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Original Research Article

Pharmacognosy for efficient natural therapy

In Vitro Antioxidant and Anti-Acetyl cholinesterase Activities of wild *Ocimum Sanctum* L. and Endangered Herb *Swertia Chirayita* (ROXB. EX Fleming) H. Karst

Annie Jessica Toppo and Sheela Chandra*

Department of Bio-Engineering, Birla Institute of Technology, Mesra, Ranchi, Jharkhand-835215

Abstract: Medicinal plants is the essence of today's therapeutic culture. In the due course of time, it a great need to produce such therapeutic agents which has multidi mentional medicinal value. These medicines should have ability to cure oxidative stress related disorders which includes cognitive ailments, cancer etc., with no or lesser amount of side effects. The present study aims to analyze stem extracts of *Swertia chirayita* and *Ocimum sanctum* in various solvents to understand the ability to cure oxidative stress related disorders. The capability of extracts has been analyzed by total phenol, flavonoid content, antioxidant and anticholinergic activities. Antioxidant activity of all extracts fractions was measured in terms of ABTS, DPPH and metal chelating properties (FRAP). Enzyme inhibitory properties was measured using AChE (Acetylcholinesterase). *O. sanctum* in methanol extract exhibited maximum content both in phenol (152.7±0.0004 mg GAE/g DW) and flavonoid content (38.38±0.0085 mgQE/g DW). Maximum free radical scavenging activities was observed in *O. sanctum* through DPPH inhibition assay (IC_{50} 12.55 µg/ml). Similarly in ABTS inhibition assay, total antioxidant level was IC_{50} 41.8 µg/ml in methanolic extract of *O. sanctum* and in metal ion chelating assay, it showed 2.494 mg TE/g DW of the sample. Acetylcholinesterase inhibition was highest in methanolic extract of *O. sanctum* (IC_{50} 12.066 µg/ml) followed by *S. chirayita* chloroform (IC_{50} 15.86 µg/ml). The results suggest that the stem methanolic extracts of these two species possess natural antioxidants and anti-cholinergic potential, which may be beneficial for the ailment of neurodegenerative disorders.

Keywords: *O. sanctum*; *S. chirayita* ; Antioxidant ; Acetylcholinesterase ; Neurodegenerative

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***Corresponding Author**

Sheela Chandra , Department of Bio-Engineering, Birla Institute of Technology, Mesra, Ranchi, Jharkhand-835215

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1. INTRODUCTION

According to the long history of plant and plant-derived products in relevance to clinical trials of various diseases, and their utility in the traditional medicine system in different parts of the world is being re-examined. In the present day, there's a boom of naturally originated products especially from medicinal plants for health risks which are exclusively used as synthetic food supplements like antioxidant are in highlights[1]. Phenolic compounds have multiple characteristics in plants, plays both as antioxidant and prooxidant deputed at high to mild health issues, flavonoids, phenolic acids, diterpenes, and lignans are the examples for having these properties[2]. *Ocimum sanctum* L., which is also known as *Ocimum tenuiflorum* or Tulsi, belongs to the family Lamiaceae. *Ocimum* species are well documented for its medicinal value and essentially known plant for curing central nervous system disorders like, Alzheimer's disease (AD) which was estimated in well–endorsed models using neurotoxins like ibotenic and colchicine deficits and elevated oxidative stress[3]. *Swertia chirayita*(Roxb. ex Fleming) H. Karst (family Gentianaceae) is prized herb which is commonly available in India, Nepal, and China. The plant is found at an altitude of 1200-3000 m and available throughout the year[4]. The plant has been reported to possess hypoglycemic activity, wound healing activity as well as antibacterial activity on selected microbial strain and most important antimalarial activity[5]. The present investigation was aimed to determine the total phenol and flavonoid contents, antioxidant assays like 1,1-diphenyl-2-picrylhydrazyl radical scavenging (DPPH), 2,2-azinobis (3-ethyl bezoline-6-sulfonic acid (ABTS) and metal chelation activity has been determined by ferrous reduced antioxidant power (FRAP) assay with anti-acetylcholinesterase activities of different solvents' fractions of *O. sanctum* and *S. chirayita* stems pieces which were least studied till far.

2. MATERIALS AND METHODS

2.1 Preparation of Extracts

Stems of *Ocimum sanctum* (Accession No:- T0134, authenticated from MGM University, Institute of Biosciences and Technology, Aurangabad, Maharasthra, India) were obtained from the medicinal garden of Birla Institute of Technology, Mesra, Ranchi, Jharkhand India and *S. chirayita* stem was collected from their natural habitat of Darjeeling, West Bengal, India. Authenticated *S. chirayita* plant samples were purchased from Ms. Parmila Choudhary, Director, Organo india Organisation, Tung, Darjeeling, West Bengal, India and both plant samples were already submitted to taxologist. Plant materials were shade dried, putrefies in the mortar and each powdered material weighs 500 gm were extracted in 1000 ml of solvents (100%) and extraction was done successively from non-polar to polar solvent (n-Hexane, Diethyl ether, Chloroform, Methanol) for 48 h. Then extracts were concentrated using rotary vacuum evaporator (yield obtained approx. 35mg) and freeze dried for further analysis[6].

2.2 Total Phenol and Flavonoid content

Total phenol content was determined by the Folin-Ciocalteu (FC) method with some modifications by Muddathir et al,[7]. The varied solvent fractions have been estimated by using reaction mixture consisting of 1.5 ml (10%) FC reagent and 1 ml (2%) of sodium carbonate was added, methanol was taken as blank. The reaction mixture was spawned at room temperature for 15 mins and by employing spectrophotometer absorbance at 765 nm was measured. Total phenol content evaluated as gallic acid equivalent per gram dry weight [6]. Estimation of total flavonoids content was done by the aluminum chloride method employed by Laghariet al.[8]. The reaction mixture contains 2 ml sample, 0.6 ml (5% w/v) $NaNO_2$, 0.5 ml (10% w/v) $AlCl_2$, 3 ml (4.3% w/v) NaOH and make volume up to 10 ml by distilled water. In every step, 6 min shaking was done, and then the solution was spawned for 15 min at room temperature. Then absorbance was measured at 500 nm employing spectrophotometer. On the basis of the calibration curve equation ($y=0.005x$, $R^2=0.991$, where x is the absorbance and y is the quercetin equivalent (mg/g) (QE), total flavonoid content were evaluated.

2.3 Antioxidant Assays

The assessment of scavenging vitality of sample by counteracting DPPH radicals was with some modifications[9]. 0.1 mM DPPH methanol solution was prepared and absorbance adjusted to 0.90±0.001 units and then 2.9 ml of it was added with the sample (20µg-80µg), the mixture was incubated for 30 mins at room temperature. Absorbance was graduated at 517 nm and Trolox was used for the standard. The equation for inhibition as follows: % inhibition = [(absorbance of control – absorbance of sample)/ absorbance of control] X 100. IC_{50} values were evaluated based on the percentage of DPPH scavenging. In ABTS assay, stock solutions consist of 7 mM ABTS solution and 2.4 mM $K_2S_2O_8$ solution, these two were blended in a proportion of 2:1, then it was constrained for 12 h in dark for reaction. Then the solution's absorbance was adjusted to 0.708±0.001 units at 734 nm. Then, the solution was diluted in ratio 1:60 using methanol, 4 ml of solution was added to sample (0-80 µg), the mixture was incubated for 2h in dark, absorbance was measured at 734 nm. Trolox was used for standard curve assimilation, percentage inhibition was calculated by: I% = [($A_{control}$- A_{sample}) / $A_{control}$] X 100 ($A_{control}$- absorbance of ABTS radical + methanol used as control; A_{sample}- absorbance of ABTS radical + sample extracts/standard). IC_{50} was based on the percentage of ABTS scavenging activity[9]. The FRAP methodology was incorporated using standard protocol[10]. The stock solution consists of 300mM acetate buffer (pH 3.6), 20mM TPTZ 2,4,6-Tri(2-pyridyl)-s-triazine in 40mM of HCl, 20mM $FeCl_3.6H_2O$. The working stock was a solution acquainted with 25 ml of acetate buffer, 2.5 ml TPTZ and 2.5 ml $FeCl_3.6H_2O$. In reaction, the mixture, 2850 µl FRAP solution, sample (0-80 µg) was taken and incubated for 2h in the dark. The absorbance of the ferrous tripyridyltriazine complex was taken at 593 nm. On the basis of mg Trolox equivalent/g dry mass, the conclusion has been drawn.

2.4 Acetylcholinesterase inhibitory assay

Acetylcholinesterase inhibition assay was taken from protocol by Lee et al.[11]. In 96 wells Microplate each well contains 125 µl (3mM) DTNB in buffer (50 mMTris-HCl, pH 8, containing 0.1M NaCl and 0.02M $MgCl_2$. $6H_2O$), 50 µl buffer (50 mM.TrisHCl, pH8). 0.1% BSA and samples (0-80 µg), MeOH was taken as blank and Galantamine was taken as control absorbance was measured at 405 nm. AChE inhibition % equation follows:-

$(Abs_{sample}-Abs_{Control} / Abs_{control}) X 100$

(Abs_{sample}– Absorbance of the sample, $Abs_{control}$– Absorbance of the Abs_{Blank}).

3. STATISTICAL ANALYSIS

All experiments were done in triplicates and reported as mean values, IC_{50} and graphs were done using Graph Pad Version 5.0 for Windows (Graph Pad Software Inc).

4. RESULTS

4.1 Total Phenol and Flavonoid Content

Stem fraction of both plants, maximum phenol and flavonoid content were observed in *O. sanctum* methanolic and lowest was in diethyl ether fraction. The evaluated results of phenol and flavonoid content variegated from 98.22±0.0050 to 152.7±0.0004 mg GAE/g DW(dry weight) and 4.1±0.00035 to 38.38±0.0085 mg QUER/g DW of samples, respectively (Table 1 and 2). The methanolic fraction of *S. chirayita* showed 136.28±0.002 mg GAE/gDW, 25.2±0.017 mg QE/gDW as compared to *O. sanctum* 152.7±0.0004 mg GAE/gDW, 38.38±0.0085 mg QE/gDW in 80 µg/ml concentration in total phenol and flavonoid content (Table 1,2).

Table 1: Total phenol content of *O. sanctum* and *S. chirayita* in different solvents, determined in various concentrations.

Plant Material	Solvent	Concentration (µg/ml)				
		20	40	60	80	
S. chirata stem (SCC)	CHLOROFORM	108.61±0.0036	112.14±0.039	123.85±0.0039	134.32±0.0055	Total Phenol Content (mg GAE/g)
O. sanctum stem (OSC)		118.85±0.0047	126.57±0.0021	135.85±0.0079	142.64±0.0035*	
S. chirata stem (SCD)	DIETHYL ETHER	98.22±0.0050	104.28±0.0042	112.71±0.013	125.74±0.011	
O. sanctum stem (OSD)		109.4±0.0013	113.17±0.0825	120.18±0.004	135.68±0.0078*	
S. chirata stem (SCM)	METHANOL	108.5±0.003	118.08±0.002	125.8±0.001	136.28±0.002	
O. sanctum stem (OSM)		127.7±0.004	138.71±0.017	144.02±0.003	152.7±0.0004*	

*Values are mean (n=5), *P<0.05 when compared with control*

Table 2: Total flavonoid content of *O. sanctum* and *S. chirayita* in different solvents, determined in various concentrations

Plant material	Solvent	Concentration (µg/ml)				
		20	40	60	80	
S. chirata stem (SCC)	CHLOROFORM	6.18±0.0121	11.78±0.0028	17.52±0.0016	24.64±0.0025	Total Flavonoid Content (mg QUER/g)
O. sanctum stem (OSC)		9.18±0.006	15.14±0.0014	23.86±0.0017	33.1±0.024*	
S. chirata stem (SCD)	DIETHYL ETHER	4.1±0.00035	7.46 ±0.018	14.66±0.0015	22.32±0.00117	
O. sanctum stem (OSD)		8.34±0.0005	13.4±0.00036	20.5±0.00012	28.5±0.00047*	
S. chirata stem (SCM)	METHANOL	5.76±0.0013	10.08±0.0077	22.66±0.0004	25.2±0.017	
O. sanctum stem (OSM)		10.48±0.0085	19.48±0.006	27.92±0.0089	38.38±0.0085*	

*Values are mean (n=5), *P<0.05 when compared with control*

4.2 Antioxidant Assays

To evaluate the strength to scavenge the free radicals, amongst discrete chemical based assays, in this study ABTS and DPPH has been employed. For the measurement of metal ion chelation potential of antioxidants, FRAP assay was used for this purpose. DPPH scavenging activity was seen in stem extracts of both plants in all fractions in which percentage inhibition of *O. sanctum* in methanol fraction was maximum (92.74%, IC_{50} value was 12.55 µg/ml in 80µg/ml concentration) and the minimum was showed in *S. chirayita* diethyl ether fraction (16.99%, 267.83 µg/ml in 80µg/ml), standard compound Trolox IC_{50} was 46.22 80µg/ml(Figure 1).

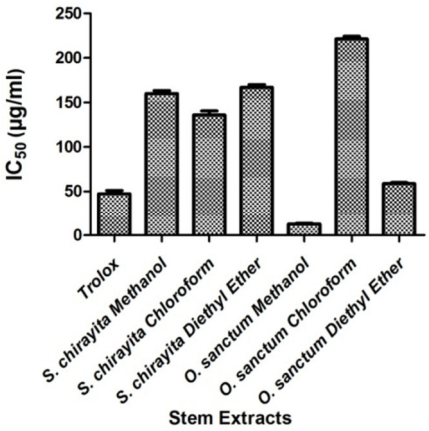

Fig 1: DPPH Scavenging Activity of Stem Extracts

The results of ABTS radical scavenging assay, says that *O. sanctum* stem tissues possess a significantly higher percentage of inhibition than *S. chirata* stem (20 to 80 µg/ml conc. of samples). Percentage inhibition observed highest in the methanolic fraction of *O. sanctum* at80 µg/ml (91.78%) followed by diethyl ether (68.04%) and chloroform fraction (22.57%). The methanolic fraction of *O. sanctum* showed an IC_{50} value of 41.8 which was found to be lower than trolox which had IC_{50} value of 48.22 (Figure 2). Thus, it can be concluded that the methanolic fraction has an efficacy as inhibitor of ABTS.

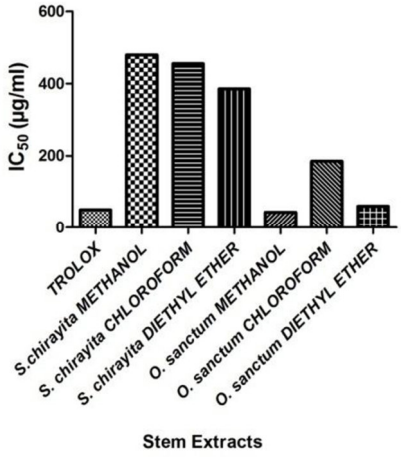

Fig 2: ABTS Activity of Stem Extracts

An obligatory antioxidant mode of action can be contemplated as chelating transition metal potential. Lipid oxidation leads to the high reactivity of iron which is known as most pro-oxidant metal, amongst the transition metals. This reducing ability of these fractions by FRAP assay has been shown in Table 3. Among all fractions, the methanolic fraction had higher FRAP value followed by diethyl ether and chloroform. The methanolic fraction of *O. sanctum* had 2.494 µgTE/g DW than *S. chirayita* which had 0.748 µgTE/g DW.

Table 3: Metal ion chelating activity (FRAP) of *O. sanctum* and *S. chirayita* extracted in different solvents, determined in various concentrations.

Plant material	Solvent	Concentration (µg/ml)				
S. chirayita stem (SCC)	CHLOROFORM	20	40	60	80	FRAP (mg TE/g dry weight of sample)
		0.139	0.319	0.471	0.552	
O. sanctum stem (OSC)		0.281	0.434	0.654	0.852	
S. chirayita stem (SCD)	DIETHYL ETHER	0.398	0.403	0.513	0.622	
O. sanctum stem (OSD)		0.948	1.170	1.387	1.447	
S. chirayita stem (SCM)	METHANOL	0.133	0.319	0.534	0.748	
O. sanctum stem (OSM)		1.048	1.455	1.899	2.494	

*Values are mean (n=5), *P<0.05 when compared with control*

4.3 Acetylcholinesterase inhibitory assay

Dementia, which can be visualized by the weakness and memory loss mostly found in aged people, but in contemporary works of literature suggests that young and middle-aged were also affected poorly. Therefore, for treating cognitive damage, the new exploration of drugs which have the capability to heal dementia is in high interest. In acetyl-cholinesterase inhibitory activity study, different fractions of the sample were screened at different concentrations using a 96 well microplate reader. *O. sanctum* was found to be the most potent inhibitor against AchE with an IC_{50} values of 12.066 µg/ml in methanol fraction, chloroform fraction (18.82 µg/ml) and in diethyl ether fraction (41.47µg/ml) as compared to *S. chirayita* which shows IC_{50} values in methanol fractions (68.09 µg/ml), chloroform fraction (15.86 µg/ml) and diethyl ether (34.07µg/ml) shown in Figure 3. It is extremely important to point out that *O.sanctum* stem's inhibitory trend line was the same as that of its antioxidant activity. In our study, galantamine was taken as the positive control, which showed an IC_{50} value of 4.69µg/ml. The ascending arrangement of AchE inhibitory

activity for stem extract's fractions on the basis IC_{50} values was as follows OSM (*O.sanctum* Methanol)>SCC (*S.chirayita* Chloroform) > OSC (*O. sanctum* Chloroform) > SCM (*S.chirayita* Methanol)> SCD (*S.chirayita* Diethyl ether)>OSD (*O. sanctum* Diethyl ether. Hence, from all these studies, a conclusion can be drawn that OS can be effectively mitigate the negative effects of oxidative stress. In many studies, the effect of AchE activity has not been studied profusely but in present experimentations *in vitro* conditions on the effect of AchE with respect to extracts has been explored. In our study, *S. chirayita's* chloroform fraction showed effective antioxidant potential, but in the study of Rashid et al.[15] methanolic extract showed antioxidant potential more than 50%. These results conclude that different concentrations of major bioactive compounds of *S. chirayita (*xanthonoids, amarogentin, and swertiamarin) present in different fractions shows significant potential for antioxidation.

5. DISCUSSION

5.1 Total Phenol and Flavonoid Content

Phenolics and flavonoids work together with the endogenous antioxidant defense system and a higher amount of polyphenol content has been associated with the higher antioxidant ability[18]. In table 1 and 2, we have shown the *O. sanctum* methanolic fraction's high elevation of total phenolic and flavonoid content. Previous studies illustrated that, due to the polar nature of extract with existence of aromatic rings, the feature of OH groups substitution and molecular weight, phenols and flavonoids attributed as free radical scavengers [19,20]. According to the work of Garg and Garg,[21], the total flavonoid content of *O. sanctum* stem was 1.81 mg/100 mg which was an appreciable amount of flavonoid present in a sample.

5.2 Antioxidant Assays

As most of the natural antioxidants are multifunctional for that, the dependable antioxidant protocol is requisite to measure more than one character. In the present study, we tried to explain the antioxidant conduct of crude extracts against free radicals which manifests the potential of phenols and flavonoids which would be helpful to cure dementia. As per the observations natural antioxidants scavenge free radicals which dwells hydrogen peroxide and singlet oxygen[22]. In a coeval study, the initial agglomeration DPPH is scaled down by the concentration of the sample extract up to 50% (IC_{50}) under experimental predicament which has been calculated (Figure 1). Highest DPPH scavenging activity in IC_{50} value was showed by methanol extract of OS i.e 12.55 µg/ml and diethyl ether extract showed 58.9µg/ml, which was notably higher over positive standard trolox (IC_{50} 47.48 µg/ml). Similar results were also found by Hakim et al.,[23] in which OS stem's methanolic extract shows higher IC_{50} value than leaves. Studies done by Agarwal et al.,[24] was found that *O. sanctum* exhibits better scavenging potential (>80% in 80µg/ml) against DPPH free radical that it's other species namely *O. kilimandscharicum*. Roy et al.,[12] also found that, in comparing different species of *Swertia* and its fractions, methanolic extract showed better scavenging and percentage inhibition which was about 22.75% for *Swertiachirayita.* In case of ABTS scavenging activity, according to the studies of Tachakittirungrod et al.,[25]found that in comparison of 34 different samples, though Guava leaves, stem and fruit pulp were highest in antioxidant activity but amongst all samples when *O. sanctum* was compared with other species of *Ocimum* i.e. *O. basillicum* the antioxidant activity is higher slightly higher in case of *O. sanctum* ethanolic extract i.e. 0.877 mM trolox equivalent/mg extract, though there are few works reported about stem (methanolic fraction) potential which showed better scavenging than other fractions which was about 22.75%. Studies by Nair et al.,[26]in which leaves of *O. basillicum* showed higher scavenging potential than other species which was 82.76%. These studies support that the *Ocimum* species contains an abundance of compounds which have the potential to donate a hydrogen atom to a free radical and makes them unstable. Therefore, we can assume that these plants can be a promising treatment for radical associated pathological tissue disability. Depicting FRAP values, the similar trend to that of DPPH and ABTS was observed, wherein the polar solvents' extract performed better as antioxidants in comparison to non-polar extracts. According to Hakkim et al.,[23]studies, the stem extract of *O. sanctum* had shown good chelation when compared with plants' inflorescence. Similarly, studies by Sethi et al.,[27],in which *O. sanctum* showed the good chelating activity of 64 mg/ml in the methanolic fraction. Kaur et al.,[28], Aydemir and Becerin,[29]observed the highest metal chelating activity of *O. tenuiflorum* seeds in methanolic solvent amongst the other solvents i.e 9.51±0.04 mM Similarly, in the present study the chelating activity of *O. sanctum* stem performed better followed by *S. chirayita,* it could be due to the presence of more antioxidant active compounds. Therefore, for retarding Fe^{2+} ions which catalyzes lipid oxidation, by this action it can be concluded as an effective agent.

5.3 Acetylcholinesterase inhibitory assay

Dementia, which can be visualized by the weakness and memory loss mostly found in aged people, but in contemporary works of literature suggests that young and middle-aged were also affected poorly. Therefore, for treating cognitive damage, the new exploration of drugs which have the capability to heal dementia is in high interest. In acetyl-cholinesterase inhibitory activity study, different fractions of the sample were screened at different concentrations using a 96 well microplate reader. Kushindarta et al.[13], has studied the ethanolic extract of *O. sanctum* which shows an increase in the amount of the cells, induces proliferation or possible neurogenesis in the middle-aged mouse model. Similar results had been observed by Raghavendra et al.[14], in which rat model induced with isotonic acid and colchicine, showed alleviated depression due to the dosage of the methanolic extract of *O. sanctum* Hence, all these results say that OS will be able to mitigate the effects of oxidative stress in animal models. Many of the studies anticholinesterase activity had not been observed but in this study, *in vitro* condition, particularly in stem tissues as methanolic extracts has been explored which were least studied. Further, we can say that OS will be able to alleviate the neuropsychological system symptoms associated with AD. In the present studies, *S. chirayita* showed antioxidant potential in chloroform fraction, but in reported study by Rashid et al.[15] in which *S. chirayita* showed effective antioxidant potential more than 50% in methanolic extract consist of aerial part. Presence of some important bioactive compounds such as xanthonoids, amarogentin, and swertiamarin have been considered as significant phytochemical constituents of *Swertia chirayita*[16], but in our study, the methanolic fraction of OS showed better inhibition of acetylcholinesterase than chloroform fraction of SC stem.

According to studies by Swamy et al.[17] due to high polarity, methanol was found to exhibit better efficiency in extracting various polar phytocompounds (phenolics and flavonoids

6. CONCLUSION

In conclusion, the study has demonstrated that methanolic fraction of stem of *O. sanctum* gave the highest antioxidant activity in all *in vitro* assays studied which may be due to the presence of the abundance of (single or combination) phenols or flavonoids. In this particular solvent's fraction which can be used as a nutritional source for overcoming brain-related disorders. In our study, it has been concluded that *O. sanctum's* stem methanolic extract can be employed as an accessible expert of natural antioxidants. Therefore, these plant fractions need to confirm for their *in vivo* activity and bioavailability, so that the good advantageous effects of the plant extracts may indeed be used in the neurodegenerative diseases.

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9. AUTHORS CONTRIBUTION STATEMENT

Dr. Sheela Chandra has conceptualized the work and Ms. Annie Jessica Toppo gathered all the datas required for the study. Data were analyzed by both of them with necessary inputs required for designing manuscript. All authors discussed and the methodology and results and contributed to the final manuscript.

10. CONFLICT OF INTEREST

Conflict of interest declared none.

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