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ASSESSMENT OF *In-vivo* AND *In-silico* ANTIINFLAMMATORY ACTIVITY OF *Lactuca virosa* LEAVES

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ABSTRACT

Medicinal plants have been used since time immemorial to treat many ailments and offer a promising source for discovering new therapeutic leads. *Lactuca virosa*, one of the traditional plants used for managing inflammation, still lacks comprehensive scientific investigation. Therefore, the present study investigates its *in vivo* and *in silico* anti-inflammatory effects to assess its potential as a plant-based lead for the treatment of inflammation. The methanol extract of *Lactuca virosa* was analysed via GCMS to identify the phytoconstituents. The extract's antioxidant and anti-inflammatory activities were assessed *in vitro*, while *in vivo* anti-inflammatory potential was evaluated in carrageenan-induced acute and cotton pellet-induced chronic inflammation in rats. Molecular docking of five GCMS-identified triterpenoids was performed using CB-Dock2 against the COX inflammatory receptor. The extract of *Lactuca virosa* exhibited potent scavenging effects with IC_{50} values of 26.9 µg/mL against nitric oxide free radicals and significantly inhibited albumin denaturation, indicating *in vitro* anti-inflammatory activity. The extract significantly ($p < 0.05$) reduced carrageenan-induced paw oedema and pellet weight in the granuloma model ($p< 0.01$), at doses of 200 mg/kg and 400 mg/kg comparable to indomethacin. Molecular docking revealed strong binding affinities of olean-12-ene (-8.7), β-amyrin (-8.9), and lupeol (-8.6) with COX proteins responsible for inflammation. The above findings support the traditional claim of *Lactuca virosa* as an anti-inflammatory agent and align with Sustainable Development Goal 3, which ensures safe and effective therapeutic alternatives for promoting good health and well-being for all.

Keywords: *Lactuca virosa*, Anti-inflammatory, Antioxidant, Carrageenan, Molecular Docking.
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INTRODUCTION

Inflammation is a normal protective mechanism of the body in response to external stimuli, which occurs due to physical trauma, harmful chemicals or various biological pathogens or infectious agents.[1] It is classified into acute and chronic phases. Acute inflammation can persist for a short period of time, for 2-3 hours, whereas chronic inflammation persists in long-term medical conditions[1]. Various NSAIDs are used to treat inflammatory conditions. However, these have been linked with unwanted side effects, which have led to a search for alternative treatments, particularly those derived from medicinal plants.[2,3] Medicinal plants have been utilised for therapeutic purposes since ancient times and form an integral part of traditional and complementary systems of medicine. The World Health Organisation (WHO) has recognised the importance of these traditional remedies, which are regarded as a rich source of active compounds for the prevention and treatment of a wide range of illnesses.[4,5] *Lactuca virosa* (Family: Asteraceae) is one of the well-known traditionally used anti-inflammatory biennial herbs, widely distributed in West Asia, Southwest Europe and North Africa. It is a perennial herb with thick, fusiform roots, rosette leaves, an upright, branched stem, and yellow, ligulate blooms in pyramid-shaped panicles.[6] The plant exudes a white latex, which darkens in the open air, known as lactucarium, resembles the opium poppy latex, which is a drug used to treat pain. Lactucarium contains the sesquiterpinoid lactone, lactucin, with mannitol, lactucerol, and volatile oil.[7-8] The use of wild lettuce dates back to 1815, when it was investigated for its potential as a pain reliever and whooping cough treatment. Modern products made from wild lettuce are now available in the form of pills, liquids, powders, and oils and are marketed as remedies for a wide range of ailments, including respiratory issues, joint pain, anxiety, and insomnia.[9] The

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plant is reported for antitussive, anti-inflammatory, analgesic and expectorant activities in the form of aqueous extracts of a di-herbal formulation with the whole plant of *Euphorbia hirta*. Based on the literature survey, there were no reports on the anti-inflammatory activity of this plant, which is a need of the hour.[10] The present study aims at the evaluation of *in vivo* and *in silico* anti-inflammatory activity of *Lactuca virosa* leaves, and it directly contributes to SDG-3 by promoting plant-based therapies to improve health outcomes that can reduce reliance on synthetic drugs.

EXPERIMENTAL

Collection and Preparation of Plant Extract

The plant was collected from roadside wet places in the month of December, in regions of Hyderabad. The plant was taxonomically authenticated. The leaves were separated, dried and ground into a coarse powder. 250 gm of powder was extracted by soxhlation technique using methanol for 72 hrs, concentrated and finally evaporated on a water bath at 40-50^{0}C to obtain the residue and the percentage yield was calculated. It was stored in a refrigerator at 4^{0}C for later use.

Phytochemical Screening and GC-MS Analysis

Freshly prepared crude methanolic extract of *Lactuca virosa* (MELV) was subjected to phytochemical analysis to indicate the presence of various chemical constituents like phenols, glycosides, sterols, flavonoids, terpenoids, alkaloids, saponins and tannins, using standard procedures.[11] Then crude MELV was subjected to GC-MS analysis, dissolving it in a small quantity of methanol. The GCMSQP2010, SHIMADZU instrument and a column, BP5, were utilised, with helium (He) as a gas carrier (99.999%, 1 mL/min) and 1 µL as the injection volume of the extract. The compounds separated in GC-MS were identified by comparison with the GC-MS NIST21 library.[12]

Animals

Rodents were kept in polypropylene cages and provided with a 12 hr light and 12 h dark cycle with a consistent temperature up to 25±2°C and a humidity level of 50–60%. Animals were provided with a constant pellet diet and palatable water and were acclimatised to the environmental conditions of the laboratory for seven days. They were taken care of and used according to the ethical guidelines for laboratory animals. Ethical permission was acquired from the IAEC (Institutional Animal Ethics Committee), GRCP, Hyderabad, to perform the work.[13]

Acute Toxicity Study

OECD (Organisation for Economic Cooperation and Development) 425 guideline was used for performing acute toxicity studies. For this, a 2000 mg/kg dose was administered to the animal, and they were monitored for any symptoms of toxicity over a period of 14 days.

In vitro Antioxidant Activity

Nitric oxide (NO) scavenging activity was used to study the antioxidant activity of MELV. Both standard (ascorbic acid) and MELV concentrations ranging from 10 - 50 µg/mL were prepared. Each concentration was incubated with 2 mL of sodium nitroprusside for 150 minutes at 250^{0}C. From each incubated concentration, 1.5 mL was pipetted and mixed with 1.5mL of Griess reagent and again incubated at room temperature for thirty minutes. The absorbance of each concentration was estimated (546 nm), and the following formula was used to determine nitric oxide scavenging activity.[14]

$$\% \text{ Nitric oxide scavenging assay} = \frac{T0 - T1}{T0} \times 100$$

T_0 - control absorbance and T_1 - test absorbance

In vitro Anti-Inflammatory Activity

Various concentrations of MELV and standard were prepared (10, 20, 40, 60, 180 µg/mL). 0.05 mL of each concentration of the test, standard, and negative control solutions were pipetted, and then 5 mL BSA (bovine serum albumin, 0.2%) solution was added. The solutions were incubated for 45 minutes at 100^{0}C and, after cooling for twenty-five minutes, the solutions were mixed for one minute, and absorbance was recorded at 660 nm.[15]

In vivo Anti-Inflammatory Activity

Carrageenan-Induced Paw Oedema in Rats

Animals (180-220 g) grouped into six (n=6) were fasted for the whole night and given water before the experiment. An injection of 0.1mL carrageenan solution (1%) was administered to the rat's left hind paw of group II (disease control), group III and IV (Treatment group) and group V (positive control) following an hour of treatment with MELV (groups III and IV) and Indomethacin (group V). Group I was the normal control, administered with distilled water. Using a plethysmometer, paw volume was recorded one to five hours after the medication was administered.[16] The protection of paw (%) was determined using the following formula:

$$\% \text{ Protection} = \left[1 - \left(\frac{Bt}{Bc} \right) \right] \text{ x } 100$$

Bt= treated animal paw volume, *Bc* = control animal paw volume.

Cotton Pellet Induced Granuloma

Cotton pellets (10 mg) was autoclaved for 30 minutes to disinfect them. The albino Wistar rats (180–220 g) were grouped into five groups (n=6), and the fur of the animal's dorsal surface was removed using depilating cream. After making a 2 cm incision under ether anaesthesia, rats' axillae was subcutaneously implanted with sterile cotton pellets. The treatment was administered 30 minutes before the placement of the cotton pellet. After that, the treatment was given consistently for seven days. The animals were sacrificed on the eighth day, and pellets were extracted aseptically and dried at 60˚C. The difference between the weights of cotton pellets before and after drying was measured.[17] The given formula was used to statistically calculate % change in granuloma weight:

$$\% \text{ inhibition} = \frac{Wpc - Wpt}{Wpc} \text{ x } 100$$

Wpt = pellet weight of treated group; Wpc = pellet weight of control group

In silico Molecular Docking

A molecular docking study was carried out for GC-MS-identified triterpenoid compounds, particularly Olean-12-ene, β-Amyrin, Lupeol, Norcholestan-3-one, and Urs-12-ene, to screen anti-inflammatory activity against COX, an inflammatory enzyme. Indomethacin and diclofenac sodium were used as reference drugs. The 3D structures of all these compounds (ligands) were retrieved from PubChem in the SDF (structure data file) format. Crystal structures of the COX enzyme (PBD ID: 4IMO) were extracted from the PDB (Protein Data Bank, https://www.rcsb.org/). The enzymes were cleaned of specific water molecules, cofactors, co-crystallised ligands, and energy-minimised prior to docking analysis. All protein target structures were then prepared. CB-Dock 2 tool was used to dock ligands over 4IMO. Both receptors (pdb files) as well as ligand molecules (SDF format) were uploaded to the CB-Dock tool. The 2D and 3D interactions were visualised using BioVIVA Discovery Studio software.[18]

Statistical Analysis

It was carried out using one-way analysis of variance (ANOVA), and data were represented by mean± SEM. Multiple comparisons between the treatment groups and normal control were performed using Dunnett's test. Statistical analyses were conducted using the GraphPad Prism program.

RESULTS AND DISCUSSION

Phytochemical Screening and GCMS Analysis

The amount of MELV was found to be 9.78% w/w. The MELV indicated the presence of alkaloids, terpenes, tannins, phenols, flavonoids, steroids, and saponins where-as absences of cardiac and anthraquinone glycosides were absent. According to GC-MS analysis, MELV was found to have 43 phytochemical compounds. A few significant active constituents are listed in Table-1, and among these, five compounds were docked *in silico* to assess the anti-inflammatory activity.

Acute Toxicity Study

MELV had not shown any toxicity signs or lethality at 2000 mg/kg for 14 days and was considered safe. Based on this, doses were fixed (200 and 400 mg/kg) to carry out the study.

Table-1: List of Some GC-MS Identified Compounds in MELV

Compounds	Biological activity
Triterpenoids Olean-12-ene	Anti-inflammatory, anti-cancer and anti-microbial [19]
β-Amyrin	Anti-inflammatory and hepatoprotective [20]
Urs-12-ene	Anti-inflammatory, anti-cancer, anti-HIV and anti-microbial [21, 22]
Lupeol	Anti-inflammatory, anti-diabetic, anti-microbial, skin protective agent, cardio-protective agent and anti-cancer, hepato-protective [23, 24]
Lup-20(29)-en-3β-ol	
Triterpene alcohol Lupenol	
Diterpene Clerodol	Cytotoxic, anti-tumour
Coumarin 2H-1-Benzopyran-2-one	Anti-coagulant, anti-oxidant, anti-inflammatory[25]
Phytosterol Fagarasterol	Anti-inflammatory[26]

Evaluation of Antioxidant Activity

Nitric oxide plays a potential role in cancer, inflammation and other pathological diseases in addition to reactive oxygen species (ROS).[27] The ability of the plant or plant-derived products to mitigate the impact of NO production may be of significant interest in shielding the human body from the negative consequences of excessive NO production. Additionally, the scavenging activity might aid in stopping the series of harmful reactions to human health that are brought on by an excess of NO formation. In the study, MELV showed potential NO scavenging activity as the concentration of MELV increased (Table-2). In comparison to the standard IC_{50} value of 25.64 µg/mL, the extract exhibited similar potential antioxidant activity with an IC_{50} of 26.9 µg/Ml.

Table-2: NO Scavenging Activity of MELV

Conc. (µg/mL)	% Inhibition (Mean ± SEM)	
	MELV	Ascorbic acid
10	21.54 ± 0.85	22.51 ± 1.58
20	36.89 ± 0.55	33.63 ± 0.67
30	50.21 ± 1.31	53.26 ± 1.14
40	71.12 ± 0.98	67.14 ± 0.33
50	81.36 ± 0.65	83.92 ± 0.58
IC_{50} (µg/mL)	26.9 ± 0.88	25.64 ± 0.67

Anti-inflammatory Activity

Protein Denaturation Assay

Various anti-inflammatory drugs have been demonstrated their capacity to inhibit protein denaturation caused by heat in a dose-dependent manner.[28] BSA will be denatured when heated, leading to the expression of antigens associated with type III hypersensitivity and illnesses such as serum sickness, glomerulonephritis, systemic lupus erythematosus and rheumatoid arthritis. Therefore, this assay was used to find chemicals that can stop the denaturation process of proteins.[29] MELVs have shown potent anti-inflammatory effects by suppression of denaturation of proteins, in a concentration-dependent manner (Table-3). MELV's inhibition (89%) was similar to the 92% inhibition rate of conventional Indomethacin.

Carrageenan-Induced Paw Edema

Carrageenan is a phlogistic agent. It causes a two-phase reaction of inflammation. Serotonin and histamine will be released during the first phase, which is when edema creation is at its highest, while prostaglandins and bradykinins will be produced during the second phase. MELV significantly decreased ($p < 0.05$) paw volume at 200 and 400 mg/kg doses from the first to the fifth hour following a one-hour carrageenan injection (Fig.-1). The percentage protection was found to be 49.3% and 67.2%, respectively, with a maximal protection of 71.8% at the 400 mg/kg dose. MVLV has demonstrated an effect similar to that of conventional Indomethacin, 25 mg/kg. By blocking the cyclooxygenase (COX) enzyme, the extract might prevent the release of pro-inflammatory chemicals. Thus, the extract is shown to have possible anti-inflammatory properties.[30]

Table-3: Protein Denaturation Assay of MELV

Conc. (µg/mL)	% Inhibition (Mean ± SEM)	
	MELV	Indomethacin
10	15.18 ± 1.10	17.23 ± 1.54
20	25.13 ± 0.43	31.58 ± 1.28
40	45.06 ± 0.82	49.32 ± 0.35
80	65.32 ± 0.93	63.21 ± 0.92
160	89.71 ± 0.44	92.10 ± 0.70
IC_{50} (µg/mL)	65.15± 0.44	63.21± 0.44

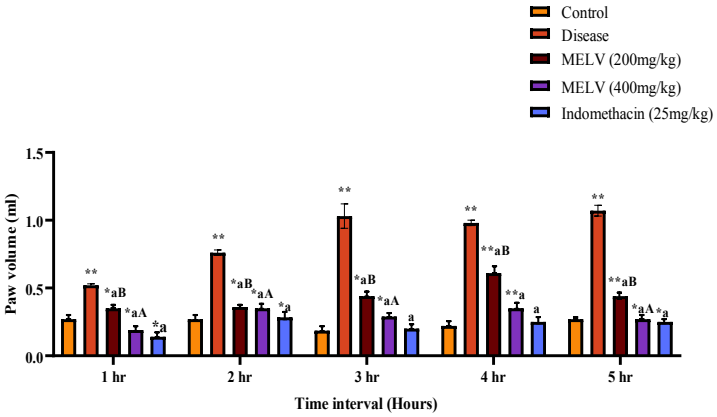


Fig.-1: Effect of MELV on Paw Edema Between the Time Intervals from 1 to 5 hrs

Values: Mean ± SEM (n=6). In comparison to control group (* = $p < 0.05$, ** = $p < 0.01$,), disease control group (a = $p < 0.01$), and standard group (A = $p < 0.01$, B = $p < 0.05$)

Cotton Pellet Induced Granuloma

It is the most widely used technique for assessing chronic inflammation, involving three primary phases of a reaction to a subcutaneously placed cotton pellet: transudative, exudative, and proliferative phases.[31] The wet weight of the granuloma is strongly influenced by the amount of fluid absorbed by the pellet, and the dried weight is directly proportional to the amount of granuloma tissue that develops.[32] As shown in Fig.-2, the weight of the pellets was reduced by 200 mg/kg (57.36 ±1.90) significantly ($p< 0.05$) where-as 400 mg/kg (44.53 ±1.57) and standard Indomethacin has significantly reduced ($p < 0.01$) the pellet weight (38.71 ±1.86), with 31.5%, 46.8% 53.77% inhibition respectively.

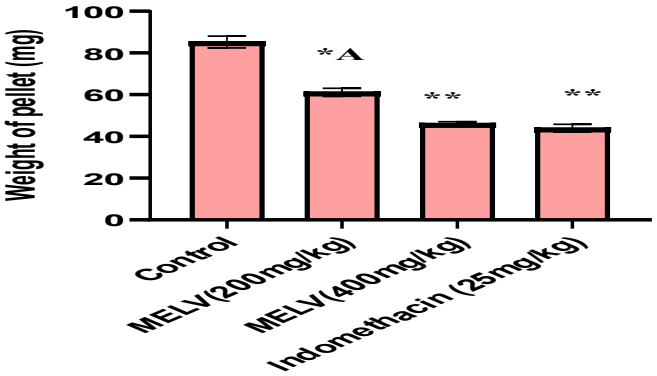


Fig.-2: Effects of MELV in Cotton Pellet-Induced Granuloma Model

Values: Mean ± SEM. In comparison to control group (* = $p < 0.05$, ** = $p < 0.01$), and standard group (A = $p < 0.01$)

This action might be due to the flavonoids, phenolic compounds and steroids present in the plant, which may decrease the generation of collagen, fibroblast number and mucopolysaccharides, which are preferred natural proliferative factors of granulomatous tissue formation.

***In silico* Molecular Docking**

Among forty-three GC-MS-identified MELV compounds, five compounds were selected for docking. The literature survey of these compounds showed that they have potent anti-inflammatory activity (Table-1). The compounds successfully docked with the selected active amino acid residues of the COX enzyme protein. The active residues of the COX enzyme were Leu48, Leu62, Cys65, Leu79, Phe83, Met94, Trp112, Val118, Phe143, Met145, Leu180, Arg85, Arg92 (hydrophobic) and Ser45, Ser52, Ser67, Ser81, Ser133 (hydrophilic) were considered as the important binding sites for protein inhibition. It is a crucial and rate-limiting enzyme in the inflammation that catalyses prostaglandin production, which in turn forms inflammatory mediators. The results were compared with the most widely used NSAIDs, Indomethacin and Diclofenac sodium, which almost directly inhibit COX enzymes and are the first line of treatment for both acute and chronic inflammation. The Glide score was higher for four of the five docked compounds, β-amyrin (-8.9), olean-12-ene (-8.7), Lupeol (- 8.6), and Urs-12-ene (-7.9) and A-Norcholestan-3-one (-7.1) (Table-4). However, the binding scores of all docked compounds of MELV were found to be higher compared to the standard drugs Indomethacin (-7.2) and Diclofenac sodium (-6.2), indicating their potency compared to the standards.[33,34] The ligand molecules that form complexes with the COX protein were shown in Figure 4A–4I.

Table-4: Ligand Docking Scores With 4IMO

Ligand	Vina score	Center (x, y, z)	Size (x, y, z)	Interaction residues
β-amyrin	-8.9	21, 31, 12	23, 32, 23	Ser45, Leu48, Ser52, Trp54, Ala61, Leu62, Ser63, Met64, Cys65, Ser67, Ser81, Phe83, Arg85, Arg92, Met94, Tyr107, Ser109, Trp112, Tyr116, Ser133, Gly140, Phe143, Met145, Leu180
Olean-12-ene	-8.7	21, 31, 12	22, 22, 22	Ser45, Leu48, Ser52, Trp54, Lys58, Ala61, Leu62, Ser63, Cys65, Ser67, Leu79, Ser81, Phe83, Leu84, Arg92, Met94, Leu96, Tyr107, Ser109, Trp112, Tyr116, Val118, Ser133, Phe143, Met145, Thr147, Tyr149, Leu180
Lupeol	-8.6	21, 31, 12	22, 22, 22	Ser45, Leu48, Ser52, Trp54, Lys58, Ala61, Leu62, Ser63, Cys65, Ser67, Ser81, Thr82, Phe83, Leu84, Arg85, Arg92, Met94, Tyr107, Ser109, Trp112, Tyr116, Phe143, Met145,Leu180
Urs-12-ene	-7.9	21, 31, 12	23, 23, 23	Ser45, Leu48, Ser50, Ser52, Trp54, Leu55, Ala61, Leu62, Ser63, Met64, Cys65, Lys66, Ser67, Leu79, Ser81, Phe83, Leu84, Arg85, Arg92, Thr93, Met94, Ser109, His111, Trp112, Tyr116, Val118, Ser133, Phe143, Met145, Thr147, Tyr149, Leu180
A-Norcholestan-3-one	-7.1	21, 31, 12	24, 24, 24	Ser45, Leu48, Ser50, Ser52, Trp54, Lys58, Ala61, Leu62, Ser63, Cys65, Ser67, Leu79, Ser81, Phe83, Arg85, Arg92, Met94, His111, Trp112, Tyr116, Val118, Ser133, Gly140, Phe143, Met145, Thr147, Tyr149, Leu180,
Indomethacin	-7.2	21, 31, 12	21, 21, 21	Ser45, Gly47, Leu48, Ser50, Ser52, Trp54, Leu55, Leu62, Cys65, Lys66, Ser67, Leu79, Ser81, Phe83, Arg85, Met94, Leu96, Tyr107, His111, Trp112, Ser114, Tyr116, Val118, Leu131, Ser133, Pro139, Gly140, Phe143, Met145, Thr147, Tyr149, Leu180,
Diclofenac sodium	-6.2	21, 31, 12	19, 19, 19	Ser45, Leu48, Ser50, Ser52, Trp54, Leu55, Leu62, Ser67, Leu79, Ser81, Arg92, Met94, Leu96, Tyr107, Ser109, Trp112, Tyr116, Val118, Leu131, Ser133, Phe143, Met145, Thr147, Tyr149

CONCLUSION

In summary, *Lactuca virosa* clearly exhibited efficient, dose-dependent anti-inflammatory effects based on the results from acute and chronic animal models and was further supported by an *in-silico* study. The triterpenoids found in the leaves, detected by GC-MS analysis, may be responsible for the activity. It can

be concluded that the investigation of the anti-inflammatory activity of *Lactuca virosa* using *in vivo* and *in silico* models validates its traditional use.

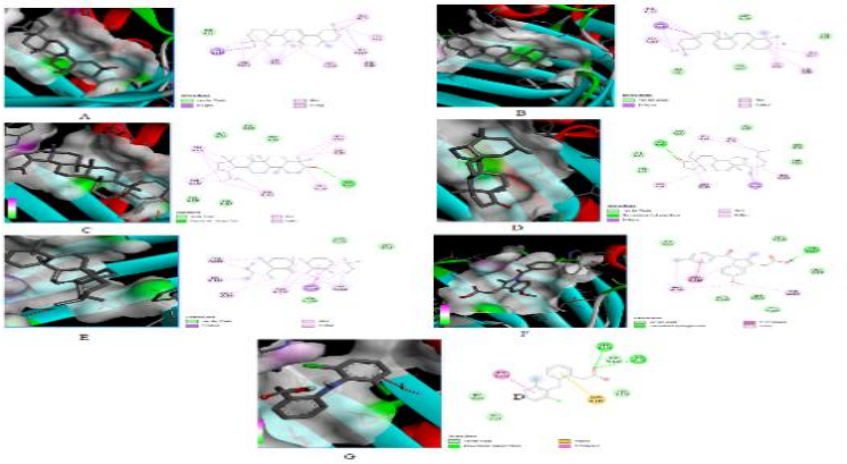


Fig.-4: Binding Complex of Protein (4IMO) with A-Olean-12-ene, B-β-amyrin, C-Lupeol, D-Norcholestan-3-one, E- Urs-12-ene, F-Indomethacin, G-Diclofenac Sodium

Furthermore, the detailed phytochemical and mechanistic studies are required to substantiate the current findings and could open new opportunities for the management of inflammation.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3: Good Health and Well-being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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