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RESEARCH ARTICLE

Bioactive compounds and antioxidant potential of *Viola cinerea* and *Pseudogaillonia hymenostephana* from Surghar Range, Pakistan

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Abstract

Medicinal plants are key source of bioactive compounds possessing antioxidant potential. The current research work was conducted to evaluate the bioactive compounds and antioxidant potential of two plants viz. *Viola cinerea* (*V. cinerea*) and *Pseudogaillonia hymenostephana* (*P. hymenostephana*) collected from Surghar Range, Pakistan in 2020. For determination of bioactive compounds, the extracts of both plants were evaluated for total phenolic, flavonoids and tannin content. For antioxidant potential three assays were applied viz. FRAP, DPPH and ABTS method. The results depicted that total phenolic content (TPC) for *V. cinerea* was found to be 38.65 ± 0.93 GAE mg g^{-1}. Total flavonoid content (TFC) was 17.37 ± 0.87 Catechin eq. mg g^{-1} and total tannin content (TTC) for *V. cinerea* was 2.88 ± 0.32 Catechin eq. mg g^{-1}. The results for *P. hymenostephana* indicated 33.16 ± 1.32 GAE mg g^{-1} (TPC), 15.21 ± 0.73 Catechin eq mg g^{-1} (TFC), while 3.12 ± 0.11 Catechin eq mg g^{-1} of total tannin contents (TTC) were observed. The results of DPPH, ABTS and FRAP assay for *V. cinerea* were 38.21 ± 2.37, 38.34 ± 2.87 Trolox equivalent µmol g^{-1} and 19.57 ± 1.05 µmol g^{-1} respectively. The value of DPPH assay for *P. hymenostephana* was 43.41 ± 3.34, ABTS assay valued at 39.21 ± 2.22 Trolox equivalent µ mol g^{-1} and FRAP assay analysis showed the value 18.23 ± 1.11 µmol L^{-1} g^{-1} for *P. hymenostephana*. The results indicated that like other violets and medicinal plants of family Rubiaceae both the plants which were under consideration shown good antioxidant potential.

Keywords: ethanol; medicinal plants; Pakistan; phytochemistry; Surghar Range

Introduction

Plants are innumerable source of bioactive compounds that have been used directly or indirectly in traditional medicine since time immemorial (1). Formerly, people lack information regarding bioactive compounds, but use of these molecules was enhanced with time. Usually, bioactive compounds are generated as secondary metabolites in plants (2). It was further analysed that secondary plant metabolites exhibit both toxic and curative effects in humans and wildlife (2). These compounds are categorized into three main kinds, viz. phenolic, alkaloids and terpenes (approximately 8000, 12000 and 2500 types, respectively) (3). These unique compounds are worth mentioning due to their antioxidant potential and stand vital for enhancing the damaging results of oxidization in vegetation (4, 5). Substantial role is played by reactive oxygen species (ROS) in the expansion of various chronic and degenerative diseases like heart diseases, cancer, diabetes, aging and nervous disorders (6). Antioxidants play crucial part opposing free radicals and subsequently stop harms produced by reactive oxygen species (7, 8). Antioxidant compounds obtained naturally by the organisms are useful to overcome various diseases for example inflammation, cancer, dementia and cardiac diseases (5, 7). Nearly, 400000 species of plants on earth possess huge quantity of bioactive compounds, but only a small fraction of them has been explored to date (9). Hence, the interest in evaluating the antioxidant nature of wild flora as natural sources emerge as prevalent amongst investigators meanwhile the pharmacological industry demands natural sources possessing great antioxidant capability. Hence, the key concern of current research is not merely about synthesizing the new antioxidants but also to report and highlight the new ways and directions for extraction and identification of antioxidants from wild flora (10).

Viola cinerea (family Violaceae) and *Pseudogaillonia hymenostephana* (family Rubiaceae) were selected as the study plants in this paper due to their ethnobotanical applications in healing among the local populace residing in the Surghar Range of Pakistan, as well as their taxonomic classification within the family exhibiting the greatest diversity of antioxidants.

Viola cinerea is a rare medicinal herb in Pakistan, infrequently gathered and has been long utilized in folk medicine for its anti-inflammatory, expectorant and febrifuge properties. Local healers frequently utilize the entire plant in decoctions, which is regarded as a crucial component in the treatment of traditional respiratory and dermatological ailments. Members of the Viola genus are known to contain a variety of bioactive compounds, particularly flavonoids and cyclotides, with reported antioxidant, antiviral, anticancer and neuroprotective activities (2, 13).

Pseudogaillonia hymenostephana, a distinct Rubiaceae species recognized by its attractive and colorful calyces, is locally valued for treating gynecological disorders, liver and respiratory ailments and fevers (12). Although the Rubiaceae family is globally acknowledged for its phytochemical diversity, particularly alkaloids, flavonoids, coumarins and terpenes, this species remains scientifically unexplored for its bioactive and antioxidant properties (14, 15).

To the best of our knowledge, there is no previous literature reporting the phytochemical composition or antioxidant potential of *V. cinerea* and *P. hymenostephana*. Therefore, this study aims to provide the first scientific account of the bioactive compounds and antioxidant activity of these two medicinal plants using standard spectrophotometric assays. The findings are expected to not only validate traditional claims but also contribute to the search for natural antioxidants with potential applications in therapeutic development.

Materials and methods

Chemicals and reagents

Ethanol, aluminum trichloride, sodium nitrite, Folin-Ciocalteu reagent (FCR), gallic acid, caffeic acid, 2,2-diphenyle 1-1-picrylhydrazyl (DPPH), sodium carbonate, 2-2-azinobis (3-athylbenzothiazoline-6-sulphonic acid), linoleic acid, ferric chloride, Potassium ferricyanide, potassium persulfate, potassium phosphate buffer, quercetin, sodium hydroxide, sodium phosphate buffer, trichloroacetic acid, hydrochloric acid from Sigma, Germany or BDH. Standard antioxidant used for the FRAP, ABTS and DPPH assays was Trolox.

Data collection

The plants (*Pseudogaillonia hymenostephana* and *Viola cinerea)* samples were collected from the study area (Surghar Range, Pakistan) during the field work from March 2017 to September 2020 (Fig. 1). The scientific names of collected plant specimens were identified following the Flora of Pakistan (11). The names were further updated after verification from the online website of "World Flora Online" (WFO) (https://worldfloraonline.org). Voucher specimens were preserved and deposited in the Herbarium of the Botany Department (SARGU), University of Sargodha, Pakistan for future reference.

Sample preparation

Leaves were washed with water and subjected to dried under shade. Dried plant material (100 g) was crushed, ground and powdered followed by sieved using 10 µg pore size sieves. Powdered material was stored in dark at 4 °C. Powder (10 g) was subjected to ethanolic maceration (250 mL) in an orbital shaker overnight. The resulting mixture was filtered and the solvent was removed from the filtrate under reduced pressure using a rotary evaporator. The resulting extract was stored at 4 °C until further analysis.

Determination of total phenolic content

By using the modified Folin-Ciocalteu assay (16), the ethanolic leaves extract was diluted 1:4 in 70 % ethanol. An aliquot of 0.1 mL diluted extract was mixed with 0.2 mL of feed conversion ratio (FCR), 2 mL of double distilled water and 1 mL of sodium carbonate-saturation solution (20% w/v in H_2O). The extract was incubated at 25 °C in the dark for 30-60 min. Reaction mixture absorbance was estimated at 765 nm using Shimadzu UV-1800 spectrophotometer. Following the same method, blank samples were prepared. Gallic acid used as the calibration standard (0-200 µg/mL) and the results were reported as milligrams of gallic acid equivalent (GAE) per gram of dry extract. The standard curve exhibited significant linearity ($R^2 > 0.995$), ensuring reliable quantification.

Determination of total flavonoid content

Flavonoid content was estimated using the aluminium tri-chloride assay with minimal modifications (16). After adding 1 mL of each extract (1 mg/mL) and 6.4 mL of distilled water to test tubes, 0.3 mL of 5 % sodium nitrite was added and left for 5 min. Subsequently, 0.3

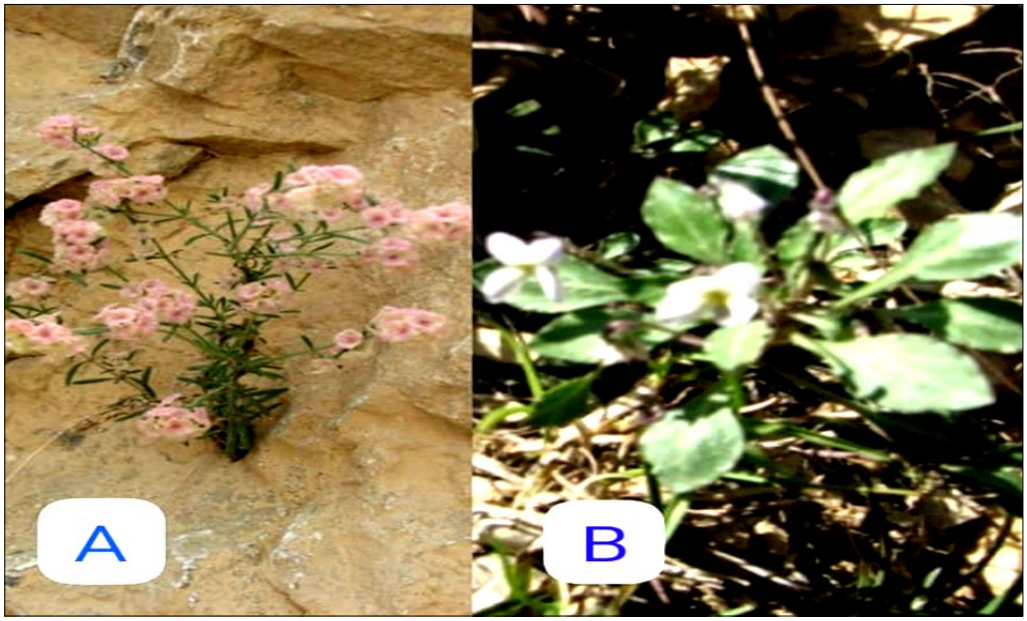

Fig. 1. Plant collected from study site (Surghar Range, Pakistan) *Pseudogaillonia hymenostephana* (A) and *Viola cinerea* (B).

mL of 10 % aluminium tri-chloride was added and the mixture was allowed to stand for 6 min. The solution was shaken for 30 min after adding 2 mL of 1M NaOH. A spectrophotometer measured absorbance at 510 nm. Rutin was standard under the same protocol. A calibration curve (0-200 µg/mL) was established using rutin and the results were expressed as milligrams of rutin equivalent (RE)/g of extract.

Determination of total tannins content

Total tannin content was determined using the method described in previous study (17). Briefly, 50 µL of each extract was mixed with 1.5 mL of 4% vanillin, followed by the addition of 750 µL of HCl. The mixture was incubated at 25 ℃ for 20 min in dark. The Catechin calibration curve (0-100 µg/mL) was established. The data were shown as milligrams of Catechin equivalent (CE)/g of dry extract.

Antioxidant assay

FRAP method

The ferric reducing antioxidant power (FRAP) was measured using the method described in previous study (18). The increase in HCl concentration to 50 mmol/L dissolving 10 mmol/L 2,4,6-tri-pyridyl-s-triazine (TPTZ) was noticed. An aqueous Fe (II) sulphate heptahydrate solution in the 0-900 mmol/L range was fine-tuned at 600 nm (r = 0.9997). All reactions were carried out at 25 ℃. All reactions were carried out at 25 ℃. The reaction mixture was pipetted twice into test tubes, thoroughly mixed and incubated for 5 min. Absorbance was measured at 700 nm using a spectrophotometer. A gallic acid solution of 10 mmol L^{-1} was used for comparison. The ferric reducing of plasma (FRAP) value of the sample (µmol/L) was calculated using the following formula:

$$\text{Ferric reducing of plasma} = \text{(Sample - blank) x 500 (Standard - blank)}$$

A calibration curve was developed using standard solutions of $FeSO_4 \cdot 7H_2O$ (0-1000 µM), with results expressed as µmol Fe^{2+} equivalents/g of extract.

DPPH method

Using a modified method described in previous study (19), 1 mg/mL of the extract was produced with methanol to achieve a concentration range of 10 to 1000 µg/mL 1 mL of sample or standard was added to 0.5 mL of methanol-based 0.2 mM DPPH solution. Trolox is a standard under these settings. After 30 min of dark incubation, 517 nm absorbance was calculated. The radical scavenging ability (RSA) was measured in % by equation:

$$RSA\% = \frac{\text{Abs control - Abs sample}}{\text{Abs x 100}}$$

Trolox was used as the positive control and a calibration curve was established in the range of 0-200 µg/mL. The results were expressed as micromoles of Trolox equivalents (TE) per gram of extract.

ABTS method

Extract was analyzed for antioxidant activity using 2-2-azino-bis(3-ethyl-benzothiazoline, 6-sulphonic acid (ABTS) technique by following significantly modified methodology (20). ABTS (2 mM) was mixed with 70 mM potassium persulfate. It was dark-incubated for 12-16 hr. Methanol was used to dilute the solution to calibrate absorbance at 0.700 ± 0.005 at 734 nm. To achieve a concentration of 25 to 1000 µg/mL, 1 mL of extract or standard at 1 mg/mL was added to 2 mL diluted ABTS solution. About 30 sec were spent incubating it. A spectrophotometer measured absorbance at 734 nm. Trolox was utilized as the standard and a calibration curve was constructed (0-200 µg/mL). The antioxidant capacity was stated in µmol Trolox equivalents (TE)/g of the extract.

Data analysis

All the experimental work and measurements were made in triplicate (n = 0.3) and results were presented as mean ± SD (Table 1). Calibration curves for all standard compounds exhibited strong linearity (R^2 > 0.99) and all blank readings were subtracted from sample reading to ensure accuracy and reproducibility.

Results

Bioactive compounds

The results depicted that total phenolic content (TPC) in ethanolic extract for *V. cinerea* were found to be 38.65 ± 0.93 GAE mg g^{-1}. Total flavonoid content (TFC) was 17.37 ± 0.87 Catechin eq. mg g^{-1} and total tannin content (TTC) for *V. cinerea* were 2.88 ± 0.32 Catechin eq. mg g^{-1}. The results for *P. hymenostephana* indicated 33.16 ± 1.32 GAE mg g^{-1} (TPC), 15.21 ± 0.73 Catechin eq. mg g^{-1} (TFC), while 3.12 ± 0.11 Catechin eq. mg g^{-1} of total tannin content (TTC) were observed (Fig. 2). Presence of these bioactive compounds (phenolics, flavonoids and tannins) is the major reason for antioxidant activities of plants. So that antioxidant potential of these plants was also measured by selected assays.

Antioxidant potential

Leaves possess a wide array of antioxidants. Consequently, the individual assessment of the antioxidant potential for each chemical presents significant challenges. Various techniques have been devised to assess the antioxidant potential of diverse botanical specimens (21). Typically, these methodologies assess the capacity of antioxidants within a certain botanical specimen to effectively neutralize specific radicals through the inhibition of lipid peroxidation or the chelation of metal ions. This work employs three distinct methods to assess the antioxidant potential of extracts. The

Table 1. Comparison of phytochemical contents and antioxidant activities of *Viola cinerea* and *Pseudogaillonia hymenostephana*

Parameter	*Viola cinerea*	*Pseudogaillonia hymenostephana*	Units
Total phenolic content (TPC)	38.65 ± 0.93	33.16 ± 1.32	GAE mg g^{-1}
Total flavonoid content (TFC)	17.37 ± 0.87	15.21 ± 0.73	Catechin eq. mg g^{-1}
Total tannin content (TTC)	2.88 ± 0.32	3.12 ± 0.11	Catechin eq. mg g^{-1}
DPPH radical scavenging activity	38.21 ± 2.37	43.41 ± 3.34	Trolox eq. µmol g^{-1}
ABTS radical scavenging activity	38.34 ± 2.87	39.21 ± 2.22	Trolox eq. µmol g^{-1}
FRAP assay	19.57 ± 1.05	18.23 ± 1.11	µmol g^{-1}

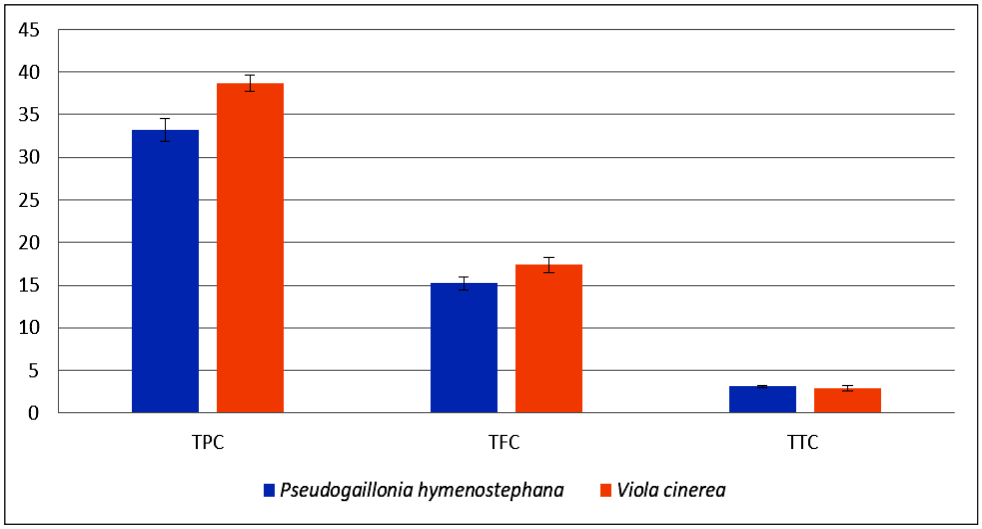

Fig. 2. Determination of TPC, TFC and TTC for *P. hymenostephana* and *V. cinerea*.

results of DPPH, ABTS and FRAP assay for *V. cinerea* were 38.21 ± 2.37, 38.34 ± 2.87 Trolox equivalent µ mol g^{-1} and 19.57 ± 1.05 µmol g^{-1} respectively. The value of DPPH assay for *P. hymenostephana* was 43.41 ± 3.34, ABTS assay valued at 39.21 ± 2.22 Trolox equivalent µ mol g^{-1} and FRAP assay analysis showed the value 18.23 ± 1.11 µmol g^{-1} for *P. hymenostephana.* These results show that both plants have antioxidant potential (Fig. 3).

Discussion

This study is the first ever preliminary account on the biologically active compounds and antioxidant potential of *P. hymenostephana* and *V. cinerea* collected from Surghar Range of Pakistan. As there is no literature available about the phytochemical analysis and antioxidant potential on *V. cinerea* and *P. hymenostephana*. The comparative analysis of ethanolic extracts of both these plants indicated that total phenolic content in *V. cinerea* was higher as compared to *P. hymenostephana.* The same result was observed for total flavonoid and tannin content. It is considered that the major reason for antioxidant potential of medicinal plants is the richness of bioactive compounds.

The antioxidant potential of plants was also evaluated by specific assays. The results of antioxidant activities are not only dependent on the extract composition but also on the test system selected for antioxidant potential determination. Due to various affecting factors a single selected assay is not considered to be perfect for determination of antioxidant activity (22). Because of unpaired valence electron in Nitrogen Bridge, the DPPH molecule is deliberated as an extremely stable free radical (23).

To measure and determine the radical scavenging activity of various important plant extracts, DPPH is one of the most important method (24, 25). In the present study, the relatively high DPPH radical scavenging capacity observed for extract of *P. hymenostephana* as compared to *V. cinerea*. The reason of lesser DPPH scavenging nature of the extract may be due to the reaction preference of DPPH as the polyphenols are favored for radical reaction (26).

The antioxidant capacity of both plant extracts was also assessed via the ferric reducing antioxidant power (FRAP) assay. In this assay, the antioxidant capacity is measured based on the capacity to reduce ferric (III) ions to ferrous (II) ions. The FRAP assay is a modest technique. The perceived antioxidant potential by FRAP assay was higher for *V. cinerea* as compared to *P. hymenostephana*. Trolox equivalent antioxidant capacity (TEAC) assay is one of the leading commonly used methods for describing antioxidant capacity. The TEAC assay calculates the capability of a compound to scavenge ABTS˙ radicals. In the present study high ABTS˙ radicals scavenging activity was observed for *P. hymenostephana* as compared to *V. cinerea.*

The literature survey conducted for comparative analysis shown that until now a total of 370 compounds has been isolated

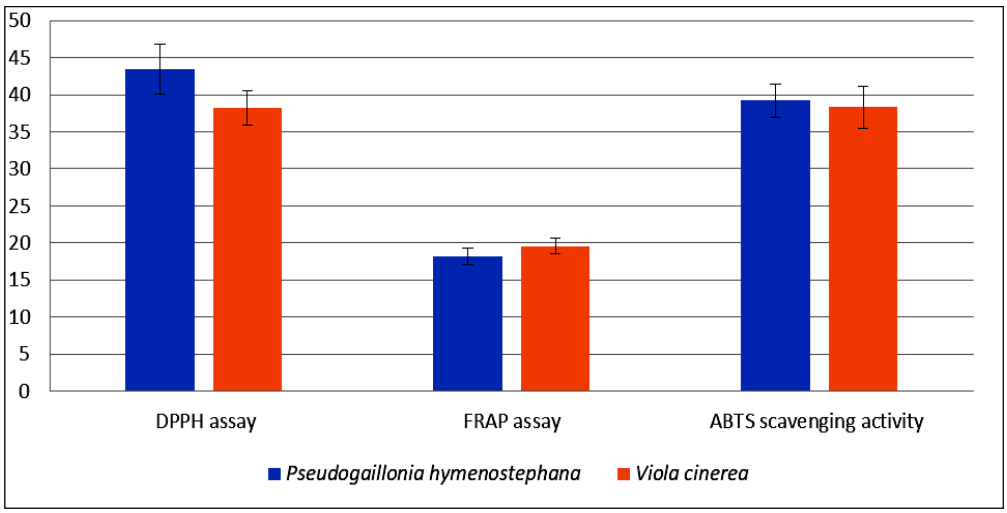

Fig. 3. Antioxidant activities determination of *P. hymenostephana* and *V. cinerea* by DPPH, FRAP and ABTS assay.

from genus *Viola*. These bioactive compounds include alkaloids, coumarins, lignans, sesquiterpenes, fatty acids, phenolic acids, flavonoids and others. From these reported compounds flavonoids and cyclotides are reported with the most species isolated from the genus *Viola*. Cyclotides are also one of the major components of plants from *Viola*. It is also reported that there is a high content of coumarins and flavonoids in this genus. The genus *Viola* is reported to have several curative and therapeutic activities including antioxidant, antiseptic, antiviral, anti-inflammatory, neuroreparative, anticancer and other activities (27, 28).

Among the angiosperm's family Rubiaceae is one of the largest families known for containing a diverse range of bioactive compounds and secondary metabolites. Plants belonging to this family have recognized to be an auspicious source for expansion of new potential metabolites and medical drug samples due to diverse pharmacological potential (29). Alkaloids, flavonoids, coumarins, terpenes and anthraquinones are the extensive variety of phytochemicals reported from plants of family Rubiaceae are the reason of therapeutic activities of plants (30). Though various factors like plant age, growing conditions and extraction methods can affect the exact composition and concentration of phytochemicals.

Conclusion

The persistent global interest in discovering bioactive plant chemicals underscores the importance of exploring unexamined plants as potential sources of medicinal prospects. This work presents the preliminary phytochemical analysis and evaluation of the antioxidant properties of *V. cinerea* and *P. hymenostephana* found in the Surghar Range, Pakistan. The findings suggested that both species had elevated concentrations of phenolics, flavonoids and tannins, which are closely associated with their antioxidant activities, as demonstrated by DPPH, ABTS and FRAP assays. Given the significant antioxidant activity observed, the two plants may serve as promising sources of natural antioxidants in the pharmaceutical, nutraceutical and functional food industries. Their historic medical application is corroborated by the presence of bioactive chemicals, highlighting the potential therapeutic uses of plants within the genus *Viola* and the family Rubiaceae.

To enhance these findings, additional experiments are strongly advisable to incorporate bioassay-guided fractionation and the isolation of specific active chemicals to further elucidate their roles and mechanisms. Structural analysis, along with in vitro and in vivo antioxidant activity screening of these compounds, may yield novel antioxidant agents or lead compounds for therapeutic development. Furthermore, pharmacological research and toxicological assessments are required to guarantee the efficacy and safety of clinical applications. The present work aids in the conservation and sustainable usage of such therapeutic plants and offers a pertinent backdrop for future research.

Authors' contributions

SR conducted experimental work compiled the manuscript. AS and SI helped in tabulation of data. GS and MA contributed to statistical analysis. MK and PRDR helped in the finalization of paper. All the authors approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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