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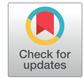
Original Article

Free Radical Scavenging Activity of *Cymbopogon Citratus* (Stapf) and Evaluation of its Antiproliferative Effect on MG-63 Human Osteosarcoma Cells



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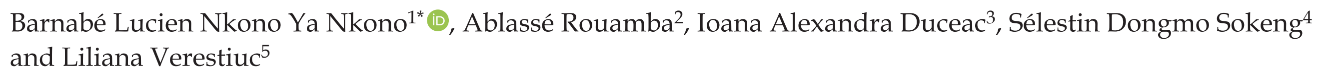

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Abstract

Background and objectives: In the etiology of a large number of metabolic disorders, free radicals are involved in the first line, in particular in the physiopathology of cancers. This interventional study aimed to evaluate *in vitro* the DPPH radical scavenging activity of a hydroalcoholic extract of *Cymbopogon citratus* (HAECC) and its antiproliferative activity in human osteosarcoma.

Methods: The antiproliferative activity of HAECC was assessed *in vitro* in MG-63 human osteosarcoma cells. Normal rabbit fibroblasts were used to evaluate the biocompatibility of a plant extract. Cell viability was assessed by the 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT test). The antiradical activity of HAECC was also evaluated *in vitro* with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Moreover, total flavonoid content of HAECC was quantified by the aluminum chloride colorimetric method.

Results: At low concentrations, the HAECC had high antiradical activity ($p < 0.001$), although it was lower than that of the ascorbic acid standard. HAECC (final concentration of 125 and 250 µg/mL) compared with the vehicle (DMSO 1%) did not affect normal fibroblast viability ($p > 0.05$) but significantly inhibited the proliferation of MG-63 tumor cells ($p < 0.5$) inducing a delay in tumor growth without complete suppression. HAECC had more pronounced cytotoxic activity on MG-63 cancer cells ($p < 0.05$) than on normal cells ($p > 0.05$). The HAECC had a high total flavonoid content of 4.127333 ± 0.205 mg quercetin equivalent/100 mg extract.

Conclusions: The HAECC contained active antiproliferative compounds with synergistic cytotoxic effects on a cancer cell line.

Keywords: *Cymbopogon citratus*; DPPH scavenging activity; MG-63 human osteosarcoma; antiproliferative; cancer.

Abbreviations: DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; HAECC, hydroalcoholic extract of the leaves of *Cymbopogon citratus*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl62H-tetrazolium bromide.

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Introduction

Cancer is mainly characterized by uncontrolled proliferation as well as the loss of a programmed death of malignant cells called apoptosis. Bone tissue cancer or osteosarcoma is a primary bone cancer that starts in bone or cartilage cells. Malignant cells are a group of cancer cells that usually invade and destroy nearby tissue and can also spread to other parts of the body. The incidence rates and 95% confidence intervals of osteosarcoma for all races and both sexes are 4.0 (3.5–4.6) for the range of 0–14 years and 5.0 (4.6–5.6) for the range of 0–19 for each year per million people.[1] The risk of being diagnosed positive for cancer increases with age,

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especially since approximately 77% of all types of cancer are diagnosed in people aged 55 and over. The lifetime probability of an individual developing cancer is just under one in two for men and just over one in three for women.[2] According to the National Vital Statistics System, in the United States, all the cancers identified constitute the second leading cause of death. Overall, one in four deaths is due to cancer.[2]

Free radicals are generally composed of reactive oxygen species (ROS) and reactive nitrogen species. They are generated in the body by various endogenous and exogenous systems. Throughout the world, the use of medicinal plants for primary health needs is a common practice. Since the 20th century, more than 80% of the world's population has depended on traditional medicine for treatment.[3] Synthetic anticancer compounds such as cis-platin and derivates exist but their cytotoxic effects on surrounding normal cells limit their use in cancer chemotherapy. Medicinal plants have played an important role in the treatment of cancer over the past five decades because most of the new clinical applications of plant secondary metabolites and their derivatives have been applied to the fight against cancer.[4] Flavonoids are secondary metabolites belonging to the phenol type. Daily consumption of flavonoids has been shown not only to significantly reduce the amount of free radicals in the body, but also the risk of death from cancer and cardiovascular disease.[4,5]

In sub-Saharan Africa, the leaves of *Cymbopogon citratus* (lemongrass) are generally consumed as an infusion like tea throughout the day because of the multiple aromas they contain. In traditional pharmacopeia, the leaves of *C. citratus* are used in the form of a decoction or infusion in the treatment of many conditions such as antimicrobial, antifungal, anti-inflammatory, antineoplastic, antidiabetic, antioxidant, against cardiovascular problems, insecticides, and pesticides.[6] Furthermore, in Mauritius and Thailand, a decoction of the leaves of *C. citratus* is used as bath water to relieve bone and joint pain, and to reduce or stop postpartum hemorrhage and diarrhea.[7,8] Previous studies have evaluated the anticancer activity of the essential oils of *C. citratus* but comparative studies of the anticancer activity and the biocompatibility of its nonvolatile phytomolecules are less well documented. The purpose of this study was to evaluate *in vitro*, the DPPH free radical scavenging activity of the hydroalcoholic extract of the leaves of *C. citratus* (HAECC) as well as its biocompatibility and its antiproliferative activity on MG-63human osteosarcoma cells.

Methods

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), Quercetin, aluminum chloride, dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT), methanol, and ethanol were purchased from Sigma Aldrich (Steinheim, Germany). All chemicals used where of analytical grade.

Plant materials

After harvesting, leaves, they were washed properly with tap water and drained. Using a kitchen knife, the leaves were cut into 1 cm pieces and placed on kraft paper away from sunlight and at ambient laboratory temperature for 2 weeks. After drying, the leaves were ground with an electric mill and sieved using a mesh size of < 0.5 mm. The powder thus obtained was stored in a dark glass jar for further use.

Extraction by decoction

The HAECC was prepared by decoction (30% water and 70% ethanol). The HAECC decoction was obtained by introducing 250 g powder into an Erlenmeyer flask with a total volume of 1 L, where we then topped up with the water-ethanol mixture (30:70) up to the mark heated at 80°C for 15 m. After cooling to room temperature, the mixture was filtered using Whatman No. 4 paper and then concentrated using a vacuum rotary evaporator (model Büchi Rotavapor R-114, Büchi Labortechnik AG, Flawil, Switzerland). The extract was reconstituted with a 100% DMSO solution in order to be purified and then filtered using a microporous filter with 0.22 µm pores. Using the purified HAECC solution, three concentrations were prepared (125, 250, and 500 µg/mL) to perform MTT test and seven concentrations were prepared (3.90625, 7.81250, 15.62500, 31.25000, 62.50000, 125.00000, 250.00000 µg/mL) for the evaluation of antiradical activity.

DPPH free-radical scavenging activity

In a 96-well plate, 200 µL of the solution of the DPPH radical dissolved in methanol (20 µg/mL) were mixed with 100 µL of HAECC at different concentrations (3.90625–250 µg/mL). A well without extract but containing 100 µL of methanol and 200 µL of the DPPH solution served as a control. The plate was incubated for 15 m away from light and the optical density (OD) was measured at 517 nm using a UV/Visible spectrophotometer. Ascorbic acid was used as the reference compound. The DPPH radical scavenging activity of the plant extract was expressed as a percentage relative to the control without extract.

Total flavonoid content assay

The total flavonoid content quantification was based on the ability of these compounds to form chromogenic complexes with aluminum trichloride. In wells of a 96-well microplate, 75 µL of solutions to be assayed (samples at 1 mg/mL in methanol or quercetin at 0.1 mg/mL) are mixed with an equal volume of $AlCl_3$ (2% in methanol). The optical density were measured at 415 nm, after 15 m of incubation using a UV/visible spectrophotometer, against a quercetin standard curve ($y = 39.8x - 3.5$; $r^2 = 0.99$, $p < 0.0001$). The results were expressed as mg quercetin equivalent (EQ) per 100 mg of dry extract.

Cells culture assays

MTT cell viability

The cell viability assay (Fig. 1) was performed as previously described for fibroblasts (Fig. 1a) and MG-63 human osteosarcoma cells (Fig. 1b).[9,10] After purification of the HAECC, different concentrations (125, 250, and 500 µg/mL) were tested using a standard MTT test following ISO 10993-5. Three parallel samples of each concentration of extract were put in contact with the cell culture (fibroblasts or MG-63 cancer cells) in the culture plate wells. After 24, 48, and 72 h of incubation, the medium was aspirated and 100 µL of 0.5% MTT solution was added. The plate was then incubated for 3 h. The MTT solution was removed and 100 µL of 2-propanol were added to dissolve the formazan crystals metabolized by the viable cells (Fig. 1c). The OD of the formazan solution was evaluated by spectrophotometry at 570 nm. Cell viability (%) was expressed as relative growth rate.

Statistical analysis

The statistical package for GraphPad Prism software version 5.0.3 was used for the statistical analysis. All tests were performed in triplicate and the data are expressed as means ± standard error of the mean (SEM, n = number of experiment). Analysis of variance,

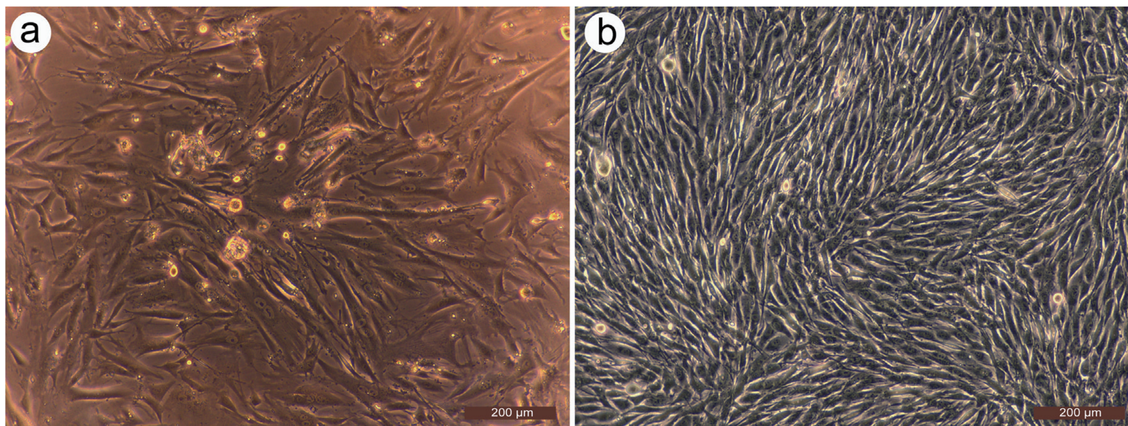

Fig. 1. Cells culture. (a) Primary dermal fibroblasts on passage six after confluence (48-well plate); (b) MG-63 osteosarcoma cells at passage six after confluence (48-well plate).

Bonferroni *post-hoc* tests) and paired *t*-tests were done to test significance and $p < 0.05$ was considered statistically significant.

Results

Extraction yields of C. citratus and total flavonoid content

The concentrated extract obtained by infusing 250 g of dry *C. citratus* powder in 1 L 70% ethanol was 16.8 g, giving a yield of 6.72%. This hydroalcoholic extract of *C. citratus* had a total flavonoid content of 4.127333 ± 0.205 mg EQ/100 mg extract.

Free radical scavenging activity

As shown in Figure 2, the extract at low concentrations (3.90625–250 µg/mL) had good DPPH radical-scavenging activity. The extract at concentrations of 125 and 250 µg/mL trapped more than 50% of the DPPH radicals, suggesting that the 50% inhibitory concentration (IC50) of the DPPH radical of the extract was < 125 µg/mL. Although the extract had good DPPH scavenging activities, it was less active than the ascorbic acid reference compound.

Biocompatibility of hydroalcoholic extract of C. citratus on normal rabbit fibroblasts

Normal rabbit fibroblasts were used to study the biocompatibility of the plant extract. As shown in Figure 3, the extract at concentrations of 125 and 250 µg/mL did not induce significant cell mortality compared with the vehicle (DMSO 1% in DMEM) ($p > 0.05$) regardless of the incubation time. However, the extract at a concentration of 500 µg/mL caused significant fibroblast mortality compared with DMSO 1% ($p < 0.001$). These finding suggest that HAECC was biocompatible at low concentrations.

Antiproliferative activity of hydroalcoholic extract of C. citratus in MG-63 human osteosarcoma

To assess the antiproliferative property of HAECC, MG-63 human osteosarcoma cells were exposed to different concentrations of the

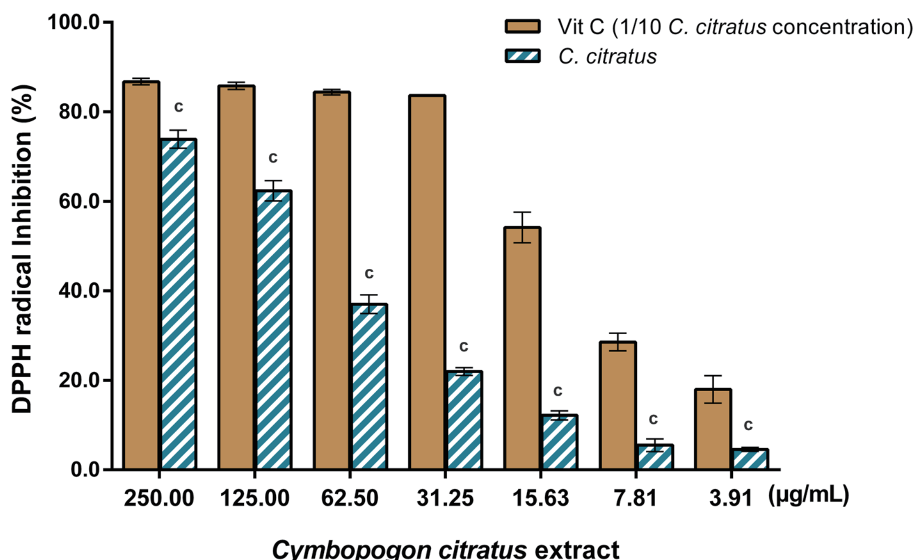

Fig. 2. *In vitro* DPPH free radical scavenging activity of hydroalcoholic extract of *Cymbopogon citratus*. The results means ± SEM (n=3). $^{c}p < 0.001$ versus vitamin C (1/10 of *C. citratus* concentration). DPPH, 2,2-diphenyl-1-picrylhydrazyl.

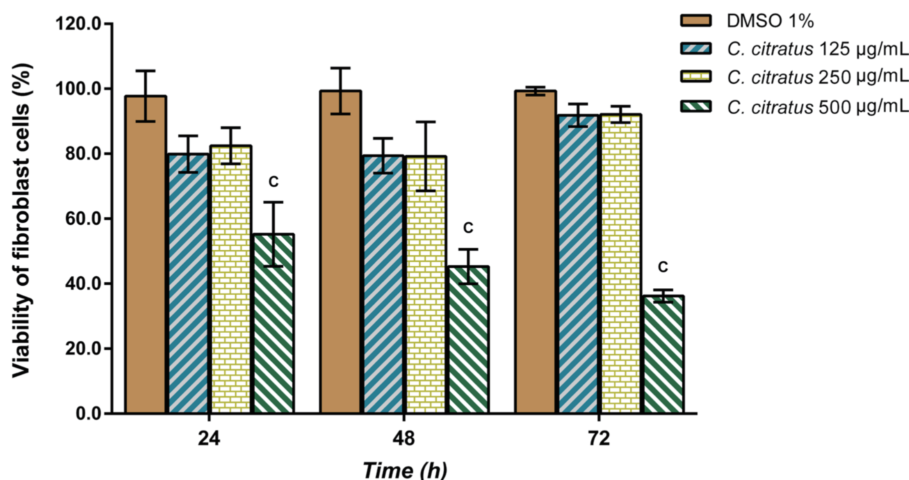

Fig. 3. Biocompatibility assessment of the hydroalcoholic extract of *Cymbopogon citratus* on the normal rabbit fibroblasts. The results are means ± SEM (*n*=3). $^{c}p < 0.001$ versus the vehicle (1% DMSO in DMEM). DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide.

plant extract (125, 250, and 500 µg/mL) for different incubation times (24, 48, and 72 h) and the cell viability was measured (Fig. 4). Compared with the vehicle (1% DMSO in DMEM), the extract at all tested concentrations caused significant cell mortality after 24 h of incubation. The antiproliferative activity of the extract was much higher at the high concentration of 500 µg/mL ($p < 0.001$). Also, the antiproliferative activity of the extract gradually decreased as the incubation time increased. At 72 h of incubation, only the extract at a concentration of 500 µg/mL remained active in osteosarcoma cells. All these results showed that HAECC was more active in osteosarcoma cells at a high concentration and a relatively short incubation time.

Discussion

Free radicals are implicated in the etiology of many metabolic disorders including high blood pressure, rheumatism, and cancer. When free radicals accumulate in cells, damage other molecules such as DNA, lipids, and proteins, which can increase the risk of cancers and other diseases including type 2 diabetes mellitus. Increased production of free radicals by drugs is also associated with apoptosis of cancer cells, indicating the dual nature of free radicals.[11] As shown by some recent studies, the harmful effect of ROS can also be beneficial to the body.[12] Indeed, at low doses, ROS also have an important role as a signaling molecule. They are then produced by a group of enzymes, including the enzyme Nox4. The Nox4 enzyme is present in almost all cells in the body and produces small amounts of hydrogen peroxide, which allows the maintenance of many specialized signaling functions contributing, for example, to the inhibition of inflammatory reactions.

Our study focused on the evaluation of the antiradical and antiproliferative properties of a hydroalcoholic extract of the leaves of *Cymbopogon citratus*. In this study, the extract at concentrations of 125 and 250 µg/mL trapped more than 50% of the DPPH radicals, suggesting that the 50% IC50 of the DPPH radical of the extract was < 125 µg/mL.

The relationship between diabetes and cancer has been clearly demonstrated. Indeed, we observed in diabetes patients an increase in the occurrence of different types of cancer, in particular pancreatic, hepatic, colorectal, mammary, urinary tract as well as the

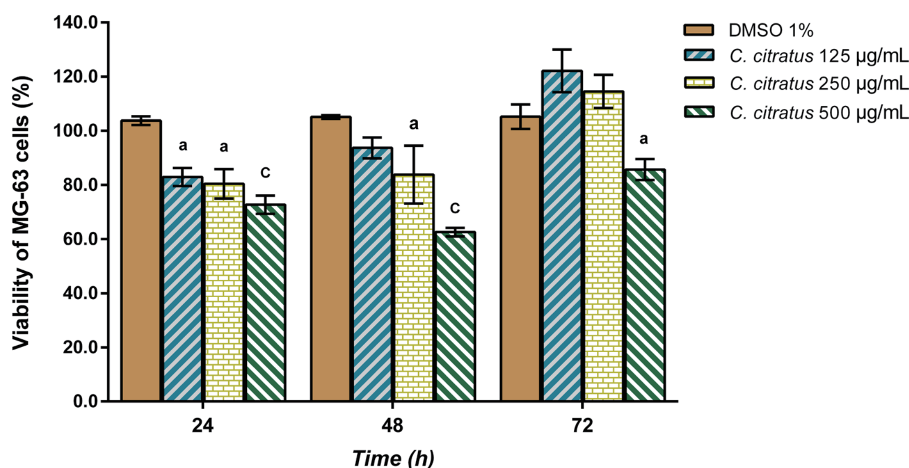

Fig. 4. Antiproliferative activity of the hydroalcoholic extract of *Cymbopogon citratus* on human osteosarcoma MG-63 cells. The results are means ± SEM (*n*=3). $^{a}p < 0.05$; $^{c}p < 0.001$ versus vehicle (1% DMSO in DMEM). DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide.

endometrium. In type 2 diabetes, insulin and insulin-like growth factor 1 (IGF-1) are two hormones that seem to have a key role in the genesis of cancer. In type 2 diabetes, the rise in circulating levels of these hormones could indeed stimulate cell proliferation in certain organs.[13,14] *C. citratus* is a medicinal plant used in traditional medicine in the southern Sahara to treat various conditions including various types of cancer and diabetes mellitus.[15–18] It has also been reported that the essential oil of *C. citratus* is also used in the treatment of diabetes.[19]

The cancerous disease is mainly characterized by the uncontrolled proliferation as well as the escape from apoptosis of the malignant cells. Osteosarcoma derives from primitive bone-forming mesenchymal cells and is the most common primary malignant bone tumor. It is the most frequent bone cancer occurring in children and adolescents between 10 and 20 years of age.[20] The treatment of osteosarcoma, apart from chemotherapy modifications, has not changed for about 30 years. Similarly, as in other tumors, mutations in the Tp53 gene are often seen in osteosarcoma.[21] Currently, its treatment aims at the complete eradication of these cells through their ablation by inhibiting their proliferation and inducing their death. To do this, several means are used alone or in combination, including surgery, radiotherapy, and drug treatments. Drug treatments include cytotoxic chemotherapy,[22] immunotherapy,[23] hormone therapy, and targeted therapies.[24]

In this study, we discussed the method of drug treatment which relies on cytotoxic herbal medicine which is similar to cytotoxic chemotherapy. It appears from this study that HAECC at low concentrations (125 and 250 µg/mL) and relatively short relative incubation times (24 h and 48 h) caused significant MG-63 osteosarcoma cell death compared with the vehicle. The anticancer activity of the extract may be due to sufficient induction of cell cycle arrest or inactivation of mitochondrial apoptosis. The inhibition of p53 protein expression could be responsible for the selectivity of HAECC on cancer cells. Nevertheless, the HAECC lost its cytotoxicity in cancer cells when the incubation time was prolonged (72 h). This finding could be due to a possible development of resistance of cancer cells or a loss of activity of anticancer compounds from the extract. HAECC is an antiproliferative extract that has been shown to act in synergy with cytotoxic products.[1] Most of the phytochemicals evaluated as antidiabetic agents in medicinal plants are flavonoids, alkaloids, and triterpenoids.[25] It has been reported that flavonoids are potentially anticarcinogenic and antioxidant compounds.[26] This could suggest that the cytotoxic and antiproliferative effects of HAECC observed may be due to flavonoids that act either by inhibiting the mutation of the *Tp53* gene,[21] or by reducing the activity of the hormone IGF-1,[13] which inhibits the appearance or proliferation of tumor cells.

In this study, the lowest concentrations tested (125 and 250 µg/mL) of HAECC were biocompatible with normal cells but the same concentrations had cytotoxic activity in MG-63 human osteosarcoma cells as well as very high DPPH radical scavenging activity. Most natural compounds have antioxidant activity in normal cells, implying that they may help prevent carcinogenesis. These compounds can also act as a pro-oxidants in cancer cells and might be associated with the induction of apoptosis.[21,27]

Conclusions

At low concentrations (125 and 250 µg/mL), the HAECC had high antiradical activity (62.35 ± 2.28% and 73.86 ± 2.00%, respectively, but was lower than that of a 1:10 dilution of the ascorbic acid standard. In addition, these low concentrations of HAECC had significant cytotoxic activity for up to 48 h of exposure in MG-63 cancer cells but not normal cells. These observed effects could be due to the presence of high content of total flavonoids present in the HEACC (4.127333 ± 0.205 mg EQ/100 mg extract). HAECC would possess active antiproliferative compounds like flavonoids which could exert antiradical activity on normal cells and synergistic cytotoxic effects on cancer cells' line. Further biochemical investigations are necessary to determine the anticancer mechanism of extract as well *in vitro* and *in vivo*.

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Conflict of interest

There is no conflict of interests to declare.

Author contributions

Defined the intellectual content (BLNYN, AR, IAD, SDS, LV), conceived and designed the study (BLNYN, AR, SDS, LV), carried out literature search, experimental studies and data acquisition (BLNYN, AR, IAD), carried out data analysis and statistical analysis (BLNYN, AR, SDS), prepared and edited the first draft of the manuscript (BLNYN, AR), revised the manuscript (IAD, SDS, LV), guarantors of the manuscript (BLNYN, AR). All the authors have read and approved the final version of the manuscript.

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