of extract. The formation of yellow color indicates the presence of protein in the sample.

Biuret test

Extract was treated with sodium hydroxide. Copper sulfate solution was added dropwise. If a violet color is formed, then it indicates the presence of proteins.

Test for steroids and triterpenoids**Salkowski test**

To the solution of extract, 2 ml of chloroform and few drops of sulfuric acid were added, boiled, and the moisture was shaken and allowed to stand for some time. Red color indicates the presence of steroids and triterpenoids.

Test for phenols and tannins**Ferric chloride test**

Extract was treated with 1 ml of 5% ferric chloride solution. Bluish or black color indicates the presence of tannins.

Lead acetate test

Extract was treated with 1 ml of 10% lead acetate solution in water. Yellow color precipitate indicates the presence of phenols.

Test for flavonoids**Shinoda test**

Extract was treated with few magnesium turnings and concentrated hydrochloric acid was added dropwise. Formation of pink scarlet,

crimson red, or occasionally green to blue color indicates the presence of flavonoids.

Lead acetate test

Few milliliters of extract solution were treated with 1ml of 10% lead acetate solution in water. If a yellow color precipitate is formed, it indicates the presence of Flavonoids.

Proximate composition**Estimation of moisture content**

Approximately 5 g of the crude drug was weight in a glass stopper shallow weighing bottle and kept in an oven at 105°C for 5 h, after which it was kept to cool at room temperature in a desiccator. Then, the weight of the bottle and the sample were taken. The loss in weight is considered as a measure of moisture content in the sample [8].

Moisture % = (weight of the original sample-weight of the dried sample) \times 100/weight of the original sample.

Estimation of ash content

Approximately 2 g of the powdered crude drug was weight in crucible silicon and was heated in a muffle furnace at a temperature of 500°C for 4-5 h. The crude drugs were cooled, and weighed and were heated again in a muffle furnace for another half an hour. This was repeated until the weight of the crucible and the crude drugs become constant (ash became white or grayish white) [9].

Ash content (%) = (weight of the Ash/weight of the sample) \times 100.

Fluorescence analysis

Crude drug shows its own characteristics properties when exposed to ultraviolet radiation and is dependent on its chemical constituents. This analysis is useful to identify adulterants during crude drug evaluation. One gram of crude drug was taken in a watch glass and was treated with different reagents and observed under the TLC chamber for the presence of their fluorescence characters [10].

Evaluation of *in vitro* antioxidant activity of methanolic extract of *Lindernia ruellioides* (Colsm.) Pennell.**Determination of total phenolic content**

The total phenolic content was determined using Folin-Ciocalteu reagent. To 1 ml of extract, 5 ml of Folin-Ciocalteu reagent were added. After 3 min, 4 ml of 0.7M Na₂CO₂ solution were added. In the same manner, different concentrations of standard gallic acid solutions (20, 40, 60, 80, and 100 μ g/ml) were prepared. Both the sample (extract) and the standard (gallic acid) were kept at room temperature for 1 h. The absorbance of the solution was taken against the blank at 550 nm using UV-Visible Spectrophotometer. The total phenol content was expressed in GAE (mg/g) of the extract [11,12].

Determination of total flavonoids content

One milliliter of the extract was taken and added 2 ml of methanol. Keep for 5 min, add 3 ml of 5% sodium nitrite and 0.3 ml of 10% aluminum chloride, keep for 6 min. Add 2 ml of NaOH (1M) and make up the volume up to 10 ml with methanol and keep for 1 h. In the same manner, the concentration of standard quercetin (20, 40, 60, 80, and 100 μ g/ml) was prepared. The absorbance of the solution was taken against blank at 510 nm using a UV-Visible spectrophotometer. The total flavonoids were expressed in QE (mg/g) of the extract [13,14].

Determination of DPPH (2,2-diphenyl-1-picrylhydrazyl) Scavenging activity

The free-radical scavenging activity of the plant samples and ascorbic acid as a positive control was determined using the stable DPPH (2,2-diphenyl 1-picrylhydrazyl). Aliquots (20-100 μ g/ml) of the test samples are prepared in the test tube, 3 ml of each concentration of the test samples were taken and 0.5 ml of freshly prepared DPPH in methanol were added.

Incubate the test samples at 37°C for 30 min. The absorbance was measured using a UV-Visible spectrophotometer at 517 nm. Ascorbic acid is used as a standard. The DPPH scavenging radical was calculated using the following equation [15].

$$\text{DPPH scavenged (\%)} = \frac{(\text{Ac} - \text{At})}{\text{Ac}} \times 100$$

Determination of reducing power

The reducing power of the methanolic extract was determined using ascorbic acid as the standard. One milliliter of extract and 1 ml of standard with various concentrations (20, 40, 60, 80, and 100 µg/ml) were mixed with 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 30 min, after which 2.5 ml of 10% trichloroacetic acid were added. Centrifugation was done at 3000 rpm for 10 min. Take the supernatant (2.5ml) which was formed after centrifuged was diluted with 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride and shake well. The absorbance was measured at 700 nm using a UV-Visible spectrophotometer. The higher absorbance of the reaction mixture indicated that reducing power is increased [16].

Determination of Nitric oxide scavenging activity

To 0.5 ml of 10 Mm sodium nitroprusside in phosphate-buffered saline (pH 7.2), 0.1 ml of different concentrations (20–100 µg/ml) of sample extract and standard of ascorbic acid were added. Incubate the mixture at 25°C for 180 min. To this, add 1.5 ml of freshly prepared Griess reagent. At 510 nm, the absorbance was measured using a UV-Visible spectrophotometer. Control was prepared using phosphate buffer + sodium nitroprusside and Griess reagent [17].

$$\text{Nitric oxide scavenged (\%)} = \frac{(\text{Ac} - \text{At})}{\text{Ac}} \times 100.$$

RESULTS

The extractive yield of different solvent

Six hundred and forty-two grams of powdered leaves of *Lindernia ruellioides* (Colsm.) Pennell were successively extracted using petroleum ether, chloroform, and methanol. The concentrated extract was kept in the refrigerator for further activity use. The extractive yield is given in Table 1.

Preliminary phytochemical screening

The result of the phytochemical study is shown in Table 2. Most of the plant’s extracts contained a rich source of secondary metabolites. Methanolic extract of *Lindernia ruellioides* (Colsm.) Pennell shows the presence of amino acid, flavonoids, tannins, steroids, and triterpenoids.

Proximate composition

The powdered leaves of *Lindernia ruellioides* (Colsm.) Pennell was taken for proximate composition analysis. Moisture analysis is an

Table 1: Extractive yield of different solvent (mean±SEM)

S. No.	Extract	% yield
1.	Petroleum ether	1.45
2.	Chloroform	2.804
3.	Methanol	7.025

Table 2: Result of phytochemicals screening of methanolic extract of *Lindernia ruellioides* (Colsm.) Pennell

S. No.	Phytoconstituents	Results
1.	Alkaloids	-
2.	Amino acid	+
3.	Carbohydrates	-
4.	Flavonoids	+
5.	Tannins	+
6.	Steroids	+
7.	Triterpenoids	+

(-) Indicates absent, (+) Indicates present

important and widely employed determination which is widely used during formulation, processing, and testing of food products. The amount of moisture content in food products is a measure of yield and quantity and is of economic importance. The chemical, physical, and microbial stability of foods are affected by the properties of water. Less moisture or removal or dehydration of moisture is a technique used for improving food storage stability [18]. The result shows the moisture content 12.7% which has less content of moisture and can improve food storage stability. If the ash content is found to be in high amount, it is considered that the plant is rich in minerals which can provide a substantial amount of minerals to our diet. The plant contains rich sources of proteins that could be beneficial and helpful in the functioning of antibodies preventing infection [19]. The proximate composition of *Lindernia ruellioides* (Colsm.) Pennell is shown in Table 3.

Fluorescence analysis

The chemical constituents present in plant material exhibited various fluorescence which is an important phenomenon. If the substances themselves are not fluorescent, they may be often converted into fluorescent derivatives by reagents; hence, some crude drugs are often assessed qualitatively in this way and it is an important parameter of pharmacognostic evaluation [20].

The result for fluorescent studies of the powdered material using different chemical reagents was studied and given in Table 4.

Evaluation of *in vitro* antioxidant activity

Determination of total phenol content

Phenolic compounds are the most abundant secondary metabolites in plants and play an important role in pigmentation, growth, and reproduction of the plant, together with resistance to pathogens and predators. This is largely due to their phytoalexin properties and potent astringency. They have been shown to provide anti-allergic, anti-inflammatory, antioxidant, hepatoprotective, antiviral, and anticarcinogenic activities [21]. The

Table 3: Proximate composition of *Lindernia ruellioides* (Colsm.) Pennell (n=3)

S. No.	Proximate composition	Amount
1.	Moisture content (%)	12.7
2.	Ash content (%)	0.43

Table 4: Fluorescence analysis of *Lindernia ruellioides* (Colsm.) Pennell using different reagent (n=3)

S. No	Reaction mixture	Visible light	UV Fluorescence	
			254 nm	366 nm
1.	Powder as such	Pale green	brown	Pale green
2.	Powder + 1N NaOH in water	Dark brown	Dark brown	Yellowish-green
3.	Powder + glacial acetic acid	Yellowish green	Dark brown	Brown
4.	Powder + 1% picric acid in water	Yellow	Light yellow	Yellow
5.	Powder + 5% ferric chloride	Dark brown	Dark brown	Brown
6.	Powder + conc. H ₂ SO ₄	Reddish-brown	brown	Light green
7.	Powder + conc. HNO ₃	Reddish-brown	orange	Light brown
8.	Powder + conc. HCL	Pale brown	Pale brown	Brown
9.	Powder + methanol	Light green	Pale green	Orange
10.	Powder + Ammonia	Dark brown	brown	Light yellow
11.	Powder + water	Pale green	Pale green	Green

antioxidant activity of phenolics is considered mainly because of the presence of their redox properties, which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers [22]. Phenolic content was measured using the Folin–Ciocalteu reagent in the extract. The results were derived from a calibration curve ($y = 0.0153x - 0.1107$, $R^2 = 0.999$) of gallic acid (0–250 µg/mL) and expressed in gallic acid equivalents (GAE) per gram dry extract weight. The result of the total phenolic content of the extract was found to be 152 mg GAE/g, as shown in Fig. 1.

Determination of total flavonoids content

Flavonoids are secondary metabolites with antioxidant activity, the potency of which depends on the number and position of free OH groups [23]. Flavonoids such as flavones, flavanols, and condensed tannins are considered as plant secondary metabolites which are antioxidant activity and it depends on the presence of free OH groups, especially 3-OH. Plant flavonoids have antioxidant activity *in vitro* and also act as antioxidants *in vivo* [24]. Flavonoid contents in plant extracts were determined using aluminum chloride in a calorimetric method. The results were derived from the calibration curve ($y = 0.0015x + 0.0334$, $R^2 = 0.9904$) of quercetin (0–100 µg/mL) and expressed in quercetin equivalents (QE) per gram dry extract weight. The result of the total flavonoids content of the extract was found to be 80 mg QE/g, as shown in Fig. 2.

Determination of 2,2-diphenyl -1-picrylhydrazyl (DPPH) radical scavenging activity

Free radicals are highly reactive compounds that can produce oxidative stress on the body causing diseases such as atherosclerosis or cancer.

To achieve atomic stability, free radicals are oxidized surrounding molecules to obtain a pair of electrons. This oxidative damage can be counteracted with primary or secondary antioxidants [25]. DPPH is a free radical which is stable at room temperature and this method is often employed to determine the antioxidant activity of many plant extracts. The concentration in g/ml of the sample to scavenge 50% of the DPPH radical is called IC50 and lower IC50 values indicate the higher antiradical activity [26]. The % inhibition of extract and the standard is shown in Table 7. The IC50 of *Lindernia ruellioides* (Colsm.) Pennell was found to be 9.50 µg/ml, while the IC50 of the standard was found to be 50.59 µg/ml, as shown in Fig. 3. Thus, the results indicate that the methanolic extract of *Lindernia ruellioides* (Colsm.) Pennell showed significantly higher antioxidants than the standard ascorbic acid.

Determination of reducing power

Reducing power is associated with an antioxidant activity which serves as a significant role of the antioxidant activity. Compounds that are reducing power are electron donors and can able to reduce the oxidized intermediates of lipid peroxidation processes and can act as primary and secondary antioxidants. In this essay, the yellow color of the test solution changes to various shades green and blue depending on the

Table 5: Mean absorbance value of total phenolic content (mean±SEM)

Concentration	Standard Gallic acid (MEAN ± SEM)
20	0.2033±0.0020
40	0.496±0.0005
60	0.809±0.0005
80	1.09±0.0036
100	1.435±0.0008

Table 6: Mean absorbance value of total flavonoids content (mean±SEM)

Concentration	Standard (Quercetin) (MEAN ± SEM)
10	0.041±0.0012
20	0.066±0.0015
40	0.099±0.0006
60	0.130±0.0008
80	0.159±0.0010
100	0.181±0.00057

Table 7: % inhibition of DPPH of the methanolic extract of *Lindernia ruellioides* (Colsm.) Pennell and ascorbic acid. Data analyzed by one-way ANOVA followed by Dunnett's: Compared all versus control test for n=3

Concentration	% Inhibition of Ascorbic acid (MEAN ± SEM)	% Inhibition of Extract (MEAN ± SEM)
20	45.623±0.5451**	54.248± 0.326**
40	48.366±0.6232**	55.215±0.1429**
60	52.614±0.3268**	60.130±0.326**
80	54.248±0.3268**	65.418±0.326**
100	55.556±0.3268**	72.000±0.5660**

*p<0.05, **p<0.01, ***p<0.001, are considered to be very significant and extremely significant

Table 8: Rate of reducing the power of methanolic leaf extract of *Lindernia ruellioides* (Colsm.) Pennell and ascorbic acid

Concentration	Standard ascorbic acid (MEAN ± SEM)	Extract (MEAN ± SEM)
20	0.129±0.0011	0.1236±0.0006
40	0.187±0.0003	0.152±0.0005
60	0.302±0.0005	0.215±0.0003
80	0.431±0.0005	0.246±0.0003
100	0.597±0.0005	0.299±0.0006

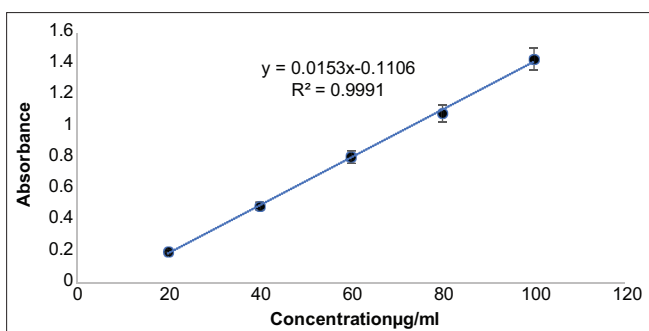


Fig. 1: Standard calibration curve of total phenolic content

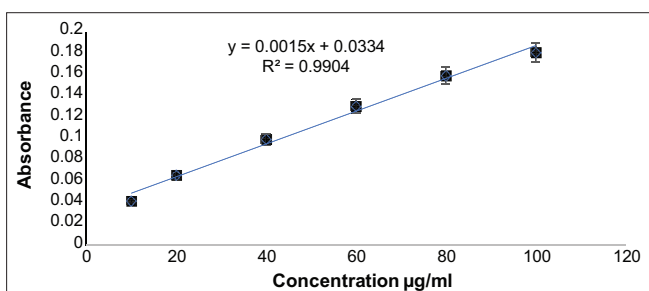


Fig. 2: Standard calibration curve for total flavonoid content

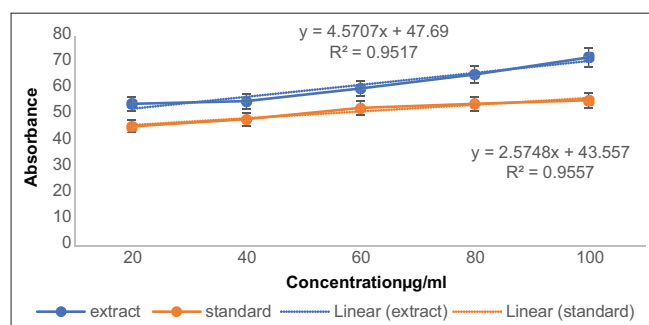


Fig. 3: Calibration curve for DPPH Scavenging Activity

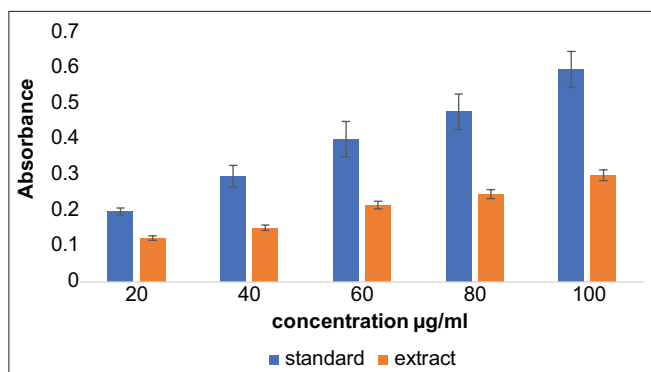


Fig. 4: Calibration curve of reducing power activity

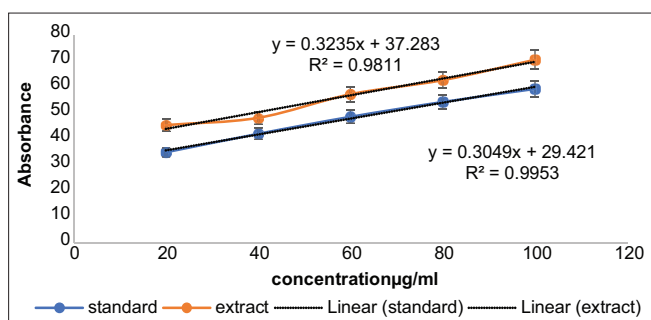


Fig. 5: Calibration curve of nitric oxide scavenging activity

reducing power of each compound. The presence of reducers causes the conversions of the ferricyanide complex in this method to the ferrous form [27]. The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron. In this assay, the presence of antioxidants in the sample resulted in a reduction of the ferric cyanide complex (Fe³⁺) to the ferrous cyanide form (Fe²⁺). Higher the absorbance of the reaction mixture, higher would be the reducing power, suggesting that the sample was able to terminate the radical chain reaction [28]. The reducing power of the standard ascorbic acid was significantly higher compared to the sample *Lindernia ruellioides* (Colsm.) Pennell, as shown in Fig. 4.

DETERMINATION OF NITRIC OXIDE SCAVENGING ACTIVITY

Nitric oxide (NO) is a free radical that is derived from the interaction of NO with oxygen or reactive oxygen species. Nitric oxide is a free radical because of its unpaired electron and plays an important reactivity with certain types of proteins and other free radicals such as superoxide. Low concentrations of NO are sufficient in most cases to affect the physiological functions of the radical. The plants exhibited antioxidant activity by competing with oxygen to scavenge for the nitrite radical at physiological pH in an aqueous environment. An increase in the concentration of the extract increases the antioxidant activity. Nitric oxide scavenging activity can be estimated using a Griess illosvoy reaction. The compound sodium nitroprusside gets decompose in aqueous solution at physiological pH (7.2) producing NO [29]. The % inhibition of extract and standard is given in Table 9. From the result, the IC₅₀ of the methanolic extract of *Lindernia ruellioides* (Colsm.) Pennell was found to be 39.31 µg/ml, while the standard of IC₅₀ was found to be 67.49 µg/ml, as shown in Fig.5.

DISCUSSION

The study suggests that the plant extract contains phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage. Presents of flavonoids derivatives show that the plant may have a wide range of antibacterial, antiviral, anti-inflammatory, anticancer, and anti-allergic activities. So comparable with the findings in the literature for other extracts of plant products, our result suggested that phenolic and flavonoids may be the major contributors for

Table 9: % Inhibition of nitric acid of the methanolic leaf extract of *Lindernia ruellioides* (Colsm.) Pennell and Gallic acid. Data analyzed by one-way ANOVA followed by Dunnett's: Compared all versus control test for n=3

Concentration	% Inhibition of Ascorbic acid (MEAN ± SEM)	% Inhibition of extract (MEAN ± SEM)
20	34.827±0.1867**	45.284±0.093**
40	41.923±0.093***	48.085±0.093***
60	48.459±0.038**	57.142±0.161**
80	54.155±0.093**	62.558±0.093**
100	59.197±0.093**	70.401±0.093**

*p<0.05, **p<0.01, ***p<0.001, are considered to be very significant and extremely significant.

the anti-oxidant activity as the IC₅₀ of the plant extract and the contents of phenolics or flavonoids exhibited significant correlation. From the result of moisture content, the crude drug shows less percentage, which can be concluded that the growth of bacteria, yeast, or fungi has a chance of minimum growth during storage. The result of total ash indicates less presence of impurities such as silicate, carbonate, and oxalate in the crude drug. Furthermore, the total ash of a crude drug reflects the purity of crude and the prepared drug. DPPH scavenging activity determined that methanolic extract of leaves of *Lindernia ruellioides* (Colsm.) Pennell plant showed better anti-oxidant potential by the DPPH method when compared to standard ascorbic acid and IC₅₀ value was found to be 9.50 µg/ml and 50.59 µg/ml for ascorbic acid and methanolic extract, respectively. Hence, we can say this plant has significant antioxidant activity. Reducing power of standard was significantly higher when compared to the plant extract, which shows the antioxidant present in the sample cause the reduction of Fe³⁺ to Fe²⁺ and thus proving its reducing power. From the result of nitric oxide scavenging activity, the plant shows greater inhibition than the standard which may be due to the anti-oxidant principles in the extract, which compete with oxygen to react with the nitric oxide thereby inhibiting the generation of nitrite. Our findings suggest that the plant has the property to counteract the effect of nitric oxide formation due to the presence of tannins and flavonoids.

CONCLUSION

The extraction of the crude drug of *Lindernia ruellioides* (Colsm.) Pennell was done successively by the Soxhlet method using petroleum ether, chloroform, and methanolic.

The preliminary phytochemical screening of the methanolic extract of leaves of *Lindernia ruellioides* (Colsm.) Pennell was performed and it was found to be the presence of amino acids, tannins, flavonoids, steroids, and triterpenoids. From the phytochemicals, results obtained it can be said that it is useful in the detection of bioactive principles which may lead to drug discovery and development.

From the result of the estimation of proximate composition, the high amount of ash content is rich in minerals and could provide a substantial amount of mineral elements to our diet.

The methanolic extract of *Lindernia ruellioides* (Colsm.) Pennell shows a significant anti-oxidant activity which may be due to the presence of phenol and flavonoids in plants. The findings of the present study showed that this plant has a potential source of natural anti-oxidant.

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CONFLICTS OF INTEREST

The author's declared that they have no conflicts of interest in publishing this research article.

AUTHOR'S CONTRIBUTIONS

The corresponding author performed a collection of plants, extraction, experimental work, and wrote the manuscript and analyzed the data. The coauthor helped in evaluating the final manuscript. All the authors have read and agreed on the final approval of the manuscript.

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