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Original Research Article

BIOCHAR IMPROVES TOMATOES GROWTH CHALLENGED WITH KHOKHRAN VIRUS INFECTION

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Abstract *Tomato production in Pakistan is being seriously hampered by begomoviruses. To sustain tomato production and to contribute the growing economy of Pakistan, the researchers should focus on the innovative and organic disease prevention/management options. In this regard, the utilization of biochar as a soil amendment and to activate the defense mechanism of plants against invading pathogens emerged to be a reasonable solution. In this study, biochar produced from available organic waste through pyrolysis was used to tackle the begomovirus called the Khokhran virus. Various levels of biochar were applied alongside the soil. Five treatments were used, each involving the addition of biochar to soil: only soil with no biochar served as control, then treatments with 5 and 10% Green Biochar each, as well as 5 and 10% Wood Biochar amended soil substrate. According to the results of root and shoot mass, the treatment with 5%, Green Biochar yielded the best outcome. Plants grown with Green Biochar were taller and had higher yields compared to those in other treatments. Additionally, tests including PCR assays, RNA extraction, and DNA quantification were conducted to confirm the presence of viral load in each treatment. In conclusion, 5% Green Biochar was found to be most effective against the Khokhran virus, by boosting plant growth and inducing resistance.*

Keywords*:* *Begomoviruses; Organic amendment; Induced resistance; Sustainable agriculture*

Introduction

Viruses are non-cellular, obligate intracellular parasites found in plants and other living organisms (Singh, 2023). They were initially considered xenobiotics due to their crystal structure and ability to replicate within host cells (Harish et al., 2021). Today, viruses are recognized as rich, and universal, and play a crucial role in the health of living organisms, including humans, plants, animals, and protists (Maraz and Khan, 2021). Martinis W. Beijerinck, known as the father of virology, discovered viruses and concluded that they were not toxins but rather infectious agents in fluid (Méthot, 2016). Dmitri Ivanovski also studied viruses but found a small bacterium to be the cause of tobacco mosaic disease (Dehghani et al., 2022). Lwoff's 1957 definition of viruses emphasizes their non-cellular nature, dependency on host cell metabolism, and the reduction of a virus's material to nucleic acid (Kostyrka, 2016). Hull's 2002 definition identifies viruses as composed of individual or supplementary nucleic acid templates enclosed in a protective coat of protein or lipoprotein, enabling replication only within appropriate host cells (Hull, 2013). Plant viruses differ from other disease-causing organisms in their morphology, physical structure, chemical composition, and methods of multiplication, translocation, invasion, dissemination, and symptom production (Sastry, 2013). They damage and cause disease by using cellular substances through multiplication and disrupting cellular metabolism, leading to abnormal growth (Goodlett and Horn, 2001). Viruses can also initiate protein production through the host, disrupting the host's common metabolism (Girdhar et al., 2021). They also disrupt

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hormone production, disrupting growth regulatory or inhibitory activity (Pener and Dhadialla, 2012). Virus diseases of plants often result from a deficiency in soluble nitrogen, and mosaic diseases may decrease carbohydrates in plant tissues (Zanini et al., 2021). Plant viruses are transmitted through various methods, including insects, mites, nematodes, and fungi (Butter, 2018). Classification involves categorizing biological entities into taxonomic categories based on similarity or relations (Richards, 2016). The International Committee on Taxonomy of Viruses (ICTV) defines virus classification into nine orders of 109 families (King, 2012). Plant viruses have either DNA or RNA genomes, with 90% having ssRNA genomes. Some have double-stranded DNA, while nano viruses and geminiviruses are ssDNA viruses (Shafiq et al., 2020). Geminiviridae viruses, first described by Goodman in 1977, are round ssDNA molecules encapsulated in twin icosahedral capsids. These plant viruses are obligate intracellular and cause dwarfing, normal reproductive organ formation, curling, foliage deformation, and vein swelling (Akhila, 2020). Geminiviruses' high mutation and recombination stages increase viral diversity (Tatineni and Hein, 2023). They develop insecticide resistance, allowing them to attack new regions and pose threats to crop plants (Hawkins et al., 2019). Gemini viruses are classified into nine genera based on genome makeup, hosts, and insect vectors (Bhattacharjee and Hallan, 2022). Mastreviruses, a type species of Maize streak virus, have a 2.7 kb monopartite genome and infect monocots and dicots (Marwal et al., 2019). Leafhoppers are obligate transmitters. The genome contains four genes: coat protein (CP), movement protein (Navas-Castillo et al., 2011), and complementary sense strand genes (Marwal et al., 2019). Aphids transmit capsule viruses, consisting of four members with C1 and C2 genes encoding Rep and Rep A, and C3 inserted with the C1 gene (Matole, 2018). Grapevine red blotch virus is a 3.2 kb granulovirus with a genome of 3.2 kb, three virion sense proteins, and a c-sense protein, with a TAATATTAC motif (Hallwachs, 2022). Over 80% of known Geminiviridae viruses belong to the genus Begomovirus, characterized by twinned incomplete icosahedra and single-stranded DNA (Brown et al., 2015). They are divided into Old World (Africa, Asia, Australia, and Europe) and New World (American continents) categories based on genome union, genetic variety, and environmental distribution (Balfourier et al., 2019). Most New World (NW) begomoviruses arose more recently than Old World (OW) viruses, possibly due to whiteflies moving from Asia to the Americas transferring viruses (Navas-Castillo et al., 2011). However, recent studies have shown that NW-like begomoviruses are present in the Old World (Briddon et al., 2010). Corchorus Yellow Vein Virus (CoYVV) and Corchorus Golden Mosaic Virus (CoGMV) were identified in Vietnam, displaying characteristics of NW viruses, including the absence of the AV2 gene and N terminal PWRLMAGT motif in the coat protein. Begomoviruses can be classified as monopartite or bipartite based on their genome organization. The sizes of DNA A and B, are around 2.8 kb (de Souza, 2020). The study was planned to find the biochar role in plant development and inducing protection against Khokhran virus.

Materials and Methodology

Experimental Setup

Tomato seedlings were grown (Angulo-Bejarano et al., 2021; Murtaza et al., 2017). The soil was taken from University of the Punjab fields and autoclaved (Hanafy and Sadak, 2023; Hou et al., 2020). Pots were filled and kept under controlled conditions (Chen et al., 2013; Kaur et al., 2021). Green and wood biochar were combined in the first four treatments, and no biochar was added to the fifth treatment, which functioned as a control. Every possible combination of soil mixture was put into sixteen pots. With great care, the tomato seedlings were gently moved into cups with the ready-made soil combinations on January 22, 2018, making sure the plants would not be adversely affected. For the transplantation procedure, 80 cups were used, and to help the soil settle around the root systems, a tiny amount of water was applied. Plants were housed in a 24–30℃ temperature range.

Agro-Infiltration

Agroinfiltration is a widely used technique for virus inoculation in plant leaves (Bos and Bos, 1970; Chen et al., 2013; Zhang et al., 2010). Inoculum was introduced into plant leaves using a needleless syringe, ensuring no damage to the leaves. Approximately 0.5 ml of inoculum was injected into each plant leaf (Zhao et al., 2020).

Viral Symptoms on the plant

Symptoms began to manifest on the plant surface in varying manners, as not all areas were affected

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equally (Bhattacharyya et al., 2015). While the leaf yellowing was non-specific and could be attributed to environmental factors, the curled leaves showed a direct response to the introduced virus through agroinfiltration (Egamberdieva et al., 2016; Schulz et al., 2013). Additionally, small brown spots speckled the upper leaf surface in a scattering of discoloration.

Effect of biochar on the growth of plants

Observations were made regarding the effect of various biochar treatments on plant growth (Kim et al., 2010; Sood et al., 2020). All five biochar treatments were compared concerning their impact on overall growth. The green biochar at 5% promoted healthier and more substantial growth than the other treatments containing 10% green biochar, 5% or 10% wood biochar, or no biochar. Most remarkably, the plants nourished with the 5% green biochar developed longer stems and a lusher appearance relative to those in other treatment groups. Samples of plants were grown and then collected in a lab environment under controlled conditions, utilizing liquid nitrogen to preserve the samples (Freschet et al., 2021; Ma et al., 2020).

Harvesting of plants

Plants were harvested to record growth parameters (Li et al., 2023). Leaves were stored at -80°C for future use (Chiavaro et al., 2012; Li et al., 2023; Nebbioso and Piccolo, 2013; Xiao et al., 2024).

Labelling of the samples

Give each sample of leaves, roots, and stem a unique label. While samples of the roots and stem were gathered to determine their dry weight, the leaf samples were prepared for molecular characterization (Soukoulis et al., 2014).

Root and stem mass analysis

First, the root samples were put into paper bags while they were still wet. After that, these bags were placed in an oven and roasted for two days at 60°C (Böhm, 2012; Korir et al., 2017). The roots were completely dried out using the dry evaporation technique in this procedure (Korir et al., 2017). After that, each dried root sample was weighed individually, and the weights were carefully recorded (Li and Keller, 2023; Wu et al., 2014). Similarly, the initially wet stem samples were also enclosed in paper bags and subjected to the- same baking process at 60 degrees Celsius for two days. Once the drying was complete, the weight of each dried stem sample was measured individually, and the measurements were diligently documented.

DNA Extraction

Initially, tomato leaf frozen plant tissue was pulverized into a fine powder using a mortar and pestle, followed by transfer into 1.5 ml Eppendorf tubes (Li et al., 2019; Rogstad, 1992). This was followed by the addition of 400 µl of buffer PL to the powder, whereby vigorous vortexing was done. Incubation of the sample in a water bath at 65°C was then followed for 10-15 minutes with intermittent mixing (Lee et al., 2012). This involves cell lysation, where 140 µl buffer PD is added and vortexed, followed by 5 minutes incubation on ice (Mesapogu et al., 2012). The lysate was then passed through EzSep™ blue filters via centrifugation at maximum speed for 2 minutes. Careful pipetting was employed to transfer the pass-through to new tubes, avoiding disturbance to the cell debris pellet. Following this, 750 µl of buffer BD was added to the lysate and immediately mixed by inversion. Subsequently, 700 µl of the mixture was applied to Gene All save green columns and centrifuged for 30 seconds, discarding the pass-through and reusing the collection tube. This step was repeated for the remaining sample. The SV column was then washed with 700 µl of buffer CW, followed by centrifugation for 30 seconds, pass-through disposal, and re-insertion into the collection tube. To do further purification, 300 µl of buffer CW was added to the SV column, and centrifuging for two minutes was required. Centrifugation was done in the last to finalize the sample processing

Agarose Gel electrophoresis

Gel electrophoresis was performed to confirm the virus (Renkawitz et al., 2019). We can employ different gel concentration (Dong et al., 2016; Tan and Yiap, 2009). TAE buffer was mixed with 0.5 gr of agarose (Green and Sambrook, 2018). After cooling and boiling for two to three minutes in defrost mode in the oven, the mixture was then supplemented with six microliters of ethidium bromide solution. Since ethidium bromide is radioactive, it binds to DNA and RNA and fluoresces when exposed to UV light, which helps to see bands. Then, a gel tray was ready, and the ends were sealed with masking tape to make sure everything was sealed. The agar slab was poured into the taped tray, which was then placed on a level surface. After that, two combs were placed on one side of the gel tray to form wells for loading RNA samples and DNA samples. Each comb makes 16 wells. It was then left on a level surface until it was completely solidified. Combs were removed very

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carefully by pulling straight up. Wells were ready for loading samples. The tape was also removed from the ends of the gel tray. In addition, the apparatus was filled with enough 1X TAE buffer just to cover the gel.

PCR (Polymerase Chain Reaction)

The reaction mixture was prepared by using "PCR Master Mixture" (Green and Sambrook, 2018). That master mixture consists of $MgCl_2$ and Taq. Buffer, dNTP"s, and Taq polymerase enzyme. The reactionmixture was prepared by,

Master mixture = 15µl

1. Forward primer = 1µl
2. Reverse primer = 1µl
3. DNA template = 1µl
4. Double distilled Water (ddH2O) = 7µl

DNA Quantification

Quantification of all the DNA samples was concluded by taking the absorbance at 260 nm in a times beam spectrophotometer (Gallagher, 2017; Garwi et al., 2024; Green and Sambrook, 2018; Saile et al., 1997). DNA samples were diluted by taking 50 µL of autoclaved deionized distilled water and 2 µL of DNA sample. Non-refundable cuvettes were used for this principle. Dilution was done according to the following formula: C1V1=C2V2. This was the greenhouse experiment. Five treatments were subjected. Treatments were made based on different concentrations of biochar. After one week, tomato plants were transferred into cups (George et al., 2012).

Results

Symptoms

The tomato plants exhibited symptoms on their leaves, although not all parts were affected. While the yellowing of the leaves could be attributed to non-living environmental factors, leaf curling was a result of virus application through agro-infiltration. Additionally, small brown spots appeared on the upper leaf surface. The severity of symptoms on the tomato plants indicated the presence of the virus.

DNA analysis

After DNA isolation, agarose gel electrophoresis was conducted to validate the presence of genomic DNA. A 1 kb DNA ladder served as a reference standard across all gel runs. The images provided illustrated the distinctions observed between inoculated and non-inoculated plant DNA samples from those without biochar treatment, those treated with 5% Green Biochar, and samples treated with 10% Green Biochar. Notably, sharp DNA bands were discernible within the agarose gel, as depicted in Fig 1 and 2.

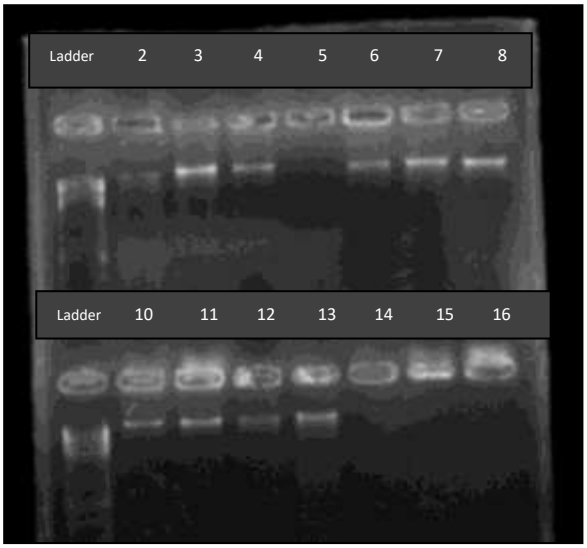

Fig 1: 2nd well-shows DNA of inoculated sample from treatment of 10% Green Biochar. 3-7 wells: DNA of inoculated samples except the 5th well from the treatment of 5% Wood Biochar. 8, 10-13 wells showed DNA of inoculated samples from the treatment of 10% Wood Biochar, while 14-16 wells were empty.

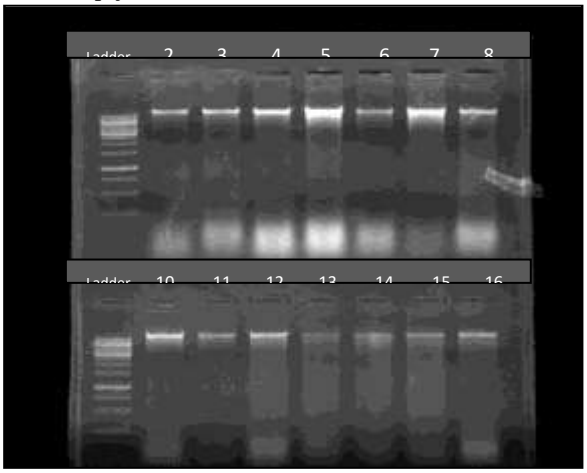

Fig 2: 2-6 wells shown DNA of non-inoculated samples from the treatment of No Biochar. 7, 8, 10-12 wells showed DNA of non-inoculated samples from the treatment of 5% Green Biochar. 13-16 wells showed DNA of non-inoculated samples from the treatment of 10% Green Biochar.

PCR Analysis

Amplification was carried out using universal primers. A 2.8 kb band of begomovirus-infected cotton was obtained. The 1 kb ladder was employed as a reference standard. Positive results were observed in the inoculated samples across different

treatments: the first well, representing samples without biochar treatment; the second well, representing samples treated with 5% biochar; the third well, representing samples treated with 10% Green Biochar; the fourth well, representing samples treated with 5% Wood Biochar; and the fifth well, representing samples treated with 10% Wood Biochar as represented in Fig 3.

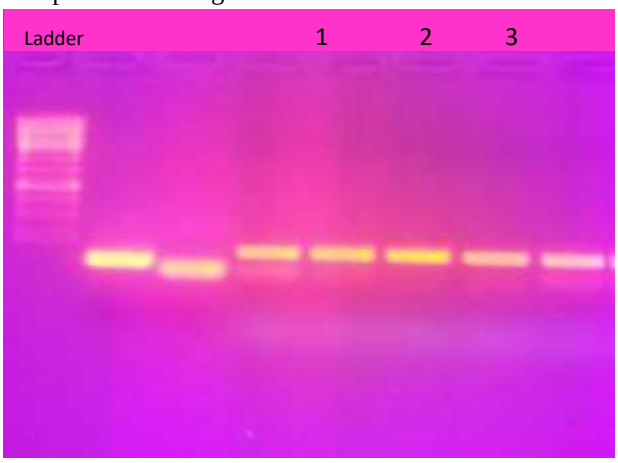

Fig 3:1kb Ladder is used. In 1st well inoculated sample of No Biochar showed positive results. In 2nd well inoculated sample of 5% Biochar showed positive results. In 3rd well inoculated sample of 10% Green Biochar showed positive results. In 4th well inoculated sample of 5% wood biochar showed positive results. In 5th well inoculated sample of 10% Wood Biochar showed positive results.

Stem and Root Mass Analysis

Stem Mass Analysis

The stem weight analysis revealed varied responses to different biochar treatments. Samples without biochar exhibited a consistent average stem weight of 0.224 grams, with low variability (Table 1). Conversely, 10% Green Biochar treatment resulted in a significantly lower average stem weight of 0.088 grams, indicating reduced plant growth. Samples treated with 5% Green Biochar displayed a similar average weight but with slightly higher variability. Both 10% and 5% Wood Biochar treatments showed lower average weights (0.21 grams and 0.166 grams, respectively) with noticeable variability. Overall, higher biochar concentrations, particularly 10% Green Biochar and 10% Wood Biochar, appeared to inhibit plant growth. Conversely, no biochar and 5% Green Biochar treatments showed potential stimulatory effects. Further investigations are warranted to comprehend the mechanisms underlying these observations in tomato plants.

Table 1 Average Stem Weight in grams of Tomato Plant Sample with or without virus inoculation

Treatment	Stem Weight (g), Inoculated Plants	Stem Weight (g) Non-inoculated Plants
No Biochar	0.224± 0.09	**0.174±0.05**
10% Green Biochar	0.088±0.04	**0.095±0.05**
5% Green Biochar	0.224±0.10	**0.201±0.12**
10% Wood Biochar	0.21±0.15	**0.189±0.13**
5% Wood Biochar	**0.166±0.08**	**0.169±0.09**

Root Mass Analysis

In Table 2, the root weight of tomato plants ranged from 0.041 to 0.067 grams across various biochar treatments. Notably, the treatment with 5% Green Biochar displayed the highest average root weight (0.067 g) among the treatments. Table 5 shows the root weight of non-inoculated tomato plant samples, ranging from 0.038 to 0.07 grams. Interestingly, the treatment with 5% Green Biochar exhibited the highest average root weight (0.07 g) among non-inoculated samples, indicating a potential stimulatory effect on root growth. In Table 6, the root weight of inoculated tomato plant samples varied from 0.038 to 0.064 grams. The treatment with 5% Green Biochar demonstrated the highest average root weight (0.064 g) among inoculated samples, suggesting a positive influence on root development even under inoculated conditions. Within the same biochar treatments, there was a modest difference in the root weights of the infected and non-inoculated samples; in general, the non-inoculated samples had somewhat greater root weights. According to these results, biochar treatments—specifically, 5% Green Biochar—may promote root development in tomato plants, which might have an impact on agricultural techniques meant to increase plant resilience and production. To clarify the underlying processes causing these reported effects, more research is necessary.

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Table 2 Average Root Weight in grams of Tomato Plants grown in different biochar amendments

Treatment	Root Weight (g) Inoculated plants	Root Weight (g) Non-inoculated plants
No Biochar	0.046±0.03	**0.055±0.02**
10% Green Biochar	0.045±0.02	**0.043±0.02**
5% Green Biochar	0.067±0.04	**0.07±0.05**
10% Wood Biochar	0.041±0.02	**0.038±0.02**
5% Wood Biochar	**0.059±0.04**	**0.065±0.06**

Discussion

Pakistan's economy is based mostly on agriculture, and the country's vitality is closely linked to this industry (Luedeling et al., 2016). However, there are ongoing challenges to the major crops in the agricultural landscape, which inevitably reduce production (Azam and Shafique, 2017; Elmer and White, 2018). As Pakistan's population continues to rise, more and more people will require staple crops. This implies that we must come up with solutions for this issue (Ahmed et al., 2017). To address these issues, a variety of plant pathology disciplines have been investigated, leading to treatment options that range from chemical pesticides to natural cures (Yu et al., 2019). Although chemical treatments work well, they can be expensive and have environmental dangers (Rashid et al., 2023). However, organic methods like biological controls or using biochar can be a useful strategy to control phytopathogens (REIS, 2020). Begomovirus possesses betasetallites that inhibit host defense and increase the replication of helper DNA. The protein is rich is the A- region (Briddon et al., 2010). βC1 modifies plant response to virus interaction. DNA β has a vital role in symptom development and determining host range of viruses (Hussain et al., 2005), and required for the replication as well (Rashid et al., 2023).

Cotton plants were also reported to have another satellite virus component called DNA-1, self-replicating with a single gene (Molin et al., 2020). Gossypium plants are now known to harbor a diverse range of alpha satellites infected by cotton leaf curl disease (CLCuD). There main role is also in inducing symptoms in their host plants. Beta satellites are usually single-stranded (Zhou, 2013). White fly (*Bemisia tabaci*) has a wide host range and potent vector of begomoviruses, thus mainly responsible for the widespread presence of begomoviruses (Matole, 2018). They enter the host plant's vascular system and enter the mesophyll cells, where they enter the nucleus for DNA replication and transcription (Ziegler-Graff, 2013).

The coat protein of monopartite begomovirus transfers viral DNA into the nucleus and cytoplasm, while bipartite begomovirus uses Nuclear Shutter Protein for movement (Castillo Gonzalez, 2017). Begomovirus, like other geminiviruses, lacks DNA polymerase and uses the replication machinery of its host to amplify its genomes in infected plant cells. Replication occurs through rolling circle replication and recombination-dependent mechanisms, with three stages: initiation, elongation, and termination. Initiation occurs when the virus's IR binds with the host factor Rep, forming a nonamer sequence with a replication fork. Elongation begins at the 3' -OH end, where nick produced by Rep acts as a helicase. Termination is the final process, where ssDNA re-ligates where Rep cuts to make a circle. The newly synthesized ssDNA passes through various passages, including reentry into DNA replication, encapsulation, and spreading to nearby cells for infection with viral movement proteins. In a recent study, researchers looked into how two types of biochar made from wood and green sources could help tomato plants fight against the Khokhran virus. Tomato plants infected with the virus showed severe symptoms like leaf curling. On the other hand, plants treated with biochar displayed reduced disease symptoms and improved growth and resistance. Tomato plants grown with green biochar produced more biomass. PCR analysis detected the virus infection in plants. Thus, biochar plays a role in immunizing plants against invading viruses. By using biochar, one can reduce disease losses and save the environment from pesticides as well.

Conclusion

To safeguard crops, natural methods like biochar combined with soil amendments enhance plant defenses and yields. Research targeting the Khokhran virus shows 5% Green Biochar to be most effective, boosting plant growth and resistance. These findings offer eco-friendly solutions to protect Pakistan's

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tomato crops and sustain agricultural benefits. Biochar application, particularly at 5% concentration, emerges as a promising strategy against begomovirus infections, ensuring economic stability for tomato growers.

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IS, MK, MTM performed experiments, SA, MTA, AA hypothesized and planned the study, MF, SA, WA, ZM finalized methodology, AA, MTA, MK, MTM data analysis, IS, MF, ZM, WA wrote the initial draft manuscript. .All authors approved final version of manuscript.

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