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Research Article

Antioxidant activity of aqueous extract of leaves from *Hypericum tomentosum*

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ABSTRACT

Hypericum tomentosum (*H. tomentosum*) is a medicinal plant distributed in Mediterranean region, it's commonly used in folk medicine for different health problems. Thus, this study aimed to investigate the antioxidant properties of aqueous extract of leaves of *H. tomentosum*. The aqueous extract was obtained by boiling *H. tomentosum* leaves in distilled water. The total phenolics and flavonoids content were determined using folin-ciocalteu's reagent and aluminum chloride, respectively. The aqueous extract of *H.tomentosum* was tested for antioxidant activity as determined by DPPH• radical and hydrogen peroxide scavenging assays, reducing power assay, ferrous ion chelating and the ability of this extract to prevent hemolysis were also performed. The result showed that aqueous extract of *H.tomentosum possess* high levels of polyphenols (303.10 ± 5.14 µg GAE/mg extract) and flavonoids (53.56 ± 1.74 µg QE/ mg extract). *H .tomentosum* exerted a powerful scavenging activity against DPPH• radical and hydrogen peroxide with EC_{50} values of 9.32 ± 0.96 µg/mL and 3.13 ± 0.49 µg/mL, respectively. Moreover, it exerted a powerful reducing ability and inhibits ferrous ion chelating .The extract exhibited a significant protective effect against AAPH-induced erythrocytes hemolysis. This study suggests that *H.tomentosum* may represent a prospective source of natural antioxidants to prevent and /or to treat oxidative stress related diseases.

INTRODUCTION

An imbalance in the oxidant/antioxidant system, either resulting from excessive reactive oxygen species (ROS) production and/or antioxidant system impairment, leads to oxidative stress that cause damage to cells and tissue [1,2]. The reactive oxygen species include radical species such as superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$), and non-radical species such as hydrogen peroxide (H_2O_2) [3]. ROS may be generated by ultraviolet rayon [4], xenobiotic as alcohol, medicament and tobacco; transited metal as plumb and cobalt may induce the formation of this species [5]. ROS may be generated also by intracellular sources. In living organism, electron-transport chain mitochondrial is the main part of ROS production. Other enzyme like xanthine oxidase and NADPH oxidase participate in their formation [6–8].

The excess of uncontrolled ROS production leads to cell damage and homeostatic disruption. They can react with polyunsaturated fatty acids of cell membranes and induce lipid peroxidation causing the generation of secondary free radicals [6, 9]. They can also attack protein and DNA [10, 11] bringing about different pathologies such as cancer, cataract, edema pulmonary, accelerated aging, diabetes and cardiovascular diseases [12, 13]. Synthetic antioxidants play an important role to protect human body against damage caused by ROS; however, they cause many side effects [14]. Plants have been used for traditional medicine throughout the world since ancient times. Most of 80% countries in the world used medicinal plants for healthy purposes [15]. In recent years, interest has increased in naturally bioactive compounds that can preserve human health from oxidative stress damage, and inhibit enzymes involved in several diseases [16].

Hypericum tomentosum (Clusiaceae) is a medicinal plant which is mainly distributed in

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Mediterranean region [17]. Its distribution areas were restricted to the humid forest [18]. *H. tomentosum* is used by traditional healers for its anti-inflammatory, antioxidant, antiviral and analgesic effects [19], it is employed also for treatment of depression, ulcer, rheumatism and hysteria [20]. According to Naboli et al. [21] *Hypericum* species have many important chemical compounds possessing biological activities. Therefore, the current study was designed to evaluate the antioxidant potency of *H.tomentosum* aqueous extracts against oxidative damages.

Materials and methods

1. Chemicals

2,2′ -azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), ferrous sulfate ($FeSO_4$), peroxidase, folin-ciocalteu's reagent, 2,2'-diphenyl-1-picrylhydrazyl (DPPH•),quercetin, gallic acid, potassium ferricyanide [$K_3Fe(CN)_6$], trichloroacetic acid (TCA), ferric chloride (III) ($FeCl_3$), butylated hydroxytoluene (BHT), EDTA, ferrous chloride ($FeCl_2$), ferrozine and Trolox were obtained from Sigma-Aldrich (Darmstadt, Germany). Aluminium chloride ($AlCl_3$) and sodium carbonate (Na_2CO_3) were purchased from Prolabo (Paris, France). Sodium dihydrogen phosphate (NaH_2PO_4), Disodium hydrogen phosphate (Na_2HPO_4) and Sodium chloride (NaCl) were acquired from Panreac (Panreac, Spain). Hydrogen peroxide (H_2O_2) and methanol of the greatest available chemical purity were provided from Riedel-de Haen (Illkirch, France).

2. Plants Material

Hypericum tomentosum was collected in June 13th, 2020 from Serdj elghoul, Setif in Algeria. The plant was identified by Pr. H. Laouer, sétif 1 university, Algeria. The aerial parts were dried in room temperature and in shadow. Then Air-dried leaves were ground using an electric grinder (sayona szj-1306, china) in order to get a fine powder.

3. Animals

Female *Swiss Albinos* mice were obtained from Pasteur Institute of Algeria. Procedure using animals were performed in accordance with European Union Guidelines for Animals Experimentation (2007/526/EC).

4. Preparation of Extraction

Aqueous extract was prepared according to Gülçin et al. [22]. 50 g of powdered dried leaves were boiling in 800 mL of distilled water for 15 min (at solid-liquid ration of 1:16). After cooling the mixture was filtrated and the extract was lyophilized to obtain a red fine powered which was stored at -32°C until use.

5. Phytochemical Analysis

5. 1. Determination of Total Phenolic Content

Total phenolic content of aqueous was determinate as describe by Messaoudi et al. [23]. Practically, 100 µL of extract were mixed with 500 µl of folin-ciocalteu's reagent (10%). After 4 minutes, 400 µl of Na_2CO_3 (7.5%) was added and the reaction mixture was incubated in obscurity at ambient temperature for 2 hours followed by the measuring of absorbance at 765 nm using UV-VS spectrophotometer (Thermo Spectronic, USA). Gallic acid was used as standard for the calibration curve. Results were expressed as µg Gallic Equivalents per mg of extract (µg GAE/ mg of extract). The sample was analyzed three times and the mean value was calculated.

5. 2. Determination of Total Flavonoid Content

Flavonoid content was evaluated using colorimetric method according to Kada et al. [24]. An aliquot of 0.5 mL of sample was mixed with the same volume of $AlCl_3$ (2% prepared in methanol). After incubation in obscurity for 10 min at ambient temperature, the absorbance was measured at 430 nm. The standard curve was established using quercetin as standard and the results were expressed as µg Quercetin Equivalent (QE) per mg of extract (µg QE/ mg of extract).

6. Antioxidant Activities

6. 1. DPPH• Radical Scavenging Activity

The ability of extract to scavenge DPPH• radical was evaluated as described by Que et al. [25]. Practically 0.5 mL of DPPH• (0.1 mM dissolved in methanol) was mixed with 0.5 mL of different concentrations of *H.tomentosum* aqueous extract (2- 65 µg/mL). The mixture was incubated in obscurity for 30 minutes. The absorbance was measured at 517 nm. Trolox was used as standard and the percentage of radical scavenging activity was calculated using following formula:

$$\text{DPPH• scavenging activity (\%)} = (A_{control} - A_{sample}) \times 100 / A_{control}$$

Where:
$A_{control}$: absorbance of the control reaction mixture without the test compounds, and,
A_{sample}: absorbance of sample reaction mixture with test compounds.

6. 2. Hydrogen Peroxide Scavenging Activity

The capacity of *H.tomentosum* to trap the non-radical reactive specie hydrogen peroxide was estimated as described by Kherbache et al. [26]. Briefly, in 96 wells microplate, 20 µl of extract at different concentrations was incubated with 100 µL of sodium phosphate buffer solution (PBS) (0.1 M, pH 5) and H_2O_2 (20 µL; 10 mM) for 5min at 37°C, followed by the addition of 30 µl ABTS (1.25 mM) and 30 µl of peroxidase (1 U/mL) then incubated again for 15 min at 37°C. The absorbance was read at λ = 405 nm using a microplate reader (ELX 800, Bio-TEK instruments, Winooski, VT, USA), ascorbic acid was used as standard in this test.

6. 3. Reducing Power Activity

Reducing power of aqueous extract of *H. tomentosum* was determined according to Kada et al. [24]. 200 µl of extract at different concentrations (2.5-50 µg/ mL) was incubated with 425 µl of PBS (0.2 M, pH 6.6) and 625 µl of potassium ferricyanide 1% (prepared in PBS) for 20 min at 50°C. After cooling 625µl of TCA (10% in PBS) was added. 625 µl of each mixture was transferred in other tubes that contain 625 µl of distilled water and 125 µl $FeCl_3$ 0.1 % and the absorbance was measured subsequently at 700 nm against blank without extract. BHT (2.5-50 µg/ mL) was used as standard antioxidant.

6. 4. Ferrous Ion Chelating Activity

H. tomentosum chelating ferrous ion was evaluated as describe by Le et al. [27]. Briefly, 700 µL of extract at different concentrations (0.1 – 3 mg/ mL) were added to 50 µl $FeCl_2$ 0.6 mM. After 5 min of incubation, the reaction was initiated by addition of 50 µl of ferrozine 5 mM (prepared in 80% methanol). The reaction was incubation again for 10 min, a necessary time for complex (Fe (II)-Ferrozine) formation with violet color which has a high absorbance at 562 nm. EDTA (1- 30 µg/mL) was used as reference.

The chelating activity of extract and EDTA was calculated according to the equation:

$$\text{Chelating activity}(\%) = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

$A_{control}$: absorbance of the control
A_{sample}: absorbance of sample

6. 5. Anti-Hemolytic Activity

Anti-hemolytic effect of *H. tomentosum* aqueous extract was assessed according to the procedure described by Meziti et al. [28]. Briefly, blood was collected in heparinized syringe through direct heart puncture from anesthetized female *Swiss albinos* mouse and then diluted to 2% in physiological buffer (NaCl 125 mM, sodium phosphate 10 mM, pH 7.4). In a 96 well plate, 120 µl of erythrocytes suspension were pre-incubated with 60 µl of different concentrations of the extract (5, 10, 25 and 50 µg/mL) or Trolox (25 and 50 µg/mL) for 30 minutes at 37°C. To each well, 120 µl of AAPH (120 mM dissolved in cold physiological buffer) were then added and the mixture was incubated at 37°C. The kinetic of hemolysis was monitored for 4 hours by measuring reductions in turbidity at 630 nm, using a 96-well microplate reader (ELX 800, Bio-TEK instruments, Winooski, VT, USA). The physiological buffer served as the control while erythrocytes not treated by AAPH were used as negative control to remove auto hemolysis of erythrocytes.

7. Statistical Analysis

Experiments were performed in triplicate. The results are expressed as mean ± standard error. The significance of differences (P <.001) among treatment means was determined by analysis of variance (one-way ANOVA) using GraphPad Prism 8 software.

RESULTS AND DISCUSSION

1. Phytochemical Analysis

Amount of total phenols and flavonoids in the *H. tomentosum* leaves aqueous extract are reported in Table 1. The content of polyphenols are reported as Gallic Acid Equivalents (GAE) by reference to standard curve (y = 0.0099x - 0.0997, R^2= 0.9981). Amount of total flavonoids are reported as Quercetin Equivalents (QE) by reference to standard curve (y = 0.037x -0.0085, R^2 = 0.9993). There are a very few studies in the literature that focused on phenolic content of *H. tomentosum.* Although, almost 500 species of this genus have been documented. But only 40 % of them have been phytochemically characterized [29].

Table1: Total phenols and flavonoids content of aqueous extract of leaves of *H. tomentosum*

	Polyphenols	Flavonoids
Amount	303.10 ± 5.14 µg GAE/ mg of extract	53.56 ±1.74 µg QE/ mg of extract
Standard curve	y = 0.0099x-0.0997	y = 0.037x-0.0085
R^2	0.9932	0.9993

2. Antioxidant Activiyt

2. 1. DPPH• Radical Scavenging

DPPH• assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH•. This method is a widely used to evaluate the free radical scavenging ability of various samples. It is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation [30]. Fig. 1 shows that DPPH• scavenging activity of extract increased with increasing of concentrations and at 30 µg/mL the *H. tomentosum* is able to scavenge until 90% of DPPH• radical. The EC_{50} value of this extract is 9.32±0.96 µg/mL. Our value is in accordance with Bouratoua et al.[18], who reported that butanolic extract of *H. tomentosum* aerial part shows a potent DPPH• scavenging with EC_{50} = 14 µg/mL, this result may be justified by the high content of polyphenols and flavonoids. Trolox shows also a high DPPH• scavenging activity with EC_{50} value of 4.60 ±0.15 µg/mL; the same value was reported by Boudoukha et al.[31]

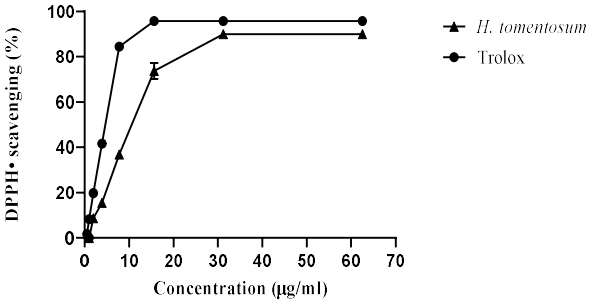

Figure 1: DPPH• scavenging activity of *H. tomentosum* leaves aqueous extract and Trolox. Values are means ± SD (n=3).

2. 2. Hydrogen Peroxide Scavenging Activity

The human exposure to H_2O_2 is very high, because of its incorporation into personal care products as bleaching agent or disinfectant. In addition, H_2O_2 can also be generated *in vivo* due to the activities of some enzymes such as superoxide dismutase. It has the ability to cross the cell membrane, thereby causing the oxidation of a number of compounds in the cytosol [32]. Fig. 2 shows that aqueous extract of *H. tomentosum* exerted a considerable dose dependent hydrogen peroxide scavenging activity with EC_{50}=3.13±0.49 µg/mL. This activity is close to that obtained by standard ascorbic acid (EC_{50}=1.23± 0.09 µg/mL). The potential scavenging activity is distributed to the hydroxyl groups in the aromatic ring of the phenolic components [33]. This group can act as proton donor thus it neutralizes the ROS. It donates the hydrogen to the hydrogen peroxide and cleaves it into water molecules [34].

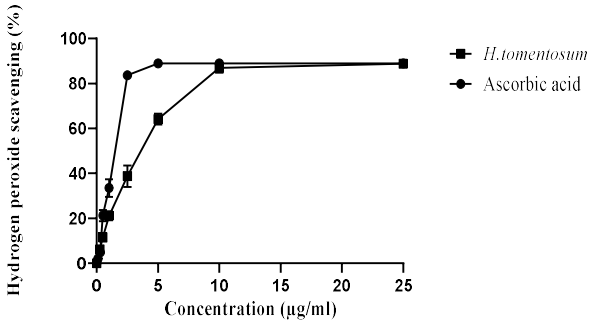

Figure 2: Hydrogen peroxide scavenging activity of *H. tomentosum* aqueous extract and ascorbic acid. Data represent means ± SD (n=3).

2. 3. Reducing Power Activity

The antioxidant activities of natural components may have a reciprocal correlation with their reducing powers. The reducing power assay is often used to evaluate the ability of the natural antioxidant to donate an electron [35]. In this assay, the presence of antioxidants in the samples would result in the reducing of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) by donating an electron. The amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance indicates an increase in reductive ability [30]. Fig. 3 shows the dose-response curves for the reducing power of extract and BHT, a standard used in this test. Reducing power of the extract increases with the increase of its concentrations. *Hypericum tomentosum* exercises a powerful reducing ability which is close to the activity of BHT with EC_{50} values of 19.64±0.01µg/mL and 14.26±0.02µg/mL, respectively. This property is probably due to the presence of active components with hydroxyl groups that act as reductants [36] giving them a reducing power and they can be used as electron donors [37].

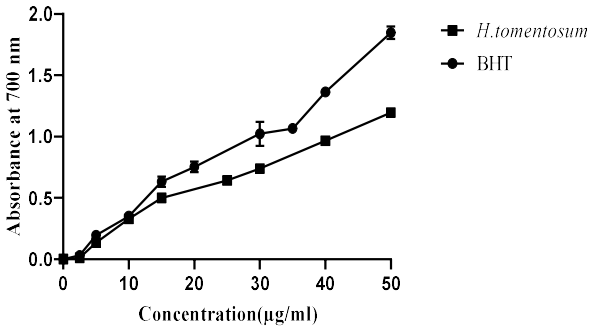

Figure 3: Reducing power capacity *of Hypericum tomentosum* and BHT. The values are expressed as means ± SD (n= 3).

3. 2. 4. Ferrous Ion Chelating

Metal ion chelating capacity plays a significant role in antioxidant mechanisms by inhibition ROS formation and radical production [14]. *H. tomentosum* inhibited the formation of Fe^{2+} - ferrozine complex in a dose dependent manner (Fig. 4).

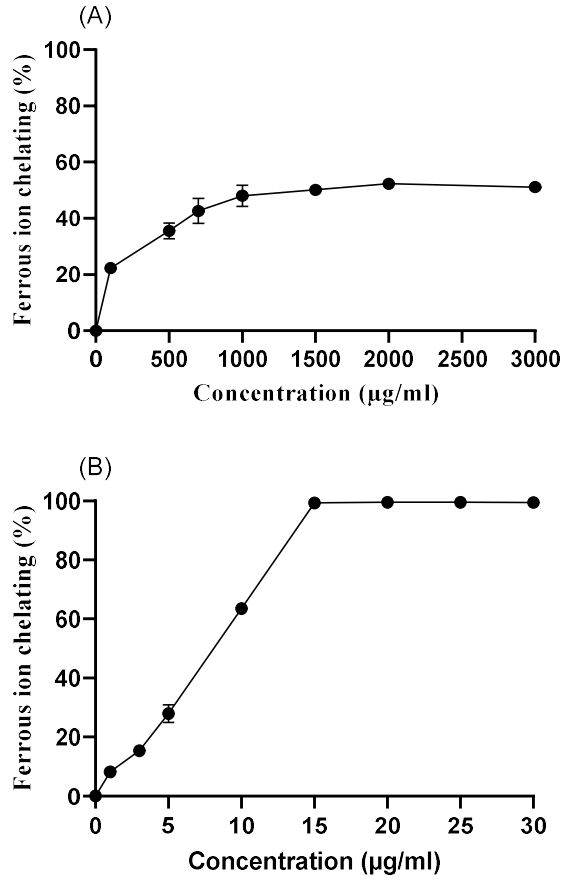

Figure 4: Ferrous ion chelating activity of *H. tomentosum* aqueous extract (A) and EDTA (B). The results are expressed as means ± SD (n=3).

Therefore, this activity was less important than EDTA which exerted a highest chelating activity. This results probably due to the richness of extract by flavonoids with weak chelating activity, it has been reported that some flavonoids, such as naringin, pelargonidin, phloridzin, and hesperitin had no chelating activity [38]. Fe2+ catalyzes the conversion of H2O2 into a highly reactive and damaging hydroxyl radical, a reaction known Fenton's reaction. Hence, *H. tomentosum* can block this reaction by their potent ability to scavenge hydrogen peroxide proved above.

2. 5. Anti-Hemolytic Activity

Hemolysis of erythrocytes by AAPH serves as an *ex vivo* model for the oxidation damage of biological membranes [39]. The aim of this test was focused on the ability of extract to prevent hemolysis caused by aqueous peroxyl radicals generated by thermal decomposition of AAPH at 37°C [40]. This hemolysis was followed continuously at 630 nm to verify changes in the turbidity of erythrocytes. Fig. 5 shows that *H. tomentosum* aqueous extract protect erythrocyte against hemolysis in a concentration dependent manner proving efficiency to scavenge radical species in the medium before they attack the erythrocytes.

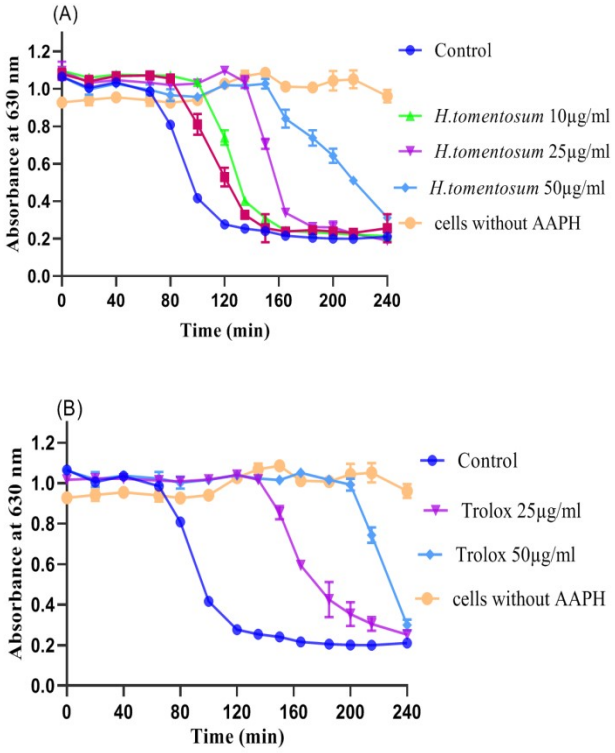

Figure 5: Changes in turbidity of erythrocytes samples during 4 hours in response to AAPH following incubation with *H. tomentosum* aqueous extract (A) and standard Trolox (B). Recording was started immediately following the addition of AAPH. Values are means ± SD (n = 3).

The results show a significant (P<.001) anti-hemolytic activity at the concentrations of 5, 10, 25 and 50 µg/mL, with half hemolysis time (HT_{50}) of 110.5±2.04 min, 123.5±2.09 min, 152.1±2.18 min and 206.7±2.31 min, respectively (Fig. 6). These results are close to those obtained by standard, Trolox. Likely, this anti-hemolytic activity is due to the richness of extract on flavonoids which enhance erythrocytes' resistance against free radical species, mainly by their capacity to capture them, by donating electrons and quenching radicals formed in the aqueous phase before they can damage the erythrocytes' membrane [41].

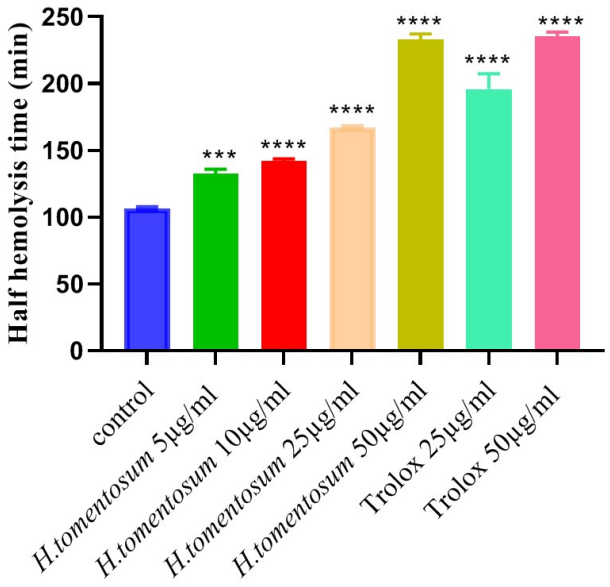

Figure 6: Half hemolysis time of *H.tomentosum* aqueous extract and Trolox. Data are expressed as mean ± SD. P< .001.

CONCLUSION

This study first focused on *in vitro* antioxidant activity of *Hypericum tomentosum*, which showed that aqueous extract of leaves of this plant exhibits a high antioxidant activity and prevents against erythrocytes hemolysis. These effects may be due to their high content of phenolics and flavonoids compounds. Hence, *H. tomentosum* could be a promising potential source of natural antioxidant for the prevention and/or treatment of oxidative stress-related diseases.

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COMPETING INTERESTS

The authors declare that they have no conflicts of interest

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors 'Author LA' designed the study, performed work, wrote the protocol, and wrote the first draft of the manuscript. 'Author MD' and 'Author KS' managed the analyses of the study. 'Author HB' and 'Author SA' designed the study, supervised the laboratory work. All authors read and approved the final manuscript.

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